A WISE NMR Approach to Heterogeneous Biopolymer Mixtures: Dynamics and Domains in Wounded Potato Tissues

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ABSTRACT: Two-dimensional $^1H-^{13}C$ wide-line separation (WISE) NMR, a technique developed for the characterization of synthetic copolymers and blends in the solid state, has been used to study the dynamic behavior and domain structure of the biopolymer suberin deposited within the wound periderm of potato tubers. This blend consists of cell-wall polysaccharides and cross-linked aromatic−aliphatic polyesters that regulate water transport and protect the plant tissue from pathogenic attack. Though most of the suberin chemical moieties are rigid, about 20% of its chain-methylene groups exhibit surprising flexibility on the 50-kHz frequency scale. The resulting motional narrowing is propagated rapidly by spin diffusion to other residues within the suberin domain, but much more slowly to the spatially separated cell-wall region. Using a selective spin-diffusion experiment with a dipolar filter, the suberin domain size has been estimated as 50 nm and compared with prior results from electron microscopy. Finally, the resistance of suberin to water has been demonstrated in WISE experiments conducted on hydrated potato wound periderm tissue.

Introduction

Suberin is a biopolymer that forms at the boundaries of plant organs and develops within the cell walls of vegetables during the wound-healing process. On the basis of various chemical and spectroscopic investigations, suberized tissue is thought to consist of a cross-linked aromatic−aliphatic polyester associated with the carbohydrate cell-wall matrix to form a polymer blend.[1−5] Solid-state NMR spectra and spin-relaxation times have proven uniquely capable of revealing the molecular structure, dynamics, and supramolecular arrangements of intact suberized cell walls in potato wound periderm. For instance, the proton relaxation parameters ($T_{1\rho}$(H)) derived from variable-contact-time cross-polarization magic-angle-spinning (CPMAS) ^{13}C NMR[3,6] demonstrated efficient $^1H-^1H$ crosstalk among functional groups of each molecular constituent, but poorer spin communication between the spatially separated suberin and cell-wall polymeric species. Moreover, the observation of two distinct T_1(C) values for both aromatic and aliphatic carbons suggested that the suberin polyester is connected covalently to the cell wall at discrete sites. Finally, the values found for $T_{1\rho}$(C), $T_{1\rho}$(H), and T_1(C) indicated that the spectral density of the cell-wall carbohydrates shifts toward lower frequencies upon suberization, possibly leading to greater resiliency at the tissue surface. Overall, these structural properties are in accord with suberin's function as a protective barrier to the invasion of fungal and bacterial pathogens.

Many significant unanswered structural and functional questions about suberin are amenable to further investigation using solid-state NMR methods. First, a more precise dynamic profile of suberized wound periderm is desirable: it would be useful to observe separate NMR signals directly from the rigid and mobile populations of a given carbon type; and it would be valuable to rapidly screen the mobilities of particular functional groups in a suberin or polysaccharide domain without the complications of proton−proton crosstalk during the experiment. Second, an understanding of this biopolymer's protective function requires determinations of which chemical moieties in suberin lie at the interface with the cell-wall polysaccharides and the dimensions of the suberized domain. Finally, an examination of suberin−water interactions is of interest to determine which portion(s) of the suberin structure account for its protective capabilities and response to humid conditions.

A detailed site-specific dynamic profile may be built up for suberized cell walls using measurements of $T_{1\rho}$(C) and T_1(C), though a full analysis is technically and computationally demanding and provides no distance information. Spin communication between polymeric domains may be assessed from values of $T_{1\rho}$(H), but neither the specific interacting functional groups nor the domain sizes are available. Estimates of the suberin domain size have been made by electron microscopy,[7] though they require laborious sample staining and are limited to the examination of domain sizes larger than 10 nm.[8]

For these reasons, we undertook a study of dynamic behavior and spin diffusion in suberized potato tissue, using both two-dimensional wide-line-separation (2D WISE) NMR and a one-dimensional dipolar-filtered experiment. Originally developed by Spiess et al.,[9,10] WISE NMR uses a two-dimensional spectrum to display the ^{1}H spectral patterns associated with each of the well-resolved ^{13}C chemical shifts in the material of interest. Because ^{1}H line widths reflect the size of the homonuclear dipolar coupling, the more rigid segments will exhibit wider lines. Thus, a single WISE spectrum can correlate information on local mobility and chemical structure. Moreover, with the insertion of a mixing-time period to allow spin diffusion among different ^{1}H spin pools, WISE NMR can provide information on the 0.5−200-nm domains formed by designated components of a block copolymer or polymer blend.[11]

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WISE NMR, including both $^1H-^{13}C$[9,10,12,13] and $^1H-^{19}F$[14,15] correlations, has been applied successfully to synthetic copolymers of different block size in order to correlate their dynamics and structure. In the current work, WISE NMR results have been obtained for both dry and hydrated potato suberin mixtures. To our knowledge, this is the first application of the WISE technique to characterize the dynamic behavior of a natural polymer. In addition, the rate of ^1H spin diffusion from the aliphatic−aromatic polyester to the cell-wall polysaccharides has been used to estimate the domain size of the suberin layer in potato wound periderm. The latter results are compared with studies using electron microscopy.

Materials and Methods

Potato Tissue. According to previously published procedures,[3,16] fresh potatoes (*Solanum tuberosum L.* cv. Russet Burbank) were cleaned and peeled under sterile conditions. The tubers were cut into 5 × 20 × 30-mm^3 sections and aerated in a dark incubator at 25 °C for a period of 7 days. As described by Bernards et al.,[5] the brown layer of wound periderm on the surface of the potato disks was collected by blade peeling in order to streamline the removal of unsuberized cell walls. These latter materials were removed by standard cellulase (ICN Biochemical Inc.) and pectinase (Sigma Chemical) enzymatic treatments.[3,16] Subsequently, soluble lipids and wax were removed using a 2:1 (v/v) mixture of methylene chloride−methanol and exhaustive extraction with a Soxhlet apparatus (Fisher Scientific) for 48 h. Finally, the suberin was extracted with 1,4-dioxane/H_2O (96:4) at room temperature overnight[17] to remove any residual glucose. The resulting pieces of suberized periderm tissue were powdered at liquid-nitrogen temperature in a freezer mill. Dry suberin samples were obtained by thorough drying in a SpeedVac concentrator (Savant Instruments, Farmingdale, NY) and lamp heating for more than 2 h until there was no further weight loss. Typically, 1 kg of potatoes yielded approximately 2 g of dry suberin. The whole process took 16 days.

Hydrated suberin samples were prepared by saturation of dried suberin in deionized H_2O overnight. Excess water was removed by using gravity filtration and pressing on a filter paper, resulting in a sample that was damp in appearance.

NMR Spectroscopy. All solid-state NMR spectra were acquired on a Varian (Palo Alto, CA) UNITY*plus* spectrometer operating at a ^1H frequency of 300.001 MHz and a ^{13}C frequency of 75.445 MHz. The experiments were conducted with a Varian 7-mm MAS probe at a regulated temperature of 25 °C. Typically, 150 mg of suberin (dry weight) was used for the NMR experiments. The 90° pulses for ^1H and ^{13}C were both set to 5.5 μs. The rotor spinning speed was maintained at 3000 ± 2 Hz by a Varian speed-controlling device. All ^{13}C chemical shifts were referenced to tetramethylsilane via hexamethylbenzene as a secondary substitution reference.

^{13}C WISE experiments were performed using a pulse sequence illustrated in Figure 1a[9,11] with a cross-polarization contact time of 500 μs. Generally, the data matrix had a size of 64 points in the t_1 (^1H) dimension and 1568 data points in the t_2 (^{13}C) dimension. The t_1 and t_2 dimensions were zero-filled to 1024 and 2048 points, respectively, before two-dimensional Fourier transformation was carried out. The spectral widths were 100 and 26 kHz in the t_1 and t_2 dimensions, respectively. A recycle delay of 1.5 s was inserted between successive data acquisitions. For WISE spectra acquired with a spin-diffusion period, the mixing time was varied from 1 to 500 ms. A typical suberin WISE experiment lasted about 4 h.

Quantitation of the spin diffusion was carried out by first selecting the proton magnetization in mobile domains using a so-called "dipolar filter" pulse sequence with twelve 90° pulses as the filter and an interpulse delay of 14 μs to adjust the filter strength[10,12] (Figure 1b). One dipolar filter was sufficient to suppress wide lines ∼50 kHz in breadth, as judged by observation of a negligible residual signal at zero mixing time. Two phase-cycled 90° pulses equivalent to alternating 0° and 180° pulses were used to flip the magnetization to the (−z) axis on every other scan and eliminate the effects of T_1 relaxation.[10,11] The mixing times for these experiments were varied from 0.5 to 50 ms, and the cross polarization time was 1.0 ms. Signal intensities of the cell-wall peak at 72 ppm were plotted versus the square root of the mixing time using SigmaPlot software (Jandel Scientific, San Rafael, CA). Linear curve fitting was performed using the same software in order to measure the initial rate of spin diffusion from the suberin layer to the cell-wall polysaccharides.[11]

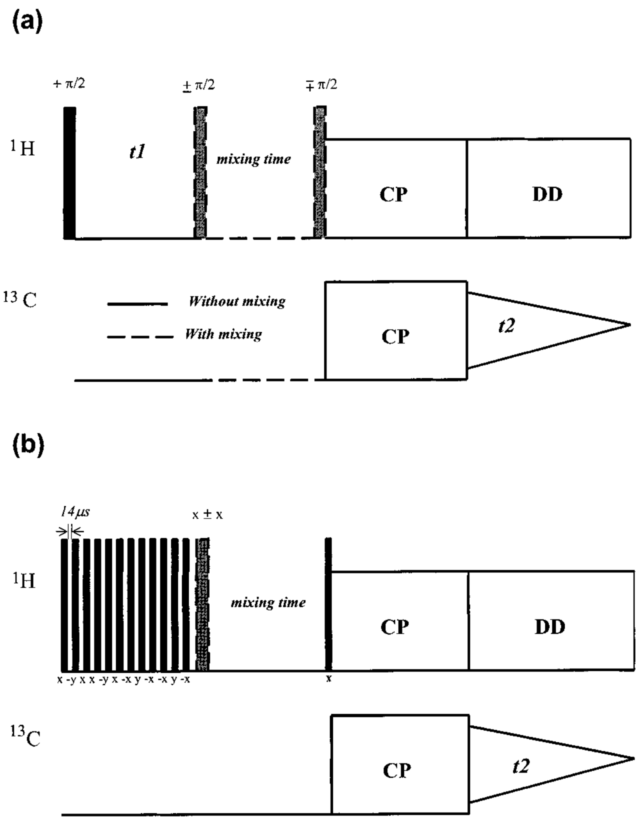

Figure 1. Pulse sequences used for (a) WISE NMR,[9] including an optional mixing time; (b) dipolar-filtered ^1H spin diffusion observed through ^{13}C signals.[10,12]

Results and Discussion

A. Dynamic Profile of Dry Suberin. As expected, the CPMAS ^{13}C NMR spectrum of the purified potato wound periderm is nearly identical to that reported previously (data not shown).[3] The buildup of cross polarization was complete after 0.5 ms for each protonated carbon resonance and after 1.0 ms for the carboxyl carbons. The corresponding 2D WISE results are summarized in Figure 2, which shows each chemically distinct ^{13}C resonance correlated with a ^1H powder pattern reflecting the mobility of that moiety. A more detailed presentation of the results in Figure 2 appears in Figure 3a, which shows ^1H spectral slices of differing line width for each of the carbon chemical environments. The ^{13}C resonances centered at 72 and 104 ppm, which arise from cell-wall carbohydrates, both have a large ^1H line width at half-height that is indicative of a highly rigid cell-wall domain. Similarly, the aromatic (130 ppm) and carboxyl (173 ppm) carbons of the polyester exhibit substantial rigidity on the 50-kHz time scale. In contrast, the aliphatic methylene carbon signals at 30 ppm are correlated with relatively narrow ^1H lines.

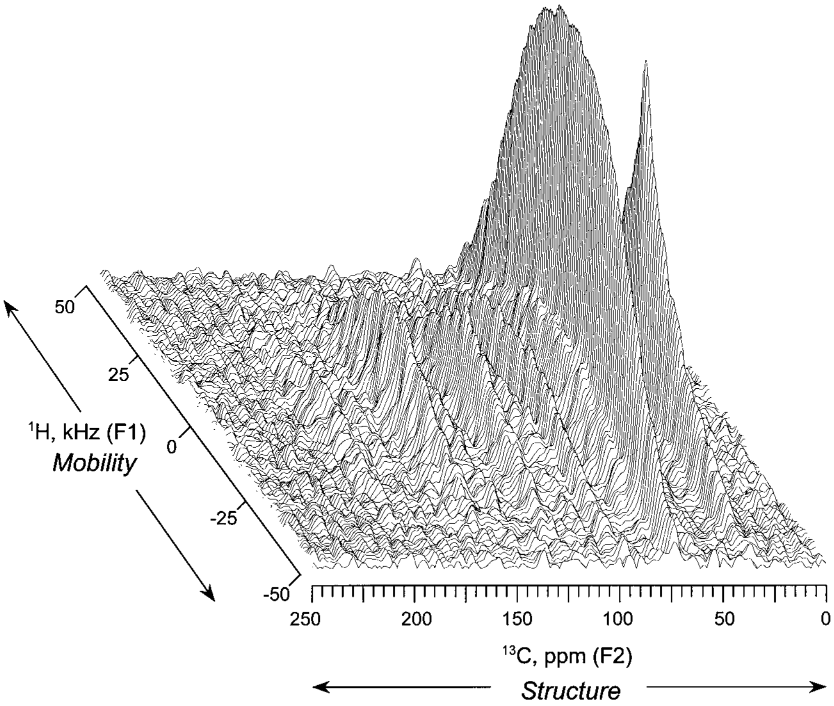

Figure 2. WISE stacked plot of dry suberized potato tissue. Spin diffusion was permitted only during the 500-μs cross-polarization period. ^{13}C signals at 72 and 104 ppm are assigned to the cell-wall polysaccharides; signals at 30, 130, and 173 ppm correspond to aliphatics, aromatics, and carboxyls of the suberin polyester, respectively.[3]

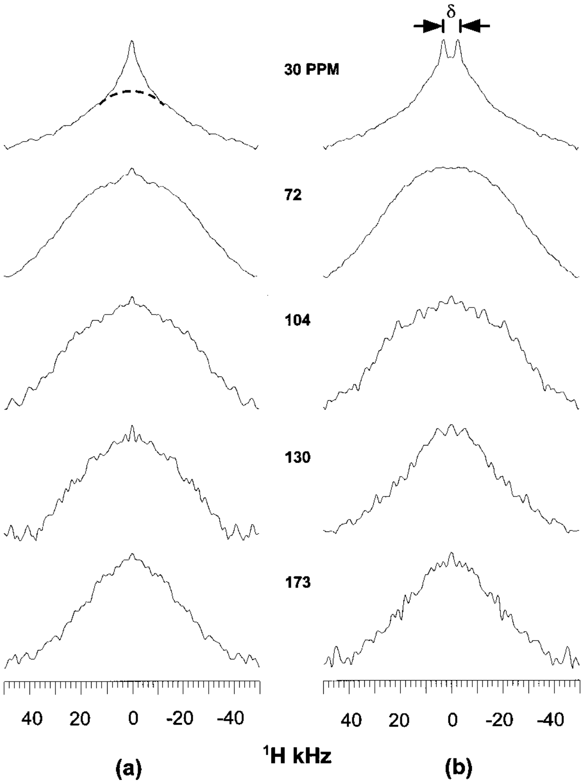

Figure 3. ^{1}H slices corresponding to ^{13}C chemical shifts in the WISE spectrum of dry potato suberin (Figure 2). ^{1}H frequency offsets with respect to the chain-methylene protons are set to (a) zero or (b) 2.7 kHz. The splitting (δ) of the 30-ppm peak in series (b) is estimated to be 5.4 kHz. Further adjustments of the ^{1}H offset produce corresponding changes in the peak splitting (data not shown).

The $(CH_2)_n$ resonance illustrates the ease with which WISE NMR can reveal two components that have different mobilities but the same chemical shift: a narrow line with half-height width of ~5 kHz and a wide line of ~30 kHz, present at an approximate ratio of 1:4. This result, which confirms and extends previous findings of biexponential spin−lattice relaxation behavior,[3] suggests that about 15−25% of suberin's chain-methylene segments are unrestricted by polyester cross-links or cell-wall attachments. Neither the values of $\langle T_{1\rho}(\mathrm{H})\rangle$, which are averaged by proton spin crosstalk throughout the suberin polyester, nor values of $\langle T_{1\rho}(\mathrm{C})\rangle$, which indicate enhanced average mobility on this time scale for the aliphatic chains,[3] can provide information on the two carbon populations so straightforwardly and definitively.

It is possible to obtain additional information on the motionally averaged population of chain methylenes by examining the response of the ^{1}H WISE spectra to resonance offset. If the ^{1}H frequency is set on resonance, signals from both cosine and sine data sets may be observed in the customary fashion by quadrature detection in the t_1 (^{1}H) domain.[11] As demonstrated previously in WISE experiments on a synthetic polymer,[9] a ^{1}H frequency offset allows observation of just the cosine data set. After Fourier transformation, there appear along the t_1 (^{1}H) axis two peaks symmetrically disposed about the midpoint of the spectrum with a total splitting of double this offset. For suberized potato tissue, a ^{1}H frequency offset of 2.7 kHz from the bulk-methylene protons results in a 5.4-kHz peak splitting for the ^{1}H slice corresponding to the ^{13}C chemical shift near 30 ppm (Figure 3b). Since the splitting pattern is discernible only if the ^{1}H line width is smaller than the offset, this result puts an upper limit on the ^{1}H line width of the motionally narrowed chain methylenes. Thus, despite the fact that suberin is thought to be a heavily cross-linked polymer embedded within the plant cell walls, the WISE spectra reveal that one in five chain methylenes retain sufficient motional freedom to produce ^{1}H lines as narrow as 5 kHz.

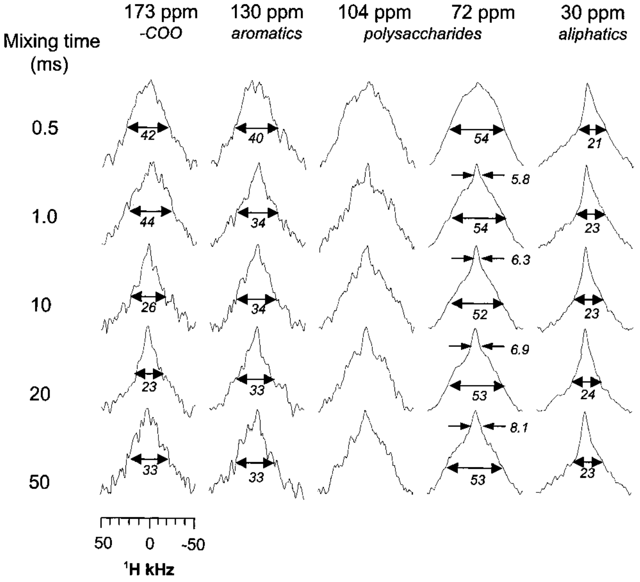

Figure 4. ^{1}H slices from the WISE spectrum of dry suberized potato, obtained with progressively increasing mixing times and zero ^{1}H frequency offset for the $(CH_2)_n$'s. Full line widths at half-height (kHz) are shown as a function of spin-diffusion time for selected polyester peaks (aliphatics, aromatics, and carboxyls) and for the major polysaccharide resonances. The minor component of the polysaccharide peak, which appears with a width of 5.8 kHz after 1 ms of mixing, broadens progressively to ~9.5 kHz as the mixing time is increased to 500 ms (data not shown).

B. Polyester and Polysaccharide Domains. If a mixing time is permitted to allow for ^{1}H spin diffusion during the WISE experiment, the motional narrowing associated with the aliphatic ^{1}H lines can be transferred to the signals of the aromatic and cell-wall carbohydrates. Figure 4 shows that within 10 ms, the ^{1}H line widths associated with the aromatic and carboxyl residues (^{13}C at 130 and 173 ppm, respectively) narrow to ~30 kHz. This common line width is observed for mixing times as long as 100 ms, exemplifying spin diffusion across short distances (<5 nm).[11] The magnetization behavior also demonstrates the close proximity of suberin phenolics and acyl chains, consistent with the working hypothesis that suberin's molecular structure includes fatty-acid esters of ferulic acid.[2,4]

A more complex pattern of line-narrowing behavior is exhibited for the cell-wall polysaccharide resonances at 72 and 104 ppm. As spin diffusion is allowed to proceed, there appear in the ^{1}H traces two components: a rapidly developing narrow feature with a line width of ~6 kHz and a broad feature that remains ~50 kHz wide. Since the spin diffusion originates from aliphatic chains within the suberin, these results indicate that portions of the cell-wall polysaccharides are located in close proximity to the biopolyester. The remaining carbohydrates (~92%, as judged by a spectral subtraction of Gaussian lines) have a more remote location with respect to the suberized domain of the potato tissue. Nevertheless, the two types of cell-wall polysaccharides can exchange spin magnetization between themselves—the narrow minor component displays a noticeable increase in line width as mixing proceeds, though the major component presumably includes too many spins to show any narrowing effects. In physical terms, it may be hypothesized that the population of cell walls nearest to the suberin protective layer is nevertheless connected covalently to other polysaccharides of the cell-wall matrix.

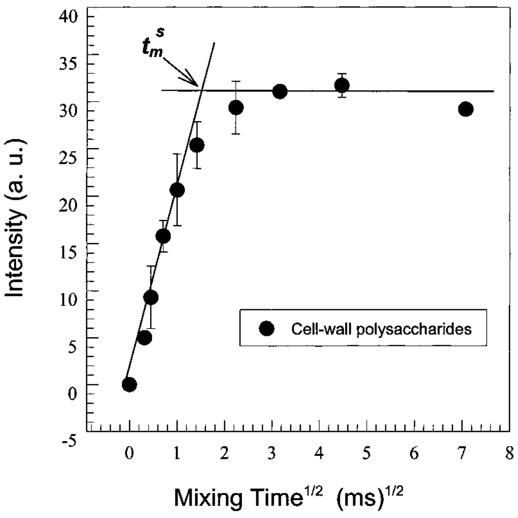

Figure 5. The growth of cell-wall polysaccharide ^{13}C signal intensity at 72 ppm upon spin diffusion from the suberin domain. Using the dipolar-filtered sequence in Figure 1b and mixing times less than 50 ms, this experiment monitors spin communication to that portion of the cell wall exhibiting a narrowed ^{1}H powder pattern. The extrapolated value t_m^s is estimated to be 2.56 ms.

C. Size of the Suberin Domain. Once the presence of spatially separate domains has been established, spin-diffusion rates and coefficients may be used to measure the polymer domain size as outlined by Schmidt-Rohr et al.[11] In the case of our biopolymer system, spin magnetization diffuses from the suberin domain (S) to the cell-wall polysaccharides (C). Using a dipolar-filtered ^{13}C detection sequence corrected for T_1 effects (Figure 1b), the buildup of the rigid cell-wall signals by spin diffusion from the flexible suberin aliphatics can be plotted versus the square root of the mixing time. As shown in Figure 5, the time at which the initial linear buildup intersects with a back extrapolation of the long-time signal intensity is designated as t_m^s. Since the mixing time never exceeds 50 ms, the prior WISE results (Figure 4) lead us to expect that the broadline components of the 72 and 104-ppm signals will exhibit no changes in appearance during the dipolar-filtered experiment. Thus, the measured value of t_m^s is associated only with the narrow component (i.e., about 8% of the cell-wall polysaccharides that are in closest proximity to the suberin layer).

The domain size d_S of the suberin layer is estimated as follows:

$$d_S = \frac{\epsilon}{f_C}\sqrt{4D_S t_m^s/\pi} \qquad (1)$$

where f_S and f_C are the volume fractions of suberin and cell wall in the potato wound periderm ($f_C = 1 - f_S$), ϵ is the "dimensionality" relevant to the polymer morphology, and D_S is the diffusion coefficient (diffusivity) of the suberin layer. Assuming equivalent spatial density and CPMAS ^{13}C NMR spectral sensitivity for all molecular functional groups, f_S and f_C are derived from spectral intensity measurements of the respective cell-wall signals (72 and 104 ppm) and suberin signals (30 and 130 ppm). The spectral intensities reveal a polymer blend composed of 45% suberin and 55% cell

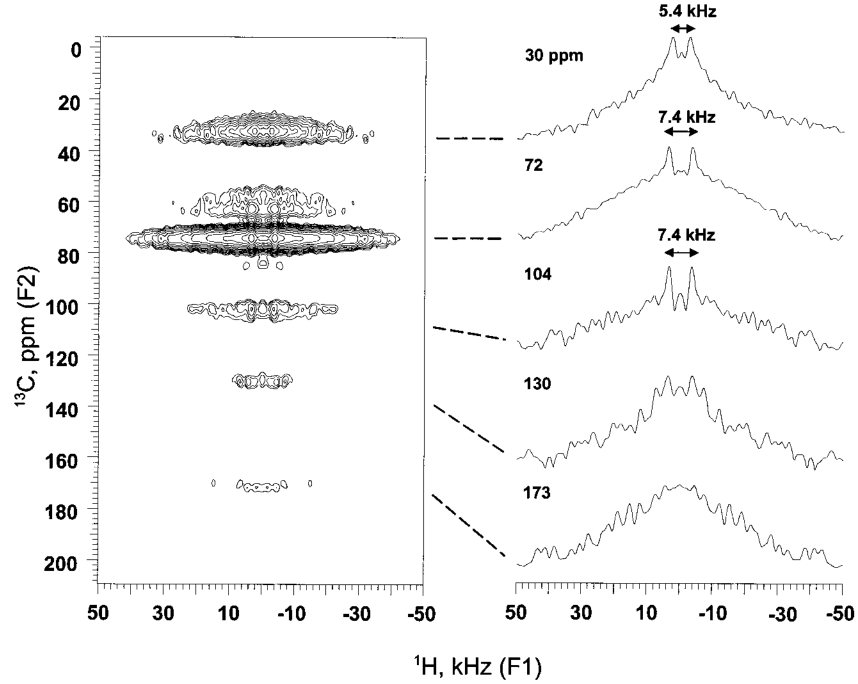

Figure 6. Contour plot and ^{1}H spectral slices for the WISE spectrum of hydrated potato suberin, obtained with spin diffusion occurring only during the 500-μs cross-polarization period. The ^{1}H frequency offset is set 2.7 kHz from the chain CH$_2$'s and 3.7 kHz from H$_2$O, respectively.

walls, in excellent agreement with prior estimates.[6] Since only 8% of the cell wall is detected in the dipolar-filtered spin-diffusion experiment, the effective ratio is 45 suberin to 4.4 cell wall and the normalized values of f_S and f_C are determined to be 0.91 and 0.09, respectively. Finally, because the suberin layer is deposited upon the wounded cell-wall tissue in a three-dimensional fashion within the plant cells, the dimensionality ϵ is taken to be 3.

To estimate the diffusivity D_S, we relied on prior results for synthetic polymers below their glass-transition temperatures.[12] Polystyrene (PS) was found to be a good model for suberin diffusivity. The first criterion involved ^{1}H line widths, which are indicative of dipolar couplings and spin-diffusion efficiency. Both PS[9,12,18] and the aromatic groups of suberin display ^{1}H line widths of ~50 kHz at ambient temperature, an appropriate comparison since the interface with cell-wall polysaccharides consists of suberin phenolics[2] (vide infra). The second criterion used the similarity of the spin-relaxation times $T_{1\rho}$(H), which were 5.2 and 5.0 ms for PS and suberin, respectively.[3] Inserting the PS spin-diffusion coefficient $D_S = 0.8$ nm^2/ms[12,18] into eq 1, it is straightforward to obtain a suberin domain size of 50 nm. By comparison, electron micrographs yield values that range from 100 to 200 nm.[1,2] The discrepancy may be attributed to variations in sample preparation, as well as to the fact that we are measuring the *effective domain size* for spin diffusion, which could be smaller than the dimensions of the entire suberized region.

D. Hydration of Suberized Potato Periderm Tissue. In addition to spin diffusion within the suberin-cell wall assembly, the WISE experiment may be used to monitor the propagation of ^{1}H line narrowing from external spin sources such as water. This strategy opens the way for exploration of the molecular interactions between suberized plant tissues and water-associated pathogens, herbicides, or insecticides. Figure 6 shows the WISE spectrum of hydrated suberin, obtained with no mixing time other than the 500-μs cross-polarization period. As compared with dry suberin examined under similar conditions, the presence of water is accompanied by the appearance of symmetrically split narrow features on the carbohydrate peaks at 72 and 104 ppm. As before, the aliphatic-chain protons of the suberin polymer have been set 2.7 kHz off resonance and thus produce a splitting of 5.4 kHz in the ^{1}H NMR spectrum corresponding to the 30-ppm carbon resonance. In the hydrated sample, moreover, the "external" water protons are 3.7 kHz off resonance and can thus produce a 7.4-kHz splitting in the ^{1}H slices corresponding to any proximal carbon moieties. In particular, WISE demonstrates that H$_2$O resides near the cell-wall carbohydrate region in potato wound periderm. This result is reasonable because the cell walls are very hydrophilic as compared with the aromatics and aliphatics of the suberin polyester.

When a mixing time is inserted into the WISE experiment, the water-related line-narrowing effect should be transferred from the cell walls to the suberin via the polysaccharide−polyester interface. Figure 7 displays ^{1}H slices from WISE spectra of hydrated suberin obtained with progressively increasing mixing time. Spin diffusion from the water protons reaches the aliphatic−aromatic polyester after about 2 ms, as evidenced by splitting of the peaks at 30, 130, and 173 ppm. This observation is consistent with the hypothesis that cell walls and suberin are situated in proximal but separate physical domains.[3] The spectral slices observed at 1 ms also suggest that spin diffusion proceeds more rapidly to the aromatic than the aliphatic groups, as expected if suberin is attached to the cell wall through its phenolics.[2,5] After 10 ms of mixing in the WISE sequence, it appears that complete H$_2$O spin diffusion has occurred at all carbon types of the biopolymer mixture. Additional experiments are in progress to examine ^{13}C−^{13}C dipolar couplings and covalent

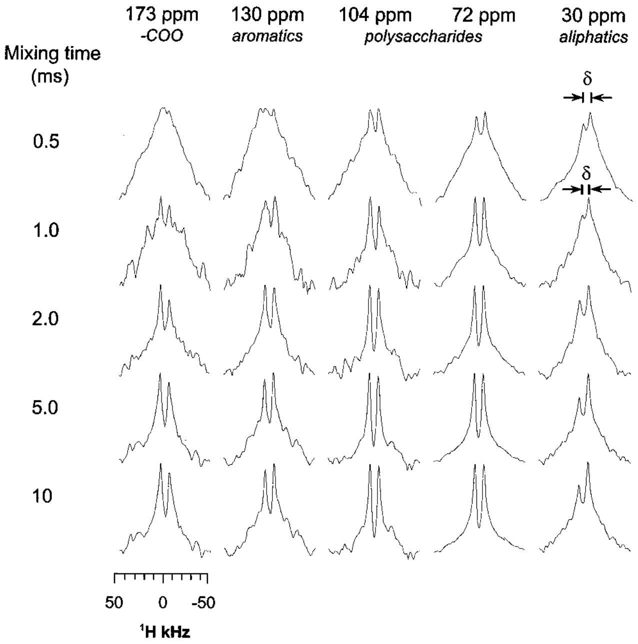

Figure 7. ^{1}H slices from the WISE spectrum of hydrated potato suberin, obtained with varying mixing times as shown. As in Figure 6, the ^{1}H frequency offset is set 2.7 kHz from the chain CH_2's and 3.7 kHz from H_2O, respectively. The splitting (δ) of the CH_2's is estimated to be 5.4 kHz, whereas all other splittings are measured as 7.4 kHz and attributed to spin-diffusion effects from H_2O.

bonding patterns between suberin and its cell-wall matrix.

Conclusions

The foregoing results demonstrate the utility of WISE NMR and associated spin-diffusion measurements[9,10] for the characterization of dynamics and domain structures in solid biopolymers. Since WISE experiments can reveal the motional behavior of distinct carbons that nevertheless possess the same chemical shift, they offer a level of detail that goes beyond the polymer's average spin-relaxation parameters. First, previous NMR-based findings[3] regarding the mobility of the aliphatic chains on the 50-kHz time scale and the physical separation of suberin and cell-wall domains have been confirmed conveniently and definitively using the WISE sequence. Second, new structural information has been derived for this natural polyester−polysaccharide blend: the 1:4 ratio of rigid and mobile chain-methylene groups, the dramatic motional narrowing of the flexible moieties, the small percentage of polysaccharides in close spin communication with the suberin polyester, and the possible proximity of suberin aromatic groups to cell-wall carbohydrates. Although suberin growing within the plant cell walls is generally immobilized, its surprisingly significant population of motionally unrestricted aliphatic chains may confer flexibility upon the healing surface or facilitate interactions with waxes in vivo.

By monitoring the diffusion of spin magnetization from the suberin polyester to its surrounding cell-wall polysaccharides, the thickness of the protective layer has been estimated as 50 nm in periderm tissue 7 days after wounding. Though this procedure relies upon estimates of the suberin diffusivity by analogy with polystyrene, it nevertheless provides a straightforward measure of domain size without the complications of sample preparation required for electron microscopy. Suberin domain sizes determined in this fashion could be monitored during the course of wound healing or compared for various internal suberized plant tissues.

Finally, WISE experiments on hydrated potato wound periderm have provided new structural information regarding the interactions of the plant tissue with water in its environment. Although the suberin polyester is deposited at the exposed surface of the cut tissue, the absorbed water becomes localized within the polysaccharide domain. Thus, the barrier role of suberin[19] is supported by its ability to repel water and water-borne pathogens. By contrast, exposed cell-wall moieties, which are very hydrophilic, interact preferentially with water. In retrospect, this affinity of suberized cell walls may not be surprising, since more than 90% of the polysaccharides are located more than 5 nm from the protons of the polyester, as judged from spin-diffusion experiments on the dry potato tissue. Controlled hydration experiments are now in progress to explore the structural and dynamic impact of water on both suberin and cutin membranes.

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