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Hyperpolarised Organic Phosphates as NMR Reporters of Compartmental pH†

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Organic phosphate metabolites contain functional groups with pKa values near the physiologic pH range, yielding pH-dependent 13C chemical shift changes of adjacent quaternary carbon sites. When formed in defined cellular compartments from exogenous hyperpolarised 13C substrates, metabolites thus can yield localised pH values and correlations of organelle pH and catalytic activity.

Different pH values of cellular compartments help to maintain optimal conditions for the specialised biological processes occurring in these compartments.1 Normal catalytic functions are supported by compartmentalisation itself, which ensures controllable catalyst, substrate and ion (not least H+) concentrations.2 Accordingly, abnormal pH homeostasis is detrimental to normal catalytic (metabolic) function in the cell.

Concurrent measurements of intracellular catalytic activities and physiological parameters such as pH permit direct correlations of biocatalytic function and physiological parameters. Nuclear magnetic resonance (NMR) spectroscopy is a non-invasive modality both for the detection of pH and for probing catalytic activities. The scope of NMR measurements has been extended by hyperpolarisation methods, which both improve the sensitivity (against the noise) and the selectivity (against the cellular background) of assays that most often detect non-protonated 13C sites.3 These improvements are achieved by the temporary redistribution of nuclear magnetisation in exogenous substrates that are used as molecular probes with lifetimes of seconds.4 In the current study, we investigate the feasibility of instantaneous compartmental pH measurements with natural substrates through hyperpolarised NMR assays exploiting compartment-specific catalytic activities. Using the example of the glycolytic reaction cascade, correlations of cytosolic pH and catalytic activity are obtained: high-field NMR renders pH-dependent signal changes in phosphorylated metabolites of the glycolytic reaction cascade detectable (Figure 1). At the same time, the signal amplitude of metabolites reflects the catalytic activity leading to their formation, thus permitting to correlate pH and catalyst activities with instantaneous measurements. The conversion of hyperpolarised endogenous substrates to metabolites reporting on physiological state (pH) alleviates problems related to cell delivery, cell retention, entrapment in lysosomes via endocytosis and substrate toxicity. Notably, the subcellular pH measurements rely on non-invasive signal detection that has proven viable mammalian tissues.4

Various magnetic resonance approaches have been devised for the measurement of extracellular pH using 1H, 31P or 33P nuclei as pH indicators.5 Some problems relating to experimental duration and sensitivity as well as dose requirements have been addressed by hyperpolarised 13C NMR using bicarbonate or Good’s buffers as biocompatible pH indicators.6 As hyperpolarised NMR methods operate on the seconds timescale, the internalisation of molecular probes to intracellular sites has remained a challenge and equivalent approaches towards the study of intracellular pH by hyperpolarised NMR have been lacking. In contrast, phosphorylated endogenous metabolites and inorganic phosphate are well-established intracellular indicators for conventional 31P NMR. Even for highly concentrated cell suspension, such measurements accumulate thousands of scans and thus provide a coarse time average of pH over

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minutes or hours, while pH adaptations can occur within seconds. The extensive accumulation of $^{31}$P NMR spectra is also not easily compatible with concurrent activity assays of cellular function. In addition, subtle pH differences are hard to detect as the signals are averaged over the entire cells and different metabolite pools can only be resolved for rather extreme pH differences in different compartments.

Hyperpolarised NMR probe molecules for the sensing of Ca$^{2+}$ ions, pH and H$_2$O$_2$ have previously been designed by combining sensing groups and long $T_1$ sites in the same molecule, but biological applications of these designed probes have remained challenging. Phosphorylated metabolites often contain quaternary $^{13}$C nuclei with sufficiently long $T_1$ times to render influx of hyperpolarised signal into the metabolite pool on the $T_1$ timescale detectable. As organic phosphates have pKa values near the physiological range, phosphorylated metabolites naturally combine long $T_1$ sites and pH reporters in the same molecule (Figure 1). The formation of organic phosphates in cellular compartments thus potentially produces hyperpolarised pH probes.

In order to test the feasibility of hyperpolarised organic phosphates as pH indicators, the pH dependence of the $^{13}$C chemical shifts was determined for a mixture of relevant reference standards with thermal $^{13}$C NMR spectroscopy at 18.7 Tesla field strength (Figure 2). Principal intermediates accumulating in the glycolytic reaction cascade of various human, murine and microbial cell lines include glycerone phosphate, pyruvate, 3-phosphoglycerate and 6-phosphogluconate. The phosphate group is located in the α-position relative to the quaternary carbon in glycerone phosphate and in the β-position relative to the quaternary carbon in 3-phosphoglycerate. Thus, significant $^{13}$C chemical shift changes in the physiological pH range are observed for the quaternary carbons of glycerone phosphate ($\Delta^{13}$C$_{cph7.5-}$ $^{13}$C$_{pH5.2-7.5}$ = 1.6 ppm) and 3-phosphoglycerate.

$$\text{pH} = pK_a + \log_{10} \frac{\Delta \delta_{\text{obs}}}{6.39 \text{ ppm} - \Delta \delta_{\text{obs}}}$$

with a pKa 5.81 and maximum and minimum chemical shift differences between glycerone phosphate and pyruvate of 6.39 and 4.42 ppm at 30°C (Figure 3). This calibration was used in determining pH values in yeast cells and to probe the effect of extracellular acidification on cytosolic
pH and catalytic activity in a well-studied model. Opposite to conventional $^{31}$P NMR determinations, hyperpolarised organic phosphates are detectable almost instantaneously when using hyperpolarised carbohydrates as substrates (Figure 4). The hyperpolarised NMR assays encompass very little of the pH-dependent signal position over time as spectra are acquired as single transients during an acquisition time of less than 0.3 seconds. In addition, spatial selectivity is warranted by the formation of the hyperpolarised organic phosphate pH indicator in defined compartments, here the cytosol.

Chemical shift changes of the glyceronephosphate signal relative to the pyruvate signal were detected upon extracellular acidification with weak organic acids (Figure 5a). Resultant chemical shift changes were translated to pH values with the parametrisation of equation (1). Cytosolic pH values determined in this manner are consistent with values determined previously by relative radioisotopic distributions of $[1^{-13}C]proionic acid to measure intracellular pH (pH) in yeast (Figure 5b). The decrease of cytosolic pH below the normal physiological range coincides with reduced catalytic activity in the glycolytic cascade (Figure 5a). Less than 10% of the metabolic activity are retained after an intracellular pH decrease to pH 5.6, in agreement with previous correlations of fermentation rate and radioisotopically determined pH, by Pampulha et al. The detrimental changes to cytosolic fermentation kinetics at low pH include alterations that induce the accumulation of pernicious intermediates, as highlighted in Figure 5a.

In concluding, we conduct high-field $^{13}$C NMR measurements of hyperpolarised intracellular metabolites as prospective pH indicators. High-field NMR affords sufficiently accurate chemical shift measurements to identify cytosolic pH concurrently to catalytic activity in central carbon metabolism and to correctly reproduce correlations between cytosolic pH and catalytic activity. Cytosolic pH measurements using hyperpolarised $^{13}$C chemical shifts of organic phosphates were extended to murine and human breast cancer cells yielding cytosolic values of 6.90, while corresponding determination for the bacterium Escherichia coli yielded an intracellular pH of 6.80. We note that the hyperpolarised $^{13}$C NMR signal of glyceronephosphate has also been measured in a preclinical animal study of tumor glycolysis using hyperpolarised $[U^{-13}C,U^{-13}H] glucose as the substrate. The use of alternative substrates with longer $T_1$ times could facilitate the detection of intracellular organic phosphates with hyperpolarized NMR. As an example, hyperpolarized $[2^{-13}C]$-glycerone has recently allowed the detection of at least five different organic phosphate metabolites in a perfused liver model. These studies encourage the hope that rapid and noninvasive imaging of compartmental pH may become feasible through hyperpolarised $^{13}$C NMR of pH dependent metabolite signals.

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Notes and references


