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UV-irradiated 2-Keto-(1-¹³C)isocaproic Acid for High Performance ¹³C Hyperpolarized MR

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ABSTRACT

Enhancing the sensitivity of magnetic resonance spectroscopy/imaging (MRS/MRI) by dissolution dynamic nuclear polarization (dDNP) has expanded the scope of MRS applications to new fields of research. Most importantly, it has paved a way towards non-invasive studying of the fate of a metabolite in real time. As its name implies, in a typical dDNP experiment, the hyperpolarized (HP) sample is extracted from the polarizer in the liquid state. This procedure, limits the HP signal exploitation time window to approximately 1 min, but it is also the only way to preserve the high spin order created in the solid state at low temperature (1 – 1.5 K) and moderate magnetic field (3.35 – 7 T) by means of microwave irradiation. Indeed, although necessary for the DNP process to happen, the presence of free radicals in the sample would prevent its extraction as a solid for relatively longer-term storage and transport to remote locations. Moreover, for biological or clinical applications, the radical should be removed from the hyperpolarized (HP) solution. This limitation can be overcome by using thermally labile ultraviolet (UV)-generated radicals that have been shown to be efficient polarizing agents, provide a radical free HP solution, and most importantly, pave the way for the transport of HP solid samples to remote sites. Herein, we demonstrate that 2-keto[1-¹³C]isocaproate (KIC), an important metabolic biomarker in brain, can be highly polarized via dDNP using the non-persistent ketyl-radical generated by UV-irradiation of the substrate itself. We investigated precursor molecule and radical properties via UV-vis measurements and ESR measurements at both X-band and high field. After optimizing sample preparation and microwave irradiation conditions, we obtained 56% ¹³C liquid-state polarization in 1 h by performing dDNP at 6.7 T and 1.1±0.1 K.

I. INTRODUCTION

A dramatic increase in the sensitivity of magnetic resonance spectroscopy and imaging (MRS and MRI), obtained with hyperpolarization via dissolution dynamic nuclear polarization (dDNP),^[1] has led to entirely new *in vivo* applications based on real-time ¹³C-traced metabolism.^[2] Hyperpolarized (HP) ¹³C MR has been used widely in various preclinical models to trace the metabolism in real time, in which it has been shown to be sensitive to variations in key metabolic pathways.^[2-7] Eminent applications of HP MR counts the ability to non-invasively perform staging of cancer and monitoring therapy response as well as measuring metabolic processes in the human brain.^[6-12]

In DNP, the carbon MR polarization enhancement is created in the solid state as a non-equilibrium nuclear spin state by microwave induced transfer of polarization from unpaired electron spins, in the form of organic radicals, to the ¹³C-nuclei inside the sample. After the polarization transfer process, *in situ* fast dissolution with pressurized pre-heated buffer is performed to take out the sample from the DNP polarizer while preserving the nuclear spin polarization. Extracting the sample in the liquid state for use as a metabolic contrast agent is at the same time the strength and weakness of the technique. The more than 10,000-fold enhanced polarization created in the solid state is preserved to the liquid state; however, the hyperpolarization is decayed by the sub-minute ¹³C-nuclear longitudinal relaxation time (T_1) in solution. This inherent limitation of the dDNP technique results in the need for a dedicated and technically demanding hardware (DNP polarizer) placed in near proximity to the patient MR, which limits the widespread use of the technique. In light of the biomedical potential of dDNP, new efficient polarization methods to limit the

polarization decay as well as an easy access to crucial hyperpolarized substrates is the need of the hour for successful clinical translation of the method.^[7, 13]

To dispense of the polarizer at individual clinical sites would crave to significantly enhance polarization lifetime on the hyperpolarized ^{13}C -nuclei. One prospect is to exploit the several hours long ^{13}C -nuclear relaxation times when at cryogenic temperature and moderate magnetic field.^[14] For this to be possible, however, paramagnetic centers need to be removed from the sample because they prevent its extraction as a solid while keeping the HP state alive.^[15]

Labile radicals generated by ultraviolet (UV) light irradiation of an α -ketoacid, such as pyruvic acid (Pyr), at low temperature ($\sim 77\text{ K}$) has been shown to be a promising substitute for persistent radicals.^[16] Such type of radicals are thermally unstable and recombine into diamagnetic compounds upon dissolution and provide a radical free HP liquid. One of the main reasons to choose α -ketoacids as dDNP radical precursors is their strong UV-sensitivity.^[17-21] Additionally, the products of photolysis are typically non-toxic at low concentrations, not showing any adverse effect when employed *in vivo* or *in vitro*.^[16, 22, 23] The most intriguing property of these non-persistent radicals is that they can be quenched by raising the temperature above the radical annihilation point (i.e. $>195\text{ K}$ for Pyr).^[24] By thoroughly optimizing the temperature cycling, most of the hyperpolarization can be preserved during this process.^[24] It has been shown that this unique feature of the UV-radicals has promising scope for the extraction of solid sample followed by the transport/storage of HP molecules (at low magnetic field and low temperature) far away from the production site.

Although, UV-induced radicals have shown a great potential in HP MR, only three types of precursors have been explored to date. Among those, Pyr plays a special role since it acts as a

metabolic substrate as well as an endogenous radical precursor.^[16, 25, 26] The non-metabolic UV-precursors trimethylpyruvic acid (TMP) and deuterated TMP (dTMP) have both been shown to be very efficient for hyperpolarizing glucose mitigating the unwanted metabolic activity of pyruvate.^[22] When hyperpolarizing UV-sensitive substrates such as dihydroxyacetone phenylglyoxylic acid (PhGA) with its extended absorption in visible light range (>400 nm) has proven useful.^[23] However, the radical concentration generated from PhGA UV-photolysis is low, which may make it less desirable for dDNP at higher field than the *HyperSense* standard.^[23] Indeed, a higher radical concentration in the sample would require higher precursor molecule concentration (>5 M) raising toxicity issues for *in vivo* applications. Each UV-radical precursor is different and requires an optimized sample preparation based on its chemical and physical properties such as solubility, glassing properties, absorbance, radical generation ability, and ESR spectral parameters. Considering the potential of UV-radical based dDNP, the further investigation and characterization of new UV-precursors, that similarly to Pyr can act as radical and substrate within the same molecule, is of high interest and would increase the scope of applications.

Besides Pyr, another alpha-keto acid, 2-keto[1-¹³C]isocaproate; [1-¹³C]KIC, has shown great potential as a HP metabolic contrast agent. HP [1-¹³C]KIC has been demonstrated to efficiently probe branched chain amino acid transferase (BCAT) activity with its metabolic conversion to the essential amino acid [1-¹³C]leucine.^[27] It has been used to assess molecular signatures of tumors in rodents based on vastly different levels of BCAT activity.^[27] HP [1-¹³C]KIC has also been implemented for imaging of cerebral metabolism in normal rats and has been suggested to be a promising substrate for evaluation of cerebral BCAT activity, linked to neurodegenerative diseases.^[28] Investigating brain metabolism by means of HP MRS, as a path to identify metabolic

signatures for various brain diseases, is of growing interest. Therefore, HP [1-¹³C]KIC metabolism is highly topical.

Herein, we report the thorough investigation of [1-¹³C]KIC as an endogenous UV-radical precursor. Challenges arising from the physical properties of the molecule have been identified and tackled. These includes the hydrophobic nature of this radical precursor, which limits the employment of widely used aqueous solvents for DNP samples preparation. The latter had implications on radical generation and DNP efficiency.

II. EXPERIMENTAL METHODS

All chemicals were purchased from Sigma-Aldrich, 2605 Brøndby, Denmark.

a. UV-Vis absorption measurements

The ultraviolet–visible (UV-Vis) absorption of KIC and [1-¹³C]KIC (8.0 M, neat) was investigated. Measurements were performed on a DH-2000 (Ocean Optics, Dunedin, FL, USA) UV–vis spectrometer equipped with a 0.1 mm light path quartz SUPRASIL cuvette (Hellma Analytics, Germany) to minimize the light path across the sample. This allowed us to measure non-diluted dDNP sample solutions. The precursor molecules absorption was investigated from 300 nm to 600 nm in steps of 1 nm.

b. ESR and DNP sample preparation

ESR samples. All ESR and DNP experiments were performed on samples irradiated using the setup reported by Capozzi *et al.*^[26] Radical generation efficiency and glassing properties of different solutions were investigated. The UV-radical precursors, KIC and [1-¹³C] KIC, were

employed in their neat form or diluted in glycerol, ethanol, DMSO, and polyethylene glycol (PEG) 400. The liquid sample was prepared in an Eppendorf vial and vortexed for 2 min followed by 10-min sonication/degassing at 40 °C to ensure a homogeneous mixing and a better glass quality of the matrix upon freezing. After sonicating the solution, a volume of $4.0 \pm 0.2 \mu\text{L}$ was taken from the vial by means of a micropipette (1–10 μL) or a syringe, depending on the viscosity of the sample, and poured as a droplet in a Dewar filled with liquid nitrogen (LN_2) to form a single frozen bead. Before starting the UV-irradiation, the diameter of the sample bead was measured using a precooled caliber tool. Finally, the bead was transferred to a synthetic quartz Dewar (Miniscope MS 5000 ESR spectrometer compatible, Magnettech, Berlin, Germany) filled with LN_2 as well for UV irradiation. UV-irradiation was performed by means of a broadband UV-source (Dymax BlueWave 75, Dymax Europe GmbH, Wiesbaden, Germany) operated always at maximum power (19 W/cm²).

DNP samples. After glassing and ESR properties investigation, three formulation were chosen to prepare DNP samples: neat [$1\text{-}^{13}\text{C}$]KIC, [$1\text{-}^{13}\text{C}$]KIC in ethanol (80:20, v/v), and [$1\text{-}^{13}\text{C}$]KIC in glycerol (60:40). For DNP experiments, 10-15 sample beads were prepared and UV-irradiated in one single round. The UV-irradiated sample could either be stored in liquid nitrogen for future use or directly loaded into the dDNP polarizer.

c. X-band ESR measurements and quantification of radical concentration

The ESR spectrum was acquired at 77 K using a benchtop X-band ESR spectrometer (MiniScope MS5000, Magnettech GmbH, Berlin, Germany). Measuring parameters were optimized to resolve the hyperfine structure of the spectrum and avoid saturation: (center of the field = 338 mT, sweep range = 20 mT, sweep time = 20 s, modulation amplitude = 0.1 mT, modulation frequency = 100

kHz, microwave power = 0.2 mW). The radical concentration was quantified from the ESR spectrum double integral by means of a calibration curve obtained from a series of $4.0 \pm 0.2 \mu\text{L}$ frozen beads of known concentrations of 4-hydroxy-TEMPO (12.5–100 mM) in glycerol:water 60:40 (v/v). The measured radical concentration was corrected for the sample bead volume calculated from the measured diameter. The radical buildup curve of each sample was obtained by measuring the ESR spectrum after each successive irradiation period. The error shown in quantification of radical concentration is an estimation of the standard deviation from replicates ($n=3$).

d. LOD-ESR measurements

The ESR spectrum and electron spin lattice relaxation time (T_{1e}) were measured at DNP conditions (6.7 T and 1.1 ± 0.1 K) using a homemade LOD-ESR setup only for the KIC:glycerol 60:40 (v/v). The ESR spectrum was obtained by sweeping the microwave frequency in steps of 5 MHz, while the T_{1e} was obtained in a single shot measurement. Details about the hardware and methods were described in detail earlier.^[26,29,30]

e. Thermal stability of the radicals

The thermal stability of the non-persistent ketyl-radical was investigated using a variable temperature unit of the MiniScope 5000 ESR spectrometer. The same ESR parameters were used and the temperature was varied from 100K - 270K. A frozen bead of $4.0 \pm 0.2 \mu\text{L}$ of $[1-^{13}\text{C}]\text{KIC:glycerol 60:40 (v/v)}$ was irradiated for 400 s and transferred to a 100 K precooled 5 mm quartz ESR tube placed inside the resonant cavity of the spectrometer. The different ESR spectra were measured in temperature steps of 5 K and the sample space allowed to stabilize for 5 s at

each step. Acquired data was processed in MATLAB (Mathworks, Natick, MA, USA) and OriginPro (OriginLab Corporation, Northampton, MA, USA). Each data point was multiplied by the corresponding temperature to compensate for the Boltzmann factor.

f. Solid-state DNP

All solid-state DNP measurements were performed on a home-built dDNP polarizer (magnet from Magnex, Oxford, UK) working at 1.1 ± 0.1 K and 6.7 T. The UV-irradiated sample beads were loaded using a custom fluid path (CFP) following methods described earlier.^[26,30] Microwaves were delivered from a 94 GHz solid-state source VCOM-10/94-WPT (ELVA-1, St. Petersburg, Russia) coupled to a 200×2R4 frequency doubler (VDI, Charlottesville, VA, USA), which provided an output power of 55 mW at 188 GHz. The source, digitally controlled through NI-DAQ device USB-6525 (National Instruments, Austin, TX, U.S.) has a tuning range of ± 0.6 GHz and the possibility to modulate the output frequency at a rate up to 2 kHz and with an amplitude of up to 100 MHz. All ^{13}C NMR acquisitions were performed using a Varian INOVA console (Palo Alto, CA, USA) connected to a low-temperature probe, placed inside the polarizer VTI (variable temperature insert), and remotely tuned to 71.8 MHz. The flip angle for all acquisitions was set to 2° (pulse length of = 5 μs for an amplifier power of 30 W). The optimal microwave frequency for DNP enhancement was identified by sweeping the latter in steps of 10 MHz. At each microwave frequency, the buildup lasted for 60 min, the NMR signal acquired, and finally destroyed before moving to the next frequency step.

The polarization buildup was monitored by pulsing every 300 s. Once the maximum polarization was achieved, the integral of the NMR signal was measured. The thermal equilibrium buildup was carried out overnight without microwaves. The signal was acquired every hour until complete

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2
3 relaxation was achieved. The DNP enhancement was calculated by dividing the DNP signal by
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5 thermal equilibrium signal.
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9 The acquired data were processed with MNOVA (Mestrelab Research, Santiago de Compostela,
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11 Spain) and OriginPro (OriginLab Corporation, Northampton, MA, USA). The standard deviation
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13 in the measurement of solid-state polarization is estimated from three replicates (n=3).
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15

16 17 **g. Dissolution and liquid state NMR** 18 19

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21 The dissolution head was loaded with 8.0 mL phosphate buffer, pressurized to 4 bar using helium
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23 gas, and finally heated up to 170 °C. Once maximum solid-state polarization was achieved, the
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25 sample vial was lifted above the liquid helium level (~10 cm) and the hot pressurized buffer was
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27 released inside the CFP. A chase gas pressure of 4 bar was used to blow over the HP solution and
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29 transfer it directly into a 10 mm NMR tube waiting inside a 9.4 T high resolution NMR magnet
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31 (Agilent, Palo Alto, CA, USA). The transfer from the dDNP polarizer to the 9.4 T magnet took 2
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33 s. Details about the CFP and dissolution system and procedure were extensively described
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35 earlier.^[30] The NMR tube contained a calculated amount of 10 M sodium hydroxide (NaOH)
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37 solution to obtain a pH of 7.3-8.0 in the final solution. In case of manual transfer to the 9.4 T
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39 magnet of the HP solution (10 s elapsed time), the latter was collected in a receiver placed close
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41 to the polarizer and containing the NaOH solution. The decay of the ¹³C HP signal was measured
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43 recorded every 3 s for 3 min using a 5° pulse angle. The sample was then doped with 50 µL of
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45 Dotarem and the same 5° pulse was used to measure the signal corresponding to thermal
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47 equilibrium (400 averages with 100 ms repetition time). The DNP enhancement was calculated as
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49 described earlier and the standard deviation in the measurement of the liquid state polarization is
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51 estimated from three replicates (n=3).
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III. RESULTS AND DISCUSSION

a. UV-sample optimization

Different α -ketoacids show different chemical and physical properties mainly dependent on their characteristic functional group. For instance, Pyr is nicely self-glassing and can be used as such in DNP sample preparations. Differently, TMP, dTMP, and PhGA require an aqueous solution involving a glassing co-solvent to obtain a good sample matrix upon freezing. Similarly, the amount of the non photo-active hydrate form of the precursor also depends on the functional group adjacent to α -ketoacid unit.^[31] Consequently, the sample preparation is unique for each specific precursor molecule and requires particular care.

Figure 1A UV-vis absorption spectrum of neat KIC (red color) and (1-¹³C)KIC (blue) at room temperature and the area colored in violet represents the wavelength range and spectral irradiance (right Y-scale) covered by the Dymax

BlueWave 75 at maximum power (19 W cm^{-2}). Diluted KIC samples also showed similar results. **B** Radical generation time evolution measured on a $4.0 \pm 0.2 \text{ }\mu\text{L}$ frozen bead of neat KIC (red circles) and ($1\text{-}^{13}\text{C}$)KIC (blue circles). The dotted lines represents the mono-exponential fit of the data points. **C** X-band ESR spectrum with normalized signal intensity measured on a $4.0 \pm 0.2 \text{ }\mu\text{L}$ frozen bead of UV-irradiated ($1\text{-}^{13}\text{C}$)KIC and KIC samples. The chemical structure of the active radical species is shown in respective colors of the ESR spectrum.

We report in **Figure 1A** the UV-vis absorption spectrum of neat KIC and neat [$1\text{-}^{13}\text{C}$]KIC. The two compounds had maximum absorption happening at the same energy, but the natural abundance one showed a slightly higher peak. This behavior nicely correlated with the two radical generation buildups (**Figure 1B**). Natural abundance KIC plateaued at $59.8 \pm 3 \text{ mM}$, while [$1\text{-}^{13}\text{C}$]KIC at $56 \pm 2.8 \text{ mM}$. Although we have no mechanistic explanation for that, this feature seems to be recurrent for UV-induced radicals, providing further demonstration that the radical yield increases with the absorption coefficient of the precursor and the UV-source power.^[22,26] The X-band ESR spectra (**Figure 1C**) clearly showed that the generated radical is of the ketyl type: being the unpaired electron mainly localized on the C2 carbon, labelling the precursor molecule in C1 position provided a relatively small broadening of the spectrum. Identical conclusions were established in a previous report from our group.^[22]

Figure 2 Radical generation as a function of UV irradiation time measured on a $4.0 \pm 0.2 \mu\text{L}$ frozen bead of: **A** $(1-^{13}\text{C})\text{KIC}$ solutions in EtOH **B** $(1-^{13}\text{C})\text{KIC}$ solutions in PEG; **C** $(1-^{13}\text{C})\text{KIC}$ solutions in DMSO; **D** $(1-^{13}\text{C})\text{KIC}$ solutions in glycerol. The dotted lines represents the mono-exponential fit of the data points and ratio of $(1-^{13}\text{C})\text{KIC}$ in solvent (v/v) is shown in brackets on the figures.

Although KIC forms a glass upon freezing,^[27, 28, 32] the amorphous matrix was not stiff enough to withstand UV-irradiation at high power, causing the shattering of the sample. This made the radical quantification as well as sample handling very difficult. Therefore, we first investigated the possibility to mix $[1-^{13}\text{C}]\text{KIC}$ with a suitable glassing solvent to improve the sample glass quality. To this end, we investigated the organic and biocompatible solvents ethanol (EtOH), glycerol (Gly), PEG400, and dimethyl sulfoxide (DMSO). It is worth mentioning that we used the solvents in their neat form and not as aqueous solutions, as it is custom in dDNP, because KIC is

hydrophobic. At the same time, this allowed us to limit the generation of the non-photoactive hydrate form of the radical precursor.^[31]

The purpose was to find a sample formulation having a concentration of $[1-^{13}\text{C}]\text{KIC}$ as high as possible along with a glassing matrix strong enough to withstand UV-photolysis. The radical generation time course for such frozen samples is reported in **Figure 2**. Numerical results are summarized in **Table 1**. The samples prepared in EtOH produced the highest concentration of radical. However, this was because of sample shattering during irradiation. For a given penetration depth of the light, small sample fragments offered higher useful volume for UV-radical generation. This also explains the very erratic radical buildup time constant trend in case of samples prepared in EtOH (**Table 2**). Surprisingly, increasing too much the content of EtOH in the sample made the glass more fragile. Next, the samples prepared in PEG were investigated and the generated radical concentration measured. Although in general the sample glass quality improved compared to EtOH, the matrix was still not able to withstand a full cycle of UV-irradiation to obtain at least 40 mM radical concentration. We assumed this value as lowest threshold to perform efficient DNP at 6.7 T using UV-induced radicals.^[22,26]

(1- ¹³ C)KIC concentration (%)	Radical concentration (mM)			
	(1- ¹³ C)KIC:EtOH	(1- ¹³ C)KIC:PEG	(1- ¹³ C)KIC:DMSO	(1- ¹³ C)KIC:Gly
90	55.0±2.8	50.0±2.6	38.6±2.0	-
80	54.0±3.0	43.0±2.1	30.0±1.5	54.3±2.7
70	55.0±3.0	42.0±2.2	-	46.3±2.3
60	-	-	-	52.0±2.5

50	78.0±3.5	45.0±2.2	-	49.0±2.4
30	66.0±3.8	39.0±2.0	-	-

Table 1. Radical concentration plateau values for (1-¹³C)KIC samples in different solvents.

The preparation in DMSO neither improved the sample quality nor generated a high enough concentration of radical. The latter is because DMSO is also photoactive and absorbs part of UV-light emitted by the source, thus reducing the neat power delivered to the KIC.^[23]

The samples prepared in glycerol provided the best results. The glass quality improved for increasing solvent concentration in the sample. Already at 30 % glycerol, the sample could withstand UV-photolysis without observing any shattering of the frozen beads. At the same time, the generated radical concentration was appropriate to perform DNP at 6.7 T.^[22, 26] The only drawback of using glycerol concerned the solution handling because of high viscosity.

(1- ¹³ C)KIC concentration (%)	Radical Buildup time constant (s)			
	(1- ¹³ C)KIC:EtOH	(1- ¹³ C)KIC:PEG400	(1- ¹³ C)KIC:DMSO	(1- ¹³ C)KIC:gly
90	35±20	132±11	184±6	-
80	60±60	192±16	92±12	222±126
70	40±22	166±26	-	203±20
60	-	-	-	192±25
50	254±45	60±10	-	207±30
30	76±16	327±34	-	-

Table 2. Radical buildup time constant obtained from the fit of raw data for (1-¹³C)KIC samples in different solvents.

A good compromise was represented by the sample containing 60% $[1-^{13}\text{C}]\text{KIC}$ in glycerol. The solution was not prohibitively viscous and provided 52.0 ± 2.5 mM of radical with a generation build-up time constant of 192 ± 25 s. Samples with a higher amount of KIC were too fragile (fraction of the sample breaking on photolysis) and samples with higher fraction of glycerol were too viscous to be handled properly.

Figure 3 The normalized ESR signal as a function of temperature for 60% $(1-^{13}\text{C})\text{KIC}$ in glycerol. The ESR signal diminishes above 200 K.

On the same formulation we investigated the thermal stability of the UV-radicals (**Figure 3**). The sudden drop in ESR signal was observed at about ~ 200 K confirming the quenching of the UV-radicals via recombination into diamagnetic molecules. The quenching temperature was slightly higher than UV-irradiated aqueous solutions containing Pyr and TMP derived radicals. Being the quenching process related to the onset of ice diffusivity, the absence of water in the formulation, with a melting temperature lower than KIC and glycerol, might justify the observed behavior.^[19]

b. dDNP results

The $[1-^{13}\text{C}]\text{KIC}:\text{glycerol}$ 60:40 (v/v) sample formulation was also our best choice for dDNP experiments (see Supporting Information for the other samples and for numerical solid-state polarization data). The microwave frequency sweep was carried out to establish best DNP conditions. The highest enhancement was obtained for a microwave irradiation frequency of 188.215 GHz with microwave modulation at the rate of 1 kHz and an amplitude of 35 MHz (sine function modulation profile). The results are reported in **Figure 4A**. On applying microwave frequency modulation, the DNP spectrum became broader with respect to the non-modulated one and the DNP enhancement improved by the factor of 2.73. For lower or higher values of modulation amplitude the DNP enhancement resulted slightly smaller. The beneficial effect of microwave frequency modulation suggests inefficient spectral diffusion within the electron spin system at these experimental conditions (6.7 T, 1.1 ± 0.1 K).^[33, 34]

Figure 4 A ^{13}C DNP spectra of $(1-^{13}\text{C})\text{KIC}:\text{glycerol}$ 60:40 (v/v) UV-irradiated for 400 s acquired with no modulation (violet circles), 1 kHz frequency microwave modulation, and 25 MHz amplitude (red circles), 35 MHz amplitude (green circles), and 40 MHz amplitude (blue circles) superimposed to the LOD-ESR spectrum (grey circles) of

KIC:glycerol 60:40 (v/v) measured at 6.7 T and 1.1 ± 0.1 K. **B** Electron relaxation time (T_{1e}) of KIC UV-radicals in identical conditions.

A validation of our hypothesis came from T_{1e} LOD-ESR measurements. Its value, measured at 188.215 GHz, resulted to be 76 ± 1 ms (**Figure 4B**). The latter was one order of magnitude lower than, for instance, trityl radicals measured at same experimental conditions.^[29] In that case microwave modulation had no effect on the DNP enhancement. The positive effect of applying microwave frequency modulation and the overlap of the DNP and LOD-ESR spectrum suggest that thermal mixing is the dominant mechanism for our system.^[35, 36]

Figure 5 ^{13}C polarization buildup at optimal DNP conditions (188.215 GHz irradiation frequency, 1 kHz modulation frequency, 35 MHz modulation amplitude) at 1.1 ± 0.1 K and 6.7 T for UV-irradiated $(1-^{13}\text{C})\text{KIC:glycerol}$, 60:40 (v/v).

The solid-state polarization buildup was recorded at optimal DNP conditions. The sample was irradiated with microwaves for at least three times the buildup time constant (T_b). The sample reached a ^{13}C solid-state polarization of 58.5 ± 5.0 % with a T_b of 1590 ± 70 s. Decreasing the radical

concentration caused the polarization to reduce from 58.5% to 38% and the buildup time constant increased by a factor of almost two (see Supporting Information, **Table S1**).

Figure 6 HP solution transfer from the dDNP polarizer to NMR spectrometer and decay of the ^{13}C polarization inside the 400 MHz spectrometer for the sample containing 60% $(1-^{13}\text{C})\text{KIC}$ in glycerol. Data were fitted with a mono-exponential curve.

After the solid-state polarization reached the plateau, dissolution was performed, and the HP solution transferred to the 9.4 T vertical NMR magnet. The sample was transferred to the NMR tube directly (transfer time ~ 2 s) or manually (transfer time ~ 10 s). The ^{13}C polarization decay is shown in **Figure 6**. With a direct transfer, we lost approximately 13% of the solid-state value ending up in a ^{13}C liquid-state polarization of 55.6 ± 2.7 %. The liquid state ^{13}C T_1 of 45 ± 1 s was calculated by fitting the data to a mono-exponential curve. In case of manual transfer a liquid-state ^{13}C polarization of 42 ± 2 % was measured. With both direct and manual transfer, the polarization losses correlated nicely with the time elapsed to transport the HP solution from the polarizer to the 9.4 T magnet.

IV. CONCLUSIONS

Herein, we have presented a detailed investigation of UV-irradiated KIC samples as endogenous radical precursor for dDNP applications. [1-¹³C]KIC is a molecule of high interest for the biological and medical community, especially for HP brain metabolic studies. A thorough investigation of sample formulation, UV-irradiation procedures, radical generation, ESR properties at X-band and at DNP conditions allowed us to optimize the system and obtain a liquid-state ¹³C polarization as high as 56% with at T₁ of 45±1 s.

In a future study, we will employ our optimized HP bio-probe to investigate the BCAT activity in the brain and the difference in BCAT activity between healthy and pathological tissues.

ASSOCIATED CONTENT

- ESR spectrometer calibration
- Additional solid-state DNP data

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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