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Communication

Dissolution DNP NMR with solvent mixtures: Substrate concentration and radical extraction

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ABSTRACT

Dynamic nuclear polarization (DNP) followed by sudden sample dissolution, is a topic of active investigation owing to the method's unique prospects for the delivery of NMR spectra and images with unprecedented sensitivity. This experiment achieves hyperpolarization by the combined effects of electron-nuclear irradiation and cryogenic operation; the exploitation of these states occurs following a sudden melting and flushing of the resulting pellet from its original environment into a conventional, liquid-state setting. This melting and flushing usually demands using the equivalent of a few milliliters of hot solvent, a procedure which although well suited for *in vivo* studies leads to an excessive sample volume when considering typical analytical settings. The present study explores a way of reducing the ensuing dilution of the hyperpolarized analytes, by employing a combination of immiscible liquids for performing the melting and flushing. It is shown that suitable combinations of immiscible solvents – both in terms of their heat capacities and densities – allow one to melt the targeted cryogenic pellet and dissolve the hyperpolarized analytes in a fraction of the solvent hitherto required. By tailoring the resulting volume to the needs of a conventional 5 mm NMR probe, a substantial sensitivity enhancement can be added to the hyperpolarization process. An extra benefit may arise from using radicals that preferentially dissolve in the immiscible organic phase, by way of a lengthening of the relaxation time of the investigated analytes. Examples of these principles are given, and further potential extensions of this approach are discussed.

1. Introduction

Whereas the capabilities of nuclear magnetic resonance (NMR) to elucidate molecular structures in both the liquid and solid states are well known, NMR's relative lack of sensitivity puts it at a competitive disadvantage vis-à-vis other modern instrumental techniques. Contributing to NMR's insensitivity is the small proportion of nuclei that become polarized – and hence contribute to the observable signal – in the experiments. Indeed, even if operating at the highest available magnetic fields, conventional room-temperature nuclear polarizations rarely exceed a few ppm's. Challenged by this limitation, recent years have witnessed growing efforts in the development of alternatives capable of increasing nuclear polarizations beyond their Boltzmann-dictated room-temperature values. Nuclear hyperpolarization methods in particular, rely on a variety of physics-based phenomena to create metastable states yielding signals that are orders of magnitude more intense than conventional counterparts. Appealing in its efficiency and generality is the *ex situ* dissolution method put forward by Ardenkjær-Larsen and coworkers in 2003 [1]. Based on well-established principles of dynamic nuclear polarization (DNP), this experiment achieves a unique performance due to a combination of key features. These include transferring an electron's polarization to bulk nuclei by microwave irradiation at the sum/difference of their Larmor frequencies, and an enhancement of these polarizations by operating at relatively high magnetic fields ($\geqslant$3.3 T) and low temperatures ($\sim$1.4 K). The setting used to implement this experiment involves a cryogenic pellet where both the analyte to be observed by NMR and the polarizing radical are co-trapped in a frozen, glassing solvent; DNP is thus executed in the solid state. But, in a crucial step for ensuing liquid-phase applications, this cryogenic pumping process is followed by a sudden dissolution of the sample. The resulting solution is then transferred to the environment where the NMR or MRI observations are to take place. By implementing this melting and transfer of the sample rapidly relative to the nuclear T_1 relaxation time, dissolution DNP avoids significant losses of polarization. It can thus yield liquid signals that have been enhanced several orders of magnitude, opening up new, hitherto untapped opportunities that have been applied successfully in analytical NMR and preclinical MRI applications [2–8].

One drawback of this liquids-oriented *ex situ* DNP, relates to the sample dilution that is inherent to it. This method requires

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sufficient volume of hot solvent for both the sudden melting of the targeted pellet from its cryogenic environment to near room-temperature conditions, as well as for rapidly transferring the resulting sample from the hyperpolarization to the NMR/MRI environs. An ~30× dilution ratio, from a ca. 0.1 mL initial pellet up to a 3.0 mL liquid sample, is thus not uncommon in this kind of experiments. While ending up with sizable, diluted sample volumes is sometimes desired in clinical and preclinical applications [9], these demands can be taxing in analytical applications for two main reasons. One is that the when translated into normal (e.g., ~1 mM) final analyte concentrations, the ensuing dilution implies that the starting concentrations needed for the initial pellet preparation often have to be in the 0.1–10 M range – a concentration which may be limited by a substrate's solubility. Furthermore, an ~30× dilution means that the magnetization observed (even if not the actual polarization) will have been effectively diluted by 1–2 orders of magnitude, with much of the polarized sample often remaining outside the region of observation – in the neighborhood of 0.4 mL for a conventional NMR measurement. None of these are desirable features in analytical applications. Possible solutions put forward to deal with this feature include carrying out the DNP pumping process at room-temperature and liquid-state conditions [10], rapid temperature jumps where melting is achieved without dissolution [11], and promising dual-centered approaches where the sample is actually moved physically from its cryogenic DNP to an ambient-like NMR environment [12].

The present Communication addresses the dilution problem inherent to *ex situ* DNP NMR, by exploring a modified dissolution in which the cryogenic pellet is flushed out by a mixture of aqueous and organic solvent vapors. These in turn are chosen so that: (i) Although immiscible and readily phase separable, both solvents have similar heat-transfer and material properties. (ii) One of the solvents will preferentially dissolve the analyte being targeted and position it in the NMR observation coil region, with an optimal dilution as required by this hardware. (iii) The remaining, majority solvent used for performing the melting will have a preference for dissolving the co-polarizing radical, so as to effectively partition it away from the observable sample. As illustrated below the main advantages foreseen from a dilution employing a reduced effective volume and a simultaneous radical extraction, involving a more concentrated sample and longer T_1 times for the nuclear hyperpolarization, are born out in the final liquid-state NMR sensitivity.

2. Experimental

All NMR measurements were performed using 5 mm NMR tubes on a 500 MHz Varian Inova® spectrometer, equipped with an inverse HCN triple-resonance triple-axis gradient probe. The active coil size targeted for these experiments was ca. 1.8 cm long, requiring in turn ≈0.4–0.5 mL of sample for optimal sensitivity and shimming. All DNP processes in this study were executed using an Oxford Instruments Hypersense® polarizer operating at 3.35 T and nearly 1.5 K. Nuclear hyperpolarization was achieved by irradiating the targeted samples using 100 mW microwave powers. These samples were flushed out off their cryogenic environments by ca. 4 mL of overheated solvent vapors, pushed by approx. 7 bars of pressurized 99.999% He gas blowing for 1.2 s. In general, no special precautions were taken for synchronizing the NMR acquisitions with these sample transfer processes; instead, NMR scans were began concurrently with the sample's dissolution and an array of acquisitions run at a 1–3 s recycle delay. The specimens targeted in these DNP experiments included (i) 50 μL samples of a 4 M natural abundance *d*-fructose solution in D_2O (an intrinsic glass forming solution) commixed with 2,2,6,6-tetramethylpypiridine-1-oxyl (TEMPO) at a 20 mM concentration as polarizing radical; (ii) 5 μL of neat $^{13}C_1$-pyruvic acid commixed with Oxford Instrument's Trityl radical OX63 at a 15 mM concentration; and (iii) aliquots of 1:1 H_2O:DMSO samples containing either 25 mM TEMPO or 25 mM 4-OH-TEMPO as co-polarizing radicals. The first two of these samples were geared at hyperpolarized ^{13}C NMR determinations, and in them the DNP process was implemented by irradiating with 100 mW of microwaves at ~93.94 GHz frequencies for 1 h. Protons were polarized in the last of these samples for ~20 min using 94.02 GHz microwaves at 180 mW. All chemicals were commercial and used as received.

3. Results and discussion

As mentioned, the goal of this study is to explore the possibility of utilizing a mixture of immiscible organic and aqueous solvents: letting one of these phases take most of the burden involved in heating and flushing the cryogenic pellet from the hyperpolarizer to the NMR, while using the second one to actually dissolve the targeted sample in the minimum volume demanded by the NMR coil for an optimized sensitivity and line shape. A convenient mixture to use when considering the observation of hydrophilic analytes is offered by water and toluene. Both of these solvents have similar boiling points (100 and 116 °C respectively), comparable gas-phase heat capacities (2.0 and 1.3 J/g K), and can reach a vapor pressure of ≈10 bar (a reasonable value for flushing the cryogenic pellet) at similar temperatures: 180 and 216 °C respectively. Both of these solvents are fairly immiscible with one another, and will spontaneously break the emulsion that may form upon flushing them simultaneously with a pressurized gas in ca. 1 s. Moreover water is substantially more dense than toluene, implying that upon filling a syringe or an NMR tube with this mixture the aqueous phase – and in it the eventual hydrophilic substrate that one is trying to target – will preferentially concentrate in the lower part of the vessel. This is in turn the one that would be normally injected if dealing with a syringe, or preferentially observed if filling up a conventional 5 mm tube in an NMR probe.

Fig. 1 (shown as a full video in the Supplementary S1 material) illustrates some of these considerations, with the aid of a series of photographs taken from an actual "dummy" dissolution performed with a water/toluene mixture, inside a 5 mm NMR Shigemi® tube placed outside the NMR magnet. Targeted in this dissolution was a pellet placed inside the Hypersense's insert, made up of 10 μL of water/DMSO and including a small amount of a hydrophilic, brightly colored red food dyestuff. This colored glass was cooled to 1.5 K, and then dissolved with 4.5 mL of overheated fluid involving ≈4 mL of toluene + ≈0.5 mL of H_2O. The first of these solvents does most of the sample heating and subsequent flushing, whereas the latter actually dissolves the analyte for its eventual observation. Both of these solvents were placed in the Hypersense's usual heating chamber, and pressurized to 11 bar before their sudden release. As can be seen in the ensuing series of pictures, this release leads to a rapid filling of the 5 mm NMR tube, and to a nearly instantaneous concentration of the targeted dye into the lower, aqueous phase. The effective volume in which the liquids NMR observation takes place is then confined to the ≈0.5 mL that the probe is meant to accommodate. Inspection of the sample container where the pellet was originally placed (not shown) reveals no coloring and thereby no leftover of the original sample in the hyperpolarizer.

It follows from these arguments that repeating a DNP NMR experiment using such mixture of solvents, should give NMR experiments on hydrophilic compounds an enhanced sensitivity vis-à-vis a standard water-only dissolution. That this is indeed the case is illustrated in Fig. 2A and B, which shows two

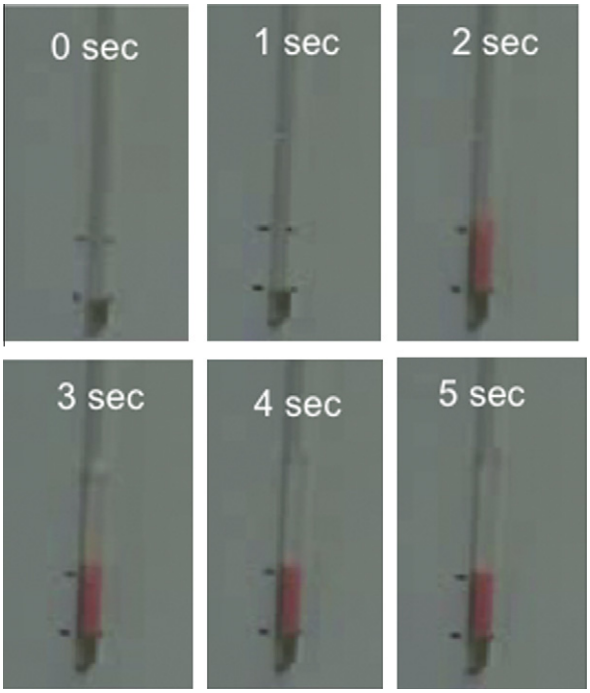

Fig. 1. Snapshots taken one second apart, showing how the rapid ejection of a water/DMSO cryogenic pellet from the DNP hyperpolarizer using a water/toluene mixture, manages to extract the hydrophilic analyte into a lower, aqueous-only phase. The analyte in question was a red food colorant, and the sample began reaching the tube ca. 1 s after time = 0. Also shown with black marks on the background are the 1.8 cm subtended by the typical coil used in 5 mm NMR probeheads. (Supplementary material shows a full 10 s video of this Shigemi® tube fill-up process, illustrating the nearly complete phase separation occurring between the phase to be targeted and the carrier solvent in a ≈1 s timescale).

complementary ^{13}C-based measurements. One involves hyperpolarization of fructose with TEMPO; the other hyperpolarization of $^{13}C_1$-pyruvic acid with OX63. In both of these examples it can be seen that replacing 3 mL of water by a 0.5/4 mL water/toluene mixture possessing a similar "flushing" capacity, leads to dissolution DNP NMR spectra whose sensitivity has been increased by ca. 3×. This is close to the factor that could have been *a priori* expected, as under the conditions that were here assayed only ≈1.8 mL of the flushing volume actually reached the 5 mm NMR tube – with the rest remaining (mostly free of analyte) in the tubing along the hyperpolarizer → spectrometer path. That the sensitivity enhancing effects displayed by the modified dissolution experiments arise due to their reduction in the analyte's dilution, can be further appreciated from the insets shown in Fig. 2B. These show ^{13}C NMR spectra collected on the $^{13}C_1$-pyruvic acid samples arising from these two injection modes, following the spins' return to normal thermal equilibrium. As can be seen these thermally-equilibrated spectra display – aside of a much poorer sensitivity related to the disappearance of the nuclear hyperpolarization – the same sensitivity ratio as their single-scan hyperpolarized counterparts. Given that all other factors in these quantitative experiments remained constant, such differences in sensitivities need to be ascribed to different pyruvic acid concentrations among the sample that was dissolved purely in water, vs. the one dissolved in the 1/8 water/toluene mixture.

An additional benefit that may arise from this kind of mixed-solvent dissolution experiments, concerns the partition and extraction of the radical away from the sample being analyzed. Separating the free radical needed for the DNP portion of the experiment has been shown useful for the NMR stage of the observation, both from a point of view of extending the liquid-state T_1 times that dictate the lifetime of the hyperpolarized states, as well as for toxicity reasons when dealing with *in vivo* measurements. A number of

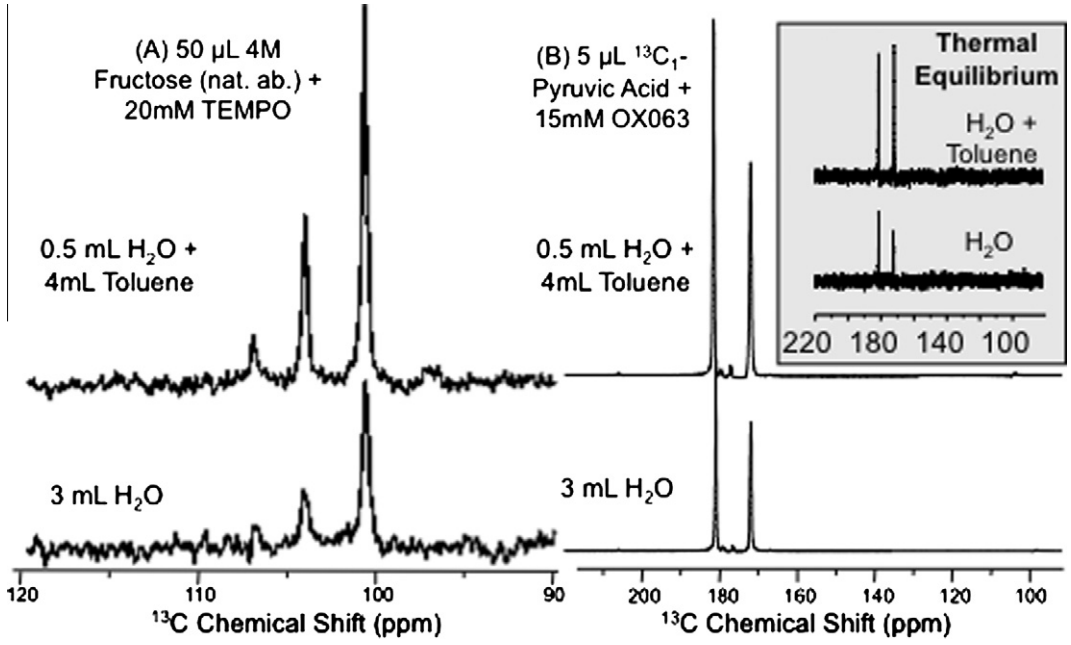

Fig. 2. Examples of the consequences of using mixed-solvent dissolutions for enhancing sensitivity in *ex situ* DNP, illustrated for ^{13}C NMR experiments carried out on two simple hydrophilic analytes. (A) Dissolution experiments on hyperpolarized fructose showing the absolute-scale differences observed for the three anomeric peaks arising from the C_2 site (which being a carbonyl is the only site endowed with relatively long T_1s) upon executing the dissolution with the indicated solvents. (B) Idem but for the C_1-carbonyl of labeled pyruvic acid, whose spectra are characterized by two peaks from the acid and from its hydrate. Shown in the inset of this panel are multi-scan thermally-equilibrated ^{13}C NMR spectra arising following the injection of these hyperpolarized samples, collected with a long recycle delay. The quantitative nature of these data confirm that most of the sensitivity gain observed in the hyperpolarized NMR experiments arises due to an increased concentration of the analytes in the coil volume (All hyperpolarized spectra were collected ca. 3 s following the samples' dissolution; further details can be found in the Experimental section).

experimental options capable of achieving this and their ensuing advantages have been demonstrated, using such strategies as immobilized radicals, molecular filtering setups and scavenging agents [13,14]. In the present scenario, we find that an additional benefit of using a combination of immiscible solvents for the sample dissolution and transport is the possibility to extract the polarizing radical away from the solution that is involved in dissolving the analyte. The effect that this has on the T_1's of the resulting compounds was investigated by focusing the dissolution DNP NMR experiment on protons; these are fast-relaxing systems where the difference between fast and slow relaxation may have a critical outcome on the applicability of the technique. ^{1}H polarization upon dissolving a 1:1 DMSO/H_2O pellet was thus measured upon using two different radicals: TEMPO, a mostly hydrophobic radical, and it's more hydrophilic analog 4-OH-TEMPO. Both the water and the DMSO protons could be polarized by factors on the order of $\approx$500$\times$ by either one of these radicals; the performance of both radicals was apparently the same. Still, as summarized in Fig. 3, the decay of the ensuing hyperpolarizations was quite sensitive depending on whether the dissolution was performed with only water (3 mL), or with a water/toluene 1/6 mixture (3.5 mL). In the pure water dissolution case, the life times of the hyperpolarized protons in D_2O was given by an apparent T_1 decay of $\approx$8 s (a value which decreased by ca. 50% when doing the dissolution in H_2O, and which may also reflect to some extent details of the tube fill-up process). Upon dissolving a TEMPO pellet with the water/toluene mixture, however, the hyperpolarization's apparent lifetime increases by about 6 s; by contrast, doing a water/toluene dissolution upon using 4-OH-TEMPO as polarizer *decreases* these apparent T_1's. This differential behavior can in turn be associated with the differential extraction that the D_2O/C_7H_8 dissolution would do on a pellet containing either one of these radicals: for the more hydrophobic TEMPO the radical would be preferentially extracted into the toluene and the remaining aqueous phase would be depleted from the latter's relaxation-enhancing properties; by contrast, the more hydrophilic 4-OH-TEMPO the radical would actually concentrate in the aqueous phase upon dissolving the pellet with the organic/aqueous mix and, owing to the aqueous phase smaller resulting volume, lead to a more rapid destruction of the hyperpolarization for all protons that remain in that (observed) phase. This in turn means that, if targeting an aqueous phase or a hydrophilic metabolite, carrying out the DNP with a hydrophobic radical and then doing the dissolution with an hydrophobic/aqueous solvent mix, might enhance the metabolite's/water signal via two parallel mechanisms: a decrease in the sample's dilution as detailed in Figs. 1 and 2, and a concomitant extraction of the radical and thereby lengthening of the hyperpolarization life time. These two factors are shown in action together in Fig. 3 for the case of ^{1}H-detection on hyperpolarized water; they are also illustrated in the paper's Graphical Abstract for a ^{13}C-detection experiment on sodium acetate. Such sensitivity-enhancing factor will only be important if the partition coefficients of the targeted analyte and of the co-polarizing radical are sufficiently different among the two solvents employed; still, it is likely that suitable solvents and/or radical combinations can often be found, to maximize this effect.

We believe the present study illustrates but some out of the many opportunities which could be opened by the use of mixed solvent mixtures in the *ex situ* dissolution DNP NMR and MRI experiments. The results shown involved the use of toluene, an affordable and convenient solvent available in both per-protio and per-deutero forms – albeit one that may not be optimal for biological applications. On the other hand many other organic/aqueous alternatives exist; at the moment, for example, we are exploring the use of perfluorinated solvents for this kind of experiments [15]. Such commercially-available liquids have a number of distinct advantages including thermal properties that are similar to those of water, the possibility of controlling their density to make

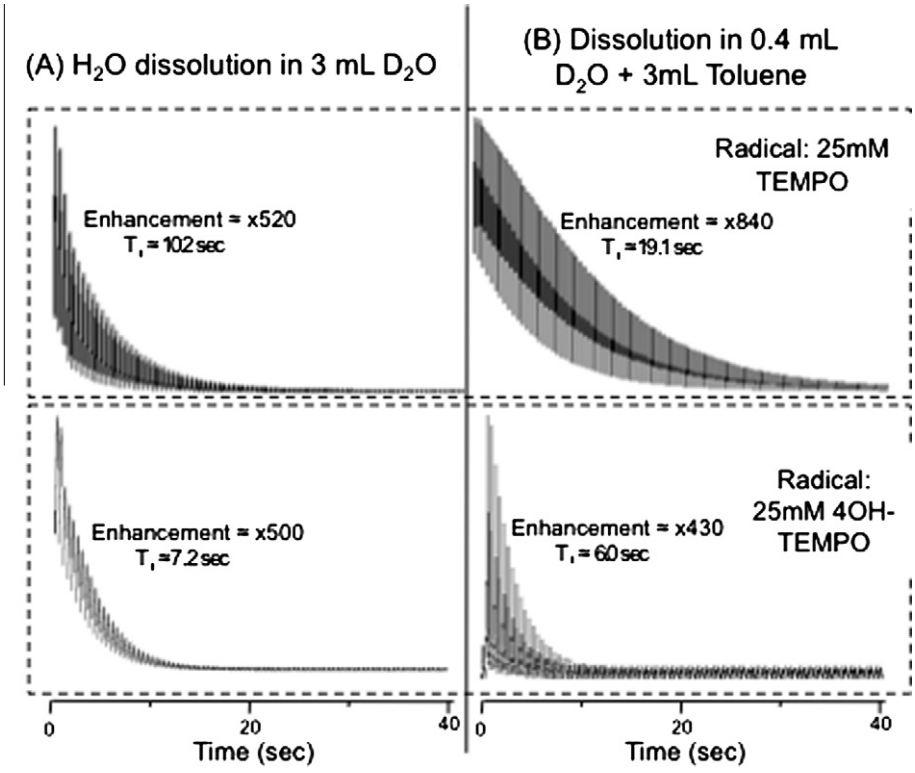

Fig. 3. Differential enhancements and relaxation effects observed in DNP-enhanced ^{1}H NMR experiments, when executed with the indicated combinations of radicals and of dissolution solvents. Further experimental details can be found in the main text.

them slightly "heavier or lighter" than water (so as to better target the mixture to the observation of hydrophilic or hydrophobic substrates), and biological inertness. From the spectroscopic standpoint they also exhibit important added values in their absence of major NMR-active nuclides other than ^{19}F, and of suitable susceptibility matching vis-à-vis aqueous solutions [16,17]. Another interesting option to consider involves combining the present approach with suitable radical quenchers, in the hope of bypassing the shortening of the hyperpolarization's T_1 should this problem be exacerbated by the use of a solvent mixture. These and other alternatives are currently under scrutiny in both NMR and MRI settings.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmr.2011.04.001.

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