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COMMUNICATION

Deuterium incorporation in biomass cell wall components by NMR analysis†

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A commercially available deuterated kale sample was analyzed for deuterium incorporation by ionic liquid solution ^{2}H and ^{1}H nuclear magnetic resonance (NMR). This protocol was found to effectively measure the percent deuterium incorporation at 33%, comparable to the 31% value determined by combustion. The solution NMR technique also suggested by a qualitative analysis that deuterium is preferentially incorporated into the carbohydrate components of the kale sample.

Introduction

In an effort to probe biomass recalcitrance and mechanisms of enzymatic deconstruction, the structure of native and pretreated lignocellulosics has been characterized before, during, and after thermochemical and enzymatic treatments by a variety of analytical techniques. Neutron scattering techniques have recently been proposed for the investigation of the properties of lignocellulosic materials.[1,2] The availability of deuterated materials, in which the non-exchangeable hydrogen atoms are replaced with deuterium, is crucial for obtaining structural information using neutron scattering techniques because deuterium and hydrogen interact very differently with neutrons.[3–6] However, in order to effectively use neutron scattering to probe biomass recalcitrance and mechanisms of enzymatic deconstruction of deuterated lignocellulosic materials, it is necessary to develop approaches to quantify the degree of deuterium substitution in the complete and individual components of the cell wall.

In many ways, isotopic enrichment of plant material is much simpler than in synthetic polymer systems because plants incorporate labeled material from their environment *via* photosynthesis. As a result, deuterium incorporation into plants often is achieved *via* deuterium enriched hydroponic cultures.[7] However, there are many technical issues associated with deuterium enrichment of plants (such as altered plant metabolism and growth), particularly for the high levels of labeling needed for neutron scattering and contrast matching techniques.[8–10] Hence, it is fairly important to have tools which can quickly and accurately assess deuterium incorporation on micro-quantities of sample resulting from a variety of modified germination and growth conditions.

A fairly relevant study utilizing NMR was conducted tracing stable ^{2}H isotope levels of tree rings in an effort to reconstruct climate history. The analysis of deuterium incorporation into cellulose was accomplished by isolating the cellulose and hydrolyzing it to glucose which gave a highly resolved, quantifiable deuterium NMR spectrum.[11] However, this technique cannot be readily employed on small samples sizes and involves intensive chemical isolation techniques. Typically, % deuterium incorporation is determined by soft-ionization mass spectroscopy techniques for simple polymeric compounds or for small molecules with well defined structures. Although, in complex polymer systems such as those that exist in biomass the traditional methods rely on combustion/pyrolysis and analysis of evolved water vapour/hydrogen by mass spectrometry,[12] infrared spectroscopy[13] or reduction of water to hydrogen followed by fractionation.[14]

In an attempt to determine the percent deuterium within deuterated biomass prepared specifically for neutron scattering studies, while also determining whether deuterium incorporation occurs preferentially within the various biomass components, NMR methods were examined. The most reliable method involves whole cell dissolution in an ionic liquid for solution NMR analysis.[15,16]

Experimental

Partially deuterated, hydroponically grown kale (*Brassica oleracea*) with an analyzed deuterium content of 31%, determined by combustion, was obtained from Oak Ridge Research Reagents, Oak Ridge, TN. A kale sample with natural isotopic abundance grown locally in Oak Ridge, TN was used as a control.

The biomass samples were air dried in a hood until moisture content of ~30% was obtained. The samples were then Soxhlet extracted with a benzene–ethanol (2 : 1, v/v) mixture for 24 cycles to remove extractives.

D-Glucose-1,2,3,4,5,6,6-D_7, (98 atom% D), dimethyl sulfoxide (DMSO)-d_6 (99.9 atom% D) and trifluoroacetic acid (TFA)-d (99.5 atom% D) were purchased from Cambridge Isotopes. D-Glucose (≥99.5%), DMSO (anhydrous, 99.9%), TFA (reagent grade, 99.0%) and pyridinium chloride (98%) were purchased from Sigma–Aldrich. Deuterated pyridinium chloride was synthesized *via* published literature procedures.[15]

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External standard preparation

Employing a glove-box under dry nitrogen pressure at RT, the external standard of TFA and TFA-*d* was added in a 1 : 1 ratio to a 2 mm stem coaxial insert to ensure no exchange of protons or deuterium at the acid site with the atmosphere (to maintain the H/D ratio for the external standard). The stem coaxial insert was then sealed before removing from the glove box. A mixture of TFA and TFA-*d* was chosen as a standard because the resonances occur outside the normal chemical shift range associated with plant cell wall biopolymers at ~11.5 ppm.

NMR sample preparation

The extracted biomass samples were lyophilized for 4 days to remove residual H_2O and D_2O. To facilitate sample dissolution, the dried biomass was ball-milled for 5 min at 25 Hz in a vibrational ball-mill and further dried overnight under vacuum at 40 °C.

NMR analysis

A ball-milled biomass sample (~10 mg) was dissolved in a 1 : 3 anhydrous pyridinium chloride/DMSO or pyridinium chloride-d_6/DMSO–d_6 mixture to form a ~2.5 wt. % solution. This was accomplished in a dry vial with a micro-stir bar at 60 °C in a silicone oil bath under positive N_2 pressure overnight to ensure complete dissolution and quantitative results. The biomass solution was then transferred to a 5 mm NMR tube *via* a glass pipette. The NMR tube was backfilled with N_2 gas and the 2 mm stem coaxial external standard insert was added before capping.

Solution NMR spectra were acquired on a Bruker Avance-400 spectrometer operated at 61.4 (^{2}H) or 400.1 (^{1}H) MHz. ^{2}H NMR spectra were measured at 55 °C with 32k data points, 4.1-kHz spectral width, 15-s recycle time and 1 K scans. ^{1}H NMR spectra were measured at 55 °C with 64k data points, 8.3-kHz spectral width, 15-s recycle delay and 16 scans.

To ensure the quantitative nature of the spectra, average spin–lattice times of D-kale dissolved in ionic liquid (^{1}H 0.8 s and ^{2}H 0.02 s) was measured using a saturation recovery experiment which in turn utilized a train of 64 pulses separated by 500 μs to fully excite all nuclei present. We chose a saturation recovery experiment because of the difficulty in finding exact 90 and 180 degree pulses in these highly heterogeneous systems.

Solid-state NMR analysis is detailed in the Supplemental Material.†

Results and discussion

The ability to label biomass with deuterium is a promising methodology to advance the structural characterization of native and processed lignocellulosics through the application of neutron scattering and diffraction techniques. A commercially available sample of deuterated kale was obtained and determined to contain ~31% deuterium at hydrogen sites *via* combustion/mass spectroscopy analysis of the evolved H_2O/D_2O mixture by the supplier of the kale. NMR performed using the proper recycle delay can determine the concentrations of nuclei, in this case either deuterons or protons, within a sample. Therefore, the recycle delay for all NMR acquisitions was set using a standard saturation recovery experiment.

In previous studies, we have reported the direct dissolution and NMR analysis of the plant cell walls by a bi-solvent system consisting of perdeuterated pyridinium molten salt and DMSO-d_6.[15,17] This solvent system can readily dissolve ball-milled and Wiley-milled biomass and was shown to facilitate direct ^{1}H, ^{13}C and 2D correlation NMR analysis. Accordingly, the non-deuterated version of this solvent system also provides an excellent route to probe the deuterium incorporation of deuterium enriched cell wall material. A clear advantage in utilizing solution NMR experiments is chemical shift resolution, which could potentially allow for site specific quantification of deuterium incorporation.

In an effort to independently confirm the combustion analysis results and develop a new NMR technique which can in turn provide molecular site specific information on deuterium incorporation, a solution-based NMR technique was employed. By dissolving the deuterated biomass in a deuterated ionic liquid system, then generating a separate NMR sample in a protonated solvent system and using a coaxial insert containing a deuterated trifluoroacetic acid (TFA-*d*)/protonated trifluoroacetic acid (TFA) mixture as an external standard, a quantitative analysis of percent deuteration is possible. TFA was found to be an appropriate choice for the external standard because its chemical shift is ~11.5 ppm well outside the range of chemical shifts for biomass components. The coaxial insert allows for the use of the same external standard in all samples and facilitates shimming in the protonated solvent system. Based on the integration of the TFA peaks, the known ratio of TFA-*d* to TFA, and recorded concentration of biomass in the ionic liquid solution a % deuterium ratio was readily calculated.

Fig. 1 displays the resulting ^{2}H and ^{1}H spectra of the D-labeled kale. The control non-labeled kale spectra are shown in Fig. 2. The control sample does not display any detectable deuterium signal while the enriched or D-labeled kale does, validating this general approach.

The peak intensity from the ^{1}H and ^{2}H spectra in the range of δ 0.0–7.9 ppm was measured and this data was normalized by the integration of the TFA or TFA-*d* peak and adjusted by the relative concentration of TFA-*d* to TFA. This normalized intensity is again normalized by the recorded concentration of biomass in solution. The

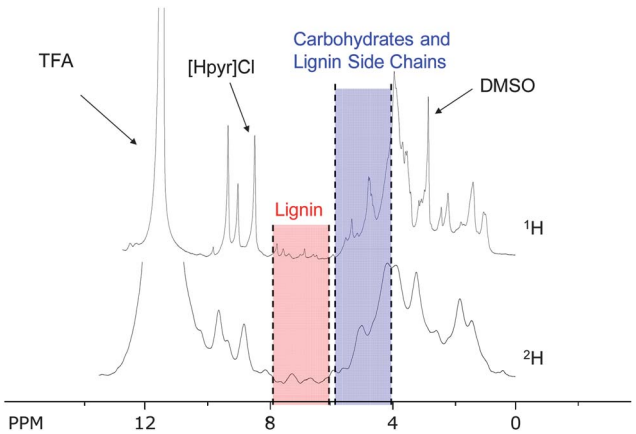

Fig. 1 ^{1}H and ^{2}H solution spectra of D-labeled kale whole cell dissolution in ~2 : 1 mixture of DMSO-d_6/pyridinium chloride-d_6 (^{1}H spectra) and DMSO/pyridinium chloride (^{2}H spectra) with 5 mm stem coaxial insert containing ~1 : 1 TFA-d/TFA mixture at 60 °C. The region in red represents protons/deuterium solely from lignin and the region in blue represents protons/deuterium solely from hemicellulose and cellulose.

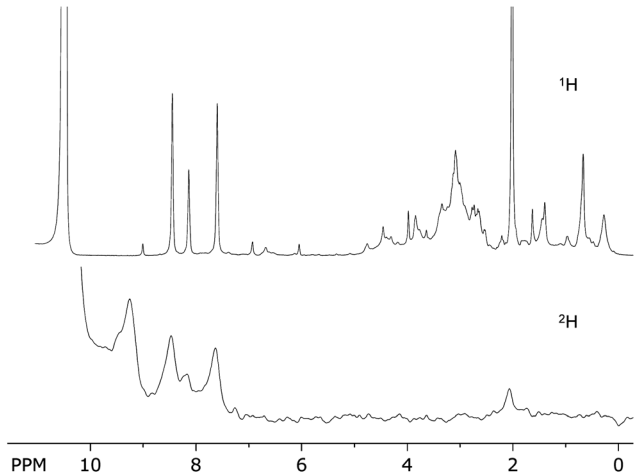

Fig. 2 ^{1}H and ^{2}H solution spectra of a control H-kale whole cell dissolution in ~2 : 1 mixture of DMSO-d_6/pyridinium chloride-d_6 (^{1}H spectra) and DMSO/pyridinium chloride (^{2}H spectra) with 5 mm stem coaxial insert containing ~1 : 1 TFA-d/TFA mixture at 60 °C.

resulting total ^{1}H and ^{2}H peak area was used to calculate the ^{2}H/^{1}H ratio. The ^{2}H/^{1}H ratio for the D-labeled kale was determined as described above and outlined in Supplementary Figure 1.† The results indicate the deuterium incorporation for the D-labeled kale is ~33% (the standard deviation of two trials is 3.3%), which is comparable to the 31% value acquired by combustion/mass spectroscopy analysis (combustion methods developed to analyze D/H ratio of non-exchangeable hydrogen in plant cellulose have been cited to display standard deviations between 1–4%[12]).

^{1}H and ^{2}H peaks attributed primarily to carbohydrates have chemical shift between δ 3.3–5.8 ppm. The chemical shift range of δ 4.1–5.8 ppm (δ_C, denotes the chemical shifts related to carbohydrates and is shown in blue in Fig. 1) is further dominated by proton or deuteron sites related to C_1 carbons on carbohydrates, though some signal intensity is due to aliphatic lignin side chain moieties. On the other hand, the ^{1}H and ^{2}H chemical shift range of δ 6.1–7.9 ppm (δ_L, denotes the chemical shifts related to aromatic lignin structures and is shown in red in Fig. 1) can be primarily attributed to aromatic lignin sites. Therefore, the relative incorporation of deuterium into the carbohydrates and lignin can be qualitatively estimated depending of the relative ratio of integration for these two regions. This can only be done qualitatively because resonances in the range of δ 4.1–5.8 ppm do not solely result from carbohydrates, and as deuterium *versus* proton incorporation may vary from cell wall biopolymer to biopolymer, it may also vary from individual chemical site to site with those biopolymers. Further complicating this type of analysis are the broad line-shapes associated with NMR of ionic liquid dissolution of biomass and overlapping nature of the spectra. Although the integration range identified for carbohydrates does not solely contain resonances from plant polysaccharides the ^{1}H integration ratio of δ_L/δ_C represents a characteristic measure of protons associated with lignin in comparison to the amount of protons associated with carbohydrates. If this ratio significantly changes for the ^{1}H and ^{2}H spectra, it will indicate a change in the relative proton/deuteron incorporation between lignin and carbohydrates. Integration of chemical shift regions for carbohydrates and lignin for the D-labeled kale sample suggest deuterium is preferentially incorporated into the carbohydrate components.

We also investigated a second NMR methodology to analyze deuterium incorporation specifically designed to require no sample preparation and be non-destructive. To accomplish this non-spinning consecutive 1D ^{1}H single-pulse and ^{2}H solid-echo NMR experiments were preformed on freeze-dried biomass samples. The ratio of the integrals resulting from the subsequent ^{1}H and ^{2}H spectra was then correlated to known molar ratios of protons to deuterons using a calibration curve generated by taking several calibration standards formed from mixtures of glucose/glucose-d_7 (See Supplemental Figure 2†).

As seen in Fig. 3, the ^{1}H and ^{2}H spectra of deuterated biomass appear as very complex line-shapes with several superimposed subspectra suggesting there exist some heterogeneous chain motion along with a non-isotropic orientation distribution of polymer chains. The ratio of the integral of the two peaks in Fig. 3 was used to determine the % deuterium incorporation based on the calibration curve in Fig. 3. The D-labeled kale displayed a deuterium incorporation of ~40% as determined by solid-state NMR. We believe significant differences in the ^{2}H solid echo times for deuterated crystalline glucose and biomass along with effects from differences in excitation power levels account for the over estimation. However, this solid-state method does seem suitable as a quick screen to determine optimal growth and germination conditions to achieve a desired deuterium incorpartion on a variety of plant species, particularly when sample small size and re-use are issues.

Conclusions

Kale was grown in medium containing deuterium oxide. The plant successfully incorporated deuterium into its major biopolymer constituents at levels near high enough for further neutron studies, which was confirmed by new NMR methodology developed as an alternative to combustion analysis. A solution NMR technique was shown to successfully quantify the percent deuterium within the deuterated kale samples at 33% deuterium incorporation.

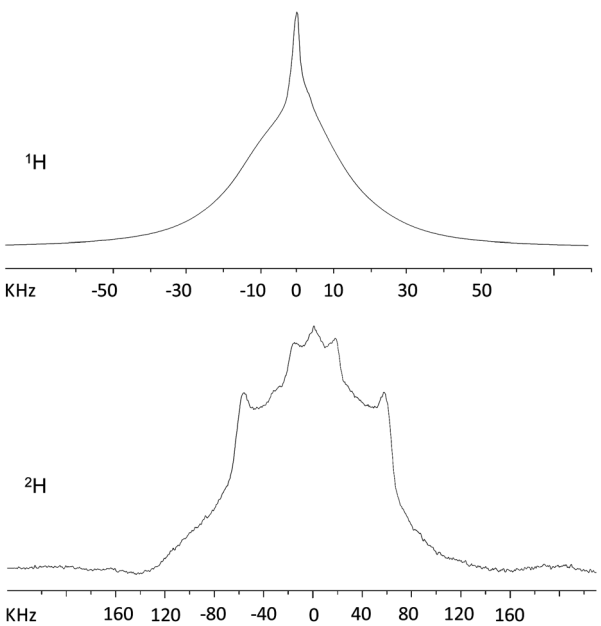

Fig. 3 ^{1}H and ^{2}H solid state spectra of D-labeled kale collected under non-spinning conditions in a 4-mm rotor.

Interestingly, the liquid NMR method which utilizes an ionic liquid solvent system, dissolving the complete biomass, provides a closer correlation between deuterium incorporation as determined by NMR with that of combustion analysis and suggests that the carbohydrate component more readily incorporates deuterium.

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