



Published in final edited form as:

*J Magn Reson.* 2010 February ; 202(2): 140. doi:10.1016/j.jmr.2009.10.008.

## A Bioreactor for In Cell Protein NMR

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### Abstract

The inside of the cell is a complex environment that is difficult to simulate when studying proteins and other molecules *in vitro*. We have developed a device and system that provides a controlled environment for Nuclear Magnetic Resonance (NMR) experiments involving living cells. Our device comprises two main parts, an NMR detection region and a circulation system. The flow of medium from the bottom of the device pushes alginate encapsulated cells into the circulation chamber. In the chamber, the exchange of oxygen and nutrients occurs between the media and the encapsulated cells. When the media flow is stopped, the encapsulated cells fall back into the NMR detection region, and spectra can be acquired. We have utilized the bioreactor to study the expression of the natively disordered protein  $\alpha$ -synuclein, inside *Escherichia coli* cells.

### Keywords

bioreactor; in-cell nuclear magnetic resonance (NMR)

## 1. Introduction

Most biophysical experiments on proteins are conducted in dilute solution. Most proteins, however, serve their physiologically relevant function in cells, which have a complex crowded environment that affect several protein properties compared to dilute solution [1;2;3;4]. For this reason, there is an increasing interest in studying proteins inside living cells. Nuclear magnetic resonance spectroscopy (NMR) has become a popular tool for experiments on living cells because it provides atomic-level information about cellular components and is nondestructive [5].

A disadvantage of NMR spectroscopy is its low sensitivity. Selective isotopic enrichment or labeling of the targeted species with an NMR active nucleus (e.g., <sup>15</sup>N, <sup>13</sup>C or <sup>19</sup>F) is one way around this problem. Serber *et al.* suggest that the minimum concentration of the protein under study should be at least ~150  $\mu$ M for <sup>15</sup>N enrichment or ~50  $\mu$ M for <sup>13</sup>C side chain enrichment [6]. Therefore, the target protein must be overexpressed or introduced into the cell by other

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means (e.g., micro injection, cell penetrating peptides [5]). To increase sensitivity further, high cell densities ( $10^9$ – $10^{11}$  cells/mL) are used, and the data are time averaged.

Current experimental setups for protein in cell NMR have several drawbacks. First, the lack of aeration and the high cell density create an anaerobic environment. Second, metabolites and waste products accumulate. These characteristics can decrease cell viability, limiting the cell types that can be used, and make it difficult to monitor temporal changes. Overcoming these challenges requires an NMR compatible device that provides a controlled environment for living cells.

Devices with these characteristics have been developed [7;8;9]. One type is an in-magnet bioreactor that enables growth of microorganisms to a high density [10;11]. Another type is a perfusion system that flows media **down** through immobilized cells [12;13]. These devices tend to be complex and difficult to fabricate. Furthermore, they are designed to study metabolism.

Here, we describe a circulating encapsulated cells (CEC) bioreactor and accessories for in-cell protein NMR. The instrument comprises parts that are commercially available or easily fabricated. The expression of the natively disordered human protein  $\alpha$ -synuclein in *Escherichia coli* is used to demonstrate its capabilities.  $\alpha$ -Synuclein is a 14.5 kDa protein implicated in the pathogenesis of Parkinson's disease [14]. The expression of the plasmid borne  $\alpha$ -synuclein gene is controlled by a lactose inducible, phage T7 promoter. We investigate the bioreactor's ability to measure the accumulation of  $\alpha$ -synuclein with time in living *E. coli* cells.

## 2. Results

### 2.1 The CEC bioreactor

The bioreactor is made from Teflon to facilitate NMR experiments involving  $^1\text{H}$  detection (We have made a similar version from Plexiglass for  $^{19}\text{F}$  detection). We designed the bioreactor for an 8 mm probe to facilitate fabrication with conventional machine tools. The bioreactor comprises three main parts; an 8 mm diameter tube, a circulation chamber, and an adjustable threaded cap (Fig. 1). An inlet for an Upchurch Scientific Super Flangeless Fitting is located on the upper part of the cap (Fig. 1E). A 1/16" OD inlet for Upchurch Scientific perfluoro alkoxy alkane tubing is located at the bottom (Fig. 1A).

Most of the components (Fig. 2) are commercially available. The liquid media is contained in a 1 L Corning three neck spinner flask with a tubing adaptor on one side arm (Fig. 2A). A peristaltic MasterFlex pump (Fig. 2B), moves the media at a rate of 45 ml/min from the flask, through the tubing adaptor, into the PFA tubing (Fig. 2, yellow lines). The tubing runs from the pump, into the bottom of an 8 mm Varian triple resonance z gradient probe through an opening created by removing the heater (Fig. 2D). The temperature is controlled with the spectrometer's FTS Systems heating apparatus (Model TC-84). PFA tubing between the pump and the bioreactor is placed in a Fisher Scientific Isotemp water bath to warm the media (Fig. 2C), which flows from the bottom of the bioreactor to the top (Fig. 1, arrows on right side). The PFA tubing at the top of the bioreactor returns the media, via a pH probe, to the 1 L the Corning three neck spinner flask. The pH probe, pump and NMR spectrometer are connected to a laptop computer (Fig. 2, green lines).

The  $^1\text{H}$ - $^{15}\text{N}$  band-selective optimized flip-angle short transient (SOFAST) heteronuclear multiple quantum coherence (HMQC) [15] pulse program provided in the Varian Biopak suite of pulse sequences was modified to send voltage outputs to the computer. The perfusion pump is controlled by one of the spare output lines on the Varian Inova console. The pump is switched on before the first steady state scan, and remains on during the perfusion delay. It is then then

switched off during the bead settling delay. After the first steady state scan, the pulse sequence skips the pump control code. The signals are interpreted by LabView (National Instruments) software, which controls the pump. The software also records the pH value at 1 min intervals.

Cells are electrostatically encapsulated into 1 mm diameter  $\text{Ca}^{2+}$  alginate spheres to keep them in the bioreactor [16]. The circulation of the encapsulated cells facilitates the delivery of nutrients and waste removal. The bioreactor (Fig. 1) has two states: pump off, and pump on. When the pump is off, encapsulated cells settle into the 8 mm diameter tube for data acquisition (Fig. 1, left panel). When the pump is on, the encapsulates travel from the 8 mm diameter tube into the wider circulation chamber. The movement of the encapsulates from a narrow to wider tube results in a reduction in pressure causing the encapsulates to circulate in the chamber (Fig. 1, right panel). The pulsed, upward motion also prevents the encapsulates from clogging the outlet of the cell chamber. Settling of the encapsulates requires approximately 90 s from the time the pump is turned off.

## 2.2 CEC bioreactor without flowing media

To assess the bioreactor's suitability for in cell NMR experiments, the  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of encapsulated *E. coli* in the bioreactor is compared to the HSQC spectrum of  $^{15}\text{N}$  enriched  $\alpha$ -synuclein obtained in a conventional 5 mm NMR probe (Fig. 3A,B). The *in vitro* spectrum of purified  $\alpha$ -synuclein (Fig. 3C) is shown as a reference. The *in vitro* spectra were acquired at a lower temperature because we have shown that the *in vitro* spectrum acquired at 10 °C is equivalent to in cell spectra acquired at 37 °C [1; 17]. The similarity of the spectra in Fig. 3 indicates that  $\alpha$ -synuclein can be detected in the bioreactor.

To assess the bioreactor's effect on spectral quality, the in cell HSQC spectrum of encapsulated *E. coli* expressing  $^{15}\text{N}$  enriched  $\alpha$ -synuclein in the bioreactor is compared to the HSQC spectrum of the same encapsulates in a 5 mm tube (Fig. 3A,D). The spectrum of the encapsulated cells in the 5 mm tube is consistent with the published spectrum [18]. The crosspeaks broaden when encapsulates are placed in the bioreactor, but the quality of spectra is only slightly degraded.

## 2.3 CEC bioreactor with flowing media

The expression of  $\alpha$ -synuclein was monitored with the  $^1\text{H}$ - $^{15}\text{N}$  SOFAST HMQC pulse sequence, rather than the HSQC sequence, to obtain better time resolution. Spectra as a function of time are shown in Fig. 4A–C. The spectrum of the encapsulates before induction (Fig. 4A) has few crosspeaks and no unambiguous  $\alpha$ -synuclein crosspeaks. After induction new crosspeaks begin to appear. With each successive spectrum, the crosspeaks increase in volume as seen at 4 h and 18 h (Figs. 4B and C). Using methods described by Slade et al. [19] we determined the intracellular concentration of  $\alpha$ -synuclein to be 0.8 mM at 18 h.

As a control, the encapsulates were removed after the experiment and a spectrum was acquired of the surrounding media (Fig. 4D). The spectrum shows only a weak crosspeak, indicating that the bulk of the signal comes from the encapsulated cells. The viability of the *E. coli* in the bioreactor experiments was determined by plating serial dilutions of dissolved encapsulates before and after each experiment. The viability was 95%. The pH of the medium perfused around the encapsulated cells remained at 7.00 for the duration of the experiment.

Although the CEC bioreactor provides an environment where encapsulated *E. coli* cells express  $\alpha$ -synuclein, our sacrifice of spectral resolution for increased time resolution (see the Experimental section) made it difficult to distinguish between metabolites and protein crosspeaks. To determine which crosspeaks corresponded to  $\alpha$ -synuclein, we collected spectra of fresh media (Fig 5A) and of *E. coli* containing a pUC18 plasmid [20] without the  $\alpha$ -synuclein

gene (Fig 5B). Overlaying these spectra with the spectrum of  $\alpha$ -synuclein expressed in the bioreactor (Fig 5C), shows that most of the crosspeaks are from  $\alpha$ -synuclein (Fig 5D).

The overlay allowed us to quantify temporal changes in crosspeak volumes (Fig 6). The crosspeak from the defined minimal media is the only crosspeak detectable at 30 min (Fig 6H). Although induction occurred at 30 min, there was a lag phase of approximately 4 h before crosspeaks could be detected (Fig 6A–B, E–G). Some crosspeaks are not detectable until approximately 7 h (Fig 6C,D),

The volumes of  $\alpha$ -synuclein crosspeaks increased with time, beginning with a lag phase before growing exponentially to a plateau (Fig 6A–E). One crosspeak deviated from this trend (Fig 6D), most likely because the poor resolution in this area of the spectrum (Fig 5D). Temporal changes in crosspeak volumes for two metabolites showed different trends. One metabolite remained constant (Fig 6F), while the other metabolite showed a time dependence that resembled the  $\alpha$ -synuclein crosspeaks (Fig 6G). The crosspeak from the defined minimal media, is the only crosspeak that showed a slight decrease in intensity with time (Fig 6H).

### 3. Discussion

The CEC bioreactor (Fig. 1) is designed to provide a controlled environment for NMR experiments involving living cells. It allows media to deliver nutrients and remove waste from encapsulated cells contained in a circulation chamber (Fig. 1, right panel). When the flow of media is stopped, the encapsulated cells settle allowing data acquisition (Fig. 1, left panel).

In the experimental setup, the CEC bioreactor is the only component located inside the spectrometer (Fig. 2). This configuration allows the external components to be altered without removing the bioreactor before or during the experiment, facilitating studies requiring different conditions in one experiment. The setup is also versatile. Different solution probes and sensors can be inserted between the external components. The tubing can be rerouted, for example, to send the media to a waste container. In addition, the material used to make the bioreactor can be changed for experiments requiring different isotopic nuclei detection. Here we use Teflon for  $^{15}\text{N}$  detection, but a Plexiglass bioreactor can be used for  $^{19}\text{F}$  NMR.

To our knowledge, the CEC bioreactor is the first bioreactor suitable for protein in cell NMR experiments (Fig. 3A,B). The design provides an environment where encapsulated cells can express protein while maintaining reasonably high quality in cell NMR spectra (Fig 3C–D). Furthermore, the bioreactor can be used to quantify temporal changes in crosspeak volumes during the experiment (Fig. 4 and 5).

Our data allow us to draw several conclusions. We showed that  $\alpha$ -synuclein was present at an intracellular concentration of 0.8 mM at 18 h. Using information from Figure 6, we conclude that the detection limit for in-cell NMR is approximately 0.14 mM. This finding is consistent with other work on the minimal intracellular protein concentration needed for in-cell NMR [6]. For most residues, the detection limit is achieved after 3 h. Two protein crosspeaks do not follow this trend in that they are not detectable until approximately 7 h (Fig. 6C,D). The crosspeaks from glycine residues that comprise the ear shaped pattern in the upper left region of  $\alpha$ -synuclein Figure 4 ( $^{15}\text{N}$  ppm 108–113,  $^1\text{H}$  ppm 8.3–8.7), follow a similar trend. The delay in detectability may be due to differential binding of  $\alpha$ -synuclein to other intracellular components, which broadens their crosspeaks. Another possibility for the delay is differential relaxation, because *in vitro* models for  $\alpha$ -synuclein dynamics show that certain residues experience less mobility [17]. Decreased mobility produces broader, weaker signals, which would explain the longer time required to detect them.

In summary, the CEC bioreactor provides a controlled environment where protein NMR spectra data can be acquired in living *E. coli* cells. Our next goal is to show that the CEC bioreactor is compatible with other cell types, and is versatile enough for metabolomic, as well as protein NMR experiments. We specifically want to focus on eukaryotic cells whose viability is adversely affected by current methods. Our long-term goal is to monitor temporal changes in protein structure and metabolism due to perturbations, such as drug interactions, in human cells, and so increase the understanding of intracellular components under physiological conditions.

## 4. Experimental

### 4.1 Purification of wild type $\alpha$ -synuclein for *in vitro* experiments

The pT7-7 plasmid containing the  $\alpha$ -synuclein gene was transformed into *E. coli* BI-21 (DE3) Gold cells (Stratagene). Plasmid containing cells were selected with 0.1 mg/mL ampicillin. A 5 mL overnight culture was grown from a single colony and used to inoculate a 50 mL culture of Spectra 9  $^{15}\text{N}$ -enriched media (Cambridge Isotope Laboratories) at 37°C in a rotary shaker (225 rpm, New Brunswick Scientific, Model I-26). The saturated overnight culture was used to inoculate 1 L of M9 minimal media [21] containing 1 g/L  $^{15}\text{NH}_4\text{Cl}$ . After reaching an absorbance at 600 nm ( $A_{600}$ ) of 0.8–1.0, the culture was induced with isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was placed in the rotary shaker (225 rpm) at 37°C.

After 5 h the cultures were pelleted using a swinging bucket centrifuge (Sorvall RC-3B, H6000A rotor) at 1600g for 30 min at 4°C and the pellet was stored at –20°C. The pellet was resuspended in 30 mL of lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 0.4 g/L lysozyme, pH 8.0). RNase and DNase were added to a final concentration of 0.02 g/L each. The samples were stirred (250 rpm) at 4°C for 20 min. The lysate was sonicated (Branson Ultrasonics, Fischer Scientific) continuously for 5 min, boiled in a water bath for 20 min, and then centrifuged at 13,000g for 30 min at 4°C (SS-34 rotor). The supernatant was subjected to streptomycin sulfate precipitation (10 g/L) and centrifuged for 30 min at 4°C. The supernatant was subjected to  $(\text{NH}_4)_2\text{SO}_4$  precipitation (361 g/L) and centrifuged again for 30 min at 4°C. The pellet was resuspended in 20 mM sodium phosphate buffer (pH 7.4) and dialyzed (Thermo Scientific, 3500 MWCO) overnight, with stirring at 4°C, against the same buffer.

The protein was further purified by anion exchange chromatography (GEHealthcare, Q Sepharose HiPrep 16/10 column) with a 0–1 M linear gradient of NaCl in 20 mM phosphate buffer (pH 7.4). Fractions were subjected to SDS-PAGE on an 18% gel with Coomassie Brilliant Blue staining. Fractions containing  $\alpha$ -synuclein were pooled and dialyzed against water overnight, with stirring, at 4°C. The protein was concentrated in a YM-3 Centricon filter (Millipore, MWCO 3500) using centrifugation at 1000g (SS-34 rotor) for 1 h at 4°C. The purity of the protein was determined by SDS-PAGE with Coomassie staining. The pure  $\alpha$ -synuclein was lyophilized (Labconco) and stored at –20°C. The yield was 35–60 mg of pure  $\alpha$ -synuclein per liter of saturated cell culture.

### 4.2 Cultivation of *E. coli* for in-cell NMR experiments

A 5 mL overnight culture was grown from a single colony and used to inoculate a 500-mL Erlenmeyer flask containing 50 mL of isotopically enriched media, as described above. After the culture reached an  $A_{600}$  of 0.8–1.0, the cells were induced with IPTG to a final concentration of 1 mM. Expression was allowed to proceed for 4 h. The cells were gently harvested by using the swinging bucket centrifuge for 30 min at 4°C. The pellet was resuspended in 1 mL of spent media.



### 4.3 Cultivation and encapsulation of *E. coli* for NMR bioreactor experiments

A 5 mL overnight culture was grown from a single colony as described above and used to inoculate 150 mL of Luria Broth (10 mg/mL Tryptone, 5 mg/mL yeast extract, 10 mg/mL NaCl) at 37°C. The culture was grown in the rotary shaker (225 rpm) to an  $A_{600}$  of 0.8–1.0. The cells were gently harvested in the swinging bucket centrifuge for 20 min at 4°C and resuspended in 1 mL of spent media. The resuspended cells were mixed with a 2% w/v alginate (Sigma) solution in 20 mM phosphate, 150 mM NaCl (pH 7.4) to give a final concentration of 1% alginate (50:50 mixture alginate:cell slurry).

The electrostatic encapsulation device (Fig. 7) comprised a 1 mL insulin syringe (BD), a 24 gauge winged angiocatheter (0.7 × 19 mm tip, Braun), a 23 gauge needle (BD), a syringe pump (Braintree Scientific 8000), and an adjustable high voltage power supply (Spellman SL10). The insulin syringe, equipped with the needle, was loaded with the cell/alginate mixture. The other needle, which was inserted horizontally through the center of the angiocatheter, was connected to the positive pole of the power supply. The negative pole of the power supply was placed into the 150 mM CaCl<sub>2</sub> solution. The syringe containing the mixture was inserted into the top of the angiocatheter and placed onto the pump. The syringe pump was set to a rate of 0.714 mL/min, the power supply voltage to 3.35 kV, and the stir-plate to approximately 300 rpm. The tip of the angiocatheter was centered 1.2 cm above a 250 mL beaker containing 150 mL of 150 mM CaCl<sub>2</sub>. The mixture was forced through the tip of the angiocatheter and streamed into the CaCl<sub>2</sub> solution. The Ca<sup>2+</sup> polymerizes the alginate which, in turn, forms encapsulated beads containing the cells. The encapsulated cells were retrieved with suction and placed in a 15 mL Falcon tube containing 150 mM CaCl<sub>2</sub> solution for transport to the NMR spectrometer.

The CaCl<sub>2</sub> solution was removed and the encapsulated cells were washed with the phosphate-free minimal medium. The phosphate-free minimal medium consisted of 100 mM HEPES (pH 7.4), 150 mM CaCl<sub>2</sub>, phosphate-free M9 salts [1 mg/mL <sup>15</sup>NH<sub>4</sub>Cl, 2 mM MgCl<sub>2</sub>, 1 μg/mL thiamine, 2 % v:v 10x <sup>15</sup>N-enriched Bioexpress 1000 media (Cambridge Isotope Laboratories)] and 0.1 mg/mL ampicillin. After washing, the encapsulated cells were placed inside the bioreactor, which was then placed into the spectrometer. After acquiring the initial spectrum, lactose was added to a final concentration of 1% w/v. The lactose acts as an inducer and the sole carbon source. For each spectrum, the pump circulated medium through the system at a rate of 45 mL/min for 30 min. Five min were allotted for the encapsulated cells to settle into the detection region of the bioreactor. As a control the procedure was repeated for *E. coli* containing the pUC18 plasmid.

### 4.4 NMR

Data were acquired at the UNC Biomeolcular NMR facility on a Varian Inova 600 MHz NMR spectrometer. Data were processed and visualized with NMRpipe and NMRviewJ, respectively [22;23].

Samples for dilute solution spectra comprised a 90:10 (v:v, pH 7.4) mixture of purified 200 μM α-synuclein solution: D<sub>2</sub>O in a standard 5 mm NMR tube. <sup>1</sup>H-<sup>15</sup>N HSQC spectra were acquired at 10°C [1] with a 5 mm Varian Triax triple resonance probe (<sup>1</sup>H sweep width: 11990.40 Hz; <sup>15</sup>N sweep width: 2100 Hz, 8 transients, 128 increments). Each spectrum required 35 min.

Samples for simple in-cell NMR experiments comprised 90:10 (v:v) mixture of resuspended cells: D<sub>2</sub>O in a standard 5 mm NMR tube. <sup>1</sup>H-<sup>15</sup>N HSQC [24;25] spectra were acquired as described above, except with 12 transients and 128 increments. Each spectrum required 1 h.

Samples for encapsulated in-cell NMR experiments comprised encapsulates in a 90:10 (v:v) mixture of 150 mM CaCl<sub>2</sub>: D<sub>2</sub>O in a standard 5 mm NMR tube. For encapsulates in standard

5 mm NMR tubes,  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were acquired as described above. In the bioreactor,  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were acquired unlocked at 37°C with an 8 mm modified Varian Triax triple resonance z gradient probe as described above. Samples for NMR bioreactor experiments comprised encapsulated cells in phosphate-free media supplemented with Bioexpress 1000.  $^1\text{H}$ - $^{15}\text{N}$  SOFAST HMQC spectra were acquired at 37°C as described above, except with 48 transients and 96 increments. The spectra were acquired unlocked due to the adverse effects of  $\text{D}_2\text{O}$  on cell growth and protein expression. Each spectrum required 35 min.

## Acknowledgments

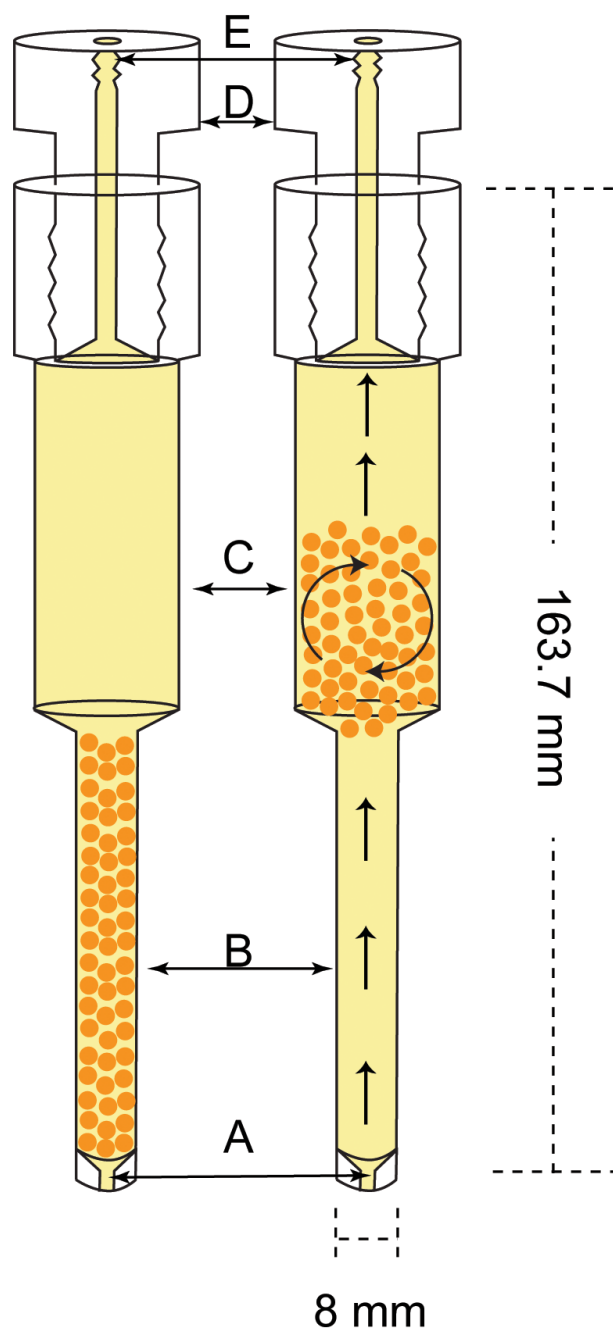
We thank Peter Lansbury, Jr., for the  $\alpha$ -synuclein expression system, Freddy Pinero of the UNC Chemistry Instrument Shop for fabricating the bioreactor and other small parts, Collin J. McKinney of the UNC Chemistry Electronics Facility for software support, Christopher Seagle for insightful input on the design of the bioreactor and the members of the Pielak laboratory for helpful comments on the manuscript.

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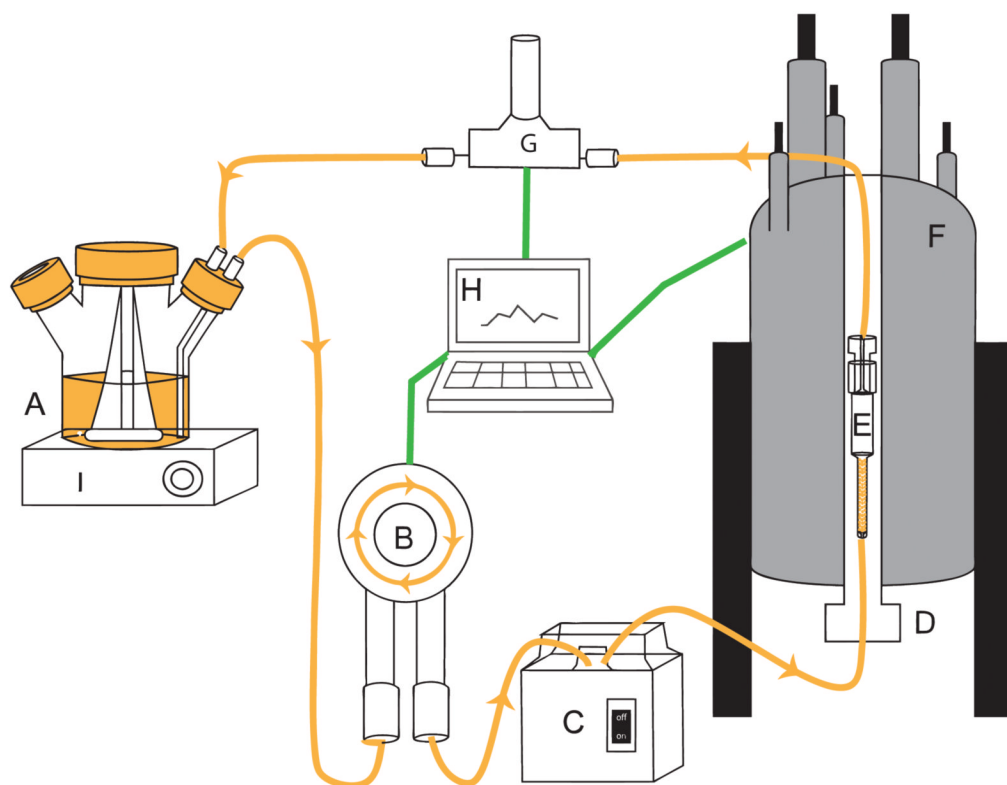
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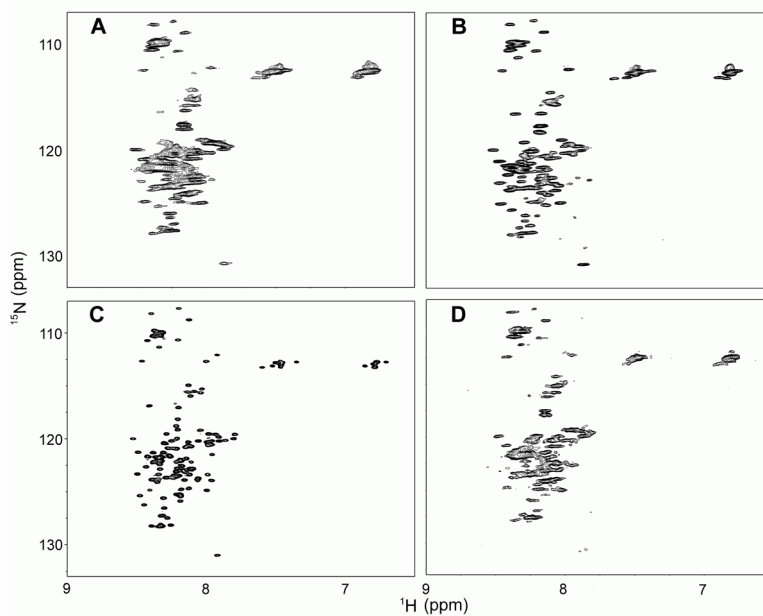




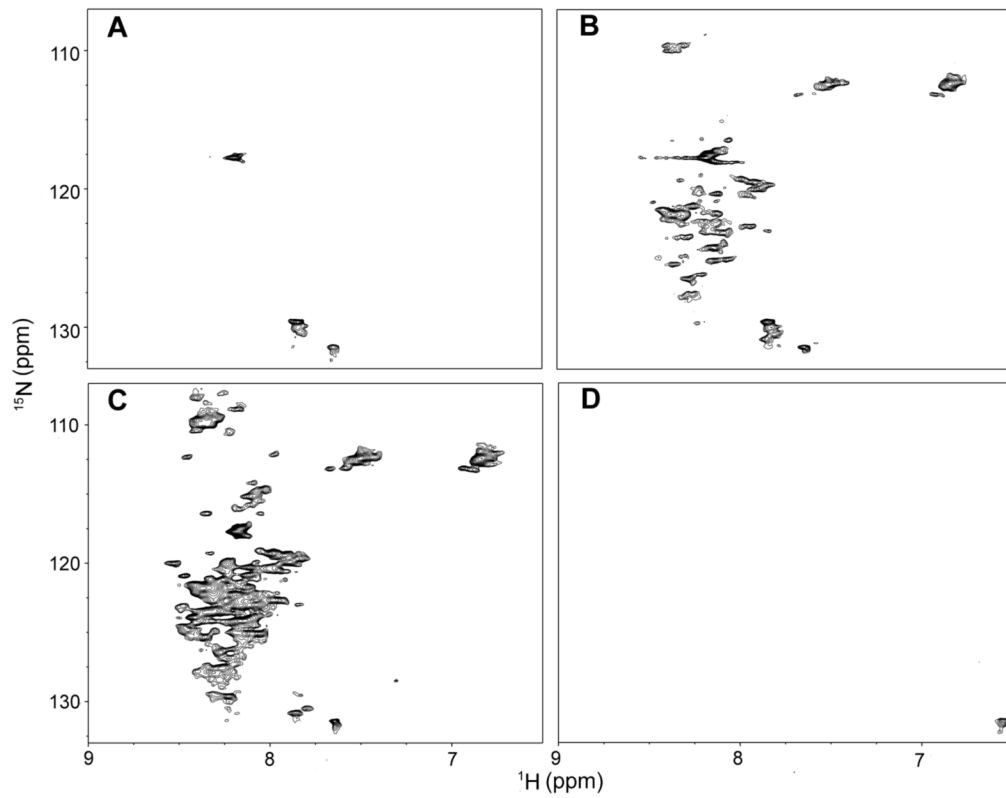
**Fig. 1.** The CEC bioreactor. On the left, the pump is off and the encapsulates are settled. On the right, the pump is on and the encapsulates circulate at a steady state in the upper chamber. A: tubing inlet, B: NMR detection region, C: circulation chamber, D: adjustable threaded cap, E: fitting inlet. Orange circles represent encapsulates containing *E. coli* cells.



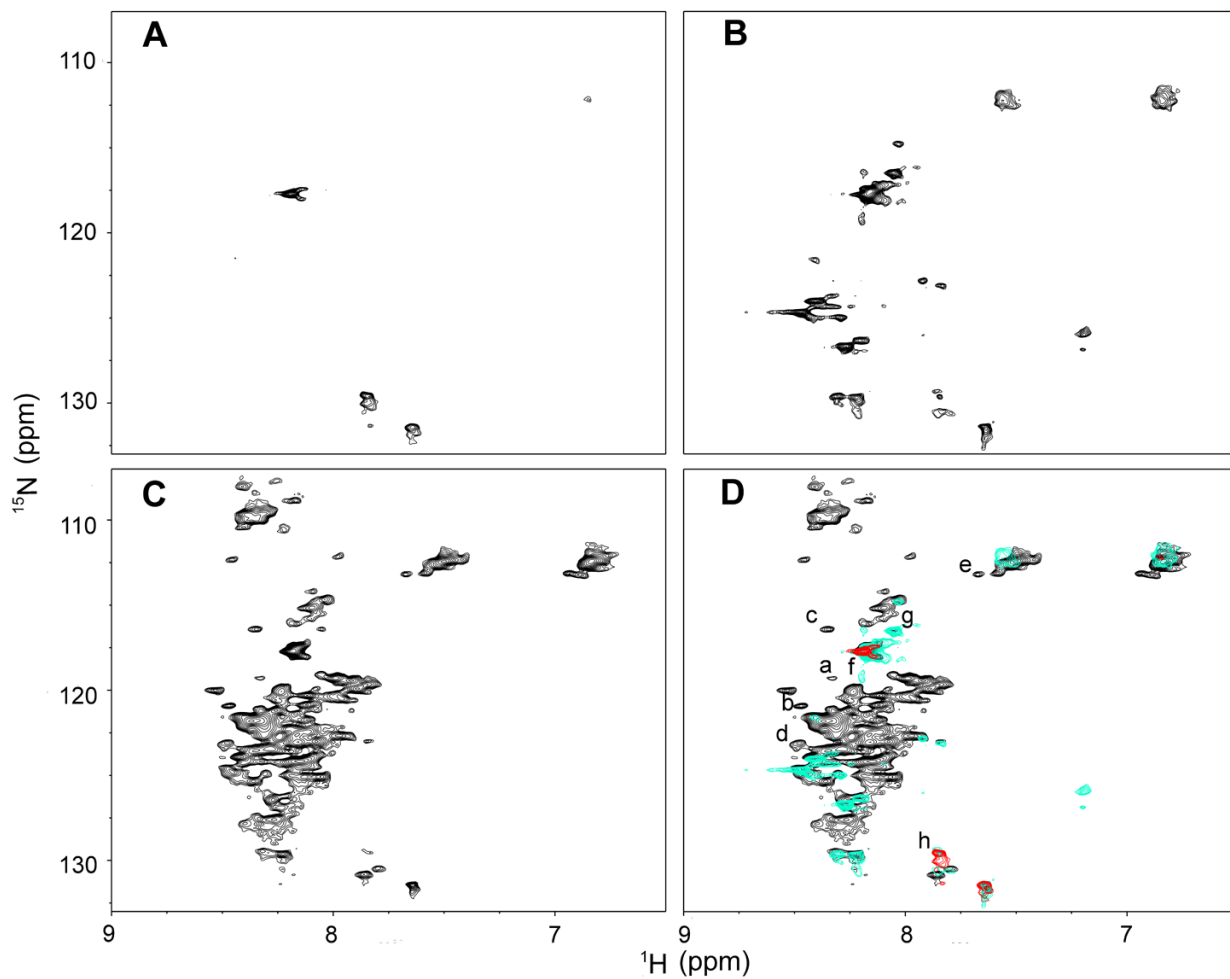
**Fig. 2.** The experimental set-up. A: Corning spinner flask fitted with a vented cap on one side arm and tubing adaptors on the other, B: peristaltic pump, C: water bath, D: 8 mm probe with heater removed, E: bioreactor, F: magnet, G: pH probe, H: computer, I: stir plate.



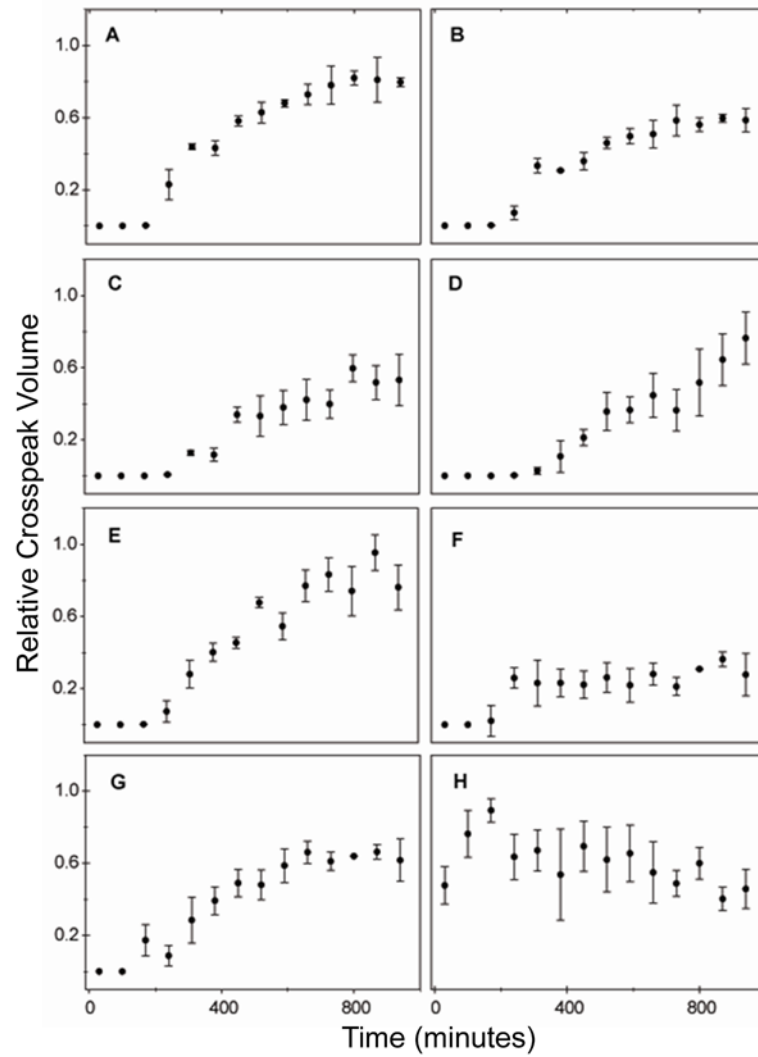
**Fig. 3.** Comparing  $\alpha$ -synuclein spectra. A) In cell HSQC spectrum of alginate encapsulated *E. coli* expressing  $\alpha$ -synuclein in the bioreactor. B) In cell HSQC spectrum of *E. coli* expressing  $\alpha$ -synuclein. C) *In vitro* HSQC spectrum of 200  $\mu$ M purified wild type  $\alpha$ -synuclein in HEPES buffer, pH 7.2 at 10°C D) In cell HSQC spectrum of alginate encapsulated *E. coli* expressing  $\alpha$ -synuclein. The spectra shown in panels A, B & D were acquired at 37°C. The spectra in panels B–D were acquired in a 5 mm NMR tube using a 5 mm probe. The spectrum in panel A was acquired in the 8 mm bioreactor using an 8 mm probe.



**Fig. 4.** In-cell SOFAST  $^{15}\text{N}$ - $^1\text{H}$  HMQC [12] spectra (37°C) of *E. coli* expressing  $\alpha$ -synuclein in the bioreactor. A) Spectrum collected before induction. B) 4 h post induction. C) 18 h post induction. D) Spectrum of the spent medium.

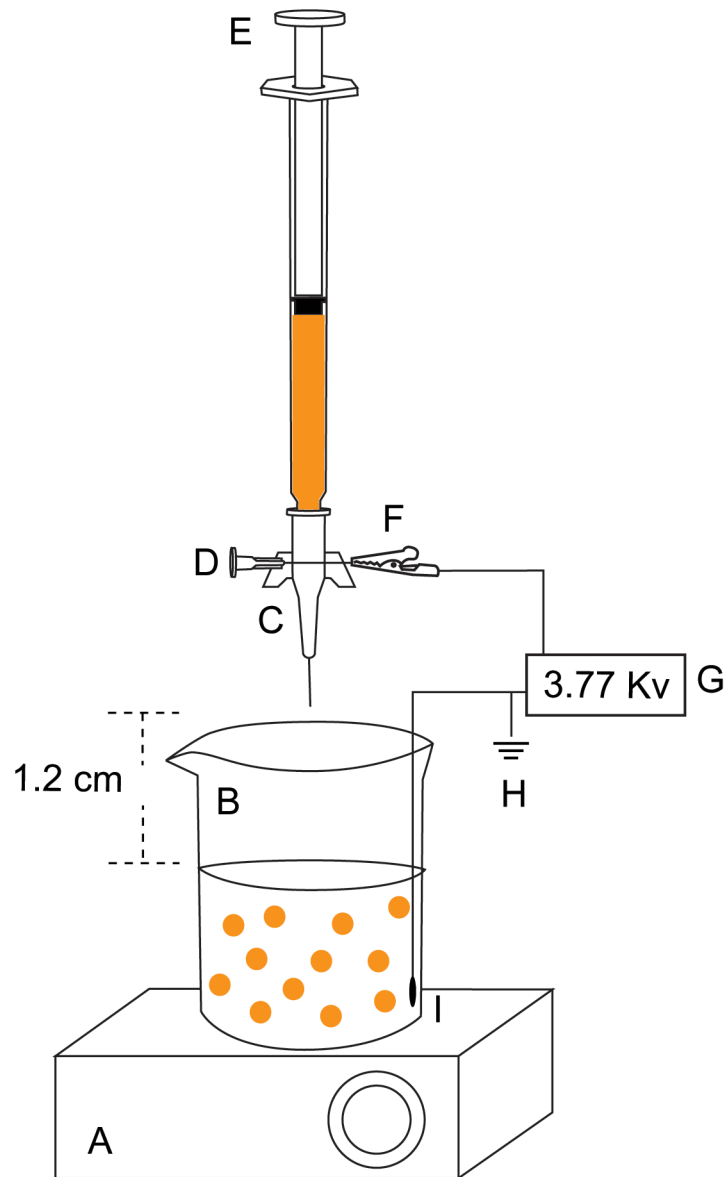


**Fig. 5.** Determining which crosspeaks correspond to  $\alpha$ -synuclein. A) In-cell SOFAST  $^{15}\text{N}$ - $^1\text{H}$  HMQC spectrum of the defined phosphate-free minimal media. B) Spectrum of  $^{15}\text{N}$  enriched encapsulated *E. coli* cells containing the control pUC18 plasmid. C) Spectrum of encapsulated *E. coli* expressing  $\alpha$ -synuclein. D) Overlay of the spectra [medium (red), puc18 control cells (cyan), and  $\alpha$ -synuclein (black)]. Crosspeaks used in subsequent analysis are labeled a-h. Spectra were acquired in the 8 mm bioreactor using an 8 mm probe at 37°C.



**Fig. 6.** Temporal changes in crosspeak volume after inducing  $\alpha$ -synuclein expression in the bioreactor. A–E)  $\alpha$ -Synuclein crosspeaks, F–G) Metabolite crosspeaks. H) Crosspeaks from the defined phosphate free minimal media. Crosspeak volumes are normalized to the largest volume and are labeled in Figure 5D. Error bars represent the standard error from three independent experiments.





**Fig. 7.** Schematic of the electrostatic encapsulation device. A: stir plate, B: beaker containing 150 mM  $\text{CaCl}_2$ , C: anion catheter, D: needle, E: insulin syringe, F: alligator clip (connects the positive power supply terminal to the needle) G: high voltage power supply, H: ground, I: negative end