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TITLE:

Monitoring Protein-Ligand Interactions in Human Cells by Real-Time Quantitative In-Cell NMR Using a High Cell Density Bioreactor

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in-cell NMR, real time, bioreactor, multivariate curve resolution, ligand binding, carbonic anhydrase II, acetazolamide, methazolamide, protein oxidation, superoxide dismutase 1, ebselen

SUMMARY:

This protocol describes the setup of an NMR bioreactor to keep encapsulated human cells viable for up to 72 h, followed by time-resolved in-cell NMR data acquisition and analysis. The methodology is applied to monitor intracellular protein-ligand interactions in real time.

ABSTRACT:

In-cell NMR is a unique approach to observe the structural and dynamic properties of biological macromolecules at atomic resolution directly in living cells. Protein folding, chemical modifications, and conformational changes induced by ligand binding can be observed. Therefore, this method has great potential in the context of drug development. However, the short lifetime of human cells confined in the NMR spectrometer limits the application range of in-cell NMR. To overcome this issue, NMR bioreactors are employed that can greatly improve the cell sample stability over time and, importantly, enable the real-time recording of in-cell NMR spectra. In this way, the evolution of processes such as ligand penetration and binding to the intracellular protein target can be monitored in real time. Bioreactors are often limited by low cell viability at high cell numbers, which results in a trade-off between the overall sensitivity of the experiment and cell viability. We recently reported an NMR bioreactor that maintains a high number of human cells metabolically active for extended periods of time, up to 72 h. This setup was applied to monitor protein-ligand interactions and protein chemical modification. We also introduced a workflow for quantitative analysis of the real-time NMR data, based on multivariate curve resolution. The method provides concentration profiles of the chemical species present in the cells as a function of time, which can be further analyzed to obtain relevant kinetic parameters. Here we provide a detailed description of the NMR bioreactor setup and its application to monitoring protein-ligand interactions in human cells.

INTRODUCTION:

In-cell Nuclear Magnetic Resonance (NMR) spectroscopy has recently emerged as a powerful approach to investigate structural and dynamical properties of macromolecules within the cellular environment[1–6]. In-cell NMR succeeded in the investigation of functionally relevant processes such as protein folding/misfolding[7–9], metal binding[7,10], disulfide bond formation[11,12], and protein–protein interaction[13], protein-ligand interaction[14–16], and nucleic acid-ligand interaction[17,18] in living human cells. One of the limiting factors of in-cell NMR applications is the short lifetime of the cells during the experiment. The solution to this problem involves the use of NMR bioreactors. In these devices, a constant flow of growth medium is applied to the cells, which are kept confined within the NMR spectrometer, in order to provide oxygen and nutrients and to remove toxic byproducts. Following the advent of in-cell NMR, several NMR bioreactors designs have been developed to improve cell viability for longer periods of time, in which either bacteria or mammalian cells are encapsulated in a hydrogel[19–22] or kept in suspension and perfused through the use of a microdialysis membrane[23]. Such bioreactors have allowed the acquisition of longer NMR experiments with increased signal-to-noise ratio (S/N)[5] and, even more importantly, could be employed to investigate cellular processes in real time[22–24]. Thanks to the high chemical and conformational sensitivity of NMR, the latter application can provide precious insights on the kinetics of functional processes within living cells at atomic resolution.

In this protocol, we show how to set up and operate an improved bioreactor recently reported[25], which was obtained by combining an existing modular bioreactor design[23] with the approach relying on cell encapsulation in hydrogel that were pioneered by other groups[19–22,26,27]. We describe the application of the bioreactor to real-time in-cell NMR studies of intracellular protein-observe ligand binding in HEK293T cells. In the bioreactor, cells are encapsulated at high density in agarose gel threads and are maintained highly viable and metabolically active for up to 72 h, during which real-time in-cell NMR experiments are recorded. The bioreactor is composed of a glass tube that fits standard 5 mm NMR probes that is watertight and connected to a tube holder so that the internal sample chamber has 4.2 mm internal diameter, 38 mm height, and a volume of 526 µL. The inlet is a 7-meter-long PEEK capillary (o.d. = 1/32”, i.d. = 0.5 mm) inserted in the sample chamber down to ~6 mm from the bottom, while the outlet is a 7-meter-long PTFE capillary (o.d. = 1/32”, i.d. = 0.5 mm) attached at the top of the tube holder (**Figure 1**). The tubing is coaxially inserted in a temperature-controlled line connected to a water bath. The inlet and outlet are connected through PEEK tubing to a 4-way, 2-position valve attached to an FPLC pump for controlling the medium flow and a waste container.

The bioreactor is applied to study the kinetics of the interaction, previously reported[14,25], between two drugs, acetazolamide (AAZ) and methazolamide (MZA), in human cells with the second isoform of human carbonic anhydrase (CA II), a pharmacologically relevant target[28–30], and the kinetics of the formation of the intramolecular disulfide bond, promoted by the small molecule ebselen[25,31], of the copper-free, zinc-bound form of human copper, zinc superoxide dismutase (SOD1), an antioxidant enzyme implicated in the onset of amyotrophic lateral sclerosis[7,8,32]. Finally, quantitative analysis of the real-time NMR data is performed in MATLAB using the Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) algorithm[33], through which the pure spectral components and the concentration profiles as a function of time are obtained for the species observed, that can be further analyzed to obtain relevant kinetic parameters.

The protocol starts from a T75 flask of HEK293T cells (~3 x 10^7 cells per flask) transiently overexpressing either human CA II (unlabeled) or human SOD1 (^{15}N-labeled). The cells were grown and maintained in T75 flasks with DMEM high glucose by 1:10 passages every 3–4 days and transfected with the cDNA encoding the protein of interest 48 h prior to the experiment. The steps involved in this phase are reported in detail elsewhere[34].

PROTOCOL:

1. Reagent and solution setup

1.1. To prepare complete DMEM, add 5 mL L-glutamine 200 mM, 5 mL penicillin–streptomycin 100x, and 50 mL fetal bovine serum (FBS, 10% vol/vol final concentration) to 440 mL DMEM.

NOTE: This solution can be stored at 4 °C for 1 month.

1.2. Prepare agarose solution by dissolving 150 mg of low-gelling agarose in 10 mL of phosphate buffered saline (PBS) at 85 °C to obtain a 1.5% (w/v) solution. Sterilize by filtration with a 0.22 µm filter. Prepare 1 mL aliquots of agarose solution in 1.5 mL capped tubes and store at 4 °C.

1.3. Prepare the bioreactor medium.

1.3.1. Dissolve 13.4 g of DMEM powder in 1 L of ultrapure H_2O.

NOTE: Depending on the application, the required final volume may differ (e.g., for 500 mL medium, dissolve 6.7 g of powder in 500 mL H_2O).

1.3.2. Add 2% FBS, 10 mM $NaHCO_3$, 1x penicillin-streptomycin (100x), and 2% D_2O (e.g., for 500 mL medium, add 10 mL of FBS, 0.4 g of $NaHCO_3$, 5 mL of penicillin-streptomycin 100x, and 10 mL of D_2O).

1.3.3. Measure the pH using a pH-meter and if needed adjust to 7.4 by adding HCl.

NOTE: Typically, the initial pH is very close to 7.4.

1.3.4. Filter the bioreactor medium with a vacuum-driven sterile filter in a sterile 250 mL or 500 mL glass bottle.

1.3.5. In the laminar flow hood, seal the bottle with a sterile steel headpiece with two hose nozzles and connect them to a FEP tubing (o.d. = 1/8”, i.d. = 1.6 mm) that will be connected to the pump and to a 0.22 µm PTFE syringe filter for air intake.

2. Bioreactor setup

2.1. Assemble the flow unit using a second flow unit NMR tube, which will be later replaced with the one containing the cells. Refer to the flow unit operating instructions for the correct assembly.

NOTE: At this point the flow unit should be already cleaned (if not, perform step 4.2).

2.2. Set the water bath connected to the flow unit temperature control to 37 °C. Place the reservoir bottle in the water bath.

2.3. Connect the FEP tubing of the reservoir bottle to the pump.

2.4. Turn the bioreactor valve to “bypass” and prefill the pump with medium.

2.5. Turn the bioreactor valve to “flow” and prefill the bioreactor with medium at 0.1 mL/min.

3. Preparation of the cell sample

3.1. Collect the cells from the CO_2 incubator.

3.1.1. Take a T75 flask of transfected HEK293T cells from the CO_2 incubator and remove the spent medium.

3.1.2. Wash the cells twice with 7 mL (each) of PBS at room temperature (~20 °C).

3.1.3. Use 2 mL of trypsin/EDTA to detach cells. After adding the solution, incubate for 5 min at room temperature to detach the cells.

NOTE: Transfected cells may take slightly longer to get detached. If necessary, incubate the cells at 37 °C.

3.1.4. Inactivate trypsin with 20 mL of complete DMEM; thoroughly resuspend the cells by pipetting up and down and transfer them in a 50 mL centrifuge tube.

3.1.5. Centrifuge the cells at 800 x *g* for 5 min at room temperature and discard the supernatant.

3.1.6. Wash the cells with 10 mL of PBS at room temperature to remove the residual medium.

3.1.7. Centrifuge the cells at 800 x *g* for 5 min at room temperature and discard the supernatant.

3.1.8. Transfer the cell pellet to a 1.5 mL capped microcentrifuge tube.

3.2. Embed cells in agarose threads.

3.2.1. Melt one aliquot of solidified agarose at 85 °C in a water bath and subsequently keep it in solution at 37 °C in a block heater.

3.2.2. With a Pasteur pipette, fill the bottom of the flow unit NMR tube with 60–70 µL of 1.5% agarose gel and place it in ice. This will create a ~5 mm high bottom plug that allows placing the cell sample within the active volume of the ^{1}H NMR coil.

3.2.3. Heat up the pellet of cells obtained in step 3.1.8 at 37 °C for 15–20 s in the thermoblock.

3.2.4. Resuspend cells in 450 µL of agarose solution. Be careful to avoid the formation of bubbles.

3.2.5. Aspirate the cell–agarose suspension into a ~30 cm long chromatography PEEK tubing (i.d. = 0.75 mm) connected to a 1 mL syringe.

NOTE: Before aspiration, the tubing and the dead volume of the syringe should be prefilled with PBS at room temperature to avoid the formation of bubbles. The length of the tubing is not critical.

3.2.6. Let the tubing cool down at room temperature for 2 min.

3.2.7. Prefill the flow unit NMR tube with 100 µL of PBS at room temperature.

3.2.8. Cast threads of cells embedded in agarose into the flow unit NMR tube by gently pushing the syringe.

NOTE: To fill the NMR tube homogenously, start by placing the end of the PEEK tubing at the bottom of the NMR tube and proceed towards the top while slowly swinging left-right.

3.2.9. Repeat steps 3.2.5, 3.2.6, and 3.2.8 until all the cell-agarose suspension has been cast.

3.3. Insert cells in the bioreactor.

3.3.1. Remove the empty NMR tube from the flow unit and increase the flow rate to 2 mL/min for a few minutes to remove residual gas bubbles in the inlet tubing.

3.3.2. Set the flow rate to 0.2 mL/min and insert the NMR tube containing the cells by pushing it upwards slowly but steadily.

NOTE: The active flow of medium avoids the backflow of tube content through the inlet, that would otherwise occur during the insertion.

4. Bioreactor operation and cleaning

4.1. Bioreactor operation during the NMR experiment.

4.1.1. Set the temperature in the NMR spectrometer to 310 K.

4.1.2. Insert the flow unit in the spectrometer.

4.1.3. Supply the bioreactor medium at a flow rate of 0.1 mL/min for the whole duration of the in-cell NMR experiments.

4.1.4. At the desired time during the experiment, inject a concentrated solution of external molecule to the medium reservoir bottle by piercing the silicone tubing with a sterile long-needle syringe.

NOTE: The final concentration of molecule in the medium should be chosen based on previous knowledge of cell toxicity and, if available, on the predicted/estimated diffusion rate through the cell membrane.

4.1.5. At the end of the NMR experiment, replace the tube containing the cells with an empty tube and rinse the flow unit with water.

4.2. Bioreactor clean-in-place.

4.2.1. Clean the flow unit by flowing the following solutions at 1 mL/min: 0.2 M sodium hydroxide (NaOH); 3 M citric acid; 0.2 M NaOH, for at least 30 min each, followed by sterile-filtered ultrapure water for >2 h.

4.2.2. Clean and autoclave the reservoir bottle and tubing assembly after each run.

5. NMR experiments

5.1. Setup of the NMR experiments.

NOTE: Perform these steps beforehand, prior to the preparation of the in-cell NMR sample, to avoid any delays between cell collection and data acquisition.

5.1.1. Create a new dataset at the NMR spectrometer and set the parameters for the desired NMR experiments.

5.1.2. Set parameters for 1D ^{1}H NMR experiments.

5.1.3. Center the ^{1}H carrier frequency at 4.7 ppm on the water signal.

5.1.4. Select the zgesgp pulse program, set the spectral width to 20 ppm, and a 1,000-µs 180° square pulse for water suppression. Set an inter-scan delay of 1 s. Acquire the spectrum with 32 scans.

5.1.5. For cells expressing unlabeled CA II: select the p3919gp pulse program, set the spectral width to 30 ppm to cover the imino region of the spectrum, and adjust the delay for binomial water suppression so that the maximum excitation is centered at the chemical shifts of the signals of interest (d7 = 20 µs at 950 MHz). Set an inter-scan delay of ≥1 s. Acquire with 512 scans.

5.1.6. For cells expressing ^{15}N-labeled SOD1: select the sfhmqf3gpph pulse program, set the ^{1}H and ^{15}N spectral widths to 16 and 50 ppm, respectively, the shaped pulse offset and excitation bandwidth to 8.5 and 6 ppm, respectively, and a 350 µs pulse for decoupling scheme (garp4 or other depending on the instrument). Set an inter-scan delay of 0.3 s. Acquire with 16 scans and 128 increments in the ^{15}N dimension.

5.2. Real-time NMR spectra acquisition.

5.2.1. Once the bioreactor is inserted in the NMR spectrometer, wait for a few minutes to allow the exchange of the medium.

NOTE: This process is easily monitored from the appearance of the lock signal as the PBS is replaced with medium containing 2% D_2O.

5.2.2. Adjust the matching and tuning of the ^{1}H channel, shim the magnet, and calculate the ^{1}H 90° hard pulse length.

5.2.3. Adjust the ^{1}H power levels in each pulse sequence according to the ^{1}H hard pulse.

5.2.4. Record a first zgesgp ^{1}H spectrum to check the sample content and the field homogeneity.

5.2.5. Copy the zgesgp and the p3919gp/sfhmqcf3gpph experiments to the desired number and queue them in the acquisition spooler.

NOTE: The zgesgp spectra are only used to control the state of the sample and the field homogeneity; therefore, they can be either skipped or recorded less frequently.

5.2.6. For cells expressing unlabeled CA II: process the p3919gp spectra by applying zero filling and exponential line broadening window function (LB = 20 Hz).

5.2.7. For cells expressing ^{15}N-labeled SOD1: process the sfhmqcf3gpph spectra by applying zero filling and squared sine bell window function (SSB = 2) in both dimensions.

NOTE: The size of the processed spectra can be further reduced by removing regions free of signals (in Topspin, this is done by setting the desired STSR and STSI values).

6. MCR-ALS analysis

6.1. For the analysis of CA II spectra, import 1D spectral regions in MATLAB R2019b.

6.1.1. In the software, create a list of experiments to be exported in the **Process Dataset List** menu.

6.1.2. Using a modified version of the au program convbin2asc, export the spectral region of interest in ASCII format for each spectrum.

NOTE: This creates a text file named ascii-spec.txt in each spectrum subdirectory.

6.1.3. In MATLAB, import the spectral regions using the custom script Load_ascii_spectra.

NOTE: This script requires the dataset directory as an input and produces a 2D array spectra containing the stacked 1D spectra and a 1D array cs containing the chemical shifts.

6.1.4 Run the Load_acqus script to extract the timestamps from the 1D spectra.

NOTE: This script produces a 1D array times_hours containing the time increment for each spectrum expressed in hours, with the initial spectrum at time = 0.

6.2. For the analysis of SOD1 spectra, import 2D spectra in MATLAB R2020b.

6.2.1. In Topspin, create a list of experiments to be exported in the **Process Dataset List** menu.

6.2.2. In MATLAB, import the 2D spectra using the custom script Load_2D_spectra.

NOTE: This script requires the dataset directory as an input and produces a 3D array Spectra containing the stacked 2D spectra and a 1D array cs containing the chemical shifts.

6.2.3. Run the Load_acqus script to extract the timestamps from the 2D spectra.

6.2.4. Specify the spectral regions of interest in the custom script Cut_2D_spectra and run the script to cut 3D sub-arrays [(^{1}H x ^{15}N) spectral intensities) x time]; reshape them as 2D arrays (time points x spectral intensities) and join them together.

NOTE: This produces a 2D array JoinSpec_flat containing the reshaped and joined spectral regions.

6.3. Run MCR-ALS 2.0 in GUI mode.

6.3.1. Open MCR-ALS 2.0 GUI by running the mcr_main script.

6.3.2. In the **Data Selection** tab, load the spectra or the JoinSpec_flat matrix. The data can be plotted for checking.

6.3.3. Evaluate the number of components either by Singular Value Decomposition (SVD) or manually.

NOTE: The number of components should correspond to the number of distinct species present in the experiment. In this case n = 2, corresponding to the free and bound protein.

6.3.4. Select a method for the initial estimation of the pure spectra. Either purest variable detection or Evolving Factor Analysis (EFA) can be used.

6.3.5. In the **Selection of the Data Set** window, select **Continue**.

6.3.6. Set the constraints for the concentrations in the **Constraints: Row Mode** window. Apply a non-negativity constraint, select fnnls (Fast nonnegativity-constrained least-squares) as "implementation and 2 species". Apply 1 closure constraint, set the constraint to 1, the closure condition as "equal to" and apply to all species.

NOTE: This forces the sum of each species concentrations to be equal to 1, so that the obtained profiles of each species are normalized with respect to the total protein concentration.

6.3.7. Set the constraints for the spectra in the **Constraints: Column Mode** window. Apply a non-negativity constraint, select fnnls as "implementation and 2 species".

NOTE: This constraint should not be applied if negative signals are present in the NMR spectra.

6.3.8. In the final window, set 50 iterations and 0.01 convergence criterion. Specify the output names for Concentrations, Spectra, and Std. deviation. Click on **Continue** to run the MCR-ALS fitting.

NOTE: A graphical window will show the result of the fitting with plots of the concentration profiles and the spectra of the pure components.

6.3.9. For the SOD1 dataset: use the custom script Rebuild_2D_spectra to reconstruct the 2D spectral regions from the 1D output of MCR-ALS and plot them.

7. Trypan blue test

7.1. Recover the NMR tube content with a Pasteur pipet, and transfer the agarose threads to a 1.5 mL capped tube.

7.2. Remove the residual medium by rinsing the agarose threads with 600 µL of PBS and centrifuge them at 4,000 x *g* for 1 min at room temperature. Discard the supernatant.

7.3. Add 250 µL of PBS and 50 µL of 0.4% Trypan blue solution.

7.4. Incubate for 2 min with continuous pipetting.

7.5. Wash twice with 600 µL of PBS discarding the supernatant.

7.6. Place a few agarose threads on a microscope slide and chop them with razor blades to create small slices of gel. Select the thinnest slices (thickness < 0.4 mm, ideally ~0.2 mm) for the analysis.

7.7. Transfer the gel slices into a self-made cell counting chamber consisting of two glass slides spaced by three layers of paraffin film (~0.4 mm total thickness) on each side.

NOTE: A chamber slide could also be used; however, the gel slices thicker than the chamber height (0.1 mm) would be squeezed, rupturing the embedded cells.

7.8. Acquire images of cells inside the agarose and count white and blue cells.

7.9. Calculate cell viability as (total cells - blue cells) / total cells.

REPRESENTATIVE RESULTS:

The above protocol allows the encapsulation of cells in threads of hydrogel to maximize cell viability for long periods of time, necessary to investigate in real time intracellular processes. In the bioreactor, the cells are maintained alive and metabolically active up to 72 h, as confirmed by Trypan blue test (**Figure 2a–c**). In principle, this protocol can be applied to observe an intracellular protein of interest undergoing any conformational or chemical changes. In the first application described above, the bioreactor is applied to monitor in real time the binding of two inhibitors, AAZ and MZA, to CA II overexpressed in the cytosol of HEK293T cells. The first ^{1}H excitation sculpting spectrum (zgesgp) recorded is used to assess the overall signal intensity (which is proportional to the number of cells), the presence of signals from the overexpressed protein and the field homogeneity (**Figure 2d**). In the case of CA II, the intracellular binding of the two inhibitors can be monitored by WATERGATE 3-9-19[35] 1D ^{1}H NMR spectra (p3919gp), by observing ^{1}H signals in the region between 11 and 16 ppm. These signals arise from the zinc-coordinating histidines and other aromatic residues of CA II[36] and are perturbed upon ligand binding[14,15]. Ligand concentrations can be chosen based on the diffusion rate or, if available, from previously determined permeability values[14]. Successful binding is confirmed visually by the appearance of an additional set of signals in the spectral region of interest, that gradually replaces the original signals (**Figure 3**). Time-dependent binding curves

are obtained by MCR-ALS analysis, which separates the two sets of NMR signals arising from free and bound CA II (**Figure 4a**) and simultaneously provides the relative concentration profiles of the two species (**Figure 4b**). In the second application, the bioreactor is applied to monitor the formation of zinc-bound SOD1 intramolecular disulfide bond promoted by ebselen, a glutathione peroxidase mimetic, in human cells. This process is monitored by observing changes in ^{1}H-^{15}N 2D SOFAST-HMQC[37] spectra (which provides a fingerprint of the protein backbone conformation) caused by perturbations of the protein structure induced by the disulfide bond formation. Additional signals arising from disulfide-oxidized SOD1 appear in the ^{1}H-^{15}N spectrum and gradually replace those from disulfide-reduced SOD1. MCR-ALS analysis on selected regions of the 2D spectrum separates the signals arising from the two species (**Figure 5a**) and provides their relative concentration profiles (**Figure 5b**). The obtained concentration curves can be further analyzed by non-linear fitting to provide information on the kinetics of the processes under study[25].

FIGURE AND TABLE LEGENDS:

Figure 1: Scheme of the bioreactor. Left: cross-section view of the empty flow unit. Right: scheme of the bioreactor setup. The PEEK inlet tubing is shown in green; the PTFE outlet tubing is shown in blue. The left panel is reproduced with permission from Luchinat et al.[25].

Figure 2: Trypan Blue Test on encapsulated cells and sample check by ^{1}H NMR. Representative slices of agarose containing embedded cells and stained with Trypan blue (**a**) immediately after casting and (**b**) after 72 h in the bioreactor; (**c**) cell viability as a function of time in the NMR bioreactor under active flow (black) and under static conditions (red), measured by Trypan Blue Test. (**d**) zgesgp ^{1}H NMR spectra recorded on agarose-embedded cells overexpressing CA II in the absence (black) and in the presence (red) of gas bubbles in the bioreactor. In the latter case, decreased field homogeneity causes line broadening and the appearance of solvent suppression artifacts. Interesting spectral features are labeled. Panels (**a–c**) are reproduced with permission from Luchinat et al.[25].

Figure 3: Representative real-time in-cell ^{1}H NMR data obtained on agarose-encapsulated cells in the bioreactor. Waterfall plots of 1D ^{1}H NMR spectra (region between 15.6 and 11.1 ppm) of HEK293T cells overexpressing CA II and subsequently treated with (**a**) 25 µM AAZ and (**b**) 10 µM MZA, recorded as a function of time in the NMR bioreactor. The time of ligand treatment is shown with an arrow. Spectral intensity (a.u.) is color-coded from blue (lowest) to yellow (highest). This figure is reproduced with permission from Luchinat et al.[25].

Figure 4: Representative MCR-ALS output from 1D NMR spectra. (**a**) ^{1}H NMR spectra of the pure components reconstructed by MCR-ALS: CA II in the absence of ligands (black) and in the complex with AAZ (red) or MZA (magenta); (**b**) relative concentration profiles of free (black) and bound CA II as a function of time upon addition of AAZ (red) or MZA (magenta) obtained by MCR-ALS. Times of ligand treatment are marked with arrows. This figure is reproduced with permission from Luchinat et al.[25].

Figure 5: Representative MCR-ALS output from 2D NMR spectra. (**a**) ^{1}H-^{15}N spectral regions (labeled I-IV) of the pure components reconstructed by MCR-ALS: disulfide-reduced SOD1 (black) and disulfide-oxidized SOD1 (red); (**b**) relative concentration profile of the pure components as a function of time upon addition of ebselen (marked with an arrow) obtained by MCR-ALS. This figure is reproduced with permission from Luchinat et al.[25].

DISCUSSION:

The aim of using a bioreactor for in-cell NMR experiment is to keep cells alive and metabolically active for a prolonged period of time. A number of critical aspects must be taken into consideration to achieve this aim. First, it is paramount to avoid bacterial contamination when preparing the cell sample and during the NMR data acquisition. If strains of *E. coli* or other bacteria commonly used for gene cloning and recombinant protein expression are used in the laboratory, they may contaminate the cells during sample preparation. Once in the bioreactor, the bacteria will grow quickly exploiting the fresh growth medium and will cause cell death due to the production of endotoxins. Bacterial contamination is only spotted at an advanced stage, when it turns the growth medium yellow and turbid. Furthermore, incomplete cleaning of the bioreactor could cause contamination of the pump or the tubing with bacteria, yeasts, or common molds.

A requirement for the success of the experiment is the avoidance of gas bubble formation. Gas bubbles trapped between the agarose threads in the active volume of the NMR coil would introduce large magnetic field inhomogeneities, causing incomplete suppression of the H_2O signal and severe loss of spectral quality (**Figure 2d**). Bubbles may be caused by air trapped in the system or by the formation of gaseous CO_2. The former can be easily avoided by flushing the system with medium prior to inserting the cell sample, while to avoid the latter it is recommended to decrease the concentration of $NaHCO_3$ in the growth medium, and to keep all parts of the system at a constant temperature to minimize differences in the CO_2 solubility. Cellular aerobic metabolism may also cause the formation of gaseous CO_2, which can be prevented by increasing the flow rate.

Cell viability should be checked after each run by Trypan Blue Test. However, that does not provide insights on the metabolic activity. To obtain a more complete picture of the metabolic state of the cells during the bioreactor operation, ^{31}P NMR spectra can be performed to assess the production of ATP as a function of time[23,25]. However, a dedicated probe is often necessary for this measurement, which may allow simultaneous recording with ^{1}H NMR.

In the case of CA II, the presence of well-resolved reporter signals in an unusual region of the ^{1}H spectrum facilitates the analysis from simple 1D NMR spectra and does not require isotope enrichment during protein expression. In general, other proteins could give rise to ^{1}H signals useful for monitoring spectral changes in other regions, such as that typical of the protein hydrophobic core between 0 and -1 ppm[11]; however, these regions tend to be crowded for folded proteins larger than ~10 kDa. In this case, as shown for SOD1, it is preferable to enrich the protein with ^{15}N, by providing uniformly ^{15}N-enriched growth medium during protein expression, and to monitor real-time changes in 2D ^{1}H-^{15}N NMR spectra. 2D spectra are imported as a 2D arrays in MATLAB, rearranged to 1D arrays and stacked prior to MCR-ALS analysis. The latter approach is generally applicable to any intracellular protein that gives rise to detectable signals, and provides information on protein conformational changes at the single residue level. In principle, the latter approach can be generalized to nD spectra and to other isotope-labeling schemes.

Concerning the application to different types of cells, the protocol should be easily adapted to different cell lines and does not require that the protein of interest is directly expressed in the cells. Therefore, other approaches to in-cell NMR can be combined with this protocol, in which the macromolecule of interest is produced recombinantly, or synthesized, and subsequently inserted into the cells by electroporation or by other delivery methods[1,9,38]. When working with different cell lines or sample preparation protocols, parameters such as the agarose concentration, the thread

thickness, and the final cell density in the agarose threads may need to be optimized empirically. Furthermore, the applicability of the protocol described here is limited to cells that tolerate agarose encapsulation. Other cell types may require different hydrogel formulations, whereas a different setup is recommended when analyzing cells that natively grow in suspension, for example, making use of a coaxial microdialysis membrane to ensure nutrient diffusion while keeping suspended cells confined in the NMR tube[23].

Compared to other NMR bioreactor designs[19–22], the device described here relies on a commercially available flow-unit, adapted with minor modifications. Therefore, the device can be easily replicated in different laboratories with high reproducibility. Furthermore, it allows standardized operation and full compliance with strict laboratory safety regulations, if needed. Overall, the flexibility and ease of operation of the bioreactor should allow many other applications of solution NMR, both in cells and in vitro, in addition to those as already reported[23,25]. Eventually, the same bioreactor design could be applied to samples that resemble more of the physiological environment of a tissue, such as spheroids or organoids, provided that appropriate scaffolds are found for keeping such samples alive—or even sustaining their growth—in the NMR spectrometer.

DISCLOSURES:

The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS:

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Figure 1

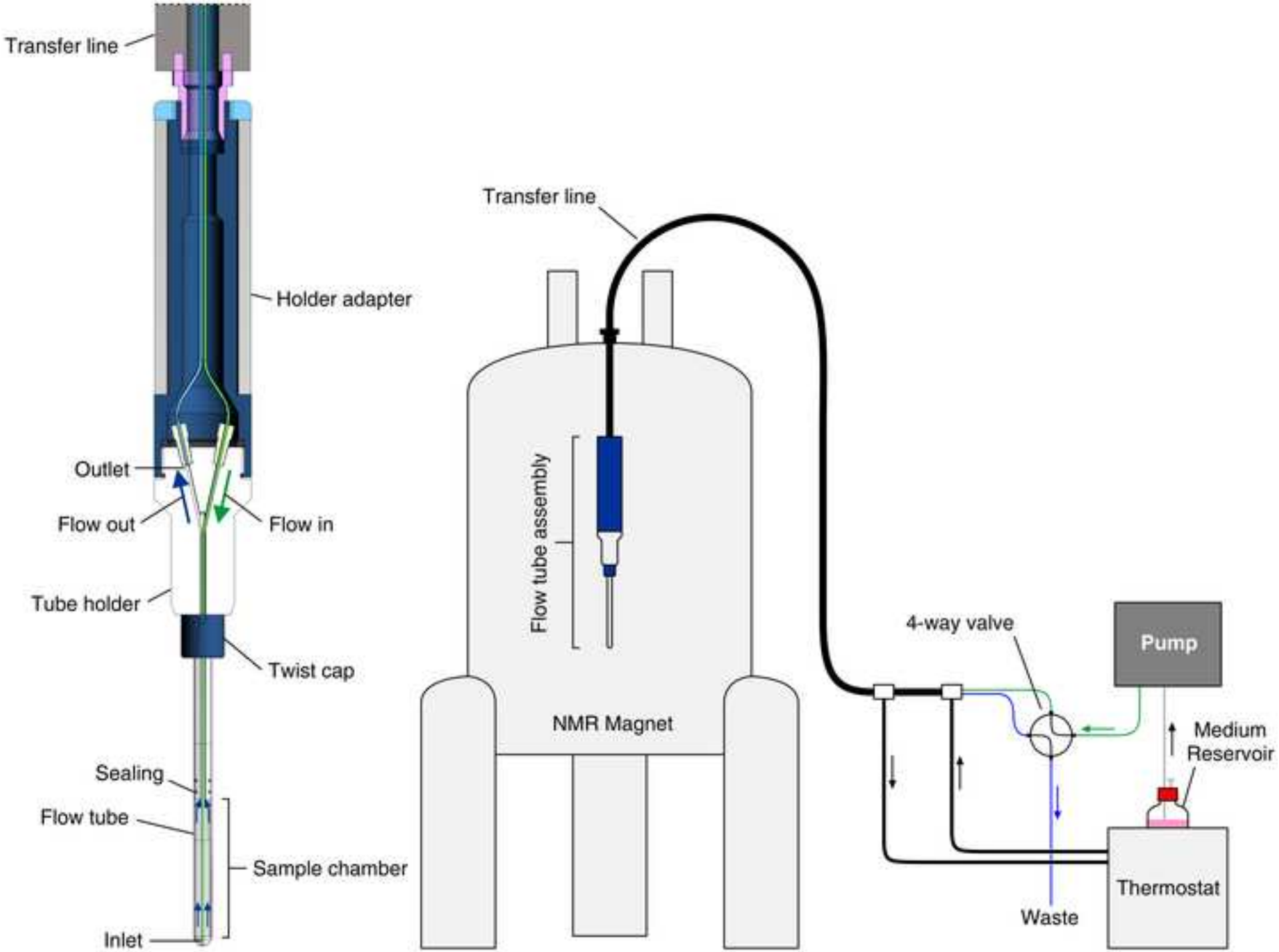


Figure 2

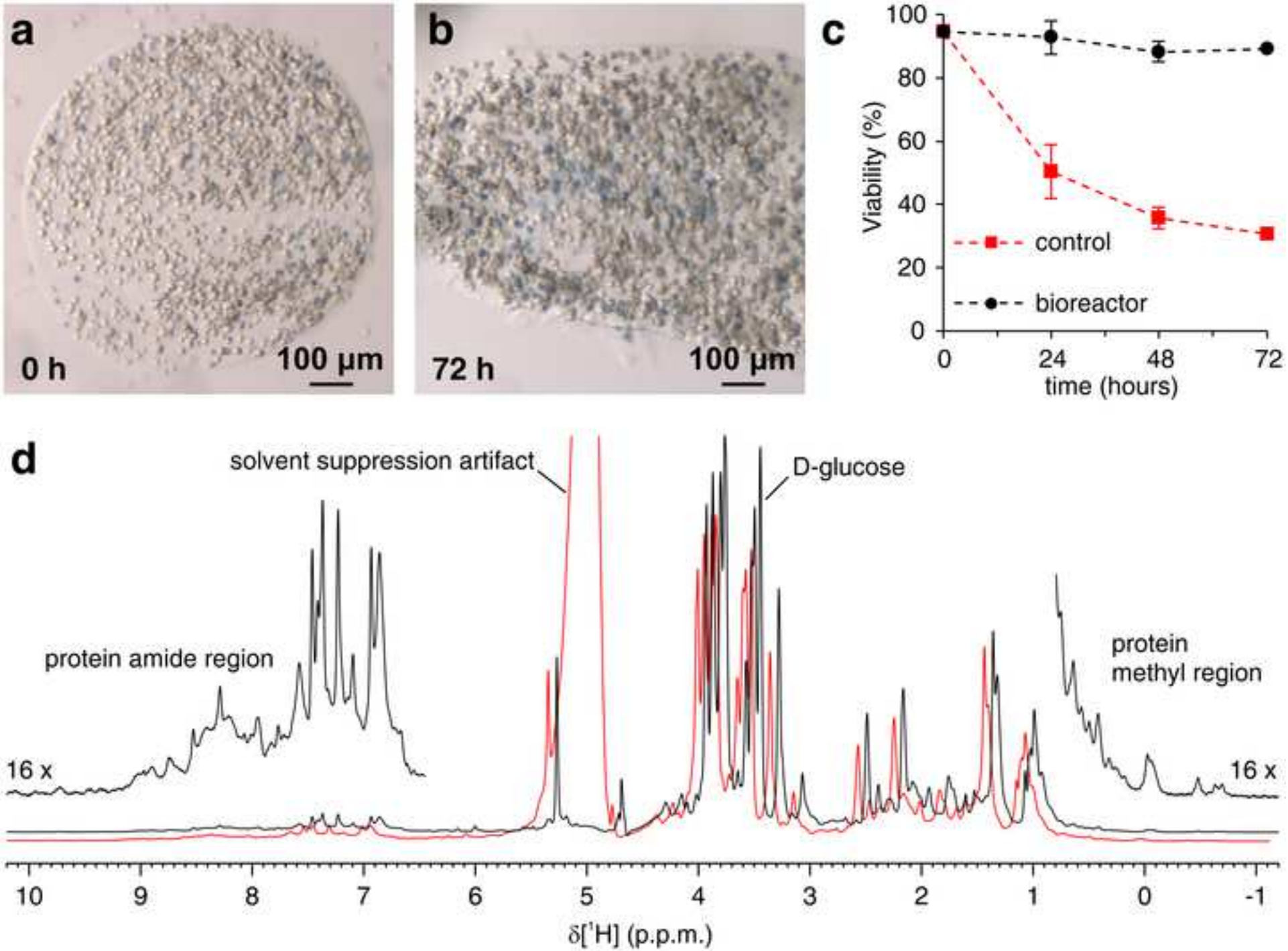


Figure 3

Click here to access/download;Figure;Figure3.tif

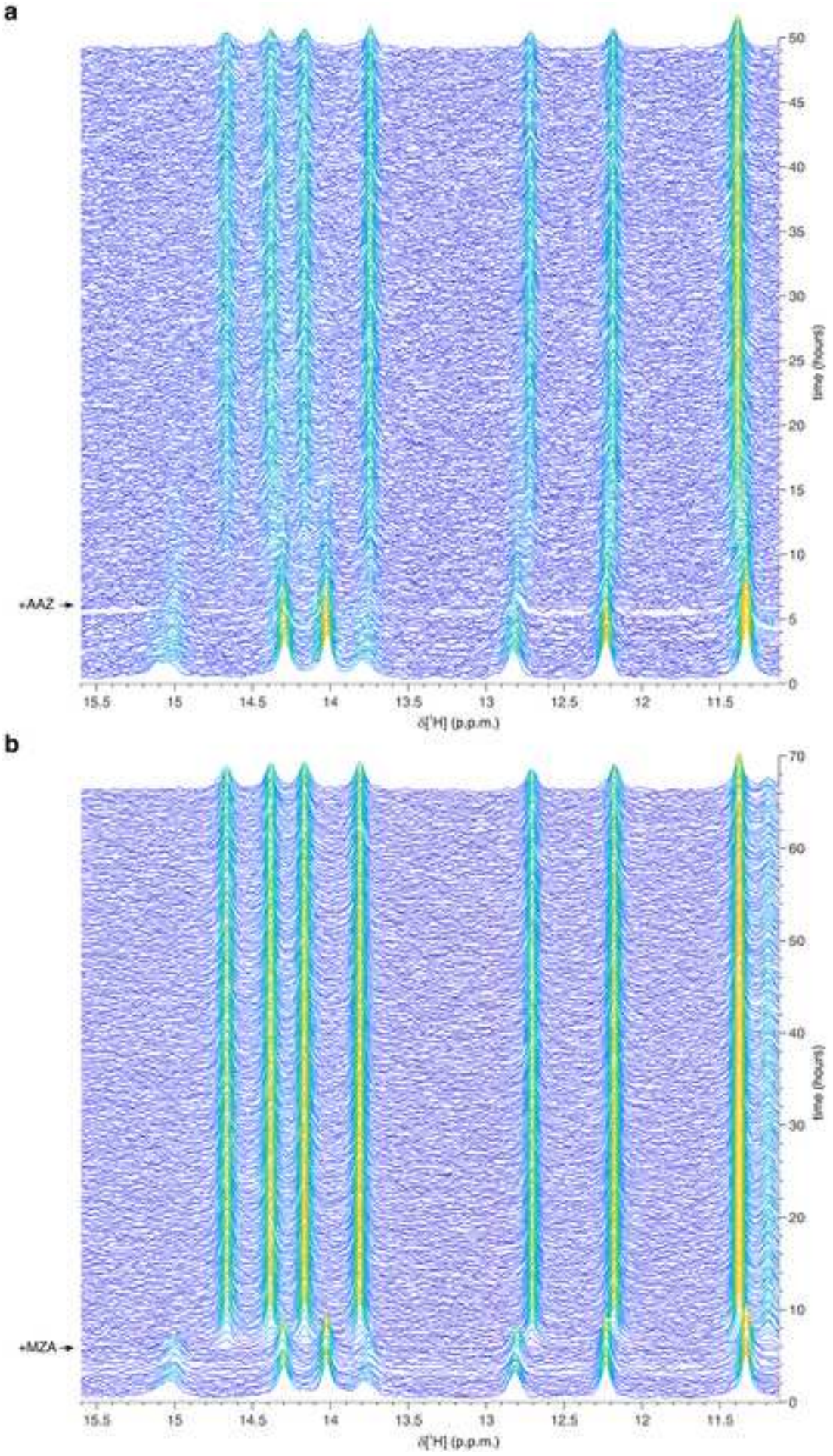


Figure 4

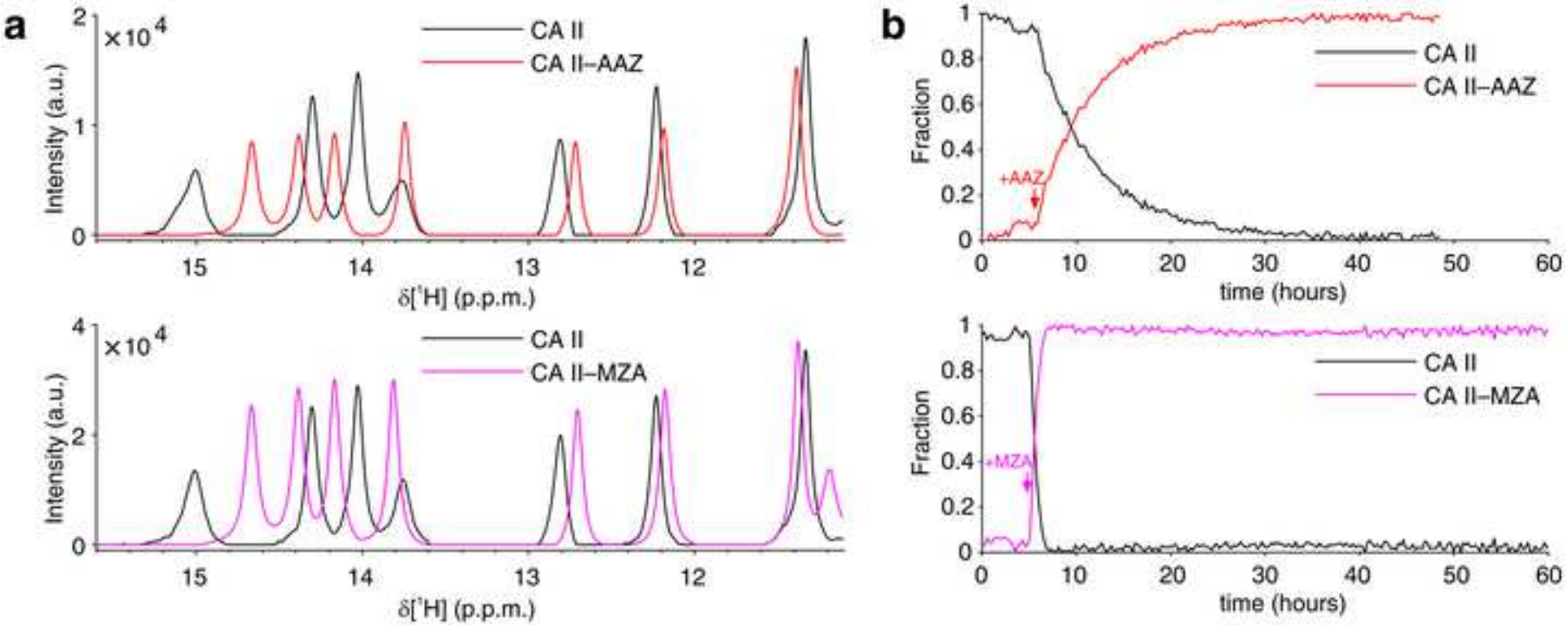
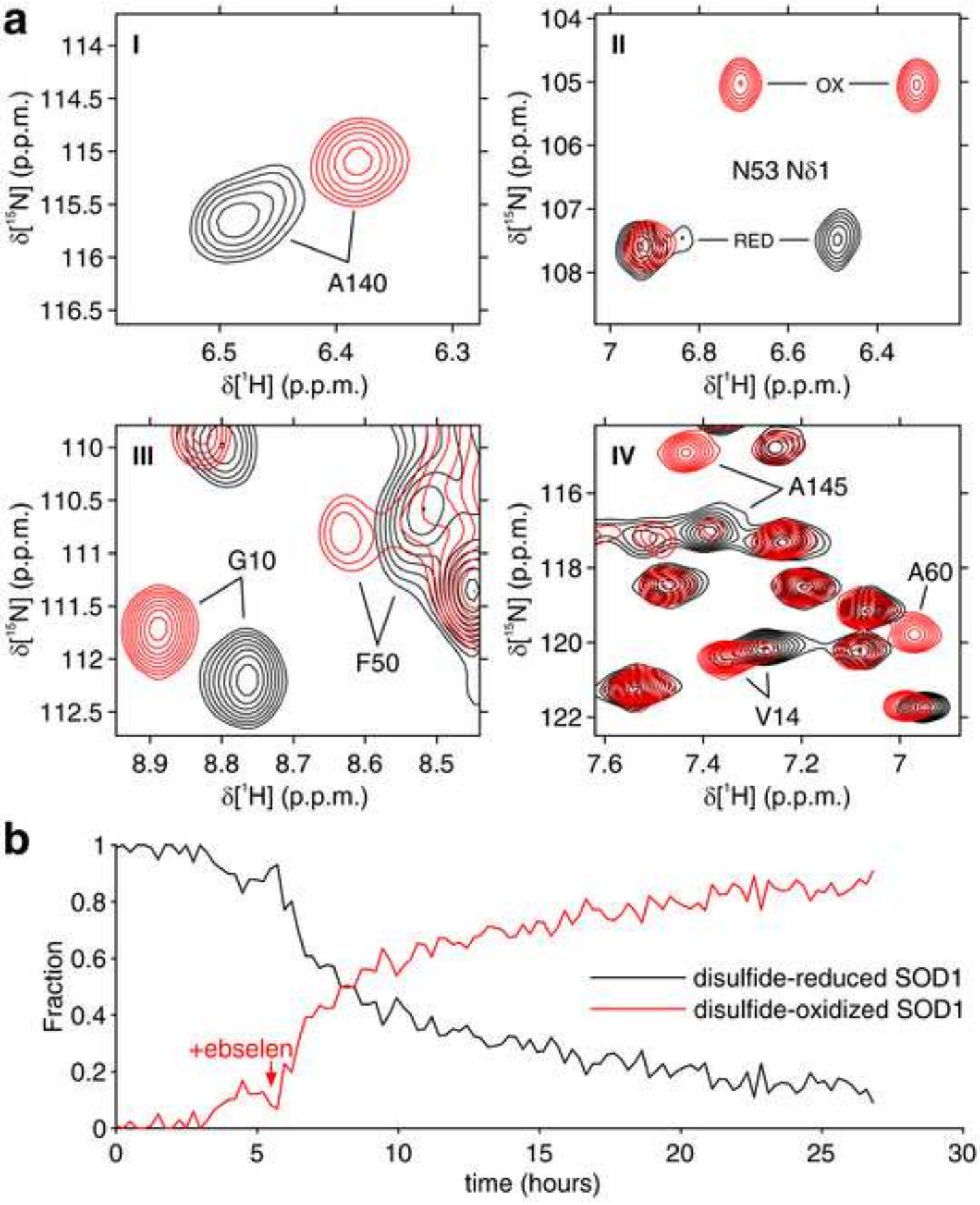


Figure 5



Name of Material	Company	Catalog Number
Citric acid	Sigma-Aldrich	251275
D2O	Sigma-Aldrich	453366
DMEM, high glucose	Life Technologies	10313-021
DMEM, high glucose, powder	Sigma-Aldrich	D5648
FBS	Life Technologies	10270
HCl	Sigma-Aldrich	30721
L-glutamine (200 mM)	Life Technologies	25030
Low-gelling agarose, powder	Sigma-Aldrich	A4018
NaHCO3, powder	Carlo Erba	478537
PBS	Life Technologies	10010
Penicillin–streptomycin (10,000 U/ml)	Life Technologies	15140-122
NaOH, pellets	Sigma-Aldrich	30620
Trypan Blue solution (0.4% (wt/vol)	Sigma-Aldrich	T8154
Trypsin–EDTA (0.05% (wt/vol))	Life Technologies	25300-054

Name of Equipment	Company	Catalog Number
Avance III Spectrometer equipped with a 5 mm CryoProbe	Bruker	n/a
InsightMR flow unit	Bruker	n/a
P-920 pump module from ÄKTA FPLC	GE Healthcare	n/a

Comments/Description

Hazard statement(s): H350 may cause cancer.

Comments/Description

All modern spectrometers and narrow-bore magnets equipped with 5 mm probes are compatible.

Any FPLC, HPLC peristaltic or syringe pump should be compatible with the flow unit.

Reply to the Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. E.g. Line 112: "…cell sample.." instead of "…cells sample.."

The manuscript has been thoroughly checked for spelling errors.

2. Please revise the following lines to avoid previously published work: Lines 47-56, 270-275, 276-280.

We have revised these lines as requested.

3. Line 80, 163: Please specify concentrations.

The concentrations have been added.

4. Please provide details about the bioreactor. E.g. dimensions, material, etc.

These details have been added in the introduction section, together with a scheme of the flow unit and of the bioreactor setup (Figure 1 in the revised manuscript).

5. Line 193-194: How is this done? Please add details e.g. mouse-clicks, options selected etc.

In writing this protocol, we had to assume that the user has sufficient knowledge to operate an NMR spectrometer and NMR software. Specifying all single steps as mouse clicks, options and commands would make the protocol extremely long, hard to read and too specific (i.e. different NMR software versions have different commands). Please advise on whether this should be stated at any point in the text.

6. Line 252-253: What is the approximate size of the slices?

This information has been added.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. E.g. Line 174

All references to company names and brand names have been removed from the main text as requested.

8. Please use the " , " separator instead of " ; " between the author names in the references according to the JoVE style: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Also, please write the journal names in full.

The reference format has been modified to comply with JoVE guidelines.

9. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Copyright permission has been included in the file copyright.docx. The figure legends have been revised as requested.

Point-by-point reply to the Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript by Barbieri and Luchinat describes an important cell based technique that permits atomic resolution studies of protein-drug interactions in live cells. The protocol is very comprehensive and contains all important details to be reproduced by a qualified graduate student.

Major Concerns:
none

Minor Concerns:
1. Page 2, line 80: Add final concentrations of Gin and pen-strep.

The concentrations have been added.

2. Page 3, line 93: Which antibiotic are you using?

Penicillin-streptomicin. This has been added in the text at lines 93-94.

3. , line 97: what is a vacuum driven sterile filter?

It is a Stericup filter. As commercial brands cannot be mentioned in this journal, we used the common name for that kind of device.

Reviewer #2:
Manuscript Summary:
The present manuscript provides a description of the NMR bioreactor setup and its application to (real-time) monitoring protein-ligand interactions in human cells. The protocol is described in a great detail and it focuses on
the practical aspects of the bioreactor-based in-cell NMR experiments. The potential pitfalls of the protocols are highlighted.
Troubleshooting of potential problems is suggested (discussed). This reviewer believes the protocol can be published as is.

Minor points (that might improve the text) are listed below:
1) reference #16 seems to be misplaced. Ref #16 doesn't relate to the application of in-cell NMR to nucleic acids.

We thank the Reviewer for noticing this mistake. We now cite ref 16 together with 14 e 15.

2) The purpose/use of "zgesgp" experiment is not clear from the text (point 5.2.4). The authors might consider to add example of the "zgesgp" experiment on a real sample and illustrate its interpretation.

The use of the zgesgp has been clarified (lines 224-225 and 310-313), and a new panel (Figure 2d) has been added to show the effect of poor field homogeneity on the zgesgp spectrum.

3) Point 4.1.4. The authors might consider to add a short discussion of parameters based on which the concentration of the external molecule is determined.

We have now discussed this in a note to point 4.1.4: "NOTE: the final concentration of molecule in the medium should be chosen based on previous knowledge of cell toxicity and, if available, on the predicted/estimated diffusion rate through the cell membrane." (lines 181-183).

Reviewer #3:

Manuscript Summary:

The submitted MS describes the use of a bioreactor (flow probe) setup for real-time (RT) in-cell NMR measurements of drug - protein interactions in HEK293 cells. Specially, the authors present the applications of consecutive, one-dimensional (1D) proton (H1) watergate experiments on agarose-embedded HEK293 cells that transient over-express the second isoform of human carbonic anhydrase (CA II) upon exogenous addition of 2 drug compounds, acetazolamide (AAZ) and methazolamide (MZA), along their original primary research papers in Angewandte Chemie 2020 (Luchinat et al) and Analytical Chemistry 2020 (Luchinat et al). They further include MATLAB routines to delineate kinetic binding profiles by Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS).

Major Concerns:

Overall, experimental steps described in the MS agree with information provided in the Materials & Methods section of the indicated papers. Nonetheless, I was surprised to find that A) the authors chose to describe the bioreactor setup using encapsulated cells and agarose threads (published and developed by Kubo et al, Angewandte Chemie 2013, and optimised by Breindel et al, Biochemistry 2018), rather than their 'own' coaxial micro-dialysis system (Cerofolini et al, Biophysical Journal 2018). Clearly, the former bioreactor system is not the expertise of the authors and it would have made much more sense if Luchinat et al had chosen to describe their own setup (for which they are actually known for).

We partly share the disappointment of the Reviewer for the fact that we did not describe the coaxial microdialysis membrane system that was developed in our lab by Cerofolini et al. While conceptually more innovative and original, the setup based on the microdialysis membrane was not optimal for ensuring good cell viability with the same cell density and experimental times as those employed here. This shortcoming was even mentioned in the original work (Cerofolini et al.): "We note that the supernatant of the bioreactor sample after 42 h of continuous flow contained some protein, suggesting that leakage had occurred to some extent". Unfortunately, we have not yet been able to improve that design to make it on par with the one described here. In parallel, we continued our work to implement a bioreactor that could ensure higher cell viability and would eventually be made available to the Structural Biology community (also as a facility to users through the trans-national access Consortia of the European Commission), and we reported our latest variant in Luchinat et al. Anal Chem 2020, which is able to sustain viable cells for up to 72 h.

Therefore, for this publication in the Journal of Visualized Experiments, we preferred to choose the protocol that worked better in our hands. If that gave the wrong impression that we appropriated someone else's approach, we do apologize. The works that first developed the hydrogel thread approach have been acknowledged more appropriately in the revised manuscript. In the future, we hope to be able to further improve the microdialysis setup to make it really useful for the community.

B) The choice of the CA II - AAZ/MZA system to exemplify the general use of a bioreactor setup for time-resolved in-cell NMR experiments is utterly unsuitable, in my opinion. The reason for this relates to the fact that Histidine imino 1H resonances at 10-15 ppm are a highly unique feature of CA II (due to Zn coordination). Hardly any other protein displays proton resonances in this spectral region so that 1D watergate experiments on non-isotope labeled in-cell NMR samples, as described in the MS, will simply not work. In other words, Lychinat et al delineate a protocol that is not usable in the general sense, but only applicable to this particular system. In my opinion, this completely defies the notion of a protocol that a broad audience can use.
Herein lies the biggest shortcoming of the MS. Inexperienced users may follow the protocol (to study their system of interest) only to realise that they fail to detect any NMR signal at all.

We partly disagree with the Reviewer on this point. In fact, it is true that the ^{1}H spectral region between 10-15 ppm does not usually contain protein signals that can be used for this kind of analysis. However, the real-time acquisition of 1D ^{1}H spectra can in principle also be applied to monitor changes occurring to aliphatic signals arising from the hydrophobic core of the protein, located in the upfield region of the ^{1}H spectrum between 0 and -1 ppm (see for example Banci et al. Chem & Biol 2013, ref. 11). In that region, the cellular proteins give rise to an almost featureless slope (likely the ‘grand average’ of the hydrophobic cores of all folded proteome), so that signals from an overexpressed target would be easily identified in that region. The applicability of this protocol with minor adaptation has now been better discussed (see below).

I strongly advise the author to choose a different exemplary system and to describe their protocol in a way that is generally applicable. In that sense, sections 5 and 6 ought to be updated accordingly as well. It doesn't make sense to provide detailed steps for only one type of NMR experiment but to provide the reader with guidelines for how to choose appropriate NMR experiments for their individual systems. Similarly, the authors should pay close attention to NMR hardware/software (field strength) requirements that may- or may not be- stringently required for the outlined experiments. The same holds true for routines to derive 'time-resolved quantitative information about binding kinetics' via MCR-ALS (section 6). To simply spell out MATLAB commands and scripts, which will only work in this one particular case is useless.

We thank the Reviewer for raising this point, as we agree that this protocol should be made as broadly applicable as possible. We have further extended the text to include the real-time analysis of the human protein copper, zinc superoxide dismutase by 2D ^{1}H-^{15}N NMR spectra, that was previously reported in the original publication (Luchinat et al. Analytical Chemistry, 2020). This example extends the range of applicability of this protocol to that of in-cell solution NMR as a whole (i.e., as long as signals can be identified in 2D spectra, they can be monitored in real time). The manuscript has been extensively revised accordingly (lines 77-80, 86, 207-211, 228-230, 246-257, 285-286, and 322-330)

We have also attached the custom scripts that were mistakenly omitted in the first submission as supplementary files.

Concerning the usefulness of following precise steps, it is a requirement of the journal to be as specific as possible when describing each step, therefore it was necessary to specify them for this particular application.

With the addition of the application to 2D spectra, we believe that the reader has now sufficient information to decide which experiments to set up and which scripts to use for data analysis.

In summary, sections 1-4 are generally applicable and useful, although I find it weird that the authors are not describing their own system (personally, I would always follow the protocol by Burz et al, Methods in Enzymology 2019, for this type of bioreactor design and encapsulated in-cell NMR samples.) Sections 5 and 6 are only applicable to the CA II - AAZ/MZA system and, as such, not useful in a general sense. Section 7 is generally applicable.

While we would like to think that the bioreactor implementation that we published in Luchinat et al. is also our "own system", we strongly believe that no one should 'own' the bioreactor protocols and variations that have been developed to date. They should all be made available to the scientific community, with appropriate acknowledgement to the previous work. On this latter aspect, we may have failed and we apologize. We now also cite Burz et al, Methods in Enzymology 2019.

It is evident that the two protocols, while conceptually analogous, are not identical: different materials, volumes, incubation times and temperatures make them unique. Different cell lines also have different requirements/optimal conditions. We think that having more protocols available is always beneficial to the community, so that one is free to choose the best for its needs and adapt it as necessary.

Finally, I find the choice of figures not very informative. Again, with this being a 'visual protocol', I would have preferred schemes and images of the bioreactor setup rather than data figures from the original publications.

We thank the Reviewer for this suggestion. We have now added a scheme of the bioreactor in the new Figure 1. This 'visualized experiment' will eventually be integrated with a video (like all publications in JoVE) where we physically show the bioreactor at work.

Minor Concerns:
One piece of information that is generally missing in sections 3 are temperature requirements for PBS 'washing' solutions i.e. 3.1.2 (line 116), 3.1.6 (line 125), 3.2.7 (line 144). In my opinion, these should be pre-warmed to 37deg C.

The temperatures have been added in the text. We actually employ PBS at room temperature or slightly lower (~20 °C).

I also wonder whether the (agarose?) sample in 3.2.6 (line 143) is still liquid.

In our experience, 2 minutes are more than sufficient to let the agarose-cell suspension solidify. It could be dependent on the type of agarose and its percentage, and on the cell line and cell density.

Reviewer #4:
Manuscript Summary:
This manuscript describes a method for real-time analysis of compound binding to cells using an NMR approach with a bioreactor based on the encapsulatio of clls in agarose hydrogels. The experimental setup is clearly described. The appraoch is however not new, agarose embedded cells have been described before using agarose or methylcellulose (for example Koczula, K. M. et al. Metabolic plasticity in CLL: adaptation to the hypoxic niche. Leukemia 30, 65-73 (2016), Alshamleh, I. et al. Real-Time NMR Spectroscopy for Studying Metabolism. Angew. Chem. Int. Ed. 59, 2304-2308 (2020).).

We thank the Reviewer for pointing out previous works where cells were encapsulated low-melting agarose, we have now mentioned them in the text at line 62. Methylcellulose-embedded cells are likely not ideal for the bioreactor design reported here as they behave like a viscous suspension (i.e. it can be pipetted) and therefore would be flushed away by the flow of medium.

The approach has an excellent underlying design and is likely to be used by others.

Major Concerns:
I don't have major concerns

Minor Concerns:
1. Usually agarose is usually not sufficient to keep adherent cells alive - why is this possible for HEK293T cells?.

Trypan Blue performed at 72 h reveals good cell viability, and 31P measurements confirm that ATP is produced at a constant level. However, based on the observation that HEK cells retain a spherical shape once embedded in the gel, we also conclude that agarose is not an ideal support for prolonged cell growth. Likely, in the short term (few days) the flow of medium allows sufficient cell viability by providing fresh nutrients and removing the byproducts of cellular metabolism. However, in the longer term, the lack of cell adhesion may prevent cell growth.

2. Whether cells are metabolically active should be readily seen in glucose consumption - this is easier than monitoring 31P signals. Bacterial growth often promotes acetate formation.The signals of the medium also provide a good control whether nutrients are sufficient and have not been used up.

It is true that glucose consumption is a straightforward measure of metabolic activity. However, practically, it was deemed unreliable in the present setup, due to the fact that a high-glucose DMEM (25 mM D-glucose) is used to grow and maintain HEK cells. At such concentration, and with a 0.1 mL/min flow rate, only a very small fraction is actually metabolized, making the measurement difficult (see also the glucose signal intensity in the ^{1}H zgesgp spectrum shown in new Figure 2d). As a side note, this suggests that the bioreactor could be operated at a much lower glucose concentration. More in general, we are currently evaluating the use of ^{1}H signals of specific metabolites (such as glucose, lactate and others) to monitor metabolic activity within the bioreactor, as this would enable an in-line measure (not requiring a dedicated broadband probe for ^{31}P NMR). However, we still do not have conclusive data at the time of writing.

3. The paper suffers from not showing what the signals in the NMR spectra are. The paper suggest that they arise from CA II. What is the concentragtion fo the protein that these signals can readily be seen? How do the authors know that these signals belong to CA-II - these are clearly aromatic signals, I would assume from some aromatic Amino acid of that protein? Are the same shifts also seen under in vitro conditions when AAZ or MZA is bound?

The intracellular concentration of overexpressed CA II is of the order of 150 µM, as reported in previous works where identically prepared cells were employed (see Luchinat et al. Angew Chem 2020 and Luchinat et al. ACS Chem Biol 2020, references 14 and 15 in the manuscript). In Luchinat et al. Angew Chem 2020 we report the available assignment of the CA II signals observed in the imino region, which are indeed aromatic $^{1}H^{N}$ from zinc-binding histidines in the protein active site, or other aromatic ^{1}H. In the same work, the chemical shift changes upon ligand binding were also shown to closely match those observed in vitro.

This is now briefly reported in the manuscript at lines 314-316.

4. Is there are good reason to use such a complex a bioreactor? What is suggested is to monitor the availability of the drug which sems to be limited by membrane penetration. But there are simpler ways to monitor membrane penetration - does the observed result actually correlate with any other known data about drug availability in these cells?
I would see a better reason for this setup to look at in vivo interference of binding that can not be seen in vitro.

Indeed, there are other cell-based assays to assess membrane penetration. However, they often need to be developed ad hoc for different targets, so that the effect of ligand penetrating the cell can be indirectly measured. Here, conversely, we directly observe ligand binding to the intracellular target. As shown in Reference 14, we observed a striking correlation between the membrane permeability measured for AAZ and

MZA and the recommended dose of each drug for treatment of glaucoma. We refer to that publication for further details.

A main advantage of this bioreactor is that it also allows to assess binding specificity, i.e. whether there are other competing binding sites within the cell. These would contribute to decrease the apparent binding affinity for the main target, which would be measurable directly or by competition binding experiments. While AAZ and MZA did not show this behavior, this was demonstrated in Ref 14 for a different molecule. Currently, we are also working to extend the application of the bioreactor to ligand-observed NMR, which would provide complementary information on the fate of the ligand as it enters the cells.

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