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Parahydrogen-enhanced pH measurements using  $[1-^{13}\text{C}]$  bicarbonate derived from non-enzymatic decarboxylation of  $[1-^{13}\text{C}]$ pyruvate- $\text{d}_3$

Non-invasive magnetic resonance methods to measure pH offer a new approach for early diagnosis of diseases characterized by acid-base imbalances. We present an optimized preparation of an hyperpolarized  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$  pH sensor *via* non-enzymatic decarboxylation with  $\text{H}_2\text{O}_2$  of  $[1-^{13}\text{C}]$ pyruvate- $\text{d}_3$ . Unprecedented degrees of  $^{13}\text{C}$  signal-enhancements of purified  $[1-^{13}\text{C}]$ pyruvate- $\text{d}_3$  were obtained using parahydrogen allowing for a secondary reaction to release the pH sensor. *In vitro* validations demonstrated accurate pH calculations. Our results highlight the efficiency of a pH sensor generated in less than one minute, with remarkable polarization, and biocompatibility suitable for future *in vivo* studies.

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# Parahydrogen-enhanced pH measurements using [1-<sup>13</sup>C]bicarbonate derived from non-enzymatic decarboxylation of [1-<sup>13</sup>C]pyruvate-d<sub>3</sub>†

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Alterations in pH are a hallmark in several pathologies including cancer, ischemia, and inflammation. Non-invasive magnetic resonance methods to measure pH offer a new approach for early diagnosis of diseases characterized by acid–base imbalances. The hyperpolarization with parahydrogen-induced polarization (PHIP) enhances inherently low signals in magnetic resonance experiments by several orders of magnitude and offers a suitable platform to obtain biocompatible markers in less than one minute. Here, we present an optimized preparation of an hyperpolarized H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub> pH sensor via non-enzymatic decarboxylation with H<sub>2</sub>O<sub>2</sub> of [1-<sup>13</sup>C]pyruvate-d<sub>3</sub> obtained by PHIP at 7 T. An improved <sup>13</sup>C polarization of purified [1-<sup>13</sup>C]pyruvate-d<sub>3</sub> in water with 36.65 ± 0.06% polarization was obtained starting from 50 mM precursor. Subsequent decarboxylation, H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub> exhibited 12.46 ± 0.01% of polarization at physiological pH, 45 seconds after the reaction start. Considering the dilution factor that [1-<sup>13</sup>C]pyruvate-d<sub>3</sub> exhibits *in vivo*, we optimized our methodology to test the accuracy of the pH sensor at single digit millimolar concentration. *In vitro* pH estimations on phantoms and cell culture media demonstrated accurate pH calculations with uncertainties of less than 0.08 units. These promising results highlight the efficiency of a pH sensor generated via PHIP in less than one minute, with remarkable polarization, and biocompatibility suitable for future *in vivo* studies.

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## Introduction

pH is robustly regulated in healthy tissues and is essential for physiological processes including tissue oxygenation, proper protein structure, and various biochemical reactions.<sup>1</sup> However, perturbations of acid–base homeostasis are a common feature of several pathological conditions including cancer, ischemia, inflammation, renal disease, and infectious diseases.<sup>2–4</sup> It is known that the acidic microenvironment generated by cancer cells correlates with aggressiveness, migration, invasion, and metastasis potential.<sup>5–7</sup> All these events are associated with the Warburg effect, which involves increased glucose uptakes, anaerobic glycolysis, increased production of lactate, and an acidic extracellular pH.<sup>8</sup> Moreover, it was reported that aberrant acidifications could contribute to

Alzheimer's disease and Amyotrophic lateral sclerosis' development.<sup>9–11</sup> Therefore, the development of safe and rapid pH measurement may aid in the early diagnosis of acid–base imbalances and subsequent pathological disorders. Although different techniques such as magnetic resonance spectroscopy (MRS), magnetic resonance imaging (MRI), and positron emission tomography (PET) have been used to measure pH in pre-clinical environments, none of them was successfully translated to routine clinical use.<sup>4,12–22</sup> Regarding MRS, <sup>31</sup>P and <sup>19</sup>F probes were used to measure pH *in vivo*. However, they present small pH dependent chemical shifts and lack of sensitivity.<sup>13,23–27</sup> Concerning MRI focused techniques, several methods were developed (e.g. CEST and the use of Gd<sup>3+</sup>).<sup>27,28</sup> Overall, the main reasons why the noninvasive developed probes are challenging to be clinically translatable are centered on, (i) the potential toxicity of the existing probes which makes them unfeasible to be injected in routine imaging studies in patients,<sup>2–4,29–32</sup> and (ii) the low signal sensitivity of the nuclear magnetic resonance techniques. For these reasons, the use of biocompatible probes combined with signal enhancement techniques offers great potential. MRS and MRI stands out as highly versatile and valuable tools for a wide array of applications including human imaging, *in vitro* and *in vivo*

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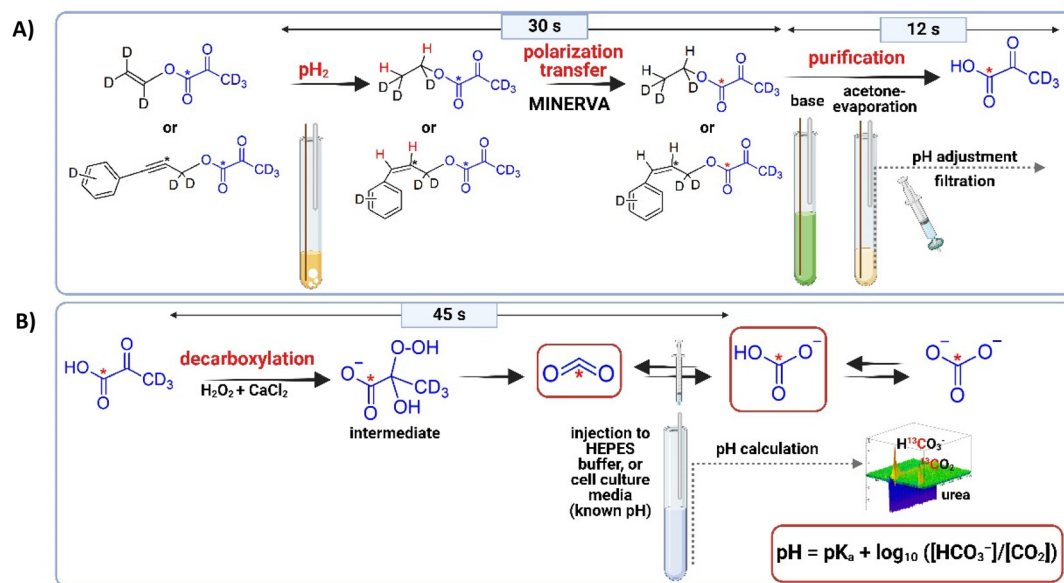
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chemical and biochemical studies, and unraveling molecular structures. Despite its remarkable capabilities, its effectiveness is hindered by its inherent low sensitivity.<sup>33</sup> However, this limitation is effectively addressed through hyperpolarized (HP) magnetic resonance. HP enhances MRS signals by over four orders of magnitude<sup>34</sup> relying on the generation of isotopically enriched, non-toxic contrast agents that enable *in vitro* and *in vivo* metabolic kinetics studies in real-time and that have been used among other for patient pH imaging.<sup>34–36</sup> To generate hyperpolarized <sup>13</sup>C metabolites, two approaches are largely employed. Between them, dissolution Dynamic Nuclear Polarization (dDNP) is a technique that has been used for producing HP pyruvate,<sup>37,38</sup> and also to produce HP bicarbonate to perform *in vitro* and *in vivo* pH measurements.<sup>2,39–44</sup> However, clinical scale dDNP is expensive and it is a slow technique that requires times between ten minutes to hours to produce HP metabolites.<sup>45,46</sup> Although several HP probes were successfully developed using dDNP, the needed of sophisticated and expensive equipment, as well as, the long polarization times are most likely the reasons why translation is challenging.<sup>2,3,40–42,47–54</sup> An alternative approach to hyperpolarize metabolites is based on parahydrogen-induced polarization (PHIP). This technique converts the nuclear spin singlet of parahydrogen (pH<sub>2</sub>) into enhanced signals within seconds. One of the pH<sub>2</sub>-based hyperpolarization methods, known as Signal Amplification by Reversible Exchange (SABRE), operates through reversible exchange processes between a target molecule and a metal catalyst.<sup>55</sup> For the classical PHIP method, an unsaturated precursor is required to which hydrogen is added. The development of PHIP-SAH (PHIP by means of the Side Arm Hydrogenation) opened up new opportunities to hyperpolarize metabolites.<sup>46</sup> Thereby, the target molecule is func-

nalized with an unsaturated side arm, *e.g. via* esterification. The molecule with the side arm serves as an efficient precursor to which the parahydrogen is added and this approach has demonstrated the highest yielding levels of polarization with pH<sub>2</sub> so far. In particular fully deuterated precursors have shown to deliver optimal polarization conditions due to the reduction of dipolar couplings to hydrogen originating from parahydrogen, prolonging *T*<sub>1</sub>.<sup>56</sup> Overall, the unsaturated bond is hydrogenated by using pH<sub>2</sub> and a catalyst, usually in organic solvents. Next, the yielded proton spin order is transferred to a target <sup>13</sup>C nucleus. In our work we use the spin order transfer sequence called MINERVA (Maximizing Insensitive Nuclei Enhancement Reached *Via* parahydrogen Amplification) for this transfer.<sup>56</sup> Following the magnetization transfer, the side chain is removed by hydrolysis enabled by the addition of a base. The organic solvent is removed by evaporation and after subsequent filtration, the pH is adjusted to physiological values, and finally, after around one minute, the HP <sup>13</sup>C-compound is obtained.<sup>34</sup> The procedures including used precursors are shown in Fig. 1A and PHIP-SAH has overall allowed to enhance important metabolites for *in vivo* studies.<sup>34–36,46,57–64</sup> Thereby <sup>13</sup>C-pyruvate is used as key metabolite for a non-invasive kinetic evaluation of conversions from pyruvate to lactate in real-time *in vitro* and *in vivo*.<sup>34,56,59,65</sup> Even though metabolites and especially pyruvate can be hyperpolarized with parahydrogen, so far, no biocompatible parahydrogen derived pH sensor is available at sufficient polarization (>10% <sup>13</sup>C polarization). The latter we are introducing in this work by performing a non-enzymatic decarboxylation in which H<sub>2</sub>O<sub>2</sub> present a nucleophilic addition to the α-carbonyl group of pyruvate to generate an unstable intermediate, which reacts to CO<sub>2</sub> that is in equilibrium with HCO<sub>3</sub><sup>−</sup>, acetate, and water at neutral pH (Fig. 1B).<sup>35,66</sup>



**Fig. 1** Schematic representation of the PHIP-SAH workflow; (A) full workup that includes: pH<sub>2</sub> bubbling (20 s), MINERVA polarization transfer (10 s), base addition (1 s), acetone evaporation (8 s), buffer addition (1 s) and filtration (3 s); (B) decarboxylation process to obtain PHIP-H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/PHIP-<sup>13</sup>CO<sub>2</sub> (reaction takes 45 s), and pH measurement at 7 T. Own Fig. created with BioRender.com.



## Materials and methods

### Chemicals

All chemicals were purchased from Sigma Aldrich, except the precursors that were synthesized according to published procedure.<sup>35,61,67</sup> Please refer to the ESI† for further information (Schemes S1 and S2†).

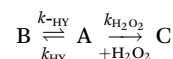
### Hyperpolarization and non-enzymatic decarboxylation: procedure and equipment

[1,4-Bis(diphenylphosphino)butane](1,5-cyclooctadien)rhodium(i)-tetrafluoroborate (Sigma Aldrich, catalog # 79255-71-3) was dissolved in acetone- $d_6$  and used as catalyst for the PHIP procedure with a final concentration of 10 mM. After mixing with the precursor (vinyl pyruvate- $d_6$  at 50 mM or phenyl propargyl pyruvate ester- $d_6$  at 5.5 mM), the samples were filled into a 7-inch 5 mm NMR tube and bubbled with  $N_2$  for 10 seconds for degassification. *para*-enriched hydrogen was obtained at 20 K with a He-cooled parahydrogen generator provided by a Cryocooler system (Sumimoto HC-4A helium compressor, Sumimoto Cold Head CH-204 with  $pH_2$  reaction chamber by ColdEdge Technologies, temperature controller Lake Shore Cryotronics, Inc.) and delivered by a home-built valve and tubing system. Hyperpolarization and polarization transfer were carried out in a Bruker Avance III HD 300 narrow bore spectrometer operating at 7.05 T field strength equipped with a room temperature broadband observe (BBO) probe head for sample diameters of 5 mm (PA BBO 300S1 BBF-H-D-05 Z). Vinyl pyruvate- $d_6$  was hyperpolarized by using the MINERVA sequence as described previously (detailed in the ESI, Fig. S2A and B†).<sup>35,65</sup> For hyperpolarization of phenyl propargyl pyruvate ester- $d_6$ , a field-independent MINERVA sequence for the transfer of longitudinal spin order of parahydrogen polarization was applied as previously published (detailed in the ESI†).<sup>36</sup> After finalization of both processes (30 s), 200  $\mu$ L of a  $Na_2CO_3$  in  $D_2O$  (75 mM for 50 mM of the vinyl pyruvate- $d_6$  precursor or 7.5 mM for 5.5 mM of the phenyl propargyl pyruvate ester- $d_6$  precursor), containing EDTA (1 mM in  $D_2O$ , which prolongs  $T_1$  and stabilize the polarization),<sup>68</sup> and sodium ascorbate (50 mM in  $D_2O$ , which decreases the polarization loss),<sup>69</sup> were added to cleave the side arm and release hyperpolarized  $[1-^{13}C]$ pyruvate- $d_3$ . Then, the NMR tube was placed outside the magnet and acetone was evaporated by passing  $N_2$  at 7 bar pressure through the tube, and by immersing the tube in warm water ( $\sim 79^\circ C$ ) for 8 seconds.<sup>34</sup> After evaporation, indicated by a reduction of the total volume by one half in these 8 s (the volume of acetone and base added were 200  $\mu$ L each, so the reduction of the acetone volume was visually checked), 50  $\mu$ L of HEPES (100 mM, pH 10 in  $D_2O$ ) were added to obtain physiological conditions in the final solution (1 s). The aqueous solution was then filtered from the NMR tube into a syringe by using a PVDF filter with a pore size of 1.0  $\mu$ m (Fisher Scientific) to obtain in 3 s the catalyst-free signal-enhanced  $[1-^{13}C]$ pyruvate- $d_3$ .<sup>34</sup> Finally, 50  $\mu$ L of  $H_2O_2$  at 326 mM (final concentration 50 mM, 0.15%) was added to induce the non-enzymatic decarboxylation. 25  $\mu$ L  $CaCl_2$  at a concentration of 3 mM was injected together

with  $H_2O_2$  to increase the decarboxylation rate. Fig. 1 and Fig. S1 (ESI)† show a schematic representation of the entire procedure. In Fig. 2 is shown the spectrum recorded after 45 s of the reaction.

### Monitoring of pyruvate decarboxylation

The decarboxylation reaction of pyruvate was followed spectroscopically at  $50^\circ C$  using a series of  $90^\circ$  single scan  $^1H$  NMR at 7 T every 5 s, after mixing 30 mM of Sodium pyruvate in  $D_2O$  (concentration of pyruvate expected in the decarboxylation experiments due to dilution by addition of buffer,  $H_2O_2$ , and  $CaCl_2$ ), with 50 mM of  $H_2O_2$ , and 3 mM of  $CaCl_2$  both in  $D_2O$ . The integral intensities of the  $^1H$  signals of the methyl group of each species were converted to concentrations. Below is detailed the model described by Tickner *et al.* (2020),<sup>70</sup> followed for the respective equations:



$$[A]_{t+\delta t} = [A]_t + (-k_{H_2O_2}[A]_t[D]_t - k_{HY}[A]_t + k_{HY}[B]_t)\delta t$$

$$[B]_{t+\delta t} = [B]_t + (k_{HY}[A]_t - k_{HY}[B]_t)\delta t$$

$$[C]_{t+\delta t} = [C]_t + (k_{H_2O_2}[A]_t[D]_t)\delta t$$

$$[D]_{t+\delta t} = [D]_t - (k_{H_2O_2}[A]_t[D]_t)\delta t$$

where A represents sodium pyruvate, B is pyruvate hydrate, C is acetate, and D is the rate of reaction between pyruvate and  $H_2O_2$  obtained by fitting the integral intensities of  $^1H$   $CH_3$  resonances of A, B, and C.  $k_{HY}$ ,  $k_{HY}$ , and  $k_{H_2O_2}$  are the rates of each reaction, and in the equations  $\delta t$  is an incremental time difference.

As described by Tickner *et al.* (2020),<sup>70</sup> rate constants were found by minimizing the differences between concentrations

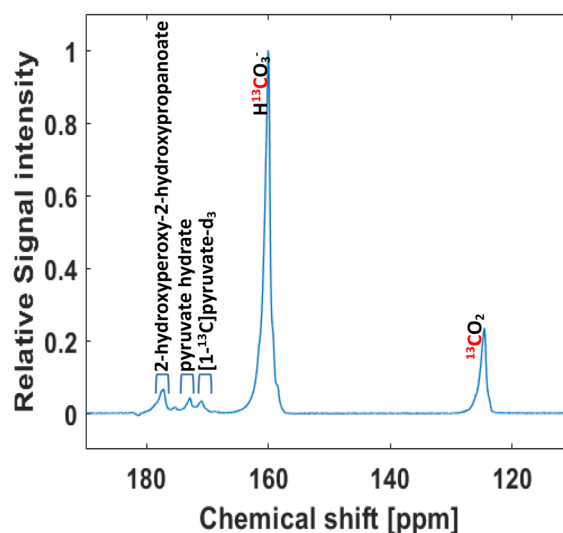


Fig. 2 Non-enzymatic decarboxylation of HP  $[1-^{13}C]$ pyruvate- $d_3$  (50 mM) with  $H_2O_2$  (50 mM) at  $50^\circ C$ . Single  $^{13}C$  spectrum recorded at 7 T after 45 s of  $H_2O_2$  injection.





determined experimentally and the calculated values. We also maintained the same assumptions, in which species as  $\text{H}_2\text{O}$ , pyruvate dimers or enol pyruvate, and the pathway that allows pyruvate hydrate reaction with  $\text{H}_2\text{O}_2$  were not included in the model. In the ESI† are detailed the obtained kinetic parameters and the fitting (Table S1 and Fig. S3†).

### PHIP $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ pH measurements in phantoms

HEPES buffer solutions in  $\text{D}_2\text{O}$  were prepared at different pH values (6.40, 6.50, 6.61, 6.70, 6.81, 6.90, 7.20, and 7.40). The pH of each solution was adjusted using HCl (100 mM in  $\text{D}_2\text{O}$ ) and NaOH (100 mM in  $\text{D}_2\text{O}$ ), as necessary, and verified using a calibrated pH electrode. The pH measurements were performed in a Bruker Avance IV 300 7.05 T wide bore spectrometer equipped with a MicWB40 rf probe and Micro 2.5 WB gradient system at room temperature. The phantom consisted in eight empty NMR tubes labelled from 1–8 and placed surrounding a urea phantom tube, which was used as a positioning reference (Fig. 4A). 350  $\mu\text{L}$  of HEPES at pH 6.4 were injected into tube 1, immediately before the injection of 175  $\mu\text{L}$  of the  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  (5.5 mM). After injection of  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ , we conducted non-localized spectroscopic experiments with low-flip angle excitations to obtain the  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  integrals. Specifically, seven seconds after injection, a train of  $20^\circ$  flip angle pulses was initiated and a spectrum was acquired every 3 seconds. After achieving the steady state (state in which the rates of the forward and reverse reactions are equal, leading to constant concentrations of the involved species), the pH value of the respective tube was calculated by using the Henderson–Hasselbalch equation (more details in the ESI†). This procedure was repeated for each subsequent tube (2–8) to conduct the pH measurements. Each experiment was separated by a 5-minute interval to ensure that no residual signal was detected from the previous tube. This interval allowed any potential HP signal to dissipate, ensuring accurate readings. Once the pH measurements were finished for all the tubes, 500  $\mu\text{L}$  of  $\text{H}_2\text{O}$  were injected into each tube to obtain a representative  $^1\text{H}$  image of the setup (Fig. 4A). For this, a FLASH sequence (fast low angle shot magnetic resonance imaging) with a field of view of  $30 \times 30 \text{ mm}^2$ , a matrix size of  $256 \times 256$ , and a central slice with 3 mm thickness in the transversal plane were used. Finally, the  $^1\text{H}$  image and pH values obtained from the spectroscopic measurements were employed to generate a pH color-representative graph using MATLAB software (Fig. 4B).

### PHIP $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ pH measurements in cell culture media

Mouse pancreatic ductal adenocarcinoma cell line (PanC02) were kindly provided by Professor Stine Pedersen (University of Copenhagen) and Professor Frauke Alves (MPI NAT, Goettingen), and they were cultured in DMEM without bicarbonate, with HEPES, supplemented with 10% Fetal bovine Serum, and 1% penicillin/streptomycin, at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  in a humidified incubator. After four days *in vitro*, cell culture media was collected from different flasks and the pH was measured with a pH-electrode. Furthermore, fresh media-pH

was also measured with the electrode. Immediately after that, we carried out pH measurement with our probe, using identical conditions as detailed before for phantoms measurements. Table S4 in the ESI† shows the individual pH values obtained for each experiment.

### Measurement of polarization and $T_1$

Polarization values were determined from six independent experiments, by comparing the signal intensity from one hyperpolarized sample recorded with a  $90^\circ$  pulse to a spectrum measured at thermal equilibrium. The thermal spectrum was measured with the same acquisition parameters,  $T = 290 \text{ K}$  using 160 scans. Peak areas from the hyperpolarized spectra were corrected according the flip angle ( $20^\circ$ ) and fitted to a decaying exponential to approximate  $T_1$  values.

### Data analysis, % polarization calculation, and statistics

The spectra were processed using TopSpin software version 4.0.8. Phase correction, line-broadening (LB 5 for EM), and baseline corrections were applied. Further analyses and 3D plot were performed using MATLAB software version R2019b. In each spectrum, the peaks of  $\text{HCO}_3^-$  and  $\text{CO}_2$  were integrated to determine the area under the curves (AUCs). From the ratio of the AUCs, pH values were calculated by using Henderson–Hasselbalch equation. Correlation and statistical analysis were performed in GraphPad Prism version 9 software.

Polarization level was calculated using the following equation:

$$P_{\text{hyp}} = \frac{I_{\text{hyp}}}{I_{\text{th}}} \times \frac{rg_{\text{th}}}{rg_{\text{hyp}}} \times \frac{ns_{\text{th}}}{ns_{\text{hyp}}} \times \frac{\sin(\theta_{\text{th}})}{\sin(\theta_{\text{hyp}})} \times P_{\text{th}}.$$

In this equation,  $P_{\text{hyp}}$  represents the polarization level of the sample in the hyperpolarized spectra, and  $P_{\text{th}}$  the polarization value of the sample in the thermal spectra.  $I$  represents the integration in the same spectra region (e.g.  $^{13}\text{C}$  NMR signal of pyruvate at around 170 ppm) in both, hyperpolarized ( $I_{\text{hyp}}$ ) and thermal spectra ( $I_{\text{th}}$ ).  $rg$  is the receiver gain of the spectrometer,  $\sin(\theta_{\text{th}}) = 1$  and  $ns_{\text{hyp}} = 1$ , as the thermal experiments were acquired with a flip angle of  $90^\circ$  degrees, and a singles scan is used in hyperpolarization experiments. A comparison between one single  $^{13}\text{C}$  scan recorded for 50 mM of hyperpolarized (HP)  $[1\text{-}^{13}\text{C}]\text{-pyruvate-}d_6$  and its corresponding thermal spectrum is showed in the ESI (Fig. S4).†

To calculate polarization levels for the  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  species, we took the most conservative calculation using  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  integrals after purification workup, and 45 s after  $\text{H}_2\text{O}_2$  injection, in at least three different experiments.

## Results and discussion

### PHIP pH probe development

In this work, a  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  probe was developed to measure extracellular pH. This probe was obtained in  $\text{D}_2\text{O}$  at physiological conditions by non-enzymatic decarboxylation of signal enhanced  $1\text{-}^{13}\text{C}\text{-pyruvate}$  (see Fig. 1B).<sup>35</sup> Fully deuterated



precursors were used to restrict the effective nuclear spin system and minimizing the loss of signal enhancement.<sup>35</sup> In a first step the hyperpolarization of  $[1-^{13}\text{C}]$ pyruvate- $\text{d}_3$  obtained from vinyl pyruvate- $\text{d}_6$  was optimized and an average polarization of  $36.65 \pm 0.06\%$  (see ESI†) was obtained starting from 50 mM  $1-^{13}\text{C}$ -vinyl pyruvate- $\text{d}_6$  precursor. It is worth to mention that the highly obtained polarization on pyruvate would enable the preparation of the metabolic probes with liquid nitrogen cooled parahydrogen for which polarization levels would be a factor of three less but still exceed  $10\%$   $^{13}\text{C}$  polarization. Note that this is the measured polarization of purified pyruvate in  $\text{D}_2\text{O}$ . The purification procedure was performed by cleavage of the side arm with  $\text{Na}_2\text{CO}_3$  (75 mM) supplemented with EDTA (1 mM), and sodium ascorbate (50 mM). After obtaining free  $[1-^{13}\text{C}]$ pyruvate- $\text{d}_3$  at  $50^\circ\text{C}$ , the pH was adjusted to biocompatible conditions with HEPES-buffered  $\text{D}_2\text{O}$ . Followed by pH adjustment,  $\text{H}_2\text{O}_2$  was added to induce the non-enzymatic decarboxylation, together with  $\text{CaCl}_2$  to increase the decarboxylation rate.<sup>2,71,72</sup> We also investigated our previously introduced Phenyl propargyl pyruvate ester precursor (PPE) at high field together with a field-independent pulsed PHIP-SAH method, which however yielded low degrees of pyruvate polarization. The complete process yielded  $2.45 \pm 0.01\%$  pyruvate polarization that might be due to reduced relaxation times as compared to low field applications.<sup>36</sup>

We attribute that remarkable polarization value achieved on  $[1-^{13}\text{C}]$ pyruvate- $\text{d}_3$  starting from vinyl pyruvate- $\text{d}_6$  to some changes implemented in the purification and pH stabilization procedure. In contrast to previous purification methodologies employed by us,<sup>34–36,56,65</sup> that involved the use of  $\text{Na}_2\text{CO}_3$  (100 mM) in  $\text{H}_2\text{O}$ , the present methodology employed the same base, but in  $\text{D}_2\text{O}$ , with a concentration of 75 mM, and supplemented with EDTA (which prolongs  $T_1$  and stabilize the polarization, according to dDNP experiments),<sup>68</sup> and sodium ascorbate (which decreases the polarization loss during cleavage as previously reported).<sup>69</sup> Notably, the evaporation procedure involved the use of  $\text{N}_2$  bubbling, differing from the conventional vacuum-assisted evaporation.<sup>36</sup>

The final adjustment of pH was performed through the addition of HEPES dissolved in  $\text{D}_2\text{O}$ , departing from the prior use of HEPES in  $\text{H}_2\text{O}$ . It was reported that retaining pyruvate in  $\text{PBS-D}_2\text{O}$ , instead of  $\text{PBS-H}_2\text{O}$  leads to lengthening of relaxation times which may play a crucial role in this process.<sup>45</sup> Moreover, in a recently published study, a similar effect was observed when  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  were obtained *via* hydrolysis of HP  $[1-^{13}\text{C}]1, 2\text{-glycerol carbonate}$ . Hydrolysis conducted with  $\text{NaOH}$  in  $\text{D}_2\text{O}$  affords superior polarization values and extended lifetimes compared to its counterpart in  $\text{H}_2\text{O}$ .<sup>52</sup> In this point, it is important mentioning that  $\text{D}_2\text{O}$  is safe for *in vivo* and humans injection, as was previously informed.<sup>45,73</sup> In a next step, we added  $\text{H}_2\text{O}_2$  (50 mM) and found that after 45 s the reaction intermediates had sufficiently been converted into  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ . A total  $^{13}\text{C}$  polarization of  $12.46 \pm 0.01\%$  was estimated based on the initial  $^{13}\text{C}$  thermal signal of the precursor. Fig. 2 illustrates a  $^{13}\text{C}$  spectrum recorded at 7 T using a single-90 degree pulse, post-purification, and sub-

sequent to 45 s of  $\text{H}_2\text{O}_2$  injection.  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  are the main species present, together with small traces of  $[1-^{13}\text{C}]$ pyruvate- $\text{d}_3$  ( $\sim 171$  ppm), the intermediate (2-hydroxyperoxy-2-hydroxypropanoate,  $\sim 176$  ppm), and pyruvate hydrate ( $\sim 178$  ppm).<sup>35,74</sup>

### $[1-^{13}\text{C}]$ pyruvate- $\text{d}_3$ decarboxylation kinetics

Furthermore, we monitored the sodium pyruvate decarboxylation rate following the model previously reported by Tickner *et al.*<sup>70</sup> Briefly, 30 mM of sodium pyruvate (concentration expected in the decarboxylation solution when starting from 50 mM in hyperpolarization experiments) were decarboxylated by the addition of 50 mM of  $\text{H}_2\text{O}_2$  in  $\text{D}_2\text{O}$  and 3 mM of  $\text{CaCl}_2$  in  $\text{D}_2\text{O}$ . The reaction was monitored spectroscopically at  $50^\circ\text{C}$  using a series of  $90^\circ$  single scan  $^1\text{H}$  NMR at 7 T every 5 s. The integral intensities of pyruvate, acetate, and pyruvate hydrate were converted to concentration and plotted over time (see ESI†). By fitting these data to the kinetic model described by Tickner *et al.* using the same assumptions (e.g. omission of  $\text{H}_2\text{O}$ , pyruvate dimers, or enol pyruvate species, as well as, omission of the reaction between pyruvate hydrate and  $\text{H}_2\text{O}_2$ ),<sup>70</sup> the rate of the reaction between pyruvate and  $\text{H}_2\text{O}_2$  was calculated. The  $k_{\text{H}_2\text{O}_2}$  value was estimated as  $(0.88 \pm 0.14) \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$  (please refer to the ESI† for further details). Even though this value demonstrate a rapid decarboxylation rate of pyruvate, and pyruvate conversion was approximately of 76%. 9 mM of uncarboxylated pyruvate (24%) were present after 45 s of  $\text{H}_2\text{O}_2$  addition. Given the signal strengths shown in Fig. 2 we do not expect interferences due to the low concentration. However, if it is considered that uncarboxylated pyruvate could be transformed *in vivo* to lactate ( $\sim 182$  ppm) and alanine ( $\sim 176$  ppm), there are no major concerns since the residual products will not interfere with the signal of the species used for the pH measurements  $\text{H}^{13}\text{CO}_3^-$  ( $\sim 160$  ppm), and  $^{13}\text{CO}_2$  ( $\sim 128$  ppm), and actually could provide valuable information about real-time metabolic kinetics. Concerning  $\text{H}_2\text{O}_2$  concentration, assuming a 1 : 1 relation between acetate formed and  $\text{H}_2\text{O}_2$  consumed, it was observed that at 45 s the remaining concentration of  $\text{H}_2\text{O}_2$  is approximately 20 mM (0.06%) in 325  $\mu\text{L}$ . Human studies exist in which  $\text{H}_2\text{O}_2$  in concentrations between (0.06–0.48)% were injected *via* intra-arterial or intravenous (evaluating its therapeutic potential on head and neck cancer, arteriosclerosis, wound healing) did not show toxicity.<sup>75</sup> On *in vivo* experiments, the reported intravenous (median lethal dose)  $\text{LD}_{50}$  in mice was founded to be 50 000 mg per kg body weight.<sup>76</sup> Therefore, given that facts, and the fact that that our solution would dilute by the blood volume ( $\sim 6$  L in humans, 77–80  $\mu\text{L g}^{-1}$  in mice), the residual concentration does not appear to pose a concern to humans or mice. Nevertheless, upon a potential advancement into clinical trials these procedures would need to be optimized by ideally further reducing the  $\text{H}_2\text{O}_2$  content and performing a toxicology assessment.

Regarding  $\text{CaCl}_2$  at the final solution, this is added during decarboxylation at 3 mM, concentration that is much lower than used safely in humans and mice for calcium replenishment in arrhythmias associated with hypocalcemia, hyperkale-

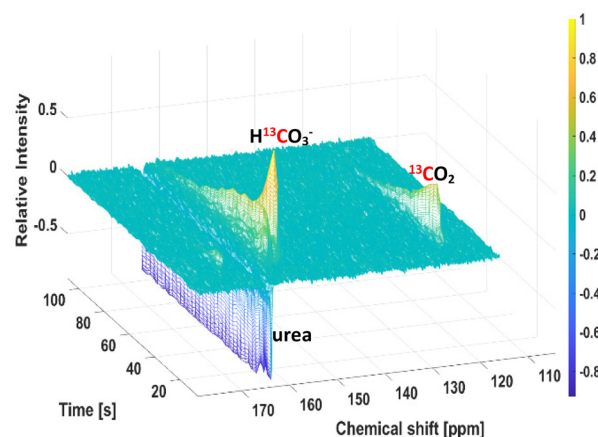


mia, an antidote for magnesium poisoning, as co-adjuvant in hemostatic agents preparations, as well as, to resolve refractory hypotension and complete heart block, *etc.*<sup>77–81</sup> Additionally, Kawai *et al.* (2011) reported the use of  $\text{CaCl}_2$  injected intravenously (*via* tail vein) in mice to show how this compound accelerate wound healing. In these experiments a dose of 0.6 mg per mouse was used without any observed side effects.<sup>82</sup> In our injections, even if all the initial  $\text{CaCl}_2$  were present in the final solution, it will represent a dose of 0.0033 mg per mouse, so side effects are unlikely. Respect to rhodium residuals, we have previously reported that the levels at the final solution after filtration is  $(17 \pm 5) \mu\text{M}$ .<sup>56</sup> This concentration have not generated any side effects *in vivo* or toxicity in cells experiments, even when double injection of the probe was used.<sup>34,56,59,65</sup> Finally, acetate is expected to be present in the final solution. However, it has been previously reported the use of hyperpolarized acetate as a tracer in different diseases and events (*e.g.* diabetes, cardiac and renal metabolism, glioma, *etc.*) on *in vivo* and human studies demonstrating that this compound does not pose challenges.<sup>83–87</sup> In addition to that, acetate-buffers were extensively used as first-line intravenous resuscitation fluids in patients, for example during hemorrhagic shock.<sup>88–90</sup>

### PHIP $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$ validation in phantoms

During *in vivo* experiments,  $[1-^{13}\text{C}]\text{pyruvate-d}_3$  is injected into the mouse's tail vein, thus suffering a dilution because of the mouse blood volume. Considering that the approximate blood volume of a mouse is  $77\text{--}80 \mu\text{L g}^{-1}$ ,<sup>91,92</sup> and that in our previous *in vivo* experiments with pyruvate usually C57BL6 mice with a weight of  $\sim 20 \text{ g}$  (10–12 weeks) are used,<sup>34,65</sup> the final concentration of biomarkers when injected into the vein tail (volume injected  $\sim 50\text{--}100 \mu\text{L}$ ) will decrease about by a factor of ten.

Taking that into account, we wanted to evaluate the accuracy of the pH sensor at a 10-fold lower concentration. Following establishment of the procedure, pH measurements of eight phantoms were performed in a 7 T imaging spectrometer (more details in the ESI†). Each phantom contained HEPES buffers at different pH (6.40, 6.50, 6.61, 6.70, 6.81, 6.90, 7.20, and 7.40) in  $\text{D}_2\text{O}$ . After filtration and decarboxylation, the solution containing  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$  was injected into the different tubes (one by one, and after an interval of 5 min, as explained in Materials and Methods section) containing the mentioned buffers. Upon injection,  $^{13}\text{C}$ -spectra were acquired with low-flip angle excitations ( $20^\circ$ ) to obtain  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$  integrals (Fig. 3 shows one example). Once achieving the steady state (state with constant concentration, according to two-way ANOVA followed by the Bonferroni's test for multiple comparisons), pH was calculated by using the Henderson–Hasselbalch equation:  $\text{pH} = 6.2 + \log([\text{HCO}_3^-] / [\text{CO}_2])$ ,<sup>93</sup> and the ratio of the  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$  integrals. Table 1 shows the means  $\pm$  SD for  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$ -measured pH values and their corresponding electrode-measured values, obtained after three independent experiments. Following spectroscopic measurements, the pH of the final solutions was determined



**Fig. 3** Example for a time series of  $^{13}\text{C}$  NMR spectra recorded with a flip angle of  $20^\circ$  upon injection of signal-enhanced  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$  in a phantom of pH 6.5, after non-enzymatic decarboxylation of hyperpolarized  $[1-^{13}\text{C}]\text{pyruvate-d}_3$ . A urea phantom was used in order to ensure correct position of the phantom tubes during the measurements, and its signal is in antiphase showing non-hyperpolarization.

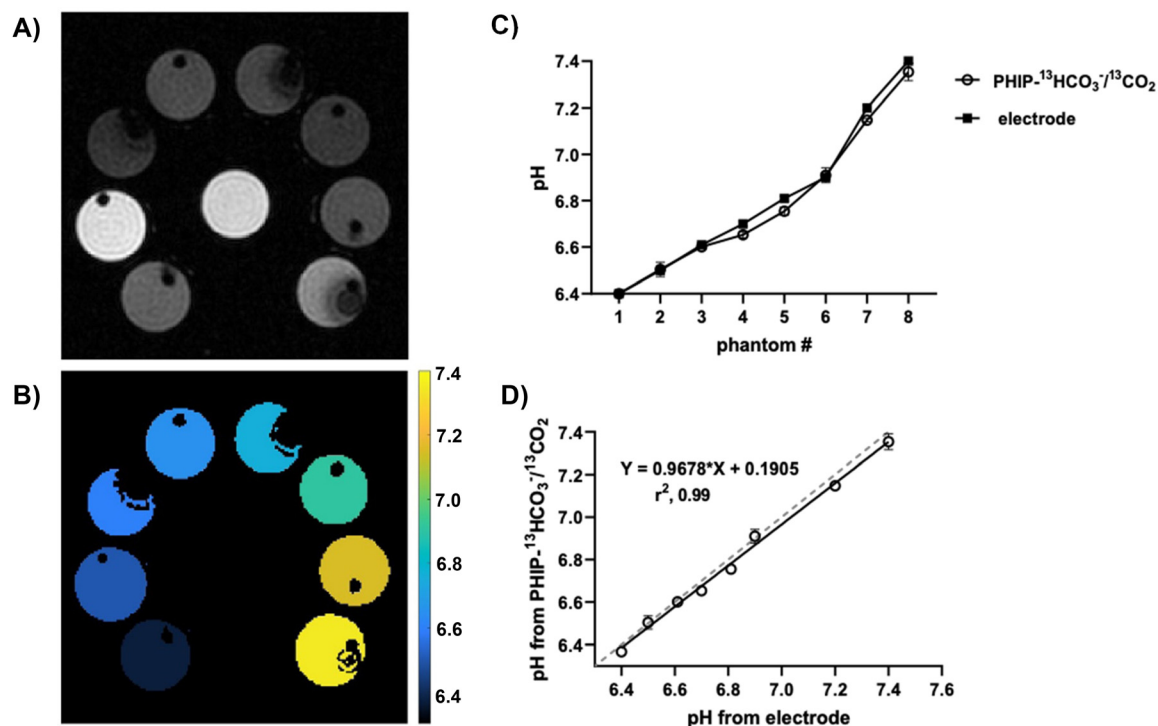
**Table 1** Measured pH by using our probe and spectroscopic data, and pH measured with an electrode

Phantom #	Electrode pH (Mean $\pm$ SD)	PHIP $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$ pH (Mean $\pm$ SD)
1	$6.40 \pm 0.01$	$6.37 \pm 0.01$
2	$6.50 \pm 0.01$	$6.50 \pm 0.05$
3	$6.61 \pm 0.01$	$6.60 \pm 0.007$
4	$6.70 \pm 0.01$	$6.65 \pm 0.02$
5	$6.81 \pm 0.01$	$6.76 \pm 0.03$
6	$6.90 \pm 0.01$	$6.91 \pm 0.06$
7	$7.20 \pm 0.01$	$7.15 \pm 0.01$
8	$7.40 \pm 0.01$	$7.35 \pm 0.07$

with a pH electrode, and the values were in good agreement with those determined from the spectroscopic data (Table 1). This pH sensor methodology demonstrates accuracy with standard deviations lower than 0.07 pH units (expanded details in the ESI†).

Likewise,  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$  pH values did not differ significantly from the electrode-measured ones, according to two-way ANOVA followed by the Bonferroni's test for multiple comparisons. Fig. 4A shows a  $^1\text{H}$  image of our measurement setup (as explained in Materials and Methods). To ensure the correct position of the tubes, a urea- $^{13}\text{C}$  phantom was placed in the center. In (B) a color map represents the pH values obtained with our probe. (C) and (D) shows the correlation between the mean of  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$  pH values obtained from three independent experiments and electrode pH. The close correlation obtained reflects a rapid establishment of chemical equilibrium and equilibration of polarization between the  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$  species. Although previous studies reported a difference of  $\sim 0.3$  pH units where the  $\text{H}^{13}\text{CO}_3^- / ^{13}\text{CO}_2$  pH was higher than the electrode-measured pH in the absence of the





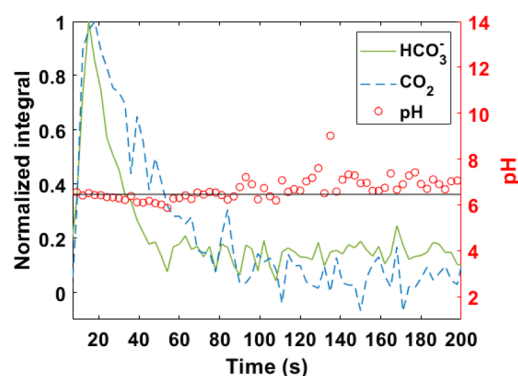
**Fig. 4** (A) Proton imaging of the setup acquired at the end of the measurements by adding H<sub>2</sub>O to all the measured tubes. The tube in the center corresponds to a urea phantom, which was used as a positioning reference. (B) Color graphical representation of the pH values generated in MATLAB software by using the pH values obtained spectroscopically from the ratio of hyperpolarized H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub> integrals, and the <sup>1</sup>H image showed in A. (C) pH vs. phantom number (#) for PHIP H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub>-pH and electrode-pH. No statistically differences were found after analysis by two way ANOVA followed by the Bonferroni's test for multiple comparisons using GraphPad Prism 9 software. (D) Correlation of the PHIP H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub>-pH from spectroscopic data with the values measured using a pH electrode. A solid linear regression line with its corresponding equation is shown for the measured pH with our probe, and a dashed line that represents direct proportionality between the two variables.

enzyme carbonic anhydrase (by using voxel by voxel estimation),<sup>39</sup> we did not observe the same effect in our spectroscopic data. Our hypothesis relies on an improvement of  $T_1$ ,<sup>45,52</sup> attributable to the generation of the signal-enhanced H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub> in D<sub>2</sub>O (instead of H<sub>2</sub>O).

The  $T_1$  values for these species in D<sub>2</sub>O at 7 T and room temperature were (54.3 ± 9.7) s for H<sup>13</sup>CO<sub>3</sub><sup>−</sup>, and (49.0 ± 3.0) s for <sup>13</sup>CO<sub>2</sub>. This might facilitate a rapid exchange between H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub>, and the rate of polarization loss for both species becomes similar (according to  $t$ -student,  $p$ -value from the comparison of the  $T_1$  values is 0.2, not statistically different). That rapid interconversion could contribute to maintain pH values during the steady state as is shown in Fig. 5, increasing the accuracy of the measurements.<sup>2</sup> Another fact that could contribute to the improved correlation between PHIP H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub>-pH and electrode-pH, is based on the use of an alkaline buffer (pH = 10) in our procedure to achieve physiological conditions (pH = 7), this step would be responsible for reducing CO<sub>2</sub> losses prior to injection.<sup>3</sup>

#### PHIP H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub> validation in cell culture media

In order to test our probe in a more biological-complex environment, pH measurements were conducted in conditioned media from pancreatic cancer cells (PanC02 cell line)

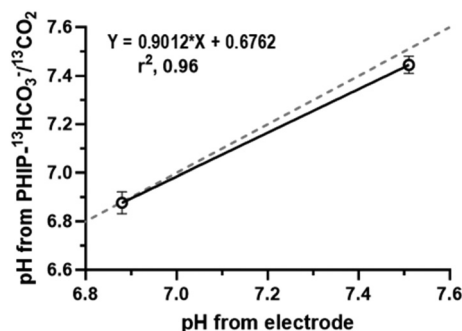


**Fig. 5** Profile of normalized integral (arbitrary unit) of H<sup>13</sup>CO<sub>3</sub><sup>−</sup> and <sup>13</sup>CO<sub>2</sub>, and pH vs. time, after injection of signal-enhanced H<sup>13</sup>CO<sub>3</sub><sup>−</sup>/H<sup>13</sup>CO<sub>2</sub> into a HEPES phantom of pH 6.5. pH values from the steady state were selected to calculate the ones showed in Table 1.

corresponding to 4 days *in vitro* (div). These measurements were compared with fresh media containing all the same supplements than the media used for PanC02 culture. Specifically, the media used was DMEM high glucose, without bicarbonate supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Fig. 6 shows the correlation of the PHIP







**Fig. 6** Correlation PHIP  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ -pH and electrode-pH values obtained from cell culture media. A solid linear regression line and equation is shown for the measured  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ -pH, the dashed line that represents direct proportionality between the two variables.

$\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ -pH from spectroscopic data with the values measured using an electrode.

The mean for pH values measured with our probe are summarized in Table 2 (please see the ESI† for more details).

In this case, accurate pH values were also found in agreement with those measured by using the electrode.

Lastly, we present a probe prepared by using highly polarized pyruvate (36.65%), and a non-enzymatic decarboxylation reaction to achieve the  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  pH sensor, with a polarization value of 12.46%. It is important to note that the polarization losses for the  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  sensor can be attributed mainly to relaxation effects ( $T_1$  54.3 s for  $\text{H}^{13}\text{CO}_3^-$ , and 49.0 s for  $^{13}\text{CO}_2$ ), which could be further improved in the future by optimized reactions in an automatized setup. We have validated our probe *in vitro* by measuring pH on phantoms and pancreatic cancer cell media with high accuracy. Previous  $\text{H}^{13}\text{CO}_3^-/\text{CO}_2$  studies were conducted with dDNP achieving similar levels of polarization: Ghosh *et al.* (2014), reached  $\text{H}^{13}\text{CO}_3^-$  with notable polarization (~16%) by employing  $\alpha$ -keto carboxylic acid-derivatives decarboxylation with  $\text{H}_2\text{O}_2$  and validated the probe on perfused rat lungs.<sup>2</sup> Besides, Mu *et al.* (2023) applied chemical reactions as hydrolysis followed by neutralization on 1,2-glycerol carbonate (GLC) to obtain  $\text{H}^{13}\text{CO}_3^-$  with a polarization of ~20%, and its validation was carried out in several *in vivo* healthy and cancer mouse models.<sup>52</sup> Other probes derived from synthetic compounds and amino acids were developed as HP chemical-shift based pH sensors.<sup>47,48,51,94</sup> As mentioned above, dDNP is facing challenges on the development to routine clinical use. With respect to pH sensors for example, polarization times of 2 h are needed (probe obtained by direct polarization on

$\text{CsH}^{13}\text{CO}_3$ ),<sup>39</sup> 1.5 h (probe obtained by decarboxylation of HP  $\alpha$ -keto carboxylic acid-derivatives),<sup>2</sup> and 7 h (probe obtained by base-catalyzed hydrolysis followed by neutralization on  $^{13}\text{C}$ -GLC),<sup>52</sup> together with all the time needed for reaction and post dilution steps. Therefore, parahydrogen based methods pose a scalable solution, as hyperpolarized agents can be rapidly produced on-site and even in portable devices. Finally, assessment of parahydrogen enhanced contrast agents in large volumes (40 mL as in dDNP) and patient studies are currently not feasible and are part of our ongoing work.

## Conclusions

In conclusion, we present an optimized methodology to obtain biocompatible hyperpolarized  $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$  pH sensor within a minute using PHIP to perform pH measurements at physiological conditions with high accuracy. To achieve the probe, purified  $[1-^{13}\text{C}]$ pyruvate- $\text{d}_3$  with a notable  $^{13}\text{C}$  polarization of  $36.65 \pm 0.06\%$  in aqueous solution and at physiological pH is decarboxylated. For this,  $\text{H}_2\text{O}_2$  with an addition of  $\text{CaCl}_2$  yield signal-enhanced  $\text{H}^{13}\text{CO}_3^-$  and  $^{13}\text{CO}_2$  in  $\text{D}_2\text{O}$  with an average  $^{13}\text{C}$ -polarization of  $12.46 \pm 0.01\%$ . We attribute these remarkable polarization values to optimizations performed in our purification and pH adjustment procedures. We envision that the presented procedures, which offer advantages in polarization efficiency, accuracy, and speed, can be rapidly adopted for preclinical investigations and future clinical parahydrogen procedures for patient imaging.

## Author contributions

Author contributions are the following: M. D. Santi, conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing-original draft; T. L. K. Hune, investigation, formal analysis, and writing-review and editing; G. G. Rodriguez, investigation, formal analysis, and writing-review and editing; L. M. Fries, investigation, formal analysis, and writing-review and editing; R. Mei, investigation, formal analysis, and writing-review and editing; S. Sternkopf, visualization, and writing-review and editing; Josef Elaßer investigation; and S. Glöggler, conceptualization, validation, funding acquisition, resources, supervision, and writing-review and editing. All authors have given approval to the final version of the manuscript.

## Data availability

The data supporting this article have been included as part of the ESI.†

## Conflicts of interest

Stefan Glöggler is co-founder of MagniKeen

**Table 2** Measured pH values in conditioned media from PanC02 cells and fresh media using our probe and spectroscopic data, and pH measured with electrodes

Media	PHIP $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ pH	Electrode pH
PanC02, 4 div	$6.87 \pm 0.08$	$6.88 \pm 0.01$
Fresh media	$7.45 \pm 0.06$	$7.51 \pm 0.01$



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