Alginate encapsulated Lactate Dehydrogenase and Hyperpolarized MR as a Platform to Characterize Enzyme Kinetics

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Introduction: Signal enhancement by dynamic nuclear polarization has facilitated the acquisition of real-time liquid state ^{13}C MRS, enabling the measurement of apparent enzymatic rates[1]. Lactate Dehydrogenase (LDH) is an enzyme often upregulated in cancers[2] and has been a popular target for such hyperpolarized studies by monitoring LDH driven pyruvate-lactate exchange rates. The apparent kinetic properties of pyruvate-lactate exchange are obtained by fitting the integrals of hyperpolarized spectra to the modified Bloch equations[3]. These properties are dependent upon several factors, including hyperpolarized (HP) substrate delivery, LDH activity, NAD/NADH (LDH cofactors) availability, monocarboxylate transporter (MCT) expression and activity, and HP signal loss due to T_1. The compounding effect of these factors makes isolating any particular parameter extremely difficult, resulting in limitations when attempting to model *in vivo* HP data. By modulating kinetic parameters ex vivo one can assess the impact of parameters on the HP MR signal for improved dynamic modelling. Utilization of a controlled MR-compatible bioreactor system [4] provides a means to separate variables (such as transport and T_1 relaxation) and interrogate kinetic properties of LDH with HP MR. Additionally, by encapsulating LDH in an alginate microsphere, LDH can be confined to the sensitive coil region and be continually perfused for NMR study. Thus the aim of this study was to develop a platform to interrogate the kinetic properties of LDH in a controlled, 'cell-like' environment by encapsulating enzyme within alginate beads and perfuse HP [1-^{13}C] pyruvate.

Methods: Isolated lactate dehydrogenase-A enzyme from bovine heart (Sigma Aldrich) and 200µL of a red dye were encapsulated in 1mL of 2.5% alginate and cross-linked in 150mM $CaCl_2$ solution[5]. LDH bead experiments were performed in a custom designed 10mm NMR-compatible bioreactor system[4] utilizing an enclosed perfusion system, providing a continuous flow of 37°C buffer at 1.5mL/min (10mM Tris, pH 7.5, 3.5mM NADH). NMR data was acquired on a narrow-bore 11.7T Varian INOVA (125MHz ^{13}C, Varian Instruments) equipped with a 10mm broadband probe. [1-^{13}C]pyruvate and ^{13}C urea were co-polarized using the Hypersense™ (Oxford Instruments) [6] and dissolved in 5mL 10mM Tris buffer containing 14.8mM NADH, resulting in a solution of 7.4mM pyruvate and 20mM urea, of which 1mL was injected into the bioreactor over 30s. Pyruvate and NADH concentrations were chosen such that LDH would be saturated during the experiment. ^{13}C NMR spectra were acquired at intervals of 3s using 10° pulses. NAD/NADH ratio was measured from ^{31}P spectrum(202MHz) with a 90° pulse and acquire sequence (nt=32, at=1s, TR=3s) prior to ^{13}C experiments. ^{13}C peak integrals were calculated and the lactate signal is normalized to the sum of the integrated areas under the curves of pyruvate and lactate for quantification. After the hyperpolarized signal had decayed, the bioreactor flow was stopped and ^{31}P spectrum (nt=512) were acquired.

Results and Discussion: **Figure 1a** shows the 10mm bioreactor tube loaded with LDH encapsulated microspheres. Alginate microspheres are uniformly distributed throughout the volume and are perfused with the buffer during the experiment. They are stained with red tissue dye for the purposes of visualization and have an average diameter of 450µm (**Figure 1b**, 10× magnification). This diameter was calculated to ensure that the buffer would readily diffuse into the entire encapsulate volume, given the diffusion properties of alginate [7]. **Figure 2** shows the dynamic spectra acquired from an injection of co-polarised [1-^{13}C]pyruvate and ^{13}C urea into microspheres containing 0.5kU LDH with time resolution 3s and 10° flip angle. The build up of [1-^{13}C]lactate from pyruvate is clearly visible. Peaks were integrated and the initial rates of lactate produced by three different enzyme concentrations: 0.5kU (red), 0.25kU (green) and 0. 1kU (blue) are shown in **Figure 3**. The system was continuously perfused throughout the experiment, and from these results we can infer that LDH was successfully encapsulated and remained confined to the beads. The sub-figure shows the linear relationship between enzyme concentration and ratio of total integrated signal from lactate/pyruvate, R^2=0.985 in the first 20 seconds indicative of initial rates. **Figure 4** shows the ^{31}P spectra acquired before and after the hyperpolarised ^{13}C experiment. Prior to the injection of [1-^{13}C]pyruvate, signal solely arises from NADH from the perfusion buffer. The conversion of pyruvate to lactate via LDH causes the production of NAD from NADH, which can be seen after the hyperpolarised experiment as the bioreactor flow is stopped, further validating the enzyme activity. This developmental system has great potential to allow the characterisation of enzyme kinetics in a cell-like environment under controlled conditions. The kinetic model describing this system consists of intra-bead and extra-bead compartments; outside the bead, the hyperpolarized signal decays with T_1 (measured to be 48.3s for pyruvate and 30.8s for lactate at 11.7T), but the intra-bead T_1 will be reduced, owing to its higher density (and therefore increased relaxation mechanisms) than the surrounding buffer. Rate constants are defined to describe the diffusion of hyperpolarized pyruvate into and out of the bead, and within the bead the enzyme-mediated conversion takes place at a known rate, given the known enzyme concentration. Co-polarization with urea allows the input function of hyperpolarized material to be measured and incorporated into the kinetic model. In future studies, this platform will be used to encapsulate multiple enzymes as a means to further mimic perfused cellular or tissue environments to help elucidate the complicated kinetic system governing HP ^{13}C kinetics for translation *in vivo*.

Fig. 1a

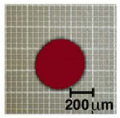

Fig. 1b

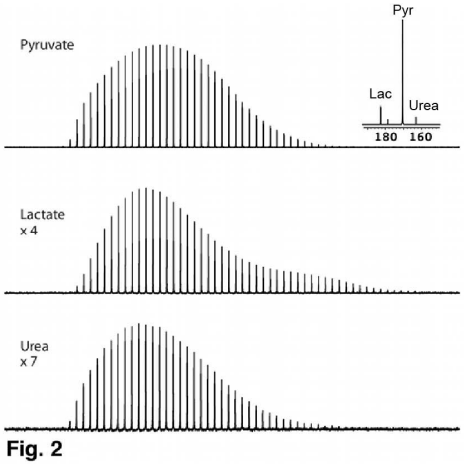

Fig. 2

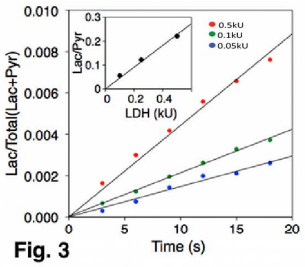

Fig. 3

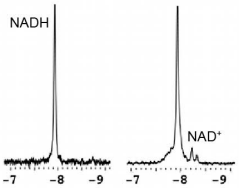

Fig. 4

Figure 1.(a) LDH encapsulated microspheres perfused in 10mm MR-compatible bioreactor (b) representative microsphere demonstrating size. **Figure 2**. Representative LDH kinetics from perfused co-polarized 7.4mM HP [1-^{13}C] pyruvate and 7.4mM ^{13}C-urea with 0.5kU of LDH enzyme in the presence of excess NADH. Inlay demonstrates the ^{13}C signal observed at 60 secs. **Figure 3.** LDH kinetics with varying enzyme concentration. Inlay demonstrates the linear correlation of initial rates with LDH. **Figure 4.** ^{31}P spectra before (left) and after perfusion (right) of HP pyruvate demonstrating the conversion of NADH to NAD^+.

References. **(1)** Ardenkjaer-Larsen, J.H., *et al. Proc Natl Acad Sci* (2003) **(2)** DeBerardinis, R. J., *et al. Cell Metabolism Review* (2007) **(3)** Day, S.E., *et al. Nat Med* (2007) **(4)** Keshari, KR. *Magnetic Resonance in Medicine* (2010) **(5)** Klokk, T. I., *J. Microencapsulation,* (2002) **(6)** Wilson DM et al. J Magn Reson (2010) **(7)** McClelland RE et al. Biotechnol Bioeng (2003).

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