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Competitive Displacement Restores the Hyperpolarized ^{15}N NMR Signal in Blood Plasma

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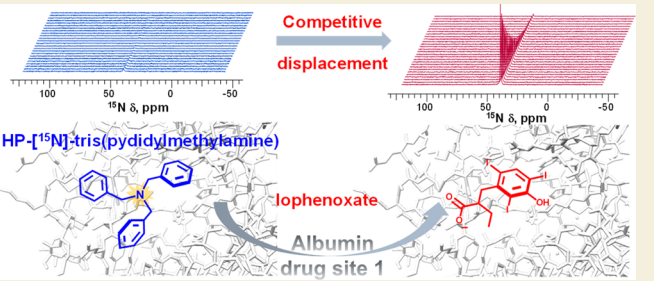
ABSTRACT: Hyperpolarized (HP) NMR can improve the sensitivity of conventional NMR experiments by several orders of magnitude, thereby making it feasible to detect the signal of low sensitivity nuclei such as ^{13}C and ^{15}N nuclei in vivo. Hyperpolarized substrates are usually administered by direct injection into the bloodstream, and interaction with serum albumin can cause rapid decay of the hyperpolarized signal due to the shortening of the spin−lattice (T_1) relaxation time. Here we report that the ^{15}N T_1 of ^{15}N labeled, partially deuterated tris(2-pyridylmethyl)amine decreases dramatically upon binding to albumin to such an extent that no HP-^{15} signal could be detected. We also demonstrate that the signal could be restored using a competitive displacer, iophenoxic acid, which binds stronger to albumin than tris(2-pyridylmethyl)amine. The methodology presented here eliminates the undesirable effect of albumin binding and should widen the range of hyperpolarized probes for in vivo studies.



KEYWORDS: *hyperpolarized NMR, dynamic nuclear polarization, ^{15}N NMR, albumin binding, competitive displacement, T_1 relaxation*

Hyperpolarized (HP) NMR encompasses innovative NMR technologies aiming at improving the sensitivity of conventional NMR experiments by generating nonequilibrium spin distributions on the nuclear Zeeman levels. These techniques include dynamic nuclear polarization (DNP), parahydrogen induced polarization (PHIP) and spin exchange optical pumping (SEOP).[1] Of these, DNP is used most frequently because it is applicable to all NMR active nuclei. Dissolution DNP is improves the sensitivity of liquid state NMR by the microwave driven transfer of high electron spin polarization to coupled nuclear spins at around 1 K followed by rapid dissolution of the sample.[2] The signal enhancement can be dramatic, several orders of magnitude, depending on the nucleus and experimental setup allowing for the rapid detection of low sensitivity nuclei at low concentration. Regardless of the HP-technique used or the nucleus polarized, the resulting hyperpolarized magnetization is not persistent but returns to thermal equilibrium by spin−lattice (T_1) relaxation. The inexorable decay of HP-magnetization is a severe limitation of the technique. As the HP-signal can be observed for about a 5 T_1 period at most, unlike in conventional NMR, long T_1 values are advantageous for HP-NMR experiments.[3] The strong signal enhancements make it feasible to perform in vivo MRI of nuclei that have long T_1, in particular, ^{13}C, ^{129}Xe and ^{15}N. Hyperpolarized NMR/MRI offers a convenient and very elegant method to follow the in vivo fate of injected hyperpolarized probes including metabolic substrates, perfusion markers, drugs and sensors of various biomarkers (pH, redox, metal ions, etc.).[4] A prominent application of HP-NMR is metabolic imaging with HP-[1-^{13}C]pyruvate, which is currently in clinical trials at several sites.[5] Long T_1 values are particularly important for in vivo studies to preserve sufficient hyperpolarized magnetization for the successful observation of the signal. The hyperpolarized solution is generally administered by direct injection into the bloodstream and consequently, the HP-probe immediately comes into contact with plasma proteins. The most abundant plasma protein is albumin, whose main physiological role includes transporting various molecules.[6,7] Albumin can reversibly bind a wide variety of structurally diverse molecules including fatty acids, amino acids, and various aromatic compounds. The structure and remarkable binding ability of albumin has been studied in great detail.[6,8−10] The protein has two primary drug binding sites capable of binding various heterocyclic and aromatic compounds ("drugs") with high affinity (K_a approximately 10^4 to 10^6 M).[6,11,12]

HP-^{15}N as well as HP-^{13}C labeled versions of drug molecules would be immensely useful to follow the in vivo metabolic fate of these compounds. However, the majority of drugs contain aromatic rings and/or nitrogen heterocycles, structural units

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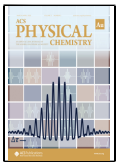
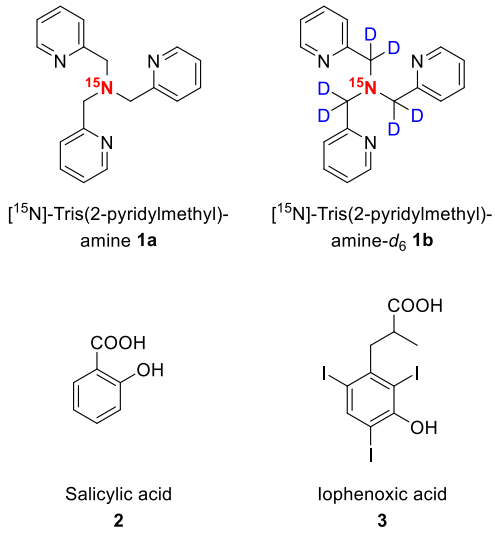


that can strongly bind to albumin.[13,14] Interaction with albumin can speed up both the spin−lattice and spin−spin relaxation of NMR active nuclei present in these moleules.[15−17] Relaxometry of the aromatic protons of acetylsalicylate was used to study the interaction of this drug with albumin.[16] Shortening of the T_1 and increased line width of the ^{13}C resonance in hyperpolarized [8-^{13}C]Aspirin (acetylsalicylic acid) due to binding and exchange with albumin was observed both in vitro and in vivo.[18,19] These undesirable effects were reduced somewhat in the presence of 3-fold excess of unlabeled Aspirin.[19] ^{15}N generally offers longer T_1 relaxation times than ^{13}C due to its lower gyromagnetic ratio. ^{15}N labeled heteroaromatic molecules such as nicotinamide and metronidazole can be efficiently hyperpolarized using parahydrogen based methods (SABRE).[20−24] These compounds are also known to bind to albumin with a K_a of around 10^5 M^{-1} and consequently, albumin binding is expected to shorten the ^{15}N T_1 values.[25,26]

Here we report that a competitive inhibitor (displacer) can restore a the HP-NMR signal of a ^{15}N labeled probe whose ^{15}N spin−lattice relaxation time is dramatically shortened upon binding to albumin to such an extent that no signal could be detected when the albumin concentration was in the physiological range.

Recently we demonstrated that hyperpolarized ^{15}N-labeled [^{15}N]-tris(2-pyridylmethyl)amine derivatives (**1a** and **1b**, Chart 1) could sense Zn^{2+} with high selectivity and with a

Chart 1. Structure of ^{15}N Labeled Tris(2-pyridylmethyl)amine Zinc Sensors, Salicylic Acid, and Iophenoxic Acid



detection threshold between 1 and 5 μM by ^{15}N NMR spectroscopy after activation by dissolution DNP.[27] Tris(2-pyridylmethyl)amine is a tripodal ligand that forms a high stability complex with Zn^{2+} with excellent selectivity over other endogenous metal ions.[28] HP-**1b** was successfully used to detect and quantify Zn^{2+} in benign prostatic hyperplasia biopsy samples and human prostatic cell line (PNT1A) by ^{15}N NMR.[27] In the Zn^{2+} complex, the four nitrogen donor atoms of the ligand coordinate to Zn^{2+}, which causes a 20 ppm upfield shift in the ^{15}N resonance of the central ^{15}N atom. (Figure S1).[27] The dominant spin−lattice relaxation mechanism for the ^{15}N label is by dipole−dipole interactions with intramolecular ^1H spins and deuteration improved the ^{15}N T_1 from 26 s (**1a**) to 71 s (**1b**) at 9.4 T.[27] It is also worth noting that in accordance with the dipolar relaxation mechanism, the T_1 was found to be largely field independent in the range of 1 to 9.4T.[27] Several optical and ^1H MRI zinc probes contain tris(2-pyridylmethyl)amine derived Zn-sensing moieties.[29,30] These agents are known to bind to albumin.[30,31] In fact, the sensing mechanism of the Gd-based zinc responsive MR agents involves binding to albumin.[30] However, interaction with albumin is expected to be undesirable for hyperpolarized tracers. Therefore, the effect of albumin binding on the hyperpolarized ^{15}N signal of tris(2-pyridylmethyl)amine probes was studied. When the hyperpolarized probes (**1a** and **1b**) were added to plasma or human serum albumin (albumin content about 0.5 mM) the total loss of hyperpolarized ^{15}N magnetization was observed within seconds (Figures 1 and S2).

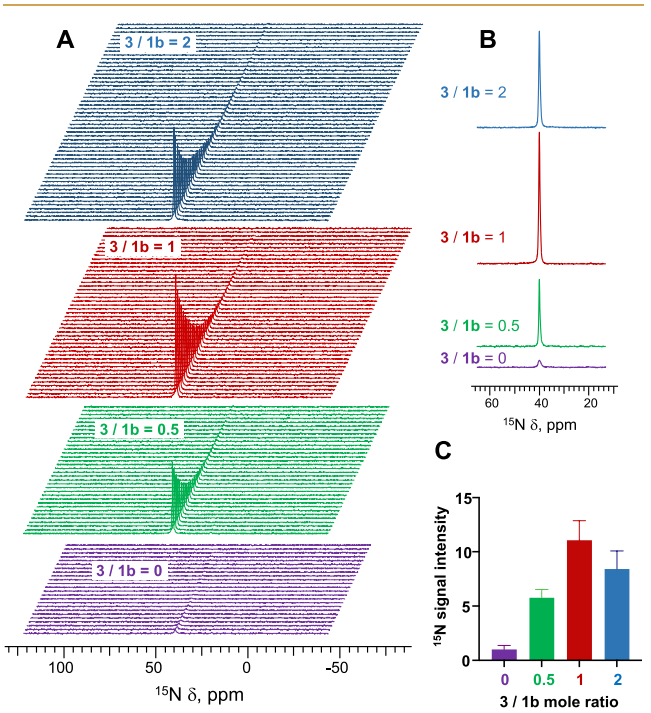


Figure 1. Effect of increasing amount of competitive displacer **3** on the ^{15}N NMR spectra of HP-(**1b**) (1.72 mM) in human plasma (HSA concentration ~0.45 mM) at 9.4 T. The **3**/**1b** mole ratio increases from 0 (no displacer) to 2. (A) Dynamic ^{15}N NMR spectra recorded every 5 s with a 5° flip angle at 9.4 T showing the decay of ^{15}N magnetization. (B) The first spectrum of each array showing the relative ^{15}N signal intensities. Maximum signal intensity was observed at 1:1 mol ratio. (C) Plot of ^{15}N signal intensity vs the mole ratio of **3** to **1b**.

To address this problem, the feasibility of using a competitive inhibitor (displacer) to eliminate or reduce the albumin binding was tested. The concept of competitive inhibition is well established in enzymology.[32] A competitive inhibitor is a compound that reversibly binds to a protein at the same site as the substrate. The inhibitor generally bears some structural similarity to the substrate. Competitive inhibition is often used to study the interaction of drugs with their target proteins.[32,33] Competitive displacement in combination with ^1H and ^{19}F NMR (including HP-[19] labeled

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reporter ligands) has become a powerful drug screening technique.[33−36]

The binding constant of unlabeled tris(2-pyridylmethyl)-amine to human serum albumin (HSA) was measured by isothermal titration calorimetry (ITC) (Figure S3). The ITC data showed that tris(2-pyridylmethyl)amine binds strongly to only one site with a K_a of about 1×10^7 M^{-1}. In preliminary experiments, we first tested the more easily available HP-(**1a**) in HSA and plasma in the absence and presence of salicylate (**2**) (Chart 1) as competitive displacer. However, salicylate only partially restored the ^{15}N signal (~19%, Figure S2), which was expected because it is an about 3 times weaker binder (K_a = 3.3 × 10^6 M^{-1}) than tris(2-pyridylmethyl)amine.[37−39] Therefore, salicylate was not further investigated. Next, we tested iophenoxic acid (**3**), which was reported to bind reversibly to site 1 of albumin with extraordinarily high affinity (it is one of the strongest binding ligands of albumin, K_a = 5.6 × 10^7 M^{-1}) and readily displaces compounds that bind to this site.[40] In addition, iophenoxic acid has low toxicity and was used as an X-ray contrast agent (Teridax). We also switched to **1b** rather than **1a** in subsequent HP-experiments because of its much longer T_1 value. This allowed the observation of a small residual signal in blood plasma even in the absence of a displacer (Figure 1). Competitive displacement experiments in which the mole ratio of **3** to HP-(**1b**) was varied from 0 to 2 revealed that equivalent amount of **3** nearly completely restored the hyperpolarized ^{15}N signal of **1b** (Figure 1). The ^{15}N T_1 of **1b** was found to be about 40 s in the presence of **3** and HSA. Although the ^{15}N NMR signal of **1b** in the albumin bound state could not be observed due to fast exchange, the T_1 of **1b** in the bound state was estimated to be around 6 s from the apparent T_1 in the absence of **3** (Table 1) using eq S2 (see the Supporting Information). Binding to albumin also led to significant line broadening, which was reduced in the presence of the displacer (Table 1). Thus, the low signal intensity upon albumin binding is due to a combination of enhanced spin−lattice and spin−spin relaxation in the bound state. Interestingly, the maximum ^{15}N signal was observed when **3** and **1b** were present in equimolar ratio. Further increases of the **3** to **1b** mole ratio resulted in a slight decrease in the ^{15}N signal intensity. The exact reason for this is not clear but may be due to the interaction between **1b** and **3** involving the aromatic rings.[41]

Table 1. Experimentally Measured T_1 Values and Line Widths of the ^{15}N NMR signal of HP-(1b) in Human Plasma in the Absence and Presence of Increasing Amounts of the Competitive Displacer (3)

3/**1b** mole ratio	T_1 (s)[a,b]	fwhm (Hz)[a,b,c]
0	34.4 ± 2.8	50.3 ± 0.8
0.5	39.8 ± 6.3	21.0 ± 0.9
1	39.8 ± 4.8	20.1 ± 3.3
2	40.2 ± 5.4	21.2 ± 4.1

[a]25 °C and 9.4 T. [b]In PBS buffer (pH 7.4). [c]Full width at half-maximum ($\nu_{1/2} = 1/\pi T^*_2$).

Interestingly, although the spin−lattice relaxation of **1b** is significantly accelerated by the interaction with HSA, the ^{15}N signal decay remains virtually unaffected when **1b** is complexed with Zn^{2+} (the ^{15}N T_1 of the zinc complex was about 40 s in the presence of HSA) (Figure 2). This must be the consequence of the diminished binding of the positively charged Zn^{2+}(**1**) complexes to albumin, which is not entirely unexpected as site 1 primarily binds hydrophobic neutral and anionic species. It should also be noted that the Zn^{2+}(**1b**) binding (log K_{ZnTPA} = 11.0) is 4 orders of magnitude higher than that of the Zn^{2+}(HSA) complex (log K_{ZnHSA} = 7.1)[28,42,43] and thus, **1b** can successfully compete with albumin for Zn^{2+}.

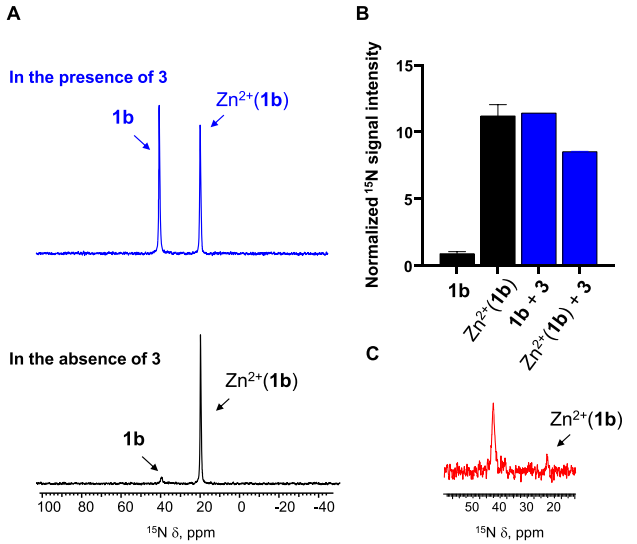

Figure 2. Effect of competitive displacer **3** on the ^{15}N NMR spectra of HP-(**1b**) (1.72 mM) in the presence of half equivalent Zn^{2+} (0.85 mM) in human plasma (0.45 mM). (A) First spectrum in the absence and presence of equimolar **3** (1.72 mM). (B) Plot of the respective ^{15}N signal intensities. (C) HP-**1b** can detect endogenous Zn^{2+} in blood plasma even in the absence of **3**.

Remarkably, HP-(**1b**) detected the endogenous level of Zn^{2+} present in plasma (Figure 2C and S4) demonstrating the high sensitivity of HP-[15] NMR.

The accelerated signal decay due to albumin binding can be attributed to the elongation of rotational correlation time (τ_C) of the albumin bound molecule and chemical exchange of the bound and free forms. Both the spin−lattice and spin−spin relaxation rates ($1/T_1$ and $1/T_2$) are characteristically dependent on τ_C.[44] Mechanistically, spin−lattice relaxation is caused by local fluctuating magnetic fields generated by molecular tumbling. The relaxation effect of these local fields is the most efficient when the frequency of the fluctuations (i.e., the molecular rotation) matches the Larmor frequency. This condition is met by medium sized molecules such as albumin at commonly encountered magnetic fields. The τ_C of albumin (around 20 ns) is well-known from extensive studies aimed at improving the efficiency of gadolinium based MRI contrast agents.[45] Increasing the τ_C of a gadolinium chelate via albumin binding results in an increase in r_1 relaxivity, which has been exploited in the design of high relaxivity agents and Zn-sensors.[46,47]

In conclusion, we showed that, for certain compounds, interaction with albumin can shorten the T_1 relaxation time to such an extent that the hyperpolarized NMR signal cannot be observed. This is clearly detrimental for hyperpolarized probes intended for in vivo studies as the hyperpolarized magnetization would rapidly decay when the probe comes into contact with albumin in the blood. However, we successfully demonstrated that the signal can be restored by the addition of a competitive displacer that can eliminate or reduce the binding of the hyperpolarized substrate to albumin. We have

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identified iophenoxic acid as a suitable displacer. This nontoxic compound prevents most substrates from binding to site 1 of albumin and should facilitate the in vivo use of hyperpolarized probes that contain aromatic or heterocyclic moieties. The methodology presented here is obviously not limited to albumin binding but can be generalized for other undesirable protein−hyperpolarized substrate interactions. It is worth noting that the competitive displacer approach combined with hyperpolarized ^{15}N NMR spectroscopy could also be applied to evaluate drug binding to target proteins and affinity screening.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsphyschemau.2c00059.

Details of ^{15}N DNP, T_1 relaxation time measurements, competitive displacement experiments, binding constant measurement by ITC, and Zn^{2+} sensing with HP-(**1b**) (PDF)

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Notes

The authors declare no competing financial interest.

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