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Hyperpolarized ^{13}C NMR for longitudinal in-cell metabolism using a mobile 3D cell culture system

T.B.W. Mathiassen [a], A.E. Høgh [a], M. Karlsson [a], S. Katsikis [a], K. Wang [a], M. Pennestri [b], J.H. Ardenkjær-Larsen [a,*], P.R. Jensen [a,*]

[a] Center for Hyperpolarization in Magnetic Resonance, Department of Health Technology, Technical University of Denmark, Kgs. Lyngby, Denmark
[b] Bruker UK Limited, Banner Lane, Coventry, United Kingdom of Great Britain and Northern Ireland, UK

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ABSTRACT

Hyperpolarization with the dissolution dynamic nuclear polarization (dDNP) technique yields > 10,000-fold signal increases for NMR-active nuclei (e.g. ^{13}C). Hyperpolarized ^{13}C-labeled metabolic tracer molecules thus allow real-time observations of biochemical pathways in living cellular systems without interfering background. This methodology lends itself to the direct observation of altered intracellular reaction chemistry imparted for instance by drug treatment, infections, or other diseases. A reoccurring challenge for longitudinal cell studies of mammalian cells with NMR and dDNP-NMR is maintaining cell viability in the NMR spectrometer. 3D cell culture methods are increasing in popularity because they provide a physiologically more relevant environment compared to 2D cell cultures. Based on such strategies a mobile 3D culture system was devised. The clinical drug etoposide was used to treat cancer cells (HeLa) and the resulting altered metabolism was measured using hyperpolarized [1–^{13}C]pyruvate. We show that sustaining the cell cultivation in cell incubators and only transferring the cells to the NMR spectrometer for the few minutes required for the dDNP-NMR measurements is an attractive alternative to cell maintenance in the NMR tube. High cell viability is sustained, and experimental throughput is many doubled.

Introduction

Dissolution dynamic nuclear polarization (dDNP) is an approach to enhance the sensitivity of liquid state NMR with orders of magnitude by transferring the spin polarization from electrons to the nuclei of interest, typically ^{13}C [1]. The increased ^{13}C signal allows real-time kinetic measurements in living cellular systems with a time resolution of less than a second. dDNP-NMR can be translated from cell to human being and the technique is now applied in the clinic as a new imaging modality [2]. Hyperpolarized pyruvate is injected into the patient and increased lactate formation can be detected in cancerous tissue due to the Warburg effect. In the past, cell studies have been used to understand underlying biochemistry and cellular mechanisms of drug treatment to drive the development of optimized imaging strategies [3–7]. The dDNP technique is challenged by the relatively short lifetime of the hyperpolarized signals that depend on the T1 of the hyperpolarized probe. For [1–^{13}C] pyruvate this is typically around 60 s. In this timeframe, the probe must be taken up over the cell membrane and metabolized to the product of interest. Metabolic changes are known to occur before morphological changes and hyperpolarized MRI, therefore has the prospect of detecting early treatment response. Longitudinal cell studies with dDNP-NMR for bio probe development are thus attractive because they provide the same metabolic information obtained as in vivo experiments and allow the same cell batch to be used as its own control in, for example, drug treatment studies. The latter being of increased importance in situations with limitations in biomaterial as, for example, when cells are derived from patients. Longitudinal cell studies are difficult to perform, and the addition of the hyperpolarized probe adds further complexity.

Since the 1980′s NMR-compatible bioreactors have been re-invented dozens of times by several labs but there are not many examples of bioreactor designs taken up by other laboratories [8]. Most commonly adhering cells are grown as a monolayer on a hard plastic surface (2D culture). This approach does not reflect tissue-architecture and is not compatible with the probe of an NMR spectrometer. Culture methods that better reflect the tissue-architecture, mechanical and biochemical cues together with cell-cell communication (3D cultures) have over the

* Corresponding authors.
E-mail addresses: jhar@dtu.dk (J.H. Ardenkjær-Larsen), peroje@dtu.dk (P.R. Jensen).

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last decade gained popularity using a variety of scaffolds [9]. Hydrogel-based cultures are versatile and have the ability to mimic the extra cellular matrix while allowing soluble factors such as cytokine, growth factors and drugs to travel through the tissue-like gel [10]. In a standard bioreactor-NMR setup for mammalian cells, the cells are immobilized on a surface in the NMR tube which is placed in the NMR spectrometer. Fresh growth medium is supplied from a reservoir using a peristaltic pump. Often high cell densities have been used to compensate for the relatively low sensitivity of NMR compared to for example mass spectrometry. As a result, rather high flow rates are often seen in NMR bioreactor systems (typically in the range of 0.1–0.2 ml/min [11–14]). In such a setting sheer stress is an inevitable issue that may lead to reduced cell viability and detachment, as well as membrane rupture in excessive cases [15,16]. This is particularly relevant when working with shear-sensitive cells, such as embryonic stem cells, primary neurons, and hepatocytes [17]. Recently it has been demonstrated that mammalian cells can be kept viable for up to three days in an NMR spectrometer in combination with the Bruker InsightMR system immobilizing the cells in agarose threads with a diameter of 0.75 mm [13] or in a collagen-based matrix with a diameter of ca. 2 mm [14]. Shorter experimental times have also been demonstrated using HeLa cells in Mebiol gel [18]. This approach is attractive because the cells are fixed in one large thread inside the active volume of the NMR spectrometer. Previously dDNP-NMR has been demonstrated to be compatible with many small spheres made of alginate [19]. Here we first demonstrate that threads of hydrogel are compatible with dDNP-NMR. However, we found that occupying the bioreactor with a single sample limited the throughput of the metabolic investigation. Thus, an alternative approach was developed where cells cast in a hydrogel can be moved between a CO_2 incubator and the NMR spectrometer. With this approach, the sample only occupies the NMR spectrometer during the metabolic measurement. In the case of hyperpolarized NMR, the measurement time per sample is 15 min. Using this flexible approach, we demonstrate that a longitudinal cell study over 48 h can be performed. We use a well-described cancer treatment drug to show the expected treatment outcome of reduced lactate production after injection of hyperpolarized [1–^{13}C]pyruvate.

Results and discussion

The immobilization of cells in agarose threads is compatible with dDNP-NMR

Gels from low-temperature gelling agarose gels have been demonstrated as a promising immobilization medium for cells in suspension and minimal surface attachment requirements, such as HEK293T cells when used with a bioreactor [20]. NMR is considered a relatively insensitive analytical technique compared to for example mass spectrometry and thus high cell densities are normally required for In-Cell NMR studies. Typically, more than 20 million (20×10^6) cells are placed in the NMR tube, which creates a high demand for nutrition and oxygen supply from the bioreactor [13,14,20]. The sensitivity of dDNP-NMR is orders of magnitude higher than conventional NMR, allowing us to decrease the cell number to 2 million cells per sample. We have previously demonstrated that a manual injection of 250 μL hyperpolarized probe via a PTFE tube (outer diameter 1/8″) into a cell suspension in 250 μL buffer provides high reproducibility [21]. The same setup was used to measure the conversion of hyperpolarized [1–^{13}C]pyruvate in 2 million HeLa cells which were cast in a hydrogel thread with a diameter of 1.5 mm (Fig. 1A). The kinetic profile of [1–^{13}C]pyruvate metabolism was acquired by a series of low flip angle ^{13}C pulses every 2 s. No significant difference was detected between experiments containing the same number of cells either in suspension or in a hydrogel when comparing the area under the curves. The decay of

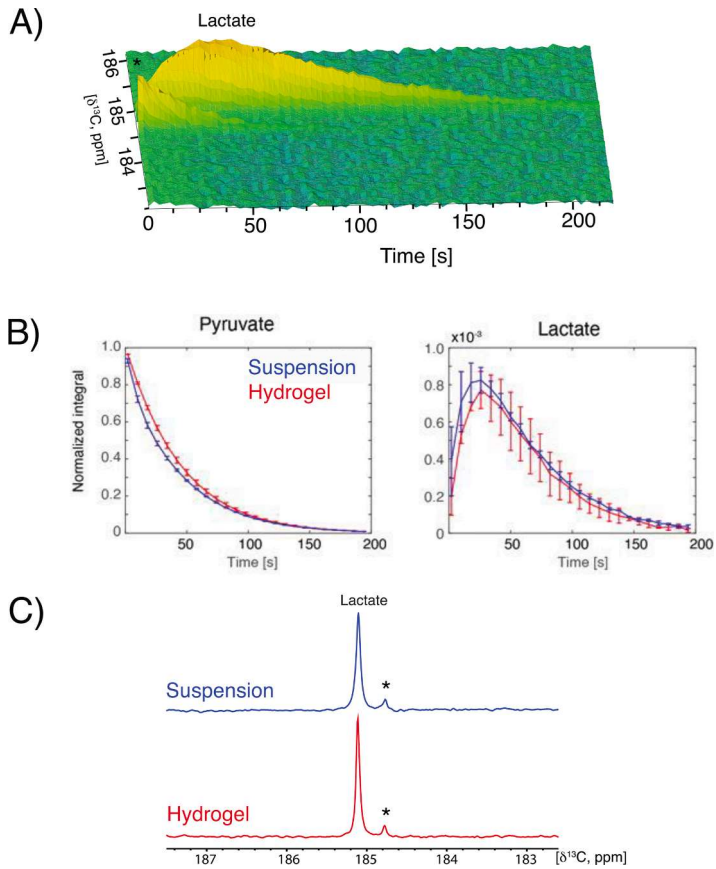

Fig. 1. (A) Lactate signal from kinetic dDNP-NMR experiment performed with hyperpolarized [1–^{13}C]pyruvate injected into 2 million HeLa cells captured in hydrogel. (B) Normalized integrals from kinetic dDNP-NMR experiment performed with hyperpolarized [1–^{13}C]pyruvate injected into 2 million HeLa cells in suspension or in hydrogel ($n = 2$). (C) Single spectrum from the kinetic dDNP-NMR experiment. Spectra show the lactate peak at the maximum intensity. The chemical shift is referenced to pyruvate at 173 ppm. *Impurity in the pyruvate sample preparation.

the hyperpolarized pyruvate signal displayed a tendency to be slightly faster in suspension, but this was within the experimental error (Fig. 1B). A comparison between single spectra after 25 s at the maximal lactate amplitude did also not show any significant difference in the line width of the hyperpolarized lactate signal (Fig. 1C). Immobilization of the cells in a hydrogel thus did not introduce increased relaxation of the hyperpolarized substrate nor any magnetic homogeneity problems. The diffusion of pyruvate into the gel was fast enough to produce similar kinetics for the two setups.

Bruker InsightMR system is compatible with dDNP-NMR

To sustain viable cells in the NMR tube, the Bruker InsightMR system can be used in combination with a small-scale fermentor that continuously adjusts pH and supplies nutrients and oxygen [13,14,20]. This setup was combined with dDNP-NMR by placing the inlet line at the bottom of the NMR tube and using this both for medium circulation and the fast manual injection of the hyperpolarized substrate. The outlet line was placed at the top of the active volume of the NMR tube ensuring correct medium volume during the experiment (Fig. 2A and Fig. S1). Outside the NMR magnet both the inlet and the outlet lines were accessible with standard connections. For the inlet line a manual valve was used, which was either connected with a female luer connection for injection of the hyperpolarized substrate via a 1 mL syringe or a fitting connected to the mini-fermentor used as the reservoir for the circulating growth medium. Two million human cancer cells cast in a hydrogel were placed in the NMR tube and connected to the bioreactor for 24 h. Hyperpolarized [1–^{13}C]pyruvate was injected at 1 h and 24 h after starting the bioreactor (Fig. 2B). To keep the gel in the active volume, a thin filter made of high-density polyethylene (HDPE) was introduced. Similar kinetics were obtained from the two dDNP-NMR experiments (respectively 1 h and 24 h) demonstrating that the full bioreactor setup was compatible with dDNP-NMR (Fig. 2B). The performance of the InsightMR system was subsequently compared to the simple setup with

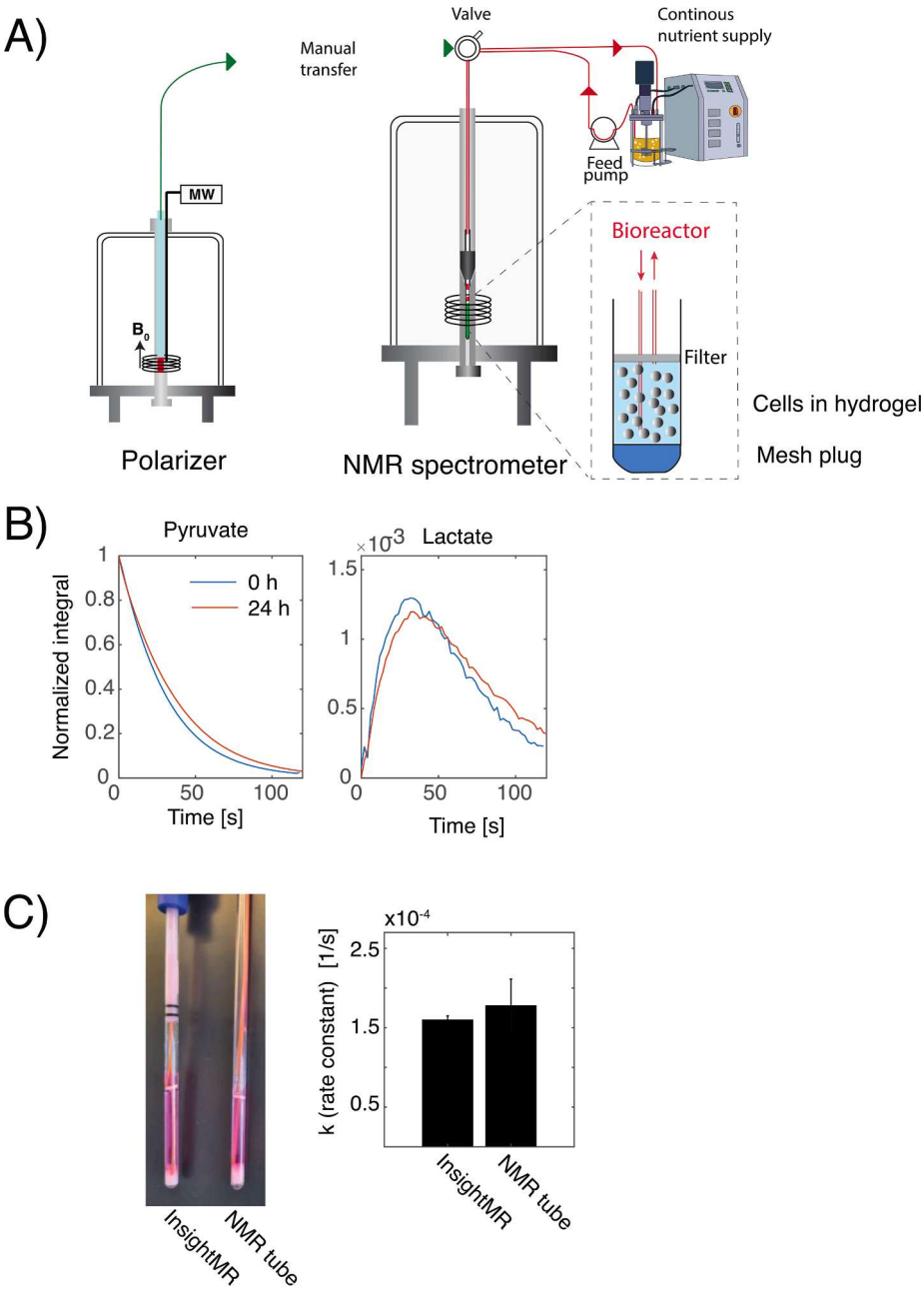

Fig. 2. (A) Bioreactor setup which can be used together either with a Bruker InsightMR system or a standard 5 mm NMR tube. A sterile piece of mesh was placed in the bottom of the NMR tube to restrict the cell containing hydrogel to the active volume of the coil. A HDPE filter was placed as an upper barrier. For detailed images see Fig. S1. (B) Kinetic dDNP-NMR experiment performed with hyperpolarized [1–^{13}C]pyruvate injected into 2 million HeLa cells in hydrogel using the bioreactor setup. The first injection was performed 1 h after starting the bioreactor and the second injection after 24 h ($n = 1$). C) Hydrogels placed in either in InsightMR or a standard 5 mm NMR tube. Fitted rate constants for pyruvate to lactate conversion for the two different setups ($n = 3$ and $n = 5$, respectively). No statistical difference was found between the two groups.

hydrogels in a 5 mm NMR tube. No statistical difference was found for the rate constant between the two injection systems (Fig. 2C). The two injection systems were set-up to have similar configurations, using the same type and length of injection line together with a HDPE filter used as an upper barrier keeping the hydrogel in the active volume during injection of the hyperpolarized substrate. The Bruker Insight system is designed to withstand high pressure with connections for tight fitting of the lines to support security against leakage if longer period of medium circulation is desired. The normal 5 mm NMR tube offers a simpler solution, which can be used for experiments without long periods of medium circulation.

Multiple injections of hyperpolarized [1–^{13}C]pyruvate in the same gel is feasible

Using the bioreactor setup poses two major limitations for drug treatment studies. Throughput is limited because only one sample can be investigated at a time and there may be a significant financial cost associated with the drug circulating throughout the entire reservoir of the bioreactor. Even in our setup, which consisted of a commercial mini-fermentor with a reservoir of 100 mL, this can pose a significant financial burden. To circumvent these limitations, we changed strategy. Instead of thin hydrogel threads ($d = 1.5$ mm) we changed to thicker threads ($d = 2.5$ mm) which easily could slide in and out of the NMR tube. Using this new setup, the gels can be maintained at optimal nutritional and oxygen conditions in a CO_2 incubator for most of the experimental time. They are only moved to the NMR spectrometer during the data acquisition period which for dDNP-NMR is a few minutes. Numerous gels can be cast simultaneously which further increases the throughput of the method. For a treatment scheme as depicted in Fig. 3A, 6 samples could be analyzed in 53 h compared to 1 sample in 48 h using the conventional bioreactor setup. The cell number and gel volume remained the same as for the previously tested gel threads. The diameter was increased to 2.5 mm and the length of the gel was two times the length of the active volume of the NMR tube. Each gel was divided in the middle and each NMR experiment was thus comprised of two pieces of hydrogel (Fig. 3B). The cell viability was characterized by confocal microscopy, following fluorescent live/dead staining with calcein-AM and propidium iodide (PI) respectively. As seen in Fig. 4A–C, cross-section images of the hydrogels indicate homogeneous viability throughout the whole gel after 48 h, and high viability was maintained. Multiple sections were examined throughout the full length of the gel to ensure cell homogeneity (Fig. S2). With this setup, multiple injections of hyperpolarized pyruvate were tested. The first injection was made 24 h after casting the cells in the hydrogel. After this injection of hyperpolarized pyruvate, the gels were transferred from the NMR tube back into the CO_2 incubator. After 48 h the gels were reintroduced into the NMR tube and a second pyruvate injection was performed. No significant difference was found for the pyruvate rate constants between 24 h and 48 h under these conditions (Fig. 4D). For insertion of the gels into the NMR tube, we found that the NMR tube must be filled with growth medium. This allowed the gel to slide smoothly and not stick to the sides. To keep the gels in the active volume of the NMR tube, a HDPE filter was fixed to the inlet line by two short pieces of silicone tubing (Fig. S1A). Once the gel was in place excess media could easily be removed by the inlet and outlet lines. To remove the gel from the NMR tube a gentle shake was applied.

Longitudinal drug treatment study showed decreased flux from pyruvate to lactate

A longitudinal drug treatment study was performed on HeLa cells using the clinical drug etoposide. Etoposide is widely used for inhibiting topoisomerase II which mediates DNA replication. Etoposide has been demonstrated to induce programmed cell death, aka apoptosis, in HeLa cells via caspase-1 and caspase-3 pathways [22]. The metabolic effect of

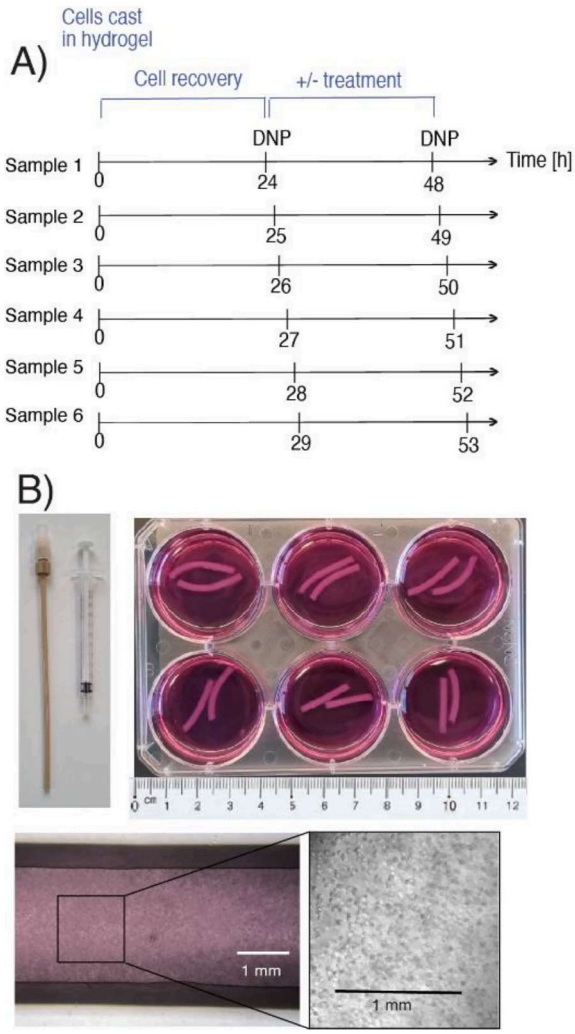

Fig. 3. (A) Example of longitudinal study outline consisting of two dDNP-NMR measurements with 24 h in between. Twelve injections can be performed in 53 h increasing the throughput compared to using the full bioreactor setup. (B) Corresponding experimental setup. Gels are cast by injection of 230 ± 30 μL of a cell/hydrogel mix through a PEEK tube into growth media in a 6-well plate. Bright-field microscopy image of a gel showing smooth surface and homogeneous cell distribution.

etoposide has been shown in several articles using [1–^{13}C]pyruvate as hyperpolarized substrate, where a decreased flux between pyruvate and lactate has been detected as a response to chemotherapy [7,23–25]. In a typical 2D cell culture protocol cells are allowed to rest for 24 h after trypsinization prior to drug treatment. We adapted this procedure. HeLa cells were cast in hydrogels, and half of the gels were treated with etoposide while the other half followed the same time schedule but received fresh medium without etoposide, thereby serving as controls. Twenty-four hours after casting, the gels were transferred one by one to an NMR tube and metabolism was measured by injection of hyperpolarized [1–^{13}C]pyruvate. The gels were returned to the CO_2 incubator where they were maintained in media with/without etoposide for 24 h whereafter a second injection of hyperpolarized pyruvate was performed (Fig. 3A). A forward rate constant was fitted to the obtained metabolic curves. In the untreated group no significant difference in pyruvate metabolism was found between the group measured at 24 h and 48 h (Fig. 5A). This result indicates that the HeLa cells have neither proliferated nor detached by the procedure. A recovery period of 24–48 h after trypsinization is normally seen for mammalian cells when they are maintained in 2D cell culture. Thus, cell numbers were not expected

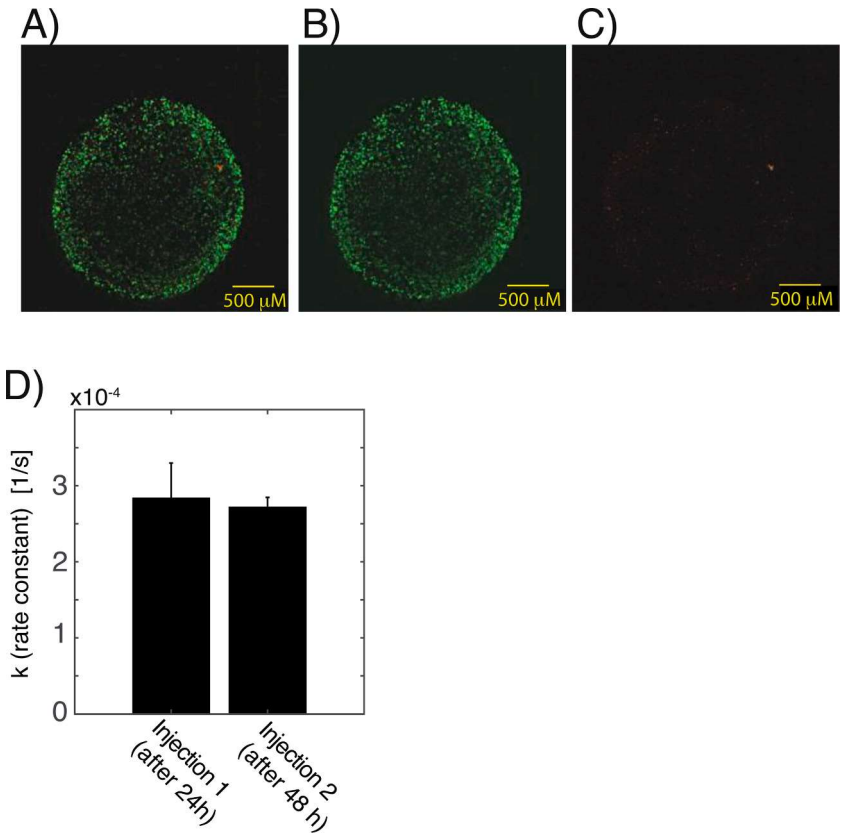

Fig. 4. Representative 4X confocal microscopy image of a cross-section of the agarose encapsulated gel; Calcein AM/PI staining of live HeLA cells (green) and dead cells (red). In order: (A) Merged image, (B) Live, (C) Dead. Scale bar 500 μm. (D) Fitted rate constants for pyruvate to lactate conversion in HeLa cells. First injection of hyperpolarized pyruvate was performed 24 h after casting the cells in the hydrogel. Second injection was performed after 48 h with maintenance of the gel in a CO_2 incubator. No statistical difference was found between the two groups ($n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to increase significantly during the experiment. In addition, the simple hydrogel used in this first demonstration of compatibility between hydrogels and dDNP-NMR is not an optimal gel with respect to cell proliferation. Hydrogels designed to promote cell proliferation are typically optimized by incorporating proteins containing an integrin binding sequence, such as the RGD motif [26]. In addition to structural proteins like collagen, adhesion ligands have been recognized for their significant impact on cell proliferation, differentiation, and adherence [27]. Hence, the absence of such ligands in our hydrogel formulation may suggest a lack of cellular adhesion and result in a metabolic behavior akin to suspended cells. This corresponds with the comparative kinetic rates between our gel and suspension, as seen in Fig. 1B. A conserved metabolism was thus the expected outcome from the control group. In the treated group a significant decrease was observed between the measurement at 24 h (before treatment) and 48 h (after treatment) (Fig. 5B). This decrease correlated with the previously observed effect of etoposide [7]. An even higher effect on pyruvate metabolism could be obtained if treatment was prolonged by an additional 24 h (to a total of 60 h) (Fig. S4). The effect of etoposide treatment on cell viability was determined by live-dead staining using calcein and propidium iodide respectively (Fig. 5C). Only a moderate decrease in cell viability was observed indicating that cell metabolism is more sensitive to drug treatment than the detection of cell death.

Conclusions

Longitudinal studies of cellular metabolism over several days are attractive because each sample serves as its own control. NMR lends itself as an obvious choice for such studies due to its non-invasive nature, but the method is limited to one sample per spectrometer. In the context of microfluidics systems, commonly used in organ-on-a-chip technology, both the cell anchoring material and flow have been extensively studied. Inducing shear stress by increasing the flow rate is often employed as a means of simulating the natural cellular environment and influencing cell differentiation and growth [17]. Inadequate control of sheer stress on the other hand can result in decreased cell growth, viability, and metabolism and thereby impacting the results of metabolic analysis. We found that maintaining 2 million HeLa cells cast in an agarose thread was feasible for 24 h using a full bioreactor setup (Fig. 2A) but this method was hampered by low throughput. We thus used a mobile 3D culturing system that only occupied the NMR spectrometer when metabolic measurements were performed. In the current setup, a simple low-melting agarose gel was used. After this first successful demonstration of compatibility between hydrogels and the dDNP-NMR method, larger efforts must be put into more advanced gel material to support even longer experimental timeframes. The approach demonstrated here is not restricted to dDNP-NMR but can be combined with conventional NMR measurements of short duration that do not deplete nutrition and oxygen during the experiment, such as typical 1D ^{1}H NMR used for metabolomics. In-Cell NMR is currently a relatively small research field with unfulfilled potential and increasing impact. Technical solutions to proper cell maintenance will hopefully provide the robustness needed to consolidate this non-invasive analytical approach for metabolic profiling.

Materials and methods

HeLa cell culture

The human cervical cancer cell line HeLa (American Type Culture Collection, ATCC CCL-2, Manassas, VA) was routinely maintained in Dulbecco's Modified Eagle Medium (DMEM; Biowest L0103) with 4.5 g/L D-glucose, stable L-glutamine, sodium pyruvate) with the addition of 10% fetal bovine serum (Biowest S1810), and 1% penicillin/streptomycin (Biowest L0022) at 37 °C in an 5% CO_2 atmosphere. For maintenance, cell medium was replaced 2 to 3 times per week and HeLa cells were grown to 80% confluence and harvested using 0.25% Trypsin-EDTA solution (Biowest X0930). For dDNP-NMR experiments, HeLa

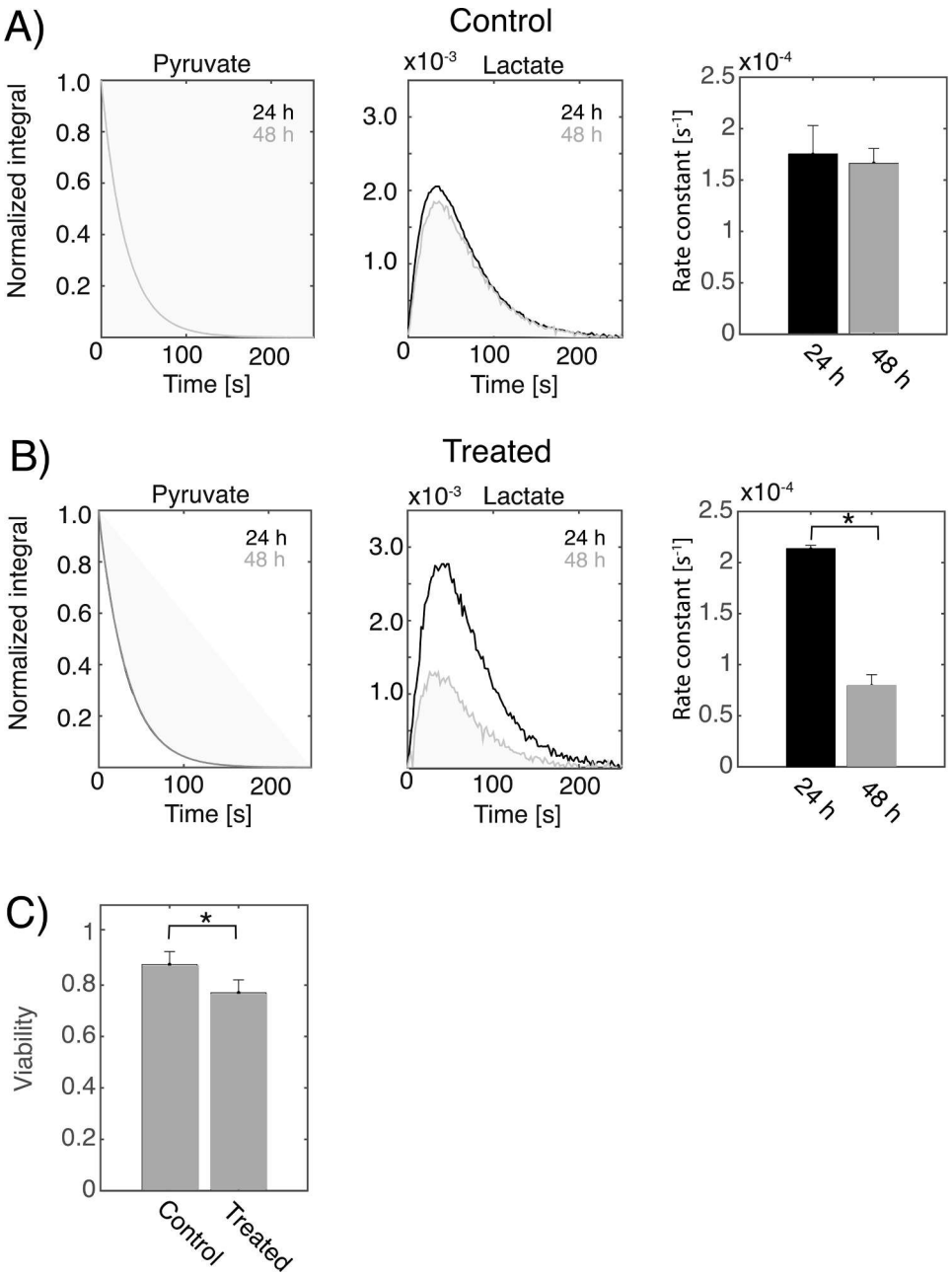

Fig. 5. (A) Example of normalized kinetic dDNP-NMR data using [1–^{13}C]pyruvate from the untreated control group, 24 and 48 h after casting in hydrogel together with fitted rate constants for the same groups ($n = 4$). (B) Example of normalized kinetic dDNP-NMR data from etoposide treated group, 24 and 48 h after casting in hydrogel together with fitted rate constants ($n = 4$). (C) Viability of etoposide treated Hela cells and non-treated Hela cells given as live cells compared to total cell count. The figure shows the mean ± standard deviation. For image analysis, ten images were analyzed from $n = 3$ biological replicates, with corresponding $n = 2$ technical replicates. $^*p \leq 0.05$.

cells were seeded at 1 million cells per T175 flask 4–5 days before the experiment day. The cell culture was harvested by trypsinization, and the detached HeLa cells were centrifuged and washed in DMEM with FBS. Hereafter the cells were washed in PBS with Ca^{2+}/Mg^{2+} (HyClone, SH30264.01) and finally the cells were resuspended to a cell stock with 60–100 × 10^6 cells/mL using PBS with Ca^{2+}/Mg^{2+}.

Production of agarose gels

Low-gelling temperature agarose (Sigma–Aldrich A4018) was dissolved to 2% w/v in PBS (with Ca^{2+}/Mg^{2}). The low-gelling temperature agarose gel was prepared in a 15 mL test tube by dissolving e.g. 37 mg agarose powder in 1.85 mL PBS. The agarose powder dissolved by heating the test tube in boiling water. When the powder had dissolved the 2% w/v mix were aliquoted into Eppendorf tubes with 200 μl in each tube. The temperature was then stabilized by incubating the agarose mix at 37 °C for more than 30 min before use.

200 μL of the warm low-gelling temperature agarose was mixed with 30 ± 10 μL cell stock solution (60–100 × 10^6 cells/mL) to obtain 2 × 10^6 HeLa cells in each gel. The variation in cell number was determined in three biological replicates over three days (as described under Confocal Fluorescence Microscopy). To confirm the cell homogeneity between gels two midsection slices were cut from the gels and ten images were taken from randomized spread-out locations on each gel resulting in an average of 117 ± 10 cells/image. The threads were formed using a 1 mL syringe and a PEEK tube with inner diameter of 2.5 mm. The agarose/cell mix was allowed to solidify for 4 min at room temperature inside the PEEK tube. Then it was released by the syringe plunger into a 6-well plate with 3 mL growth medium in each well. Each tread was divided in two equal sized pieces.

The hydrogel threads were kept in a 5% CO_2 humid incubator at 37 °C. For dDNP analysis a set of two threads was gently transferred into an NMR tube which was mounted with two PEEK tubes (1/32″ ID 0.5 mm) as inlet and outlet lines. On the inlet line, a thin HDPE filter was

placed just above the active volume and the outlet line was placed above the filter. Excess medium was drained via the outlet line before the NMR tube with the hydrogel threads was placed in the NMR spectrometer.

Etoposide treatment of HeLa cells

Etoposide, CAS no. 33,419-42-0 (341,205-25 MG from Calbiochem) was dissolved in DMSO to a concentration of 25 mg/mL. Medium was prepared either with 450 μM etoposide or vehicle (DMSO) in 6-well plates with 3 mL medium per well. Cells were incubated for 24 h in humidified conditions at 37 °C and 5% CO_2.

Cell maintenance with mini-fermentor

A mini-fermentor (Vario 500 from MDX Biotechnik, Germany) was used as medium reservoir. The inlet and outlet PEEK tubes (1/32″, ID 0.5 mm) were connected to a piece of silicone tubing and peristaltic pumps (Shenchen LabV1) ensured low inlet (10 μL/min) and outlet flow (50 μL/min) of medium (DMEM with additional 40 mM HEPES buffer (Gibco 15,630-056)). Aeration was obtained with sterile filtered atmospheric air and stirring. pH and temperature were kept at respectively 7.4 and 37 °C. A set of hydrogel threads (as described above) was placed in the NMR tube and connected to the mini-fermentor with the inlet and outlet lines.

Viability test with Calcein and Pi staining

Fluorescent staining. Gels were washed with Dulbecco's Phosphate Buffered Saline (Sigma–Aldrich D8537) and sliced into 1 mm thick flat discs. Discs were placed in a CELLview™ - Cell culture dish (GR-627,870) and stained with a solution of 2 μg/mL Calcein AM (Invitrogen™ C1430) and 0.5 μg/mL Propidium iodide (Invitrogen™ eBioscience™ Cat: BMS500PI) and left foil-covered at 37 °C for 25 min before transport to the confocal microscope.

Confocal fluorescence microscopy. Images were acquired with a Nikon CSU-W1 confocal inverted microscope with a spinning disk confocal unit (CSU-W1, Yokogawa), a Prime 95B sCMOS camera (Photometrics), and a 4x and 20x objective (CFI Plan Apochromat Lambda NA 0.75 WD 1.0 mm). Representative images were taken with Brightfield. The excitation/emission was 488/550 nm for Calcein-AM and 561/620 for Propidium Iodide. Full gel pictures were taken with the 4X objective to evaluate the homogony of the cell population, as seen in Fig. 4A–C. Using the 10X objective, each slice was photographed at no fewer than ten different locations, chosen at random to ensure a diverse representation of the area. With $N = 3$ biological replicates and $N = 2$ technical replicates, it resulted in approx. 60 images for each experimental condition. A representative image can be seen in Figure S3.

Image analysis. Cells were segmented and counted with the aid of Cell Profiler 4.2.1 (image analysis software) and Image J 2.0 (Fiji).

dDNP-NMR experiments

A substrate sample stock solution was prepared from [1–^{13}C]pyruvic acid (Sigma-Aldrich) doped with trityl radical AH111501 (GE Healthcare) to 17 mM and Gadoteridol gadolinium chelate solution (Bracco Imaging) to 1.5 mM.

Each sample (3.1 mg, 35 μmol) was hyperpolarized to equilibrium polarization in a Hypersense 3.3 T polarizer (Oxford Instruments). After complete hyperpolarization buildup (approximately 1 h) the sample was dissolved in 5 ml phosphate buffer (pH 7.4, 40 mM) with added 3 μl (approximately one acid equivalent) of a 10 M sodium hydroxide solution to ensure low pH variation between samples. The dissolution parameters were set to produce a temperature of the solution which, after transport and injection into the cell suspension was approximately 310 K. The concentration of pyruvate in the solution, after dissolution, was 7 mM, and the liquid state polarization was approximately 25%. Just before the DNP experiment the remaining growth medium was drained out of the NMR tube through the inlet line which was subsequently used for the injection of hyperpolarized pyruvate. The dissolved hyperpolarized pyruvate was collected in a 50 mL receive container at the polarizer and 400 μL was drawn into a 1 mL syringe. This was manually transferred 3 m and at the top of the magnet the hyperpolarized solution was injected into the NMR tube via the inlet line using a female luer connection.

NMR data analysis

The NMR data were recorded on a Bruker 500 MHz AVANCE NEO spectrometer fitted with a 5 mm DCH cryoprobe. After injecting the hyperpolarized substrate solution into the cell suspension, a time series of ^{13}C NMR spectra was recorded using a pulse angle of 10° and a 2 s total delay between pulses. Analysis of the NMR data was performed with the MestReNova software (Mestrelab Research). After phase and baseline correction the substrate ([1–^{13}C]pyruvate) and product ([1–^{13}C]lactate) signals in the time series were integrated. A model written in Python programming language was fitted to the integrals using a numerical solution of the ODE's describing the forward reaction. In the model, the pulse length and power were kept to a fixed value but the T1 values were allowed to vary.

1) $dS/dt = -k*S(t) - 1/T1_s*S(t) - (1-\cos(pw)^n) * S(t)$
2) $dP/dt = k*S(t) - 1/T1_p*P(t) - (1-\cos(pw)^n) * P(t)$

The reproducibility of the experiments was assessed by calculating the standard deviation of the rate constants (k) obtained from fitting the ODEs for the reaction pyruvate to lactate. For the individual fits, the rate constants were well determined with a mean relative standard error (stderr/k) of 2.4 ± 0.8%.

Statistical analysis

The statistical analysis was calculated using the *Student's t*-test method. The *p*-value ≤ 0.05 was considered a significant difference.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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