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Dynamic nuclear polarization/solid-state NMR of membranes. Thermal effects and sample geometry

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A B S T R A C T

Whereas specially designed dinitroxide biradicals, reconstitution protocols, oriented sample geometries and NMR probes have helped to much increase the DNP enhancement factors of membrane samples they still lag considerably behind those obtained from glasses made of protein solutions. Here we show that not only the MAS rotor material but also the distribution of the membrane samples within the NMR rotor have a pronounced effect on the DNP efficiency. These observations are rationalized with the cooling efficiency and the internal properties of the sample, monitored by their T_1 relaxation, microwave on versus off signal intensities and DNP enhancement. The data are suggestive that for membranes the speed of cooling has a pronounced effect on membrane phase transitions and concomitantly the distribution of biradicals within the sample.

1. Introduction

In order to overcome the inherently low sensitivity of NMR spectroscopy Dynamic Nuclear Polarization (DNP) has been developed [1]. Of all NMR approaches, solid-state NMR of heteronuclei probably suffers the most pronounced limitations in sensitivity, therefore, DNP/solid-state NMR settings that became commercially available only a few years ago promises to have a particularly large impact on such structural investigations [2]. The technique has been applied, for example, to materials, pharmaceutical formulations, proteins and biomolecular aggregates, investigations that would not have been possible without the sensitivity gained by DNP technology [1,3–11].

Membrane proteins are particularly time consuming to study because they are difficult to over-express, label and purify, they usually occur at highly dilute concentrations in their native lipid bilayer environment [12], and they tend to adopt a number of conformational states [13,14]. A widespread solid-state NMR approach to investigate membrane polypeptides is the measurement of distances and torsion angles under magic angle sample spinning (MAS) [15–19]. A complementary technique is the investigation of uniaxially oriented bilayer samples which is used to study the structure, membrane topology, dynamics, conformational heterogeneity or topological exchange [20–23]. Furthermore, it has been possible to combine both approaches by magic angle oriented sample spinning [24–26].

Because membrane polypeptides in their functional state often require their reconstitution at relatively low protein-to-lipid ratios their investigation by high-resolution structural techniques remains particularly challenging. DNP can overcome many of the concomitant sensitivity limitations and has indeed been used to study biomacromolecules [17, 27]. However, the signal enhancements obtained when cells or membranes were investigated lag behind by about an order of magnitude to those obtained in isotropic environments and the reasons for this difference are only slowly understood [3,4,6,7,28–37].

During the DNP experiments a large polarization is created for unpaired electron spins that have been introduced to the sample in the form of exogeneous free radicals. This polarization is transferred to the surrounding nuclei through microwave irradiation which results in a large enhancement of the ^1H NMR signal, in theory up to 660-fold for ^1H nuclei, corresponding to the ratio of the gyromagnetic ratios of electrons and the nucleus (γ_e/γ_n) [1,38–40]. The ^1H polarization can subsequently be cross polarized to ^{13}C and ^{15}N nuclei. Because such experiments are performed at low temperatures (e.g. around 100 K) the sample is typically embedded in a glass forming matrix to ensure a homogenous distribution the paramagnetic polarizing agent [1,41]. For matrix-free samples such as stacks of membranes oriented on solid supports additional experimental considerations arise [37,42]. These samples are prepared in the absence of bulk water and have resulted in even lower DNP signal enhancements [26,37,43,44].

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For nitroxide-based polarizing agents used in such experiments the cross effect (CE) has been described as the most efficient polarization mechanism, which involves two coupled electron spins and a nearby proton were the difference in Larmor frequencies of the two electrons matches the Larmor frequency of the proton [1,45]. The ^1H spin polarization then diffuses throughout the sample. For optimal DNP efficiency the solvent matrix [46] and the sample formulation have been found important [35,43,47,48].

Indeed, DNP has been found less efficient in the presence of the anisotropic and heterogeneous environment that lipid bilayers provide [23,26,35,43]. Therefore, a number of investigations have been performed to systematically improve the DNP efficiency of membrane preparations including sample preparation protocols [17,35,37,43], a dedicated NMR probe for DNP/solid-state NMR of static oriented samples [49], special biradicals with lipid anchors [37,50–52] as well as studies of the effect of low temperature on the properties of bioactive membrane polypeptides [7]. Despite these efforts that have much increased the enhancement factors in membranes there is still room for further improvement suggesting that their biradical distribution is not optimal in the membrane environment [26,35,37,43,50,53,54].

During our previous work at optimizing protocols, microwave irradiation and sample preparation which we performed at four different DNP locations we observed considerable differences in the enhancement factors between sites although samples were identical and the NMR spectrometer and gyrotrons for microwave production of the same magnetic field strength (400 MHz for ^1H), built and manufacturer [43]. This observation was even more puzzling because the results obtained when reference samples in glycerol/water were used to calibrate the settings were reproducible from instrument to instrument. We investigated these effects in a systematic manner and in this paper we report on the importance of sample geometry which is itself influenced by the detailed protocols of sample preparation and cooling speed, *i.e.* the manner how samples are inserted into the NMR coil at cryo temperatures. The insights gained can be used to further optimize solid-state NMR/DNP investigations of peptides and proteins under MAS conditions and in aligned lipid bilayers where no solvent or glassy matrix is present [7,42]. The latter systems are free of cryoprotectants (DMSO or glycerol) and used for NMR structural investigations also of supported lipid bilayers, for which good enhancements factors are more difficult to obtain when compared to glass-forming solvent mixtures [35,43].

2. Materials and methods

The natural abundance 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) is from Avanti Polar Lipids (Alabaster, AL), glycerol- d_8 from Sigma-Aldrich. All commercial materials were used without further purification.

2.1. Sample preparation

A homogeneous mixture of lipid, peptide, and free radical was obtained by co-dissolving the membrane components in trifluoroethanol. Typically, 15 mg of POPC lipid and dinitroxide biradical (1 mol%) were used for each sample. The solutions were dried first under a stream of N_2 gas and then under high vacuum overnight. Thereafter, the sample was equilibrated for 1 day in an atmosphere of 93% relative humidity of $\text{D}_2\text{O}/\text{H}_2\text{O}$ (90:10 v/v). The lipid paste was transferred into a 3.2 mm sapphire or zirconia MAS rotor. Thereafter, the rotor was spun at 2 kHz for 1 min at room temperature to allow mass equilibration of the viscous material inside the rotor to obtain the equilibrated sample geometry (see Fig. 1A). Alternatively, with the use of a small stick the lipid paste was pushed toward the bottom of the rotor to obtain the non-equilibrated geometry (Fig. 1A).

The mixture of ^{15}N , ^{13}C -proline in glycerol- $\text{d}_8/\text{D}_2\text{O}/\text{H}_2\text{O}$ (60/30/10 by volume) with 10 mM AMUPol was prepared as a stock solution and used as a reference. To obtain the equilibrated geometry with the liquid sample distributed on the rotor walls 10 μl sample volume was placed inside the rotor, and a tight silicon plug insert was positioned right under the cap leaving an empty space between the liquid and the insert. Thereafter the rotor was spun at 2 kHz in the warm DNP MAS probe while cooling was launched. After the temperature reaches 100 K usual DNP experiments were performed on the sample with equilibrated geometry.

2.2. DNP/solid-state NMR spectroscopy

DNP/solid-state NMR spectroscopy measurements were performed on a Bruker BioSpin wide-bore 9.4 T magnet and an Avance III solid-state NMR spectrometer equipped with a gyrotron producing 263 GHz irradiation, a microwave transmission line delivering up to 9.5 W of microwave power at the sample, a cooling unit using liquid nitrogen, and a

Fig. 1. A. shows the equilibrated or non-equilibrated sample geometries within the rotor upon freezing. After filling the rotor (middle) a first spinning at ambient temperature results in an equilibrated membrane film along the rotor wall (left). Alternatively, pushing the sample to the bottom results in membrane samples that completely fill the full inner diameter but only half the height of the rotor. B. Experimentally observed enhancements ϵ (ϵ is the ratio of μ -wave ON versus μ -wave OFF signal intensities) of POPC membranes in the presence of 1 mol% PyPol-C16 without cryo-protectant before the cooling was made more efficient and C. after a better insulated transfer line results in faster cooling of the sample. In B and C the temperature of the cooling gas was set to 93 K at the level of the variable temperature control inside the probe head. Please note that all data were obtained from the same sample preparation which was transferred into different rotors.

low-temperature triple-resonance 3.2 mm MAS probe (Bruker Biospin, Billerica MA). The spectra were obtained by using a commercial $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ MAS probe with DNP capabilities and set up for low temperatures of about 100 K (Bruker Biospin, Rheinstetten, Germany). An adiabatic cross polarization (CP) pulse sequence was used with a spectral width of 29.8 kHz and an acquisition time and CP contact of 17.2 ms and 0.65 ms, respectively. The recycle delay was set to 1.3 times ^1H T_1^{DNP} determined by the saturation recovery method with ^{13}C CP detection directly on each sample under DNP microwave irradiation. The ^1H $\pi/2$ pulse and spinal64 heteronuclear decoupling field strengths B_1 corresponded to a nutation frequency of 50 kHz [55]. To equilibrate the system before acquisition, the sample was exposed to eight dummy scans. The DNP signal enhancement was determined as the ratio of the integral signal intensity of microwave ON versus microwave OFF spectra obtained with identical parameters.

3. Results and discussion

Whereas a number of improvements have already been obtained for DNP/solid-state NMR measurements of membrane samples the enhancement factors obtained in such anisotropic, often matrix-free environments still lag behind those obtained in glassy matrices although even with the present state of the art solid-state NMR spectra have been obtained with much shorter acquisition times [37,50]. In order to further optimize the protocols for application of this promising technology here we investigated the role of sample geometry and the influence of freezing on the membrane samples. The enhancement factor which compares the signal intensity in the presence and absence of microwave irradiation was used as a first measure for DNP efficiency, but relaxation, absolute intensity, line width and other properties were also taken into consideration.

In a first step the material of the MAS rotor was under scrutiny. Indeed, our data confirm previous publications [2,56] where samples inserted into sapphire rotors give better enhancement than in the more commonly used and cheaper zirconia settings (Figs. 1 and 2, Table 1). This observation is strongly suggestive that the heat conductivity which is 30–35 W/(m·K) for sapphire and 2–3 W/(m·K) for zirconia as well as the microwave penetration characteristics are important parameters [57–59].

In a next step, we investigated how the transfer of the sample into the NMR probe and coil, a setting that is precooled with nitrogen gas at 100 K, affects the DNP efficiency. Two protocols were evaluated. In the first case the sample was frozen before undergoing fast spinning, in the second case the rotor was first equilibrated under fast MAS before the sample was frozen (Fig. 1A). Clearly, pre-equilibration of the sample at ambient temperature has an even larger effect on DNP efficiency within membrane samples (ca. 45% difference) than the material of the MAS

Table 1

DNP enhancement factors for membrane samples at 93 K gas flow when transferred to sapphire or zirconia rotors at different sample geometries (equilibrated versus non-equilibrated, cf. Fig. 1).

Sample	Sapph. eq.	Sapph. non-eq.	Zirc. eq.	Zirc. non-eq.
Wissembourg site, with an improved cooling unit (2016 and later)				
PyPol-C16	71 ^{a)}	49 ^{a)}	57 ^{a)}	31 ^{a)}
	80 ^{b)}			
AMUPOL	50.5			
	58 ^{b)}			
Wissembourg site, with cooling unit installed before 2015				
PyPol-C16	49 ^{a)}	33 (full rotor ^{c)})		
AMUPOL	41		20	
TOTAPOL	17		11	
bTbk	17		12	
bCTbk	13		8	
bPyTbk	40		20	

^a All data from same sample transferred between rotors.

^b Gas flow temperature was set to 87 K

^c Full rotor with twice the sample quantity.

rotor (Figs. 1C and 2 and Table 1). Taken together, the membrane sample measured in an equilibrated sapphire rotor gives the best enhancement and the non-equilibrated zirconia the least efficient DNP process.

Notably, the microwave irradiation not only pumps the polarization transfer but is also responsible for considerable heating of the sample [49]. When the membranes in MAS rotors were exposed to more intense microwave irradiation the enhancement factors increased until reaching an asymptotic limit (Figs. 1B and C and 3). Only the non-equilibrated zirconia rotor exhibits a reduced enhancement factor when the microwave intensity is increased beyond 4W (Fig. 1B and C) indicating that the sample heating prevents a more efficient polarization transfer. Thus, a sapphire rotor with the membrane sample spread along the walls is the best for efficiency by combining good microwave penetration [58] with good cooling efficiency (Table 2). The data are suggestive that not only the heat conductivity of the MAS rotor but also the contact of the membrane sample with its container are essential properties for good DNP efficiency.

After MAS equilibration, a film of sample spreads equally along the surface of the rotor thus allowing the maximal heat exchange with the rotor and thus the cooling gas (Fig. 1A). In contrast when the sample is frozen in a compact manner in half the container volume heat exchange is slowed down for the inner core of the sample. This is also the case when the rotor is filled completely where the enhancement is about the same as for the non-equilibrated sample, while some improvement is possible with a setting allowing lower temperatures (Fig. 3, Table 1).

These observations parallel prior studies where membranes oriented along glass slides showed rather low DNP efficiency, a situation which was much improved by supporting the lipid bilayer on sapphire and

Fig. 2. Proton-decoupled ^{13}C solid-state NMR spectra of POPC in the presence of 1 mol% PyPol-C16 measured with the more efficient cooling unit in panels A–D and with the old installation in panel E at the indicated sample geometries. The temperature of the cooling gas was set to 93 K at the level of the variable temperature control inside the NMR probe head. The corresponding, enhancement factors are shown in Fig. 1. For the μ -wave ON spectra the power was set to 8.8 W in panels A–C and E, and 4.2 W in panel D. The recycle delay was set to $1.3 \cdot T_1^{\text{DNP}}$, 64 scans were acquired after 8 dummy scans for equilibration. Dashed lines are shown to guide the eye.

Fig. 3. Enhancement factors as a function of μ -wave power for ^{13}C -proline with 10 mM AMUPol in glycerol- $\text{d}_8/\text{D}_2\text{O}/\text{H}_2\text{O}$ 6/3/1 (reference sample) with A. the older less efficient temperature device and B. the improved cooling unit. A 3.2 mm sapphire rotor was used at 8 kHz MAS.

Table 2

Published heat conductivity and microwave penetration of materials used in DNP/solid-state NMR.

Material	Heat conductivity (at ambient temperature) W/(m K)	100 K	Microwave penetration (at ambient temperature)	references	Low temperature data (reference)
sapphire	32–42		9.6	[60]	
zirconium	1.7–3		12	http://www.kayelaby.npl.co.uk/general_physics/2_6/2_6_5.html	
glass	0.8–1.1	0.72*0.58	4–7.5	https://web.archive.org/web/20081116100234/http://www.goodfellow.com/E/Silica.HTML	* [61]
HDPE	0.42–0.51		2.3	https://www.engineeringtoolbox.com/thermal-conductivity-d_429.html	
Membranes	0.21–0.31 (0.21 for low water content)			[62]	
Oil	0.100–0.212		2.7	https://www.engineeringtoolbox.com/thermal-conductivity-d_429.html	
Paraffin wax	0.25		2.2	http://www.rfcafe.com/references/electrical/dielectric-constants-strengths.htm	
water	0.606	Supercooled liquid water 250 K 0.52	80.4 (RT) 29.3 (demineralized water)	https://www.engineeringtoolbox.com/thermal-conductivity-d_429.html	
Glycerol	0.29	0.35	42 (RT)	https://www.engineeringtoolbox.com/thermal-conductivity-d_429.html	[63]
DNP juice			3.5 (glass, 100 K)	https://www.engineeringtoolbox.com/liquid-dielectric-constants-d_1263.html	[58]

HDPE polymers [49]. However, the heat conductivity of glass and HDPE are rather similar (0.65) which is suggestive that other geometrical contributions may be important. Notably whereas the thickness of the glass slides (0.05–0.07 mm) comes close to the wavelength of the microwave irradiation (1.14 mm in vacuum) the geometries of the rotors (wall thickness 0.4 mm), sapphire plates and PEEK films were considerably different (thickness of 0.4 mm and 0.01 mm, respectively) suggesting that the glass stacks may interfere with microwave penetration.

An interesting observation was made when the transfer line of the cooling cabinet of the DNP/solid-state NMR spectrometer was replaced with a better isolated one thus lower temperatures at the entrance to the probehead are reached whereas all of the spectrometer electronics and equipment remained unchanged.

Using the previous cooling device, the direct comparison of the full rotor with the equilibrated one gives only a 13% higher signal-to-noise in favor of the full rotor albeit the sample contains twice as much material. Notably, the same ^1H T_1^{DNP} relaxation time of 2.3 s was measured in both cases. Because the μ -wave OFF signals correlate well with the quantity of sample (spectra shown in grey in Fig. 2B,D,E) there must be considerable

differences in the DNP efficiency. It should be noted that ^1H T_1^{DNP} and ^1H T_1 observed with MW OFF spectrum are essentially similar for the membrane samples of this kind [37].

With the new more powerful cooling assembly the difference in μ -wave ON spectra between the equilibrated ($\epsilon = 71$) and the non-equilibrated rotors ($\epsilon = 49$) is about 17% (Fig. 2A and B black lines). It is interesting to further analyze the spectra recorded with the new cooling unit by not only considering relative enhancements but also the absolute intensities and relaxation behavior [64,65]. In the equilibrated rotors, the ^1H T_1^{DNP} is 3.0 ± 0.1 s (Fig. 2A,C), a value that drops to ~ 2.0 s for the non-equilibrated rotors (Fig. 2B and D). At the same time the μ -wave OFF signal of the non-equilibrated sample is growing by $\sim 20\%$ when compared to the equilibrated geometry (in both sapphire and zirconia, Fig. 2B,D) while the μ -wave ON signal is decreasing by $\sim 15\%$ (for sapphire rotor, compare Fig. 2A with 2B). Therefore, nearly half of the difference leading to higher enhancement values (i.e. μ -wave ON vs. μ -wave OFF) observed for the equilibrated geometry belongs to the reduction in intensity of the μ -wave OFF spectrum. This is probably due to a more homogenous distribution of the biradicals within the

equilibrated sample which results in quenching more of the NMR signals in the μ -wave OFF situation [37] but also a more uniform signal enhancement in the μ -wave ON condition.

It is interesting to note that the signal intensities and relaxation times of samples in the sapphire rotor with equilibrated geometry acquired under the conditions of the older cooling unit (Fig. 2E) are similar to the ones obtained with the new cooling set-up and non-equilibrated sapphire rotors (Fig. 2B). Indeed, the ^1H T_1^{DNP} relaxation times and in the μ -wave OFF signal intensities indicate that equilibration results in the modification not only of the sample geometry but also of its inherent properties. It is probable that the speed of freezing depends on the thickness of the membrane films as well as the instrumental settings and can result in different arrangements of the lipids, biradicals and water molecules within the membrane sample ('phases'). Notably, on a supramolecular level the lipid bilayers appear intact and oriented when frozen along solid supports [7,26]. In order to further test the effect of the speed of freezing, the non-equilibrated sample in zirconia showing the least efficient performance, was flash frozen by dropping into liquid nitrogen. The sample now exhibits a low intensity of the μ -wave OFF spectrum, similar to the equilibrated geometries (data not shown). Furthermore, the enhancement factor is much increased and even outperforms the previously tested equilibrated sapphire rotor at similar MW irradiation (<6W; see Fig. 1B).

Although this behavior parallels the good glass formation of an aqueous solution by fast sample freezing it should be mentioned that membrane samples that have been hydrated through the gas phase at 93% relative humidity do not contain bulk water. Therefore, the formation of ice crystals is not an issue. However, it is possible that membrane phase transitions such as fluid-to-gel or other changes at low temperatures play an analogous role as the water – ice transition in isotropic solvent systems. In such cases the speed of the transition can have an effect how the biradicals redistribute within the membrane interface [35,43] similar to recently published observations on water/glycerol mixtures used for DNP [66].

Indeed, the water/glycerol reference samples show similar enhancement factors when the DNP solid-state NMR instrumentation of identical built but at different locations (Billerica, Grenoble, Berlin, Wissembourg) and the same temperature was used during the experiments are compared to each other (compare Fig. 3A and B, red dots for DNP spectra acquired at 93 K). Furthermore, for the membrane sample and the water/glycerol reference the same field dependence of enhancement is observed (Fig. S1), a procedure which is part of the instrument calibration. When the enhancement factor of rotors that were half-filled with the glass formed by the glycerol containing reference solution at 100 K ($\epsilon = 293$) are compared to a completely filled rotor ($\epsilon = 256$) the difference is only 15%, which can be assigned to somewhat improved cooling and microwave penetration of the outer layers of the sample.

In contrast, when comparing the DNP enhancement observed for membrane samples the difference between the full and the only half-full and equilibrated sapphire rotors is about 45–50% regardless of the temperature unit used (equilibration increases ϵ from 33 to 49 (previous cooling) or from 49 to 71 (new cooling device); Table 1). This difference is probably related to a high sensitivity of the membrane samples on the speed of freezing which is related to the sample geometry and rotor material. We speculate that upon freezing different biradical distributions are trapped, which explains both different μ -wave OFF spectra and ^1H T_1 values (see Fig. 2). Finally, it should be noted that with the newer setting somewhat lower temperatures can be reached (87 K for the gas flow) which in itself allows for improved DNP efficiencies of up to 80 (Table 1).

4. Conclusion

It has been demonstrated that the DNP signal enhancement observed for membrane samples depends on the nature and concentration of biradicals, the membrane lipid composition [37] and water content [35,43],

the protocol of membrane reconstitution ([35], Figs. 1 and 4), the rotors (Table 1) and/or the support used to orient membranes [49], the DNP/NMR probe [49] and the sample temperature ([49,67–69], Fig. 3B). Here we demonstrate that also the exact geometry of the sample (i.e. distribution inside the rotor; Fig. 1) and the speed of freezing have a large influence on the DNP efficiency with membrane samples (Fig. 1). Therefore, the geometry and physical properties such as heat conductivity and heat capacity not only of the rotor material but also of the sample composition may have an important influence (Table 2). By optimizing the material of the MAS rotor and the sample geometry/speed of freezing the enhancement factors could be more than doubled in the present work where a pre-equilibrated sapphire setting provided the best conditions (Table 1). Furthermore, fast freezing of the sample by dropping the rotor into liquid nitrogen was favorable to performance (Fig. 1B) suggesting that the lipid phase at cryotemperatures and thereby the distribution of biradicals in the sample are affected by the protocol with important effects on DNP efficiency.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ssnmr.2019.03.004>.

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