A new low volume NMR tube for *in-vitro* hyperpolarized ^{13}C experiments of prostate cancer cell suspensions

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Introduction

Hyperpolarized NMR is an excellent tool to study molecular processes in cell suspensions. It offers optimal control of the conditions presented to the cells, allowing accurate characterization and quantification of the metabolic processes under study. The *in-vitro* experiments are mostly performed in 10 mm diameter NMR tubes as they offer easy handling, whereas the use of 5 mm NMR tubes has been avoided because of mixing problems and air bubble formation distorting the B_0 homogeneity. Typical experiments in 10 mm NMR tubes require 25 – 100 million cells (1-4). Next to cell suspensions, bioreactors are used for *in-vitro* experiments, which have the ability to perform multiple HP measurements on the same cells (5). However, they require encapsulation of the cells and a flow system. Therefore bioreactors are typically constructed in 10 mm probes or larger systems, again requiring large numbers of cells. To reduce the number of cells necessary, we present a modified 5 mm diameter NMR tube for low volume cell suspension with proper mixing and excellent linewidths, showing HP experiments of prostate cancer cell lines with as little as two million cells. This enables HP experiments in primary cell cultures, for which the amount of cells available is a limiting factor.

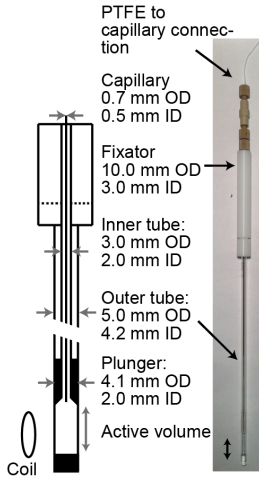

Figure 1. Schematic and photo of the small volume cell suspension setup

Description

A standard 5 mm outer diameter (OD) Shigemi NMR tube with magnetic susceptibility matched to D_2O (Shigemi Inc.) is adapted to allow fast injection of the HP metabolite (**Figure 1**). The plunger is modified with a centric hole (ID = 2.0 mm) with a conical end. The plunger is attached to the inner tube (OD = 3.0mm, ID = 2.0mm), which fits a 0.7 mm OD (ID = 0.53 mm) fused silica capillary (Polymicro Technologies). The smaller diameter compared to the inner tube allows air and excess liquid to escape. The capillary is connected to a 0.5 mm ID PTFE tube using standard PEEK components (IDEX/Upchurch Scientific). The inner tube is fixed relative to the outer tube by a custom made Teflon screw lock (fixator). The HP pyruvate is injected using a 1 ml syringe connected to the PTFE tube.

Methods

Cell culture – Prostate cancer cell lines PC3 and LNCaP were grown under standard conditions, kept at exponential proliferation, trypsinized and washed thrice in fresh RPMI 1640 medium. Between 2.2 and 5.3 million cells in 160 µl RPMI were transferred to the spectrometer. The time between the final wash step and HP pyruvate injection was less than 4 min.
Hyperpolarization – 2M of sodium 1-^{13}C pyruvate was dissolved in D_2O:d6-ethanol (2:1) mixture containing 30 mM 4-hydroxy-TEMPO, polarized for ~1hr at 1.2 K and 3.35 T using 90 mW continuous wave irradiation at 95.0 GHz using an in-house build polarizer (6). Mean polarization at liquid state was 2.5 ± 0.5 %.
Dissolution and injection – Dissolution buffer: isotonic 40 mM HEPES with 30 mM (unlabeled) lactate, pH of 7.1 after dissolution. The unlabeled lactate was used to increase ^{13}C label exchange (4). In total 160 ± 10 µl was manually injected into the NMR tube (after correction for dead volume of 700 µl). Final pyruvate concentration in the cell suspension was 15 mM.
NMR and data analysis – Metabolic conversion at 37 °C was followed using ^{13}C pulse-acquire with a 9° flip angle and WALTZ64 ^{1}H decoupling, 1 sec. acquisition time, 12.5kHz spectral width and a 3 sec TR on a 11.7T Bruker Avance III spectrometer with a 5 mm broadband inverse probe. Linewidths were measured in the spectrum as full width half height (FWHH), after zerofilling (32k points total), without linebroadening. Unidirectional forward exchange rate constants of pyruvate to lactate conversion were obtained by fitting the normalized integrals of pyruvate and lactate of 1Hz linebroadened spectra to a two-site exchange model (3).

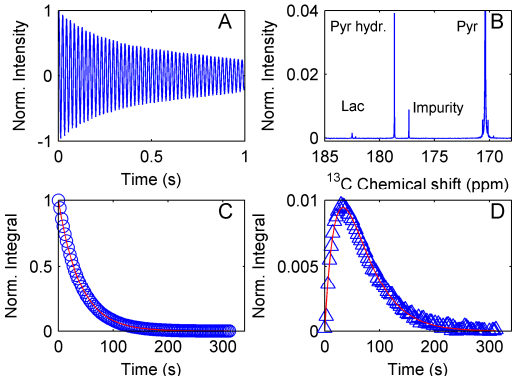

Figure 2. Example of 2.3 million PC3 cells with 15 mM HP pyruvate. FID (A) and spectrum (B) of the second FID after pyruvate injection, hence the lactate signal is not yet at maximum. Fit of the pyruvate (C) and lactate (D) signals.

Results

The injection of the HP pyruvate was performed fast (1-2s) to assure proper mixing. Maximum and stable signal was attained at the second acquisition after arrival of the HP pyruvate. As shown in **Figure 2**, the one second acquisition truncates the FID, as is the case for all experiments (n = 6). The mean linewidth, FWHH = 0.9±0.2 Hz, was measured at the pyruvate C1 keto resonance from the first stable FID. Linewidths did not change significantly, FWHH = 0.9±0.1 Hz after 120 s.
Accurate fits were obtained for all experiments (n = 6), an example is shown in Figure 2 C,D. The mean exchange rates for PC3 cells (n=3) was $2.8\pm0.5\cdot10^{-10}$ s^{-1} cell^{-1} and LNCaP cells (n=3) $3.8\pm1.6\cdot10^{-11}$ s^{-1} cell^{-1}. The higher conversion in PC3 cells is also observed when comparing the peak integral ratios of lac/pyr of the first 90 spectra added together. Normalized to one million cells the peak ratios are: 0.0078±0.0018 and 0.0009±0.0004 for PC3 and LNCaP.

Discussion

Our setup allows HP substrates to be injected into the cell suspension in a restricted volume, while retaining excellent B_0 homogeneity. We show linewidths below 1Hz and reproducible exchange rates. The exchange rates are lower than expected, especially for the LNCaP cells. We speculate that this is due to the relatively high pyruvate concentration, as the optimal pyruvate concentration for EL-4 cells was reported to be around 5 mM (4). We are currently investigating this hypothesis.
The advantages and possibilities of HP NMR in 5 mm tubes as presented here are numerous. The reduced total sample volume and therefore the low number of cells necessary not only reduces the costs of cell culture, but also opens up the HP experiments for biological systems for which millions of cells cannot easily be obtained, such as primary cultures.
The restricted volume setup reduces convection: in a normal NMR tube the sample volume must be larger than the active volume of the coil to obtain good B_0 homogeneity. Convection, due to HP substrate injection and cell sedimentation, leads to influx of HP substrates that did not feel all radio frequency pulses into the active volume, which complicates the quantitative analysis of the metabolic process. Finally, our system can be used in cryo-cooled pre-amplifier probes, nowadays offering a two to three times gain in sensitivity compared to room-temperature probes and typically only available for small diameter NMR tubes.

References [1] Day, *Nature Med.* 2007 13(11) 1382-7 **[2]** Gallagher, *Proc Natl Acad Sci.* 2009 106(47): 19801-6 **[3]** Harrison, *NMR Biomed.* 2012 25(11): 1286-94 [4] Witney, *J Biol Chem.* 2011 286(28) 24572-80 **[5]** Keshari, *MRM* 2010 63(2) 322-9 **[6]** A. Comment et al. *Concepts Magn Reson B* 2007 31:255-69
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