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Efficient Hyperpolarization of U-\(^{13}\)C-Glucose using Narrow-line UV-generated Labile Free Radicals


**Abstract:** Free radicals generated via irradiation with UV-light of a frozen solution containing a fraction of pyruvic acid (PA), have demonstrated their dissolution Dynamic Nuclear Polarization (dDNP) potential providing up to 30% \([1-^{13}\text{C}]\)PA liquid-state polarization. Moreover, their labile nature has proven to pave a way to nuclear polarization storage and transport. Herein, differently from the case of PA, we tackled the issue of providing dDNP UV-radical precursors, trimethylpyruvic acid (TriPA) and its methyl-deuterated form d\(_9\)-TriPA, not involved in any metabolic pathway. The \(^{13}\)C dDNP performance was evaluated for hyperpolarization of \([\text{U}-^{13}\text{C}]_\text{c,1,2,3,4,5,6-d}_\text{6}-\text{D}_\text{2}-\text{D}-\text{glucose}\). The generated UV-radical proved to be a versatile and highly efficient polarizing agent providing, after dissolution and transfer (10 s), a \(^{13}\)C liquid-state polarization up to 32%.

During the last decade, \(^{13}\)C hyperpolarized (HP) magnetic resonance imaging (MRI) and spectroscopy (MRS) have encountered a tremendous development, showing convincing demonstrations in detecting and monitoring biochemical changes in real time in both clinical and preclinical studies.[1] Among the different hyperpolarization techniques used to increase the NMR sensitivity of the substrate, dissolution Dynamic Nuclear Polarization (dDNP) is the most widespread one, because of its versatility in biomedical applications.[2] In particular, hyperpolarization of \(^{13}\)C enriched molecules shows a combination of features that make it the ideal nucleus for real-time metabolic studies:[3] ubiquitous presence in biomolecules, large chemical

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Supporting information for this article is given via a link at the end of the document.
The generation of labile paramagnetic species from UV-light illumination of α-keto acids has been explained by photoexcitation of the $n - \pi^*$ transition (300 – 350 nm) of the α-carbonyl group, followed by efficient intersystem crossing (ISC) to an excited triplet state ($3\pi^*$).[10] When PA is the photoactive precursor, "PA" can react with another PA molecule and two paramagnetic intermediates are expected to appear: the ketyl radical CH$_3$C(OH)C(O)OH (R1 in Figure 1) and the acyloxyl radical CH$_3$C(O)C(O)Ȯ (R2 in Figure 1); the latter can then decarboxylate into the acetyl radical CH$_3$C(Ȯ)(OH).[11] These radicals are extremely unstable at room temperature, but can be, at least partially, "captured" when the UV-irradiation takes place in liquid nitrogen (77 K).[12] Having a clear understanding of which intermediates survive is crucial for the appropriate choice or chemical synthesis of UV-radical precursors with improved $^{13}$C dDNP properties. To clarify this point UV-irradiation was performed in liquid nitrogen on frozen solutions of glycerol:water 50:50 (v/v) (GW55) containing 10% of natural abundance PA or PA with site specific $^{13}$C labelling.

In Figure 2A to 2C, the X-band ESR spectrum measured at 77 K (black line) and the corresponding fit (dashed red line) are reported for UV-irradiated PA:GW55 1:9 (v/v), [1-]$^{13}$C-PA:GW55 1:9 (v/v) and [2-$^{13}$C]-PA:GW55 1:9 (v/v), respectively. While the same g-tensor = [2.0036 2.0027 2.0007] could be used for fitting all three spectra, it is interesting to see how the hyperfine coupling changes as a function of the $^{13}$C labelling of the PA molecule. In panel A the peak quartet spectrum was reproduced through an isotropic coupling $a_{1H} = 1.67$ mT to the magnetically equivalent methyl protons, in good agreement with previous studies.[12] In panel B the $^{13}$C labelling in C1 position generated a peak quintet and it was taken into account by adding an extra isotropic coupling $a_{13C} = 1.07$ mT. In panel C the strong ($a_{13C} = 2.82$ mT) coupling to the $^{13}$C nucleus in C2 position changed dramatically the ESR spectrum appearance generating almost a doublet of quartet (for more details about spectra fitting see Supporting Information). Thus, $^{13}$C labelling affected the spectrum in both cases with a stronger effect on C2. On the basis of the foregoing discussion, we can state that the radical stabilized by the cold environment and thus, active DNP wise is the ketyl one. It appears clear now that the choice of TriPA as UV-radical precursor provides a more isolated electron environment: by pushing the methyl protons two carbon positions away, the hyperfine coupling was reduced ($a_{1H} = 0.11$ mT) generating a sharp single line X-band ESR spectrum (see Figure 2D). Deuteration of the methyl groups decreased the hyperfine coupling ($a_{1H} = 0.02$ mT) and the ESR linewidth even further (data not shown). Moreover, the g-anisotropy was less pronounced when TriPA and d$_5$-TriPA were the radical precursors: g-tensor = [2.0031 2.0022 2.0012] (see Supporting Information for spectra comparison at 6.7 T).

As previously described,[5] also UV-irradiated dDNP samples were prepared in liquid nitrogen outside from the polarizer. We studied frozen solutions consisting of 0.7 M TriPA and d$_5$-TriPA with 2M [U-$^{13}$C,d]-D-glucose dissolved in GW55. Measurements were performed on a single 4.0±0.2 µL frozen bead immersed in liquid nitrogen (A). Room temperature UV-vis absorption measurements for the same sample solutions are shown in (B) (left y-scale); the light blue coloured area represents the spectral irradiance (right y-scale) of the UV-source (Dymax, BlueWave 75) at maximum power (19 W/cm²) used to photo-generate the radicals.

In Figure 2A to 2C, the X-band ESR spectrum measured at 77 K (black line) and the corresponding fit (dashed red line) are...
found experimental evidence that the TriPA light absorption in difference in hydrate amounts was observed between the other present work, the physical chemistry behind the origin of the isotopic effect characterizing d9-TriPA remains unclear. Indeed, when in water solution, hydration of the carbonyl group of ketones is a well-known phenomenon. The hydrate form of the precursor molecule is not photoactive and does not contribute to the generation of any radical. We estimated the amount of trimethylpyruvate, d5-trimethylpyruvate and pyruvate hydrate in the three liquid mixtures via 13C NMR. A higher amount of hydrate corresponds to the d9-TriPA sample, but no significant difference in hydrate amounts was observed between the other two (Supporting Information). Although beyond the scope of the present work, the physical chemistry behind the origin of the isotopic effect characterizing d9-TriPA remains unclear. In order to obtain efficient DNP, it is important to achieve a homogeneous radical distribution inside the frozen sample. This is a minor issue when using chemical doping, but may be more challenging when radicals are photo-induced. Using a methodology previously described, we demonstrated that for this low concentration of precursor the paramagnetic centres were homogeneously induced inside the sample volume (see Supporting Information).

Figure 4. The longitudinal detected (LOD) ESR spectrum (grey curve) and the 13C DNP microwaves sweep without modulation (blue curve) and with modulation (red curve) are reported for TriPA_DNP-sample (panel A) and d9-TriPA_DNP-sample (panel B). All measurements were performed at 6.7 T and 1.1 K. Samples were UV-irradiated in liquid nitrogen for 300 s.

In Figure 4 the ESR spectrum and 13C DNP microwaves sweep measured at 6.7 T and 1.1 K are shown for TriPA_DNP-sample and d9-TriPA_DNP-sample. Even at high field, where the anisotropic Zeeman interaction dominates the linewidth, the deuterated sample showed a slightly narrower ESR spectrum (grey curves) because of the reduced hyperfine coupling to the methyl groups (numerical values are reported in Table 1). Nevertheless this feature did not generate any significant difference between the widths of the two 13C DNP sweeps (blue curves). For both samples the maximum positive and negative DNP enhancement (DNP+ and DNP-) appeared at 187.98 GHz and 188.17 GHz, respectively, with |DNP+| > |DNP-|.

Modulation of the microwaves frequency improved the DNP enhancement (red curve). A stronger effect was observed on the protonated sample: at optimal conditions (20 MHz of modulation amplitude, 1 kHz of modulation frequency), the DNP value improved by 37% for TriPA_DNP-sample and 23% for d9-TriPA_DNP-sample. This difference is in good agreement with the different radical concentration generated in the two samples. Indeed, a higher radical concentration yields stronger electron spin dipolar coupling. The latter, especially at high field, represents a key parameter to efficiently saturate the radical ESR line and provide better DNP performance, thus decreasing the microwaves modulation effect.

Figure 5. The 13C polarization solid-state build-up (left panel) and liquid-state relaxation (right panel) are shown for TriPA_DNP-sample, d9-TriPA_DNP-sample and Trityl_DNP-sample. Solid-state measurements were performed at 6.7 T and 1.1 K by mw irradiation at 188.19 GHz (20 MHz amplitude modulation and 1 kHz frequency modulation) for TriPA_DNP-sample and d9-TriPA_DNP-sample; Trityl_DNP-sample was polarized at the 187.94 GHz (no microwaves modulation) corresponding to the optimum for Trityl (see Supporting Information). Liquid-state measurements were acquired on 9.4 T high resolution magnet equipped with a 5 mm NMR probe kept at 40 °C. The transfer of the HP solution from the polarizer to the high resolution magnet took 10 s. In the inset the HP[U-13C,d7]-D-glucose NMR spectrum corresponding to the first point of d9-TriPA_DNP-sample signal decay is reported (C2-C5 DNP enhancement = 41000).

TriPA_DNP-sample and d9-TriPA_DNP-sample were polarized at optimal conditions (microwaves frequency 188.19 GHz with ±20 MHz frequency modulation at a rate of 1 kHz) to estimate the maximum achievable DNP enhancement. The build-up time constants (Tb) were 1836±228 s and 1230±30 s, respectively (n=3). Once at the plateau the samples were quickly dissolved and transferred (Ttrans = 10 s) to a 9.4 T high resolution magnet kept at 40 °C (see Figure 5). The 13C liquid-state polarization (PSS) was 26.8±1.1 % for TriPA_DNP-sample and 30.1±1.8 % for d9-TriPA_DNP-sample, in both cases the liquid state T1 was close to 20 s (n = 3). Because of the excessively long spin-lattice relaxation time in the solid state (>13 h), the polarization value at the moment of dissolution (PSS) was measured only once for each sample. The result was in good agreement with the value back calculated from the liquid-state polarization: PSS = PSS(exp(Ttrans/T1)). We obtained solid-state polarizations of 42.9±1.8 % and 49.5±3.0 % for TriPA_DNP-sample and d9-TriPA_DNP-sample, respectively. To test the versatility and improved DNP performance of the new UV-radicals we polarized the reference substrate 1,1-bis(hydroxymethyl)cyclopropane-1-13C,d9 (HP001), at the same DNP conditions of d9-TriPA_DNP-sample where 2 M HP001 replaced the labelled glucose in the preparation. HP001 is a well suited “polarization probe” since its
liquid state $T_m = 123.0 \pm 1.0$ s. The measured $^{13}$C liquid-state polarization of HP001 was 53.7±2.0 % (n = 2) (see supporting Information).

We compared the results achieved using UV-TriPA and UV-d9-TriPA to the routinely used trityl radical. The Trityl$_{DNP}$-sample was prepared by dissolving 30 mM trityl radical AH111501 and 2 M labelled glucose in GW55; DNP was performed at 187.94 GHz corresponding to microwave sweep positive lobe maximum for trityl (see Supporting Information). Although 30 mM trityl radical represents the optimal concentration to perform dDNP on $[1^{-13}$C]PA at 6.7 T (with 70% $^{13}$C polarization routinely obtained),[17] Trityl$_{DNP}$-sample liquid-state polarization was not any higher than 21.1±1.5 % (n = 3) in good agreement with previous results.[8a] All relevant dDNP data are summarized in Table 1.

![Table 1](image)

We verified the radicals persistency in vision of establishing a robust protocol for storage and transport of HP glucose samples.[7] In Figure 6 we report how the UV-TriPA (panel A) and UV-d9-TriPA respond to temperature. In order to compensate for the Boltzmann factor and take into account the number of paramagnetic centres only, the ESR signal intensity multiplied by the Boltzmann factor and the measured 13C liquid-state polarization and liquid-state relaxation time $T_1n$.

![Figure 6](image)

We finally injected d9-UV-TriPA hyperpolarized [U-$^{13}$C,d7]glucose into live prostate adenocarcinoma cells to judge the spectral influence of the presence of radical precursor (see Figure 7). At the current concentration the signals from the precursor are large relative to the metabolite signals in a cell experiment with limiting biological material (7 million cells). However, none of the precursor signals overlapped with metabolites from the glycolysis and had thus no influence on a kinetic analysis.

The results herein show that UV-TriPA and UV-d9-TriPA, are valuable polarizing agents for $^{13}$C DNP at high magnetic field. Compared to UV-PA, they benefit from a higher radical yield and improved DNP performance. These two features, respectively, are a consequence of stronger light absorption in correspondence to the $n-n^*$ electron transition and a narrower ESR spectrum due to reduced g-anisotropy and hyperfine coupling to the C2 position. The $^{13}$C polarization level achieved was comparable to or better than trityl radical for the same sample. Moreover, their unique property of quenching above 190 K (see Figure 7). At the current concentration the signals from the precursor are large relative to the metabolite signals in a cell experiment with limiting biological material (7 million cells). However, none of the precursor signals overlapped with metabolites from the glycolysis and had thus no influence on a kinetic analysis.
and the absorbance of TriPA and d9-TriPA is relatively small. As recently demonstrated, efficient photo-generation of these labile radicals is strictly related to the photon density at the radical precursor light absorption peak.\[16\]

### Experimental Section

Chemicals were purchased from Sigma-Aldrich, (2605 Brøndby, Denmark) excepted for the radical precursor d9-TriPA (synthesized in house, see Supporting Information) and the Trityl radical AH111501 (GE Healthcare, Amersham, UK). All experimental methods and hardware used were described previously.\[3\]

The UV-source (Dymax, BlueWave 75) spectral irradiance was kindly provided by the manufacturer. In the present work the UV-source was always operated at its maximum power (19 W/cm²). Indeed, as previously demonstrated for the case of PA,\[5\] the maximum radical yield was achieved at these experimental conditions. It is worth pointing out that the HP sample was finally dissolved in 10 mL of hot 40 mM phosphate buffer.

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### Conflict of interest

Dr. Arnaud Comment is currently employed by General Electric Medial System, Inc.

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Trimethylpyruvic acid is a non-expensive and effective UV-induced radical precursor for dDNP. Its molecular structure provides high radical yield and a narrow ESR linewidth beneficial for high performance [U-\textsuperscript{13}C]glucose hyperpolarization.

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