Spatially resolved DNP-assisted NMR illuminates the conformational ensemble of α-synuclein in intact viable cells.

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ABSTRACT

The protein α-syn adopts a wide variety of conformations including an intrinsically disordered monomeric form and an α-helical rich membrane-associated form that is thought to play an important role in cellular membrane processes. However, despite the high affinity of α-syn for membranes, evidence that the α-helical form of α-syn is adopted inside cells has thus far been indirect. In cell DNP-assisted solid state NMR on frozen samples has the potential to report directly on the entire conformational ensemble. Moreover, because the DNP polarization agent can be dispersed both homogenously and inhomogenously throughout the cellular biomass, in cell DNP-assisted solid state NMR experiments can report either quantitatively upon the structural ensemble or can preferentially report upon the structural ensemble with a spatial bias. Using DNP-assisted MAS NMR we establish that the spectra of purified α-syn in the membrane-associated and intrinsically disordered forms have distinguishable spectra. When the polarization agent is introduced into cells by electroporation and dispersed homogenously, a minority of the α-syn inside HEK293 cells adopts a highly α-helical rich conformation. Alteration of the spatial distribution of the polarization agent preferentially enhances the signal from molecules nearer to the cellular periphery, thus the α-helical rich population is preferentially adopted toward the cellular periphery. This demonstrates how selectively altering the spatial distribution of the DNP polarization agent can be a powerful tool for preferential reporting on specific structural ensembles, paving the way for more nuanced investigations into the conformations that proteins adopt in different areas of the cell.

INTRODUCTION

In-cell structural biology enables the study of protein conformation in environments that maintain the identity, stoichiometry, concentrations and organization of the myriad of biomolecules that can interact with a protein of interest.(1-4) Nuclear magnetic resonance (NMR) is uniquely suited to study proteins in these complicated contexts.(2, 5-8) NMR has the resolution and specificity to study atomic-level protein conformations of isotopically-labeled proteins in complex environments that contain molecules with a wide range of molecular sizes and dynamic properties.

Both solution and solid states NMR are well-suited to investigate molecules inside cells and can provide highly complementary insights. Solution state NMR excels in the

characterization of small, dynamic biomolecules. However, it is less well-suited for larger, slower moving biomolecules because the formation of protein complexes and/or interactions with membranes results in signal attenuation. In contrast, magic angle spinning (MAS) solid state NMR, particularly when performed under cryogenic conditions, can report directly on the entire conformational ensemble – including protein-protein complexes and proteins that associate with membranes(9-11). While solid state NMR has historically been limited by experimental sensitivity, with the sensitivity gains conferred by dynamic nuclear polarization (DNP), solid state NMR has the sensitivity to detect proteins at their endogenous concentrations(1, 12-16). Thus, DNP-assisted MAS solid state NMR is particularly well-suited to directly investigate the conformations of proteins in cellular settings because it can report directly on the ensemble of sampled protein conformations, including the conformations for proteins in complex with membranes or other cellular constituents. We recently developed protocols for in cell DNP-assisted NMR that result in efficient DNP enhancements and are compatible with cellular viability(17-20).

Because DNP increases the sensitivity of NMR spectroscopy through the transfer of the large spin polarization of an unpaired electron to nearby nuclei(21), the sensitivity enhancements from DNP rely upon proximity to the polarization agent.(14) Thus, DNP-enhanced MAS NMR experiments are biased towards observation of molecules that are accessible to polarization agents. These polarization agents are typically introduced into a sample by doping with millimolar concentrations of stable biological radicals (22-24). In our recent work describing methods for DNP MAS NMR on intact viable cells (19, 25), we examined two of many potential approaches to deliver polarization agents to intact cells. In that work, we introduced the polarization agent, AMUPol (24), to cells by electroporation of intact cells in the presence of AMUPol and by incubation of intact cells with AMUPol and compared the distribution of the polarization agent throughout the cellular biomass for cells (25). AMUPol was homogenously distributed inside cells when it had been introduced by electroporation. Thus, data from experiments on such samples report quantitatively on the entire structural ensemble. In contrast, AMUPol was inhomogeneously distributed in cells when it was delivered by incubation; the signal intensity from DNA in the nucleus was lower than the signal intensity from proteins and RNA in the cytoplasm. Thus, data from experiments on such samples report qualitatively, not quantitatively, on the structural ensemble. Any observed conformation in such samples exists,

but the population of that conformation relative to any other cannot be determined directly from integrated peak intensities. Collectively, this work indicated DNP-assisted MAS NMR could be used both to understand the conformational ensemble of a protein and to uncover spatial biases in the distribution conformations of a protein throughout a cell.

The protein α-syn adopts a wide variety of conformations which include an α-helical rich membrane-associated form that is thought to play an important role in cellular membrane processes(26) and an intrinsically disordered monomeric form that is the dominant form in solution (27-29). Purified α-syn can be introduced into cultured mammalian cells by electroporation and remains uniformly distributed throughout the cell for days (30). Initial solution state NMR studies of α-syn inside cells indicated that it was a compact intrinsically disordered monomer (30). Surprisingly, despite the high affinity of α-syn for membranes in purified settings (31), there was no evidence of membrane-associated α-syn inside cells (30). A subsequent in-cell solution state NMR investigation of α-syn found that the signal attenuation observed at the first 12 amino acids of α-syn resulted from transient interactions of α-syn inside cells with chaperone proteins (2). Moreover, they found that chaperone association and membrane-binding of α-syn are mutually exclusive. Reduction of the cellular chaperone levels resulted in co-localization of α-syn with mitochondria and signal attenuation of the first 90 amino acids of α-syn inside cells(2), a signature of membrane-associated α-syn (32-34). While this strongly suggests that α-syn can adopt an α-helical rich membrane-associated form inside cells, α-helical rich conformations were not directly observed; their presence was only inferred from the pattern of signal attenuation. Moreover, the signal attenuation pattern was only observed in the setting of a cell with a highly perturbed chaperone environment. Thus, the conformations that α-syn adopts in cells remain poorly understood, and the α-helical rich form, which is thought to be the functional conformation, has not yet been directly observed in unpertubed cells.

In this work, we introduce α-syn to HEK293 cells, freeze the cells and directly assess the entire conformational ensemble using DNP-assisted MAS NMR. Using purified samples, we find that the membrane-associated α-helical rich conformation and the intrinsically disordered conformation are easily distinguished by their chemical shifts under DNP conditions. Using these two spectra, we model the conformational ensemble of α-syn inside cells as a linear combination of the membrane-associated α-helical rich conformation and intrinsically disordered conformation of α-syn to quantify the population of α-helical rich α-syn present in healthy cells.

Furthermore, because delivery of the polarization agent by electroporation enables quantitative assessment of the entire structural ensemble while delivery of the polarization agent by incubation favors peripheral regions, we determine whether the distribution of α-syn conformations is uniform throughout the cell or if the distribution of conformations has a spatial bias.

RESULTS

To determine if the α-helical rich nanodisc-associated form and the frozen intrinsically disordered monomeric form of α-syn could be distinguished under the experimental conditions required for efficient DNP-enhanced experiments, we collected two-dimensional ^{13}C-^{13}C correlation spectra of uniformly ^{13}C labeled α-syn associated with nanodiscs and frozen in solution. To do so, we compared the peak shapes and centers for the glycine C^{α}-C', non-glycine carbonyl C^{α}-C', alanine C^{α}-C^{β} and threonine C^{β}-C^{γ} regions because cross peaks for those sites are distinguished by 10 ppm or more from other cross peaks. For nanodisc-associated α-syn, the peak centers of these regions were consistent with α-helical chemical shift value (Figure 1, red; Table S1). For frozen monomeric α-syn, as expected(35), these regions had composite peaks that spanned a wide range of chemical shifts (Figure 1, blue), and the central values of these broad peaks were consistent with random coil values (Table S1). The peak centers of the α-helical-rich nanodisc-associated form of α-syn and the frozen intrinsically disordered monomeric forms of α-syn differ by an average of 2 ppm. These two forms are distinguishable by ^{13}C-^{13}C correlation spectroscopy by both peak center and shape under DNP conditions (Figure 1, left column).

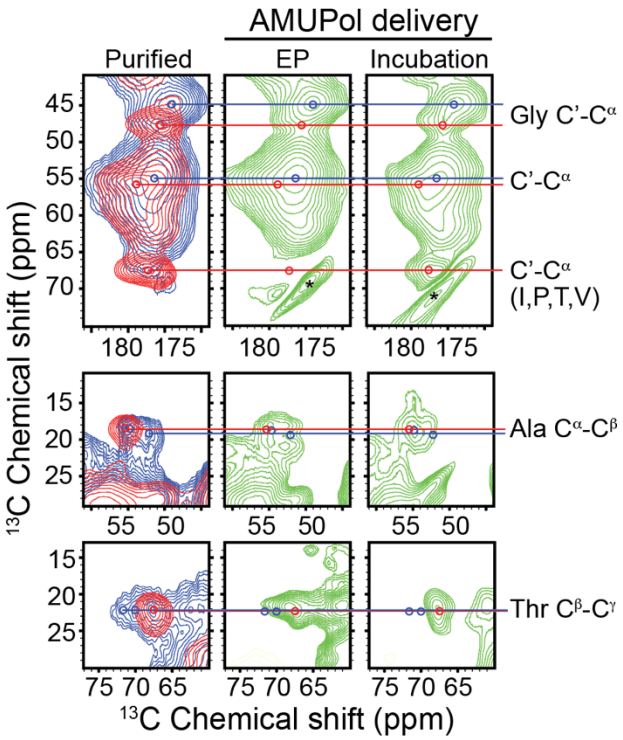

***Figure 1**: Purified samples of uniformly ^{13}C labeled α-syn in the nanodisc-associated form (red) and the frozen intrinsically disordered form (blue) were distinguished by ^{13}C-^{13}C correlation spectroscopy (DARR, 20 ms mixing) under DNP conditions (left column). The spectra of 75 µM ^{13}C labeled α-syn inside intact HEK293 cells where the polarization agent AMUPol was introduced by electroporation (center column) resembles the spectra of the purified intrinsically disordered monomer while the spectra of 75 µM a-syn inside cells where AMUPol was introduced by incubation in 10 mM AMUPol (right column) shares features with the nanodisc associated form. Peak centers for the nanodisc-associated form are annotated with a red circle and peak centers for the frozen monomer are annotated with a blue circle. Spinning side bands are marked with an *. All spectra were recorded at 600 MHz with 12 kHz MAS at 104 K.*

To assess the structural ensemble of α-syn inside HEK293 cells, we introduced uniformly ^{13}C labeled α-syn into HEK293 cells and then collected ^{13}C-^{13}C correlation spectra under DNP conditions. We added isotopically enriched α-syn to HEK293 cells using established protocols resulting in a 75 µM intracellular concentration of α-syn (Figure S1) then prepared the cells for analysis by DNP NMR (25). A concentration of 75 µM is double the endogenous concentration of α-syn in neurons and similar to the endogenous concentration of chaperone proteins in cells (36). The introduced α-syn did not form of puncta on the timescale of this experiment (Figure 4). To assess the conformational ensemble of α-syn in a spatially unbiased manner, we delivered the polarization agent AMUPol by electroporation to homogenously disperse AMUPol throughout the cellular biomass(25). The ^{13}C-^{13}C correlation spectra of cells containing uniformly

isotopically enriched α-syn inside cells had broad peaks. The peak shapes and centers for the glycine C^{α}-C', non-glycine carbonyl C^{α}-C', alanine C^{α}-C^{β} and threonine C^{β}-C^{γ} regions were most consistent with those of a frozen region of intrinsic disorder (Figure 1, middle column, Table S1). Thus, while neither the presence nor the absence of α-helical rich form of α-syn can be ruled out, the conformation of α-syn inside cells is most consistent with that of an intrinsically disordered monomer.

When AMUPol is delivered to cells by incubation, the distribution of AMUPol through the cellular biomass is heterogenous and the resulting NMR spectrum is biased towards the cellular periphery and away from the nucleus (25). To determine whether the conformational ensemble of α-syn inside cells has a spatial bias, we prepared cells as above but delivered AMUPol by incubation rather than electroporation. When cells containing 75 µM uniformly isotopically enriched α-syn were incubated with AMUPol, the peaks in the ^{13}C-^{13}C correlation spectra were consistent with those of both α-helical and intrinsically disordered conformations (37) (Figure 1, right column, Table S1). For example, the glycine and backbone C'-C^{α} region had maxima with peak centers consistent with those of both nanodisc-associated and intrinsically disordered α-syn (Figure 1, right column). Thus, α-syn adopts at least two distinct conformations inside HEK293 cells; an α-helical rich form and an intrinsically disordered form. Moreover, the strong α-helical chemical shifts in this sample suggest that the α-helical rich form may be preferentially adopted in the peripheral regions of the cytoplasm.

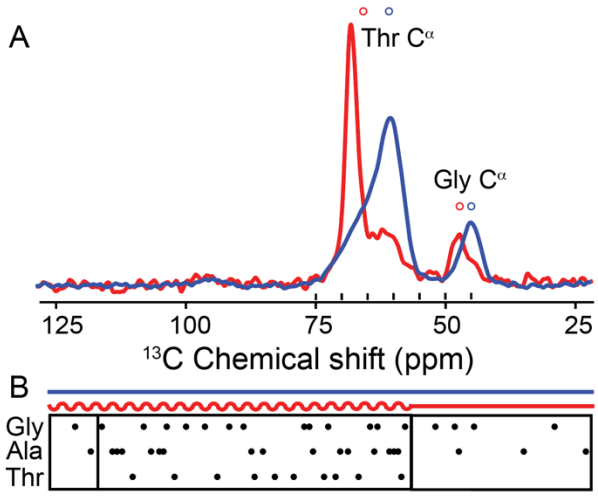

***Figure 2**: A) α-syn in the nanodisc-associated α-helix rich (red) and frozen monomeric (blue) forms are differentiated by ^{15}N-filtered ^{13}C spectra collected under DNP conditions on*

specifically threonine labeled samples. Forward labeling with threonine resulted in ~10% isotope scrambling to glycine. Open circles indicate database chemical shifts for α-helical (red) and random coil (blue) conformations for both threonine and glycine C^{α}. B) Cartoon representation of the primary sequence of α-syn with the predicted regions of intrinsic disorder (lines) and α-helices (squiggles) are annotated with the location of glycines, alanines and threonines. Boxes indicate the chaperone binding region for the intrinsically disordered monomeric form (left) and the disordered tail of the membrane associated form of α-syn (right).

To better visualize and quantify the structural ensemble of α-syn inside cells, we developed a more specific reporter of α-helical α-syn conformation. Threonine residues are uniformly distributed throughout the region that has α-helical propensity when associated with nanodiscs, are absent from the amino terminal chaperone interaction region and the disordered carboxy terminal region (Figure 2B), and have C^{α} chemical shift values that are distinct from those of most other amino acids. Thus, we specifically isotopically labeled the threonine residues in α-syn. This approach resulted in α-syn that was ^{13}C and ^{15}N labeled at the threonine residues with 10% scrambling of ^{13}C to glycine but no scrambling to other amino acids. We collected 1D ^{15}N-filtered ^{13}C spectra of α-syn associated with nanodiscs and frozen in solution. The nanodisc-associated form had a major narrow peak centered near the database value for threonine C^{α} at 68.3 ppm and two minor peaks; one centered at the random coil value for threonine C^{α} and one centered at the α-helical chemical shift of glycine C^{α} (Figure 2, red). The frozen intrinsically disordered monomeric form had a broad major peak that covered the range of threonine C^{α} chemical shift values with a maximum at the random coil value and a minor peak centered at the random coil value for glycine C^{α} (Figure 2, blue). The nanodisc-associated and intrinsically disordered conformations of α-syn were distinguished by a difference in the chemical shift of the major peaks by 7.7 ppm and of the peak width of the major peaks by 4.7 ppm. Thus, these two conformations are easily distinguished by 1D spectroscopy of specifically threonine labeled α-syn.

To determine the relative populations of the α-helical rich and the intrinsically disordered forms of α-syn present inside cells, we collected highly signal averaged 1D ^{13}C spectra of threonine labeled α-syn inside cells. To specifically isolate signals from these sites in 1D from the natural abundance isotopes in the cellular biomass, we used a pulse sequence that reported only on ^{13}C sites that are within one bond of an ^{15}N site (38) to increase the specificity for the labeled sites in α-syn by 200-fold(39, 40). We collected ^{15}N-filtered ^{13}C spectra of HEK293 cells

containing 75 μM of threonine-labeled α-syn when AMUPol was introduced either by electroporation or incubation. We found that the method of AMUPol delivery altered the spectra. Delivery of AMUPol by electroporation resulted in spectra that largely resembled that of the frozen intrinsically disordered monomer with a small additional feature consistent with nanodisc-associated α-syn. Delivery of AMUPol by incubation resulted in spectra that resembled the nanodisc-associated α-syn with a minor feature centered at the random coil value. To quantify the difference, the spectra were fit to a linear combination of the spectrum of purified α-syn associated with nanodiscs, which is highly α-helical, and the spectrum of purified frozen intrinsically disordered α-syn (Figure 2). We found that in cells where AMUPol was introduced by electroporation, α-syn was mostly intrinsically disordered (92% ± 1) but had a small (8% ± 2) α-helical population (R^2 = 0.75) (Figure 3A). In contrast, in cells where AMUPol was introduced by incubation, α-syn was mostly α-helical (60 ± 1%) but had a sizable intrinsically disordered population (40 ± 1%) (R^2 = 0.80) (Figure 3B). The AMUPol delivery method altered the α-helical population of the spectra by an order of magnitude.

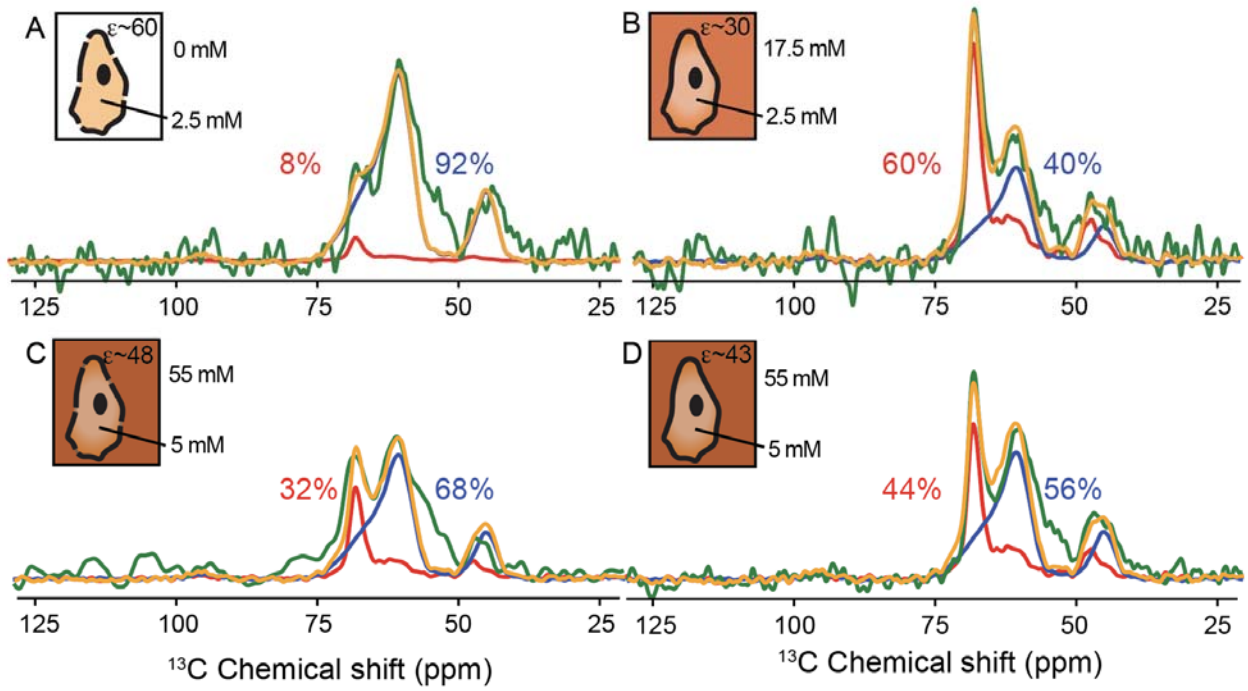

Figure 3*: Altering the spatial distribution of AMUPol in cellular samples highlights conformations present in different regions of the cells. A) Delivery of AMUPol via electroporation of cells in the presence of 20 mM AMUPol followed by removal of the extracellular AMUPol prior to data collection reports quantitively on the entire structural*

ensemble. In contrast, delivery of AMUPol by other methods results in a spatial bias in the resulting spectra. B) Delivery of AMUPol via incubation of cells in 10 mM AMUPol. C) Delivery of AMUPol to cells that had been electroporated in buffer and allowed to recover for 15 minutes before delivery via incubation in 30 mM AMUPol. D) Delivery of AMUPol to cells via incubation in 30 mM AMUPol. The ^{15}N-filtered ^{13}C spectra of threonine labeled α-syn inside cells (green) is plotted with the spectra of the nanodisc-associated α-helical rich α-syn (red) and the frozen intrinsically disordered monomeric forms (blue) that are scaled by the weighting that resulted in the best fit linear combination (orange). Insets in each panel are cartoon representation of the AMUPol distribution (brown) in the cell and the interstitial space for each AMUPol delivery method. Darker shades represent higher AMUPol concentrations. DNP enhancements (top right corner) and estimated extracellular (top right) and intracellular (bottom right) AMUPol concentrations are annotated. Spectra were recorded at 600 MHz with 12 kHz MAS at 104 K.

While the method used to introduce AMUPol to the cells altered the observed population of α-helical rich α-syn in the cells by an order of magnitude, this change could be a result of an alteration of the conformational ensemble by the AMUPol delivery method, an alteration in the distribution of delivered AMUPol throughout the cellular biomass or a result of the combination of both perturbations.

To determine if the distributions of α-syn inside HEK293 cells were altered by the method used to introduce the polarization agent to the cells, we compared the localization of α-syn in HEK293 cells using fluorescent microscopy. Cells were prepared identically to those for NMR spectroscopy except cells were fixed and imaged, rather than packed into the NMR rotor and frozen. The fixed cells were immunostained with an anti-α-syn antibody and nuclei were visualized with DAPI. When AMUPol was introduced by electroporation, the α-syn in most of the cells was homogenously dispersed throughout the interior (95.5%), while in a minority of cells, the α-syn was more concentrated in the ~1 μm at the periphery of the cell than in the interior (4.5%) (Figure 4, n = 223). When AMUPol was introduced by incubation, the α-syn in most of the cells was homogenously dispersed throughout the cellular interior (80%) while the α-syn in some of the cells (20%) was more concentrated near cellular periphery (Figure 4B, arrowheads, n = 155). Thus, the method of AMUPol delivery altered the localization of α-syn in a sub-population of HEK293 cells, potentially altering the structural ensemble of the sample.

To determine if the altered localization of α-syn in a sub-population of cells could account for the magnitude of the conformational change observed by NMR, we calculated the degree of alteration in conformational ensemble based upon the observed alteration in α-syn

localization. We found that the increase in the proportion of cells with peripherally localized α-syn (15%) was not sufficient to account for the increase in α-helical content in the structural ensemble (52%), even in the extreme case where we assumed that all the α-syn in cells with peripherally localized α-syn was completely α-helical. Therefore, the alteration of the observed population by NMR does not result from only the alteration of the conformational ensemble by the AMUPol delivery method. Thus, the observed alteration must either result the alteration in the distribution of delivered AMUPol alone or a combination of an alteration of the conformational ensemble and the spatial distribution of AMUPol.

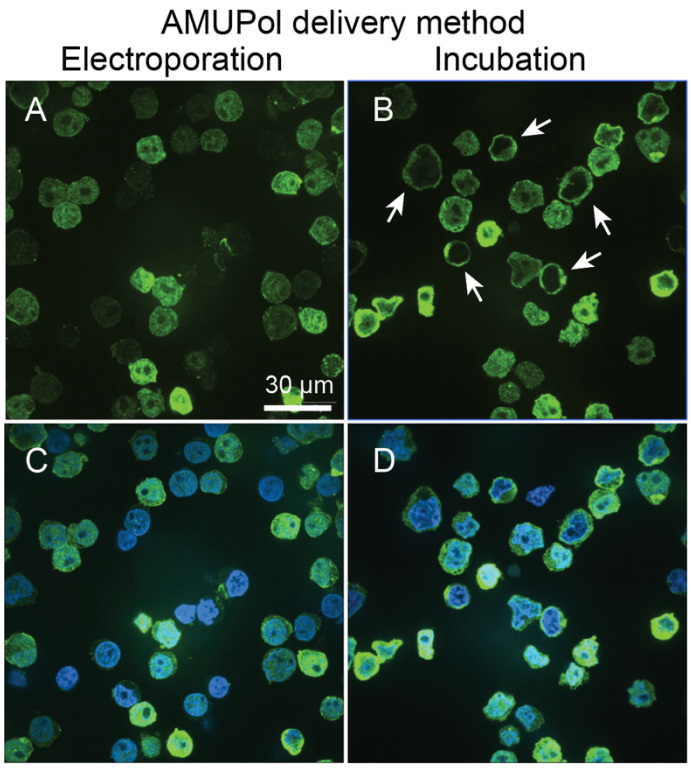

Figure 4*: Representative fluorescent microscopy images depicting the distribution of α-syn inside HEK293 cells when AMUPol was delivered by electroporation (A&C) and incubation (B&D). Cells were prepared identically to those used for NMR spectroscopy except cells were fixed and immunostained for α-syn (green) and stained with DAPI (blue). The a-syn was homogenously dispersed throughout most of the cells regardless of AMUPol delivery method, but the a-syn was more concentrated near the cellular periphery (arrowheads) for cells when AMUPol was delivered by incubation.*

To determine if the differences in the relative contributions of different conformations of α-syn to the NMR spectra arise from the difference in the distribution of AMUPol, we altered the

distribution of AMUPol without altering the conformational ensemble of α-syn. To do so, we altered the distribution of AMUPol by increasing the concentration of AMUPol added upon delivery by incubation. Increasing the incubation concentration from 10 mM AMUPol to 30 mM AMUPol increases the intracellular AMUPol concentration - as assessed by $T_{B,on}$ and DNP enhancements - but does not alter the degree of inhomogeneity of the distribution of AMUPol throughout the cellular biomass (as assessed by the amount of stretch, β, required to fit the build-up curve to a stretched exponential) (25, 41, 42). Using $T_{B,on}$ and DNP enhancements to estimate intracellular AMUPol concentration and assuming cells occupy half of the volume of the rotor, incubation of cells in 10 mM AMUPol results in an intracellular concentration of ~ 2.5 mM and an extracellular concentration of ~17 mM while incubation of cells in 30 mM AMUPol results in intracellular concentrations of ~ 5 mM and an extracellular concentration of ~55 mM AMUPol (25) (Figure 3B,D). Thus, because the degree of inhomogeneity is the same for cells incubated with 10 mM and 30 mM AMUPol, but the concentrations of AMUPol in the intracellular and extracellular space differ, the spatial distribution of AMUPol in these samples must differ. While the exact spatial distribution of the polarization agent when AMUPol is delivered by incubation is unknown, delivery by incubation results in an AMUPol gradient that preferentially enhances more peripherally-localized molecules(25). The highest DNP enhancements occur at intermediate concentrations of polarization agents because very high concentrations of radicals result in signal attenuation from paramagnetic relaxation. Therefore, because of the intracellular and extracellular concentrations of AMUPol, the spatial distribution of AMUPol resulting from incubation in 10 mM AMUPol is likely to favor the cellular periphery more than incubation in 30 mM AMUPol.

As reported above, when AMUPol was delivered by incubation in 10 mM AMUPol, the NMR spectrum was best fit (R^2 = 0.81) by a linear combination of 0.60 ± 0.01 times the spectrum of purified α-syn on nanodiscs and 0.40 ± 0.01 times the frozen intrinsically disordered monomer (Figure 3B). In contrast, when AMUPol was delivered by incubation in 30 mM AMUPol, the spectrum was best fit (R^2 = 0.75) by a linear combination of 0.44 ± 0.01 times the spectrum of purified α-syn on nanodiscs and 0.56 ± 0.01 times the frozen intrinsically disordered monomer (Figure 3D). The relative contributions of the different conformations of α-syn to the NMR spectra differed by 16% and the α-helical population was more prominent for the sample with an AMUPol distribution that more strongly favors the cellular periphery. Therefore, in the

absence of alterations to the underlying conformational ensemble, altering the spatial distribution of the polarization agent throughout the cellular biomass altered the relative contributions of different conformations of α-syn to the NMR spectra. These data indicate that two conformations of α-syn have different sub-cellular localizations and suggest that the α-helical conformation is more represented near the cellular periphery.

To determine if the α-helical rich conformation of α-syn is preferentially sampled near the cellular periphery, we again altered the spatial distribution of the polarization agent, this time in cells subjected to electroporation. Delivery of AMUPol to cells by electroporation results in a homogenous distribution of AMUPol throughout the cellular biomass (25). To alter the spatial distribution of the polarization agent in electroporated cells, instead of subjecting cells to electroporation in the presence of AMUPol, we subjected HEK293 cells to electroporation in the absence of AMUPol (e.g. a blank electroporation), allowed cells to recover for 15 minutes and then delivered AMUPol via incubation in 30 mM AMUPol. We assessed the spatial distribution of AMUPol in these blank electroporated cells by determining the DNP enhancements, build-up times and AMUPol dispersion homogeneity. The DNP enhancements (47 ± 6) and $T_{B,on}$ values (4 s ± 1 s) were indistinguishable from those for cells incubated with 30 mM AMUPol (p > 0.22), indicating that the delivered concentration of AMUPol for cells subjected to electroporation before incubation in 30 mM AMUPol was similar to delivered concentration of AMUPol by incubation in 30 mM AMUPol. The AMUPol distribution in blank electroporated cells had a β-factor of 0.82 ± 0.06, which was indistinguishable from the degree of inhomogeneity observed for cells where the AMUPol was delivered by incubation alone (p = 0.62) and distinct from that of cells where AMUPol was delivered by electroporation (p < 0.001). (Supplemental Table 2). Thus, AMUPol dispersion in cells subjected to electroporation before delivery of AMUPol by incubation in 30 mM AMUPol was similar to the dispersion from delivery of AMUPol by incubation in 30 mM AMUPol alone and distinct from the dispersion from delivery of the AMUPol by electroporation.

Because delivery of AMUPol by electroporation results in a uniform distribution of AMUPol through the cellular biomass and the resulting NMR spectra report quantitatively on the conformational ensemble, we know that 8% of the α-syn in the ensemble in electroporated cells adopts α-helical rich conformations. We collected ^{15}N filtered ^{13}C spectra of HEK293 cells containing 75 μM of threonine-labeled α-syn on cells that were electroporated before delivery of

AMUPol by incubation with 30 mM AMUPol. The spectrum was best fit (R^2 = 0.74) by a linear combination of 0.32 ± 0.01 times the spectrum of purified α-syn on nanodiscs and 0.68 ± 0.01 times the frozen intrinsically disordered monomer (Figure 3B). The relative population of the α-helical population was four times more prominent when the AMUPol distribution favors the cellular periphery. Because the spatial distribution of AMUPol in cells subjected to electroporation before delivery of AMUPol by incubation in 30 mM AMUPol was indistinguishable from delivery of AMUPol by incubation in 30 mM AMUPol alone, the peripheral regions of cells incubated in 30 mM AMUPol without electroporation will also be favored by four-fold. Thus, we can estimate that electroporation followed by a 15-minute recovery period reduced the α-helical population of the conformational ensemble by 3%. Delivery of AMUPol to cells by electroporation reduced the α-helical population of α-syn inside cells by a few percent relative to cells where AMUPol was delivered by incubation.

DISCUSSION

The protein α-syn adopts a wide variety of conformations including an intrinsically disordered monomeric form and an α-helical rich membrane-associated form that is thought to play an important role in cellular membrane processes. However, despite the high affinity of α-syn for membranes, evidence that the α-helical form of α-syn is adopted inside cells has thus far been indirect. In contrast to in cell solution state NMR spectroscopy, which can only directly report on the chemical shifts of dynamic molecules, in cell DNP-assisted solid state NMR on frozen samples has the potential to report directly on the entire conformational ensemble. Moreover, because the DNP polarization agent can be dispersed both homogenously and inhomogenously throughout the cellular biomass, in cell DNP-assisted solid state NMR experiments can report either quantitively upon the structural ensemble or can preferentially report upon the structural ensemble with a spatial bias(25). Using DNP-assisted MAS NMR we establish that the spectra of purified α-syn in the nanodisc-associated and intrinsically disordered forms have distinguishable spectra. When the polarization agent is introduced into cells by electroporation and homogenously dispersed, a minority of the α-syn inside HEK293 cells adopts a highly α-helical rich conformation. Alteration of the spatial distribution of the polarization preferentially enhances the signal from molecules nearer to the cellular periphery. We find that the α-helical rich population is preferentially adopted toward the cellular periphery.

Previous in cell NMR experiments had established that α-syn behaved as a compact intrinsically disordered monomer in a variety of cultured mammalian cell lines, including the HEK293 line used here (30). This result is consistent with many biophysical and structural experiments of α-syn in both purified and complex biological contexts. Here we found that α-syn in HEK293 cells adopted at least two distinct conformations, one of which is α-helical rich and the other is intrinsically disordered. Because the α-helical form accounts for only 8% of the total ensemble, it is possible that this conformation was present in solution state experiments, but the decrease in peak intensity could not be discerned with confidence above experimental noise (30). However, while the α-syn in both experiments was diffuse throughout the cell, the cellular α-syn concentration used in this work was four times higher than that of the solution state NMR experiments. Therefore, the discrepancy may also result from a difference in the underlying conformational ensembles. Nonetheless, we find that α-syn can adopt at least two distinct conformations inside cultured mammalian cells, one of which is rich in α-helices.

While the NMR data strongly indicate the presence of an α-helical form of α-syn that is preferentially localized in the regions near the cellular periphery, they do not provide direct evidence for membrane associated α-syn. There are multiple lines of evidence that indicate that α-syn associates strongly with membranes in both purified(31) and complex biological settings (43, 44). The alignment of the major peak center of the in cell spectra and the nanodisc-associated spectra indicates that the nanodisc-associated form of α-syn captures the major environmental features of the form adopted inside cells. Nonetheless, α-syn selectively binds to membranes with specific curvatures (45, 46) and α-syn may also form α-helical rich tetramers inside cells (47). Experiments employing mutant versions of α-syn, different isotopic labeling schemes, and additional purified reference samples could potentially determine which α-helical rich forms of α-syn are adopted in both this and other cell lines.

In this work, we delivered isotopically labeled α-syn to mammalian cells by electroporation, allowed the cells to recover for 5 hours and then delivered AMUPol to the samples shortly before data collection. While the time period after the delivery of isotopically labeled α-syn by electroporation is sufficient for the cells to recover(30, 48), the effects of a 15 minute recovery period after delivery of AMUPol by electroporation were unclear. In the absence of functional assays for α-syn activity, we used microscopy to assess the effect of electroporation on the cellular localization of α-syn. There was an electroporation-dependent

alteration in the cellular localization α-syn; electroporation increased the number of cells with homogenous distributions of α-syn by of 15%. However, NMR investigations revealed that this alteration in protein location was resulted in a small (3%) decrease in the α-helical population of the conformational ensemble. In contrast, it appears that effects of electroporation on membrane integrity are more transient. The permeability of the plasma membrane to AMUPol shortly after electroporation was indistinguishable from that of un-electroporated cells. Interestingly, because the inhomogeneity in the dispersion of AMUPol is likely to be rooted in the kinetics of radical reduction rather than in the semi-permeability of the plasma membrane (19, 20, 25), development of efficient biostable polarization agents (49-51) could further mitigate cellular perturbations by allowing longer recovery times for cells post-delivery and/or alleviate the necessity for electroporation for homogenous delivery of the polarization agent to cells altogether.

Understanding the underlying distribution of the polarization agent throughout the cell has emerged as a critical factor in interpreting DNP-assisted MAS NMR spectra (19, 25, 49, 52, 53). Most recently, sub-cellular information was obtained about ubiquitin in nuclei from DNP-assisted NMR studies on isolated nuclei (49). Here, we obtain sub-cellular specificity via alteration of the spatial distribution of the polarization agent in intact viable cells, demonstrating the importance of understanding the spatial distribution of the polarization agent as well as the potential for unique insights into protein conformations that may emerge from doing so. By altering the spatial distribution of the polarization agent(25), we found that α-syn preferentially adopts α-helical rich conformations towards the cellular periphery. Altering the spatial distribution to determine protein conformations in a cellular sub-structure will benefit from precise descriptions of the spatial distribution of the polarization agent. Fluorescent microscopy of tagged polarization agents will make an important contribution(49) although it is most effective for agents resistant to cellular reduction, as fluorescence reflects both active and inactive molecules. Direct assessment of the DNP enhancements, build-up times and amount of stretch in the build-up time for the cellular biomass, which is captured by the β-factor likewise describe the distribution of the polarization agent and is an appropriate approach for all polarization agents (25), regardless of their biostability. More precise delivery of polarization agents to cellular samples has the potential to enable structural characterization of proteins with

organelle-specific structural insights in the realm of structural biology. This study pioneers spatially resolved DNP NMR in intact viable cells.

SUPPLEMENTAL INFORMATION:

Supplemental information is available for this article.

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Methods:

α-syn expression and purification

The *E. coli* strain BL21 DE3 was used to express isotopically labeled wild-type α-syn. Uniformly isotopically labeled protein was expressed by growth of cells in 4 L LB to an OD_{600} of 0.6 followed by concentration and resuspension in 1L of M9 media containing 1 g/L of ^{15}N chloride and 4 g/L of ^{13}C enriched glucose (54). The protocol for specific isotopic labeling of threonines was based on previously published protocols (55, 56). First, the *E. coli* cells were grown in 4 liters of M9 supplemented with natural abundance threonine (50 mg/mL), α-ketobutyrate (100 mg/mL), and natural abundance glycine (500 mg/mL). Cells were grown at 37 °C until the OD_{600} of the culture reached 0.6 – 0.8. The *E. coli* cells were then harvested, washed once with 1x M9 salts (54), and transferred to 4 liters of M9 supplemented with ^{13}C,^{15}N-threonine (50 mg/ml), α-ketobutyrate (100 mg/ml), and natural abundance glycine (500 mg/ml). Cells were then grown at 37 °C until they reached OD_{600} of 1.5. Expression of α-synuclein was induced with addition of 1 mM IPTG. Cells were harvested after 3 hours of expression at 37 °C.

Purification was performed as described (57). Briefly, cells were frozen after the expression, resuspended in lysis buffer containing a detergent (20 mM Tris pH 8.0, 1 mM EDTA, 0.1% Triton-X) and incubated for 30 minutes at 37 °C. After the incubation, DNA and RNA were digested by adding 2.5 µL Omni Nuclease and 2.5 uL DNAse while supplementing

the solution with 10 mM $MgCl_2$ and 10 mM $CaCl_2$ final concentration. After 1 hour incubation at 37 °C the excess metal was chelated by addition of 5 mM EDTA. Insoluble cell debris was separated from the supernatant by centrifugation at 4,000 x g for 15 minutes. The concentration of NaCl in the supernatant was adjusted by mixing 6 parts of supernatant with 1 part of 5 M NaCl. The supernatant was heated above 90 °C for 10 minutes in a water bath, after which the supernatant was cooled in a room temperature water bath and on ice. The proteins that precipitated during the boiling step were pelleted by centrifugation at 20,000 x g for 20 minutes at 4 °C. The α-synuclein in the soluble supernatant was precipitated by addition of cold saturated ammonium sulfate at 1:1 ratio (final concentration of 50 % saturated ammonium sulfate at 4 °C) and incubation overnight at 4°C with gentle stirring. The precipitated protein was separated from the supernatant by centrifuging at 4000 x g for 20 minutes at 4 °C. The pellet was dissolved in 20 mM Tris pH 8.0, 20 mM NaCl. The solution was loaded onto a Q-sepherose column and α-synuclein was eluted with a salt gradient of 0-500 mM NaCl. The α-synuclein containing fractions were pooled and concentrated to 1 mM and then run over a size exclusion column (Superdex 75 Increase HiScale 26/40, 40 cm). Protein purity was assessed by gel electrophoresis and was determined to be greater than 98%.

Preparation of frozen intrinsically disordered monomers

The stock α-synuclein solution at 1 mM was diluted in D_2O to result in 10 mM sodium phosphate buffer at pH 7.0 at a 12:88 ratio of H_2O:D_2O. Glycerol was added to a final concentration of 15 % d_8-^{12}C-glycerol resulting in a 10:75:15 ratio of H_2O : D_2O : d_8-^{12}C-glycerol. AMUPol was dissolved in the sample to result in a final concentration of 10 mM AMUPol. The sample was transferred to a rotor and stored at -80 °C until measurements. Prior to measurement samples were briefly warmed to room temperature to mark and cap the rotor. Room temperature rotors were inserted into the probe that was pre-equilibrated to 100 K. Sample temperature was inferred from the temperature of the stator and decreased from room temperature to 200 K in ~2 minutes followed by a slow decrease to 104 K over 15 minutes.

Preparation of complex with nanodiscs

The nanodisc scaffold MSP1E3D1 was expressed and purified as previously described (58). POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol) was used to assemble the nanodiscs. Buffer used for the nanodisc samples was 20 mM sodium phosphate, 50 mM NaCl, pH 7.4.

Nanodiscs and α-synuclein were mixed at equimolar ratio, and D_2O was added to obtain an 88:12 D_2O:H_2O ratio. The sample was concentrated to the final concentration of 100 μM α-synuclein. Depleted deuterated glycerol (d_8-^{12}C-glycerol) and AMUPol were added as the last step for a final composition of 15:75:10 for d_8-^{12}C-glycerol:D_2O:H_2O (*v*/*v*/*v*) respectively with 6.8 mM AMUPol.

Preparation of natural abundance and isotopically enriched HEK293 cells

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum and 1% PenStrep (Gibco). For quantification of the AMUPol distribution through cellular biomass, uniformly isotopically labeled HEK293 cells were cultured in ^{13}C, ^{15}N labelled media (BioExpress 6000 Mammalian U-^{13}C, 98%; U-^{15}N, 98%, Cambridge Isotope Laboratories, USA) with 10% (v/v) fetal bovine serum (FBS, qualified, Gibco) and 1% (v/v) PenStrep (Gibco) at 37 °C and 5% CO_2.as previously described (25).

Introduction of α-syn into HEK293 cells by electroporation

Delivery of α-syn to HEK293 cells by electroporation was performed as described (30). Briefly, confluent adherent HEK293 cells were rinsed with PBS, detached from the culture dish with trypsin/EDTA (0.05 %/ 0.02 %) and collected by centrifugation at 233 x g for 5 min at room temperature. The 100 µL cell pellet was resuspended in 10 mL of PBS and 10 µL of this suspension was mixed with 10 µL of trypan blue (0.4% solution) to determine the number of live cells as assessed by trypan blue membrane permeability with a Countess automated cell counter (Life Technologies) using the manufacturer's instructions. The cells then were pelleted, washed with electroporation buffer then pelleted again before being mixed with α-syn.

Cells were suspended in 100 mM sodium phosphate, 5 mM KCl, 15 mM $MgCl_2$, 15 mM HEPES with freshly added 2 mM ATP, 2 mM reduced glutathione and 900-1000 µM purified α-syn at 40×10^6 cells per mL. 100 µL aliquots (4×10^6 cells) were electroporated with an Amaxa Nucleofector I (Lonza) using the HEK293 pulse sequence. Cells were pulsed twice with gentle mixing followed by a 5 minute room temperature incubation between the two pulses. Ten minutes after electroporation, 0.5 mL of pre-warmed (37 °C) growth medium was added to each cuvette and samples were transferred to a cell culture dish. Cells were returned to the incubator

and allowed to recover for 5 h. Once cells regained their adherent morphologies, dishes were washed with PBS and cells were harvested by trypsinization.

Western blotting

Harvested electroporated cells were counted, pelleted and lysed with RIPA buffer. Samples were fractionated by SDS-PAGE, transferred to polyvinylidene fluoride membrane probed with the anti-a-syn antibody (BD Bioscience 610786). Samples were denatured by incubation at 95 °C for 10 minutes in the presence of 2% SDS before separation. Secondary antibodies were coupled to horseradish peroxidase. Blots were visualized by a standard ECL analysis and band intensities quantified using Image Lab (Bio-Rad).

Delivery of AMUPol to HEK293 cells

AMUPol was delivered to HEK293 cells by either incubation or electroporation as previously described (25). Briefly, for delivery by incubation, a 50 µL cell pellet was mixed with 50 µL perdeuterated 1x PBS (85% D_2O + 10% H_2O, pH 7.4) containing AMUPol (Cortecnet, USA) and 18 µL of d_8-glycerol. The 118 µL cell suspension had a final composition of 15% (v/v) d_8-glycerol, 75% (v/v) D_2O and 10% (v/v) H_2O. For delivery by electroporation, a 50 µL cell pellet was mixed with 100 µL electroporation buffer (SF cell line solution, Lonza) containing AMUPol and electroporated (HEK293 pulse sequence, Lonza 4D-nucleofactor) using manufacturer's instructions. Post electroporation, cells were allowed to recover for 10 minutes in electroporation buffer containing AMUPol inside the tissue culture hood. Next, cells were washed twice with 50-100 µL (depending on cell pellet volume) of 1x PBS to eliminate the electroporation buffer and any extracellular AMUPol from the sample and the 50 µL cell pellet was resuspended in perdeuterated 1x PBS and d_8-glycerol for a final composition of 15% (v/v) d_8-glycerol, 75% (v/v) D_2O and 10% (v/v) H_2O. For blank electroporation samples, cells were subjected to electroporation in the absence of AMUPol in electroporation buffer, allowed to recover for 10 minutes and then incubated in perdeuterated PBS containing 30 mM AMUPol. For blank electroporation samples, cells were subjected to electroporation in the absence of AMUPol in electroporation buffer, allowed to recover cells for 10 minutes and then incubated in perdeuterated PBS containing 30 mM AMUPol. The DNP matrix had a final composition of 15% (*v/v*) d_8-glycerol, 75% (*v/v*) D_2O and 10% (*v/v*) H_2O. Cell pellets were transferred to 3.2

mm sapphire rotors by centrifugation, sealed with a silicon plug and then frozen at a controlled rate of 1 °C/min (25).

Fluorescence microscopy

Cells for fluorescence microscopy were treated identically to those prepared for DNP NMR except that after the manipulation for the introduction of the radical. Cell pellets were fixed in PBS containing 4 % (*w/v*) paraformaldehyde (PFA) for 15 min at room temperature rather than being transferred to rotors and frozen. The cells were washed in PBS twice then permeabilized by incubation in 0.1 % (*v/v*) Triton-X in PBS for 3 min. Cells were pelleted by centrifugation, the supernatant was removed, and the pellet was incubated in fresh PBS for 10 minutes three times before cells were blocked by incubation in 0.1% (*w/v*) BSA (Sigma) in PBS for 1 h. Cells were then incubated for 2 h with primary antibody anti-α-Syn sc-69977 (Santa Cruz, 1:100 dilution) in blocking buffer. Cells were pelleted by centrifugation, the supernatant was removed, and pellet was incubated in fresh PBS for 10 minutes three times before cells were incubated with anti-mouse IgG Alexa-488, (Sigma, 1:1,000 dilution) for 1 h in blocking buffer. Cells were again pelleted by centrifugation, the supernatant was removed, and pellet was incubated in fresh PBS for 10 minutes three times. Suspended cells were allowed to settle onto 44 mm diameter glass bottom dishes (Nunc) for 30 minutes then mounted using ibidi mounting medium containing DAPI. The plates were stored at 4 °C. The cells were imaged by confocal microscopy (Nikon CSU-WI spinning disc confocal) with a 100x objective lens. Excitation and emission were 488/496 nm and 406/460 nm for Alexa488 and DAPI, respectively. Images were analyzed using Fiji software.

NMR spectroscopy

Rotors were transferred in liquid nitrogen directly into the NMR probe that had been previously equilibrated to 104 K as described (18, 25). All dynamic nuclear polarization magic angle spinning nuclear magnetic resonance (DNP MAS NMR) experiments were performed on a 600 MHz Bruker Ascend DNP NMR spectrometer/7.2 T Cryogen-free gyrotron magnet (Bruker), equipped with a ^{1}H, ^{13}C, ^{15}N triple-resonance, 3.2 mm low temperature (LT) DNP MAS NMR Bruker probe (600 MHz). For ^{13}C cross-polarization (CP) MAS experiments, the ^{13}C radio frequency (RF) amplitude was fixed at 60 kHz and an ^{1}H RF amplitude was 72 kHz. The 90° ^{1}H

pulse was 100 kHz, the 90° ^{13}C pulse was 62.5 kHz, and ^{1}H TPPM at 85 kHz for decoupling with phase alternation of ± 15° during acquisition of ^{13}C signal. ^{13}C-^{13}C 2D correlations were measured using 5 ms or 20 ms DARR mixing with the ^{1}H amplitude at the MAS frequency. A total of 280 complex points in the indirect dimension were recorded with an increment of 25 μs. DARR experiments were apodized with a Lorenz-to-Gauss window function with IEN-to-GB ratio of 2.5 and the IEN between 20 and 80 for both the t_1 and t_2 time domains. For ^{13}C-^{15}N 1D spectra either a TEDOR or NCa double CP pulse sequence was used. In the case of TEDOR (38), the mixing time was 1.92 ms with a recycle delay of 3.9 s. For 1D NCa double CP (59) experiments, the contact time was 6 ms with a recycle delay of 3.0 s. The DNP enhancements were determined by comparing 1D ^{13}C CP spectra collected with and without microwave irradiation. For $T_{\mathrm{B,on}}$ measurements, recycle delays ranged from 0.1 s to 300 s. To determine the $T_{\mathrm{B,on}}$, the dependence of the recycle delay using saturation recovery on both ^{13}C peak intensity or volume was fit to the stretched-exponential equation $I_t = I_0 \times \left[1 - e^{-\left(\frac{t}{T_{\mathrm{B,on}}}\right)^{\beta}}\right]$.

Fitting:

In-cell spectra were fit to a linear combination of the experimental spectra of nanodisc bound α-syn, which is α-helical, and purified frozen intrinsically disordered α-syn using the generalized least squares regression function in statsmodels.api. The coefficients of the linear regressions were used to weight the monomer and nanodisc-bound data, and a numpy trapezoidal approximation of the integral (trapz) was calculated to determine the relative populations of α-helical and intrinsically disordered α-syn. Code available at https://github.com/kendrakf/Spotlight2023

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