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Site-specific dynamic nuclear polarization of hydration water as a generally applicable approach to monitor protein aggregation

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Abstract

We present a generally applicable approach for monitoring protein aggregation by detecting changes in surface hydration water dynamics and the changes in solvent accessibility of specific protein sites, as protein aggregation proceeds in solution state. This is made possible through the Overhauser dynamic nuclear polarization (DNP) of water interacting with stable nitroxide spin labels tethered to specific proteins sites. This effect is highly localized due to the magnetic dipolar nature of the electron–proton spin interaction, with >80 % of their interaction occurring within 5 Å between the unpaired electron of the spin label and the proton of water. We showcase our tool on the aggregation of tau proteins, whose fibrillization is linked to neurodegenerative disease pathologies known as tauopathies. We demonstrate that the DNP approach to monitor local changes in hydration dynamics with residue specificity and local contrast can distinguish specific and neat protein-protein packing leading to fibers from non-specific protein agglomeration or precipitation. The ability to monitor tau assembly with local, residue-specific, resolution, under ambient condition and in solution state will help unravel the mechanism and structural characteristics of the gradual process of tau aggregation into amyloid fibers, which remains unclear to this day.

Introduction

We introduce a generally applicable approach for monitoring protein aggregation by detecting hydration water dynamics at individual protein sites via nitroxide radical-based spin labels. This method relies on the principle that the binding of any two molecules with sufficiently high binding affinity will either alter the surface hydration dynamics at the interface, or even exclude surface water at the spin-labeled segment that gets buried into a hydrophobic core. The measurement of hydration water dynamics at specific protein sites is realized by utilizing a physical principle generally known as dynamic nuclear polarization (DNP) to enhance nuclear magnetic resonance (NMR) signal of protons that are in direct dipolar interaction with electron spins of stable radical spin-labeled protein sites ^{1, 2, 3}. Among the different DNP mechanisms, the relevant one for the presented method is the Overhauser effect, where the coupling between electron and proton spins is mediated by the translational molecular motion of the interacting molecules in solution state. Part of the higher polarization of unpaired electron spins is transferred to proton nuclei, here of water, through the saturation of the electron spin's paramagnetic resonance (EPR) signal and effective short-range electron-proton cross-relaxation, resulting in large (e.g. up to 130 fold at 0.35 Tesla field) ¹H NMR signal enhancement. The dipolar nature of the motional coupling between the electron spin and the proton spin manifests itself as enhanced NMR signal with negative signal amplitude. While sensitivity enhancement of NMR through signal amplification, also widely known as hyperpolarization, by itself is an important

goal^{4,5,6}, this is not the focus of this work. Our goal is to access unique information through the amplitude of the Overhauser-induced NMR signal enhancement, E , that depends on the interaction between the electron and proton spins as follows:

$$E = 1 - \rho f s \frac{\gamma_s}{\gamma_I}, \quad (1)$$

where ρ is the coupling factor that describes the dipolar interaction between the electron and proton, f is the leakage factor that describes the electron's ability to relax the proton, s is the saturation factor that describes the saturation of the electron Zeeman transition, γ_s and γ_I are the magnetogyric ratios of the electron and proton, respectively, with $\gamma_s/\gamma_I = 658$. Here, we can extrapolate the measurements of E for maximum EPR saturation of the affected EPR hyperfine lines of the nitroxide radical, create or estimate conditions for s to approach 1^{7,8}, and determine f through NMR T_1 relaxation measurements, leaving ρ as the key variable upon appropriate analysis. An important idea to note is that burial versus exposure of radical spin labels from and to hydration water will not necessarily manifest itself in differences in the leakage factor, because NMR T_1 relaxation effects are long-range and can migrate through space. However, the coupling factor, ρ probes local changes in dynamics, since motionally restricted water will exhibit a different dipolar relaxation, than mobile water; this relaxation is only effective at atomic collisional distances within 3–5 Å of the spin label. Further details and methods for the quantitative analysis of f and s , when employing (free or tethered) nitroxide-based spin labels as the source for unpaired electron spins are presented elsewhere^{7,9}. Using models and analysis methods previously described^{7,9,10,2}, the translational correlation time between the unpaired electron and proton τ , can be obtained from ρ , and is defined as:

$$\tau = \frac{d^2}{D_I + D_S}, \quad (2)$$

where d is the distance of closest approach of the two spins, D_I is the diffusion coefficient of the solvent molecules and D_S is the diffusion coefficient of the electron spin-bearing molecules. In our earlier work we have shown that the distance of closest approach⁷, which depends on the Van der Waals attraction between the electron and proton, is 4.5 Å. So, when employing protein-tethered spin labels, only spin labels closely interacting with dynamic water that displays significant motional freedom with translational correlation times (τ in Eq. 2) of ~550 ps or less will display measurable Overhauser enhancement, but not the spin labels buried in hydrophobic cores. It is important to note that the determining factors are the translational dynamics of the water (D_I in Eq. 2) and the proximity with which the water can approach the unpaired electron of the spin label (d in Eq. 2), not the frequency of collisions between water and the spin label.

Unlike the widely used Nuclear Overhauser Effect (NOE), the Overhauser effect for enhancing NMR or EPR spectroscopy is an unexplored technique for biophysical analysis. Unique to our approach is the utilization of the Overhauser DNP effect in combination with site-directed mutagenesis and spin labeling of proteins to approach localized information of surface hydration water dynamics. We state that probing inter-protein interaction through surface hydration water dynamics is especially well-suited for monitoring protein aggregation processes because the binding affinity tends to be greater compared to other binding events, and thus the bonding life time is sufficiently long, allowing for electron spin-proton cross relaxation to be dominated by the characteristics of the bound species during build-up of the DNP-enhanced NMR signal. Characteristically, the DNP signal

contrast is only *on* when the spin label is interacting with water, but terminally turned *off* as soon as oligomerization and/or fiber formation buries the spin label, making this approach particularly sensitive to earlier aggregation events. This is in contrast to light scattering that will equally display non-specific agglomeration of proteins or other water-penetrated soft assemblies, as long as these agglomerates scatter light, or Thioflavin T (ThT) fluorescence that will continue to detect increasing amounts of amyloid fibrils¹¹, both of which methods are capturing rather macroscopic aggregation events.

Another important advantage of our Overhauser DNP approach is that EPR analysis can be carried out concurrently on the same spin-labeled protein sample to obtain information on segment mobility, polarity or inter-spin interactions and distances, in addition to changes in surface hydration dynamics⁸. EPR analysis via site-specific spin labeling has greatly aided the structural study of protein aggregation and structure of amyloid fibers over the last decade^{12,13,14,15}. The analysis of EPR spectra of spin labeled side chains reveals valuable information on mobility and the local secondary and tertiary environment of the labeled side chains of proteins^{16,17,18,19}. EPR spectra, however, does not always reveal direct information on protein-solvent interactions and the solvent dynamics, which is crucial for understanding the tertiary fold of the protein segment. The EPR power saturation method for spin-labeled protein in the presence of various paramagnetic colliders (Ni-DDTA, O₂ or CrOx) was proven very useful in studying solvent accessibility of membrane-docked protein segments^{20,21,22,23}. However, such studies are not easily implemented for studying hydration of proteins and protein-protein complexes in solution, and are not feasible for probing dynamic biochemical events. Overhauser DNP, on the other hand, is able to probe protein-solvent interactions with fast (ms) temporal resolution, and can thus monitor the solvent dynamics at specific protein sites throughout the course of protein aggregation to amyloid fibers in solution state under ambient conditions.

We demonstrate the validity and feasibility of the DNP tool, complemented by EPR analysis, as a biochemical tool by applying it to the study of *in vitro* fibrillar aggregation of tau proteins. Tau is a microtubule-associated protein in healthy subjects, but also found as a main components of neurofibrillary tangles and plaques that are surrogate markers of neurodegenerative pathologies of Alzheimer's disease (AD) and other taupathies^{24,25}. Little is known about the cause-effect relationship between the complex disease pattern of taupathies and fibrous deposits, complicated by the fact that *tau* aggregation *in vitro* does not occur spontaneously, but requires external inducer molecules such as polyunsaturated fatty acid micelles or heparin that carry extended negative charges²⁶. Systematically studying the kinetics of and contributors to tau assembly will help unravel the mechanism by which tau undergoes self-aggregation to form mature fibrils.

Because the Overhauser-induced ¹H NMR signal contrast is local and specific to one protein site, effects of large inducer species such as heparin, fatty acid micelles or lipid membranes can be introduced without perturbing the detected signal contrast, unlike with most other techniques. The molecular-level and protein residue-specific characterization of the tau aggregation event in solution state through DNP and EPR analysis will help elucidate details of aggregation mechanisms, e.g. finding the core protein segments that may undergo structural changes and/or participate in binding at an earlier or later stage of aggregation. The aim of this study was to answer the following basic questions in order to first validate our novel tool. (1) Is our Overhauser-DNP measurement indeed monitoring the change of hydration water dynamics on aggregating protein surfaces, as opposed to change in segment mobility, spin-spin interaction or NMR relaxation contrast? (2) If so, is probing surface hydration dynamics with protein site specificity a viable tool for monitoring protein aggregation? (3) What generic features of the protein aggregation process are we capturing by probing surface hydration dynamics throughout aggregation?

Experimental

Expression and Purification

The expression and purification of tau¹⁸⁷ has been previously described²⁷. Briefly, tau¹⁸⁷ contains residues 255–441 of the longest human tau isoform, cloned into the pET28 vector. In addition, our constructs contained the substitutions C291S, C322S and S413C to create S413C-SL tau¹⁸⁷, or only C291S to create C322-SL tau¹⁸⁷²⁷. Ecoli BL21 (DE3) culture was grown from frozen glycerol stock overnight in 10 mL luria broth (LB). This culture was used to inoculate 1 L of fresh LB and grown at 37°C with shaking. At A₆₀₀ optical density of 0.6–0.8, tau¹⁸⁷ expression was induced by incubation with 1 mM isopropyl-β-D-thiogalactoside for 3 hours, then the bacterial product was harvested by centrifugation for 20 min at 4500 × g (Beckman J-10). The pellets were stored at –80°C until further use.

The bacteria from the 1 L culture were washed and resuspended in 20 mL lysis buffer (20 mM MOPS (pH 7.4), 500 mM NaCl, 10 mM imidazole, 0.1 mM EDTA, and 1 mM DTT, supplemented with PMSF (1 mM), leupeptin (0.5 ug/ml), aprotinin (2 ug/ml) and pepstatin A (0.7 ug/ml). After incubation for 30 min on ice with lysozyme (2 mg/mL), DNase (20ug/mL) and MgCl₂ (10 mM), the samples were sonicated for 6 × 20 seconds at power setting 5, with 1 min cooling on ice between each sonication, using a Branson Sonifier 250. The lysate was cleared by centrifugation for 30 min at 10,000 rpm in a Beckman J-20 rotor. After addition of another 1mM PMSF, the cleared lysate was heated for 15 min in a 65° C water bath to denature bacterial proteins. After adding another 1mM PMSF, the lysate was cooled and cleared again by centrifugation, as described above. The supernatant was then loaded at 4°C onto a Ni-NTA agarose column preequilibrated with lysis buffer. The column was washed with 20 mL of lysis buffer followed by 40 mL lysis buffer containing 1M NaCl to remove contaminating nucleotides. Finally, the column was washed again with lysis buffer, and subsequently tau¹⁸⁷ was eluted with lysis buffer containing 300 mM imidazole. Elution fractions were analyzed by SDS-PAGE, and the fractions containing pure tau proteins were pooled. Protein concentration was determined by absorbance at A₂₇₄ using an extinction coefficient $\epsilon_{274} = 2.8 \text{ cm}^{-1}\text{mM}^{-1}$. The eluted protein was reduced with 5 mM DTT, and monomeric tau was precipitated by addition of equal volume of ice-cold EtOH and incubation at –20 °C overnight. The tau protein precipitate was collected by centrifugation at 14,000 × g.

Spin Labeling tau

Precipitated Tau was solubilized in 6M guanidinium hydrochloride and labeled with 10 fold molar excess of (1-oxyl-2,2,5,5-tetramethylpyrrolidin-3-yl) methyl methanethiosulfonate (MTSL) spin-label (Toronto Research Chemicals) or diamagnetic analog of the MTSL (1-Acetyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl methanethiosulfonate (dMTSL, Toronto Research Chemicals) for 1–2 hours at room temperature. Excess spin label was removed by buffer exchange using a PD-10 column (Amersham Pharmacia Biotech) preequilibrated in storage buffer (20 mM sodium phosphate @ pH 7, 100 mM NaCl, 0.1 mM EDTA). To determine the extent of labeling, 20 μM tau¹⁸⁷ was reacted with 20 μM 2,2'-dithiodipyridine, and quantified by absorbance at A₃₄₃ to be >95%. The protein was concentrated using a centrifugal filter (MW cut off 10,000 kDa, Amicon). The final spin-labeled protein was 95% pure as determined by SDS-PAGE.

X-band cw EPR Spectroscopy

Cw EPR spectra were acquired at 295K on a Bruker EMX X-band spectrometer equipped with a rectangular TE₁₀₂ cavity using a center field of 0.35 Tesla and microwave frequency of 9.8 GHz. Typically, a modulation amplitude of 0.25–1 Gauss, a modulation frequency of

100 kHz and a field sweep width of 150 Gauss were used. A quartz capillary with a 0.66 mm inner diameter was used as the sample tube.

Overhauser-enhanced ^1H NMR spectroscopy

DNP-enhanced NMR experiments were carried out using the EPR instrument components as described above. A home-built U-shape NMR coil made of 0.013" diameter teflon coated silver wire was introduced into the TE_{102} EPR cavity. The NMR coil is connected via a coaxial cable to a simple LC tuning box (14.8 ± 0.1 MHz), and connected to a broadband channel of a Bruker Avance NMR spectrometer. The sample temperature was kept constant throughout the DNP experiment by flowing nitrogen gas over the sample at an optimized rate for a given power setting at 9.8 GHz²⁸. Consecutive NMR spectra were recorded into a two dimensional experiment over a 10–15 hour period.

Aggregation of tau¹⁸⁷

Aggregation of tau¹⁸⁷ was induced by mixing MTSL-labeled tau¹⁸⁷ and heparin (18kDa, Sigma) at a molar ratio of 8:1 under ambient conditions. For ^1H DNP experiment, the final tau¹⁸⁷ concentration was adjusted to 500 μM . Time-resolved DNP-enhanced ^1H NMR or EPR spectra were recorded, immediately after heparin addition, continuously over 10–15 hours.

Turbidity Measurements

To monitor aggregation, 120 μL of 100 μM tau¹⁸⁷ protein in storage buffer was placed in a (100 μL) microcuvette at room temperature. Immediately after inducing aggregation with heparin, optical density at 350 nm was monitored using a spectrophotometer (Shimadzu UV-1601) equipped with an automated multi-cell changer.

Fluorescence Spectroscopy

For fluorescence measurement, 25 μM of tau¹⁸⁷ in an assay buffer (20 mM sodium phosphate (pH 7), 100mM NaCl, 100uM EDTA) was first incubated with 20 μM ThioflavinT dye (Sigma). This was followed by mixing tau¹⁸⁷ with heparin, and monitoring changes in fluorescence intensity over time. Fluorescence was measured at room temperature with a Varian Cary Eclipse Fluorimeter with excitation at 450 nm and emission at 484 nm. Excitation and emission slit widths were set to 5 nm.

Transmission Electron Microscopy

TEM experiments were performed as described previously²⁵. Briefly, 2 μL of tau¹⁸⁷ fibrillar solution (aggregation induced >10 hours) was applied to a 300 mesh Formvar/carbon coated copper grids, and then the excess liquid removed by blotting on filter paper. Samples were stained with 10 μL of 1.5% uranyl acetate followed by removing the excess uranyl acetate by blotting. Finally, the samples were washed with 10 μL of deionized water and air-dried. Samples were analyzed using a JOEL JEM-1230 transmission electron microscope.

Results and Discussion

We present studies on an N-terminally truncated tau fragment, that contains residues 255–441 of the longest human tau isoform. This fragment contains four microtubule binding (MTB) repeat regions, R1–R4, and a hexahistidine tag fused to the N-terminus, resulting in tau¹⁸⁷ (Fig. 1A)²⁷. Tau belongs to a class of intrinsically disordered proteins described as unstructured and unfolded in the native state²⁹. Upon aggregation to its pathological form, tau forms paired helical filaments (PHF) with cross beta structure that are characteristic of amyloid fibers. The MTB repeat regions constitute the core of PHF, in agreement with the

studies of aggregated truncated tau fragments, proteolysis studies, fluorescence and NMR studies^{30,31,32,33}. We have examined two spin labeled mutants of tau¹⁸⁷ at sites C322 (tau¹⁸⁷C322-SL), that is a part of a third repeat region and S413 (tau¹⁸⁷C413-SL) that is located outside the MTB region, close to the end of the carboxy terminus. In monomeric tau, both spin labeled sites are exposed to solvent due to the unfolded nature of tau protein in its native state. The C322 site is expected to be part of or close to the tau residues, which constitute the filament core with characteristic beta sheet fold^{34,35}. Site S413 is expected to be outside the aggregating core, and thus is expected to remain relatively flexible and largely water-accessible, before as well as after aggregation.

Both spin labeled mutants were analyzed for their ability to form amyloid fibers upon incubation with heparin (see experimental details). TEM images (Fig. 1C) confirmed the formation of amyloid fiber by tau¹⁸⁷C322-SL, which was further successfully verified by ThioT stain fluorescence measurements (data not shown, see experimental details). However, aggregates formed by the tau¹⁸⁷S413C-SL mutant were shown to be morphologically unstructured, as revealed by TEM image (Fig. 1C), and were amyloid fiber-deficient as verified by ThioT stain fluorescence measurements.

Part of the first question posed, whether our Overhauser-DNP approach monitors changes of hydration water dynamics at the surface of aggregating tau proteins, is addressed by comparing the kinetics of tau¹⁸⁷C322-SL and tau¹⁸⁷C413-SL aggregation, as probed through spin labels at the C322 or S413 site of tau¹⁸⁷. Upon addition of heparin and throughout aggregation, tau¹⁸⁷C322-SL shows distinct and gradual decrease in ¹H DNP NMR enhancement (about 5 fold), with the effect starting to level off at around 200 minutes, while tau¹⁸⁷S413C-SL does not display any distinct change in the DNP signal enhancement over ~600 minutes (Fig. 1B). However for both mutants, the formation of some type of supramolecular complexes were confirmed using TEM (Fig. 1C) or turbidity analysis (data not shown, see experimental details). Then, why is it that only minimal changes in the ¹H DNP Overhauser effect were observed during tau¹⁸⁷S413C-SL aggregation, but a dramatic effect was observed during tau¹⁸⁷C322-SL aggregation? The answer can be found by considering that the ¹H DNP enhancement effect of water relies heavily on the local dynamics (within 5 Å of the unpaired electron) around the spin label attached at sites C322 or S413C of the tau¹⁸⁷ fragment. So, only if the surface hydration water dynamics at these locations is altered e.g. due to protein folding, packing or neat inter-protein binding involving the spin labeled segment, will the ¹H DNP signal enhancement be modulated. Thus, our results show that the tau segment that contains the S413C site does not become embedded in the aggregating filament core or becomes a part of a protein network formed through non-specific protein-protein interactions. In these scenarios, the S413C-SL site remains largely water accessible and/or there exists a random distribution of varying degrees of water accessible and excluded sites. Either way, the ¹H DNP enhancement will be minimally modulated, while 350 nm light will still display strong scattering intensities (data not shown) due to the formation of large protein-protein or protein-heparin complexes with unstructured morphology as shown in Fig. 1C.

Our next task is to elucidate and confirm in greater detail the origin of the ¹H DNP signal modulation occurring during the fibrilization of tau¹⁸⁷C322-SL, as displayed in Fig. 1-A. The DNP signal enhancement is driven by on-resonant continuous wave (cw) irradiation of the EPR signal, and thus depends on the extent of saturation of the spin label's EPR signature⁷. Thus, one question we would like to address here is whether the extent of EPR saturation is contributing to or dominating the DNP signal modulation. Closely related to that is the question whether the change of the EPR absorption signal intensity during the aggregation process is the origin of the change in DNP enhancement observed throughout

the aggregation of tau. We verified that this is not the case with following sets of experiments.

First, we compared the change of ^1H DNP signal enhancement and EPR signal amplitude of the center hyperfine line by applying the same microwave power of 128 mW, on-resonant to the center hyperfine line for ^1H DNP as well as for cw EPR measurements (Fig. 2). The trend of decrease in EPR signal intensity displays completely different kinetic features compared to the decreasing trend of ^1H DNP enhancement throughout the same time course of tau¹⁸⁷ aggregation. A much greater depth of signal modulation is observed over the time course, in this case, for EPR compared to ^1H DNP analysis. Note that 128 mW corresponds to far less than (<100%) saturating power condition for DNP, but significant (e.g., >30%) oversaturating condition for EPR detection. Thus at this non-saturating power for DNP, saturation factor s (of Eq.1) will not have approached s_{max} . This level of saturation, however, is already sufficient to significantly alter the intensity of the detected EPR signal, because different degree of (dipolar or exchange) coupling and/or mobility will result in different saturability of the EPR signal. However, the key finding and emphasis here is that even under this experimental condition, where the DNP measurements would clearly depend on the EPR signal saturation factor s , the DNP effect does not mirror the changes of EPR absorption signal, but displays distinctly unique contrast.

The next test was to determine and compare the DNP factor $\rho \cdot s_{\text{max}}$ for the tau¹⁸⁷ species with various levels of hydration at the C322-SL site. From this measurement we can extract and compare the translational correlation times between hydration water and spin label (Eq 2). We do this by first determining the DNP enhancement at extrapolated, infinite microwave power, where s then approaches s_{max} . Extrapolation to maximum DNP enhancement will account for the differences in saturability of EPR transitions that arise from various EPR line broadening effects throughout protein aggregation, with the assumption that the number of spins in the sample remains constant. This assumption has been confirmed as the total EPR signal stays constant as a function of time (double integral of differential EPR signal). Thus, $\rho \cdot s_{\text{max}}$ (Eq. 1) can be extracted upon extrapolation of s to s_{max} and measurement of f (Table 1). Monomeric tau¹⁸⁷ is freely dissolved in solution as an unfolded protein, where the C322-SL site is mobile and exposed to solvent, and thus larger values for ρ are expected. The $\rho \cdot s_{\text{max}}$ values have been determined here for two scenarios that display significant changes of EPR line broadening. In the first scenario, EPR line shape changes in the beginning (10–15 minutes) versus after >10 hours of aggregation, among others reasons, due to changes of segment mobility as the C322-SL site gets incorporated into the aggregate. In the second scenario is where EPR line broadening is distinctly different due to varying degrees of inter-spin interaction, as tested by diluting the paramagnetically labeled tau¹⁸⁷ with tau proteins labeled with diamagnetic analog label (see experimental details)^{21,12}. We produced tau¹⁸⁷C322-SL, composed of 100%, 50% and 20 % paramagnetically labeled C322-SL diluted with diamagnetically labeled protein, so that the total tau¹⁸⁷ protein concentration remains the same. These three sets of samples would demonstrate distinctly different EPR line broadening effects due to different inter-MTSL labeled site interaction (dipolar or exchange), which effect is especially dominant if parallel tau stacking to a tight hydrophobic core would occur in solution state. The result of the quantitative ^1H DNP analysis, to obtain $\rho \cdot s_{\text{max}}$, and estimate values for τ , are summarized in Table-1 for all scenarios and samples mentioned. While the detailed analysis of EPR spectra of the tau aggregation mechanism is the subject of an ongoing study, we are currently able to draw the clear conclusion that the ^1H DNP contrast is predominantly represented by the change of the coupling factor, and not by the differences of the saturation factor at maximum power, s_{max} , or the leakage factor, f (Table-1). All three preparations containing 100%, 50% or 20% tau¹⁸⁷C322-SL samples show comparable $\rho \cdot s_{\text{max}}$ of 0.13 before protein aggregation was induced. All three sample preparations after >15 hours of

aggregation display again comparable $\rho \cdot s_{\max}$ values, this time reduced by about 6 fold to 0.022 ± 0.005 , compared to the $\rho \cdot s_{\max}$ value for monomeric tau. This observation proves that the DNP contrast is inherent to changes in ρ , and thus changes in hydration water dynamics at the surface of proteins at site C322. This also provides indirect evidence that $s_{\max}$ approaches 1, and the altered inter-spin interactions are not changing the $\rho \cdot s_{\max}$ contrast in the case of nitroxide spin-labels attached to proteins. This is an expected outcome, given that the rotational correlation time of tau¹⁸⁷ is slow enough to cause rapid ¹⁴N nuclear spin T_1 relaxation of the nitroxide's hyperfine lines, leading to complete mixing of hyperfine states as we have previously shown^{9,8}. Thus, assuming $s_{\max}=1$, we can model τ from ρ , as listed in Table-1.

The final question that we would like to address is: what is the general feature of the protein aggregation process probed through changes of the surface hydration water at spin labeled protein segments versus, e.g., the restriction of the same segment's rotational mobility obtained from EPR analysis? We performed simple EPR analysis by monitoring the change in EPR signal intensity during aggregation, which is a first and simple indicator of the variation in the immediate environment of the spin labeled site^{36,21,37}. The quantitative analysis of the individual EPR spectra that were obtained for kinetic monitoring is beyond the scope of this paper, among other reasons, due to the unfolded nature of tau¹⁸⁷. Therefore, we tracked the changes of the EPR signal intensity of the $I(^{14}\text{N})=0$ center field hyperfine line of tau¹⁸⁷C322-SL throughout the time course of aggregation. This was possible because the EPR spectra of tau¹⁸⁷C322-SL, even after many hours of aggregation, displayed significant features of motional averaging due to the relatively high mobility of the C322SL site throughout aggregation. As we verified that the contribution of monomeric tau is ceased early in the time course and that the total number of detected electron spins does not change with time, we can attribute the ~5–6 fold decrease in amplitude for the EPR line intensity to decreasing mobility, increasing tertiary contact and/or increasing interspin (dipolar or exchange) interaction of the spin labeled segment during aggregation of monomeric tau into the characteristic global fold to form amyloid fibers. We observed and compared the time dependence of the EPR signal intensity of the $I(^{14}\text{N})=0$ hyperfine line to the time dependence of the ¹H DNP signal (Fig. 3). We observe that changes of the ¹H DNP signal modulation are initially greater than EPR signal modulation, and additionally reaches an earlier plateau. So, DNP observes that the C322 site of tau¹⁸⁷ experiences a decrease in surface water hydration dynamics or water exclusion at an early stage of aggregation. This effect levels off earlier, while the EPR signal intensity continues to decrease (or the EPR linewidth continues to broaden). This indicates that the segment mobility continues to decrease, and the interaction between two C322-SL sites or the C322-SL site and another part of the tau¹⁸⁷ protein (tertiary contact) continues to increase. The observation that the EPR signal continues to change, while the DNP effect has nearly ceased also confirms, once more, that EPR is not detecting (after an initial period) the disappearance of monomeric tau populations, but the change of the environment near the spin-labeled site as the tau species undergo structural changes. So, a possible interpretation for this observation (Fig. 3) is that all monomeric tau¹⁸⁷ species become early incorporated into protein-protein complexes, where hydration water dynamics at site C322 is slowed beyond 550 ps or water access at site C322 is excluded beyond 5 Å, so that the ¹H DNP effect ceases to occur. Yet, these protein-protein complexes may continue to undergo structural rearrangements to eventually form amyloid fibers. Thus the segments continue to undergo tighter packing and/or experience increasing inter-protein interaction, as registered by EPR. So, the answer to the earlier posed question is that monitoring of hydration water dynamics with site-specificity through ¹H DNP analysis, complemented with more quantitative EPR analysis, has the potential to detect early aggregation events and oligomeric protein complexes in solution state.

Conclusions

We introduced a novel approach to dynamically monitor protein aggregation processes and mechanisms with protein residue-specific resolution, under ambient condition and in solution state. We utilize generic features of protein aggregation, namely the alteration of hydration water dynamics at the surface of protein sites, if these segments undergo inter-protein binding and progressively get buried in the hydrophobic cores of protein aggregates. This mapping is made possible through DNP-enhanced ^1H NMR signal of hydration water that is closely, within 5 Å, interacting with site-specific nitroxide spin labels tethered to proteins. We validated, on the example of the aggregation of a tau protein fragment, that our Overhauser-DNP measurement indeed monitors the change of hydration water dynamics on the protein surfaces, as opposed to changes in segment mobility, spin-spin interaction or NMR relaxation contrast. We demonstrated that the protein aggregation process can be mapped with site-specific resolution, and found that site C322 gets significantly buried in a hydrophobic complex during progressing aggregation. Finally, we demonstrate that probing protein amyloid fiber aggregation mechanism through the alteration of surface hydration water dynamics is a unique complementary method to EPR derived structural information based on analyzing the extent of segment mobility and changes in the local protein environment. Potentially, probing aggregation through surface hydration water allows for the characterization of early aggregation event and oligomeric protein complexes. The ability to monitor protein aggregation dynamically and with residue-specific resolution provides a powerful approach to unraveling, in the future, the role of aggregation inducer molecules as well as the mechanisms and structural characteristics of protein aggregation to amyloid fibers.

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Fig 1.

A. Diagrammatic representation of tau¹⁸⁷: the N-terminally truncated tau fragment that contains residues 255–441 of the longest human tau isoform and includes all four MTB repeat domains R1–R4. **B.** Time dependence of ¹H DNP-enhanced signal of hydration water as probed through MTSL spin-labeled cites C322 and S413C of tau¹⁸⁷. **C.** Transmission electron micrographs showing the different morphologies of the aggregates formed by tau¹⁸⁷C322-SL and tau¹⁸⁷S413C-SL after 16–24 hours of heparin-induced aggregation.

Fig. 2. Time dependence of ^1H DNP signal intensity and EPR signal intensity of the center ($I^{14}\text{N}=1$) hyperfine line probed at site C322-SL, during 10 hours of aggregation of tau^{187} with application of the same microwave power of 128 mW for DNP saturation and detection of EPR resonances. This intermediate power setting provides insufficient power for DNP, but too much power to obtain an optimally narrow EPR signal.

Fig. 3. Time dependence of ^1H DNP signal intensity and EPR signal intensity of the center ($I^{14}\text{N}=1$) hyperfine line probed at site C322-SL, during 10 hours of aggregation of tau^{187} with application of optimal (close to extrapolated maximum) power setting for ^1H DNP and optimal (non-saturating) power setting for EPR/

Table 1

DNP parameters, leakage factor f and coupling factor ρ for spin labeled tau¹⁸⁷ at site C322 for 100%, 50% and 20% paramagnetic spin labeled species (diluted with diamagnetic spin labeled species) before and after fibrilization.

Sample	Monomeric Tau			Aggregated Tau		
	f	$\rho^*s_{\max}$	τ (ps)	f	$\rho^*s_{\max}$	τ (ps)
100 % C322-SL	0.19	0.127	143	0.29	0.023	511
50 % C322-SL	0.15	0.124	147	0.24	0.02	500
20 % C322-SL	0.06	0.132	140	0.15	0.028	450