

Quality assurance of PASADENA hyperpolarization for ^{13}C biomolecules

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Abstract

Object Define MR quality assurance procedures for maximal PASADENA hyperpolarization of a biological ^{13}C molecular imaging reagent.

Materials and methods An automated PASADENA polarizer and a parahydrogen generator were installed. ^{13}C enriched hydroxyethyl acrylate, 1- ^{13}C , 2,3,3- d_3 (HEA), was converted to hyperpolarized hydroxyethyl propionate, 1- ^{13}C , 2,3,3- d_3 (HEP) and fumaric acid, 1- ^{13}C , 2,3- d_2 (FUM) to hyperpolarized succinic acid, 1- ^{13}C , 2,3- d_2 (SUC), by reaction with parahydrogen and norbornadiene rhodium catalyst. Incremental optimization of successive steps in PASADENA was implemented. MR spectra and in vivo images of hyperpolarized ^{13}C imaging agents were acquired at 1.5 and 4.7 T. **Results** Application of quality assurance (QA) criteria resulted in incremental optimization of the individual steps in PASADENA implementation. Optimal hyperpolarization of HEP of $P = 20\%$ was achieved by calibration of the NMR unit of the polarizer (B_0 field strength ± 0.002 mT). Mean

hyperpolarization of SUC, $P = [15.3 \pm 1.9]\%$ ($N = 16$) in D_2O , and $P = [12.8 \pm 3.1]\%$ ($N = 12$) in H_2O , was achieved every 5–8 min (range 13–20%). An in vivo ^{13}C succinate image of a rat was produced.

Conclusion PASADENA spin hyperpolarization of SUC to 15.3% in average was demonstrated (37,400 fold signal enhancement at 4.7 T). The biological fate of ^{13}C succinate, a normally occurring cellular intermediate, might be monitored with enhanced sensitivity.

Keywords Hyperpolarization · PASADENA · Fumarate · Succinate · Parahydrogen · ^{13}C MRI

Introduction

PASADENA (Parahydrogen and synthesis allow dramatically enhanced nuclear alignment [1, 2]) is unique in its ability to achieve an extremely high degree of hyperpolarization at liquid state temperatures within seconds and at relative low cost. This is achieved by catalytic addition of molecular parahydrogen to a precursor molecule, while spin order is manipulated consecutively for the generation of net polarization on a designated nucleus, in this case ^{13}C . Nuclear spin polarization depicts the alignment of spins exposed to a magnetic field, and corresponds to the fraction of all spins, that contribute to NMR signal (Eq. 1):

$$P = \frac{N(\uparrow) - N(\downarrow)}{N(\uparrow) + N(\downarrow)} \quad (1)$$

At ambient temperature, the thermal energy exceeds the transition energy of the spin states by orders of magnitudes, leaving only ~ 1 –100 ppm of all spins contributing to the overall NMR signal. This is described mathematically by the Boltzmann distribution (Eq. 2):

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$$P_{\text{Boltzmann}} = \tanh\left(\frac{\gamma\hbar B_0}{2k_B T}\right) \approx \frac{\gamma\hbar B_0}{2k_B T}, \quad (2)$$

where γ is gyromagnetic ratio characteristic for a nucleus of interest, \hbar is the Plank constant divided by 2π , B_0 is the field strength, k_B is the Boltzmann constant and T is the temperature.

Hyperpolarization techniques hold the potential to increase the polarization to the order of unity, which is an enhancement of 4–5 orders of magnitude (η_{observed}) with respect to the thermal polarization originating from the magnetic field (Eq. 3):

$$\eta_{\text{observed}} = \frac{P_{\text{HP}}}{P_{\text{Boltzmann}}}. \quad (3)$$

The polarization achieved in total, P_{HP} , provides a measure for the efficiency of the hyperpolarization technique, independent of the specific system for detection. It is predominantly used in this paper (Eq. 4):

$$P_{\text{HP}} = \eta_{\text{observed}} \cdot P_{\text{Boltzmann}}. \quad (4)$$

Heteronuclear PASADENA hyperpolarization employs the spin order added to the precursor molecule by addition of parahydrogen to form spin polarization on a third, designated nucleus, such as, but not limited to, ^{13}C or ^{15}N [1–3].

The system of two spin 1/2 particles of parahydrogen was predicted 1924 by Born and Heisenberg [4], and experimentally measured three years later by Bonhoeffer and Harteck [5]. The singlet state holds spin zero (Eq. 5), while the triplet states has spin one (Eq. 6).

$$|0, 0\rangle = 1/\sqrt{2} (|\uparrow\downarrow\rangle - |\downarrow\uparrow\rangle) \quad (5)$$

$$|1, 1\rangle = |\uparrow\uparrow\rangle$$

$$|1, -1\rangle = |\downarrow\downarrow\rangle \quad (6)$$

$$|1, 0\rangle = 1/\sqrt{2} (|\uparrow\downarrow\rangle + |\downarrow\uparrow\rangle)$$

The r.f. pulse sequence to form scalar order in the ^{13}C nucleus in a parahydrogenated molecule is described in Goldman et al. [6]. Since the evolution of the spin system is affected by the J-couplings characteristic to the molecular environment, the spin order transfer sequence is specific to each individual molecule.

The method has previously been restricted, as early examples were non-biological molecules, soluble only in acetone. A water-soluble molecule which was toxic at the doses employed, and confined to the vasculature after injection in vivo was employed in a demonstration of ^{13}C angiography [7,8]. A further selection of hyperpolarizable molecules is described by Kuhn and Bargon [9]. A metabolizable molecule, ^{13}C sodium acetylenedicarboxylate which is converted to ^{13}C succinate by two hydrogenation steps, has also been reported from this laboratory [10]. In a recent report, we described hyperpolarization of $1\text{-}^{13}\text{C}$ in succinate [11].

Succinate is an intermediate in the tricarboxylic acid cycle and other significant metabolic pathways, including gluconeogenesis and the recently defined HIF1 α oncogene [12,13]. With the advent of imaging reagents with the potential to broaden the use of parahydrogen hyperpolarization in biology, comes the need to standardize. In a previous manuscript, we described the equipment necessary to perform individual steps of the experiment: PASADENA generation, chemistry and hardware of the hydrogenation reaction and the MR electronics for the spin order transfer [14]. Here we present the user with procedures of quality assurance necessary for the setup and optimization of polarization yield, and introduce standard operational protocols developed for day-to-day hyperpolarization experiments in vivo.

Theoretically, PASADENA is realized in three steps (Fig. 1a–c). In practice, a number of factors determine the overall success of hyperpolarization. First, the quality of parahydrogen, the source of the spin order, limits the level of hyperpolarization. Second, the addition of parahydrogen to the ^{13}C -enriched precursor molecule, the catalyst and the reaction conditions determine whether or not the target molecule is formed efficiently and in such a way that parahydrogen spin order is available to the third step, the manipulations of the spins and transfer of polarization to ^{13}C (or ^{15}N). The efficacy of these manipulations is determined by the accuracy with which the J-couplings of the target molecule are known, and how well the designated manipulations are applied to the spins by r.f. hardware. We describe a technique whereby highly reproducible operation of the PASADENA polarizer can be achieved and using succinic acid, $1\text{-}^{13}\text{C}$, $2,3\text{-d}_2$ (SUC), demonstrate its efficacy in vivo.

Materials and methods

Table 1 establishes a protocol for PASADENA hyperpolarization. For convenience, the materials prerequisite (column A) we describe in Methods while installation, calibration (column B) and the experimental routine (column C), all of which lead to Quality Assurance guidelines, are presented in “Results”.

Prerequisites for PASADENA

Parahydrogen

Commercially available ultra-pure hydrogen (Gilmore, South El Monte, USA) was catalytically converted to parahydrogen (pH_2) by slow passage over granular hydrous ferric oxide (IONEX-type O–P catalyst; Molecular Products Inc., Lafayette, CO, USA). After the gas was converted to parahydrogen it was stored in 7L aluminum cylinders at room

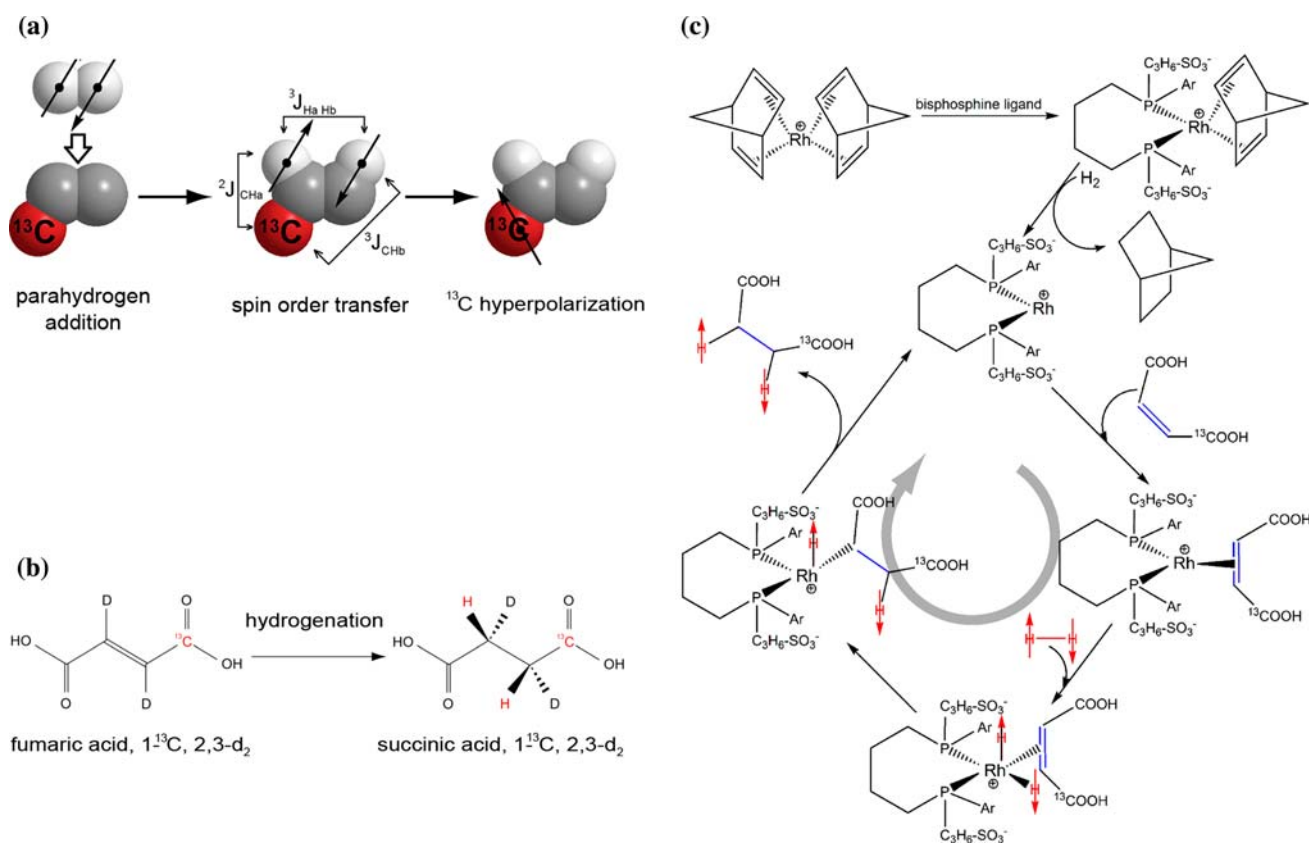


Fig. 1 Chemistry and spin physics of the PASADENA experiment. **(a)** (left) molecular parahydrogen (pH_2) is added to the precursor (^{13}C) by catalytic hydrogenation; (center) manipulations of the spins of the molecule by spin order transfer r.f. pulse sequence, involving the $^2J_{\text{CHa}}$, $^3J_{\text{CHb}}$, $^3J_{\text{HaHb}}$ couplings; (right) net spin polarization is formed on ^{13}C . **(b)** Hydrogenation of precursor FUM forms the product SUC.

temperature at a pressure of 33 bar. The quality of pH_2 was determined to be $>97\%$ by high resolution NMR [7]. Each batch was used within 7 days, with no measurable decrease in the yield.

Imaging reagent precursors

For initial calibration, a previously validated PASADENA reagent, hydroxyethyl acrylate, $1\text{-}^{13}\text{C}$, $2,3,3\text{-d}_3$ (HEA), was prepared as an aqueous solution. 2–5 mg HEA (Isotec Sigma-Aldrich, USA) was dissolved in a small volume of phosphate buffer, pH 7.0, mixed with the catalyst solution and de-aerated using a vacuum line (Schlenk). A second PASADENA reagent [11] introduced for further calibration and biomedical studies; fumaric acid, $1\text{-}^{13}\text{C}$, $2,3\text{-d}_2$ (FUM); (Cambridge Isotope Laboratories, Andover, MA, USA) was dissolved in a small volume of phosphate buffer at pH 2.9. Low pH was necessary for well-defined J-couplings in the resulting hydrogenation product, SUC (as described further below).

Hydrogenation catalyst

A unique catalyst developed for the purpose of hydrogenation which preserves the desired spin order [15, 16] was freshly prepared by mixing two components. The bisphosphine ligand, 1,4-bis-[(phenyl-3-propane sulfonate) phosphine] butane disodium salt (Q36333, Isotec, OH, USA), was dissolved in $\text{H}_2\text{O}/\text{D}_2\text{O}$ to yield 2.5–3.0 mmol/L concentration followed by removal of oxygen using vacuum and nitrogen connected via a manifold. The rhodium catalytic moiety was then introduced to the reaction mixture under nitrogen (N_2) atmosphere as a solution of bis(norbornadiene)rhodium (I) tetrafluoroborate (catalog number 45-0230, CAS 36620-11-8, Strem Chemicals, MA, USA) in acetone with 5% molar excess of bisphosphine ligand with respect to rhodium. The resulting solution was vigorously shaken and acetone was removed under vacuum at room temperature. Excess of bisphosphine is necessary for complete removal of rhodium.

The imaging reagent solution was added to the catalyst and alternately exposed to inert gas (N_2) and vacuum. The

Table 1 Protocol for PASADENA hyperpolarization

A. Prerequisites	B. Installation and calibration	C. Quality assurance routine
1. Parahydrogen (>95%)	1. Polarizer fluid control system	1. Flush the polarizer
2. Precursor molecule(s)	2. Calibration of the low field NMR system	2. Flush delivery system
3. Hydrogenation catalyst	3. Determine center frequency	3. Prepare MRI scanner
4. PASADENA polarizer	4. Calibration of the low field NMR system: adjust flip angles ^1H , ^{13}C	4. Check temperature
	5. J couplings for target molecule	5. Check gas pressure (N_2 , pH_2)
	6. B_0 optimization	6. Check B_0 value
	7. B_1 optimization	7. Check r.f. amplitudes of B_1 pulses

(A) materials required for successful hyperpolarization, (B) tasks required to establish PASADENA hyperpolarization, optimize the operating equipment and (C) quality assurance routines in daily use are summarized under these headings

For A, see “Methods”; for B and C, see “Results”

resulting mixture contained 1–3 mmol/L FUM and 2.0–2.5 mmol/L catalyst concentrations in 50 mmol/L pH 2.9 phosphate buffer. The aqueous mixture of catalyst and molecular precursor was prepared fresh, prior to each hyperpolarization procedure. The completed PASADENA solution of precursor and catalyst was drawn into a 20 mL plastic syringe and connected to valve 2 of the PASADENA polarizer, for injection of the desired amount of imaging reagent precursor for each experiment (3.5 mL unless otherwise noted). When prepared in these proportions, hydrogenation of the precursor was carried to completion and no residual precursor (HEA or FUM) was detected by ^{13}C NMR.

PASADENA polarizer

The very detailed description of the central instrument required are provided in an accompanying manuscript [14].

Other methods

Determination of J-couplings of the PASADENA agents

As indicated in Fig. 1 and described in some detail by Bowers and Weitekamp [1,2], Natterer and Bargon [17] and more recently by Goldman et al. [6,18] significant transfer of spin order from parahydrogen to the third nucleus is achieved through design and implementation of a spin transfer sequence which is then applied to the mixture of ^{13}C reagent and rhodium catalyst. In order to achieve ^{13}C hyperpolarization by r.f. radiation, the spin order transfer (SOT) sequence should be tailored to the J-couplings of the newly added hydrogen and the nucleus to-be-polarized ($^2\text{J}_{\text{CHa}}$, $^3\text{J}_{\text{CHb}}$, $^3\text{J}_{\text{HaHb}}$) in the product molecule. For the experiments described, two SOT sequences designed for HEP [6,18] and SUC [11], respectively, were programmed on the synthesizer incorporated into the polarizer and delivered as r.f. pulses to the B_1 coil of the low field NMR.

Quantification of hyperpolarization

A number of strategies have been employed to quantify the extent of hyperpolarization [19]. In the present paper, the following convention was applied. The signal enhancement (η_{observed}) achieved by hyperpolarization was quantified in respect to the signal of a thermally polarized sample (100% ethanol, 188 mM natural abundance ^{13}C at each site (Eq. 7)). The signal intensities were determined by numerical integration (xWINNMR software, Bruker Biospin, Germany):

$$\eta_{\text{observed}} = \frac{P_{\text{HP}}}{P_{\text{Boltzmann}}} = \frac{I_{\text{HP}}}{I_{\text{ref}}} \cdot \frac{c_{\text{ref}}}{c_{\text{HP}}} \quad (7)$$

where I_{HP} , I_{ref} , c_{HP} and c_{ref} are integral intensities and molar concentrations of hyperpolarized and reference samples, respectively.

T_1 decay of hyperpolarization

The decay of ^{13}C polarization caused by longitudinal relaxation (T_1) of the hyperpolarized sample during the delivery to the detection system was determined (see “Results”). This allows for the determination of the nascent signal enhancement (Eq. 8), and nascent level of achieved polarization (Eq. 9):

$$\eta_{\text{observed}} = \eta^{t=0} \cdot \exp\left(-\frac{\text{delivery time}}{T_1}\right) \quad (8)$$

$$P_{\text{HP}}^{t=0} = \eta^{t=0} \cdot P_{\text{Boltzmann}} \quad (9)$$

Unless otherwise indicated, $P_{\text{HP}}^{t=0}$ and $\eta^{t=0}$ are reported in the work presented, using a ^{13}C polarization of $P_{\text{Boltzmann}} = 0.00041\%$ or 4.1 ppm at 298 K and 4.7 T. T_1 values for the PASADENA reagent concerned in calibration and quality assurance (QA) were determined directly. A series of small angle ($\alpha \approx 8^\circ$) pulse-and-collect experiments was employed to probe the decay of the magnetization of the hyperpolarized agents (small excitation angle approximation, SEA). The T_1 values were extracted by the fit of (Eq. 10) to the data points,

taking into account the loss of magnetization caused by the excitation pulses:

$$I(t) = I_0 \cdot e^{-\left(\frac{t}{T_1}\right)} \cdot e^{-\left(\frac{1-\cos(\alpha)t}{TR}\right)}. \quad (10)$$

Results

Installation and calibration of the PASADENA experiment

Seven steps are indicated (Table 1, Column B) for the set-up and calibration of the polarizer. Together these constitute a Quality Assurance routine which ensures maximal hyperpolarization and optimal reproducibility for each PASADENA reagent.

Step 1: polarizer fluid control system

An automated fluid control system delivers reagents to the reaction chamber. The steps of the hyperpolarization experiment, including the fluid control system were controlled by custom software (LabView platform, National Instruments, Austin, TX, USA) able to load a r.f. pulse file, and to store the sequence of timings and events separate file. The following procedure was optimized to produce hyperpolarized agent in less than 1 min: (1) flush the system with N₂ gas (14 bar), (2) load the aqueous solution of precursor and catalyst in the N₂ path, (3) fill the previously flushed reactor with 10 bar pH₂ and pressurize N₂ path, (4) close pH₂ path, inject precursor solution in pH₂ atmosphere while applying ¹H r.f. saturation pulses (to maintain the singlet state of hydrogen attached to molecules), (5) apply the SOT, (6) deliver the hyperpolarized solution to the detector (eg. small animal NMR or clinical MRI scanner) employing residual pressure. The reader can refer to [14] for a description of the valves function.

Step 2: calibration of the NMR system of the polarizer

An initial calibration of the NMR system of the polarizer is necessary to perform accurate r.f. manipulations on the spins of the PASADENA agent in the reactor. Since the r.f. electronics and coil were designed for transmission only, a second, external high-field NMR system was employed for the detection (7 T Mercury spectrometer, Varian, Palo Alto, USA). A sample of saturated 1-¹³C sodium acetate CH₃-¹³COONa in D₂O was pre-polarized at high field for 3 min, delivered to the polarizer for a ¹³C inversion pulse of arbitrary length, followed by delivery back to the high field unit, where the resulting magnetization was detected by a 90° pulse-acquisition sequence.

Step 3: determine center frequency of NMR system of polarizer

To center the frequency, a ¹³C sample was subjected repeatedly to a “prepolarize_{Varian} – pulse_{polarizer} – delay – pulse-break acquisition_{Varian}” experiment while the strength of the low-field in the polarizer was incremented by 0.1 mT, and the r.f. was kept constant: the optimal field for the 18 kHz carbon pulse was found to be ~1.76 mT at the position of the Hall sensor of the gauss meter (Fig. 2). Once the optimum field for the carbon frequency was found, the corresponding proton frequency was calculated (75 kHz).

Step 4: flip angle calibration

For the calibration of the flip angles of the r.f. excitation pulses (*B*₁), the previous protocol for the combination of low-field pulse and high field detection (Fig. 3) was employed. At constant *B*₀, the width of the r.f. pulse given in the polarizer was varied for ¹³C and ¹H frequencies independently. The acquired data was fitted using a T₁ corrected cosine function, providing an estimate for the *B*₁ fields.

Step 5: determination of J-couplings

While the J-coupling constants for HEP employed in calibration of the polarizer have previously been reported [10], the relevant J-couplings for succinate were unknown and were determined by computations employing GAMMA simulations [20] and confirmed by experiment [11]. The ¹³C spectra (Fig. 4) were acquired using a saturated (100 mM) aqueous

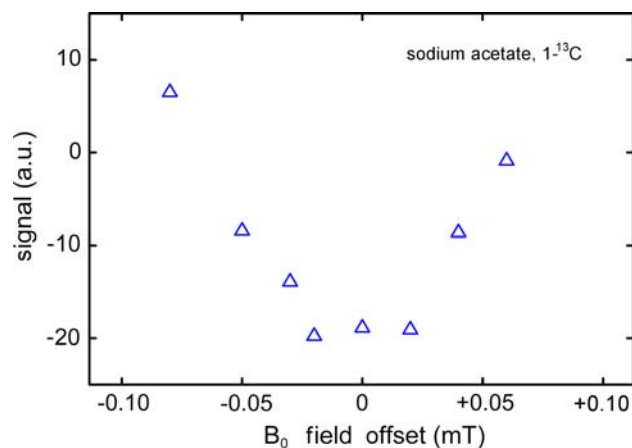


Fig. 2 Experiment to center the frequency of the low field MR unit of the polarizer. A constant ¹³C excitation pulse was applied to a sample of saturated 1-¹³C sodium acetate in D₂O, followed by the transfer (~14 s) and a pulse-acquisition detection in a high field spectrometer. This procedure was repeated for eight settings of the static magnetic field to determine the optimum (1.763 mT). The scale refers to the offset from this optimum

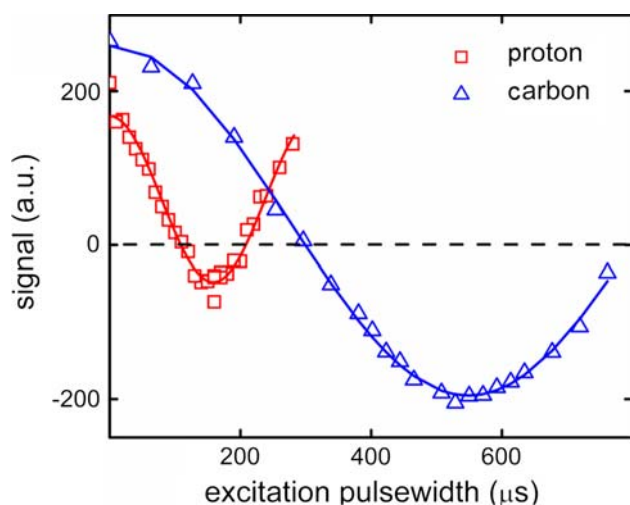


Fig. 3 ^{13}C and ^1H flip angle calibration of the low field MR unit in the polarizer. A ^{13}C or ^1H square excitation pulse was applied to a sample of saturated $1\text{-}^{13}\text{C}$ sodium acetate in D_2O , followed by the transfer (~ 14 s) and a pulse-acquisition detection in a high field spectrometer. This procedure was repeated with 22 and 31 different pulse widths for ^{13}C , ^1H , respectively, at constant amplitudes (^1H 31.8 V, ^{13}C 9.6 V). Inversion pulses were determined to be 157 μs (^1H) and 550 μs (^{13}C), respectively

solution of succinic acid (natural abundance, Isotec, Sigma Aldrich, USA) at a 14 T high resolution spectrometer (Varian, H/C/N probe, 256 acquisitions) without decoupling.

At pH 7.4 (Fig. 4b), substantial line broadening attributed to chemical proton exchange was observed on the carboxyl group (~ 185 ppm) which prohibited the resolution of line splitting by J-couplings. All experiments were therefore performed at pH 2.95 \ll pKa (Fig. 4c).

For the simulations, the spectra of a ^{13}C spin coupled to two protons (C, Ha, Hb) were calculated under the effect of Zeeman interaction and indirect couplings ($^2J_{\text{CHa}}$, $^3J_{\text{CHb}}$, $^3J_{\text{HaHb}}$).

The simulations were performed iteratively varying the J-couplings until the best correspondence of the simulated to the experimental spectra (Levenberg–Marquart least-square fit) was found ($^2J_{\text{CHa}} = -7.15$ Hz, $^3J_{\text{CHb}} = 5.82$ Hz and $^3J_{\text{HaHb}} = 7.41$ Hz).

These constants were employed to tailor the spin order transfer sequence to the target molecule, SUC.

Step 6: optimization of spin order transfer: B_0 calibration with hyperpolarization

Introducing parahydrogen and substituting a PASADENA reagent and catalyst, acceptable levels of hyperpolarized signal were achieved as detected in the high-field spectrometer. The optimization of B_0 to center the frequency was repeated over a smaller range, recording the yield of hyperpolarization at each field (Fig. 5). Maximal polarization of

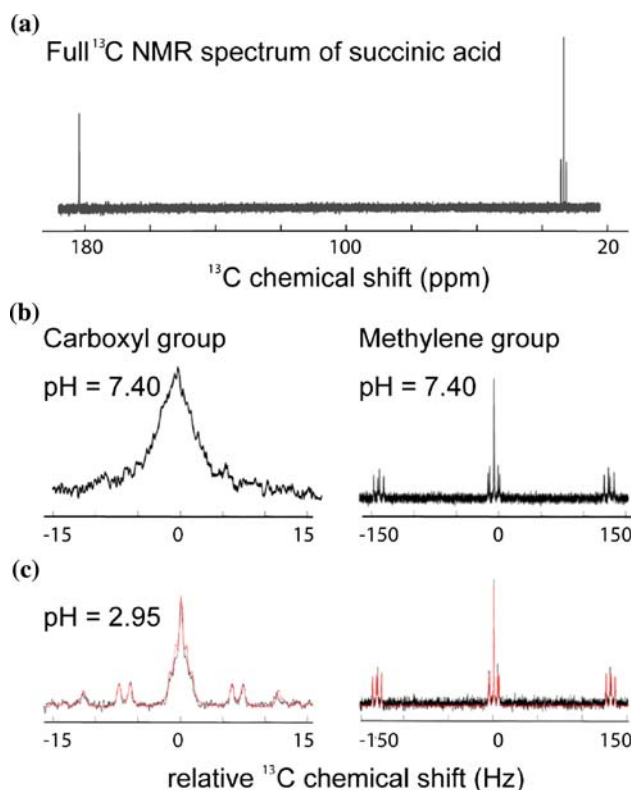


Fig. 4 Determination of the relevant J-couplings for the hyperpolarization of succinate. (a) ^{13}C NMR spectrum of natural abundance succinic acid at pH 7.4 (14 T Varian spectrometer, 256 acquisitions, H/C/N probe, no decoupling). (b) ^{13}C NMR spectra of carboxyl (1, 4- ^{13}C , ~ 185 ppm) and methylene (2, 3- ^{13}C , ~ 35 ppm) groups at pH 7.4. Note the line broadening of the former. (c) Simulations (red) and measured ^{13}C NMR spectra of carboxyl and methylene groups at pH 2.95. Note that at pH 2.95 improved resolution of the line splittings in carboxyl resonances are observed. The J-couplings extracted of the simulated spectrum were $^2J_{\text{CHa}} = -7.15$ Hz, $^3J_{\text{CHb}} = 5.82$ Hz and $^3J_{\text{HaHb}} = 7.41$ Hz. These values were employed to calculate the spin order transfer sequence for succinate

$P_{\text{HP}}^{t=0} = 21\%$ (nominal ^{13}C signal enhancement $\eta^{t=0} \approx 50,000$ at 4.7 T) was found at 1.763 mT.

As expected, hyperpolarization was extremely sensitive to deviations from the precise low magnetic field applied during spin order transfer. At ± 0.009 mT from the optimal field, the polarization declined by 50% (FWHM = 0.018 mT). Marked variations persisted in the polarization yield at the same field strength, which were attributed to remaining variables in r.f. amplifier performance, reagent preparation technique and, because signal decays rapidly before detection, to prolonged and variable sample delivery times to the spectrometer. Delivering hyperpolarized sample and triggered detection in the NMR scanner were further optimized. The polarizer was moved to the in vivo suite, where it was positioned carefully close to a small-animal horizontal bore, high field MR (7.63 m \pm 0.5 cm distance, 4.7 T Bruker MRI scanner). An automated system was implemented, whereby the

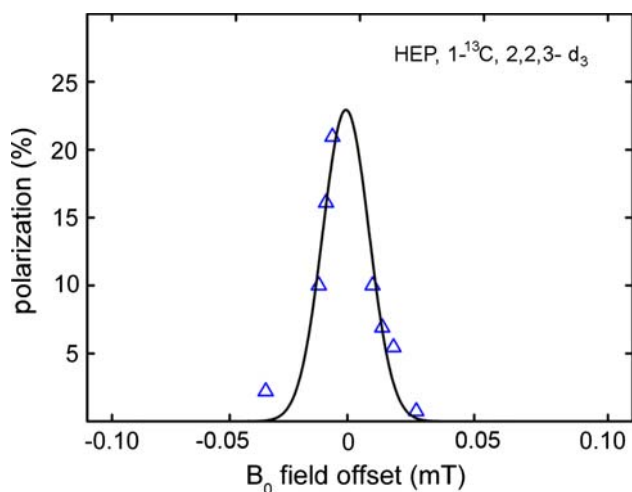


Fig. 5 Calibration of the center frequency of the low field MR unit. Maximal polarization achieved in this experimental set-up was 21%, somewhat lower than previously reported [18]. The PASADENA experiment was conducted at different strengths of the static field using HEP (0.1 mM). This was very sensitive to variations of the static field: Half of the polarization was lost at a field offset of ± 0.009 mT (full width at half maximum (FWHM) = 0.018 mT). The amplitudes and durations of the B_1 pulses of the SOT used are shown in Fig. 3

hyperpolarized solution was driven by remaining gas pressure in the reactor, through polyethylene (PE) tubing from the polarizer to an NMR-tube in a dual-tuned $^1\text{H}/^{13}\text{C}$ solenoid coil in the MR scanner, where after a precise interval (33 s) acquisition was triggered. The solution was then drained to permit successive experiments without coil repositioning. Together, these measures proved to be essential for the reliability of the level of achieved polarization. The modifications implemented improved the stability of the B_0 and provided stronger B_1 , allowing for better r.f. performance and less sensitivity of hyperpolarization of HEP to off-resonant B_0 , as demonstrated in Fig. 6 (left).

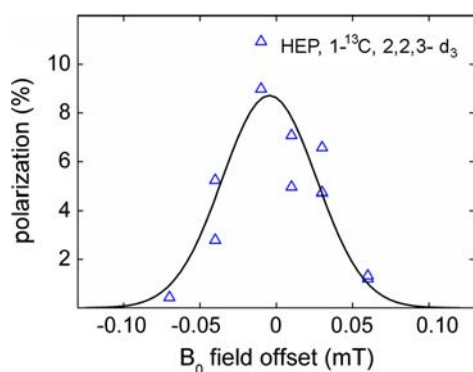


Fig. 6 Impact of r.f. amplifier power to hyperpolarization. (left) New r.f. hardware allowed for increase in r.f. power (previously 25V/16V in Fig. 5, now 25V/50V (for carbon, hydrogen, respectively): 3.1-fold increase) and less sensitivity of hyperpolarization to off-resonance fre-

Step 7: B_1 optimization with hyperpolarization

With the goal of in vivo application, the next group of calibrations were performed with the non-toxic, water soluble PASADENA biomolecule SUC (Fig. 6, right).

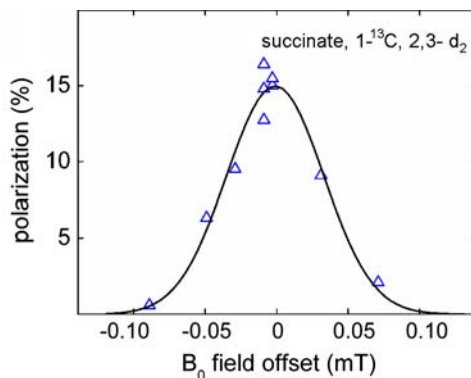
The hyperpolarization yield was incrementally increased by further optimization of the individual pulse widths of ^{13}C , ^1H in the SOT sequence, at constant B_0 (Fig. 7). First, the pulse width of the ^1H pulses in the SOT was varied (Fig. 7a). The optimum was determined to be at 115 μs (50 V). Next, keeping the ^1H pulse width constant, the experiment was repeated while varying the ^{13}C pulse widths in the SOT sequence (Fig. 7b). Each experiment was conducted eight times. The optimum ^{13}C pulse width was 230 μs (25 V). Fig. 7c demonstrates the multidimensional character of this optimization.

The maximal polarization achieved with $1\text{-}^{13}\text{C}$ succinate under these conditions was 18%.

T_1 of short-lived hyperpolarization

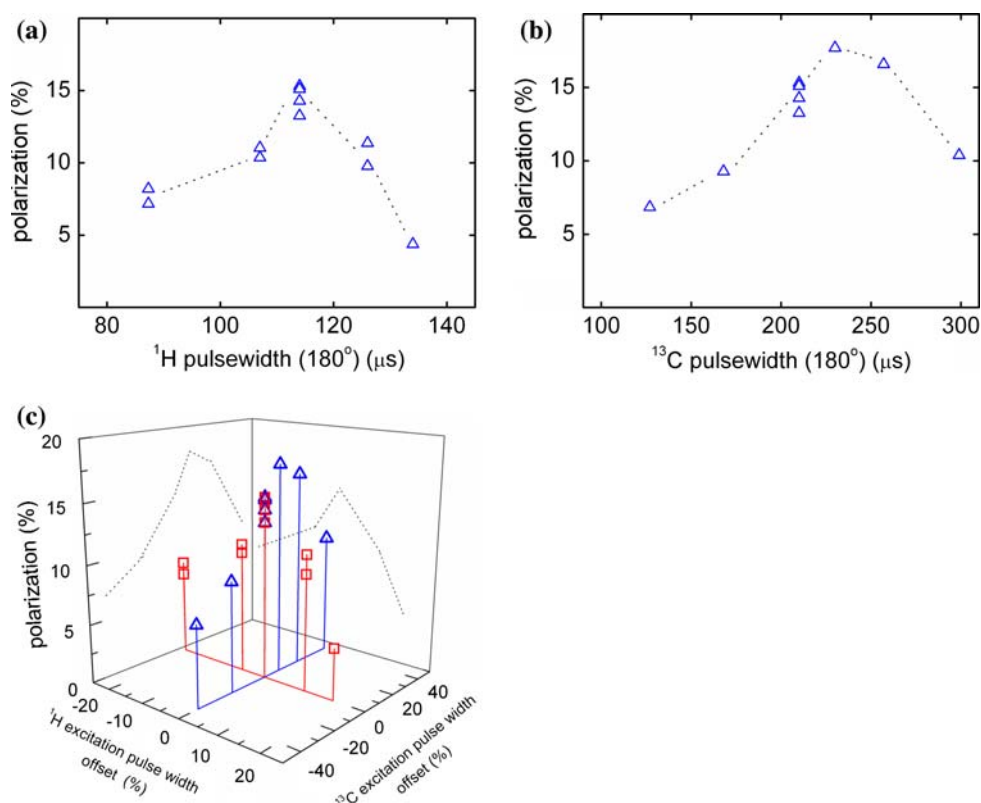
Using these QA methods, we defined two important properties of PASADENA for a biologically appropriate molecular imaging reagent. First, T_1 of hyperpolarized MR signal is critical in defining duration of enhanced ^{13}C (or ^{15}N) MR signal after delivery. We determined the maximum polarization achieved in PASADENA as relevant for quantitative comparison with competing reagents and hyperpolarization techniques. Secondly, the effect of D_2O prolonging the lifetime of polarization is a general property, and of value for most hyperpolarization techniques. The T_1 decay time for HEP was 70 s in D_2O , as compared to 50 s in H_2O (pH 7, $B_0 = 4.7$ T).

The relaxation constant of hyperpolarized SUC was determined to be $T_1 = [27 \pm 3]$ s ($N = 3$, pH 3, $B_0 = 4.7$ T) in H_2O solvent (at a concentration of 1–3 mM of SUC, 2.5 mM



quency (previously FWHM = 0.018 mT, now FWHM = 0.062 mT, 3.4-fold increase). Adaptation of the spin order transfer sequence allowed for hyperpolarization of SUC, which was conducted ten times at different strengths of the static field (right)

Fig. 7 Optimization of SUC hyperpolarization yield by two-dimensional optimization of the B_1 flip angles. First, the PASADENA experiment was conducted 8 times. Each time the pulse width of the ^1H pulses in the SOT was varied (a). The optimum was determined to be at $115\ \mu\text{s}$ (50 V). Next, the experiment was repeated under variation of the ^{13}C pulse widths in the SOT (b). The optimum found was $230\ \mu\text{s}$ (25 V). (c) demonstration of the multidimensional character of this optimization



of catalyst). $T_1 = [40 \pm 2]\ \text{s}$ ($N = 4$, pH 3, $B_0 = 4.7\ \text{T}$) was significantly prolonged when measured in D_2O (Fig. 8). Preliminary results indicate a further increase in measured T_1 to $\sim 50\ \text{s}$, when the hyperpolarized reagent–catalyst mixture was buffered to physiological pH 7 at 4.7 T (not shown).

Quality assurance routine

A check-list is provided (Table 1, column C) which is recommended to be performed as quality assurance each day. These seven steps were found to ensure a high level of cleanliness of the entire polarizer circuit and reaction chamber as well as accurate function of the electronics necessary for delivery, circulation and SOT-spin physics.

Quantification and reproducibility of polarization

Repeated hyperpolarization experiments of SUC in D_2O were carried out to determine the level and stability of the polarization produced by the PASADENA polarizer. A representative ^{13}C spectrum of hyperpolarized SUC is demonstrated in Fig. 9a. Peak amplitude was quantified by comparison with the spectrum of ethanol (Fig. 9b), using the equations described in “Methods”. Polarization of ^{13}C succinate in D_2O achieved 19%, in this representative experiment, cor-

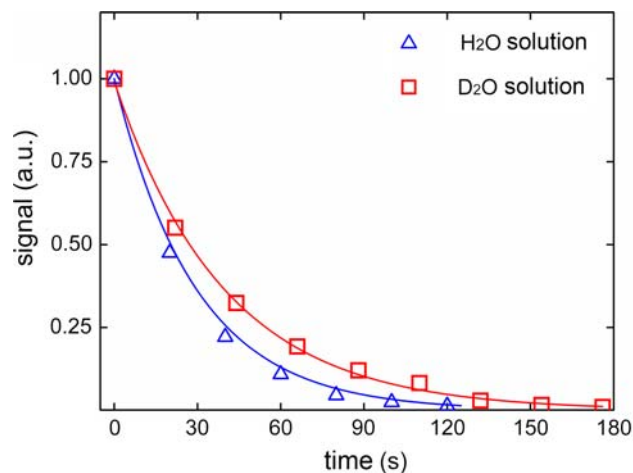


Fig. 8 T_1 measurement of hyperpolarized SUC in D_2O ($T_1 = [40 \pm 2]\ \text{s}$) and in H_2O ($T_1 = [27 \pm 3]\ \text{s}$). The decaying polarization was probed by small angle pulse-acquisition experiments in a high field system (4.7 T)

responds to an overall enhancement of $\eta^{t=0} \approx 49,000$ -fold at 4.7 T.

To define the reproducibility of polarization, 28 further studies were performed. Note that since each experiment takes only 2–3 min, with allowance for rinsing and reloading of the polarizer, the automated polarizer permitted as many as ten PASADENA hyperpolarizations each hour.

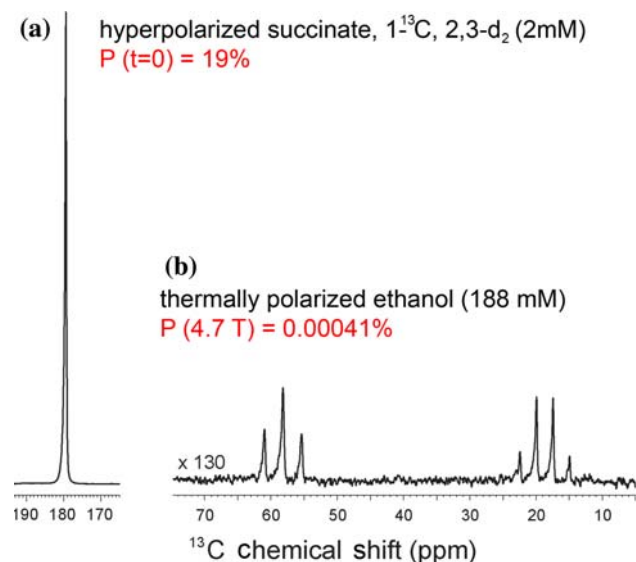


Fig. 9 Quantification of polarization: (a) ^{13}C spectra of SUC (1.95 mM) hyperpolarized to 19%, corresponding to an enhancement of $\sim 47,000$ at 4.7 T and (b) unlabeled thermally polarized ($P = 4.1 \times 10^{-4}\%$) ethanol (^{13}C concentration 188 mM per site natural abundance)

Greater reproducibility and higher absolute polarization were achieved after B_1 calibration and when the procedure was performed in D_2O (Table 2). Hyperpolarization. $P_{\text{HP}}^{t=0} = [15.3 \pm 1.9]\%$ was reproducibly achieved in 16 hyperpolarization experiments on SUC, in D_2O . This corresponds to a

relative enhancement of $\eta^{t=0} = 37,400 \pm 4,600$ at 4.7 T. The intra day variability was found to be $P_{\text{HP}}^{t=0} = [16.5 \pm 3.3]\%$, $N = 3$, $P_{\text{HP}}^{t=0} = [15.9 \pm 2.0]\%$, $N = 4$, $P_{\text{HP}}^{t=0} = [14.8 \pm 1.3]\%$, $N = 5$, $P_{\text{HP}}^{t=0} = [14.5 \pm 0.9]\%$. Detailed results are presented graphically in Fig. 10. More relevant to the conduct of experiments was the degree of hyperpolarization at the point of measurement. As expected with a mean delay of 33 s for delivery and $T_1 = 27$ s to 40 s, enhancement was significantly lower, $P_{\text{HP}}^{t=33\text{s}} = [6.4 \pm 0.8]\%$, $N = 16$, in D_2O (enhancement $\eta^{t=0} \approx 37,400 \times [6.5/15.3] = 15,900$ -fold at the point of measurement).

No effect of the concentration of SUC on the level of hyperpolarization was found over a narrow range of concentrations (0.96–2.93 mM in D_2O ; Table 2) or (1.65–2.89 mM in water; Table 3). Although it is anticipated that injection of small amounts of D_2O will be harmless, hyperpolarization was also determined in aqueous solution (Table 3). The average polarization achieved in H_2O , calculated from T_1 to be that at the point of production, was $P_{\text{HP}}^{t=0} = [12.8 \pm 3.1]\%$ on SUC (2–5 mM) for 12 experiments in three series, not significantly different ($P > 0.05$) from results in D_2O ($P_{\text{HP}}^{t=33\text{s}} = [5.4 \pm 1.3]\%$ in water).

Efficacy of hyperpolarized $1\text{-}^{13}\text{C}$ succinate in vivo

Figure 11 demonstrates, in a representative ^{13}C image, overlaid on a standard proton image, the efficacy of hyperpolarization

Table 2 Reproducibility and performance of the polarizer, D_2O

(A) Number	(B) $P_{\text{HP}}^{t=0}$ (%)	(C) $P_{\text{HP}}^{t=33\text{s}}$ (%)	(D) c (SUC) (mM)	(E) c (Rh) (mM)
1	15.5	6.5	1.71	2.2
2	20.1	8.4	1.71	2.2
3	13.8	5.8	1.71	2.2
Mean ($N = 3$)	16.5 ± 3.3	6.9 ± 1.3		
4	17.1	7.2	1.4	2.2
5	15.9	6.7	1.4	2.2
6	17.5	7.3	1.4	2.2
7	13.0	5.5	0.96	2.2
Mean ($N = 4$)	15.9 ± 2.0	7.1 ± 0.3		
8	14.9	6.2	2.93	2.1
9	15.4	6.4	2.93	2.1
10	16.3	6.8	1.24	2.2
11	14.7	6.2	1.24	2.2
12	12.7	5.3	1.24	2.2
Mean ($N = 5$)	14.8 ± 1.3	6.5 ± 0.3		
13	15.3	6.4	1.59	2.2
14	14.3	6.0	1.95	2.2
15	15.1	6.3	1.59	2.2
16	13.3	5.6	2.07	2.2
Mean ($N = 4$)	14.5 ± 0.9	6.2 ± 0.2		
Total ($N = 16$)	15.3 ± 1.9	6.4 ± 0.8		

Four different sets of experiments of hyperpolarization of SUC in D_2O were performed as indicated (Fig. 10). From left to right: (A) number of experiment; (B) level of nascent polarization; (C) level of polarization after 33 s; (D) concentration of SUC in mM; (E) concentration of Rh-catalyst complex in mM. Level of polarization after 33 s (C) is equivalent to that available at the point of delivery to an experimental animal

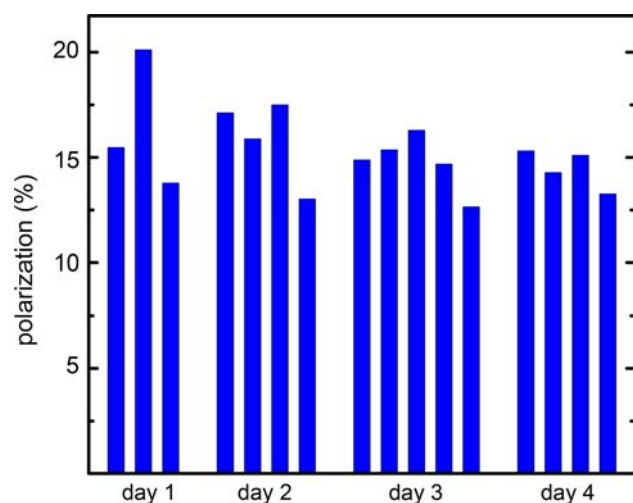


Fig. 10 Reproducibility of hyperpolarization. $P_{\text{HP}}^{t=0} = [15.3 \pm 1.9]\%$ was achieved in 16 hyperpolarization experiments on SUC, in D_2O conducted on successive days. Details are provided in Table 2

zed ^{13}C succinate for in vivo imaging. After rapid ante-grade injection into the common carotid artery, hyperpolarized ^{13}C succinate appeared in the anatomical distribution coincident with the rat brain, as outlined by ^1H MRI.

Discussion

A reliable method for the hyperpolarization of a biomolecule for routine applications in bio-medical research is presented in this work. The performance is demonstrated on SUC, a ^{13}C

PASADENA metabolic agent with the potential to diagnose brain cancer [10]. This molecule was polarized to an average of $P_{\text{HP}}^{t=0} = [15.3 \pm 1.9]\%$ in 16 experiments, corresponding to a 37,400-fold enhancement at 4.7 T, with a maximum enhancement of $\eta^{t=0} \approx 49,000$ -fold.

The robust design of the polarizer, reflected in the achieved levels and reproducibility of polarization, qualifies (after reagent-sterility is confirmed) for routine bio-medical/clinical application. However, the theoretical maximum of polarization is not reached, and it is worthwhile to consider the remaining limitations of the experiment.

Three major factors govern both the reproducibility and level of polarization achieved by PASADENA: (A) The primary source of spin order, parahydrogen, (B) the hydrogenation of the precursor molecule, and (C) the manipulation of the spins in the hydrogenated molecule to generate a net polarization (by r.f. Spin order transfer sequence, SOT).

- (A) The parahydrogen is routinely enriched to $>97\%$, thereby guaranteeing an excellent source of spin order. It proved to be important to allow a minimum of 6 h for the cryo-system to cool down, prior to the generation of parahydrogen.
- (B) It was noted that an inert atmosphere was a key aspect in the preparation of the PASADENA precursor solution. This is attributed to the sensitivity of the Rhodium (Rh) based catalyst to oxygen and moisture. Therefore, bis(norbornadiene)rhodium (I) tetrafluoroborate should be used fresh and with minimum air and light exposure during the chemical preparation of catalyst solution. An automated setup under inert atmosphere

Table 3 Reproducibility and performance of the polarizer, H_2O

(A) Number	(B) $P_{\text{HP}}^{t=0}$ (%)	(C) $P_{\text{HP}}^{t=33s}$ (%)	(D) c (SUC) (mM)	(E) c (Rh) (mM)
1	16.2	6.8	1.85	2.2
2	19.0	8.0	1.85	2.2
3	17.5	7.3	1.85	2.2
Mean ($N = 3$)	17.6 ± 1.4	7.4 ± 0.6		
4	10.1	4.2	2.89	2.2
5	13.1	5.5	2.89	2.2
6	12.4	5.2	2.89	2.2
7	12.2	5.1	2.89	2.2
Mean ($N = 4$)	12.0 ± 1.3	5.0 ± 0.6		
8	9.8	4.1	1.65	2.2
9	10.2	4.3	1.65	2.2
10	11.6	4.9	1.65	2.2
11	11.8	5.0	1.65	2.2
12	10.6	4.4	1.65	2.2
Mean ($N = 5$)	10.8 ± 0.9	4.5 ± 0.4		
Total ($N = 12$)	12.9 ± 3.1	5.4 ± 1.3		

Three sets of experiments of hyperpolarization of SUC in H_2O were performed as indicated. For details, see Table 2

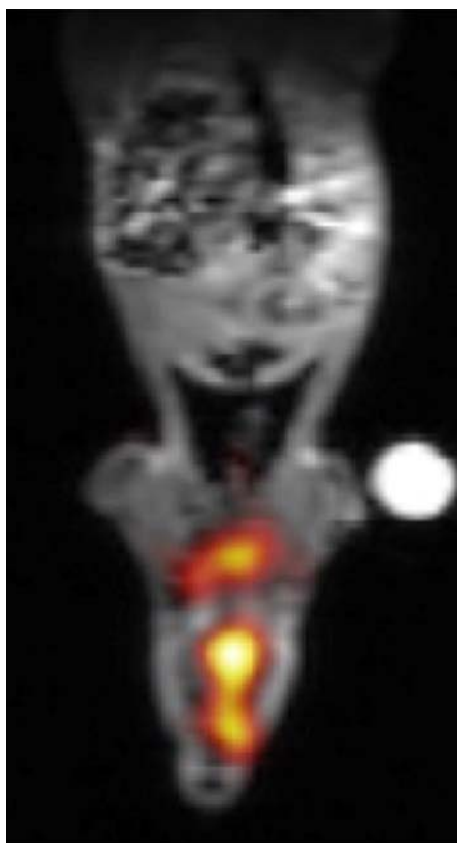


Fig. 11 In vivo ^{13}C sub-second image (0.3 s) of a rat brain, acquired 9 s after close-arterial injection of 1 ml, 25 mM hyperpolarized SUC (in color). The ^{13}C image was overlaid on a coronal ^1H fast gradient echo image with matching field-of-view (FOV) and slice location acquired prior to infusion to provide anatomical correlation (3D FIESTA, TR/TE = 6.3/3.1 ms, $5 \times 5 \times 5 \text{ mm}^3$ spatial resolution, FOV = 220 mm/320 mm, 44 phase encoding steps/64 readout points, respectively)

would be desirable to minimize the exposure of the Rhodium catalyst to atmospheric oxygen, accelerate the preparation process and reduce experimental variability (currently under development in our laboratory). The duration of the hydrogenation reaction may hold further potential for improvements, as preliminary results indicate. This may be attributed to the progress of the hydrogenation reaction, and the loss of spin order state of molecular and bound parahydrogen while the reaction is going on. However, the hydrogenation reaction was deemed to be complete to >99% within 4 s under 10 bar pH_2 and 62°C , which is supported by the fact that no precursor signal was found in high field NMR spectrum of the hydrogenated sample.

- (C) This leaves the spin manipulations by r.f. to explain why the theoretical value of unity, >90%, ^{13}C polarization was not achieved. The pattern of the spin manipulations tailored to the molecule, and how it is realized by NMR electronics, are essential to achieve hyper-

polarization. While the first requires knowledge of the coupling constants of the target molecule, the latter is mostly governed by experimental imperfections of the setup.

The static field of the 4.7 T MR unit used for the detection of hyperpolarization was found to perturb the relatively low field in PASADENA polarizer. At a distance of $(7.6 \pm 0.1) \text{ m}$, the stray field of the 4.7 T adds a $\sim 0.1 \text{ mT}$ horizontal component to the vertical 1.7 mT field of the polarizer. This effect is manifested in a complete loss of hyperpolarization if the polarizer is moved in to close proximity of the non-shielded magnet. While this challenge may not persist in the proximity of modern, actively shielded high field magnets, active (field compensation gradients) or passive shielding for the PASADENA polarizer are potential solutions under investigation in our laboratory. To avoid thermal drift of the B_0 field, a minimum of 4 h was allowed for the polarizer to reach operational temperature (62°C).

The sensitivity of the hyperpolarization towards fluctuations in B_0 could be reduced by the application of even stronger B_1 pulses. However, possible limitations arise: first, large (with respect to B_0) B_1 fields could make the pulse sequence perform sub-optimally as conventional NMR theory was used for the development (approximation $B_0 \gg B_1$). Second, at strong B_1 , interaction of ^{13}C and ^1H has to be taken into account: There is increased cross-talk between the two, and the pulses applied at one Larmor frequency may affect the spins at the other. Moreover, ^2H nuclei present in the SUC may be affected by the r.f. pulses, too, a matter not yet investigated in detail. Preliminary simulations suggest that the cross-talk between the two channels can be as large as 9% under the conditions of the present study. These challenges may be significantly alleviated by (1) conducting the hyperpolarization experiment at a higher field ($\sim 10 \text{ mT}$) and/or (2) design of shaped pulses for selective excitation at low field.

Conclusion

A PASADENA polarizer is presented, capable of achieving reproducible polarization of a test reagent, custom-designed for application in bio-medical research. The protocols employed for the production of hyperpolarized substrate are provided, along with a detailed description of the optimization and quality assurance procedures. The performance of the apparatus is demonstrated on succinate, an intermediate of the Krebs-TCA cycle and many other vital processes. In 16 experiments, performed over 4 different days, an average polarization of $P_{\text{HP}}^{I=0} = [15.3 \pm 1.9]\%$ was achieved. The fully automated set up can be operated by a single person, and allows continuous production of hyperpolarized agent

within seconds at liquid state temperatures. Limitations to the hardware are discussed, and potential improvements outlined.

While the unique chemical specificity of nuclear magnetic resonance holds great potential in bio-medical research, it is limited by the inherently low polarization (order of 10^{-5}) to in vivo detection of major moieties (\sim mM). Routinely available PASADENA hyperpolarization will encourage exploration of metabolic events involving metabolites in the micro or even nanomolar range.

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