**DNP-Enhanced MAS NMR of Bovine Serum Albumin Sediments and Solutions**

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DNP-Enhanced MAS NMR of Bovine Serum Albumin Sediments and Solutions

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ABSTRACT: Protein sedimentation sans cryoprotection is a new approach to magic angle spinning (MAS) and dynamic nuclear polarization (DNP) nuclear magnetic resonance (NMR) spectroscopy of proteins. It increases the sensitivity of the experiments by a factor of ∼4.5 in comparison to the conventional DNP sample preparation and circumvents intense background signals from the cryoprotectant. In this paper, we investigate sedimented samples and concentrated frozen solutions of natural abundance bovine serum albumin (BSA) in the absence of a glycerol-based cryoprotectant. We observe DNP signal enhancements of ε ∼ 66 at 140 GHz in a BSA pellet sedimented from an aqueous solution containing the biradical polarizing agent TOTAPOL and compare this with samples prepared using the conventional protocol (i.e., dissolution of BSA in a glycerol/water cryoprotecting mixture). The dependence of DNP parameters on the radical concentration points to the presence of an interaction between TOTAPOL and BSA, so much so that a frozen solution sans cryoprotectant still gives ε ∼ 50. We have studied the interaction of BSA with another biradical, SPIROPOL, that is more rigid than TOTAPOL and has been reported to give higher enhancements. SPIROPOL was also found to interact with BSA, and to give ε ∼ 26 close to its maximum achievable concentration. Under the same conditions, TOTAPOL gives ε ∼ 31, suggesting a lesser affinity of BSA for SPIROPOL with respect to TOTAPOL. Altogether, these results demonstrate that DNP is feasible in self-cryoprotecting samples.

INTRODUCTION

Bovine serum albumin (BSA) is a highly soluble globular protein of 67 kDa molecular weight. BSA is known to stabilize biomolecules under otherwise denaturing conditions; for example, it has been shown to have cryoprotecting properties, reducing damage of enzymes during storage at low temperature.1 Furthermore, with centrifugation, BSA forms a concentrated sediment (or pellet,2–6 reported protein content values are about 600–700 mg/mL2,4), which is composed of a significant volume of protein which reduces the amount of free water within the sample.4,7,8 This in turn is likely to limit the formation of neat ice crystals, at least in close proximity of the protein molecules (i.e., bound water is limited to the surface and pores of the protein, inhibiting degradation from freezing). The cryoprotective properties together with the tight packing of the protein molecules in a sediment layer might preserve the protein itself from cold denaturation processes.

Nuclear magnetic resonance (NMR) is an excellent spectroscopic technique to examine protein structure, function, and dynamics, especially in noncrystalline environments. In particular, NMR has the ability to locally probe the nuclei of interest providing both short- (<4 Å) and medium-range (4–7 Å) length scales. Unfortunately, because of the small nuclear Zeeman polarization, NMR is a low sensitivity technique, and therefore studies of low abundant nuclei (e.g., 15N and 15C) are often challenging. A highly successful method to increasing sensitivity is dynamic nuclear polarization (DNP), a concept initially proposed by Overhauser9 and demonstrated soon thereafter by Carver and Slichter.10 DNP relies on the transfer of electron polarization (typically from an organic based polarizing agent)11–18 to neighboring nuclei, and for 1H, a polarization enhancement of up to ∼600 can in principle be achieved.19 In the 1990s, magic angle spinning (MAS) DNP utilizing gyrotrons as high power microwave sources20–28 was introduced, and this led to widespread applications of DNP in MAS NMR studies,29,30 especially of biological systems such as globular proteins, membrane proteins, nanocrystals, amyloid fibrils, and DNA31–44,69 and more recently in materials science.45–50

For biological systems, the analyte is typically dispersed in a cryoprotecting solution containing the polarizing agent. Although homogeneous solutions of globular proteins can be investigated,31 the ideal analyte forms a heterogeneous solution that is phase-separated from the cryoprotecting solvent/polarizing agent, for example, proteins embedded in a bilayer membrane,32,36,37 amyloid fibrils33,34,51 or insoluble nanocryst-
The cryoprotecting properties of the glass-forming matrix prevent the phase separation of solvent and polarizing agent, and also prevent formation of grain boundaries due to crystallization upon freezing. The inhibition of crystallization allows for efficient dispersal of polarization from the bulk to the analyte. The sediment is to some extent separated from the bulk solvent; thus, an amorphous glass-like environment may be formed at cryogenic temperatures. Recently, we demonstrated the possibility of studying NMR of sedimented solutes (SedNMR) with DNP experiments. In particular, the sediment has proven as an ideal matrix for dispersing biradical polarizing agents and inhibiting crystallization, and therefore is an extremely suitable target for DNP, an approach which we termed SedDNP.

In this study, we investigate BSA sedimented 

**RESULTS AND DISCUSSION**

**BSA Sedimented DNP.** Cryoprotection is often required when temperature cycling a protein below 273 K in order to avoid cold denaturation and to maintain the integrity of the protein structure at low temperatures (< -75 °C). The addition of a glass-forming solvent, often glycerol, is used to inhibit bulk ice crystallization, enabling the formation of an amorphous solid that protects the protein, and disperses the polarizing agent if it is present. However, if, due to self-crowding, the tightly packed soluble protein forms a glassy state upon freezing of the water matrix, then the addition of a cryoprotectant is superfluous. The feasibility of cryoprotectant-free DNP by sedimenting BSA was tested using a solution with an initial concentration of 100 mg/mL in 90/10 (v/v) D2O/H2O to which 5 mM TOTAPOL was added. Following centrifugation (75 000 rpm for 24 h), the sediment (~50 μL) was packed into a sapphire rotor and inserted into the NMR probe, which had been precooled to cryogenic temperatures (between 85 and 90 K). Irradiation with 8 W of 140 GHz microwaves resulted in a 66-fold enhancement (ε) of the protein CPMAS NMR signal (Figure 1).

![Figure 1. DNP-enhanced (“mw on”, blue) and thermal equilibrium (“mw off”, red) polarization 13C-CPMAS spectrum of natural abundance BSA sedimented from a 100 mg/mL solution in 90/10 (v/v) D2O/H2O with 5 mM TOTAPOL. The thermal equilibrium spectrum has also been multiplied by a factor of 10 (“off ×10”, red) for better comparison.](image-url)

The magnitude of this enhancement is comparable to typical DNP experiments on proteins, where samples have been prepared by dissolving the protein in a glycerol/water mixture. However, the 1H polarization buildup time constant of T_{1B} = 1.8 s is short compared to a conventional approach. The rapid buildup of 1H polarization is most likely caused by the high protein 1H density in the sediment in combination with an increased biradical concentration due to potential protein–TOTAPOL interactions. In an earlier study, we have observed preferential enrichment of TOTAPOL in the sediment layer in SedDNP. In order to further investigate this situation, the TOTAPOL concentration of the BSA solution was varied prior to sedimentation. Three samples...
were prepared with 200 mg/mL BSA each and 2.5, 5, and 10 mM TOTAPOL concentration, respectively, and the results are shown in Figure 2. DNP enhancements increase, $\varepsilon = 29, 48,$ and 64, with increasing TOTAPOL concentration, while $^1H$ buildup times showed an inverse trend with $T_B = 3.6, 2.6,$ and 1.6 s, respectively. In a control experiment, the spin–lattice relaxation time constant, $T_1 = 6.3$ s, was measured for a sample prepared in an identical manner sans TOTAPOL.

Interestingly, when comparing enhancements as well as buildup rates (i.e., $T_B^{-1}$) in Figure 2B and C, we observe very similar values for the sample sedimented from 100 mg/mL BSA doped with 5 mM TOTAPOL (blue, squares) and BSA sedimented from 200 mg/mL doped with 10 mM TOTAPOL (red circles). This suggests that it is not the absolute TOTAPOL concentration in the solution prior to centrifugation that is determining the TOTAPOL in the sediment but rather the TOTAPOL to protein concentration ratio (doping ratio) that is preserved during sedimentation. That would be the case if TOTAPOL were tightly or transiently binding to the protein, with the equilibrium much in favor of the protein–TOTAPOL complex in the solution. During centrifugation, TOTAPOL is then sedimented together with the protein (Figure 3A). A similar case has been observed during SedDNP for apoferritin and TOTAPOL. Further parameters and details for all samples are provided below in Table 1. The last three columns of Table 1 assist in describing sensitivity by taking into account the final sedimented protein concentration (i.e., 600 mg/mL) and appropriate scaling for both DNP $^1H$ enhancement ($\epsilon_{BSA}$) and repetition rate ($T_B^{-1/2}$) and combining all parameters ($\epsilon \times \epsilon_{BSA}/(T_B)^{1/2}$) to provide an overall enhancement factor. It is important to point out the enhancement previously recorded within sedimented apoferritin is most probably nonspecific due to the fact that the hydrophobic patches present on the protein surface are more concentrated in the sediment. This would provide a more suitable environment for the biradical TOTAPOL to partition within the sedimented protein layer with respect to the bulk solution (Figure 3B). Figure 3C represents a situation that has not yet been encountered where the protein does not interact with the radical. In this case, it is expected that the concentration of the radical will be uniform throughout the sample, regardless of the gradient formed by the protein, and this situation is not different from the radical distribution observed in the usual DNP sample (i.e., $d_8$-glycerol/D$_2$O/H$_2$O).

Direct binding between BSA and TOTAPOL is not unexpected. BSA contains two hydrophobic binding sites that could provide a preferential environment for the partially hydrophobic TOTAPOL. Furthermore, TOTAPOL possesses a relatively flexible structure, allowing it to adopt a conformation suitable for binding. The combination of amphiphilicity and flexibility could further improve TOTAPOL’s tendency to interact with the protein, allowing a molar ratio between bound TOTAPOL and BSA larger than 2. At the same time, it is important to emphasize that radical binding to the protein is not an intrinsic feature of MAS DNP but rather an intrinsic
Table 1. BSA Sample Conditions and DNP NMR Results for a Series of BSA/TOTAPOL Sedimented Mixtures (SedDNP)

<table>
<thead>
<tr>
<th>ε_{BSA} (mM)</th>
<th>ε_{TOTAPOL} (mM)</th>
<th>ε_{TOTAPOL}/ε_{BSA} (doping ratio)</th>
<th>ε</th>
<th>T_B (s)</th>
<th>ε × ε_{BSA} (mM)</th>
<th>ε/(T_B)^{1/2} (s^{-1/2})</th>
<th>ε × ε_{BSA}/(T_B)^{1/2} (mM s^{-1/2})</th>
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<td>3.03 (200 mg/mL)</td>
<td>0.00</td>
<td>0.00</td>
<td>6.3</td>
<td>9.1</td>
<td>0.4</td>
<td>3.6</td>
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<tr>
<td>3.03 (200 mg/mL)</td>
<td>2.5</td>
<td>0.83</td>
<td>29</td>
<td>3.6</td>
<td>263.6</td>
<td>15.2</td>
<td>138.1</td>
</tr>
<tr>
<td>3.03 (200 mg/mL)</td>
<td>5.0</td>
<td>1.65</td>
<td>48</td>
<td>2.6</td>
<td>436.3</td>
<td>29.5</td>
<td>268.3</td>
</tr>
<tr>
<td>3.03 (200 mg/mL)</td>
<td>10.0</td>
<td>3.29</td>
<td>64</td>
<td>1.6</td>
<td>581.8</td>
<td>50.6</td>
<td>460.1</td>
</tr>
<tr>
<td>1.52 (100 mg/mL)</td>
<td>5.0</td>
<td>3.29</td>
<td>66</td>
<td>1.8</td>
<td>601.9</td>
<td>49.2</td>
<td>448.6</td>
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Table 2. BSA Sample Conditions and DNP NMR Results for a Series of BSA/TOTAPOL Sedimented Mixtures (SedDNP)

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<thead>
<tr>
<th>ε_{BSA} (mM)</th>
<th>ε_{TOTAPOL} (mM)</th>
<th>ε_{TOTAPOL}/ε_{BSA} (doping ratio)</th>
<th>ε</th>
<th>T_B (s)</th>
<th>ε × ε_{BSA} (mM)</th>
<th>ε/(T_B)^{1/2} (s^{-1/2})</th>
<th>ε × ε_{BSA}/(T_B)^{1/2} (mM s^{-1/2})</th>
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<td>0.00</td>
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<tr>
<td>1.14 (75 mg/mL)</td>
<td>4.39</td>
<td>35</td>
<td>0.44</td>
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<td>52.7</td>
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<tr>
<td>1.52 (100 mg/mL)</td>
<td>3.29</td>
<td>50</td>
<td>0.55</td>
<td>75.8</td>
<td>67.2</td>
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<tr>
<td>3.03 (200 mg/mL)</td>
<td>1.65</td>
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<tr>
<td>6.06 (400 mg/mL)</td>
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<td>2.47</td>
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Initial concentration before sedimentation. The BSA concentration in the sediment is assumed to be 600 mg/mL (i.e., a factor of 3–6 times larger than presedimented starting material).
respectively. The small difference between the two latter samples lies well within the experimental uncertainty. Figure 5C illustrates the problem of significant $^{13}$C background from residual $^{13}$C-glycerol overlapping with the $\alpha$ spectral region in this case of the natural abundance BSA. This background can be circumvented either by utilizing isotopically depleted solvent or by application of this novel method of cryoprotectant-free DNP.

**Protein/Radical Interactions.** Figure 6 summarizes the effect of specific protein–radical binding on the DNP performance under the distinct conditions of sediment and highly concentrated solutions. When the binding is strong as is the case in the study at hand, the radical that is bound in solution remains with the protein also when the solution is frozen. On the contrary, if the interaction is weak, the radical tends to partition into the more hydrophobic sediment rather than staying in the aqueous solution, but when the sediment is not present the radical will tend to segregate. The latter case has been observed in a previous article by the same authors on the protein complex apoferritin.

The data at hand clearly raises the question of the sample preparation method that optimizes the DNP-enhanced NMR sensitivity. This sensitivity is not only determined by DNP enhancement factors but also depends strongly on the optimal recycle delay between acquisitions and therefore on the buildup time constant. Another simpler factor determining sensitivity is the analyte concentration. Generally, a shorter recycle delay allows for faster acquisition of the spectra; however, in many cases, the minimum recycle delay is determined by instrumental limitations. Although sample heating is of minor concern during DNP experiments due to active sample cooling and low dielectric properties of the frozen sample, high-power decoupling of protons results in a significant rf duty cycle at short recycle delays. In cases where the recycle delay is instrumentally limited, a quantitative assessment of sensitivity cannot be straightforwardly given. Several measures of sensitivity are given in Tables 1 and 2, including effects from DNP enhancement ($\varepsilon$), the size of the recycle delay ($T_B^{-1/2}$), and analyte concentration ($c_{BSA}$). In Figure 7, the overall DNP-enhanced NMR sensitivity ($\varepsilon \times c_{BSA}/(T_B)^{1/2}$) is shown for sediments (Table 1) and concentrated solutions (Table 2) of BSA in 90:10 (D$_2$O/H$_2$O) with 5 mM TOTAPOL (dashed line, gray).
solutions with large BSA concentrations and BSA sediments yield sensitivities superior to those obtained with a glycerol/water solution; in particular, a sensitivity gain of almost 5-fold can be obtained with the sediment.

The potential for signal quenching induced by strong paramagnetic interactions is always of concern, although challenging to measure accurately due to variable issues. To account for paramagnetic quenching effects within the sedimented samples, four $^{13}$C-CPMAS experiments were acquired under identical conditions (i.e., sample volume ($50 \pm 5 \mu L$), BSA concentration (200 mg/mL), temperature, recycle delay ($1.3 \times T_B$), spectrometer parameters (e.g., gain, CP parameters, coadded transients, etc.), and performing all experiments without microwaves). Even with this careful attention to detail, we expect our uncertainty in these measurements to be approximately 10% of the observed signal intensity. Using the off signal from the nondoped sample scaled to 1, we ascertain that doping ratios < 1 are well within experimental error and minimize quenching effects; heading toward a doping ratio of 3, a loss of 40 ± 10% is observed (Figure 8). These interactions have been recently studied extensively by Corzilius et al., whereby a loss in signal intensity occurs while under magic-angle spinning conditions but does not occur for nonspinning samples. These losses in signal have been seen for both the narrow-line radical, trityl (∼35%), and the wide-line nitroxide biradical, TOTAPOL (∼45%), in agreement with our study on sedimented BSA. Although the paramagnet induces some quenching of the $^{13}$C signal intensity, Figure 8A illustrates the significant gain in overall sensitivity when the overall DNP-enhanced sensitivity ($E = \varepsilon \times c_{BSA}/(T_A)^{1/2}$) is taken and multiplied by paramagnetic quenching observed from the off spectrum (Figure 8B). This treatment was not applied to the DNP-enhanced BSA concentrated solutions due to the drastic differences in protein concentrations (i.e., 50 mg/mL vs 400 mg/mL could lead to maintaining identical probe efficiency due to the drastically different physical (i.e., dielectric) properties of a low (e.g., <100 mg/mL) versus a high (>200 mg/mL) viscosity sample.

Direct binding interactions between TOTAPOL and BSA potentially raise concerns about paramagnetic interactions and resonance broadening of protein NMR signals. Quantifying paramagnetic broadening was attempted by analyzing the line width of the carbonyl resonance. Although the line shape represents the envelope of all individual carbonyl resonances (∼582) of the protein and is therefore due mainly to inhomogeneous effects, it serves as an acceptable first approximation to measure for possible homogeneous broadening. Results are shown in Figure 9. The full width at half-

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Paramagnetic signal quenching effects on the 200 mg/mL sedimented BSA samples. (A) Paramagnetic signal quenching for the carbonyl (C$\beta$) and aliphatic (C$\alpha$ and C$\gamma$) regions, determined from a prepared sample without radical (NB: off-signal were compared for four sedimented BSA samples ranging from 0 to 3.29 doping ratio). (B) DNP-enhanced NMR sensitivity of BSA samples with increased TOTAPOL to protein doping ratio, $^1$H DNP enhancement (open circles, red), absolute sensitivity with paramagnetic quenching (open diamonds, blue), and absolute sensitivity without paramagnetic quenching (closed diamonds, blue).

![Figure 9](https://example.com/figure9.png)

**Figure 9.** Effect of increasing doping ratio on paramagnetic broadening of BSA. BSA solutions (filled circles, black), sedimented samples from 200 mg/mL (open circles, red), sedimented sample from 100 mg/mL solution (open square, blue), and the 160 mg/mL BSA in 60/30/10 (v/v/v) $d_2$-glycerol/D$_2$O/H$_2$O with 5 mM TOTAPOL (dashed line, gray).

maximum (fwhm) is found to vary between ∼7.5 and 10 ppm. Interestingly, both the sedimented BSA sample as well as the solution with the lowest doping ratio of ∼0.83 ($c_{TOTAPOL}/c_{BSA}$) show line widths slightly below the line width found for the BSA solution in glycerol/water (7.6 ppm). On the basis of these data, we conclude that line broadening is of little or no concern in comparison to the “standard” DNP sample preparation, as long as the doping ratio is kept low. As such, a three-way balance is achieved in order to optimize radical concentration for buildup times, enhancement, and resolution at cryogenic temperatures. The study of natural abundance BSA limits our ability to probe specific sites in order to ascertain signal quenching or broadening in the hydrophobic region of the protein are not possible at this time. By applying selective labeling protocols and moving toward high-field DNP NMR spectrometers (i.e., 600/395, 700/460, and 800/527 MHz/ GHz), it will be possible to achieve further resolution and further details regarding protein–radical interactions.

The clear interaction between BSA and TOTAPOL prompted an evaluation of the binding behavior in the presence of a different radical, SPIROPOL (Figure 10), that has been shown to yield ∼20% larger enhancement for model systems as compared to TOTAPOL while still being soluble in glycerol/water mixtures. However, the solubility in pure water is limited to 3 mM. Like TOTAPOL, SPIROPOL is a bis-nitroxide-
based radical but it is bulkier and less flexible, and thus may show different binding affinity toward BSA (Figure 10). In a control experiment, SPIROPOL did yield a larger enhancement in a 5 mM 60/30/10 d<sub>c</sub>-glycerol/D<sub>2</sub>O/H<sub>2</sub>O solution containing 160 mg/mL BSA. In particular, ε = 89 is about 14% larger than ε = 78 found for TOTAPOL under otherwise identical conditions. However, when comparing the DNP behavior in a 90/10 (v/v) D<sub>2</sub>O/H<sub>2</sub>O solution containing 400 mg/mL BSA, the enhancement obtained with 2.5 mM SPIROPOL (ε = 26) was lower than that measured using the same concentration of TOTAPOL (ε = 31). The reason for this is not yet clear. As already mentioned, the differences in flexibility and hydrophobicity might lead to different binding behavior. At the same time, SPIROPOL is used at the upper limit of its solubility in water and might undergo a more pronounced phase separation within the bulk water during freezing. On the basis of these results, we suspect that TOTAPOL may have a higher affinity for BSA than SPIROPOL. This leads to an improved H DNP enhancement of the former within the concentrated solution, whereas SPIROPOL is more effective in the traditional glassing matrix (glycerol/water).

**SUMMARY**

Using a model globular protein, BSA, a high-throughput method using sedimentation preparation has been demonstrated, which reduces the need for a cryoprotecting matrix in DNP experiments. The sedimentation approach provides efficient DNP enhancements while circumventing unwanted background signals, which can affect systems in natural abundance, limited sample volumes, or sparsely labeled large biological solids. Utilizing various sample preparation approaches and radical concentrations, we have proposed three radical/protein interaction models, which can affect the SedDNP approach. BSA/TOTAPOL binding was determined to be site-specific, whereas our previous study on apoferritin/TOTAPOL exhibited nonspecific binding, thus requiring in situ sedimentation for effective DNP. Substituting the type of polarizing agent (i.e., SPIROPOL vs TOTAPOL) may allow adjustment of radical/protein interaction to maintain effective sensitivity gain and minimize broadening effects on NMR spectra. We have shown the SedDNP method provides a facet for high sample throughput in order to achieve significant gains in sensitivity, while maintaining clean, background-free 13C spectra. With further investigations of the sedimentation process for DNP NMR, this approach may be applied to selectively labeled biological systems for improved access to determination of structure and function.

**REFERENCES**
