Signal enhancement of hyperpolarized $^{15}$N sites in solution— increase in solid-state polarization at 3.35 T and prolongation of relaxation in deuterated water mixtures

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Hyperpolarized $^{15}$N sites have been found to be promising for generating long-lived hyperpolarized states in solution, and present a promising approach for utilizing dissolution-dynamic nuclear polarization (dDNP)-driven hyperpolarized MRI for imaging in biology and medicine. Specifically, $^{15}$N sites with directly bound protons were shown to be useful when dissolved in D$_2$O. The purpose of the current study was to further characterize and increase the visibility of such $^{15}$N sites in solutions that mimic an intravenous injection during the first cardiac pass in terms of their H$_2$O: D$_2$O composition. The $T_1$ values of hyperpolarized $^{15}$N in [${^{15}N}_2$]urea and [${^{15}N}$]NH$_4$Cl demonstrated similar dependences on the H$_2$O:D$_2$O composition of the solution, with a $T_1$ of about 140 s in 100% D$_2$O, about twofold shortening in 90% and 80% D$_2$O, and about threefold shortening in 50% D$_2$O. [${^{13}C}$]urea was found to be a useful solid-state $^{13}$C marker for qualitative monitoring of the $^{15}$N polarization process in a commercial pre-clinical dDNP device. Adding trace amounts of Gd$^{3+}$ to the polarization formulation led to higher solid-state polarization of [${^{13}C}$]urea and to higher polarization levels of [${^{15}N}_2$]urea in solution.

**KEYWORDS**
ammonium, carbon-13, D$_2$O, dissolution dynamic nuclear polarization, nitrogen-15, urea

1 | INTRODUCTION

Hyperpolarized $^{15}$N sites have been found to be promising for generating long-lived hyperpolarized states in solution and present a promising approach for utilizing dissolution-dynamic nuclear polarization (dDNP)-driven hyperpolarized MRI for imaging in biology and medicine.$^{1-6}$ The purpose of the current study was to further characterize and increase the visibility of such $^{15}$N sites in solution, specifically for those sites with directly bound deuterium atoms.$^1$ This work has been done as a preparation for translation of these potentially useful agents to intravenously (IV) injected hyperpolarized compounds. We aimed to provide a basis for predicting the effect of the inevitable mixing of the bolus injection of the hyperpolarized medium (which is D$_2$O based) with the water-based blood, further to the injection. Only the solvent-protonation-related $T_1$ shortening effect was tested here, as the interaction with whole blood and the inevitable resultant overall $T_1$ shortening has been previously reported.$^{1,2}$ Two separate directions were investigated: (1) the effect of D$_2$O concentration in the water solution on the hyperpolarized $^{15}$N $T_1$

**Abbreviations:** CPS1, carbamoyl-phosphate synthetase 1; dDNP, dissolution-dynamic nuclear polarization; IV, intravenous; MW, microwave.

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and (2) the effect of adding trace amounts of Gd\(^{3+}\) agent to the polarization formulation on the \(^{15}\)N polarization in the solid state and the \(T_1\) in solution.

Hyperpolarized \(^{15}\)N sites that are bound to exchangeable protons have shown prolonged relaxation time when dissolved in D\(_2\)O.\(^5\) However, in a potential medical application, these solutions will have to be injected into the bloodstream, where mixing with non-deuterated water-based blood will lead to a shorter relaxation time. However, upon bolus injection, dilution of the bolus by the total body blood content is not immediate, with increasing dilution with each cardiac recirculation. The first (cardiac) pass of the bolus volume may be recorded and utilized as a diagnostic factor. The same strategy has been used with Gd-based contrast agents on MRI\(^7\) and with other exogenous agents and imaging modalities.\(^8\) Such studies suggest that the IV injected bolus has a peak arterial concentration at about 10 s after the injection on passing through the heart for the first time, before cardiac recirculation, in a human-size body, and a second lower peak at about 30 s after the injection\(^9\) due to the second cardiac pass. During the duration of the first pass, we estimate that the hyperpolarized compound may be surrounded by 80–90% of the original bolus composition mixed with 20–10% volume of blood, respectively. To estimate the effect of such mixing on the \(T_1\) of the hyperpolarized agent during the first pass, when injected as a bolus (in D\(_2\)O), we studied the \(T_1\) relaxation of hyperpolarized \(^{15}\)N agents bound to exchangeable protons in various D\(_2\)O concentrations in water. To this end, we tested two \(^{15}\)N-labeled agents—\([^{15}\text{N}_2]\)urea, whose potential as a perfusion agent has been suggested multiple times,\(^1\) and \([^{15}\text{N}]\)ammonium chloride, which is not intended for in vivo use and is used here only as a model system with the maximal number of exchangeable protons directly bound to the hyperpolarized \(^{15}\)N site.

In order to optimize the \(^{15}\)N polarization in the solid state one must be able to monitor it in that state. Otherwise, multiple polarization and dissolution experiments are required for this, as previously reported.\(^1,2\) Besides being very costly in terms of liquid helium consumption, such studies consist of additional variables (e.g. solution composition, temperature, duration between the end of the solid-state polarization and the measurement in solution), and are therefore prone to further errors. Ideally, one would like to have a solid-state \(^{15}\)N NMR probe within the dDNP device; however, this is not a very common experimental set-up and has been reported in the literature only once.\(^10\) We show here a potential bypass for this problem, originally designed only to show that the sample is in place but which turned out to provide a potential qualitative indication of the solid-state \(^{15}\)N polarization.

2 | MATERIALS AND METHODS

2.1 | Chemicals

The OX063 radical (GE Healthcare, Chalfont Saint Giles, UK) was obtained from Oxford Instruments Molecular Biotools (Oxford, UK). \([^{15}\text{N}_2]\)urea, \([^{13}\text{C}]\)urea, \([2-^{13}\text{C}]\)glycerol, \([^{13}\text{C}_3]\)glycerol, and \([^{13}\text{C}_6]\)D-glucose were purchased from Sigma-Aldrich (Rehovot, Israel). \([^{13}\text{C}_6]\)2-deoxy-D-glucose was purchased from Omicron Biochemicals (South Bend, IN, USA). \([^{15}\text{N}]\)NH\(_4\)Cl was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Gd\(^{3+}\) as gadoteric acid–gadoterate meglumine (Dotarem) was obtained from Guerbet (Villepinte, France).

2.2 | Spin polarization

Spin polarization was performed in a dDNP spin polarizer (HyperSense, Oxford Instruments Molecular Biotools) operating at 3.35 T. For \([^{15}\text{N}_2]\) urea and \([^{13}\text{C}]\)urea, the polarization was achieved by irradiating the sample at a microwave (MW) frequency of range of 94.104–94.150 GHz. For \([^{15}\text{N}]\)NH\(_4\)Cl, the polarization was achieved by irradiating the sample at an MW frequency of 94.116 GHz (\(n = 2\)) or 94.136 GHz (\(n = 21\)). Both polarization protocols were carried out with a power of 100 mW for the MW irradiation, at 1.5 K, for about 2 h. The MW source of the spin polarizer was replaced towards the end of the study (prior to acquiring the data shown in Figure 6 later). To eliminate possible differences in MW source frequency calibrations, the \([^{13}\text{C}]\)urea irradiation frequencies were referenced to that of \([1-^{13}\text{C}]\)pyruvic acid P\(^+\) (the first maximum in the DNP MW intensity profile), as shown in Figure 3 later.

2.3 | NMR spectroscopy

\(^{15}\)N NMR spectroscopy was performed using a 5.8 T high resolution NMR spectrometer (RS2D, Mundolsheim, France), equipped with a 10 mm broad-band NMR probe. Hyperpolarized \(^{15}\)N spectra were acquired using a hard pulse with a flip angle of 10°, a repetition time of 5 s, and 16 384 points.
2.4 | Calculation of hyperpolarized $[^{15}\text{N}_{2}]\text{urea}$ and $[^{15}\text{N}]\text{NH}_4\text{Cl} T_1$

The $T_1$ was determined from the decay curve of the hyperpolarized $^{15}$N signal at 35–44 °C. Curve fitting was performed using MATLAB (MathWorks, Natick, MA, USA), taking into account the decay of the signal due to the time from the first spectrum and the cumulative effect of the RF pulses, according to

$$A_t = A_0 e^{-t/T_1 \cos \theta}$$

where $A_t$ is the intensity of the signal at time $t$, $A_0$ is the intensity of signal on the first spectrum, $t$ is the time from the first spectrum, $T_R$ is the repetition time (5 s), $T_1$ is the spin–lattice relaxation time, and $\theta$ is the flip angle of the RF pulse excitation (10°).

2.5 | A $^{13}\text{C}$ solid-state tracer

To systematically characterize the polarization of $^{15}$N, in the absence of a solid-state probe for $^{15}$N in the polarizer, multiple $^{15}$N polarizations and dissolutions would have to be performed with a high solid-state polarization, as previously described. Unfortunately, the commercial dDNP device used here, which is common in non-clinical dDNP laboratories, can only detect $^{13}$C polarization in the solid state. This prohibits monitoring the solid-state polarization build-up of other nuclei and also puts the procedure and the instrument at risk, as one cannot monitor the MW irradiation process or the presence of the polarization cup within the polarization chamber. The latter could lead to spraying water in the polarization chamber, which will be immediately frozen (at 4 K) and prevent the instrument’s operation.

To resolve the above problems, we looked for a $^{13}$C-labeled agent that could be added to the $^{15}$N agent’s polarization formulation, and mark the progress of MW irradiation and the presence of the polarization cup in the polarization chamber.

Several attempts to use pH neutral compounds such as $[2-^{13}\text{C}]$glycerol, $[^{13}\text{C}_3]$glycerol, $[^{13}\text{C}_6]$D-glucose, and $[^{13}\text{C}_6]$2-deoxy-D-glucose for this purpose did not yield detectable $^{13}$C polarization in the solid state at sufficiently short time (about 15 min) and low quantity (<20 mg formulation). As the main agent we wished to investigate was $[^{15}\text{N}_{2}]$urea, we also investigated the use of $[^{13}\text{C}]$urea for this purpose.

2.6 | Composition of polarization formulations

2.6.1 | Formulation 1—$[^{15}\text{N}_{2}]$urea without Gd$^{3+}$

A typical formulation for $[^{15}\text{N}_{2}]$urea polarization consisted of OX063, 3.88 mg (15.0 mM); $[^{15}\text{N}_{2}]$urea, 79.4 mg; and 181.0 μL of a D$_2$O:glycerol mixture at a respective ratio of 0.6:0.4. The concentration of urea in this formulation was 4.42 μmol/mg formulation.

2.6.2 | Formulation 2—$[^{13}\text{C}]$urea without Gd$^{3+}$

A typical formulation for $[^{13}\text{C}]$urea polarization consisted of OX063, 3.48 mg (15.1 mM); $[^{13}\text{C}]$urea, 70.4 mg; and 161.9 μL of a D$_2$O:glycerol mixture at a respective ratio of 0.6:0.4. The concentration of urea in this formulation was 4.40 μmol/mg formulation.

2.6.3 | Formulation 3—$[^{15}\text{N}_{2}]$urea with Gd$^{3+}$

A typical formulation for $[^{15}\text{N}_{2}]$urea polarization in the presence of Gd$^{3+}$ consisted of OX063, 3.46 mg (15.0 mM); $[^{15}\text{N}_{2}]$urea, 70.86 mg; and 186.58 mg of a mixture of 1.3 mM Gd$^{3+}$ in D$_2$O:glycerol at a respective ratio of 0.6:0.4. The total concentration of urea in this formulation was 3.8 μmol/mg formulation.

2.6.4 | Formulation 4—$[^{13}\text{C}]$urea with Gd$^{3+}$

A typical formulation for $[^{13}\text{C}]$urea polarization in the presence of Gd$^{3+}$ consisted of OX063, 2.64 mg (14.9 mM); $[^{13}\text{C}]$urea, 53.69 mg; and 141.28 mg of a mixture of 1.3 mM Gd$^{3+}$ in D$_2$O:glycerol at a respective ratio of 0.6:0.4. The concentration of urea in this formulation was 4.5 μmol/mg formulation.
2.6.5 | Combinations of Formulations 1–4 for studies of $^{15}$N$_2$urea

A typical formulation loaded into the dDNP cup for studies without Gd$^{3+}$ consisted of 20.25 mg of Formulation 1 combined with 19.93 mg of Formulation 2. A typical formulation loaded into the dDNP cup for studies in the presence of Gd$^{3+}$ consisted of 19.85 mg of Formulation 3 combined with 12.25 mg of Formulation 4. The $^{13}$C$\text{urea}$ formulations were added to confirm that the cup is in the correct position in the polarization device and that the MW source is working, by monitoring the $^{13}$C build-up in the solid state (i.e., as a $^{13}$C solid-state tracer).

2.6.6 | Formulation 5—combined formulation of $^{13}$C$\text{urea}$ and $^{15}$N$_2$urea without Gd$^{3+}$

This formulation consisted of 96.90 mg of Formulation 1 combined with 96.45 mg of Formulation 2.

2.6.7 | Formulation 6—combined formulation of $^{13}$C$\text{urea}$ and $^{15}$N$_2$urea with Gd$^{3+}$

This formulation consisted of 72.35 mg of Formulation 3 combined with 71.68 mg of Formulation 4.

2.6.8 | Formulation 7

A typical formulation for $^{15}$N$\text{NH}_4\text{Cl}$ polarization consisted of OX063, 2.67 mg (14 mM); $^{15}$N$\text{NH}_4\text{Cl}$, 26.16 mg; 74.2 μL D$_2$O; and 40.19 mg glycerol. The total concentration of NH$_4$Cl in this formulation was 3.18 μmol/mg formulation.

2.6.9 | Addition of a $^{13}$C tracer to Formulation 7

In some of the experiments, $^{15}$N$\text{D}_{2}$ glucose (15–18 mg) was added as $^{13}$C solid-state marker to the $^{15}$N$\text{NH}_4\text{Cl}$ formulation. In other experiments the $^{15}$N$\text{NH}_4\text{Cl}$ formulation was added to a cup that had a plastic-wrapped droplet of $^{1-13}$C pyruvic acid formulation attached to the bottom (not mixed with the $^{15}$N$\text{NH}_4\text{Cl}$ formulation), for the same purpose. These experiments were carried out before establishing $^{13}$C$\text{urea}$ as a useful $^{13}$C solid-state tracer.

2.7 | Fast dissolution

For studies in solution, following polarization, the formulation was dissolved in 4 mL of D$_2$O:H$_2$O mixtures and directly injected into a 10 mm NMR tube located inside the NMR spectrometer. A heating tape was wrapped around the dissolution discharge line and set to 40 °C. The concentration ranges of urea and NH$_4$Cl in the dissolution media were 21–48 mM and 16–20 mM, respectively.

2.8 | Temperature monitoring during $^{15}$N hyperpolarization decay in the NMR spectrometer

An NMR compatible temperature sensor was fixed inside the NMR tube, at a position corresponding to the middle of the NMR detection probe, to monitor the temperature throughout the experiment (OSENSA, Burnaby, British Columbia, Canada).

2.9 | Statistical analysis

Statistical analysis was calculated with Excel (Microsoft, Ra’anana, Israel). Significance for all comparisons was tested using a one-tailed, non-paired Student t-test.

3 | RESULTS

3.1 | The effect of D$_2$O concentration and the presence of Gd$^{3+}$ on $^{15}$N$_2$urea $T_1$ at body temperature

Figure 1 and Table S1 show that, as regards the effect of D$_2$O percent in the water solution on the $T_1$ of $^{15}$N$_2$urea, reducing the concentration of D$_2$O from 100% to 90% and 80% led to a $T_1$ shortening of about twofold (from 144 ± 57 s ($n = 4$) to 75 ± 12 s ($n = 3$) and 86 ± 20 s ($n = 3$),
FIGURE 1  The $T_1$ of $^{15}$N in $[^{15}$N$_2$]urea in D$_2$O:H$_2$O mixtures with and without Gd$^{3+}$. Each point represents an individual $T_1$ measurement of hyperpolarized $[^{15}$N$_2$]urea in saline (H$_2$O based, red), or in various concentrations of D$_2$O in H$_2$O, with Gd$^{3+}$ or without. The red, green, and black bars are the respective means. The standard deviations are shown in grey. Further information on the individual experiments is provided in Table S1. *In this condition (100% D$_2$O without Gd$^{3+}$) there were two measurements with the same $T_1$ value (127 s), $n = 4$ altogether.

TABLE 1  Polarization parameters, $T_1$ in solution, and enhancement factor of hyperpolarized stable-isotope-labeled urea

| Gd$^{3+}$ concentration in the formulation (mM) | Sample number in Figures 4 & 5 | MW irradiation frequency (GHz) | Concentration of $[^{15}$N$_2$]urea in the solution (mM) | $^{15}$N enhancement factor in solution |
|-----------------------------------------------|-------------------------------|--------------------------------|------------------------------------------------|-----------------------------------------|
| 1.3                                           | 1                             | 94.130                         | 18.9                                           | 22 456                                  |
|                                               | 2                             | 94.140                         | 18.7                                           | 18 441                                  |
|                                               | 3                             | 94.150                         | 18.5                                           | 14 505                                  |
|                                               | Average ± standard deviation   |                                |                                                | 18 467 ± 3246*                          |
| 0                                             | 4                             | 94.140                         | 24.2                                           | 10 749                                  |
|                                               | 5                             | 94.140                         | 22.4                                           | 11 025                                  |
|                                               | 6                             | 94.140                         | 22.4                                           | 9 761                                   |
|                                               | Average ± standard deviation   |                                |                                                | 10 512 ± 543                            |

*a.u., arbitrary units. The polarization levels of $[^{13}$C$_2$]urea in a.u. were normalized to the mass in the cup. The levels of the normalized polarizations are reported at 75 min of polarization due to malfunction of the MW irradiation source after that time on one of the time courses; see also Figure 4.

*p = 0.013.

**p = 0.012.

***p = 0.034. All comparisons made with a one-tailed, non-paired Student t-test.

TABLE 1  (Continued)

| Gd$^{3+}$ concentration in the formulation (mM) | $^{15}$N polarization in solution (%) | $[^{15}$N$_2$]urea $T_1$ in solution (s) | Polarization level of $[^{13}$C$_2$]urea after 75 min of polarization (a.u.)* |
|------------------------------------------------|---------------------------------------|------------------------------------------|--------------------------------------------------------------------------------|
| 1.3                                           | 7                                     | 114                                      | 818                                                                            |
|                                               | 5.8                                   | 134                                      | 696                                                                            |
|                                               | 4.6                                   | 107                                      | 335                                                                            |
|                                               | 5.8 ± 1.0**                           | 118 ± 11                                | 616 ± 205***                                                                 |
| 0                                             | 3.4                                   | 127                                      | 259                                                                            |
|                                               | 3.5                                   | 127                                      | 255                                                                            |
|                                               | 3.1                                   | 84                                       | 258                                                                            |
|                                               | 3.3 ± 0.2                             | 113 ± 20                                | 257 ± 2                                                                       |

*a.u., arbitrary units. The polarization levels of $[^{13}$C$_2$]urea in a.u. were normalized to the mass in the cup. The levels of the normalized polarizations are reported at 75 min of polarization due to malfunction of the MW irradiation source after that time on one of the time courses; see also Figure 4.

*p = 0.013.

**p = 0.012.

***p = 0.034. All comparisons made with a one-tailed, non-paired Student t-test.
respectively). A reduction to 50% D2O led to a T1 shortening of about threefold (from 144 ± 57 s (n = 4) to 49 ± 4 s (n = 3)). Figure 1, Table 1, and Table S1 show that the addition of Gd3+ to the formulation for polarization did not change the T1 relaxation time in solution, as determined in 100% D2O to avoid solvent proton relaxation effects, (144 ± 57 s (n = 4) without Gd3+ and 118 ± 11 s (n = 3) with Gd3+).

3.2 | The effect of D2O concentration on [15N]NH4Cl T1 at body temperature

Figure 2 and Table S2 show that, as regards the effect of D2O percent in the water solution on the T1 of [15N]NH4Cl, reducing the concentration of D2O from 100% to 90% and 80% D2O led to a T1 shortening of about 1.8-fold (from 136 ± 26 s (n = 3) to 75 ± 15 s (n = 3) and 73 ± 10 s (n = 3), respectively). A reduction to 50% D2O led to a T1 shortening of about 2.9-fold (from 136 ± 26 s (n = 3) to 46 ± 17 s (n = 3)).

3.3 | A 13C solid-state tracer for 15N polarization on a dDNP device with a 13C only spectrometer

Studies with multiple 13C-labeled traces (Section 2) did not yield 13C solid-state SNR at sufficiently short time (~15 min) and sufficiently low quantity (<20 mg formulation). However, studies with [13C]urea showed promising results. To optimize the performance of [13C]urea as a solid-state tracer we first characterized its MW irradiation profile. Such studies were done on large samples (typically 180 mg to 260 mg formulations) using irradiation intervals of 4 min for every frequency point. Figure 3 shows the MW irradiation profile of a [13C]urea formulation that did not contain Gd3+ ions and of formulations that contained 0.7 mM, 1.3 mM, and 2.3 mM Gd3+ ions (as gadoterate meglumine). It can be seen that the addition of Gd3+ shifts the center of the first lobe of this profile and narrows it. The range of Gd3+ concentrations that was tested here resulted in similar MW irradiation profiles. The formulation with 1.3 mM Gd3+ (a Gd3+ concentration previously found favorable for [13C6,D7]D-glucose polarization as well11) was tested three times to ensure reproducibility of the profile.

In order to choose the best formulation for the solid-state [13C]urea tracer, we recorded the solid-state build-up of these formulations. The formulations that contained Gd3+ at concentrations of 0.7 mM and 1.3 mM showed a 2–2.5 times higher polarization level and an apparent shorter build-up time constant (Table 2: 76 and 73 min for 0.7 and 1.3 mM Gd3+, respectively, versus 108 min without Gd3+). However, while the build-up time is about the same in both formulations that contained Gd3+, the maximal polarization level was higher for the formulation that consisted of 1.3 mM Gd3+. The formulation that contained 2.3 mM Gd3+ showed a lower maximal polarization level on the MW intensity profile. Although this profile is shown in Figure 3, this lower intensity is not apparent due to the normalization of the data. For this reason, its use was discontinued and the formulation with 1.3 mM Gd3+ was selected for further studies.

Figure 4 shows the solid-state 13C polarization build-up characteristics of [13C]urea when polarized together with formulations of [15N2]urea, without or with Gd3+ (a combination of Formulations 1 and 2 or Formulations 3 and 4, respectively, see Section 2). Each combined formulation was polarized for the duration shown in Figure 4 and then rapidly dissolved and transferred to the NMR tube, which was situated inside the spectrometer, to record the hyperpolarized 15N signal of [15N2]urea in solution. The enhancement factor of these samples in solution is provided in Figure 5 and Table 1. On one of the polarizations with Gd3+ (no 1 in Figure 4), the MW source stopped irradiating after 75 min due to malfunction.
that was later confirmed. This malfunction likely led to the decrease in $^{13}$C solid-state polarization level that was observed in the later time points of this time course.

The experiments marked 1-3 in Figure 4 (which were performed with formulations that contained 1.3 mM Gd$^{3+}$) were performed with various MW irradiation frequencies in an attempt to optimize the hyperpolarized $^{15}$N signal in solution (the enhancement factor reported in Table 1). The variability in $^{15}$N enhancement factor (Table 1) and solid-state $^{13}$C polarization (Figure 4) of these samples is likely attributable to this variation in irradiation frequency.

The increase in solid-state build-up level and shorter build-up times shown in Figure 4 and Table 2 for the formulations that contained Gd$^{3+}$ was favorable for tracing the process of MW irradiation and for making sure the cup with the polarization formulation was in the polarization chamber. As shown in Figure 1 and Table 1, the minute concentration of 6-7 $\mu$M Gd$^{3+}$ dissolved in the hyperpolarized medium (1.3 mM $\times$ ~20 $\mu$L/4 mL) did not shorten the $T_2$ of the hyperpolarized $^{15}$N site. We then examined the polarization level of $[^{15}$N$_2]$urea in these studies (Table 1). To our surprise, it appeared that the conditions that benefitted $[^{13}$C$]$urea polarization in the solid state also benefitted the
When examining the $[13C]$urea solid-state polarizations at 75 min (Figure 4, before the MW source malfunction on polarization build-up 1) in comparison with the polarization of $[15N_2]$urea in solution derived from the same samples and polarization processes, it can be seen that these polarization levels correlate (Figure 5). The irradiation at various MW frequencies was serendipitous in revealing this correlation.

To make sure that, when irradiating at the same MW frequency, the variability is lower among build-up time courses for formulations that consist of both $[13C]$urea and $[15N_2]$urea, we performed another set of experiments. This time we recorded build-up time courses from the same sample, which was situated at exactly the same place in the polarizer. In this set of experiments only two (large) samples were used (Formulations 5 and 6). Three build-up time courses were recorded from each sample. The polarization was given a day to relax in between recordings, while the sample remained inside the polarizer. Figure 6 and Table 3 show that in this set of recordings the variability in build-up time constant and maximal polarization is lower. Of note, this set of experiments was carried out with a different MW source from the rest of the experiments reported here.
The MW frequency was adjusted with respect to the P of [1-13C]pyruvic acid (based on the data shown in Figure 3). In this set of experiments, to save on liquid helium, the irradiation was performed with a power of 10 mW. This is in contrast to the all other measurements reported here, which were carried out with a power of 100 mW. In our experience, this reduction in irradiation power does not change the polarization of [1-13C]pyruvic acid. To the best of our knowledge, the effect of this change is as yet unknown for other compounds.

As opposed to the studies using 100 mW irradiation power that were done on formulations that contained only [13C]urea and not [15N2]urea, Table 3 shows that the build-up time constant was shorter for the sample that did not contain Gd3+. The maximal polarization was still higher for the samples that contained Gd3+, with a larger increase upon 1.3 mM Gd3+ doping (7.7-fold in Table 3 versus 2.5-fold in Table 2).

4 | DISCUSSION

To the best of our knowledge, this is the first study reporting the use of Gd3+ doping of the polarization formulation for enhancing 15N polarization. We note that in the solid state the concentration of Gd3+ is high and therefore affects polarization build-up, most likely through modulation of polarization transfer mechanisms already described before.12–14 However, in the resulting hyperpolarized solution, the concentration of Gd3+ is much lower (6–7 μM) than in the formulation for polarization. The concentration of Gd3+ in the solutions studied here is also much lower than the concentration used in MRI studies to relax blood/body water protons (about 2 mM, considering a dose of 20 ml (0.5 M) and a blood volume of 5 L). This likely explains the finding that the T1 values of hyperpolarized [15N2]urea in solution were similar with or without Gd3+. The maximal polarization was still higher for the samples that contained Gd3+, with a larger increase upon 1.3 mM Gd3+ doping (7.7-fold in Table 3 versus 2.5-fold in Table 2).

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| Gd3+ concentration in the formulation (mM) | [13C]urea build-up time constant (min)c | [13C]urea maximal polarization level (a.u.)d | Number of samples tested |
|---------------------------------------------|----------------------------------------|-------------------------------------------|-------------------------|
| 1.3                                        | 89 ± 4                                 | 2555 ± 355                                | 3                       |
| w/o                                        | 19 ± 2                                 | 333 ± 31                                  | 3                       |

aThe build-up time courses were recorded using MW irradiation at 94.104 GHz with a power of 10 mW.
bThe polarization level was normalized to the mass of formulation in the cup.
w/o, without. Comparing the results w/o Gd3+ with 1.3 mM Gd3+: c \(p = 5.6 \times 10^{-4}\), d \(p = 1.0 \times 10^{-5}\). Both comparisons made with a one-tailed, non-paired Student t-test.
As regards the study of $T_1$ of $^{15}$N sites in H$_2$O:D$_2$O mixtures, it can be seen that decreasing the concentration of D$_2$O (and in parallel increasing the concentration of H$_2$O) leads to shorter $T_1$ for both [$^{15}$N$_2$]urea and [$^{15}$N]NH$_4$Cl, in a similar manner. This finding is in agreement with our previous report on $^{15}$N sites that are bound to exchangeable protons in solutions of pure D$_2$O and H$_2$O. However, with 90% or 80% D$_2$O, the $T_1$ values of both compounds were still long (>70 s). As hyperpolarized injections are done as a bolus, and assuming an injection of the hyperpolarized $^{15}$N-labeled compound in 100% D$_2$O, it is likely that during the first pass the concentrations of D$_2$O in the hyperpolarized bolus will be in this range (80–90%) and therefore the visibility time of hyperpolarized [$^{15}$N$_2$]urea and [$^{15}$N]NH$_4$Cl in blood will likely benefit from the bound deuterons. In this respect, it is worth noting that the $T_1$ in whole blood will be further impacted by blood-borne agents such as divalent ions and oxygen. The $T_1$ of [$^{15}$N$_2$]urea in whole blood was previously determined to be about 10 s. As a reference, we note that the $T_1$ of [$^{15}$N]nitrate, which has no bound protons, in whole blood, was previously reported to be about 30 s. For preclinical imaging in small animals with heart rates of 300–400 beats per minute, the first pass would be much faster than in human subjects and therefore the imaging should be carried out fast as well. This is in agreement with the need to image fast on hyperpolarized MRI.

It is interesting that similar effects ($T_1$ shortening of about twofold in mixtures of 90% or 80% D$_2$O and $T_1$ shortening of about threefold in mixtures of 50% D$_2$O) have been observed for both compounds: urea and the ammonium ion. This similarity is in contrast to the fact that the former has two exchangeable proton positions and the latter has four. This finding may suggest that the additional interaction with the third and the fourth protons in ammonium chloride adds little to the $T_1$ relaxation time constant.

Urea is an agent that is safe to use, and stable-isotope-labeled urea analogs have been used in pre-clinical dDNP studies. However, [$^{15}$N]NH$_4$Cl was studied here mainly as a model system that could validate the results obtained with [$^{15}$N$_2$]urea, as both consist of exchangeable protons directly bound to $^{15}$N and have previously shown prolongation of the $^{15}$N $T_1$ upon dissolution in D$_2$O. NH$_4$Cl is not recommended for development as an MRI contrast agent. NH$_4$Cl is a systemic and urinary acidifying agent that is converted to ammonia and hydrochloric acid through oxidation by the liver. Although IV injection of NH$_4$Cl is a treatment option for severe cases of metabolic alkalosis, it is generally poorly tolerated due to adverse effects including encephalopathy, metabolic acidosis, and ammonia toxicity. The LD$_{50}$ for IV injection of NH$_4$Cl to rats is 7–10 mmol/kg. To the best of our knowledge, further toxicity data and the no-observed-adverse-effect level have not been reported for IV injection of this compound. The physiological concentration of NH$_4^+$ in the blood is estimated to be between 11 and 50 μM, which is about 1000 times lower than the concentrations used in this study (16–20 mM), further limiting the potential use of this compound as a hyperpolarized MRI agent.

Nevertheless, it is conceivable that hyperpolarized [$^{15}$N]NH$_4$Cl could be used in a number of ex vivo studies in the investigation of nitrogen metabolism, such as the following. (1) To observe NH$_4^+$ and NH$_3$ metabolism (for example, in the glutamate–glutamine interconversion). (2) To study metabolism relating to the urea cycle. NH$_4^+$ ions are actively transported by RhBG and are converted to carbamoyl phosphate by carbamoyl-phosphate synthetase (CPS1). CPS1 is the rate limiting enzyme in the urea cycle in liver mitochondria and is also useful in diagnosing hepatocellular carcinoma (using the antibody HepPar1). (3) To study transport and viability in the kidney, where NH$_4^+$ enters the collecting duct via NH$_4^+$/K$^+$/ATPase. Moreover, the transport of NH$_4^+$ by various kidney cells and its secretion may be studied using hyperpolarized [$^{15}$N]NH$_4$Cl as a marker. The optimization of $^{15}$N polarization and the characterization of the $T_1$ of this agent in various H$_2$O:D$_2$O mixtures are likely useful for such potential studies.

As the commercial polarizer available in our laboratory (and in most other hyperpolarized MR laboratories) can only monitor the polarization of $^{13}$C in the solid state, we needed a $^{13}$C marker within the sample to both (1) mark that the polarization cup has reached the polarization cavity—to confirm that the dissolution stick will lock into the cup during the dissolution process—and (2) verify that the MW source is indeed working throughout the polarization time.

[$^{13}$C]urea was found to be a useful marker for MW irradiation and the presence of the polarization cup in the polarization chamber. Surprisingly, it was also found that the [$^{13}$C]urea solid-state polarization was a useful qualitative reporter for [$^{15}$N$_2$]urea polarization. This may alleviate the need for solid-state $^{15}$N polarization monitoring in further studies. However, the one sample in which the MW source stopped irradiating about 1 h prior to dissolution (Sample 1, Figure 4) suggested that the $T_1$ values of the two nuclei are not the same in the solid state. While the $^{12}$C polarization decayed after the end of irradiation, the $^{15}$N polarization, as evidenced by the liquid-state polarization (Figure 5), apparently did not decay or did not decay to the same extent. This suggests that the solid-state $T_1$ of the $^{15}$N sites investigated here is longer than the $^{13}$C site of [$^{13}$C]urea (in agreement with the difference between their $T_1$ values in solution). This observation warrants further investigation and suggests the potential of such compounds to preserve polarization if storage of the polarization is required.

By fitting the decay of the polarized signal of the hyperpolarized 15N-labeled compound in 100% D$_2$O, it is likely that during the first pass the concentrations of D$_2$O in the hyperpolarized bolus will be in this range (80–90%) and therefore the visibility time of hyperpolarized [$^{15}$N$_2$]urea and [$^{15}$N]NH$_4$Cl in blood will likely benefit from the bound deuterons. In this respect, it is worth noting that the $T_1$ in whole blood will be further impacted by blood-borne agents such as divalent ions and oxygen. The $T_1$ of [$^{15}$N$_2$]urea in whole blood was previously determined to be about 10 s. As a reference, we note that the $T_1$ of [$^{15}$N]nitrate, which has no bound protons, in whole blood, was previously reported to be about 30 s. For preclinical imaging in small animals with heart rates of 300–400 beats per minute, the first pass would be much faster than in human subjects and therefore the imaging should be carried out fast as well. This is in agreement with the need to image fast on hyperpolarized MRI.

Previously, we reported a polarization of 5.1% for [$^{15}$N$_2$]urea in D$_2$O. We show here that this polarization can be increased 1.4-fold at 3.35 T by adding Gd$^{3+}$ ions to the polarization formulation (reaching 7% polarization in solution). For [$^{15}$N]sodium nitrate a lower polarization of 1% was previously reported in solution. It remains to be seen whether the measures investigated here for [$^{15}$N$_2$]urea and [$^{13}$C]urea solid-state polarization will be useful also for [$^{15}$N]sodium nitrate polarization.

In summary, the current studies show promise for an increase in $^{15}$N hyperpolarization and suggest prolonged visibility of such hyperpolarized agents in the blood during the first pass following a bolus IV injection (in D$_2$O).
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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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