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Impact of Ho³⁺-doping on ¹³C dynamic nuclear polarization using trityl OX063 free radical

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Abstract

We have investigated the effects of Ho-DOTA doping on the dynamic nuclear polarization (DNP) of [1-¹³C] sodium acetate using trityl OX063 free radical at 3.35 T and 1.2 K. Our results indicate that addition of 2 mM Ho-DOTA on 3 M [1-¹³C] sodium acetate sample in 1:1 v/v glycerol:water with 15 mM trityl OX063 improves the DNP-enhanced ¹³C solid-state nuclear polarization by a factor of around 2.7-fold. Similar to the Gd³⁺ doping effect on ¹³C DNP, the locations of the positive and negative ¹³C maximum polarization peaks in the ¹³C microwave DNP sweep are shifted towards each other with the addition of Ho-DOTA on the DNP sample. W-band electron spin resonance (ESR) studies have revealed that while the shape and linewidth of the trityl OX063 ESR spectrum was not affected by Ho³⁺-doping, the electron spin-lattice relaxation time T_1 of trityl OX063 was prominently reduced at cryogenic temperatures. The reduction of trityl OX063 electron T_1 by Ho-doping is linked to the ¹³C DNP improvement in light of the thermodynamic picture of DNP. Moreover, the presence of Ho-DOTA in the dissolution liquid at room temperature has negligible reduction effect on liquid-state ¹³C T_1 , in contrast to Gd³⁺-doping which drastically reduces the ¹³C T_1 . The results here suggest that Ho³⁺-doping is advantageous over Gd³⁺ in terms of preservation of hyperpolarized state—an important aspect to consider for *in vitro* and *in vivo* NMR or imaging (MRI) experiments where a considerable preparation time is needed to administer the hyperpolarized ¹³C liquid.

1. Introduction

Due to its high specificity in molecular and structural elucidation of materials, nuclear magnetic resonance (NMR) is a valuable non-invasive analytical tool that is widely used in many areas of science and medicine. However, when compared to other spectroscopic methods, NMR is a rather insensitive technique especially when used on nuclei with low

gyromagnetic ratio (γ) such as ^{13}C spins. At ambient conditions and low concentrations, the time required for NMR data acquisition can become prohibitively long. This inherently low sensitivity in NMR emanates from the fact that nuclei have relatively low magnetic moments. As a consequence, the strength of the NMR signal which is proportional to polarization P or the nuclear spin population difference between the Zeeman energy levels is relatively miniscule, with P on the order of only a few parts per million (ppm).^{1,2}

On the other hand, electrons have a much larger magnetic moment than nuclei by about 3–4 orders of magnitude. Thus, electron spins can be easily polarized with P already approaching unity at low temperature close to 1 K and high magnetic field. By using slightly off-resonance microwave irradiation of samples doped with trace number of free electrons, a technique called dynamic nuclear polarization (DNP) can transfer this high electron spin alignment to nuclei with non-zero spin number.^{1,2} This method consequently creates a large surplus of nuclear spins residing in one Zeeman energy level—a “hyperpolarized” state, which implies amplified NMR signals. The use of DNP can be traced back in the 1960s where highly polarized protons and deuterons were used as targets in nuclear scattering and particle physics experiments.^{1,2}

The NMR signal amplification capability of DNP has found its practical application in chemistry and biomedicine with the invention of the dissolution DNP method by Ardenkjaer-Larsen and co-workers in 2003.³ Given that the spin-lattice relaxation time T_1 of nuclei of interest is sufficiently long, the amplified NMR signals of frozen polarized samples at cryogenic temperature can be mostly preserved in the liquid-state after rapid dissolution, translating to several thousand-fold NMR signal enhancements.³ With the enhanced NMR sensitivity afforded by DNP, low- γ nuclei such as ^{13}C , ^{15}N , ^6Li , ^{89}Y , $^{107,109}\text{Ag}$ etc. can now be observed in 1 scan with excellent signal-to-noise ratio (SNR) even at millimolar (mM) concentrations.^{3–8} The use of hyperpolarized ^{13}C -enriched biomolecules such as pyruvate, glucose, etc. has opened a new door to *in vitro* and *in vivo* metabolic NMR spectroscopy or imaging (MRI) that combines both excellent SNR and high chemical shift resolution.^{9–16} Furthermore, this new technique also provides excellent temporal resolution, allowing for real-time analyses of metabolic activities *in vitro* in cells or *in vivo* in tissues of interest, which would be of great diagnostic and prognostic values for a variety of diseases.^{9–16}

Due to the nature of this technology, the common theme in dissolution DNP is to achieve the highest polarization level in the solid-state and to preserve this polarization in the liquid-state. As such, optimization methods in sample preparation and DNP instrumentation are quite crucial to the success of *in vitro* or *in vivo* hyperpolarized ^{13}C NMR or MRI experiments. Different methods have been implemented in accordance to this theme. Among these optimization methods are the appropriate choice of free radical polarizing agents,^{17–20} deuteration^{21–23} and ^{13}C enrichment²⁴ of the glassing matrix, using polarizers operating at higher fields,^{20,25–27} DNP cross polarization from ^1H spins to low- γ nuclei,²⁸ the use of trace amounts of lanthanides in the DNP sample,^{29–32} the use of magnetic tunnel in shuttling of the liquid,³³ and dissolution in a fringe field area.³⁴ In this study, we have investigated the lanthanide doping method, in particular the effect of Ho^{3+} -doping on ^{13}C DNP. Previous studies have shown that lanthanides,^{29–32} specifically Gd^{3+} complexes can significantly improve the DNP-enhanced polarization in the solid-state by a factor of 2–4 for samples

doped with trityl OX063 free radical at 3.35 T. A previous study by Gordon *et al.* reported a set of DNP experiments on trityl-doped ^{13}C pyruvate samples in which the main finding was that only Ho^{3+} , in addition to Gd^{3+} , showed a beneficial improvement of DNP-enhanced ^{13}C polarization among the series of unchelated lanthanide ions used as dopants.³⁰ While Gd^{3+} complexes such as Gd-DOTA and Gd-HP-DO3A have been well studied and optimized as beneficial additives in DNP samples,^{29–32} the use of Ho^{3+} doping has not been extensively investigated and optimized for DNP. Thus, the main goal of this research was to optimize the use of Ho^{3+} doping on ^{13}C DNP samples via extensive studies of its effects on the ^{13}C microwave DNP sweeps, relative ^{13}C polarization levels, and liquid-state ^{13}C enhancements and T_1 . $[1-^{13}\text{C}]$ sodium acetate, a biologically important substrate,^{15–16} was chosen as the model ^{13}C compound in this study. It has a relatively lower cost and long liquid-state ^{13}C hyperpolarization lifetime comparable to that of the other popular DNP metabolic agents such as $[1-^{13}\text{C}]$ pyruvate. The main DNP optimization data of this study were compared with ^{13}C DNP using the well-known DNP-enhancing Gd^{3+} -based dopant Gd-HP-DO3A. Furthermore, W-band electron spin resonance (ESR) was employed in this study to further elucidate the physical mechanisms behind the improvement of ^{13}C DNP signals with Ho^{3+} -doping.

2. Experimental

2.1 Sample preparation

All chemicals and reagents were acquired commercially and were used without further purification. 24.9 mg $[1-^{13}\text{C}]$ sodium acetate (Cambridge Isotope Lab, MA) was added to a solution containing 1:1 v/v water:glycerol (Sigma-Aldrich, MO). 100 μL aliquots of these $[1-^{13}\text{C}]$ acetate solutions were prepared a few hours before the experiment and subsequently frozen at -20°C . Each frozen solution was thawed and then mixed with 2.14 mg trityl OX063 free radical (Oxford Instruments Biotech, MA) right before the DNP experiment. The final concentrations of $[1-^{13}\text{C}]$ acetate and trityl OX063 in the solution were 3 M and 15 mM, respectively. 100 μL aliquots of these DNP samples were prepared containing various concentrations of Ho-DOTA prepared from an aqueous stock solution of 218 mM Ho-DOTA (deionized water, pH = 7.2). Ho-DOTA was prepared by adding to Ho^{3+} solutions free ligand DOTA⁴⁺ (Macrocyclics, Dallas, TX) at M/L molar ratios of 1:1.05 and the resulting solutions were heated at 338 K under stirring for at least 12 h. The pH was maintained close to 7 by periodic addition of aqueous KOH. The absence of free Ho^{3+} ions in solution was verified by the xylenol orange test.^{35–37} The Gd-HP-DO3A contrast agent or ProHance (Bracco Diagnostics, New Jersey) was purchased as a 0.5 M solution. The optimum DNP concentration of Gd-HP-DO3A (2 mM) in the ^{13}C DNP sample was prepared for use in comparative DNP studies with the Ho-doped samples. The structures of the trityl OX063 free radical and the two lanthanide complexes used in this study are displayed in Fig. 1.

2.2 Hyperpolarization and NMR measurements

The DNP experiments were performed at the Advanced Imaging Research Center at the University of Texas Southwestern Medical Center (UTSW) using a commercial polarizer HyperSense (Oxford Instruments, UK). This hyperpolarizer operates at 3.35 T and is equipped with a rootsblower pump vacuum system (Edwards Vacuum, UK) which provides

a base temperature of 1.2 K for the cryostat sample space. A 100-mW ELVA microwave source (ELVA-1 millimeter Wave Division, RU) with 400 MHz sweepable frequency range was used to irradiate the samples. 100 μ L aliquots of ^{13}C DNP samples were inserted into the hyperpolarizer.

Prior to the polarization build-up experiments, ^{13}C microwave frequency sweeps were measured for ^{13}C samples with varying Ho^{3+} concentrations to determine their optimum microwave frequencies for the DNP, namely the positive P(+) and negative (P-) polarization peaks. For the DNP sweep, ^{13}C NMR signal was recorded after 3 minutes of microwave irradiation in 5 MHz steps using a DNP sweep program in the HyperSense. A train of hard pulses was applied after every ^{13}C NMR signal recording to avoid remnant polarization of a previous frequency step that may add up to the NMR signal of the next frequency step. The optimum P(+) and P(-) frequencies were located and normalized for comparison.

Once the optimum DNP frequencies were determined, ^{13}C polarization build-up curves were measured for each sample by recording the ^{13}C NMR signal every 3 minutes during irradiation of the sample at the P(+) microwave frequency. Due to instrumental difficulty and prohibitively long acquisition times needed in measuring the ^{13}C thermal NMR signals at cryogenic temperatures, we were not able to quantify the actual percent polarization levels for each sample in the frozen state. Instead, we have used relative polarization level as a method for evaluating the effect of this lanthanide complex on ^{13}C DNP. We note that such method has been nevertheless useful as a metric as demonstrated in previous DNP optimization studies.^{22,23,30,32} The relative maximum ^{13}C DNP signal for each sample were recorded and normalized with respect to the ^{13}C DNP signal from the undoped reference sample. The solid-state ^{13}C polarization buildup curves for samples doped with 0 mM Ho (reference sample), 2 mM Ho (optimum concentration For Ho-DOTA), and 2 mM Gd (optimum concentration for Gd-HP-DO3A) were measured in triplicate. Three separate and freshly-made samples were used in the triplicate solid-state DNP build-up measurements. Relative bar graphs of mean values were plotted and normalized with respect to the reference sample. After the samples reached their maximum solid-state polarization, dissolution was carried out on these three aforementioned sets of samples to investigate the effects of Ho^{3+} and Gd^{3+} doping on ^{13}C NMR enhancement and ^{13}C T_1 in the liquid-state. Approximately 4 mL of aqueous dissolution liquid was rapidly transferred to a 10-mm NMR tube (Wilmad-LabGlass, NJ) inside a 400 MHz Varian NMR magnet (Agilent Technologies, CA) via a 0.125 in OD PTFE tubing (Cole-Parmer, IL). The dissolution transfer was done in an automated process and the total shuttling time of the hyperpolarized liquid from the HyperSense polarizer to the adjacent 9.4 T high resolution NMR magnet was 8 s. The liquid state T_1 decay of each sample was monitored by applying an array of 2-degree tip-angle RF pulse with 2-second repetition time. The first hyperpolarized ^{13}C NMR spectrum of this array was taken right after the 8-second dissolution transfer time with a time delay of 0.001 s. This hyperpolarized NMR spectrum, along with the subsequent thermal NMR measurement, was used in the liquid-state ^{13}C NMR enhancement calculation. The liquid-state T_1 values were calculated from the hyperpolarized ^{13}C NMR decay data using methods described previously.^{6,38}

2.3 ESR measurements

ESR measurements were performed on the reference and samples doped with 2 mM Ho-DOTA to study the mechanism responsible for the DNP enhancement associated with Ho³⁺-doping. The ESR measurements were carried out at the National High Magnetic Field Laboratory (NHMFL) in Tallahassee, FL. The W-band (94 GHz) measurements were performed on a Bruker E680 ESR spectrometer (Bruker Biospin, Billerica, MA) using a Bruker TE₀₁₁ cylindrical cavity. The sample temperature was regulated using a CF1200 helium flow cryostat (Oxford Instruments, UK) down to 5 K to closely imitate the DNP polarization conditions. Samples were loaded in 0.15 mm I.D. thin quartz capillary tubes before inserting into the cylindrical cavity. The temperature-dependent trityl OX063 electron T_1 data were recorded by saturation recovery and the ESR spectra were collected using the field-swept electron spin-echo method.

2.4 Data Analysis

The NMR, ESR, and DNP data were plotted and analyzed using Igor Pro version 6.2 (Wavemetrics, Lake Oswego, OR). The liquid-state ¹³C NMR data gathered from Varian NMR spectrometer were processed using ACD labs version 12 (Advanced Chemistry Development, Toronto, Canada). Mean and standard values were calculated for samples done with $N=3$ trials.

3. Results and discussion

The source of free electrons in DNP, mainly provided by stable organic free radicals, has a major impact in achieving the highest NMR signal enhancements.^{17–20,39,40} The carbon-centered trityl OX063, the free radical used in this study, has a narrow ESR linewidth that is suited for polarizing low- γ nuclei such as ¹³C. Numerous studies have shown that at least at $B_0=3.35$ T and temperatures close to 1 K, the predominant DNP process that allows for polarization transfer from trityl OX063 electrons to low- γ nuclei such as ¹³C and ⁸⁹Y is the thermal mixing DNP mechanism.^{6,7,41,42} Thermal mixing occurs when the source of free electrons has ESR linewidth D that is larger than or comparable to the Larmor frequency ω_n of the nucleus of interest.^{1,2,40} In thermal mixing, a thermal contact is established between the electron dipolar system (EDS) and the nuclear Zeeman system (NZS) because of their compatible energies. Upon microwave irradiation, the spin temperature of EDS is lowered via dynamic cooling and due to the thermal link between the two reservoirs, the same low spin temperature is acquired by NZS.^{1,2,40} This process translates to higher nuclear spin population difference or polarization, thus amplified NMR signals.

Since the free electrons are at the nexus of the polarization transfer process, perturbations to the electronic properties of the free radicals via addition of highly paramagnetic lanthanide complexes could contribute to changes in the maximum achievable nuclear polarization levels.^{29,43} To extensively investigate the influence of Ho³⁺-doping on ¹³C DNP, we have first examined its effect on the ¹³C microwave DNP sweeps as displayed in Figure 2. The ¹³C microwave frequency sweeps, also known as ¹³C microwave DNP spectra, are quite crucial since they provide the locations of the optimum microwave frequency peaks P(+) and P(–) for DNP.^{23,29} It can be seen from Figure 2 that the positions of the peaks vary with Ho-

DOTA concentrations, where the separation between P(+) and P(−) become closer with increasing Ho-DOTA concentrations. Initially, the position of both P(+) and P(−) peaks shift by approximately 10 MHz towards the center with an addition of 0.5 mM Ho-DOTA. Further increasing the Ho-DOTA concentration, the optimum microwave frequency shifts by another 10 MHz at 2mM Ho-DOTA doping. However, at concentrations larger than 2 mM there is no significant shift in either P(+) or P(−) up to 8 mM. These optimum frequency shifts with Ho³⁺-doping are reminiscent of similar behaviour with the effect of Gd³⁺-doping on ¹³C microwave DNP spectra.²⁹ Based on our data, we note that following the exact shift of P(+) or P(−) with Ho-doping could have a profound effect in achieving the highest ¹³C DNP enhancement. For instance, it is estimated from the ¹³C microwave spectra in Figure 2 that ¹³C DNP at 2 mM Ho³⁺-doping without the corresponding 20 MHz P(+) peak shift correction from the reference sample will only yield about one-half of the maximum polarization that can be achieved if the sample was irradiated at the optimum frequency. Therefore, these peak shift corrections due to lanthanide doping are important considerations to achieve the highest NMR signal enhancements in DNP. On a separate but relevant theoretical note, the locations of P(+) and P(−) peaks can be approximately predicted by a spin-temperature model first put forth by Borghini.^{1,44,45} In this model, the shape of the microwave DNP spectrum as well the maximum polarization values at P(+) and P(−) are almost exclusively dependent upon the shape and features of the ESR spectrum of the free radical polarizing agent used, and to a lesser extent, also upon the electron and nuclear relaxation rates.^{1,44} Thus, an important question to answer is whether or not the presence of Ho³⁺ complex in the DNP samples affect the electronic properties of the trityl OX063 free radical, leading to changes in the ¹³C microwave DNP spectra. We will revisit this question in the subsequent discussion of relevant ESR data in this study.

With the optimum microwave frequencies for DNP identified, we then proceeded with the investigation of the growth of ¹³C NMR signals as a function of microwave irradiation time for trityl-doped [1-¹³C]acetate samples containing various concentrations of Ho-DOTA. Figure 3a shows the representative ¹³C solid-state polarization build-up curves of Ho³⁺-doped ¹³C samples as well as a sample doped with DNP optimum concentration of Gd-HP-DO3A. The ¹³C DNP data were taken at the corresponding P(+) microwave frequency of each sample at 3.35 T and 1.2 K using a 100 mW microwave source. These ¹³C DNP signal buildup curves were measured on the same volumes of DNP samples (100 μL) with the same [1-¹³C] acetate (3 M) and trityl OX063 (15 mM) concentrations, type of glassing solvents (1:1 v/v glycerol:water), and DNP conditions (3.35 T and 1.2 K). Thus, their maximum ¹³C NMR signals can be used as a polarimeter that reflect their relative ¹³C polarization levels. Figure 3b provides a summary of the relative ¹³C DNP signals of all ¹³C samples with varying Ho-DOTA concentrations; these data points correspond to the maximum ¹³C NMR signals of the samples normalized with respect to the ¹³C signal of the reference sample (0 mM Ho-DOTA). As can be seen from Figure 3b, there seems to be a linear increase in the relative ¹³C polarization as the Ho³⁺ concentration is increased from 0 mM to 2 mM. Then, a monotonic decrease of ¹³C DNP signal can be observed with further increase of Ho³⁺ concentration from 2 mM to 8 mM. The optimum concentration of Ho-DOTA for ¹³C DNP was found to be 2 mM. This result is similar to the optimum concentration reported for Gd³⁺-doping on ¹³C DNP.²⁹ However, the overall profile of Ho³⁺ concentration dependence

of ^{13}C DNP signal has a subtle difference from that of previously reported Gd^{3+} doping DNP results. Ho^{3+} -doping exhibits a peak in maximum ^{13}C DNP signal at 2 mM then a steady decrease beyond this concentration, whereas in a previously published report,²⁹ Gd^{3+} -doping exhibits a plateau in maximum ^{13}C DNP signal from 2 mM to 5 mM then a monotonic decrease in DNP signal at higher concentrations. In Figure 3c, the relative ^{13}C polarization levels achieved with optimum Ho -DOTA (2 mM) and Gd -HP-DO3A (2 mM) are compared as bar graphs normalized vis-à-vis the reference sample. The error bars are standard deviations for $N=3$ trials. It can be readily observed from Figure 3c that Ho -doping further enhanced the solid-state ^{13}C DNP signal of acetate by a factor of 2.7-fold, whereas Gd^{3+} -doping resulted in a 3.5-fold enhancement of the ^{13}C DNP signal of the reference sample. Thus, Gd^{3+} -doping, in this case, yielded slightly better ^{13}C solid-state polarization enhancement than Ho^{3+} -doping.

To further elucidate these ^{13}C DNP behaviour with Ho^{3+} -doping, ESR was performed on the reference sample and an optimally Ho -doped ^{13}C samples. Thus, we revisit that previous question on whether or not Ho^{3+} -doping affect the electronic properties of the free radical polarizing agent. First, inspection of Figure 4a reveals that the shape and linewidth of the trityl OX063 W-band ESR spectrum at 10 K are essentially the same with or without Ho -DOTA. This finding is reminiscent of the previously reported ESR result for Gd -HP-DO3A in which the trityl OX063 spectrum was also not affected by the presence of an optimum DNP concentration of Gd^{3+} in the sample.⁴³ In addition, the W-band ESR signal of Ho^{3+} could not be detected, even in wider ESR spectral window. This behaviour is attributed to the very short relaxation times of Ho^{3+} , and lanthanides in general, even at this temperature which pose a challenge for direct ESR detection. Within the framework of the Borghini model for DNP,^{1,44} the narrowing of the ^{13}C microwave DNP spectra with Ho^{3+} -doping shown in Figure 2 does not seem to fit with the absence of visible changes in the trityl OX063 ESR spectrum with Ho^{3+} -doping shown in Figure 4a. As stipulated before, the Borghini prediction of microwave DNP spectrum is almost entirely dependent on the shape and size of the ESR spectrum of the polarizing agent in DNP.^{44,45} There is, however, another term in the Borghini model equation that involves the ratio of the electron and nuclear relaxation times, which is often considered negligible since electron relaxation is still relatively small ranging from few μs to a second and nuclear relaxation is extremely long on the order of several-thousand seconds at cryogenic temperatures.¹ Qualitatively, it is thus suggested that the changes in the ^{13}C microwave DNP spectra due to Ho^{3+} -doping may be attributed to changes in the electron relaxation. A possible alternative explanation to microwave frequency shift with Ho -doping is that the DNP mechanism may be a combination of solid effect and cross effect as suggested by previous studies that were done at relatively higher DNP temperatures of 6.5 K or above.^{46,47} Based on this mechanism, it would seem that addition of lanthanide may cause the microwave DNP frequency shift by reducing the solid effect contribution and increasing the influence of cross effect, leading to a narrowing of the ^{13}C microwave DNP spectra. However, we note that it is also possible that the physics of DNP at 6.5 K or higher may be quite different for DNP done at $T \sim 1\text{K}$. As mentioned before, mounting experimental evidence^{6,7,41,42} suggest the thermal mixing is the predominant DNP mechanism for low- γ nuclei such as ^{13}C , ^{89}Y , etc., at least at DNP conditions of 3.35 T and $T \sim 1\text{K}$ in which we have performed the experiments. Further

combined DNP and ESR studies at these conditions may be needed to fully clarify these effects.

Next, we have also closely examined the effect of Ho^{3+} -doping on the trityl OX063 electron relaxation rate T_1^{-1} as shown in Figure 4b. Before discussing the details and analysis of the relaxation data, we note that the electron T_1 data here were obtained by fitting the electron relaxation recovery curves at different temperatures with a double-exponential build-up function $M(t) = M_a \exp(-t/T_{1,a}) + M_b \exp(-t/T_{1,b}) + C$. In this equation, C is a constant, the longer time constant $T_{1,a}$ is the actual electron T_1 value of the free radical, and the smaller time constant $T_{1,b}$ corresponds to the contribution from electron-electron cross relaxation effects.^{48,49} The electron T_1^{-1} versus temperature data shown in log-log scale in Figure 4b were fitted to the power law equation $T_1^{-1} = AT^\alpha$, with the values of α given in Figure 4b. For the reference sample, the trityl OX063 relaxation rate decreases rapidly with decreasing temperature, following $\alpha \approx 4$ dependence at high temperatures above 50 K. This result indicates that in this temperature regime, the predominant electron relaxation mechanism could be due to a combination of two-phonon Raman and other mechanisms such as three-phonon Orbach process.⁵⁰ As the temperature decreases further in the intermediate range from 50 K to around 20 K, the rate of change of T_1^{-1} for the reference sample slows down to $\alpha \approx 2$ dependence, indicating that the electronic relaxation mechanism is dominated by Raman process.⁵⁰ At lower temperatures, the relaxation rate further slows down to either $\alpha \approx 1$ or 0, indicative of the one-phonon direct process being the predominant electron relaxation mechanism.⁵⁰ Meanwhile, for the sample doped with 2 mM Ho^{3+} , the relaxation rate values almost overlap with those of the reference sample at higher temperature above 100 K. Below this temperature, it becomes apparent that the relaxation rates for the sample with Ho^{3+} is higher and this relaxation rate difference between the two samples becomes more prominent at lower temperatures. Below 60 K, the relaxation rate of the Ho^{3+} -doped sample appears to behave according to the direct process relaxation mechanism. At 5 K, which is the base temperature of the ESR cryostat, the presence of 2 mM Ho^{3+} in the sample resulted in a drastic reduction of the electron T_1 of trityl OX063 from 380 ms to about 25 ms. Following these low temperature power law trends, it seems likely that direct process would still be the dominant relaxation process for both samples at temperatures close to 1 K where DNP is performed.

Similar to the physical mechanism on DNP with Gd^{3+} -doping,²⁹ we qualitatively attribute the improvement of the ^{13}C DNP signal with Ho^{3+} -doping to the reduction in electron T_1 of trityl OX063 free radical. We invoke the thermodynamic model of DNP equation in which the theoretical maximum limit of polarization is given by the following equation:^{29,40}

$$P_{\text{DNP,max}} = \tanh \left(\beta_L \frac{\omega_e \omega_I}{4D} \frac{1}{\sqrt{\eta(1+f)}} \right) \quad \#(1)$$

where $\beta_L = \hbar/k_B T_L$ (T_L is the lattice temperature), ω_e is the electron Larmor frequency, ω_I is the nuclear Larmor frequency, and D is the ESR linewidth of the electron system. The remaining factors in Equation 1 are $\eta = T_{1,e}/T_{1,D}$, which is the ratio of the relaxation times

of the electron Zeeman system to the electron dipolar spin-lattice system and f , which is the “leakage factor”. The leakage factor f is caused by nuclear relaxation mechanisms other than the coupling of nuclei with the free electron spins involved in DNP.¹ In Equation 1, at a constant field and temperature, the factors β_L , ω_e and ω_I in this study are the same between the reference and the Ho-doped samples. Based on the trityl ESR spectra, there is no significant change in D with Ho-doping. Therefore, assuming the “leakage factor” remains unchanged, the decrease of electron T_1 is the factor responsible for the improved DNP enhancement, similar to the effect of Gd-doping.^{23,29,39} At this point, using Equation 1 as a guide, we can only provide qualitative explanation for linking the improved ^{13}C DNP efficiency with the reduction of trityl OX063 electron T_1 with Ho-doping, similar to the behaviour observed in ^{13}C DNP with Gd-doping.²⁹ Further experimental studies are needed to provide a more quantitative way of elucidating the relationship between these two aforementioned physical parameters in DNP.

Finally, we have also evaluated the effects of Ho-doping in dissolution DNP. Since the thermal and hyperpolarized NMR measurements were done on the same sample and with the same number of transients, the liquid-state enhancement relative to the thermal NMR signal was quantified using the following equation:⁶

$$\varepsilon = \frac{A_{\text{DNP}}}{A_{\text{Th}}} \frac{\sin \theta_{\text{Th}}}{\sin \theta_{\text{DNP}}} \quad \#(2)$$

where A_{DNP} and A_{Th} are the integrated areas of the hyperpolarized and the thermal signals, respectively and θ_{DNP} and θ_{Th} are their corresponding tip angles. In our experiment, the hyperpolarized and the thermal NMR signals were measured using 2° and 90° tip-angle pulses, respectively. Both thermal and DNP signal were measured with 1 transient. In addition, it should be noted that the values of liquid-state NMR enhancements reported were measured approximately 8 s after dissolution liquid transit time from polarizer to NMR magnet. Figure 5a shows the representative ^{13}C thermal and hyperpolarized NMR spectra in the liquid-state at 9.4 T and 297 K are shown in Figure 5a. A summary of the average ($N=3$) ^{13}C NMR enhancements for the reference and ^{13}C DNP samples doped with 2 mM Ho-DOTA and 2 mM Gd-HP-DO3A is depicted on Figure 5b. For the reference sample, the average liquid-state ε for $[1-^{13}\text{C}]$ acetate DNP sample was found to be $13,700 \pm 800$ ($P=11.2 \pm 0.6\%$), while the sample doped with 2 mM Ho-DOTA gives an enhancement factor of $36,600 \pm 1,100$ ($P=29.7 \pm 0.9\%$). These liquid-state ε values are roughly proportional to their relative solid-state ^{13}C DNP signals where the Ho-doped sample is about 2.7 times the DNP-enhanced polarization level of the reference sample. On the other hand, the enhancement factor for the Gd-doped sample was only $31,500 \pm 1,350$ ($P=25.60 \pm 1.1\%$), which is lower than the Ho-doped sample despite the fact that it has the highest relative ^{13}C solid-state DNP enhancement of about 3.5 times the DNP signal of the reference sample. This implies that the loss of liquid-state ^{13}C DNP signal due to T_1 relaxation during the dissolution liquid transit is more prominent in Gd-doped sample than the other 2 samples.

To determine the liquid-state ^{13}C T_1 , the ^{13}C DNP relaxation decay curves were fitted to the following equation, which describes the polarization loss due to both the T_1 relaxation effect and radiofrequency (RF) pulsing:^{6,38}

$$P(t) = P_0 \sin \theta (\cos \theta)^{t/TR} e^{-t/T_1} \quad \#(3)$$

where P , θ , t , TR , and T_1 are the polarization, the NMR tip angle, time, the repetition time, and the longitudinal relaxation time constant, respectively. In our setup, the RF tip angle θ for hyperpolarization was set as 2° with $TR = 2$ s. Figure 5a shows the normalized ^{13}C polarization decay curves of the reference sample, and those doped with Ho^{3+} and Gd^{3+} . Based on these data, the calculated ^{13}C T_1 of trityl-doped $[1-^{13}\text{C}]$ acetate reference sample is 47.6 ± 1.1 s. Meanwhile, addition of 2 mM Gd contrast agent in a 100 μL sample then dilution with 4 mL of water resulted in a reduction of liquid-state T_1 by nearly 60%, with $T_1 = 19.0 \pm 2.1$ s in the presence of 49 μM Gd-HP-DO3A. On the other hand, Ho-DOTA doping of same amount as Gd-HP-DO3A did not lead to any significant difference in T_1 of ^{13}C , with liquid-state $T_1 = 45.9 \pm 1.7$ s. This is apparent with the overlapping ^{13}C polarization decay curves of the reference sample and Ho^{3+} -doped sample shown in Figure 5c. We note that if we assume that during the dissolution transfer of 8 s the predominant cause of DNP signal loss is T_1 decay, the calculated polarization levels at $t=0$ s prior to dissolution are around 13%, 35%, and 40% for the reference, Ho-doped, and Gd-doped samples, respectively. These back-calculated polarization values are in close proportion to the relative ^{13}C solid-state polarization levels shown in Figure 3c.

On a related note, we would like to point out that previous studies have shown that the type of chelation ligands can also affect both the polarization levels and T_1 relaxation times, at least for Gd^{3+} compounds.^{30,32} The use of chelating ligands in lanthanide dopants is recommended in dissolution DNP to reduce the toxicity especially in *in vivo* experiments. In a previous study, Gd^{3+} -based contrast agents with macrocyclic ligands such as Gd-DOTA yielded less T_1 reduction effect and higher liquid-state NMR enhancements compared to samples doped with Gd^{3+} compounds with open-chain ligands such as Gd-EDTA.³² On the other hand, T_1 reduction with Ho^{3+} is generally very minimal, regardless of the type of chelation ligand.³⁰ Overall, the use of lanthanide compounds with macrocyclic ligands is a preferred choice in dissolution DNP because of better stability, enhancement levels, and hyperpolarization lifetimes in the liquid-state.

The reduced T_1 effect observed upon addition of the Gd(III)-complexes is a well-known phenomenon. Gd^{3+} ions contain seven f -orbital unpaired electrons, a symmetric S -state, and its electronic relaxation is relatively slow. Gd-complexes are extensively used in contrast-enhanced MRI because these compounds are efficient nuclear T_1 relaxation agents.^{51,52} Although its use has been mainly applied to reduce the T_1 of protons in water, similar effect can be observed in other nuclei, such as ^{13}C , ^{15}N , and ^6Li .^{33–35} Curie relaxation is actually larger for Gd^{3+} than for Ho^{3+} , but dipolar relaxation is orders of magnitude larger for Gd than for Ho. As a result, Ho^{3+} is a less efficient T_1 relaxation agent compared to Gd^{3+} .^{51,53} The strong relaxivity effect of Gd^{3+} , while beneficial for T_1 -weighted conventional MRI, will present a problem when used in hyperpolarized ^{13}C experiments where longer

preparation time (e.g. 15–30 s) is needed before administering the hyperpolarized ^{13}C liquid such as in *in vivo* hyperpolarized ^{13}C MRI. Furthermore, hyperpolarized ^{13}C experiments with bigger animal subjects may necessitate larger ^{13}C DNP sample volume (e.g. >100 μL) in which case the ^{13}C T_1 of the dissolution liquid will be reduced further in the presence of increased Gd^{3+} concentration, eventually negating the improved ^{13}C DNP polarization acquired in the solid-state due to Gd-doping. Based on our results, Ho-doping is advantageous over Gd-doping in terms of preservation of the usable enhanced liquid-state polarization in dissolution DNP.

4. Conclusion

In conclusion, we have found that inclusion of an optimum concentration of Ho-DOTA of around 2 mM in trityl-doped ^{13}C sample can significantly improve the ^{13}C solid-state polarization level, by about 2.7 times the ^{13}C DNP signal of the reference DNP sample at 3.35 T and 1.2 K. Ho^{3+} -doping resulted in the narrowing of the ^{13}C microwave DNP spectrum, so one must closely follow the shift in the optimum ^{13}C microwave frequencies to get the highest ^{13}C DNP signal. W-band ESR study has revealed that the shape and linewidth of the trityl OX063 ESR spectrum were not affected by Ho^{3+} -doping. However, the trityl OX063 electron T_1 was drastically reduced at cryogenic temperatures in the presence of 2 mM Ho-DOTA. Below 60 K, the electron relaxation rate versus temperature data for trityl OX063 in the presence of 2 mM Ho-DOTA appear to follow a power-law dependence indicative of the one-phonon direct process. Within the framework of the thermodynamic model for DNP, we ascribe the improvement of the ^{13}C DNP signal with Ho^{3+} -doping to the reduction of the electron T_1 of the trityl OX063 free radical. Similar to the DNP effect with Gd^{3+} -doping, the presence of Ho-DOTA in the trityl-doped ^{13}C sample lowers the spin temperature of the trityl OX063 electron dipolar system, which is in thermal contact with the nuclear Zeeman system, thus resulting in further enhancement of the ^{13}C DNP signal. Gd^{3+} -doping resulted in a slightly better ^{13}C solid-state DNP signal improvement than Ho^{3+} -doping, however this initial advantage of Gd^{3+} is negated by its strong reduction of ^{13}C T_1 of the hyperpolarized liquid. In comparison, the presence of Ho-DOTA in the hyperpolarized liquid, after dissolution of a typical ^{13}C DNP sample volume of 100 μL with 4 mL water, has a negligible or minimal reduction effect in liquid-state ^{13}C T_1 . At least for the ^{13}C samples and DNP conditions described here, Ho^{3+} -doping consequently results in a better ^{13}C NMR enhancements in the liquid-state than Gd^{3+} -doping right after the transit time of the dissolution liquid. It is anticipated that the reduction effect on polarization due to shorter ^{13}C T_1 with Gd-doping will become even more pronounced in hyperpolarized *in vitro* or *in vivo* ^{13}C NMR or MRI experiments where an extra preparation time and/or a larger DNP sample is needed. Therefore, especially in such cases, the use of Ho^{3+} complexes such as Ho-DOTA in a ^{13}C DNP sample may prove quite advantageous over Gd^{3+} -doping as it is expected to provide higher liquid-state ^{13}C NMR enhancement levels.

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Figure 1.

Structures of the trityl OX063 free radical polarizing agent and the lanthanide complexes used in this work.

Figure 2.

Normalized ^{13}C microwave DNP sweeps of 3 M $[1-^{13}\text{C}]$ acetate in 1:1 v/v glycerol:water with 15 mM trityl OX063 and doped with varying concentrations of Ho-DOTA. These ^{13}C DNP data were taken at 3.35 T and 1.2 K. Two ^{13}C DNP maxima can be observed as denoted by $P(+)$ and $P(-)$. Note the shift in the locations of $P(+)$ and $P(-)$ with varying Ho-DOTA concentration. The arrows indicate the direction of increasing Ho-DOTA doping.

Figure 3.

(a) Representative normalized ^{13}C polarization build-up curves in the solid-state for 100 μL aliquots of 3 M $[1-^{13}\text{C}]$ sodium acetate in 1:1 v/v glycerol:water with 15 mM trityl OX063 and doped with various concentrations of Ho-DOTA. For comparison, the build-up curve for the same ^{13}C sample doped with 2mM Gd-HP-DO3A is included. b) A summary of the relative ^{13}C maximum DNP signal for various concentrations of Ho-DOTA. The up arrow indicates the optimum Ho^{3+} concentration for DNP. (c) Comparative bar graph representations of the average relative ^{13}C DNP signals ($N=3$) for the reference sample, and

samples doped with 2 mM Ho-DOTA, and 2 mM Gd-HP-DO3A. All ^{13}C DNP data were taken at 3.35 T and 1.2 K.

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Figure 4.

(a) W-band ESR spectra of 15 mM trityl OX063 in 1:1 v/v glycerol:water with 3 M [1- ^{13}C] sodium acetate in the presence and absence of 2 mM Ho-DOTA. Both spectra were measured at 10 K. (b) The temperature dependence of the trityl OX063 electron relaxation rate T_1^{-1} at W-band for the reference sample (solid circles) and the Ho $^{3+}$ -doped sample (solid squares). The dashed lines are fits to a power-law equation mentioned in the text, with the approximate values of exponent α values given next to the fits.

Figure 5.

(a) Representative thermal and hyperpolarized ^{13}C NMR signals of $[1-^{13}\text{C}]$ sodium acetate in aqueous solutions after dissolution. The hyperpolarized signal was acquired using a 2° pulse at 9.4 T and 297 K. The thermal signal was taken using a 90° pulse with 1 transient and has been magnified by 500 times for clarity. (b) Average liquid-state ^{13}C NMR signal enhancements ($N=3$) for the reference sample and for samples originally doped with 2 mM Ho-DOTA and 2 mM Gd-HP-DO3A in the polarizer. These enhancement values were measured 8 s after the dissolution transfer to the NMR magnet at 9.4 T and 297 K. The

equivalent percent polarization level for the liquid-state ^{13}C NMR enhancement is displayed on the right axis. (c) Normalized ^{13}C hyperpolarization decay curves for the three aforementioned samples from which the corresponding liquid-state ^{13}C T_1 values were calculated.