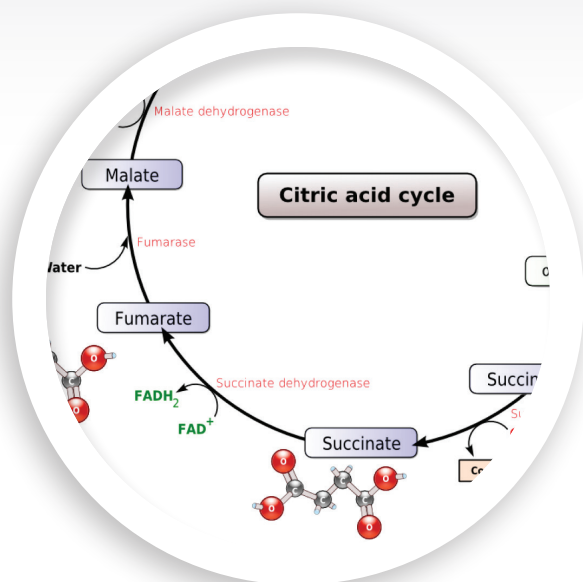# Development of Hyperpolarized Metabolic Contrast Agents Using PASADENA

Eduard Y. Chekmenev,[1,2] Pratip Bhattacharya,[2] Brian D. Ross[2]
1 California Institute of Technology, Pasadena, CA 91125 USA
2 Huntington Medical Research Institutes, Pasadena, CA 91105 USA

Nuclear magnetic resonance (NMR) is a powerful method not only for high-resolution structure elucidation methods on atomic and molecular scale, but also for *in vivo* magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) in biomedicine. Although NMR has a number of benefits for *in vivo* applications being non-radioactive and non-toxic compared to other imaging modalities, such as positron emission tomography (PET) and computed tomography (CT), the main weakness of NMR method is its relatively low sensitivity compared to the above methods. Low sensitivity is largely determined by poor alignments of the nuclear spins, which contribute to the formation of the NMR signal in a static magnetic field B_0. For example, only 10 out of one million proton spins are aligned with respect to the applied magnetic field $B_0=3$ Tesla (T) at room temperature (Figure 1). Nuclear spin alignment is even worse for other biologically relevant nuclei with low gyromagnetic ratio, γ, such as ^{13}C and ^{15}N by four and ten times, respectively, compared to protons. In addition to worse nuclear alignment, NMR detection of low-γ nuclei is further exacerbated by lower receptivity, as overall sensitivity usually scales with γ.[3] Thus, ^{13}C and ^{15}N are rarely used for direct detection in clinical practice and biomedical *in vivo* research in general. However, low-γ also yields a much longer spin lattice relaxation time T_1 for ^{13}C and ^{15}N since the dominating mechanism of dipole-dipole relaxation results in T_1 being inversely proportional to γ.[2] As a result, T_1 of ^{13}C and especially ^{15}N of some molecular sites can reach several minutes *in vivo*.[1]

While in conventional *in vivo* NMR, such long T_1 is prohibitively inconvenient for signal recording and signal averaging, it offers unique advantages and opportunities for NMR methods enhanced by hyperpolarization. The goal of hyperpolarization techniques is to increase the nuclear spin alignment from several parts per million (ppm) to the order of unity (Figure 1). Both dynamic nuclear polarization (DNP)[2] and parahydrogen and synthesis allow dramatically enhanced nuclear alignment (PASADENA)[3,4] have recently[5-13] been demonstrated to reach spin polarization of order unity on ^{13}C and $^{15}N^1$ sites. This corresponds to a signal enhancement by a factor of ~100,000 on currently utilized MRI scanners. This dramatic signal enhancement brings the sensitivity of ^{13}C and ^{15}N to the realm of research and potentially clinical *in vivo* tools.
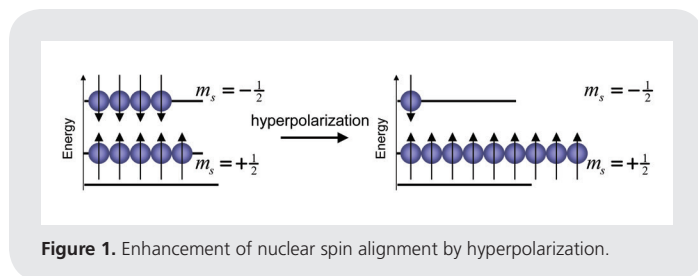
(continued)

Figure 1. Enhancement of nuclear spin alignment by hyperpolarization.

Figure 1. Proton NMR spectra of ortho- and parahydrogen mixtures at 4.7T.

While DNP inherently requires expensive cryogenic equipment, a homogeneous high-field magnet and sample polarization for a few tens of minutes, PASADENA offers an economical advantage of low magnetic field of several milliTeslas (mT) and ultra-fast sample polarization in just seconds. However, the main short-coming of PASADENA implementation in practice is the requirement for unsaturated -C=C- or -C≡C- bonds of the molecular precursor that leads to hyperpolarized ^{13}C or ^{15}N molecules after molecular hydrogenation.[3,4] This application note provides the reader with essential steps for preparation of novel hyperpolarized ^{13}C molecules using PASADENA.

Parahydrogen is routinely produced by a passage of hydrogen (25% para and 75% ortho at room temperature) gas through a catalyst chilled to cryogenic temperatures of 5-20K under 20-40 atm, allowing fast conversion of ortho-para mixture of hydrogen gas to parahydrogen. Produced in this fashion, parahydrogen gas is collected and stored in an aluminum cylinder under 20-30 atm pressure for convenient storage, transportation and production of ^{13}C hyperpolarized compounds.[6,7] The parahydrogen quality is characterized by ^1H spectroscopy. Since parahydrogen is NMR silent, proton spectroscopy measures the concentration of orthohydrogen, which is then used to calculate the percentage of parahydrogen in the mixture. An example of such measurement is shown in Figure 2.

^{13}C spin lattice relaxation time and the decay of the singlet state of the attached parahydrogen spins significantly limit the time allowed to manipulate the nuclear spins during PASADENA experiments. As a result, the molecular hydrogenation of the unsaturated bond and spin order transfer from the attached parahydrogen spins is carried out on the time-scale of several seconds. The hydrogenation scheme of 1-^{13}C-fumaric acid-D_2 (CIL #CDLM-6062) to produce 1-^{13}C-succinic acid-D_2 [13] is presented in Figure 3. We estimate that hydrogenation with water-soluble Rh-based catalyst shown on Figure 4 takes 1-3 seconds for >99% conversion[14] of the molecular precursor under conditions of 2.5 mmol/L catalyst and <20 mmol/L 1-^{13}C-fumaric acid-D_2 concentrations.[6,13] The water-soluble Rh-based catalyst allows the entire hyperpolarization procedure to be performed in aqueous medium which is mandatory for *in vivo* applications. The toxicity of the catalyst is a primary concern for extending PASADENA hyperpolarization to *in vivo* and clinical applications. We are currently investigating a variety of ion-exchangers for fast and automated design of catalyst filtration of hyperpolarized aqueous solutions. Moreover, there are studies in progress in our laboratory at Huntington Medical Research Institutes to assess the residual toxicity of the Rh-based catalyst in cell cultures and rodents injected
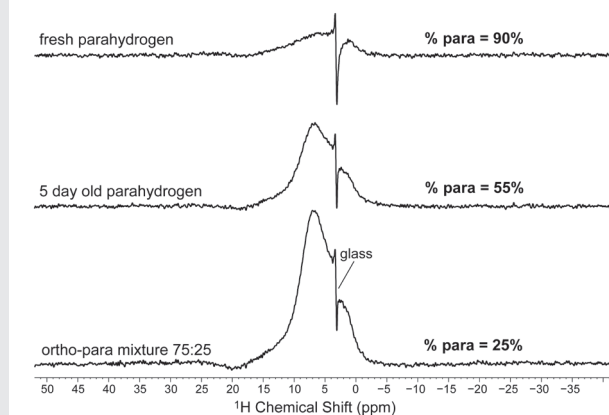
with hyperpolarized solutions after filtration (Bhattacharya, P., et al., manuscript in preparation).

The radio frequency (rf) pulses are applied after four seconds of hydrogenation to transfer the spin order from parahydrogen spins to $^{13}C_1$ of succinic acid, in a 1.8 mT magnetic field (Figure 5). The spin order transfer sequence[9,15] requires ~0.35 second, which is the result of relatively weak J-couplings between the three spins of interest shown in Figure 6. Thus, the prerequisite of rapid molecular addition of parahydrogen and spin-order transfer is fulfilled, since the time necessary for both of these steps is much shorter compared to 27 seconds $^{13}C_1$ T_1 and the singlet state[16] of the parahydrogen spins in succinate (Figure 3).

The spin-order transfer sequence[9,15] relies heavily on the prior knowledge of the spin-spin couplings between the two hydrogen spins and ^{13}C (Figure 6). These spin-spin couplings could be conveniently obtained from ^{13}C or ^1H multiplets. Here, we used proton-coupled ^{13}C spectroscopy to record (Figure 6, black trace) the multiplets, which were simulated (Figure 6, red trace) to yield three spin-spin couplings.

1-^{13}C-succinic acid is a representative example of a carboxylic acid that participates in a variety of biochemical processes. When designing the metabolic contrast agent for MRI, ^{13}C spin lattice relaxation time should be extended as much as possible to allow a substantial penetration of biochemical pathways by the
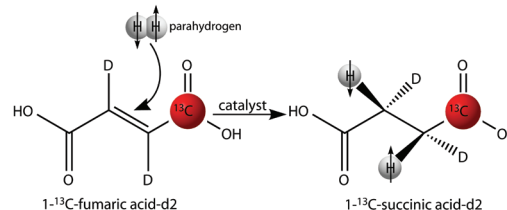

Figure 3. Molecular addition of parahydrogen to 1-^{13}C-fumaric acid-D_2 to produce 1-^{13}C-succinic acid-D_2.
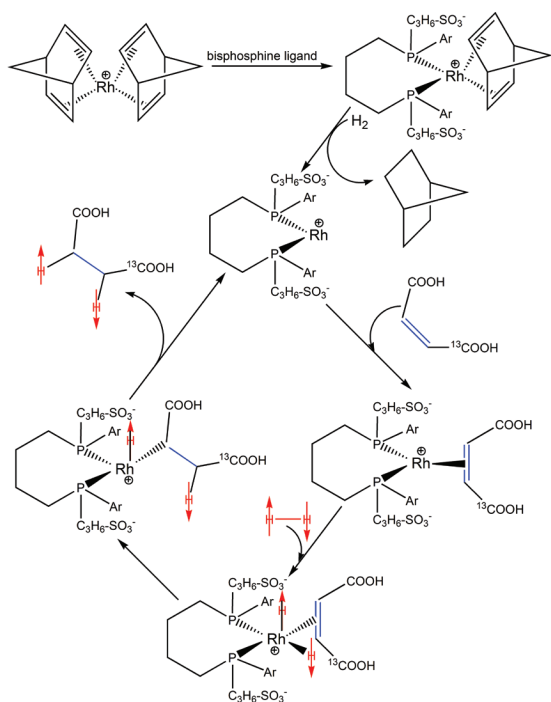
Figure 4. Molecular hydrogenation scheme using Rh-based catalyst.

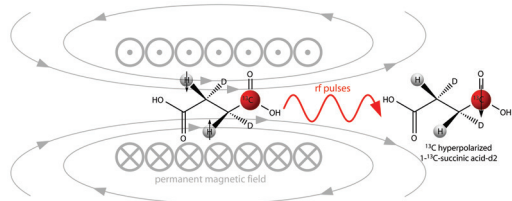**Figure 5.** The scheme of the spin-order transfer from singlet states of parahydrogen spins to $^{13}C_1$. Rf pulses[9,15] are applied to transfer the spin order inside an 1.8 mT electromagnet following by ejection of hyperpolarized 1-^{13}C-succinic acid-D_2. The entire process is automated.
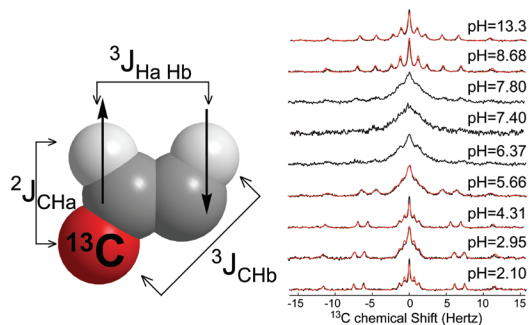

Figure 6. (Left) three spin system and three J-couplings necessary for spin order transfer sequence;[9,15] (right) proton coupled ^{13}C multiplets (experimental in black and simulated fits in red) of C_1 carbon collected in samples of natural-abundance succinic acid under various pH conditions at 14T.

hyperpolarized ^{13}C label. It is achieved most efficiently by perdeuteration of the molecular precursor. For succinic acid, this lead to the increases of $^{13}C_1$ T_1 from six seconds[6] (no deuteration) to 27 seconds (deuteration in positions 2 and 3 in fumaric acid (Figure 3).[13] ^{13}C T_1 could be further extended by deuteration of the aqueous medium used during molecular addition of parahydrogen and during the delivery of the hyperpolarized contrast agent solution *in vivo*. For example, $^{13}C_1$ T_1 can be extended from 27 seconds (in H_2O) to 56 seconds (in D_2O, >99%) in succinic acid at 4.7T.

Intermediate chemical exchange, observed as line-broadening (Figure 6), can potentially preclude accurate extraction of the J-couplings at pHs near the pKa value(s).[13] Moreover, it can also make the spin-order transfer extremely inefficient for the target chemical compound at such pH conditions. For example, no signal enhancement for $^{13}C_1$ was observed at pHn7 using a 1.8 mT magnetic field in the PASADENA polarizer. Therefore, transfer of spin order should be carried out at pH << pKa, where the J-couplings necessary for PASADENA are best resolved.

The entire procedure should be automated for routine application of the hyperpolarized metabolic contrast agents to allow reproducibility and quality assurance of PASADENA-based hyperpolarization for *in vivo* work.[17]

A number of hyperpolarized molecules have already shown early evidence of utility in biomedical research. The best known metabolic agent, hyperpolarized 1-^{13}C-pyruvic acid by DNP, measures the lactate dehydrogenase-catalyzed flux of ^{13}C label between the carboxyl groups of pyruvate and lactate in the tumor using ^{13}C magnetic resonance spectroscopy and spectroscopic imaging.[18]

Nontoxic hyperpolarized 1-^{13}C-succinate-D_2, is the most developed metabolic agent with routinely obtained polarization (P>18%) by PASADENA.[13] It can potentially assess the *in vivo* activity of succinate dehydrogenase (SDH), the enzyme that was recently tagged as an oncogene due to its crucial role in cell energetics (Figure 7).[19, 20] These mutations in SDH result in the increase of mitochondrial succinate pool that eventually leaks to cytosol, where it inhibits prolyl hydroxylase (PH) (Figure 7). The reduced level of prolyl hydroxylation stabilizes otherwise constantly depleting hypoxia-inducible factor (HIF)α, causing pseudo-hypoxia and hypoxia-inducible factor induction allowing tumor proliferation by means of anaerobic glycolysis. As a result, the metabolic profile is different from non-mutated cells, and it could be potentially measured using hyperpolarized metabolic contrast agents such as succinate. Moreover, it is possible to test the potency of these ^{13}C hyperpolarized agents by conventional ^{13}C NMR spectroscopy in cellular models of cancer.

Our preliminary results shown in Figure 8 obtained in cell cultures demonstrate different metabolic profiles when ^{13}C label of 1,4-^{13}C-succinate enters the TCA cycle in normal and cancer cells.[21] The normal product of the TCA cycle bicarbonate is detected in both pancreatic cancer cells and non-cancer fibroblast. In addition, pancreatic cancer cell line spectrum exhibits enrichment
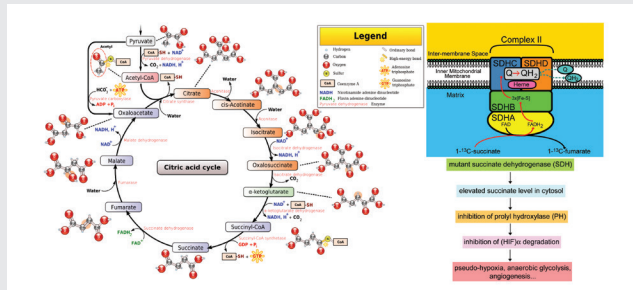
(continued)

Figure 7. Tricarboxylic acid (TCA) cycle and the effect of succinate dehydrogenase (SDH) mutations.

Figure 8. Proton-decoupled ^{13}C spectra obtained from pancreatic cancer MIA Pa Ca (top) and fibroblast (bottom) spheroids containing $2*10^7$ cells exposed to 10 mM 1,4-^{13}C-succinate (9 mL) at 37°C for 72 hours in bioreactor demonstrating production of multiple metabolites of TCA cycle. The cell metabolism was stopped by three freeze-thaw cycles using $N_2(l)$ at HMRI and spectra acquired using 128 scans at 14T.

of glutamate and citrate. Based on the direct link between TCA cycle dysfunction due to impaired SDH transmembrane assembly (Figure 7), it will therefore be possible to provide metabolic markers for dysfunctional SDH complex, thereby providing an opportunity to detect genetic mutations in action. Specifically, it is anticipated that ultra-fast localized and non-localized spectroscopy utilizing hyperpolarized ^{13}C-succinate will demonstrate the appearance of early key products of the TCA cycle, as well other mitochondrial and cytosolic pathways utilizing and TCA cycle intermediates. When translated to a clinical environment, these new metabolic markers could additionally allow detection of spatial distribution of such genetic mutations by means of ultra-fast chemical shift imaging (CSI), providing an opportunity to image active genetic mutations
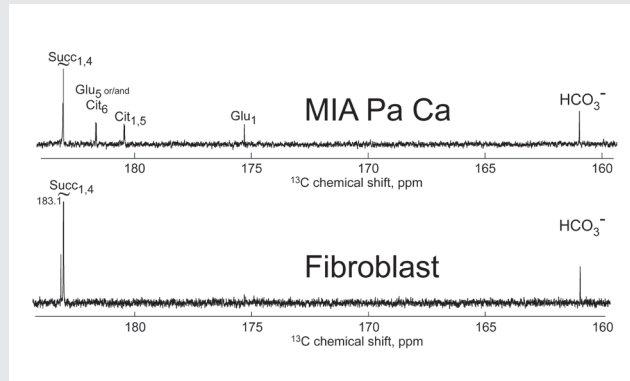
in humans. Besides an obvious utility to diagnose deficient SDH complex *in vivo*, such diagnostic methods could additionally guide surgery, radiation and especially gene therapy, as well as monitor the response to treatment, because multiple examinations would not result in additional exposure to radiation (in contrast to PET), toxic agents and would require only several minutes of patient and radiologist time.

References

1. Gabellieri, C.; Reynolds, S.; Lavie, A.; Payne, G,S.; Leach, M.O.; Eykyn, T.R. **2008**. Therapeutic Target Metabolism Observed Using Hyperpolarized ^{15}N Choline. *J Am Chem Soc, 130:*4598-4599.

2. Abragam, A.; Goldman, M. **1978**. Principles of Dynamic Nuclear-Polarization. *Rep Prog Phys, 41:*395-467.

3. Bowers, C.R.; Weitekamp, D.P. **1986**. Transformation of Symmetrization Order to Nuclear-Spin Magnetization by Chemical-Reaction and Nuclear-Magnetic-Resonance. *Phys Rev Lett, 57:*2645-2648.

4. Bowers, C.R.; Weitekamp, D.P. **1987**. Para-Hydrogen and Synthesis Allow Dramatically Enhanced Nuclear Alignment. *J Am Chem Soc, 109:*5541-5542.

5. Ardenkjaer-Larsen, J.H.; Fridlund, B.; Gram, A.; Hansson, G.; Hansson, L.; Lerche, M.H.; et al. **2003**. Increase in signal-to-noise ratio of > 10,000 times in liquid-state NMR. *Proc Natl Acad Sci, 100:*10158-10163.

6. Bhattacharya, P.; Chekmenev, E.Y.; Perman, W.H.; Harris, K.C.; Lin, A.P.; Norton, V.A.; et al. **2007**. Towards hyperpolarized ^{13}C-succinate imaging of brain cancer. *J Magn Reson, 186:*108-113.

7. Bhattacharya, P.; Harris, K.; Lin, A.P.; Mansson, M.; Norton, V.A.; Perman, W.H.; et al. **2005**. Ultra-fast three-dimensional imaging of hyperpolarized C-13 *in vivo*. *Magn Reson Mat Phys Biol Med, 18:*245-256.

8. Goldman, M.; Johannesson, H.; Axelsson, O.; Karlsson, M. **2005**. Hyperpolarization of C-13 through order transfer from parahydrogen: A new contrast agent for MFI. *J Magn Reson Imaging, 23:*153-157.

9. Goldman, M.; Johannesson, H.; Axelsson, O.; Karlsson, M. **2006**. Design and implementation of C-13 hyperpolarization from para-hydrogen, for new MRI contrast agents. *C.R. Chimie, 9:*357-363.

10. Golman, K.; Petersson, J.S. **2006**. Metabolic imaging and other applications of hyperpolarized C-13. *Acad Radiol, 13:*932-942.

11. Johannesson, H.; Axelsson, O.; Karlsson, M. Transfer of para-hydrogen spin order into polarization by diabatic field cycling. *C.R. Physique, 5:*315-324.

12. Kohler, S.J.; Yen, Y.; Wolber, J.; in't Zandt, R.; Gram A, Ellner F, et al. Carbon-13 Metabolic Imaging at 3T using Hyperpolarized ^{13}C-1-Pyruvate. *Proc Int Soc Magn Reson Med, 14:*128.

13. Chekmenev, E.Y.; Hovener, J.; Norton, V.A.; Harris, K.; Batchelder, L.S.; Bhattacharya, P. et al. **2008**. PASADENA hyperpolarization of succinic acid for MRI and NMR spectroscopy. *J Am Chem Soc, 130:*4212-4213.

14. Ahlquist, M.; Gustafsson, M.; Karlsson, M.; Thaning, M.; Axelsson, O.; Wendt, O.F. **2007**. Rhodium(I) hydrogenation in water: Kinetic studies and the detection of an intermediate using C-13{H-1} PHIPNMR spectroscopy. *Inorg Chim Acta, 360:*1621-1627.

15. Goldman, M.; Johannesson, H. **2007**. Conversion of a proton pair para order into C-13 polarization by rf irradiation, for use in MRI. *CR Physique, 6:*575-581.

16. Pileio. G.; Levitt, M.H. **2007**. J-Stabilization of singlet states in the solution NMR of multiple-spin systems. *J Magn Reson, 187:*141-145.

17. Hövener, J.B.; Chekmenev, E.Y.; Norton, V.A.; Weitekamp, R.; Harris, K.C.; Perman, W.H, et al. **2008**. Quality assurance of PASADENA hyperpolarization for ^{13}C Biomolecules. In: ENC Conference, Asilomar, CA 2008.

18. Day, S.E.; Kettunen, M.I.; Gallagher, F.A.; Hu, D.E.; Lerche, M.; Wolber, J., et al. **2007**. Detecting tumor response to treatment using hyperpolarized C-13 magnetic resonance imaging and spectroscopy. *Nat Med, 13:*1382-1387.

19. Rustin, P.; Munnich, A.; Rotig, A. **2002**. Succinate dehydrogenase and human diseases: new insights into a well-known enzyme. *Eur J Hum Genet, 10:*289-291.

20. Selak, M.A.; Armour, S.M.; MacKenzie, E.D.; Boulahbel, H.; Watson, D.G.; Mansfield, K.D., et al. **2005**. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell, 7:*77-85.

21. Chekmenev, E.Y.; Norton, V.A.; Bhattacharya, P.; Ross, B.D.; Weitekamp, D.P. **2008**. Hyperpolarized ^1H NMR Employing Low-gamma Nucleus as a Spin Order Storage. In: EUROMAR, St. Petersburg, Russia.