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Solid-State NMR Spectroscopy of Oriented Membrane Polypeptides at 100 K with Signal Enhancement by Dynamic Nuclear Polarization

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Solid-state NMR spectroscopy provides a powerful tool for the structural investigation of membrane peptides and proteins in their native lipid environment.[1−4] Polypeptides reconstituted in membranes have been studied by magic angle spinning approaches which result in spectra that resemble NMR spectra in solution and which provide chemical shifts and distance correlations that are used for structural analysis. Alternatively, static solid-state NMR approaches have been developed for oriented[5−10] or nonoriented samples[11,12] from which angular constraints are obtained for structural analysis. Whereas solid-state NMR spectroscopy has been used to study the structure, dynamics, and topology of these proteins it has been difficult to develop its full potential due to the inherently low sensitivity of NMR spectroscopy which is associated with the small energy difference between the nuclear Zeeman eigenstates. This problem is enhanced for solid-state NMR spectra where the observed line widths are commonly increased when compared to solution NMR spectra, and in particular for static oriented samples where the inherently dynamic properties of the polypeptides in lipid environments and the mosaic spread of the sample cause additional line broadening effects.

During recent years new developments have allowed for the combination of high-field solid-state NMR spectroscopy with dynamic nuclear polarization (DNP) where the samples are doped with free radicals and the unpaired electrons polarized by microwave (μ-wave) irradiation.[13] When compared to the situation of the nuclear spins the energy difference between the electron spin states is ∼3 orders in magnitude increased and the resulting polarization is therefore also much augmented. When irradiated with μ-waves this electron polarization transfers to the ^{1}H nuclear spins and can be relayed to dilute heteronuclei by conventional cross-polarization (CP). Due to the pioneering work of Robert G. Griffin's group it has been possible to demonstrate impressive DNP signal enhancements of up to 120 times (at 90 K) for biomolecular samples[14] such as amyloid fibers,[15] purple membranes,[16,17] or bacteriophages.[18] Even better enhancements have been observed for smaller model compounds or at lower temperatures.[13,19] However, to our knowledge, so far the technique has not been applied to polypeptides in lipid bilayers, oriented or nonoriented, although these provide an interesting alternative route for membrane protein structure determination. Therefore, many questions regarding this type of sample preparation remain such as the potential interference with membrane alignment due to very low temperatures or the presence of radicals. Furthermore, although oriented peptide−lipid mixtures of gel-phase lipids have been studied by solid-state NMR spectroscopy previously,[20,21] it remains possible that further decreasing the temperature causes changes in the macroscopic phase properties and/or alignment properties of the membranes. Here we present a first study where an oriented membrane has been prepared and studied in a 9.4 T NMR magnet at cryo-temperatures and irradiated with 263 GHz microwaves of 7 W. The microwaves were generated by a commercially available gyrotron operating at 9.7 T and transmitted into the NMR probe via a μ-waveguide (Bruker, Billerica, MA).

To test the effects of low temperatures and the enhancement by DNP in the context of lipid bilayers, the transmembrane model peptide hΦ17W was reconstituted in oriented membranes. The mixtures of peptide, lipid, and biradical were dissolved in 2,2,2-trifluoroethanol, spread onto 0.01 mm thick films of high density polyethylene (HDPE; dimensions 70 × 6 mm^2, Goodfellow, Cambridge, UK), dried and equilibrated at 93% r.h. of D_2O/H_2O 90/10, and rolled into cylinders to fit a 3.2 mm MAS rotor made of sapphire (cf. TOC graphics).[15,22] The use of partially deuterated "solvent" (lipid and water) channels spin diffusion toward the protonated peptide chain. These samples were investigated by magic angle oriented sample spinning (MAOSS) solid-state NMR spectroscopy at low (1−2.5 kHz) and high (8 kHz) spinning speeds.[22]

In a first series of experiments the ^{15}N signal of the peptide was investigated at high spinning speed. Although we were unsuccessful in recording room-temperature ^{15}N spectra in overnight acquisitions (typically 10 000 transients at 3 s recycle delay), in agreement with previous investigations of a similar sample,[22] decreasing the temperature reduces electronic noise and movements that interfere with cross-polarization.[11,23] By changing the temperature from 300 to 100 K, the Boltzmann distribution of Zeemann magnetization is also enhanced by a factor of 3, and at 100 K a small signal was observed after ∼7 h (Figure 1B). The ^{15}N spectrum could be further enhanced 18-fold by irradiating the sample with μ-waves of ∼7 W at 263 GHz, and the spectrum shown in Figure 1A was obtained after 50 min. Increasing the μ-wave power had no additional effect on the signal intensity (not shown) indicating that the DNP signal enhancement curve is within the plateau region.

Next, the alignment of the sample was investigated by MAOSS spectroscopy at low spinning speeds.[22,24] The good signal intensities obtained under DNP conditions at cryo-temperatures allowed us to directly investigate the ^{15}N labeled site of the peptide also in the presence of side bands. The spectra shown in Figure 1A−C were obtained using a commercial ^{1}H−^{13}C−^{15}N triple resonance MAS probe and setup for low temperature, 100 K (Bruker, Billerica, MA); at the present time flat coil probes are not available for DNP experiments. The corresponding simulation (Figure 1D) indicates a high degree of alignment for the peptide despite the low temperatures and the presence of a significant concentration of radicals in the sample. Therefore, the present investigations show

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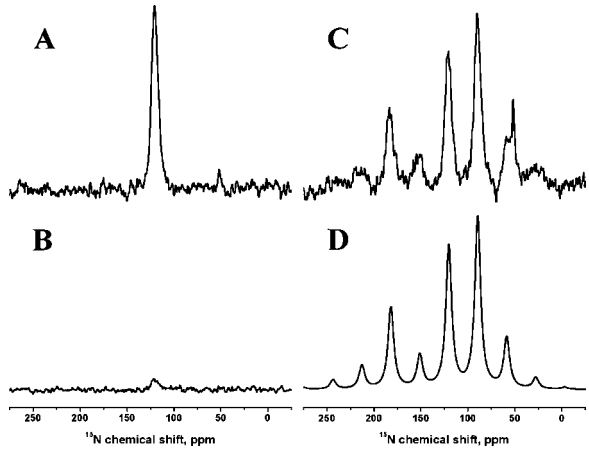

Figure 1. MAOSS solid-state NMR spectra at 100 K of 1 mg ([^{15}N-Leu5]-KKKA L* LALLALAWALALLALLAKKK) in 6 mg POPC-d_{31} (5 mol % peptide) in the presence of 40 μg bTbK[19] biradical (panels A−C) oriented on HDPE.[22] An EPR spin count revealed that the sample carried 5×10^{16} electron spins. Proton-decoupled ^{15}N CPMAS solid-state NMR spectra at 8 kHz (A, 1028 scans, B, 8192 scans, 3 s recycle delay) and 1.25 kHz (C, 4096 scans). Spectra A and C were recorded with 263 GHz μ-wave irradiation, whereas the μ-wave irradiation was switched off during acquisition of B. The ^{15}N signal at 50 ppm is probably from the lipid head groups. D is a simulation of C (cf. Supporting Information). A and B are scaled with the number of acquisitions to correctly reproduce the DNP signal enhancement.

for the first time not only that oriented membrane samples are amenable to liquid nitrogen temperatures and DNP technology but also that oriented MAOSS samples suitable for 3.2 mm MAS rotors (i.d. 2.2) can be prepared. Furthermore it should be mentioned that during these experiments we were able to diminish the sample quantities to only 1 mg of peptide labeled at a single site, and although the quantity of peptide was an order of magnitude reduced the DNP experiments nevertheless required only a fraction of the acquisition time when compared to previous investigations of a related setup.[22]

To complement these investigations the samples were also investigated by proton-decoupled ^{31}P NMR MAOSS spectroscopy at 295 and 248 K (cf. Supporting Information).

The 18-fold signal enhancement of the proton-decoupled ^{15}N CPMAS solid-state NMR spectra, between Figure 1A and 1B, arises from the transfer of polarization from the electron spins of the radicals to the ^{1}H nuclei by way of the DNP cross effect which is then transferred onto the dilute heteronuclei in the sample by cross-polarization. The DNP effect of our oriented membrane samples can probably be improved by further optimizing the sample preparation conditions to reach the higher values observed in aqueous environments.[14–18] This might be possible by tuning the radical concentration or type/structure,[19,25] sample handling, further directing the ^{1}H spin diffusion pathways using partial deuteration, and/or performing the experiments at even lower temperatures.[14,18] Furthermore, it should be mentioned that EPR spectra recorded at 9 GHz indicate that the radicals exhibit a small degree of alignment in these samples (not shown) as do for example the water molecules in stacked membranes.[26] The interactions of free electron carrying amphipathic or hydrophobic molecules with oriented membranes have interesting implications for the DNP enhancement in such systems,[25] and the development of new biradicals specially designed for their use in lipid membrane environments is currently ongoing in our laboratories.

In conclusion, by demonstrating the applicability of DNP signal enhancement techniques to oriented membrane samples, a starting point for additional improvements in sample preparation protocols is provided. In addition, these results motivate further technical developments such as flat-coil NMR probes for membranes stacked in between glass plates.[27]

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Supporting Information Available: Additional ^{31}P NMR spectra as well as details on the simulation of Figure 1D are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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