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Dynamic Nuclear Polarization of Sedimented Solutes

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Supporting Information

ABSTRACT: Using the 480 kDa iron-storage protein complex, apoferritin (ApoF), as an example, we demonstrate that sizable dynamic nuclear polarization (DNP) enhancements can be obtained on sedimented protein samples. In sedimented solute DNP (SedDNP), the biradical polarizing agent is co-sedimented with the protein, but in the absence of a glass-forming agent. We observe DNP enhancement factors $\varepsilon > 40$ at a magnetic field of 5 T and temperatures below 90 K, indicating that the protein sediment state is "glassy" and suitable to disperse the biradical polarizing agent upon freezing. In contrast, frozen aqueous solutions of ApoF yield $\varepsilon \approx 2$. Results of SedDNP are compared to those obtained from samples prepared using the traditional glass-forming agent glycerol. Collectively, these and results from previous investigations suggest that the sedimented state can be functionally described as a "microcrystalline glass" and in addition provide a new approach for preparation of samples for DNP experiments.

Sedimented solute nuclear magnetic resonance (SedNMR)[1−3] was recently proposed as a method to investigate biomolecular systems which are otherwise not detectable by either solution or magic angle spinning (MAS) NMR techniques because of long rotational correlation times[4,5] or their inability to crystallize. In particular, following ultracentrifugation, macromolecules or macromolecular complexes with MW > 30 kDa (i.e., 60% of the protein in the Swissprot database, without considering complexes) form a sedimented state, in which the correlation time is long, due to self-crowding. This state can be used for structural studies with MAS NMR. Recently, Bertini and co-workers demonstrated this approach with the 480 kD homo-24-mer apoferritin (ApoF) that undergoes sedimentation during MAS due to the centrifugal forces that accompany high-frequency sample rotation.[2,3] The sedimentation process was monitored *in situ* by comparing signal intensities obtained by solution and MAS NMR techniques. The spectra obtained in the case of sedimented ApoF are indistinguishable from those recorded from a microcrystalline sample, also available in this case. Since centrifugation provides a favorable high protein concentration (≤700 mg/mL) within the sedimented layer, it is expected to form a glass-like, amorphous state upon freezing. Concurrently, freezing at sufficiently low temperatures enables cross-effect[6−12] dynamic nuclear polarization (DNP) of the protein from a polarizing agent dispersed within the sediment. The addition of DNP to SedNMR therefore could not only dramatically boost the signal intensity of the MAS NMR experiment but also serve as a tool to probe the state of the frozen sediment.

In this Communication, we demonstrate the principle of sedimented solute DNP (SedDNP) at 140 GHz (5 T) with a study of the homo-24-mer of ApoF[13−15] ultracentrifuged *in situ* from aqueous solution inside a 4 mm MAS rotor together with the biradical polarizing agent 1-TEMPO-4-oxyl-3-TEMPO-4-amino-propan-2-ol (TOTAPOL).[16] We observe enhancements of $\varepsilon \approx 42$ from the sedimented state, whereas in a frozen solution we find $\varepsilon \approx 2$. Recently, Gardiennet et al. used a fixture specially designed for an ultracentrifuge[17] and demonstrated in elegant experiments on dodecameric DnaB helicase (708 kDa)[18] that, as predicted,[3] *ex situ* sedimentation directly into an NMR rotor is feasible. We have also sedimented bovine serum albumin into a rotor using an ultracentrifuge and observed enhancements of ∼65, illustrating that this approach is also feasible. We note that the sediment has no long-range order as seen by XRD.[18] Collectively, these results indicate that the sedimented state forms a glass that prevents phase separation of the polarizing agent from the protein. In addition, the resolution in the high-field MAS spectra suggests that the proteins behave as if they were in a microcrystalline environment. Accordingly, we suggest that *functionally* the sedimented state can be described as a "microcrystalline glass".

DNP has been shown to dramatically increases the sensitivity in MAS NMR experiments by transferring electron polarization to neighboring nuclei; for ^{1}H a polarization enhancement of up to ∼660 can in principle be achieved.[19] With the introduction of high-frequency microwave sources, DNP was recently extended to contemporary NMR frequencies/fields and used in studies on membrane proteins, nanocrystals, amyloid fibrils and virus particles in a number of different laboratories[20−27] as well as to surfaces.[28,29] In the case of the biological samples the analyte is heterogeneously dispersed in a frozen glycerol/water solution containing the polarizing agent. The cryoprotecting properties of the glass-forming matrix prevent phase separation of solvent and polarizing agent and also allows for dispersal of polarization from the bulk to the analyte. In a SedNMR

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experiment the sediment is largely segregated from the bulk solvent and consists of a highly concentrated protein solution with a reproducible protein and water content (≤700 mg/mL). This solution has a high viscosity due to self-crowding[30,31] and the water that is contained therein is likely to be bound or interacting with the protein.[32−34] Consequently, the frozen sediment is not as susceptible to ice formation within the bulk solvent as is a homogeneous frozen solution. This suggests the possibility that the sedimented protein could exhibit glass like behavior and be suitable for DNP experiments in the absence of a glass forming agent such as glycerol.

To investigate this possibility, we studied three samples: (i) ApoF sedimented by MAS at room temperature from an aqueous solution containing TOTAPOL and then frozen; (ii) aqueous solution of ApoF and TOTAPOL frozen *sans* sedimentation; and (iii) same as (i) but without the addition of TOTAPOL. All samples were prepared from solutions with an initial protein concentration of 30 mg/mL in 90/10 (v/v) D_2O/H_2O in 3 mM tris(hydroxymethyl)aminomethane (Tris) buffer. Samples (i) and (ii) also contain 2 mM TOTAPOL. The reduction of the ^{1}H concentration in the matrix to ∼10% is known to yield optimal conditions for ^{1}H DNP.[8]

For preparation of samples (i) and (iii), U-^{13}C,^{15}N-ApoF was spun at $\omega_r/2\pi$ = 10 kHz, and cross-polarization (CP) was used to monitor the sedimentation *in situ*, typically over a period of a few hours. The sedimented sample was subsequently frozen *while spinning* using cooled N_2 gas (avoiding sediment dispersion) to perform MAS DNP measurements. The spinning frequency was reduced to 4.8 kHz at cryogenic temperatures (T < 90 K). Figure 1 illustrates significant gains in signal intensity from DNP under microwave irradiation (on-signal) for sample (i), indicating the incorporation of the radical into the sediment. The ^{1}H polarization and buildup time was investigated by CP to ^{13}C and yielded a 42-fold increase in signal strength as compared to the thermal (Boltzmann) polarization signal acquired without microwave irradiation (off-signal). Direct polarization of ^{13}C was observed via a Bloch decay; the enhancement factor was determined to be 22. Due to the absence of ^{13}C in the D_2O/H_2O matrix, spin polarization has to be transferred directly and cannot be transported through the matrix via spin diffusion. Therefore, the protein must be in proximity to TOTAPOL, limiting the distance between the unpaired electron spins and the uniformly ^{13}C-labeled protein.

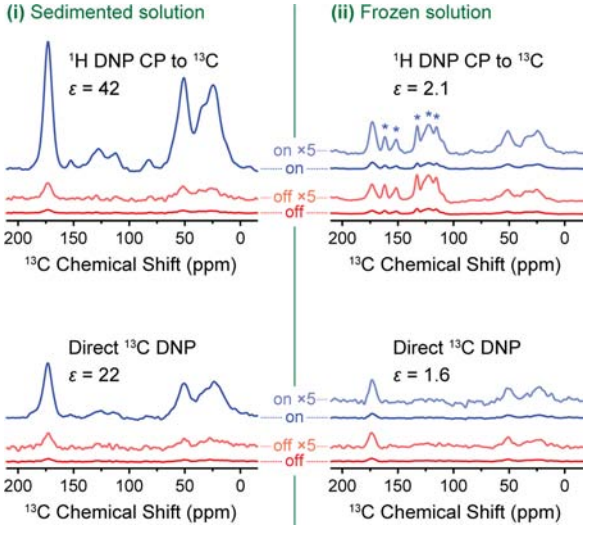

Figure 1. Comparison of DNP-enhanced signals from a frozen sedimented sample (i) and for a frozen solution (ii) using cross-polarization (^{13}C−^{1}H) and direct detection (^{13}C) under otherwise identical experimental conditions. DNP-enhanced spectra (on-signals) are given in blue, while thermal polarization spectra (off-signals) are given in red. Spectra are also scaled by a factor of 5 for better visualization (given in light red or blue color). Resonances marked with an asterisk arise from Vespel spacer material in the rotor which was used for the frozen solution.

In contrast, the frozen solution (ii) provides very poor enhancements ($\varepsilon \approx 2$) for both ^{1}H and ^{13}C polarization due to the inability to form a glass and phase separation of water, protein, and TOTAPOL, inhibiting effective electron−nuclear spin polarization (Figure 1). This shows that sedimentation provides a layer of glassy-like protein on the wall of the sapphire rotor, which enables the biradical to be homogeneously dispersed throughout the sediment, providing glass-like properties and efficient $e^- \rightarrow {}^1$H (^{13}C) polarization transfer.

We measured the polarization buildup time constants (T_B) and found them to be unusually short for the sedimented sample (i), suggesting direct protein−TOTAPOL interactions (*vide infra*). In order to assess those potential interactions, two d_8-glycerol/D_2O/H_2O (60/36/4 v/v) solutions were prepared with 2 and 15 mM TOTAPOL, respectively. Using a glass-forming agent to disperse the polarizing agent in a homogeneous *solvent* is a common approach in many DNP NMR experiments, and often provides the optimal enhancements and protects the protein from cold denaturation at cryogenic temperatures. The bulk ^{1}H concentration of 4% was chosen in order to slow homonuclear spin diffusion, and thus to enable us to partially discriminate between polarization transported from remote TOTAPOL to ApoF via spin diffusion versus direct transfer of polarization by bound TOTAPOL. Thus, samples with the appropriate TOTAPOL concentration were dissolved, and a fraction of the U-^{13}C,^{15}N-ApoF was dissolved and rapidly frozen in a MAS rotor inside the DNP NMR spectrometer.

The enhancements observed from the 2 and 15 mM solution samples were 70 and 100 (Figure 2), and biphasic buildup times on the order of 20 and 5 s, respectively, were found for the slow component. The fast component appeared with time constants of 1.1 and 0.6 s, respectively. The biphasic nature of the buildup can be explained by the existence of two distinct polarization transfer mechanisms, for example, spin-diffusion via bulk and direct transfer from protein-bound polarizing agent. This interpretation is further supported by an increase of the amplitude ratio between the slow and the fast components from 4.9:1 to 6.1:1 upon increasing the TOTAPOL concentration. Saturation of the binding sites obviously leads to a larger contribution of the bulk polarization transfer mechanism at higher TOTAPOL concentrations.

For the sedimented samples, the ^{1}H spin-polarization buildup time constants were found to be of the order of 1.2 (i) and 2.1 s (ii and iii). As suggested (*vide supra*), the difference in the observed polarization times could indicate an increased TOTAPOL concentration in the sediment with respect to the bulk solution. Using the glycerol data and relating these to the sedimented samples provides evidence that the TOTAPOL concentration is in fact higher in the sedimented samples. Buildup time constants and data from model systems suggest that the effective biradical concentration with respect to the protein is ∼10−20 mM. This provides a

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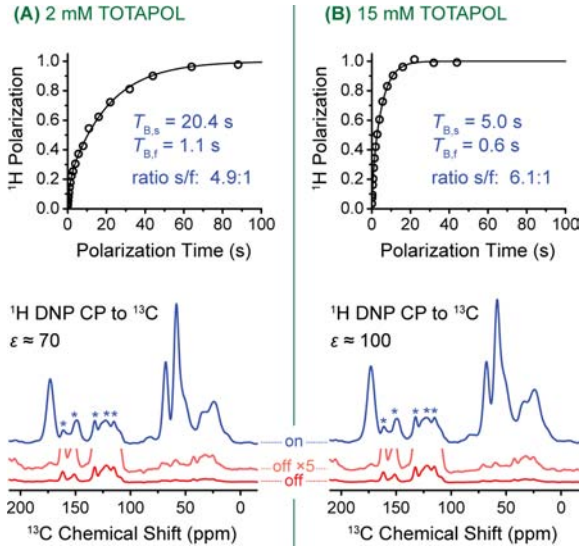

Figure 2. Comparison of DNP-enhanced signals from a cryoprotected ApoF sample (12 mg/mL) in d_8-glycerol/D_2O/H_2O (60/36/4 v/v) with ^{1}H polarization buildup time constants for 2 (A) and 15 mM (B) TOTAPOL concentration under otherwise identical experimental conditions. DNP-enhanced spectra (on-signals) are given in blue, while thermal polarization spectra (off-signals) are given in red. Spectra are also scaled by a factor of 5 for better visualization (given in light red color). Non-enhanced background signals from the Vespel spacers are marked with asterisks.

qualitative picture of the TOTAPOL preferentially binding to the surface of the ApoF 24-mer, and illustrates the importance of using low radical concentration (≤5 mM) for SedDNP studies of proteins. Radical binding to proteins was recently reported in other cases.[35] We note that biradicals were specifically developed to function at lower e^- concentrations than monomeric polarizing agents.

A summary of the ^{1}H and ^{13}C buildup times and ^{1}H polarization enhancements for all samples is provided in Table 1.

Table 1. Summary of DNP Enhancements and ^{1}H, ^{13}C Polarization Buildup Time Constants for All Samples

sample	^{1}H T_B (s)	^{13}C T_B (s)	ε (^{1}H/^{13}C)
frozen sediment, 2 mM TOTAPOL (i)	1.2	12.4	42/22
frozen solution, 2 mM TOTAPOL (ii)	2.1	13.4	2.1/1.6
frozen sediment sans TOTAPOL (iii)	2.1[a]	12.1[a]	–[b]/–[b]
cryoprotected 2 mM TOTAPOL (A)	20.4/1.1[c]	–[d]	70/–[d]
cryoprotected 15 mM TOTAPOL (B)	5.0/0.6[c]	3.5	100/~10

[a] T_B equals nuclear T_1 for non-DNP enhanced signals. [b] ε = 1 by definition for non-DNP-enhanced signals. [c] Slow and fast component of biphasic buildup. [d] Not determined.

Radical binding to the protein is not a feature of the SedNMR or SedDNP, but rather an aspect of the protein chemistry. Thus, we can expect that different proteins will interact differently with different biradical polarizing agents. Although we cannot predict the behavior *a priori*, one would expect that, if the radical were not interacting with the protein, its concentration in the sediment layer would be lowered. Assuming a 33% water content in the sediment,[34] and a non-interacting biradical, the concentration of the radical would be 0.66 mM, as compared to the 33 mM ferritin monomer, i.e., 1 radical molecule per 50 ferritin monomers (i.e., about 1 per 2 ferritin cages). In cases like these, one should then optimize the biradical concentration as is customary in a DNP experiment.

The enhancement from DNP allowed the acquisition of multidimensional spectra (Figure 3) of sedimented samples

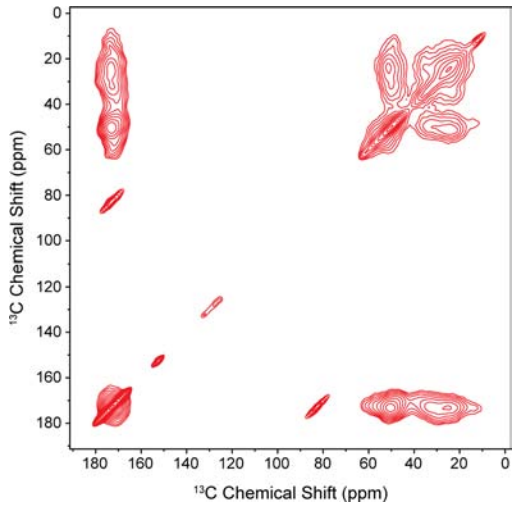

Figure 3. Representative DNP-enhanced 2D ^{13}C–^{13}C correlation spectrum (proton-driven spin diffusion, τ_{mix} = 20 ms) of ApoF sedimented from an D_2O/H_2O (90/10 v/v) solution at an initial concentration of 60 mM ApoF monomer containing 5 mM TOTAPOL. The acquisition period was ~5 h.

within hours using ~1.8 mg of a ~0.5 MDa protein complex. Recall that this was recorded at $\omega_{0I}/2\pi$ = 211 MHz and therefore does not permit resolution of individual cross peaks from a 20 kDa protein, but it does illustrate that standard 2D MAS experiments are feasible on a sedimented sample doped with TOTAPOL.

In summary, we have shown that sedimentation of the protein enables significant DNP enhancements without the addition of a glass-forming material such as glycerol, resulting in an ApoF/TOTAPOL glass at the wall of the rotor, leaving in the center of the rotor a pool of bulk water which undergoes crystallization upon freezing. The results reported here represent an important step toward DNP of proteins sedimented into an MAS rotor by ultracentrifugation—experiments that are currently underway. Enhancements are a factor of ~2 lower compared to the "standard approach", which may be attributed to short T_1 relaxation of nuclei, being induced by the high concentration of protons in the sediment, or by the increased content of paramagnetic polarizing agent within the sediment itself. The shorter T_B associated with the sedimented samples is useful in shortening the experimental acquisition time, resulting in almost identical sensitivity between SedDNP and DNP of homogeneously dispersed protein in glycerol/water. In practice these experiments will be performed by direct centrifugation of the sample into the rotor.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details for sample preparation and DNP NMR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.
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