

Generation of Hyperpolarized Substrates by Secondary Labelling with [1,1-¹³C] Acetic Anhydride

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INTRODUCTION: Recent development of techniques to retain highly polarized spins in solution via dynamic nuclear polarization (DNP) has enabled ¹³C NMR spectroscopy and MR spectroscopic imaging studies with very high signal to noise in short acquisition times [1,2]. Generally, the technique requires polarization of a low molecular-weight ¹³C labelled chemical probe in a highly concentrated sample, for example [1-¹³C] pyruvic acid or ¹³C-urea. For molecules having limited solubility, crystalline properties at low temperature, or high molecular weights, the DNP technique may not provide sufficient polarization for *in-vitro* or *in-vivo* applications. To address this limitation, this study was designed to develop a method of "secondary hyperpolarization", whereby a reactive molecule that hyperpolarizes well could be used to chemically 'tag' biomolecules of interest. To demonstrate the method, hyperpolarized [1,1-¹³C] acetic anhydride was used to resolve a mixture of secondarily hyperpolarized N-acetylated amino acid adducts in aqueous solution in a matter of seconds. This general strategy is outlined in Figure 1. Additionally, we initiated investigations of the utility of this secondary hyperpolarization technique for important biomolecules that may not be well polarized using direct DNP techniques.

METHODS: [1,1-¹³C] acetic anhydride was hyperpolarized to 5.9% using the Hypersense (Oxford Instruments) as described previously, and dissolved in anhydrous 1,4 dioxane (Sigma-Aldrich). Initial studies (N=3) involved mixing the dissolution solution containing dioxane and hyperpolarized [1,1-¹³C]-acetic anhydride with 3 mL of a buffered (100mM phosphate, 0.3 mM EDTA pH = 7.8) solution of amino acids glycine (Gly), serine (Ser), valine (Val), leucine (Leu), and alanine (Ala) at 3.5 mM each, or 17.5 mM total amino acid concentration. A two-fold excess of acetic anhydride with respect to the total amino acid concentration was used in all labelling studies. The final pH of the resulting solutions was ≈ 5.5. To determine whether or not this technique could produce reasonable SNR for a solution containing amino acids at physiologic concentrations, a buffered, aqueous solution containing 70 μM amino acids (Gly, Ser, Val, Leu, Ala) was acetylated under the same conditions used for the higher amino acid concentration studies (final pH ≈ 7.3). In an additional set of experiments, the technique was used to generate a hyperpolarized, anhydride-depleted aqueous solution of a drug of interest. Instead of using toxic dioxane as a dissolution media, a basic solution of cysteine (5x molar excess) was used in order to generate N-acetyl cysteine during the dissolution process. All NMR studies were performed on a 11.7T Varian INOVA spectrometer (125MHz ¹³C, Varian Instruments) using a 10mm ¹⁵N/³¹P/¹³C triple-tuned direct detect probe. For the acquisition of hyperpolarized ¹³C spectra of the hyperpolarized acetylated amino acids eighty proton-decoupled (WALTZ-16, 9000 Hz bandwidth, decoupling during acquisition only) pulse and acquire hyperpolarized ¹³C NMR spectra (1 average, spectral window = 4000 Hz, number of points= 16000, TR=3.5s, acq time= 2s, total acq time = 2 min 55s) were acquired every 3 sec using a 5° pulse. For the low concentration amino acid secondary hyperpolarization study, a single 90° pulse was used to acquire the hyperpolarized ¹³C spectrum. T₁'s were determined by performing a mono-exponential fit to the signal decay curve of the hyperpolarized compounds. Yields and percent polarizations were calculated by comparison to fully relaxed spectra at thermal equilibrium.

RESULTS: [1,1-¹³C] acetic anhydride is an excellent substrate for DNP hyperpolarization. At 11.7T and 37°C, the calculated T₁ for hyperpolarized carbonyl of [1,1-¹³C] acetic anhydride in dioxane was 33.9 sec. In a mixed dioxane/aqueous solvent, derivatization of amines with hyperpolarized [1,1-¹³C] acetic anhydride takes place rapidly, prior to acquisition of the first hyperpolarized spectrum, to form the corresponding acetylated products. This method takes advantage of the preferential reaction of acetic anhydride with amine nucleophiles, which occurs much more rapidly than hydrolysis [3]. In triplicate studies it was also determined that a mixture of hyperpolarized [1-¹³C] N-acetylated Gly, Ser, Val, Leu, and Ala could be well resolved with excellent SNR (Figure 2). Chemical shifts, T₁'s, %yield, and hyperpolarized signal enhancement for the N-acetyl adducts are reported. Figure 3 demonstrates the hyperpolarized ¹³C spectrum acquired after the acetylation of the mixture of amino acids at physiologic concentrations with excellent signal noise in a single acquisition. Figure 4 demonstrates that a small molecular weight drug, N-acetylcysteine, can be also be secondarily polarized using hyperpolarized [1,1-¹³C] acetic anhydride. With a five-fold molar excess of cysteine, the reaction went to completion as evidenced by the absence of acetic anhydride in the hyperpolarized ¹³C spectrum.

DISCUSSION: These studies demonstrated the feasibility of a "secondary hyperpolarization" approach combining the remarkable NMR signal enhancement provided by DNP with the high reactivity of [1,1-¹³C] acetic anhydride with amino acids to perform rapid metabolic analyses of amino acid mixtures and to hyperpolarize biomolecules of interest. To our knowledge, the secondary hyperpolarization approach has not been reported previously in the DNP literature. Future studies will focus on determining if this approach can be used to analyze metabolite levels in human biofluids and used to follow the biodistribution and metabolism of low molecular weight drugs *in vivo*.

REFERENCES:

[1] Ardenkjaer-Larsen et al. PNAS 2003; 100(18): 10158-10163. [2] Golman et al. Cancer Res 2006; 66(22): 10855-10860. [3] Shanaiah N et al. PNAS 2007; 104(28): 11540-11544.

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Fig. 1. General strategy for secondary hyperpolarization of amino acids using pre-polarized [1,1-¹³C] acetic anhydride

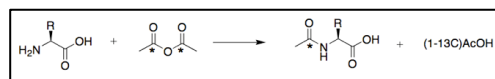
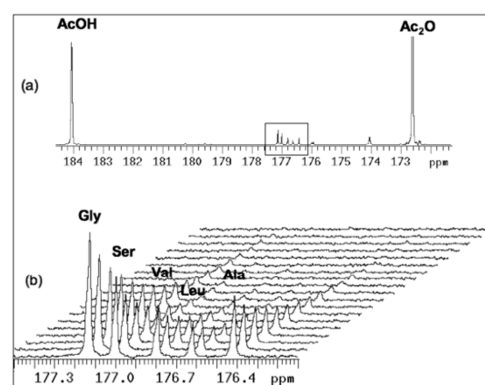


Fig. 2. Hyperpolarized dynamic spectrum when a buffered solution (100 mM phos, pH=7.8) containing the amino acids gly, ser, val, leu, ala (3.5 mM/ amino acid) is reacted with a twofold excess of hyperpolarized acetic anhydride. (a) full spectrum with acetylated amino acid products (outlined) (b) resolved amino acid products. Mean T₁'s, % yield and signal enhancements are reported (standard deviation in parentheses).



| PRODUCT | CHEMICAL SHIFT (PPM) | T1 (S) | YIELD (%) | ENHANCEMENT |
|------------------|----------------------|------------|------------|-------------|
| N-ACETYL GLYCINE | 177.13 | 14.7 (0.4) | 91.7 (4.5) | 1391 (220) |
| N-ACETYL SERINE | 177.00 | 10.9 (0.2) | 97.0 (3.0) | 775 (86) |
| N-ACETYL VALINE | 176.79 | 10.0 (0.2) | 93.3 (2.3) | 551 (43) |
| N-ACETYL LEUCINE | 176.62 | 8.9 (0.6) | 85.0 (4.6) | 412 (70) |
| N-ACETYL ALANINE | 176.41 | 14.7 (0.4) | 82.0 (3.6) | 654 (96) |

Fig. 3. Hyperpolarized N-acetyl amino acids at physiologic concentrations, single 90° acquisition

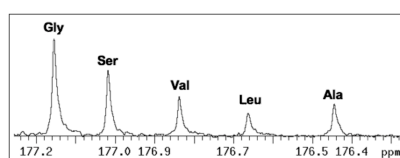


Fig. 4. Generation of hyperpolarized N-acetyl cysteine by secondary labeling.

