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Efficient Hyperpolarization of U-13C-Glucose using Narrow-line UV-generated Labile Free Radicals

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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% [1-13C]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 d9-TriPA, not involved in any metabolic pathway. The 13C dDNP performance was evaluated for hyperpolarization of [U-13C6,1,2,3,4,5,6,6-d7]-D-glucose. The generated UV-radical proved to be a versatile and highly efficient polarizing agent providing, after dissolution and transfer (10 s), a 13C liquid-state polarization up to 32%.

During the last decade, 13C 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.

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. In particular, hyperpolarization of 13C enriched molecules shows a combination of features that make it the ideal nucleus for real-time metabolic studies: ubiquitous presence in biomolecules, large chemical shift dispersion (possibility to easily distinguish between several substrates), low natural abundance (absence of a background signal) and relatively long nuclear longitudinal relaxation time ($T_1$) at specific molecular positions (e.g. carbonyl groups, tens of seconds). Indeed in a dDNP experiment, enhancement of the nuclear polarization (i.e. the relative difference between the populations of the two spin eigenstates) takes place ex-situ in the so-called DNP polarizer. The latter provides a moderate magnetic field (3.35 – 7 T) and a cold environment (1 – 1.5 K) hosting a glassy frozen sample that includes the substrate of interest (at molar concentration) and unpaired electrons (15 - 50 mM), in the form of organic free radicals. After the microwaves driven polarization transfer from the electron spins to the surrounding nuclear spins reaches a steady-state, the frozen sample is quickly dissolved in a hot buffer and ejected from the polarizer. Extracting the sample in the liquid state is at the same time the strength and weakness of the technique. On the one hand, the polarization created in the solid state is preserved, generating a high sensitivity metabolic probe ready to be injected. But on the other, a dedicated polarizer is needed as close as possible to the MRI scanner, since the HP state is $T_1$-limited. A way to circumvent this limitation is to employ labile radicals. Labile radicals, generated via irradiation with UV-light of a frozen solution containing a fraction of PA (UV-PA), have demonstrated their dDNP capability on PA itself and other substrates, providing up to 30% [1-13C]PA liquid-state polarization at optimal conditions. Moreover, their unique feature to recombine around 190 K allows recovery of a HP solution without radicals and has proven to pave the way, together with other approaches, to nuclear polarization storage and transport. Indeed, quenching the radicals when the sample is still frozen gives the possibility of extracting it from the DNP polarizer and dissolve the HP sample far away from its production site.

In the present work we tackled the issue of providing dDNP UV-radical precursors not involved in any metabolic pathway and producing labile radicals with improved 13C dDNP performance compared to UV-PA. To this end, we investigated the photochemical properties of two α-keto acids, trimethylpyruvic acid (TriPA) and its methyl-deuterated form d9-TriPA (see Figure 1). We studied their dDNP efficiency as non-persistent radicals on isotope labelled D-glucose ([15C6,1,2,3,4,5,6,6-d7]), a substrate showing increasing interest in the hyperpolarization community thanks to the richer metabolic pathways it can give access to, compared to the routinely used [1-13C]pyruvate. Narrow ESR-line radicals (e.g. Trityls or BDPA), are well known to provide good direct DNP enhancements on 13C nuclei, although UV-PA has a thinner spectrum than the well-known broad nitroxyl radicals (e.g. TEMPO and TEMPOL), it still shows an ESR-line too broad compared to the 13C Larmor frequency, providing efficient DNP on protons. Thus, it may not be the optimal precursor for direct 13C nuclei polarization in general.
The generation of labile paramagnetic species from UV-light illumination of α-keto acids has been explained by photo-excitation of the $n\rightarrow\pi^*$ transition (300 – 350 nm) of the α-carbonyl group, followed by efficient intersystem crossing (ISC) to an excited triplet state (3PA*). When PA is the photoactive precursor, 3PA* can react with another PA molecule and two paramagnetic intermediates are expected to appear: the ketyl radical CH3C(OH)C(O)OH (R1 in Figure 1) and the acyloxyl radical CH3C(O)C(O)Ȯ (R2 in Figure 1); the latter can then decarbonylate into the acetyl radical CH3ĊC(O). 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). Having a clear understanding of which intermediates survive is crucial for the appropriate choice or chemical synthesis of UV-radical precursors with improved 13C isotropic properties. To clarify this point UV-irradiation was performed in liquid nitrogen on frozen solutions of PA:GW55 1:9 (v/v) containing 10% of natural abundance PA or PA with site specific 13C labelling. The structural formula of the radical precursor is shown.

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), [1-13C]PA:GW55 1:9 (v/v) and [2-13C]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 13C 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. In panel B the 13C 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 (ambient) $g_{13C} = 2.82$ mT) coupling to the 13C 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, 13C 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 ($a_{2H} = 0.02$ mT) and the ESR linewidth even further (data not shown). Moreover, the g-anisotropy was less pronounced when TriPA and d9-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, 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 d9-TriPA with 2M [U-13C,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 3A the radical generation as a function of UV-irradiation time for frozen mixtures of 0.7 M TriPA (red curve), d9-TriPA (black curve) and PA (blue curve) with 2 M [U-13C,d]-D-glucose dissolved in GW55. Measurements were performed on a single 4.0±0.2 µL frozen bead immersed in liquid nitrogen (A).
sample for 300 s was sufficient to reach the radical concentration plateau. Surprisingly the radical yield was doubled when d9-TriPA was employed. For the sake of comparison a third sample containing 0.7 M of PA was prepared. The latter generated half of the radical concentration obtained with the TriPA sample. We found experimental evidence that the TriPA light absorption in two (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.\cite{11} The hydrate form of the precursor molecule is not photoactive and does not contribute to the generation of any radical.\cite{13} We estimated the amount of trimethylpyruvate, d9-trimethylpyruvate and pyruvate hydrate in the three liquid mixtures via 13C NMR. A higher amount of hydrate characterizes the sample containing PA, 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\cite{14} but may be more challenging when radicals are photo-induced. Using a methodology previously described,\cite{16} we demonstrated that for this low concentration of precursor the paramagnetic centres were homogeneously induced inside the sample volume (see Supporting Information).

In Figure 4 the ESR spectrum and 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.

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 and d9-TriPA_DNP-sample. In both cases the liquid state polarization 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) 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 T2 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: $P_{ss} = P_{ss,exp} \times (T_{ss,exp}/T_{ss})$. 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-\textsuperscript{13}C,d8 (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 $T_{m} = 123.0\pm1.0$ 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-d$_9$-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.

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-d$_9$-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 take into account the number of paramagnetic centres only, the ESR signal intensity multiplied by

We finally injected d$_9$-UV-TriPA hyperpolarized [U-$^{13}$C,d$_7$]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 glycology and had thus no influence on a kinetic analysis.

The results herein show that UV-TriPA and UV-d$_9$-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 has two main advantages: first it allows recovering a HP solution naturally free of radical; second and more important, it represents the key feature to move towards a protocol for polarization storage. Our next aim is to decrease the radical precursor concentration to the tens of mM range, in order to reduce as much as possible the presence of any liquid-state background signal other than the HP substrate itself in a metabolic study. Preliminary results on samples preparation show that by doubling the UV-light power using two sources simultaneously, it is possible to achieve the same radical concentration with half of the precursor amount. As shown in Figure 3B, the overlap between the irradiance of the UV-source

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**Table 1** The main dDNP parameters and results obtained for TriPA$_{DNP}$-sample, d$_9$-TriPA$_{DNP}$-sample and Trityl$_{DNP}$-sample at 6.7 T and 1.1 K are summarized. From the 1st to 7th column we report in order: sample name, radical concentration, ESR spectrum full-width at half-maximum measured at DNP conditions, solid-state buildup time constant $T_{ss}$, solid-state back calculated $^{13}$C polarization, liquid-state $^{13}$C polarization and liquid-state relaxation time $T_{1n}$.

<table>
<thead>
<tr>
<th>DNP Sample</th>
<th>Radical conc [mM]</th>
<th>ESR linewidth [MHz]</th>
<th>SS $T_1$ [s]</th>
<th>SS pol [%]</th>
<th>LS $T_1n$ [s]</th>
<th>LS pol [%]</th>
<th>LS $T_1n$ [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TriPA</td>
<td>20±1</td>
<td>142</td>
<td>1836±128</td>
<td>49.5±3.0</td>
<td>30.1±1.8</td>
<td>20.1±1.0</td>
<td></td>
</tr>
<tr>
<td>d$_9$-TriPA</td>
<td>40±1</td>
<td>131</td>
<td>1230±30</td>
<td>42.9±2.8</td>
<td>26.8±1.1</td>
<td>19.8±1.0</td>
<td></td>
</tr>
<tr>
<td>Trityl$_{DNP}$</td>
<td>30</td>
<td>108</td>
<td>1330±28</td>
<td>35.7±2.5</td>
<td>21.1±1.5</td>
<td>19.4±0.8</td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 6.** The ESR signal as a function of temperature is reported for TriPA$_{DNP}$-sample (panel A) and d$_9$-TriPA$_{DNP}$-sample (panel B). In both cases, the ESR signal suddenly disappeared above 190 K. The two samples became diamagnetic when still frozen.

**Figure 7.** UV-d$_9$-TriPA hyperpolarized [U-$^{13}$C,d$_7$]glucose conversion in live prostate carcinoma cells (PC-3). A build-up of the glycolysis derived metabolite lactate could be followed over 30 s. Intermediates in glycology are identified by $^{13}$C-$^{13}$C coupling constants due to the uniformly labelled substrate and indicated by black bars in a sum of 30 spectra: Lactate (LAC), Glutamate carbon #1 and #5 (GLU-C1 and C5), 3-phosphoglycerate (3PG), unspecified amino acid (AA). Remaining radical precursor is identified outside ppm area of interest as singlets (TriPA). The signal denoted * is likely to originate from the precursor.
and the absorbance of TriPA and de-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.[18]

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 Triyl radical AH111501 (GE Healthcare, 01494 Amersham, UK). All experimental methods and hardware used were described previously.[4]

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,[19] 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 Medical System, Inc.

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