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Rapid Distinction of Intracellular and Extracellular Proteins Using NMR Diffusion Measurements

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Supporting Information

ABSTRACT: In-cell NMR spectroscopy offers a unique opportunity to begin to investigate the structures, dynamics, and interactions of molecules within their functional environments. An essential aspect of this technique is to define whether observed signals are attributable to intracellular species rather than to components of the extracellular medium. We report here the results of NMR measurements of the diffusion behavior of proteins expressed within bacterial cells, and find that these experiments provide a rapid and non-destructive probe of localization within cells and can be used to determine the size of the confining compartment. We show that diffusion can also be exploited as an editing method to eliminate extracellular species from high-resolution multidimensional spectra, and should be applicable to a wide range of problems. This approach is demonstrated here for a number of protein systems, using both ^{15}N and ^{13}C (methyl-TROSY) based acquisition.

NMR spectroscopy, in conjunction with specific isotope labeling methods, is a powerful approach for the direct observation of biological molecules such as proteins within their natural and highly complex cellular environments, as well as in the dilute solutions commonly studied under laboratory conditions. In recent years, for example, a number of studies have succeeded in characterizing at the atomic level the structures and properties of proteins within cells.[1,2] A fundamental requirement of all in-cell NMR studies is to determine unequivocally whether specific resonances in a given spectrum originate from species that are localized within the cell rather than in the extracellular medium. As in most cases the chemical shifts of intracellular and extracellular species are likely to be very similar, this exercise is in general nontrivial; indeed, the most common approach at present is simply to centrifuge the sample and to compare spectra of the supernatant with those from the original sample.[3] A non-destructive alternative is highly desirable, and we report here a simple approach based on pulsed-field gradient NMR diffusion measurements that can provide the required information in a very short time, typically less than 2 min.

NMR spectroscopy using pulsed-field gradients provides a powerful method for the characterization of molecular diffusion,[4] and, for example, has been applied to study the diffusion of proteins within cell lysates and other crowded solutions in an effort to develop an understanding of the physical processes controlling macromolecular transport within the cell.[5] Diffusion may also be used as a means of spectral editing, for example, to suppress the resonances of small molecule metabolites from spectra of macromolecules within cell lysates.[6] However, within intact cells, the measurement of protein diffusion has been dominated by a range of fluorescence methods.[7,8] Such measurements have demonstrated that the effective viscosity of the eukaryotic cytosol is typically 3−4 times greater than that of water,[9] while in the highly crowded bacterial cytosol, containing 300−400 mg mL^{-1} of macromolecules,[10] translational diffusion of proteins is reduced by approximately an order of magnitude relative to dilute aqueous solution.[11]

An essential difference between in-cell studies and conventional solution studies is that the diffusion of any molecule under observation is restricted to a much smaller volume, by being confined within a cell or a cellular compartment. In the specific case of *Escherichia coli*, the system studied in this paper, the total cellular volume corresponds to approximately 0.5 fL. On the time scale of NMR diffusion measurements, the consequence of this confinement will be a reduction in the apparent diffusion coefficient, suggesting that diffusion-edited NMR experiments could be used to distinguish between intracellular and extracellular species.

In this communication, we first explore this approach using the intrinsically disordered protein α-synuclein (αSyn), which has previously been reported to give well-resolved NMR resonances when expressed within *E. coli* cells.[12] ^{15}N-edited stimulated-echo (STE) diffusion experiments[13] of such samples were recorded here using a long diffusion delay, Δ, of 300 ms, in order to maximize the contrast between slowly and rapidly diffusing species. The signal intensities $I(G)$ for a sample of cells expressing αSyn, and for purified αSyn added separately to the extracellular medium of a sample of cells, are plotted in

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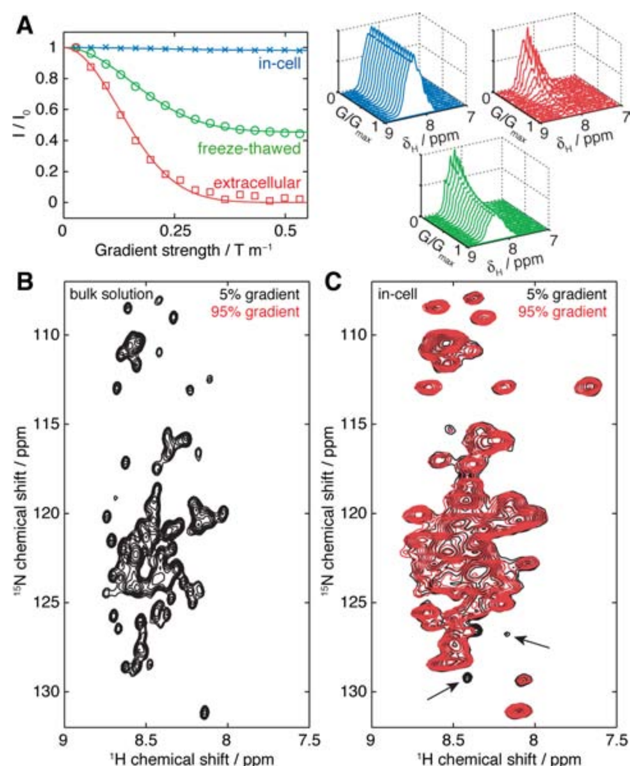

Figure 1. (A) 300 ms ^{15}N-edited XSTE diffusion NMR measurements[13] of cells in which αSyn has been expressed (blue), cells partially lysed following freeze−thawing with liquid N_2 (green), and purified αSyn added to the extracellular medium of a sample of cells (red). (B, C) Diffusion-edited (300 ms XSTE) HSQC NMR spectra[15] of (B) purified αSyn in bulk solution, and (C) cells expressing αSyn. The gradient strength used in each experiment is indicated relative to the maximum accessible gradient strength of 0.55 T m^{-1}. Arrows highlight resonances of extracellular species that are absent at higher gradient strengths.

Figure 1A, and are fitted to the Stejskal-Tanner equation[14] to determine the apparent diffusion coefficient, D_{app}:

$$I(G) = I(0)\exp[-\gamma^2 G^2 \delta^2 s^2 (\Delta - \delta/3 - \tau/2) D_{app}] \quad (1)$$

where $I(0)$ is the signal intensity in the absence of gradients, γ is the magnetogyric ratio of the proton, δ is the gradient pulse length, s is the gradient shape factor, τ is the delay between bipolar gradients, and G is the gradient strength.

The intensity of the resonances of cell samples at the maximum gradient strength applied here, $G = 0.55$ T m^{-1}, was observed to be 97 ± 1% of that in the absence of the gradient, with a fitted value of $D_{app} = 4.6 \pm 0.8 \times 10^{-13}$ m^2 s^{-1}. By contrast, resonances from exogenous αSyn are effectively completely attenuated at this maximum gradient strength. The measured value of $D = (1.2 \pm 0.1) \times 10^{-10}$ m^2 s^{-1} is only slightly reduced relative to that observed for αSyn in bulk solution, $D = (1.3 \pm 0.1) \times 10^{-10}$ m^2 s^{-1}, which is itself comparable to values from previous measurements and corresponds to a hydrodynamic radius of 25 ± 2 Å.[16] This result reveals that intracellular and extracellular protein molecules can be distinguished clearly as their apparent diffusion coefficients differ by almost 3 orders of magnitude. Each experiment in the present study was acquired within 6 min, and this time could be reduced to under a minute by omitting intermediate gradient points.

The large difference in the apparent diffusion coefficients for a protein inside and outside the cell provides an opportunity to use pulsed-field gradients to dephase selectively the resonances of rapidly diffusing extracellular species and so to use diffusion-edited experiments to observe intracellular species exclusively. This strategy is illustrated in Figure 1B,C, where diffusion-edited HSQC spectra[15] have been recorded of purified αSyn in bulk solution (Figure 1B) and of αSyn expressed within *E. coli* cells (Figure 1C). The sensitivity of these measurements was found to be approximately 40% of that of a conventional HSQC experiment, due in part to an increase in relaxation that occurs during the longer pulse sequence, but predominantly to the 50% loss of signal inherent in all stimulated echo experiments, as only a single transverse magnetization component can be stored longitudinally during the diffusion delay.

To validate further this approach, additional in-cell samples were prepared and subjected to partial lysis induced by freeze−thawing with liquid N_2. The maximum attenuation of the stimulated echo was observed to be 55 ± 2% (Figure 1A), indicating that approximately half of the protein had escaped from the cell. This value compares to 51 ± 1% determined from analysis of the supernatant following centrifugation and shows the NMR method to be highly robust, at least for the bacterial cells studied in this communication.

We have also investigated the application of diffusion-edited methods to the observation of two representative folded proteins expressed within the cell. TTHA1718 ('TTHA') is a 66-residue folded protein that has been shown to give well-resolved HSQC spectra when expressed within the cell.[2] We find here that well-resolved diffusion-edited HSQC spectra may also be acquired for such samples (Figure 2A). As was the case

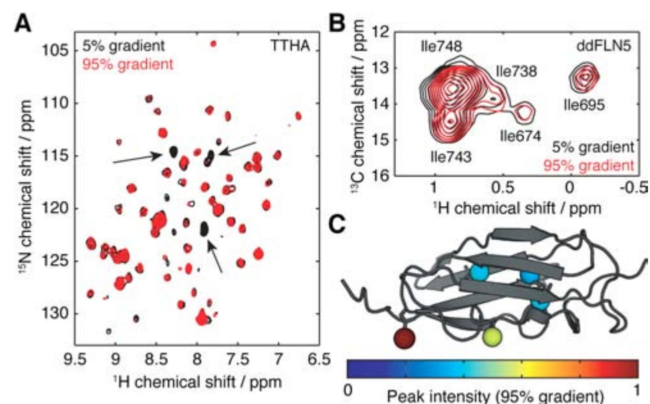

Figure 2. (A) Diffusion-edited (300 ms ^{15}N XSTE) HSQC NMR spectra of TTHA expressed within cells. The gradient strength used in each experiment is indicated relative to the maximum accessible gradient strength of 0.55 T m^{-1}. Arrows highlight resonances of extracellular species that are absent at higher gradient strengths. (B) Diffusion-edited (300 ms STE) ^{13}C HMQC (methyl-TROSY) NMR spectra of [Ile-$^{13}CH_3$]-ddFLN5 expressed within cells. (C) Crystal structure of ddFLN5 (pdb 1QFH) showing isoleucine Cδ groups, colored according to the peak intensity observed within the cell.

for αSyn, a number of resonances are absent at higher gradient strengths and therefore can be attributed to extracellular species (Figure 2A, arrows). However, in our hands we also found that TTHA samples showed higher levels of leakage than was typically observed for αSyn: the signal intensity at the maximum gradient strength was 81 ± 3% of that in the

absence of the gradient, indicating that approximately 19% of the protein was present outside the cell in this experiment. In such circumstances, diffusion-edited acquisition is particularly valuable in permitting the exclusive observation of intracellular species.

The second folded protein we examined is ddFLN5, a 113-residue immunoglobulin domain from the *Dictyostelium discoideum* gelation factor ABP-120, currently under study within our group as a ribosome-nascent chain complex for the investigation of cotranslational folding.[17,18] However, in contrast to the smaller TTHA molecule, resonances of ddFLN5 could not be observed in $^{1}H-^{15}N$ HSQC spectra of cell samples (Figure S1), indicating the presence of rapid transverse relaxation that may result from the viscosity of the cytosol, and also potentially from specific or nonspecific interactions with other components of the cell.[19,20] We therefore prepared deuterated cell samples with selective $^{13}CH_3$ labeling of isoleucine Cδ methyl groups, in order to utilize the methyl-TROSY effect to reduce the consequences of transverse relaxation.[21] Methyl-TROSY methods were also combined with STE diffusion measurements[22] which, as illustrated for αSyn, may be acquired rapidly in a 1D manner to measure echo attenuations. In addition, diffusion-edited $^{1}H-^{13}C$ HMQC spectra were acquired, and are shown in Figure 2B for a sample of cells in which isoleucine-labeled ddFLN5 has been expressed. All ddFLN5 isoleucine resonances were observed, with identical chemical shifts to those of the protein in bulk solution (Figure S2) indicating that no major structural changes have occurred within the cell. Peak intensities at the maximum gradient strength were 83 ± 2% of those in the absence of the gradient, indicating that approximately 17% of the protein was present outside of the cell.

Significant residue-to-residue variation was observed in the intensities of resonances in the diffusion-edited spectrum of ddFLN5 (Figure 2B). By contrast, intensities in the spectrum of the protein in bulk solution are close to uniform (Figure S2). To investigate this broadening effect further, the relative peak intensities were projected onto the structure of the protein (Figure 2C), from which it may be observed that the greatest intensities in the diffusion-edited spectrum are found for residues on the surface of the protein. The intensities are inversely correlated with predicted methyl S^2 order parameters[23] (r^2 = 0.90, Figure S3) and, given the slow rotational diffusion within the crowded cytosol, this suggests that the extent of local mobility on ps−ns time scales may be an important factor in determining the observability of intracellular material. However, interactions between the protonated methyl groups within the core of the protein might also contribute significantly to the observed relaxation. Future work, combining diffusion-edited acquisition with spin relaxation measurements, has the potential to enable these effects to be interpreted in more detail.

The apparent diffusion coefficient determined by NMR measurements is related to the mean square displacement during the diffusion delay, $D_{app} = \langle Z^2\rangle/2\Delta$. For an intracellular species, $\langle Z^2\rangle$ is limited by the size of the cell, and the apparent diffusion coefficient may therefore provide information on the size of the confining compartment. To investigate the possibility of obtaining such information, in a further series of experiments, we recorded a series of diffusion measurements using a probe (Bruker Diff30) that is able to provide a gradient strength of up to 11.7 T m^{-1}.

This approach provided larger echo attenuations, allowing the determination of the apparent diffusion coefficients of intracellular αSyn. ^{1}H spin−echo experiments were recorded for four values of Δ from 11 to 20 ms, and echo attenuations of up to 90% were observed in all cases (Figure 3). These

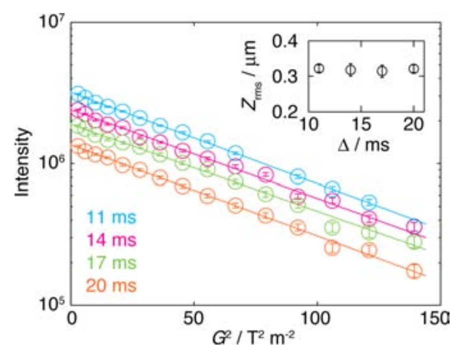

Figure 3. ^{1}H spin−echo measurements (δ = 2 ms) of an in-cell sample of αSyn, with the diffusion time, Δ, varied between 11 and 20 ms as indicated. Data are fitted to eq 1, and (inset) the rms displacements, $Z_{rms} = (2D_{app}\Delta)^{1/2}$, are plotted as a function of Δ.

attenuations were independent of Δ, indicating that diffusion is restricted, and fitting the data to eq 1 indicates rms displacements of 0.33 ± 0.02 μm (Figure 3, inset). This is comparable in magnitude to an rms displacement of 0.47 μm estimated from Brownian dynamics simulations[24] for a compartment size of 0.5 × 0.5 × 2 μm, typical for an *E. coli* cell. Thus, the characterization of restricted diffusion provides not only the ability to quantify intracellular localization, but also to provide an estimate of the size of the confining compartment. We note that we do not observe diffusion-diffraction effects in these measurements, for example, as previously observed for water diffusing in red blood cells.[25] This finding may reflect a combination of heterogeneity in the cell size and lack of alignment within the sample, and spatial averaging occurring during the finite gradient pulse length, resulting in shallower diffraction minima.[26]

Finally, we consider the role of diffusion measurements in the study of other types of cells. The diffusion-editing methodology described in this communication is particularly effective due to the effect of restricted dimensions on the apparent diffusion coefficient. However, the magnitude of this enhancement decreases when the cell size becomes comparable to the rms displacement occurring during the diffusion measurement, and simple estimates suggest that the effects described here for *E. coli* cells are not likely to be significant at length scales of 10 μm and above, for example, for mammalian cells and particularly for *Xenopus laevis* oocytes used previously for in-cell NMR studies.[27,28] However, it should remain possible to distinguish intracellular from extracellular species via the slower diffusion in the cytoplasm. More importantly, restricted diffusion is likely to be a key probe for observing the confinement of species to compartments or organelles within the cell.

In summary, diffusion measurements are a powerful component of the biomolecular NMR toolkit, with diverse applications including the study of protein aggregation[29,30] and large macromolecular complexes such as the ribosome and ribosome nascent chain complexes.[17,31] We have shown in this communication that diffusion experiments also provide a means of rigorously identifying and selectively observing protein

molecules within the living cell. We believe that such methods are widely applicable, and are able to provide important information in the emerging field of in-cell NMR.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and methods and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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