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A review of whole cell wall NMR by the direct-dissolution of biomass†

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To fully realize the potential of lignocellulosic biomass as a renewable resource for the production of fuels, chemicals, and materials, an improved understanding of the chemical and molecular structures within biomass and how those structures are formed during biosynthesis and transformed during (thermochemical and biological) conversion must be developed. This effort will require analytical techniques which are not only in-depth, rapid, and cost-effective, but also leave native cell wall features intact. Whole plant cell wall nuclear magnetic resonance (NMR) analysis facilitates unparalleled structural characterization of lignocellulosic biomass without causing (or with minimal) structural modification. The objective of this review is to summarize research pertaining to solution- or gel-state whole plant cell wall NMR analysis of biomass, demonstrating the capability of NMR to delineate the structural features and transformations of biomass. In particular, this review will focus on the application of a two-dimensional solution-state NMR technique and perdeuterated ionic liquid based organic electrolyte solvents for the direct dissolution and analysis of biomass. We believe this type of analysis will be critical to advancing biofuel research, improving bioprocessing methodology, and enhancing plant bioengineering efforts.

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Introduction

Due to the compounding negative impact of fossil fuel production and utilization on the global environment and the growth in the demand for energy and material associated with increasing populations, research on the generation of a readily available and renewable resource as an alternative feedstock for the global production of fuels and materials has become of considerable and renewed interest.¹ For some time now, lignocellulosic biomass (*e.g.*, agricultural and forestry waste residues and non-food grassy and woody dedicated energy crops) has been considered as an abundant renewable resource that could potentially displace or replace fossil fuels as the major feedstock for the production of fuels, chemicals, and materials.²

Lignocellulosic biomass is a complex composite consisting primarily of secondary plant cell wall biopolymers: cellulose (35–50%), hemicellulose (20–35%), and lignin (10–25%).³

These secondary plant cell wall biopolymers are described to exist and to be integrated in a closely associated network structure called the lignin-carbohydrate matrix.³ Lignin is an irregular, heterogeneous, highly branched, and cross-linked polyphenolic biopolymer linked to polysaccharides through what are called lignin-carbohydrate complexes (LCCs). The exact molecular nature of LCCs still remains unclear; however, the existence of LCCs has been elucidated on chemically treated wood samples.⁴ The major monomeric unit composing lignin is in the form of phenylpropanoids with varying degrees of methoxylation including: *p*-hydroxyphenyl (H) units without methoxyl groups, guaiacyl (G) units with one methoxyl group at the 3-phenolic ring position, and syringyl (S) units with two methoxyl groups at the 3- and 5-phenolic ring positions. Hemicelluloses are a class of highly branched polysaccharides with relatively short chain lengths (as compared to cellulose) which strongly adhere and bind to other cell wall components. Cellulose is linear and only contains glucose monomeric groups, whereas hemicellulose is comprised of not only glucose but also xylose, mannose, galactose, rhamnose, arabinose as well as various sugars in their acidified forms (*e.g.*, glucuronic acid and galacturonic acid).

The natural resistance of plant cell walls towards decomposition is referred to as “biomass recalcitrance”⁵ and limits the use of biomass of a feedstock. Currently, biomass is most commonly processed to produce bioethanol and other fermentative products. In this case, biomass first undergoes chemical pretreatment to reduce biomass recalcitrance,⁶ during which

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hemicelluloses are hydrolyzed, lignin structure is modified/disrupted, and cellulose ultrastructure is altered. The cellulose is subsequently hydrolyzed by cellulases (*i.e.*, cellulolytic enzymes) into monosaccharides that are then fermented into bioethanol or other products. In fermentative processing of lignocellulose, lignin is under-utilized (considered waste) as a resource for on-site process heat or electricity generation. However, recent efforts have been devoted to lignin valorization through the production of lignin-derived aromatic chemicals,⁷ carbon fibers,⁸ *etc.* Thermochemical processing routes (pyrolysis, gasification, *etc.*) can also transform biomass into a wide range of fuels and chemicals.⁹ In all these examples, product yield, product purity, and process productivity is highly correlated to plant cell wall chemical structure and associated recalcitrance. As a result, economical utilization of biomass depends on advancing research (leveraged by deep understanding of plant cell wall chemical structure) focused on the origins and circumvention of biomass recalcitrance.

Understanding plant cell wall chemical structure is critical to selecting/generating optimal biomass substrates and improving biomass processing technologies (encompassing both biological and thermochemical approaches).¹⁰ Most traditional methods for the characterization of biomass involve separation and isolation of cellulose, lignin, and hemicelluloses followed by characterization of those individual components. These routes are not only time and energy consuming, but more importantly, may alter the native structure of the cell wall components. Plant cell wall chemical functionalities or moieties have been examined by wet chemistry methods;¹¹ however, these wet chemistry methods require cell wall component isolation to attribute the origin of any specific chemical functionality. Degradative methods such as hydrolysis, acidolysis, thioacidolysis, and derivatization followed by reductive cleavage are also well-understood and are used to determine lignin monomer distribution and bonding pattern (only in isolated lignins).¹² Unfortunately, these methods analyze only the monomeric or dimeric units that result from these degradative schemes and are typically limited to an analysis of monolignols linked *via* aryl ether linkages.

However, NMR is well-suited for the analysis of complex chemical systems, such as the plant cell wall, because it is not only responsive to chemical functionality but also chemical connectivity and environment.¹³ Traditional solid state NMR, such as ¹³C cross polarization magic angle spinning (CP/MAS) on intact biomass, is hampered by poor resolution and overlapping resonances.¹⁴ Solution NMR on the other hand can distinguish between a varieties of chemical moieties producing high resolution spectra owing to the well-developed technology. Solution state 2D correlation NMR on biomass components or intact biomass has been shown to provide well resolved spectra in which overlapping resonances are spread over two-dimensions in an effort to deconvolute the complex 1D NMR dataset.^{13,15} This further enables solution NMR for in-depth characterization of hemicellulose and lignin sub-units. This methodology can be used to characterize lignocellulosic biomass components very effectively in a single step

and to estimate the structural changes occurring during different stages of biomass development and/or processing. However, non-destructive dissolution of plant biomass in common solvents is a challenge due to the highly crystalline nature of cellulose and complexity of the cross-linked cell wall construction. This review will provide a detailed overview of whole plant cell wall characterization by solution state NMR analysis particularly the application of two-dimensional solution-state NMR techniques and perdeuterated ionic liquids based organic electrolyte solvents for the direct dissolution and analysis of biomass.

Routes of biomass dissolution

Whole plant cell wall characterization by solution state NMR relies on methods that can sufficiently dissolve or swell biomass, thus we will briefly review routes of biomass dissolution that could potential be employed. Dissolution of plant biomass can be classified according to two basic categories of solvents: non-derivatizing and derivatizing.¹⁶ Non-derivatizing solvents dissolve (or swell) the plant cell wall material by disrupting intermolecular interactions only. There are two classes of non-derivatizing cellulose solvents that include: aqueous (*e.g.*, transition metal complexes with amines, transition metal complexes with tartaric acid ammonium hydroxides, alkali hydroxides, *etc.*) and non-aqueous (*e.g.*, ionic liquids, dipolar aprotic solvents/LiCl, dimethyl sulfoxide (DMSO) containing bicomponent solvents, liquid ammonia (NH₃)/sodium or ammonium salts, NH₃ or amine/salt/polar tricomponent solvents, *N*-alkylpyridinium halogenides, oxides of tertiary amine) solvents.¹⁶ For example, *N,N*-dimethylacetamide/lithium chloride (DMAc/LiCl) and dimethyl sulfoxide/lithium chloride (DMSO/LiCl) are well-known solvent systems for cellulose.

On the other hand, there are derivatizing solvents that facilitate dissolution *via* the formation of derivatives, usually converting hydroxyl groups to ether, ester, or acetal moieties.¹⁶ Modifying the ubiquitous hydroxyl functionality of the cell wall material disrupts intermolecular bonding and produces a material more amenable to dissolution in a wide array of common solvents. Non-destructive (non-derivatizing and derivatizing) dissolution of plant biomass for NMR analysis must therefore rely on these well-established methods.

A key point to note is that by the virtue of biomass being a covalently cross-linked material, the introduction of solvent without breaking all or the majority of the chemical, physical, and topological cross-links present leads to gelation (swelling) rather than dissolution. However, many of the dissolution/swelling protocols used include ball-milling, derivatization, sonication, and/or heating. Based on the assumption that most cross-links (*i.e.*, LCCs) in biomass are fairly liable linkages, it therefore seems reasonable to suggest that the processing and conditions required to cause dissolution of biomass may contribute to cell wall LCC cleavage. This change in cell wall chemistry would then produce a material that is more

amenable to true dissolution, though most likely many of the studies in this review technically involve the formation of gels. With that said, the processing of biomass, especially milling, to cause dissolution does alter the structure of biomass components other than LCCs, in particular the chemical and molecular structure of lignin. In the context of whole plant cell wall characterization, this fact presents a serious limitation and must be considered when choosing a dissolution method, sample preparation, and solvent system for NMR analysis as well as when interpreting the resulting NMR spectra.

Ionic liquids and organic electrolyte solvent systems

Ionic liquids (IL)s are low melting point and thermally stable salts, with tunable solvent properties.^{17–19} ILs can be recycled and are capable of dissolving a variety of organic, inorganic, and polymeric materials. ILs have the special property of disrupting the intermolecular hydrogen bonding in cellulose which is also beneficial for dissolving natural biopolymers such as lignin and hemicelluloses. ILs are an ideal media for the chemical modification dissolution, and preservation of biomass.^{17–24} Generally, imidazolium-based ILs are used in the dissolution, functionalization, and pretreatment of biomass. Seen in Fig. 1, Pu *et al.* reported that 1,3-dimethylimidazolium methyl sulfate ([Mmim][MeSO₄]), 1-hexyl-3-methylimidazolium trifluoromethanesulfonate ([Hmim][CF₃SO₃]), 1-butyl-2,3-dimethylimidazolium tetrafluoroborate ([Bm₂im][BF₄]) are particularly useful for the dissolution and NMR analysis of lignin.²² More recently, a study screened a series of ammonium, phosphonium, and pyrrolidinium based ionic liquids for lignin dissolution, and found that tributylmethylphosphonium methyl sulfate and *N*-butyl-*N*-methylpyrrolidinium dicyanamide displayed high Kraft lignin solubility with a rather low viscosity.²⁵ An organic electrolyte solvent system is comprised of a mixture of IL and organic

solvent. In many cases, organic electrolyte solvent systems are preferred due to their lower viscosity and reduce utilization of expensive IL.

Table 1 describes the common IL and organic electrolyte solvents used for biomass dissolution in derivatization and NMR studies. Pyridinium chloride was observed to be one of the most effective ILs for the dissolution of cellulose and lignin. The type of IL anion seems to be critical due to its capability to disrupt hydrogen bonds. Other factors contributing to the efficient dissolution of biomass in ILs are: (1) biomass particle size (the solubility efficiency of biomass in IL was observed in the following order: ball-milled wood powder > sawdust > thermo-mechanical pulped (TMP) fibers ≫ wood chips), (2) resonance time and temperature of dissolution, and (3) water content (water was found to significantly reduce the solubility of biomass in an IL).^{19,23} Kyllönen *et al.* using a ³¹P NMR technique was able to quantify the solubility of the wood samples in different IL solvents under different treatment

Table 1 IL and organic electrolyte solvents used for biomass dissolution^{23,27–29}

Ionic liquid	Preparation	Solubility conditions	Approx. solution concentration (mg g ⁻¹)
[Bmim]Cl	Wood chips	130 °C, 15 h	Partially soluble
[Bmim]Cl	Sawdust	110 °C, 8 h	8
[Bmim]Cl	TMP	130 °C, 8 h	7
[Bmim]Cl	TMP	130 °C, 8 h	5
[Amim]Cl	TMP	130 °C, 8 h	5
[Amim]Cl	Ball-milled	80 °C, 8 h (IL : DMSO, 1 : 2)	8
[APyr]Cl	Ball-milled	60 °C, 1 h (IL : DMSO, 1 : 2)	35
[APyr]Br	Ball-milled	60 °C, 6 h (IL : DMSO, 1 : 2)	<5
[CmPyr]Cl	Ball-milled	60 °C, 1 h (IL : DMSO, 1 : 2)	70
[HPyr]Cl-d ₆	Wiley-milled	80 °C, 6 h (IL : DMSO, 1 : 2)	80
[HPyr]Cl-d ₆	Ball-milled	60 °C, 1 h (IL : DMSO, 1 : 2)	80

1-Allylpyridinium: [APyr]; cyanomethylpyridinium: [CmPyr]; pyridinium: [HPyr]; benzyl-methylimidazolium: [Bzmim]; 1-allyl-3-methylimidazolium: [Amim].

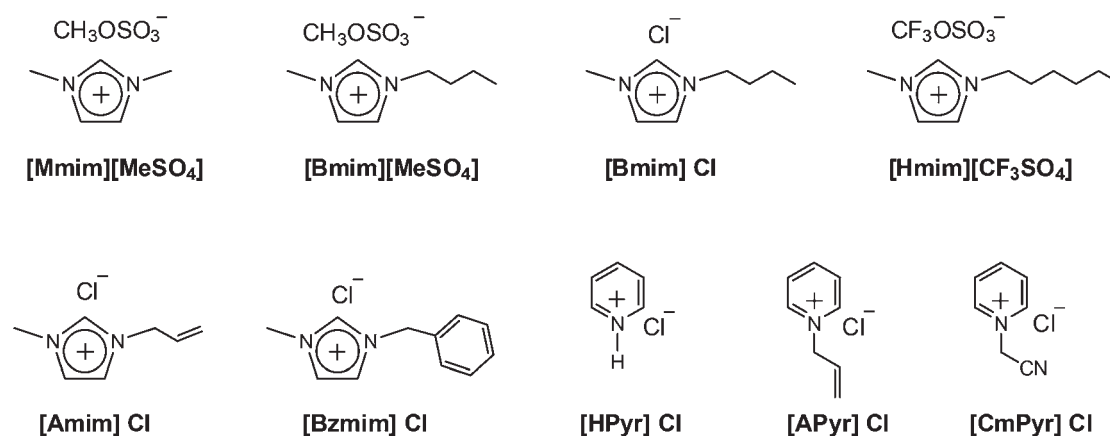


Fig. 1 Structure of ILs.

conditions.²⁶ They found all media tested required some amount of milling to facilitate solubility, which was related not only to the IL system used but also factors such as cell wall composition and ultrastructure. Ultimately, the IL system and treatment condition being used and the biomass being tested determined whether full or partial solubilization resulted. This later point about full *versus* partial solubilization would have a large effect, causing selective detection of soluble cell wall moieties as a result of direct NMR analysis.

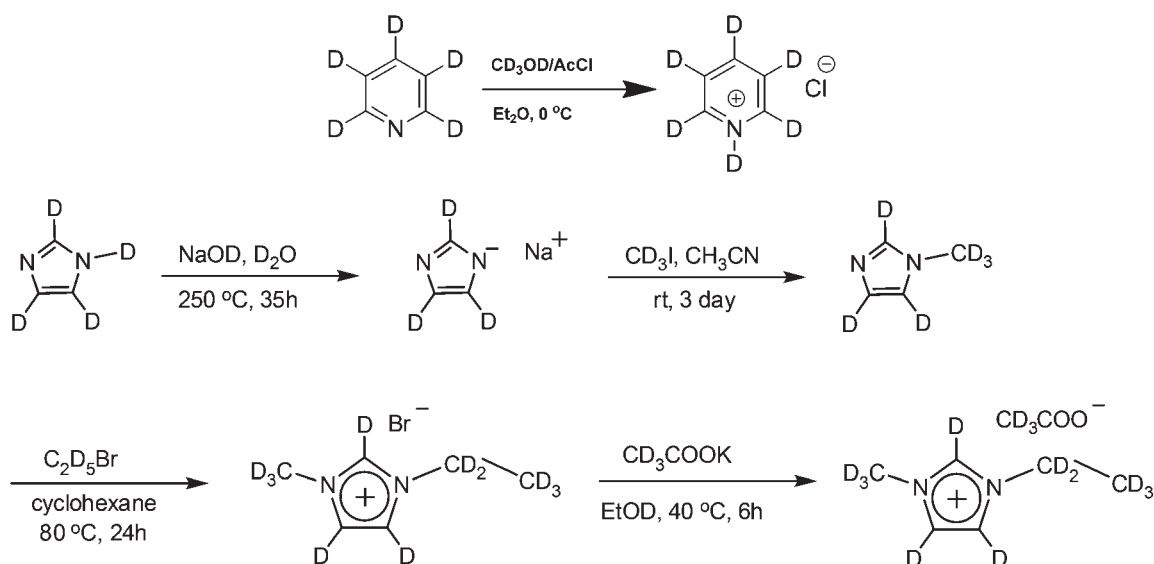
Despite the wide spread application of ILs in biomass pretreatments and functionalization, the use of IL and organic electrolyte solvents as NMR solvents have been limited due to synthetic issues surrounding the facile and economical preparation of perdeutero-materials. In addition, LCC, lignin, and hemicellulose degradation can readily occur as a result of exposure to IL and organic electrolyte solvents at elevated temperature.^{30,31} Addressing the former issue with the use of ILs as NMR solvents, Ragauskas *et al.* synthesized perdeuterated pyridinium chloride (PyCl-d₆) in a one-step procedure from commercially available deuterated pyridine in 95% yield (see Scheme 1) and established that a (1 : 2) organic electrolyte solvent of anhydrous PyCl-d₆/DMSO-d₆ is well suited for the dissolution and NMR analysis for both Wiley-milled and ball-milled biomass.^{27–29}

Beyond PyCl-d₆, Cheng *et al.* recently synthesized perdeutero-1-ethyl-3-methyl-imidazoliumacetate-d₁₄ ([Emim]OAc-d₁₄) starting with commercially available imidazole-d₄.³² Essentially, imidazole-d₄ is converted to 1-methylimidazole-d₆ in the presence of CD₃I and sodium. In a second step, 1-methylimidazole-d₆ was converted to 1-ethyl-3-methylimidazolium bromide-d₁₁ using ethylbromide-d₅ (prepared by bromination of ethanol-d₆ with phosphorus tribromide). Finally the bromide anion is exchanged with a perdeutero-acetate ion (Scheme 1).

Whole plant cell wall solution state NMR: a derivatizing approach

In this section of the review, we will highlight research that first pioneered the use of whole plant cell wall solution state NMR as well as more recent interesting applications, focusing on the utilization of biomass derivatization methods to facilitate biomass solubility in NMR solvents. Table 2 is a summary of biomass dissolution methods used for NMR analysis, including their advantages and disadvantages. One of the first demonstrations of whole plant cell wall solution state NMR by Ralph *et al.* reported utilizing a derivatizing approach (biomass acetylation).³³ In this case, a binary solvent system of DMSO with either tetrabutyl ammonium fluoride (TBAF) or *N*-methylimidazole (NMI) was used to dissolve/swell finely ball milled biomass at room temperature. The mechanism of dissolution/swelling, though not well understood especially for the DMSO/NMI system, was attributed to substantial disruptions in hydrogen bonding within the biomass along with significant swelling by DMSO. In later efforts, to clarify the mechanism of dissolution for cellulose in DMSO/TBAF, Östlund *et al.* suggest that the strongly electronegative fluoride ions of TBAF act as hydrogen bond acceptors to cellulose hydroxyl groups.³⁴ Nevertheless, the resulting solutions were too highly viscous for direct NMR analysis of biomass in either the DMSO/NMI or DMSO/TBAF solvent systems; therefore, they were used for the derivatization of cell wall hydroxyl groups and the acetylation of its cell wall biopolymers. Once acetylated, cell wall material could be precipitated quantitatively in water, and then displayed high solubility in common deuterated NMR solvents (*e.g.*, DMSO, chloroform, *etc.*).

Ralph *et al.* successfully carried out ¹H-¹³C heteronuclear single quantum coherence (HSQC) spectroscopy (*i.e.*, 2D



Scheme 1 Synthesis of perdeuterated pyridinium chloride (top) and [Emim]OAc-d₁₄ (bottom).

Table 2 Summary of the dissolution methods and their advantages and disadvantages

Solvent system	Advantages	Disadvantages	Ref.
1. Derivatizing—biomass acetylation:			
Derivatization in DMSO/TBAF or DMSO/NMI	Simple and cheap solvent system that facilitates derivatization of the entire biomass for NMR analysis in common deuterated solvents	Requires significant milling requirement and harsh conditions for derivatization	33, 34, 58 and 59
Derivatization in ILs	Higher solubilities at less harsh sample preparation and derivatization conditions	ILs are costly and may have reactive characteristics that alter biomass structure	60 and 61
2. Non-derivatizing:			
DMSO-d ₆	Cheap and simple sample preparation	Requires significant milling, has high sample viscosity, and does not solubilize crystalline cellulose	15, 58 and 63
DMSO-d ₆ /pyridine-d ₅ or DMSO-d ₆ /NMI-d ₆	Enhanced swelling compared to pure DMSO with a lower sample viscosity, better biomass solubilization, and greater polymer chain mobility		62 and 64–66
ILs	Higher solubilities with less harsh sample preparation, reduced size reduction requirements, and solubilizes crystalline cellulose	Expensive and may have reactive characteristics that alter biomass structure	22 and 25
Organic electrolyte	Reduced sample viscosity and reduced use of ILs, and thus, less costly than using pure ILs. Solubilizes crystalline cellulose		27–29, 32, 75 and 79–83

correlation NMR) on the acetylated biomass, providing chemical information about lignin and polysaccharides present.³³ The spectral chemical shift assignments were made based on years of research and extensive efforts³⁵ mainly by Ralph *et al.*;^{36–49} Lundquist *et al.*;^{50–55} and Gellerstedt *et al.*;⁵⁶ conducting a variety of 1D and both homo- and hetero-nuclear 2D NMR experiments such as correlation spectroscopy (COSY); total correlation spectroscopy (TOCSY); heteronuclear multiple bond correlation (HMBC); and HSQC experiments on isolated lignin, derivatized lignin, synthetic dehydrogenation polymer (DHP) lignin, monosaccharides, oligosaccharides, and a library of other synthesized model compounds.⁵⁷ This battery of research enabled the analysis of acetylated milled pine by ¹H–¹³C HSQC NMR, readily characterizing structures seen in Fig. 2 such as β-O-4 aryl ether (A), phenylcoumaran (B), resinol (C), dibenzodioxocin (D) aliphatic side-chain monolignol linkages (also referred to as lignin inter-unit linkages or lignin inter-monomer linkages) and confirmed that guaiacyl (G) rich lignin dominates the pine cell wall structure.

Similarly, later work by Ralph *et al.* characterized acetylated milled aspen by whole plant cell wall ¹H–¹³C HSQC NMR. The NMR results infer that the lignin within the milled aspen is syringyl (S) rich with β-O-4 ether and resinol linkages as the major lignin inter-unit linkages.⁵⁸ In this case however, phenylcoumaran and cinnamyl alcohol were also detected as minor units. Though the technique as described above produces high resolution spectra with good chemical specificity, disadvantages include: (1) inclusion of separate dissolution/swelling and derivatization steps which requires additional time and effort and causes naturally occurring acetates in hemicelluloses and lignin to be obscured, (2) inclusion of ball-milling which potentially causes changes in cell wall chemistry, and (3) lack of spectral quality in samples with a high cellulose content.

Cellulase treatments, following the ball-milling sample preparation step, can be used to facilitate the partial removal of the carbohydrate component, thereby (1) increasing the relative concentration of lignin, (2) eliminating overlapping polysaccharide resonances, and (3) facilitating the more quantitative estimation of lignin sub-units and functionality. However, cellulases can bind strongly to various cell wall components, may not be easily removed by washing with aqueous buffer, and could potentially serve as contamination, obscuring plant cell wall NMR resonances. In this case, a subsequent treatment with a protease has been employed and nitrogen content testing used to evaluate protein contamination.

Since those early efforts by Ralph and his co-workers, several other researchers have successfully applied whole plant cell wall solution state NMR facilitated by a derivatizing dissolution strategy. A more recent example of whole plant cell wall solution NMR analysis facilitated through derivatization investigates the structural heterogeneity of lignin polymers as a result of different sequential dissolution (*i.e.*, DMSO/NMI), regeneration, and enzymatic hydrolysis treatments of bamboo.⁵⁹ The lignin polymers in bamboo were demonstrated to contain (1) H-, G-, and S-type monolignols, (2) partially acetylated γ-carbon side-chains with *p*-coumarate and acetate groups, and (3) major inter-unit linkages of β-O-4, β-β, and β-5 bonds. Most importantly, various linkages believed to be covalent LCCs were observed (*i.e.*, benzyl ether and phenyl glycoside linkages). Ultimately, they determined the sequence of treatments greatly affect the amount of S and condensed units detected. Since then, the authors have published a review,¹³ superbly outlining advances in 2D solution state NMR techniques for structural analysis of lignin.

Qu *et al.* applied whole plant cell wall solution state NMR on acetylated biomass for the characterization of a softwood

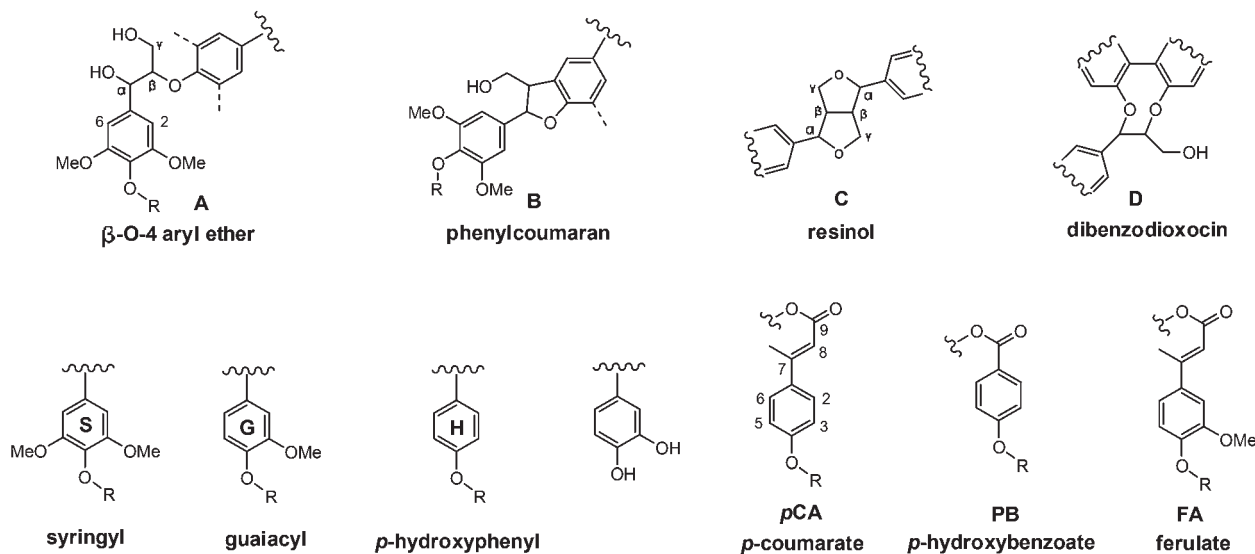


Fig. 2 Common lignin units identified in biomass.

(*Abies sachalinensis*) using an IL solvent system for the derivatization method rather than the aforementioned DMSO/NMI or DMSO/TBAF combinations. The ^1H - ^{13}C HSQC NMR spectra of the acetylated biomass were well-resolved and used to characterize cell wall polysaccharides and major lignin inter-unit linkages.⁶⁰ In this study, noteworthy efforts were taken for the characterization of polysaccharides such as arabinoxylan, galactomannan, and glucomannan, where chemical shift assignments were accomplished *via* comparison with commercial hemicelluloses. Generalizing this method, the same group studied the structural characterization of a hardwood and a bamboo.⁶¹ Hachiku bamboo and Japanese white birch were selected for dissolution in 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), an IL, facilitating first acetylation by treatment with pyridine/acetic anhydride and then ^1H - ^{13}C HSQC NMR analysis in a common NMR solvent, DMSO- d_6 . The HSQC analysis identified major sugar units β -D-glucopyranoside (β -D-Glcp) units in cellulose and β -D-xylopyranoside (β -D-Xylp) units in xylan and several other minor sugar units namely, α -L-arabinofuranose, β -D-mannopyranose, and α -D-galactopyranose. In addition to that, the majority of lignin structures such as β -O-4 aryl ether, phenylcoumaran, resinol, spirodienone, cinnamylalcohol, and cinnamylaldehyde were readily characterized. Bamboo lignin is primarily composed of G- and S-type monolignols with small amounts of H, ferulate (FA), and coumarate (CA) units. In these studies, the derivatizing dissolution strategy employs an IL solvent rather than the DMSO/NMI or DMSO/TBAF systems used by Ralph *et al.* in earlier efforts. The application of ILs may allow for higher solubilities at less harsh sample preparation and derivatization conditions, and thus more minimal changes to the biomass structure. However, many ILs are costly and not well-suited to higher throughput derivatization and NMR experiments.

Whole plant cell wall solution state NMR: a non-derivatizing approach

In this section of the review, we will highlight research on the use of whole plant cell wall solution state NMR, focusing on the utilization of biomass non-derivatization methods to facilitate biomass solubility in NMR solvents (Table 2). The dissolving/swelling the whole plant cell wall material by derivatization for NMR analysis clearly yields better resolution and more structural information than solid state NMR analysis.¹⁵ However, the NMR analysis of non-derivatized biomass has several advantages such as (1) retaining native structures and linkages which could be obscured and altered during derivatization or isolation and (2) sample preparation methodology that is relatively simple and rapid. Since the early work involving biomass derivatization for whole plant cell wall NMR analysis by Ralph *et al.*, many researchers have investigated methods utilizing deuterated solvent systems for the non-destructive (or less-destructive) and one-pot swelling of biomass, as well as, subsequent NMR analysis to characterize and quantify lignin and hemicellulose sub-units present in intact biomass. In these cases, significant swelling of the cell wall material provides enough molecular mobility in the cell wall polymers to eliminate significant dipolar couplings, increase nuclear relaxation times, and produce spectral data similar to high resolution liquid NMR spectra of completely dissolved polymer systems.

Ralph *et al.* conducted one of the first studies that employed a solvent system that facilitated both solubility of biomass without derivatization and direct NMR analysis of biomass in that same "pot". Ralph and co-workers successfully synthesized deuterated *N*-methylimidazole- d_6 (NMI- d_6) using imidazole as a precursor. This solvent was effectively used for

the swelling and NMR analysis of ball milled plant cell material, in a solvent system with a 1:4 mixture of NMI- d_6 : DMSO- d_6 at room temperature.⁶² Avoiding the complicated synthesis of deuterated NMI, swelling highly ball milled biomass in more common deuterated organic solvents (*i.e.*, DMSO) was then considered as an alternative technique.⁵⁸ However, due to the highly crystalline nature of cellulose and complexity of cell wall construction, simply swelling biomass sample in DMSO- d_6 is comparatively more difficult and generates a highly viscous suspension. The advantage of this methodology are several fold, but primarily include simple and fast sample preparation conducted directly in the NMR tube itself. A typical preparation involves producing a 50 mg mL⁻¹ concentration of biomass gel from ball milled samples in DMSO- d_6 following sonication for 1–5 h in a 5 mm NMR tube.¹⁵ A detailed outline of the protocols used for solubilization strategies for plant material of varying composition (including both gelling without and with derivatization) intended for 2D NMR acquisition are provided in a Nature Protocol manuscript by Mansfield *et al.*⁶³

Research has shown that a system consisting of pyridine and DMSO is an improved solvent for whole plant cell wall 2D NMR analysis.⁶⁴ There are several advantages to this system such as (1) enhanced swelling, (2) lower viscosity, (3) better solubilization of biomass, and (4) greater polymer chain mobility (therefore longer T_2 relaxation), all enhancing spectral intensities and resolution. In addition, Ralph *et al.* suggest this preparation is easier to handle than DMSO- d_6 gel. Accordingly, they reported the whole plant cell wall ¹H-¹³C HSQC NMR analysis of ball-milled aspen, pine, and kenaf utilizing the DMSO- d_6 /pyridine- d_5 solvent system.⁶⁴ Lignin side-chain resonances related to major sub-unit linkages including β -O-4 aryl ether, phenylcoumaran, resinol, dibenzodioxocin, and cinnamyl alcohol end groups were characterized. In addition, resonances for S-, G-, and H-aromatic units along with *p*-coumaric acid (*p*-CA) and ferulic acid (FA) units were observed. Through the volume integration of the $S_{2,6}$ and G_2 correlations, the S-:G-ratio of aspen and kenaf were estimated at 2.0 and 4.6, respectively. The semi-quantitative analysis of lignin sub-units by whole plant cell wall NMR analysis represents intact and unaltered lignin, and unlike the various degradative methods, detects condensed structures as well. Major hemicelluloses were tentatively assigned based on the well-resolved anomeric correlations.

Ralph *et al.* later made full use of this methodology, carrying out a systematic model study with the amorphous cellulose and xylan from ball-milled cotton linters.⁶⁵ Despite the conformational complexity, all carbohydrate related peaks were assigned such as glycosidic α - and β -reducing end units. In addition, the xylan from the cotton linter was identified as a 4-O-methylglucuronoxylan (typically found in hardwoods).

A recent study showed that whole plant cell wall ¹H-¹³C HSQC NMR analysis in DMSO- d_6 :pyridine- d_5 successfully characterized catechyl lignin (C-lignin) in the seed coats of vanilla orchid and demonstrates the utility of whole cell wall solution NMR in understanding lignin biosynthesis.⁶⁶ The HSQC spectra confirmed that seed lignin is entirely composed

of C-lignin and that G- and S-type lignin, typically observed in angiosperm, were absent. The sub-unit linkage (aliphatic) region displayed predominantly benzodioxane linkages, resulting from β -O-4 coupling of monomer with catechyl units. In addition, trace amounts of phenyl coumaran and resinol were identified. This structural characterization was confirmed from the HSQC spectra of synthetic dehydrogenation polymer produced *via* horseradish peroxidase-catalyzed polymerization of caffeyl alcohol. In contrast to the above results, typical lignin isolated from angiosperm lignin is composed of G- and S-units with primarily β -O-4 aryl ether linkages along with modest amounts of phenylcoumaran and resinol linkages and trace amounts of dibenzodioxocin linkages.

Whole plant cell wall solution state NMR: methodology

In this section of the review, we detail technical issues related to sample preparation, 2D NMR correlation spectral acquisition, and 2D NMR correlation spectral processing. Sample preparation is perhaps the single most important step in obtaining good quality NMR spectra of whole plant cell biomass samples. A proper solution state whole plant cell wall NMR analysis sample preparation begins with procedures to remove small molecules and non-structural material from the plant cell wall prior to analysis to prevent spectral interference. This is typically done by extraction with solvents like toluene, benzene, dichloromethane, ethanol, methanol, and/or water to remove compounds such as waxes, fats, resins, photosterols, non-volatile hydrocarbons, low-molecular-weight carbohydrates, salts, *etc.*⁶⁷ Amylase treatments can be applied to biomass sample enriched with starch.⁶³ Occasionally, biomass samples have high concentrations of paramagnetic species, which can induce the fast relaxation of nuclei in close proximity, broadening line-widths and obscuring resonances. Solid-liquid extractions of these samples with low concentration aqueous solutions of disodium ethylenediaminetetraacetic acid followed by extensive distilled water washings can alleviate this issue, a step proven particularly useful on some pre-treated biomass samples.⁶⁸

The influence of the milling procedure and ultimate particle size as a result of size reduction is also important because of the high surface area requirement for the effective dissolution of biomass in DMSO, DMSO/pyridine, IL, or organic electrolyte solvent systems.²³ For example, Ralph *et al.* utilizes a planetary ball-mill to grind particles to an average size of <5 μ m in an effort to increase the ability of solvent to swell the biomass.^{58,63,64} The presence of water greatly reduces the solubility of biomass, in particular cellulose in ILs; therefore, the effective preparation of biomass for dissolution in ILs or organic electrolyte solvent systems requires extensive drying of the biomass, drying of the solvent system, and dissolution under an inert atmosphere. The possible advantages of IL or organic electrolyte solvent systems over swelling with DMSO or DMSO/pyridine include: (1) further reduced viscosity,

(2) increased and actual solubilization, (3) greater polymer chain mobility (therefore longer T_2 relaxation), and (4) reduced size reduction requirements. Although, the viscosity of biomass in IL or organic electrolyte solvent systems is lower than if swollen with DMSO or DMSO/pyridine, a solution of biomass in IL or organic electrolyte solvent systems can still be very viscous, particularly with high biomass loading or with biomass samples high in cellulose content. Ultimately, controlling the viscosity by establishing an optimum biomass concentration, solvent composition, and NMR acquisition temperature (that avoids biomass decomposition) is important. We have determined an 8–10 weight% of dried biomass in a PyCl-d₆/DMSO-d₆ (1 : 3) solvent system is the optimal PyCl-based preparation.

2D NMR correlation experiments can enhance spectral content and provide additional cell wall chemical information by helping to resolve complex and overlapping signals *via* dispersion of chemical shift information over two-dimensions, in a ^1H - ^{13}C HSQC experiment over a proton and carbon chemical shift dimension.⁶⁹ A wide range of NMR correlation experiments can be chosen, employing direct or inverse observation, however; ^1H - ^{13}C HSQC experiments are most commonly utilized. In traditional or direct observation NMR sequences, magnetization is transferred from protons to carbon for detection, but because carbon is a relatively insensitive nuclei the resulting spectra from these types of experiments are poor.⁶⁹ On the other hand, in an indirect detection NMR sequence magnetization is transferred from protons to carbon, and then back to protons through a double inept transfer for detection. Indirect detection along with inverse probes, whose radiofrequency coil design places the proton-related coil closest to the sample greatly increases spectral quality for an HSQC NMR experiment. It should be noted that direct observation with a broadband probe would have improved direct ^{13}C signal detection (*e.g.*, 1D ^{13}C or 2D heteronuclear correlation (HETCOR) experiments) with respect to indirect detection probe. An HSQC NMR experiment is an indirect sequence which filters ^1H - ^1H couplings, detects carbons through attached protons, and produces 2D spectra with sharp cross-peaks at correlating one-bond ^1H and ^{13}C chemical shifts. An inherent issue with whole plant cell wall solution NMR is the relatively short T_2 relaxation times of the biomass, which leads to significant signal attenuation during HSQC evolution periods and poorer signal-to-noise.

A variety of studies on whole plant cell wall solution NMR use a phase-sensitive gradient-edited 2D HSQC NMR sequence with sensitivity improvement *via* echo/anti-echo gradient selection.⁷⁰ Preservation of equivalent pathway (PEP) and adiabatic pulses are sometimes applied and denote sequences which perverse coherences through gradient selection. Ultimately, optimizing sample preparation conditions (*e.g.*, particle size, moisture content, heating time, biomass concentration in IL, *etc.*), NMR acquisition parameters (*e.g.*, recycle delay, number of scans, number of time domain points (TD) collected in the direct and indirect dimensions, pulse power/duration, delay duration, *etc.*), and NMR processing protocols (*e.g.*, zero filling, application of window functions, forward/backward

linear prediction, *etc.*) are critical to maximizing spectral resolution and signal-to-noise.⁶⁹

The resolving power of an HSQC NMR experiment greatly expands the usefulness of NMR to complex systems, however a clear shortcoming is related to the fact HSQC NMR spectral signatures are difficult to quantify. 2D HSQC NMR is not quantitative mainly due to deviations in ^1H - ^{13}C coupling constants, off resonance effects, ^1H T_1 relaxation effects, ^1H and ^{13}C T_2 relaxation effects, and proton homonuclear coupling effects.⁷¹ A properly selected recycle delay ($5 \times T_1$) can remove almost all ^1H T_1 relaxation related effects. The effect of deviations in ^1H - ^{13}C coupling constants can be suppressed by modulating the polarization transfer delays of HSQC, this was demonstrated by Heikkinen *et al.* on the HSQC of milled wood lignin isolated from spruce.⁷² Similarly, Sette *et al.* have successfully applied a different quick quantitative HSQC (QQ-HSQC) technique, which also suppress the ^1H - ^{13}C coupling constant dependence of individual signals in an HSQC spectrum, to an array of milled softwood, hardwood, and technical lignins.⁷³ Zhang *et al.* examined the influence of T_2 relaxations on HSQC spectra, determining measured T_2 values on polymeric samples, such as the plant cell wall, could not be used to eliminate intensity biasing due to relaxations effects.⁷¹ They, on the other hand, suggested that quantification of 2D HSQC NMR spectra on polymer systems can be achieved *via* a properly chosen internal standard reference compound of low molecular weight while also selecting a secondary internal standard reference signal which is part of the same polymer structure being analyzed with similar structural features. This approach was shown to effectively remove errors from resonance offsets, homonuclear couplings, and deviations in heteronuclear coupling constant and transverse relaxations.

Ralph *et al.* has demonstrated that an inverse cryoprobe-equipped NMR instrument produces acceptable 2D NMR spectrum of the whole cell wall in under an hour for the purposes of chemometrics, S/G ratio estimation, *etc.*⁶⁴ In this study, optimal spectra are obtained in 6 h (16 scans per slice, 480 slices, and 0.75 s recycle delay). Samuel *et al.* have shown acquisition of high quality whole plant cell wall ^1H - ^{13}C HSQC NMR analysis in PyCl-d₆:DMSO-d₆ may require total experiment times of ~24 h (256 scans per slice, 204 slices and 1.5 s recycle delay), conducted on a traditional indirect probe and at 55 °C.^{74,75}

In the case of pyridinium chloride and many other ILs, their constituent cations and anions may have reactive characteristics, such as the acidity of a pyridinium cation or nucleophilicity of a chloride anion. As a result, IL dissolution of biomass may also be aided by “undesired” reactive mechanisms that fundamentally alter the biomass being analyzed. One might imagine pyridinium chloride at elevated temperatures would lead to significant cleavage of labile cell wall linkages. However, Fig. 3, which displays the ^1H - ^{13}C HSQC NMR spectra of ^{13}C enriched corn stover in a PyCl-d₆:DMSO-d₆ system (4 scans per slice and 128 slices for a total experiment time of 30 min) before and after heating the dissolved biomass sample at 60 °C for 24 h, clearly demonstrates minimal

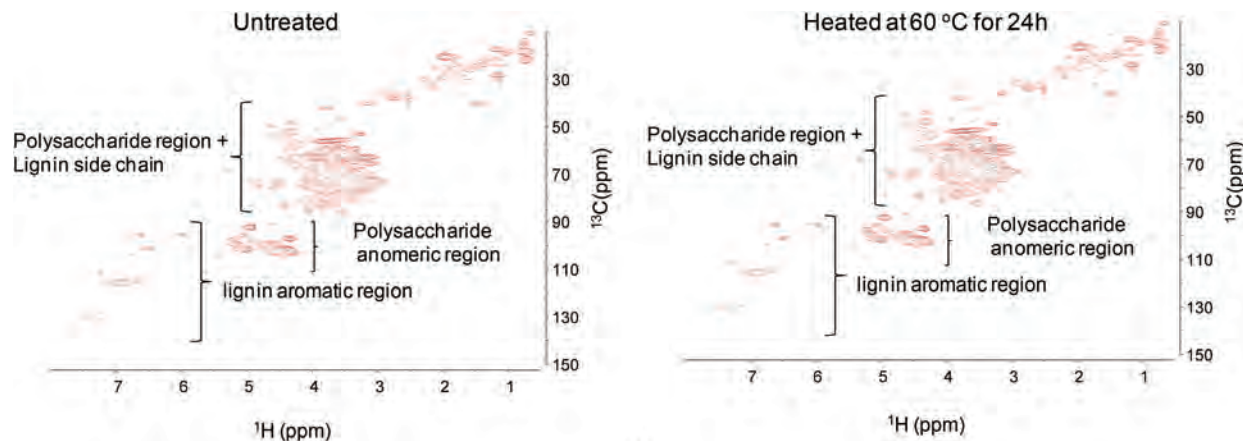


Fig. 3 ^1H - ^{13}C 2D HSQC NMR spectra of ^{13}C -enriched corn stover in PyCl-d_6 : DMSO-d_6 solvent system.

Table 3 The typical chemical shift assignments of ^1H - ^{13}C HSQC NMR spectra of biomass in PyCl-d_6 : DMSO-d_6 system⁶⁴

δ_c/δ_H (ppm)	Assignment
55.7/3.8	CH_3 in methoxyl group
60.6/3.6	C_6 polysaccharide + A_γ
71.0/4.8	$\text{C}_\alpha/\text{H}_\alpha$ in β -O-4 linkage (A)
74.0/4.5	C_2/H_2 in 2-OAc- β -D-Xylp
75.0/4.8	C_3/H_3 in 3-OAc- β -D-Xylp
81.8/3.5	C_4 polysaccharides
86.0/4.4	$\text{C}_\beta/\text{H}_\beta$ in β -O-4 linkage (A)
87.5/5.5	$\text{C}_\alpha/\text{H}_\alpha$ in phenylcoumaran substructure (B)
83.4/4.95	$\text{C}_\alpha/\text{H}_\alpha$ in dibenzodioxocin substructure (D)
102.4/4.4	Internal 1-4 linked β -D-glucopyranoside (β -D-Glucop ¹)
102.5/4.5	Internal 1-4 linked β -D-xylopyranoside (β -D-Xylp ¹)
105.3/6.6	$\text{C}_{2,6}/\text{H}_{2,6}$ in etherified syringyl units (S)
111.4/7.0	C_2/H_2 in guaiacyl units (G)
115/6.7	C_5/H_5 in guaiacyl units (G)
119.5/6.9	C_6/H_6 in guaiacyl units (G)
128.0/7.2	$\text{C}_{2,6}/\text{H}_{2,6}$ in <i>p</i> -hydroxyphenyl units (H)
145.0/7.5	<i>p</i> -Coumaric (<i>p</i> -CA) and ferulic (FA) acids

degradation occurs in most lignin and hemicellulose related units during a typical sample preparation and acquisition period at elevated temperatures. Table 3 presents ^1H - ^{13}C HSQC chemical shifts for lignin and polysaccharide related moieties in a PyCl-d_6 : DMSO-d_6 system.

Research applications of ionic liquids and organic electrolyte solvents in direct NMR of analysis of biomass

A variety of lignocellulosic biomass species have been proposed as possible bioenergy crops in future biorefineries; however, to unlock those crops full potential, a detailed understanding of cell wall composition and chemical structure must be obtained. Solution state whole plant cell wall 2D NMR analysis in IL-based solvents can profile biomass chemistry

without extensive isolation, in a rapid manner, and on a milligram scale sample. As a result, this powerful technique has a wide-range of applications in biomass research.

Applications: characterization of biomass

A 1-butyl-3-methylimidazolium chloride ($[\text{C}_4\text{mim}]\text{Cl}$)/ DMSO-d_6 solution at 90 °C was used to dissolve and obtain high-resolution 1D ^{13}C NMR spectra of cellulose and cellulose oligomers. Accurate ^{13}C chemical shifts were obtained for cellobiose, celotetraose, and cellohexaose, and spectra of dissolved cellulose revealed that it has a disordered structure in ILs, similar to that observed in water.⁷⁶ Solvent suppressed 1D ^1H NMR spectroscopy of cellulose in a non-deuterated ILs have been performed to confirm complete dissolution of cellulose and hydrogen bonding patterns between cellulose and ILs.⁷⁷ Kuroda *et al.* used similar No-D NMR and solvent suppression techniques to confirm the applicable of three representative non-deuterated ILs for the extraction cellulose and xylan from wheat bran.⁷⁸ The success of the later two studies suggest various solvent suppression techniques could be applied to both 1D ^1H and ^{13}C NMR (see ESI Table 1† for typical chemical shift assignments of 1D ^{13}C NMR spectra of biomass in DMSO-d_6) of whole cell biomass dissolved/swollen in ILs to avoid the expense and effort associated with the use of deuterated ILs, as well as overcome the signal “swamping” effect of solvents.

Jiang *et al.* initially reported a PyCl-d_6 : DMSO-d_6 solvent as an effective system for the NMR characterization of biomass.²⁷⁻²⁹ A (1:2) mixture of PyCl-d_6 : DMSO-d_6 was capable of dissolving up to 80 mg of Wiley-milled (~80–20 mesh) and ball-milled poplar and switchgrass samples by stirring at 60 °C for 6 h in 1 mL of solvent. ^1H and ^{13}C NMR spectra of both the Wiley milled and ball-milled samples provided comparable spectra, generating all relevant and representative signals from both lignin and polysacchar-

ides, subsequently demonstrating the exceptional solubility of biomass in the solvent system. Fig. 4 illustrates a representative 2D ^{13}C - ^1H HSQC NMR spectrum of ball-milled switchgrass in PyCl-d_6 : DMSO-d_6 with many of the assignments based on chemical shifts in Table 3. The major lignin inter-unit linkages identified were β -O-4 aryl ether (A), phenylcoumaran (B), and resinol (C) linkages. Lignin aromatic units S, G, H, *p*-CA, and FA were readily assigned as well.

Foston *et al.* applied this type of IL-based solvent to the whole cell wall ^1H - ^{13}C HSQC NMR analysis as a complementary technique in a comprehensive study probing the phenotypic and biochemical factors leading to the lower recalcitrance observed in tension wood (TW) versus normal wood (NW) and opposite wood (OW) in *Populus tremula* \times *alba* (PTA).⁷⁹ Solution NMR was able to determine that the observed ratio of 2-acetylated xylopyranoside versus 3-acetylated xylopyranoside was 1 : 1 in NW and that this observed ratio increased in TW and OW samples. With regards to lignin in NW, β -O-4 aryl ether linkages were seen as the major aliphatic side chain sub-units, detecting minor amounts of resinol and phenylcoumaran sub-units. Typical lignin aromatic units, S, G, and *p*-hydroxybenzoyl (PB) units were also identified in NW. Based on the relative intensities of the corresponding peaks in TW sample, NMR experiments indicated a four-fold increase in PB lignin with respect to OW and NW, while observing a corresponding decrease in G-units.

Cheng *et al.* reported that DMSO-d_6 /[Emim]OAc- d_{14} was able to dissolve lignocellulosic material (*Miscanthus* \times *giganteus*) completely and gave high-resolution HSQC spectra.³² They made comparisons of spectra taken of the same biomass prepared in DMSO-d_6 , DMSO-d_6 /TBAF, DMSO-d_6 /TBAF, DMSO-d_6 /PyCl- d_6 solvent systems, observing a broad signal at 100.1/5.09 ppm in samples prepared in DMSO-d_6 /[Emim]OAc- d_{14} that was absent in other solvent systems. Moreover, extrapolated time-zero ^1H - ^{13}C HSQC NMR was applied on the sample prepared in DMSO-d_6 /[Emim]OAc- d_{14} and enabled quantitative analysis of structural traits. The results were consistent with xylose and

glucose quantification made from gas chromatography mass spectrometry (GC-MS) cell wall neutral sugar analysis.

Applications: estimation of changes occurring during pretreatment

One of the most useful applications of IL-based solvents to whole plant cell wall NMR characterization has been estimating structural changes in lignin and hemicellulose during different stages of biomass processing. Samuel *et al.* conducted acid, steam, and lime pretreatment on *Populus trichocarpa* \times *deltoides* under increasing severity.⁷⁵ In an effort to understand the structural changes occurring, ^1H - ^{13}C HSQC NMR spectra of untreated and pretreated biomass was generated. This study demonstrated that steam pretreatment causes a decrease in hemicellulose related resonances, which was based on significant reductions in volume integrations of 2- and 3-acetyl xylopyranoside resonances. This was used to conclude that auto-catalyzed cleavage of glycosidic bonds in hemicellulose is appreciable during steam pretreatment. With respect to lignin, reduction or complete removal of lignin side chains (aliphatic) signatures and decrease in the intensity of lignin aromatic units confirmed that degradation/depolymerization also occurred. Lime pretreatments resulted in an almost complete removal of acetylated hemicelluloses along with a preferential removal of *p*-hydroxybenzoate (PB) units from lignin which was attributed to the cleavage of the ester linkages in the *p*-hydroxy benzoyl ester linkages. Finally, spectra generated from acid pretreated poplar samples displayed complete removal of hemicelluloses, severe reduction of lignin aliphatic resonances consistent with degradation of major lignin inter-unit linkages, and substantial removal of lignin aromatic units.

Li *et al.* applied multiple lignin characterization techniques including IL-based solvent whole plant cell wall NMR analysis to grasses with a diverse range of lignin structures in an effort to correlate the differences in lignin to the susceptibility to alkaline hydrogen peroxide pretreatment and subsequent enzymatic deconstruction.⁸⁰ NMR analysis concluded alkaline hydrogen peroxide pretreatment results in (1) a decrease in olefinic carbons originating from either *p*-hydroxycinnamates (*i.e.*, *p*CA and FA), (2) an apparent increase in S/G ratio, and (3) removal of 2-acetyl- β -D xylopyranose and 3-acetyl- β -D-xylopyranose units without disruption of β -D-xylopyranose units.

Applications: analysis of isotopic enrichment of biomass

As neutron scattering techniques to analyze biomass are being developed, the need to quantitatively estimate deuterium incorporation of deuterium enriched biomass has become of considerable interest. Foston *et al.* developed a methodology for the estimation of deuterium incorporation in deuterium enriched biomass on commercially available deuterated kale

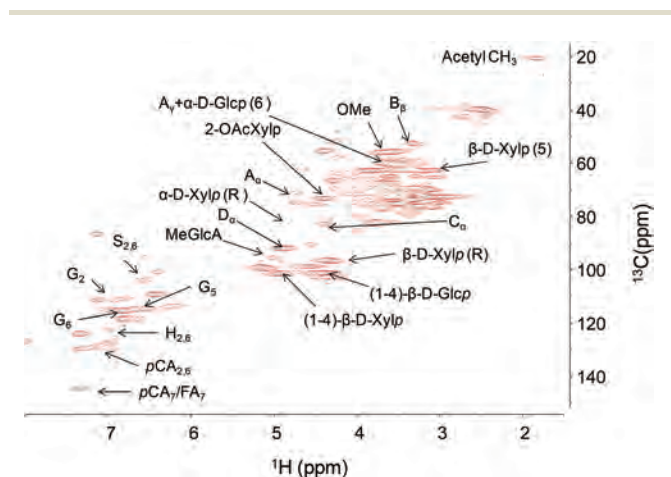


Fig. 4 Representative ^1H - ^{13}C HSQC 2D NMR spectra of a control switchgrass in PyCl-d_5 : DMSO-d_6 system.

by whole plant cell wall NMR analysis in a PyCl-d_6 : DMSO-d_6 system.⁸¹ This method included acquiring both ^1H and ^2H solution spectra utilizing a sample NMR tube with a coaxial insert containing a (1 : 1) mixture of trifluoroacetic acid-d (TFA-d) and TFA as an external standard. Based on (1) the integration of the TFA and biomass related resonances, (2) the known ratio of TFA-d to TFA, and (3) the concentration of biomass in the NMR sample, the percentage of deuterium was calculated. Bali *et al.* used a similar method to investigate deuterium incorporation into bacterial cellulose cultivated from 100% deuterated glycerol in a D_2O medium.⁸²

In a different study, Foston *et al.* highlight the application of the PyCl-d_6 : DMSO-d_6 solvent system coupled with ^{13}C enrichment of biomass in biofuel research.⁸³ A ~2 wt% solution of ^{13}C enriched corn stover in a (1 : 3) PyCl-d_6 : DMSO-d_6 system was shown to produce a well-resolved ^1H - ^{13}C HSQC NMR spectrum with a ~29 min acquisition time, effectively characterizing various lignin sub-units. In contrast naturally abundant corn stover, in a ~10 wt% solution, required ~19 h of acquisition time, only then generating comparable spectra. The authors then suggested ^{13}C enriched plants combined with whole plant cell wall solution NMR analysis was a viable route for the high-throughput characterization of biomass.

Limitations of direct NMR of analysis of biomass

This review included several examples of how solution state whole plant cell wall NMR analysis of biomass can be used to advance biomass research efforts. However, direct analysis of biomass using whole plant cell wall NMR, specifically based on 2D ^1H - ^{13}C HSQC NMR acquisition and biomass dissolution in IL and organic electrolyte solvents, has several limitations. First, sample preparation requires milling. Even with measures designed to reduce the amount of size reduction required, these mechanical methods of NMR sample preparation are known to alter certain cell wall features of interest. IL and organic electrolyte solvents with high biomass loadings can be very viscous, as a result, optimal sample dissolution and rheology therefore requires heating which in the presence of ILs (that have reactive character) will degrade certain cell wall features of interest. Whole plant cell wall NMR includes the cellulosic fraction which is chemically uninteresting and essentially serves to obscure and dilute key lignin and hemicellulose features of interest. This fact requires increased biomass loading in the NMR sample, having negative consequences with respect to sample preparation and spectral appearance. Typical 2D ^1H - ^{13}C HSQC NMR is not quantitative and has inherent resolution limitations which both seriously reduce the usefulness of the spectral data. The ^{13}C and ^1H chemical shift assignment of plant cell wall related NMR resonances is a non-trivial task which requires synthesis of complex and difficult to generate model compounds. As a result, only a few laboratories have the capability to identify and assign new or unassigned NMR resonances that, for example, that might

result from plant cell genetic modifications or chemical transformations. The spin system that defines the plant cell wall is rather diffuse and have small peak heights. Thus, without isotopic labeling, NMR acquisition times are long and small changes in cell wall structure are undetectable.

The future of direct NMR of analysis of biomass

The research presented in this review focuses primarily on the use of solution state 2D ^1H - ^{13}C HSQC NMR analysis on whole plant cell wall material. However, the successful application of other multinuclear solution state NMR experiments, especially ones probing long-range correlation, provide great promise in helping to resolve the structural components within intact biomass, specifically in LCCs. The direct NMR analysis and identification of LCCs is problematic because LCCs (1) are fairly liable and thus suitable to most of the mechanical and chemical methods of NMR sample preparation, (2) chemical shift assignments are only based on model compound that must first be proposed (and synthesized) to be evaluated, and (3) only represent a very small fraction of the complete spin-system defining biomass. In the future, direct NMR analysis and identification of LCCs will require improved (1) sample preparation methods that further avoid most common mechanical and chemical sample preparation methods and (2) NMR methods that further deconvolute overlapping spectral signatures and that are edited to contain resonances that result only from through-bond magnetization transfer from lignin to carbohydrate monomers.

Future prospects: resolving complex spectra

3D NMR spectroscopy such as heteronuclear single quantum coherence-total correlation spectroscopy (HSQC-TOSCY) or heteronuclear single quantum coherence-diffusion ordered spectroscopy (HSQC-DOSY) can be used to further deconvolute overlapping spectral signatures based on not only chemical but also physical properties. For example, Kikuchi *et al.* reported to use various pulse programs including HCCH-COSY and HSQC-NOESY for the structural characterization of commercially available ^{13}C labeled lignocelluloses, successfully characterizing xylan and xyloglucan structures.⁸⁴ The new method provided greater insight into fine structures of lignin by providing a high resolution to the aromatic signals of the β -aryl ether and resinol moieties, as well as the diastereomeric signals of the β -aryl ether. Another possible route to reduce spectral complexity and further separate overlapping spectral signatures involves employing spectral subtraction routines to eliminate cellulose only signatures. This spectral subtraction method will require determining the proportion of spectral intensity due to cellulose, a non-trivial activity because almost all cellulose resonances overlap with either a hemicellulose

and lignin resonance and cellulose related intensity in a HSQC spectrum will not be simply related to its concentration but a variety of other NMR acquisition/processing factors. Recent applications of high-resolution magic angle spinning (HR-MAS) for gel samples suggest a range of deuterium locked 2D NMR experiments on whole cell wall samples in ionic liquids can be conducted with the advantage of significantly reduced dipolar couplings and increased relaxation times, and thus increased spectral resolution and ability to detect longer-range correlations or previously unobserved correlations. HR-MAS NMR may additionally offer a route to avoid the milling requirement of whole cell wall NMR in solution spectrometers.

Future prospects: application to phosphorus NMR

King *et al.* has demonstrated the use of imidazolium chloride-based ionic liquids to dissolve lignin and cellulose samples for *in situ* phosphorylations, determining solubility and terminal chain phenolic content by ^{31}P NMR.^{85,86} A subsequent study was able to dissolve pulverized wood in [Amim]Cl, directly phosphorylate its functional groups, and quantify its hydroxyl contents by quantitative ^{31}P NMR.⁸⁷ Similarly, *in situ* phosphorylation of corn stover, *Eucalyptus Globulus* thermomechanical pulp, and *Eucalyptus* kraft pulp following ball-milling and cellulase treatment were analyzed in ionic liquids for quantitative hydroxyl content characterization.⁸⁸

Future prospects: high-throughput screening

Ralph *et al.* has shown how quickly spectra can be acquired on whole plant cell wall material swollen in DMSO- d_6 /pyridine- d_5 with an inverse cryoprobe-equipped NMR instrument.⁶⁴ A later study demonstrated the feasibility of using solution state ^1H - ^{13}C NMR analysis on ^{13}C enriched biomass as an approach for the high-throughput screening of cell wall chemistry for (1) genetic engineering, (2) plant development, and/or (3) biomass deconstruction/conversion studies.⁸³ Aiming to solve similar analysis bottlenecks, Ralph *et al.* assembled a high-throughput software platform for plant cell wall profiling that uses spectral deconvolution by Fast Maximum Likelihood Reconstruction (FMLR). The reconstructions can provide rapid and reproducible fingerprinting of numerous polysaccharide and lignin components in whole, unfractionated cell wall material.⁸⁹

Conclusion

Such future developments can provide tools to directly probe structures and conformations in intact and unaltered biomass and are critical to the advancement of biofuel research,

improving bioprocessing methodology, and bioengineering efforts.

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