High-resolution Neutron Spectroscopy to Study Picosecond-nanosecond Dynamics of Proteins and Hydration Water

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TITLE:

High resolution neutron spectroscopy to study ps-ns dynamics of proteins and hydration water illustrated with the amyloid aggregation process.

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SUMMARY:

Neutron backscattering spectroscopy offers a non-destructive, and label-free, access to the ps-ns dynamics of proteins and their hydration water. The method workflow is presented and illustrated with two studies on amyloid proteins, i.e. on the time-resolved dynamics of lysozyme during aggregation and on the hydration water dynamics of tau upon fiber formation.

ABSTRACT:

Neutron scattering offers the possibility to probe the dynamics within samples on a wide range of energies in a non-destructive manner and without labeling. In particular, neutron backscattering spectroscopy records the scattering signals at multiple scattering angles simultaneously and is well suited to study the dynamics of biological systems on the ps-ns time scale. By employing D_2O – and possibly deuterated buffer components – the method allows to monitor both center-of-mass diffusion and backbone and side-chain motions (internal dynamics) of proteins in liquid state. Additionally, hydration water dynamics can be studied by employing perdeuterated proteins in H_2O. Here, we present the workflow employed on the instrument IN16B at the Institut Laue-Langevin (ILL), to investigate protein and hydration water dynamics. The preparation of hydrated protein powder samples using vapor exchange is explained. The data analysis procedure for both protein and hydration water dynamics is described for the different type of datasets (quasi-elastic spectra or fixed-window scans) that can be obtained on IN16B. The method is illustrated with two studies involving amyloid proteins. The aggregation of lysozyme into µm sized spherical aggregates – denoted particulates – is shown to occur in a one-step process on the space and time range probed on IN16B, while the internal dynamics remains unchanged. Also, the dynamics of hydration water of tau was studied on hydrated powders of perdeuterated protein. It is shown that translational motions of water are activated upon the formation of amyloid fibers. Finally, critical steps in the protocol are discussed as well as how neutron scattering is positioned regarding the study of dynamics with respect to other experimental biophysical available methods.

INTRODUCTION:

The neutron is a charge-less and massive particle which has been successfully used over the years to probe samples in various fields from fundamental physics to biology[1]. For biological applications, small-angle neutron scattering, inelastic neutron scattering and neutron crystallography and reflectometry are extensively used[2–4]. Inelastic neutron scattering provides an ensemble-averaged measurement of the dynamics without requiring specific labeling *per se* and no dependence on the system size[5]. The measurement can be done using highly complex environment for the protein under study that mimics the intracellular medium like deuterated bacterial lysate or even *in vivo*[3,6,7]. Different experimental setup can be used to study dynamics, namely i) time-of-flight - giving access to sub-ps-ps dynamics -, ii) backscattering – giving access to ps-ns dynamics -, and iii) spin-echo – giving access to dynamics from ns to hundreds of ns. Neutron backscattering makes use of the Bragg's law $2d\ sin\theta = n\,\lambda$, where **d** is the distance between planes in a crystal, θ the scattering angle, **n** the scattering order and λ the wavelength. The use of crystals for backscattering towards the detectors permits to achieve a high resolution in energy, typically of around 0.8 μeV. To measure the energy exchange, either a Doppler drive carrying a crystal in backscattering is used to define and tune the incoming neutron wavelength[8–10] (Figure 1) or a time-of-flight setup can be used at the cost of a decrease in energy resolution[11].

[Place **Figure 1** here]

For backscattering spectroscopy, the main contribution to the signal from hydrogen proton-rich samples such as proteins comes from incoherent scattering, for which the scattering intensity $S_{inc}(\mathbf{q}, \omega)$ reads[12]:

$$S_{\mathrm{inc}}(\mathbf{q},\omega) = \frac{\sigma_{\mathrm{inc}}}{4\pi}\frac{k'}{k}\frac{1}{2\pi\hbar}\int_{-\infty}^{\infty}\left\langle e^{\mathbf{q}\mathbf{r}_j(0)}e^{\mathbf{q}\mathbf{r}_j(t)}\right\rangle e^{i\omega t} \qquad (1)$$

where σ_{inc} is the incoherent cross-section of the element considered, k' is the norm of the scattered wavevector, k the norm of the incoming wavevector, **q** the momentum transfer, $\mathbf{r}_j(t)$ the position vector of atom j at time t and ω the frequency corresponding to the energy transfer between the incoming neutron and the system. The angular brackets denotes the ensemble average. Hence, incoherent scattering probes the ensemble-averaged single-particle self-correlation of atom positions with time and gives the self-dynamics averaged over all atoms in the system and different time origins (ensemble average). The scattering function is the Fourier transform in time of the intermediate scattering function I(**q**, t), which can be viewed itself as the Fourier transform in space of the van Hove correlation function $G(\mathbf{r}, t) = \frac{1}{N}\int d\mathbf{r}'\ \langle \rho(\mathbf{r}' - \mathbf{r}, 0), \rho(\mathbf{r}, t)\rangle$ where $\rho(\boldsymbol{r}, t)$ is the probability density of finding an atom at position **r** and time t[13]. For a Fickian diffusion process, the self-diffusion function $G_s(r,t) = (4\pi Dt)^{-3/2}e^{-\frac{r^2}{4Dt}}$ results, after a double Fourier transform, in a scattering function consisting in a Lorentzian of line width given by $\gamma = Dq^2$. More sophisticated models were developed and found useful such as the jump diffusion model by Singwi and Sjölander for ps-ns internal protein dynamics[14] or the rotation model by Sears for hydration water[15–17].

On the neutron backscattering instrument IN16B[8,9] at the ILL (Figure S1), France, a setup commonly used with proteins is Si 111 crystals for the analyzers with a Doppler drive for tuning the incoming wavelength (Figure S2), thereby giving access to the momentum transfer range ~0.2 $Å^{-1}$ < q < ~2 $Å^{-1}$ and energy transfer range of -30 μeV < $\hbar\omega$ < 30 μeV – corresponding to time scales ranging from a few ps to a few nanoseconds and distances of a few Å. Besides, IN16B offers the possibility to perform elastic and inelastic fixed-window scans (E/IFWS)[10], which consist in data acquisition at a fixed energy transfer. As the flux is limited when working with neutrons, E/IFWS allows to maximize the flux for one energy transfer, and thus reduce the

acquisition time needed to obtain a satisfying signal-to-noise ratio. A more recent option is the backscattering and time-of-flight scattering (BATS) mode[11], which allows to measure a wide range of energy transfers, i.e. -150 μeV < $\hbar\omega$ < 150 μeV, with a higher flux than with the Doppler drive, yet at the cost of a lower energy resolution (Figure S3).

An important property of neutron scattering is that the incoherent cross-section σ_{inc} has a 40 times higher value for hydrogen than for deuterium and is negligible for other elements commonly found in biological samples. Therefore, the dynamics of proteins in a liquid environment can be studied by using a deuterated buffer and the powder state allows for the study of either protein internal dynamics with hydrogenated protein powder hydrated with D_2O, or the study of hydration water for perdeuterated protein powder hydrated with H_2O. In the liquid state, neutron backscattering typically allows simultaneously accessing the center-of-mass self-diffusion of proteins (Fickian-type diffusion) and their internal dynamics. The latter are backbone and side-chain motions usually described by the so-called jump diffusion model or others[3,18]. In hydrogenated protein powders, the protein diffusion is absent and only internal dynamics needs to be modeled. For hydration water, the contributions of translational and rotational motions of water molecules present a different dependence on the momentum transfer **q**, which allows for their distinction in the data analysis process[17].

Here, we illustrate the neutron backscattering method with the study of proteins that were found to be able to unfold, aggregate into a canonical form consisting of stacks of β-strands, the so-called cross-β pattern[19,20], and form elongated fibers. This is the so-called amyloid aggregation, which is extensively studied due to its central role in neurodegenerative disorders such as Alzheimer's or Parkinson's diseases[21,22]. The study of the amyloid proteins is motivated as well by the functional role they can play[23,24] or their high potential for the development of novel biomaterials[25]. The physico-chemical determinants of the amyloid aggregation remain unclear and no general theory of amyloid aggregation is available, despite tremendous progress during the past years[21,26].

Amyloid aggregation implies changes in protein structure and stability with time, the study of which naturally implies dynamics, linked to protein conformation stability, protein function and protein energy landscape[27]. Dynamics is directly linked to the stability of a specific state through the entropic contribution for the fastest motions[28], and protein function can be sustained by motions on various time scales from sub-ps for light-sensitive proteins[29] to ms for domain motions, which themselves can be facilitated by picosecond-nanosecond dynamics[30].

Two examples of using neutron backscattering spectroscopy to study amyloid proteins will be presented, one in the liquid state to study protein dynamics and one in the hydrated powder state to study hydration water dynamics. The first example concerns the aggregation of lysozyme into μm sized spheres (called particulates) followed in real-time[5] and the second a comparison of water dynamics in native and aggregated states of the human protein tau[31].

Lysozyme is an enzyme involved in immune defense and is composed of 129 amino acid residues. Lysozyme can form particulates in deuterated buffer at pD of 10.5 and at a temperature of 90 °C. With neutron scattering, we showed that the time evolution of the center-of-mass diffusion coefficient of lysozyme follows the single exponential kinetics of thioflavin T fluorescence (a fluorescent probe used to monitor the formation of amyloid cross-β patterns[32]), thereby indicating that the formation particulate superstructures and cross-β patterns occur in a single step with the same rate. Moreover, we observed that the internal dynamics remained constant throughout the aggregation process, which can be explained either by a fast conformational change that cannot be observed on IN16B or by the absence of significant change in protein internal energy upon aggregation.

The human protein tau is an intrinsically disordered protein (IDP) of 441 amino acid for the so-called 2N4R isoform, which is involved notably in Alzheimer's disease[33]. Using neutron backscattering on powders of perdeuterated protein tau, we showed that hydration water dynamics is increased in the fiber state, with a higher population of water molecules undergoing translational motions. The result suggests an increase

in hydration water entropy might drive the amyloid fibrillation of tau.

PROTOCOL:

1. Preparation of deuterated buffer for proteins in the liquid state.
   1.1. Dissolve all components of the buffer in pure D_2O.
   1.2. If the pH electrode was calibrated in H_2O, adjust the pD according to pD = pH + 0.4 using NaOD or DCl.
   NOTE: the use of D_2O instead of H_2O might affect protein solubility and the buffer conditions might need to be adapted, e.g. by a slight change in salt concentration.
2. Preparation of H_2O-hydrated powders of perdeuterated protein
   2.1. Preparation of the sample holder
      2.1.1. Clean thoroughly a flat aluminum sample holder with its indium wire seal and screws with water and ethanol and let it dry.
      2.1.2. Weigh the different parts of the sample holder, that is, bottom, lid and indium wire separately on a precision balance.
      2.1.3. Put in place the 1 mm indium wire seal in the groove of the bottom part of the sample holder, leave a small overlap where the two ends join (Figure 2).
      2.1.4. Place an appropriate amount of lyophilized protein (typically around 100 mg of protein) such that it fills the inner surface of the bottom part of the sample holder.
   2.2. Hydration of the powder
      2.2.1. Place the sample holder in a dessicator with a petri dish containing P_2O_5 powder (careful: very corrosive) for 24 h to completely dry the protein powder[34] (Figure 3). Weigh the dry bottom part of the sample holder containing the indium seal and the dry powder to get m_{dry}.
      2.2.2. Remove the P_2O_5 from the dessicator and put a petri dish with D_2O inside. Control the mass of the powder regularly to check the hydration level $h = m_{hyd} / m_{dry}$ where m_{hyd} and m_{dry} are the mass of the hydrated powder.
      NOTE: for highly hydrophobic proteins like insulin, it might be necessary to increase the temperature inside the dessicator to get a higher vapor pressure and reach the desired hydration level h.
      2.2.3. Repeat at least three times steps 2.2.1. and 2.2.2. to properly convert all exchangeable hydrogens to deuterons.
      2.2.4. Hydrate the powder to slightly above the desired level, let the bottom part of the sample holder with the indium wire and hydrated powder on the precision balance and wait for the mass to decrease slowly to the desired value to get the target h (typically 0.2 – 0.4 if a medium sized globular protein is to be covered by one complete hydration layer).
      2.2.5. Quickly put the lid on the bottom part and close the sample holder first with four screws to stop the vapor exchange (Figure S4).
      2.2.6. Place and tighten all remaining screws until no gap between the bottom part and the lid is visible (Figure S5).
      2.2.7. Weigh the sealed sample holder to be able to check for any potential hydration loss via leaks after the neutron experiment.
3. Incoherent neutron scattering
   NOTE: The following concerns an experiment conducted on the backscattering spectrometer IN16B at the ILL[8,9], using a cryofurnace as a sample environment. The instrument control system will change from one instrument to the other but the working principles remain the same.
   3.1. Liquid state sample

3.1.1. Dissolve your protein in the deuterated buffer.

3.1.2. Determine the appropriate volume of liquid to be put in the sample holder using water (there should not be any overflow when the sample holder is closed, Figure 4).

3.2. Sample insertion

3.2.1. Thoroughly dry the sample stick, remove the previous sample if any after checking that the ionizing radiation dose is lower than 100 µSv/h before handling any material (at the ILL).

3.2.2. Place your sample, check for proper centering relative to the beam center (Figure S6), insert the sample stick in the cryofurnace, turn on the vacuum pump to reach less than 10^{-3} bar and flush out the air inside the cryofurnace by repeating the following steps 3 times, fill the cryofurnace with helium gas until atmospheric pressure is reached, then remove the gas again with the vacuum pump.
NOTE: in case of a flat sample holder, the stick has to be oriented at a 45° angle relative to the incoming beam.

3.2.3. Let some helium gas in the cryofurnace such that the pressure is around 0.05 bar.

3.3. Data acquisition

3.3.1. Data will be acquired in presence of the instrument local contact for inexperienced users (at least for the beginning of the experiment). The protocol and software used depend on the facility and the instrument.

NOTE: every experiments will require the acquisition of calibration data, that is, the empty cell for subtraction or absorption corrections, the buffer alone at the different temperature used to model the background and a measurement of vanadium (or equivalently the sample at a temperature of 10 K or lower) to obtain the resolution function of the instrument.

4. Data analysis – E/IFWS

4.1. Using the Python software nPDyn v3.0.0[35], import the dataset using the following:

```
>>> from nPDyn.dataParsers import IN16B_FWS

>>> sample = IN16B_FWS(
...       "path_to_data/first_scan:last_scan.nxs",
...       detGroup="path_to_detector_grouping_file.xml"
... ).process()

>>> sample = sample.get_q_range(0.3, 1.8)
```

4.2. Data corrections (optional) can be performed with the following commands (see the documentation of nPDyn for more information, Figure 5):

```
# it is assumed that data for empty cell, vanadium, and buffer
# were imported already in dataset called 'empty_cell', 'vanadium',
# and 'buffer', respectively.

# for empty cell subtraction with a scaling factor
# (errors are propagated automatically)
>>> sample = sample - 0.95 * empty_cell

# for correction using Paalman-Ping coefficient
# (mutually exclusive with the example above)
>>> sample = sample.absorptionCorrection(empty_cell)

# for normalization
>>> sample = sample.normalize(vanadium)

# for binning along observable axis
# observable is the aggregation time here
```

```
>>> sample = sample.bin(3, axis=0)
```

4.3. The dataset – samples, empty cell, deuterated buffer, and vanadium – can be fitted using built-in models or user-defined model (see nPDyn documentation). Protein dynamics in liquids is typically modeled using the following:

$$S(\mathbf{q},\omega) = R(\mathbf{q},\omega) \otimes \beta(\mathbf{q})\left[a_0(\mathbf{q})\mathcal{L}_\gamma(\mathbf{q},\omega) + \left(1 - a_0(\mathbf{q})\right)\mathcal{L}_{\gamma+\Gamma}(\mathbf{q},\omega)\right] + \beta_{D_2O}\mathcal{L}_{D_2O} \qquad (2)$$

where $R(\mathbf{q},\omega)$ is the resolution function, β a scalar independent for each momentum transfer q, a_0 is the elastic incoherent structure factor (EISF), $\mathcal{L}_\gamma$ a Lorentzian accounting for center-of-mass diffusion with a width given by $\gamma = D_s q^2$ - D_s being the self-diffusion coefficient -, $\mathcal{L}_{\gamma+\Gamma}$ is a Lorentzian following the jump-diffusion model[14] accounting for internal dynamics with $\Gamma = D_i q^2/(1 + D_i q^2 \tau)$ - D_i being the apparent diffusion coefficient for internal dynamics and τ a relaxation time for diffusive motions – and $\beta_{D_2O}\mathcal{L}_{D_2O}$ being the fitted signal from D_2O re-scaled by its volume fraction in the sample.

```
>>> from nPDyn.models.builtins import (
...   modelPVoigt,
...   modelProteinJumpDiff,
...   modelCalibratedD2O,
... )

# builtin models use a column vector of the momentum
# transfer q values
>>> q = vanadium.q[:, None]

# the vanadium is fitted using a pseudo-Voigt profile
>>> vanadium.fit(modelPVoigt(q))

# for pure D2O, a model with calibrated linewidth
# for different temperatures is included in nPDyn
>>> buffer.fit(modelCalibratedD2O(q, temp=363))

# here, equation 2 is used for liquid samples
# convolution with resolution function and addition of
# D2O background is done automatically with the
# provided arguments
>>> sample.fit(modelProteinJumpDiff(q),
...   res=vanadium,
...   bkgd=buffer,
...   volume_fraction_bkgd=0.95
... )
```

4.4. The fitted data can be plotted using:

```
>>> from nPDyn.plot import plot
>>> plot(sample)
```

5. Data analysis – QENS

5.1. The data analysis procedure for QENS data is similar to the one described in section 4. The dataset is imported using 'IN16B_QENS.process()' function and the model for hydration water is called 'modelWater'. This model reads[17]:

$$S(\mathbf{q},\omega) = R(\mathbf{q},\omega) \otimes \left[(a_r j_0^2(qd) + a_0)\delta(\omega) + \sum_{l}^{N} (2l+1) j_l^2(qd) a_r \mathcal{L}_{l(l+1)\gamma_r} + a_t \mathcal{L}_{\gamma_t} \right] + b(q) \qquad (3)$$

where a_0, a_r, and a_t are scalars accounting for the relative contribution of elastic signal, rotational motions and translational motions, respectively, $j_l(qd)$ is the l^{th} order spherical Bessel function with q being the momentum transfer and d the O-H distance in the water molecule, $\delta(\omega)$ is the Dirac delta, which is here multiplied by the EISF, N is the highest order of spherical Bessel function used (typically around 5), $\mathcal{L}_{l(l+1)\gamma_r}$ and $\mathcal{L}_{\gamma_t}$ are the Lorentzian rotational and translational motions, respectively and b(q) is a flat background term.
NOTE: The contributions of rotational and translational motions should be convoluted to be perfectly rigorous. The success of an additive model is to be attributed to the presence of distinct populations of water on the protein surface and to the limited energy range accessible.

5.2. Similar to step 4, use the following to fit and plot the data (Figures 6 and 7):

```
>>> sample.fit(modelProteinJumpDiff(q),
...       res=vanadium,
...       bkgd=buffer,
...       volume_fraction_bkgd=0.95
... )

>>> plot(sample)
```

6. Data analysis – temperature ramp elastic fixed-window scans (EFWS)

6.1. The analysis procedure is similar to section 4, except for data processing and fitting. The temperature ramp data will be normalized by the signal at the lowest temperature (typically 10 K):

```
>>> from nPDyn.dataParsers import IN16B_FWS

>>> sample = IN16B_FWS(
...   'path_to_data/first_scan:last_scan.nxs',
...   detGroup='path_to_detector_grouping_file.xml'
... ).process()

# normalization with the 5 first points on the observable
# axis, which correspond to the temperature
>>> sample /= sample[:5].mean(0)

# the momentum transfer q range used here is smaller
# as the model used is valid for low q only
>>> sample = sample.get_q_range(0.2, 0.8)
```

6.2. The model is a simple Gaussian, the width of which is given by the so-called mean squared displacement (MSD). The model can be built and fitted by the user using the following:

```
>>> import numpy as np
>>> from nPDyn.models import Parameters, Model, Component

# a is a scaling factor
>>> params = Parameters(
...   a={'value': 1, 'bounds': (0, np.inf)},
...   msd={'value': 1, 'bounds': (0, np.inf)}
... )
```

```
>>> model = Model(params)
>>> model.addComponent(Component(
…      'gaussian',
…      lambda x, a, msd: a * np.exp(-x ** 2 * msd / 6)
… ))

>>> sample.fit(model, x=sample.q[:, None])

>>> plot(sample)
```

NOTE: The Gaussian approximation always holds for $q^2 MSD \ll 1$, but for relative comparison between samples, wider momentum transfer range can be used. More sophisticated models, that go beyond the Gaussian approximation, have been developed[36,37].

REPRESENTATIVE RESULTS:

The aggregation of lysozyme into particulates was performed at 90°C with a protein concentration of 50 mg/ml in a deuterated buffer (0.1M NaCl at pD 10.5). The formation of particulates is triggered by the temperature increase to 90°C and occurs within 6 hours (Supplementary Figure 7). The data acquisition was performed on IN16B as described in the protocol above (data are permanently curated by the ILL and accessible at http://dx.doi.org/10.5291/ILL-DATA.8-04-811). A QENS spectrum was acquired at 7 °C to fully characterize the initial state. Subsequently the temperature was increase to 90 °C (takes typically about 30 minutes on IN16B – Supplementary Figure 8) to trigger the aggregation process. The kinetics can be followed using a sliding average of QENS spectra, allowing access to the full range of energy transfer, yet with a limited resolution in time. The time resolution can be improved – to about 1 min using EFWS and to about 20 minutes using E/IFWS at four energy transfer values[5]. In the example presented we explored energy transfers of 0, 0.6, 1.5 and 3 μeV. The E/IFWS scans are acquired continuously during the aggregation process and a QENS spectrum was acquired for the final state at 90°C. The E/IFWS data were corrected for absorption using the E/IFWS of the empty cell, and normalized using the vanadium data. For the lysozyme E/IFWS, we observe an increase of the signal in time at low momentum transfer and low energy transfer, while the signal at high energy transfer and low momentum transfer decreases (Supplementary Figure 9). This qualitative observation indicates the formation of larger objects, diffusing more slowly, thereby confirming that the aggregation process took place. The analysis, according to equation 2, results in an initial center-of-mass diffusion coefficient of 15 $Å^2$/ns, in agreement with the presence of small protein clusters (supported by dynamic light scattering and HYDROPRO calculations[5,38]), which then exponentially decreases over time (Figure 8). The apparent diffusion coefficient for internal dynamics remains constant throughout the aggregation process. Hence, the formation of lysozyme particulates appears to occur in a single aggregation phase. The absence of change in internal protein dynamics suggest that either the conversion to cross-β and possible associated change in energy are too fast or that other driving effects such that increase in solvent entropy might be fully dominating within the energy range probed.

The study of hydration water dynamics around monomers and fibers of tau was performed on SPHERES at the Maier-Leibnitz Zentrum (MLZ) in Garching, Germany, using the protocol described above for powder samples. Approximately 100 mg of deuterated tau protein powder, hydrated to 0.4 gram of H_2O per gram of protein, was used. EFWS were acquired during a temperature ramp and QENS spectra at constant temperature. The EFWS were recorded starting at 20 K and increasing the temperature to 300 K at a rate of 0.2 K / min while continuously acquiring data during 5 min scans. The QENS spectra were recorded at 20 and at 280 K. The EFWS data were fitted using a simple Gaussian over the momentum

transfer range 0.2 $Å^{-1}$ < q < 0.8 $Å^{-1}$ to extract the MSD (Supplementary Figure 10). At temperatures higher than 220 K, the hydration water around fibers of tau is significantly more mobile than hydration water around tau monomers (Figure 9). The fitting of QENS data allows to obtain the linewidth and relative contribution of elastic, rotational and translational motions of hydration water in the sample (Supplementary Figure 11). It appears that the fraction of water molecules undergoing translational motion is increased around fibers and both translational and rotation diffusion coefficients of water molecules are increased around fibers. On the contrary, the ps-ns internal dynamics of the protein tau, reflecting backbone and side-chain motions, did not change upon fibrillation.

FIGURE AND TABLE LEGENDS:

Figure 1. Sketch of a neutron backscattering spectrometer with a Doppler drive. The incoming beam hits the phase space transformation (PST) chopper[39], which increases the flux at the sample position, then is backscattered toward the sample by the Doppler drive which selects an energy E_1. The neutrons are then scattered by the sample and the analyzers, made of Si 111 crystals, will only backscatter neutrons with a specific energy E_0. Hence, the momentum transfer **q** is obtained from the detected position of the neutron on the detector array and the energy transfer is obtained from the difference $E_1 – E_0$.

Figure 2. Base of a flat aluminum sample holder. The flat aluminum sample holder presents a central part exposed to the neutrons where the protein powder is maintained within a small gap – typically of around 0.3 mm - between the base and the lid. An indium wire can be used for sealing as it resists temperature increases up to 90°C. In case of higher temperatures, a Teflon seal can be used.

Figure 3. Protein drying and hydration procedure in a desiccator. The protein powder is placed in the base of the sample holder with the indium wire in place. The sample holder is placed in a desiccator in presence of either P_2O_5 for drying or H_2O/D_2O for hydration. Vacuum grease is added to avoid any leak between the bottom and the lid of the desiccator. Vacuum can be used with caution to accelerate the drying process. Heating the bottom of the desiccator can be required for highly hydrophobic samples.

Figure 4. Cylindrical sample holder for liquid samples. The protein solution is placed between the inner and outer cylinder. Make sure not to pipette too much liquid (there should be no overflow when the sample holder is closed). The indium wire is placed in the circular groove.

Figure 5. Data corrections and normalization may improve the fit. The data were imported using nPDyn as described in the Protocol. **(left)** The dataset was normalized using the data of the monitor only. **(right)** The signal of the empty can was used along with the Paalman-Ping coefficients[40] to correct for neutron absorption from the sample holder. Subsequently, the fitted model for the vanadium signal was integrated independently for each momentum transfer q and the result was used to normalized the sample dataset.

Figure 6. Plotting window generated by nPDyn showing the result of the fit. The QENS data were fitted using nPDyn as described in the Protocol. The plotting window allows to plot the data along different axes – observable, q, energies – and selectors are available to navigate along the others axes. Different types of plot are available, with the simple 'Plot' presented here.

Figure 7. Plotting window generated by nPDyn showing the fitted parameters. The QENS data were fitted using nPDyn as described in the Protocol. Similar to Figure 6, the plotting window shows here the

'Analysis – q-wise' plot. The parameters are plotted along the q values, a single horizontal line is shown for global – one value for all q – parameters. The errors are represented using a colored shaded area.

Figure 8. Lysozyme aggregation occurs in a one-step process with constant internal dynamics. The lysozyme was dissolved in the aggregation buffer (described elsewhere[5]) and E/IFWS was acquired throughout aggregation, which was triggered by temperature increase to 90°C. After absorption correction with empty cell and normalization using the vanadium signal, the data were analyzed using the jump diffusion model as described in the Protocol. The fitted center-of-mass self-diffusion coefficient is plotted as a function of time (blue triangles) along with the apparent diffusion coefficient for internal dynamics (orange squares). Reprinted with permission from Pounot et al. (2020)[5]. Copyright 2020 American Chemical Society.

Figure 9. Hydration water dynamics is increased around tau fibers. The H_2O-hydrated powders of deuterated tau fibers and monomers were sealed in a flat aluminum sample holder and EFWS data were acquired at during a temperature ramp from 20 to 300 K. After absorption correction with empty cell and normalization using the vanadium signal, the data were analyzed using the jump diffusion model as described in the Protocol. Redrawn using smaller q range ($0.2 < q < 0.8$ $Å^{-1}$) from data from Fichou et al. (2015)[31].

Supplementary Figure 1. Photographs of the instrument IN16B at the ILL. (top) The instrument IN16B as seen from the radiation controlled zone dedicated to the instrument. The incoming beam travels within the neutron guide to the vacuum chamber which contains most of the elements of the instrument (PST chopper, analyzers, sample, detectors). **(bottom)** Interior of the vacuum chamber. The PST chopper is visible as well as the analyzers surrounding the cryofurnace containing the sample. The detectors are located behind the cryofurnace. Courtesy of Laurent Thion, ecliptique.

Supplementary Figure 2. Sketch of IN16B in classical mode (with Doppler drive). The neutron beam is partly monochromatized by the velocity selector. Subsequently, the background and PST choppers will produce a single neutron pulse, from which an energy profile will be selected by the Doppler monochromator (E/IFWS or QENS mode). The neutrons are then scattered by the sample and a single energy is reflected back toward the detectors by the analyzers. Courtesy of the ILL.

Supplementary Figure 3. Sketch of IN16B in BATS mode. The BATS choppers are used to define a single neutron pulse with a defined energy range. The neutron beam is partly monochromatized by the velocity selector. Subsequently, the background chopper will remove unwanted neutrons that do not belong to the selected pulse. The neutrons are then scattered by the sample and a single energy is reflected back toward the detectors by the analyzers. Courtesy of the ILL.

Supplementary Figure 4. The sample holder is closed quickly after the powder has reached the desired hydration. The flat sample holder is quickly closed using four screws. A small gap will remain due to the indium wire. The other screws are then added and the sample holder is sealed slowly, by tightening gently each screw several times to allow the indium to relax.

Supplementary Figure 5. The sample holder properly sealed. The flat sample holder is properly sealed when no gap is visible anymore between the holder base and the lid.

Supplementary Figure 6. The sample holder is center with respect to the neutron beam. A cylindrical sample holder has been placed on the sample stick. The position of the sample holder is checked such

that the beam hits the bottom part of it.

Supplementary Figure 7. Lysozyme reproducibly forms particulates. Lysozyme was dissolved in the aggregation buffer (prepared as described in Protocol, step 1) and Thioflavin T (ThT) was added to a final concentration of 2 μM to monitor the formation of cross-β structure using fluorescence. The plot represents the average of three independent measurements and the error bars are the standard deviation. The inset shows a fluorescence microscopy photograph of the particulates formed after 6 hours of aggregation. Reprinted with permission from Pounot et al. (2020)[5]. Copyright 2020 American Chemical Society.

Supplementary Figure 8. Fast temperature ramp as available on IN16B. In the fast mode on IN16B, the temperature can be increased from 280 K to 363 K in approximately 30 minutes. Reprinted with permission from Pounot et al. (2020)[5]. Copyright 2020 American Chemical Society.

Supplementary Figure 9. E/IFWS data on lysozyme during aggregation into particulates. The lysozyme was dissolved in the aggregation buffer (described elsewhere[5]) and E/IFWS was acquired throughout aggregation, which was triggered by temperature increase to 90°C. The data – after absorption correction with empty cell and normalization using the signal of vanadium – are plotted against momentum transfer q and time for the different energy transfer measured, 0 μeV (upper left), 0.6 μeV (upper right), 1.5 μeV (lower left) and 3 μeV (lower right). Reprinted with permission from Pounot et al. (2020)[5]. Copyright 2020 American Chemical Society.

Supplementary Figure 10. Fitting of the EFWS on hydration water of tau using a Gaussian. The H_2O-hydrated powder of deuterated tau monomers was sealed in a flat aluminum sample holder and EFWS were acquired during a temperature ramp from 20 to 300 K. The experimental data (blue line with error bars) are plotted for the momentum transfer range $0.2 < q < 0.6$ $Å^{-1}$ along with the fitted Gaussian used to extract the MSD.

Supplementary Figure 11. Translational and rotational motions of hydration water obtained from QENS fitting. The H_2O-hydrated powders of deuterated tau fibers **(A)** and monomers **(B)** were sealed in a flat aluminum sample holder and QENS data were acquired at 280 K. The experimental data for fibers (blue triangles) and monomers (green dots) are plotted along with the fitted model (black solid line) and its components, background (blue), resolution function (green), rotations (red) and translations (cyan) for the momentum transfer q=0.783 $Å^{-1}$. Reproduced from Fichou et al. (2015)[31].

DISCUSSION:

Neutron backscattering spectroscopy is the only method that allows probing the ensemble-averaged ps-ns dynamics of protein samples regardless of the size of the protein or the complexity of the solution when deuteration is used[6]. Specifically, by probing self-diffusion of protein assemblies in solution, the hydrodynamic size of such assemblies can be unambiguously determined. Nonetheless, the method is commonly limited by the low neutron flux, which implies long acquisition times and the requirement of high amounts of sample (typically 100 mg of protein) to obtain a good signal-to-noise ratio within the allocated beam time.

For liquid-state samples, the minimum concentration that can be used is about 50 mg/ml. High protein concentration is associated with faster fibrillation rates, which helps fitting the measurement in the allocated beam time, but it can affect the aggregation pathway too[41]. Hence, thorough sample characterization with complementary methods such as atomic force or electron microscopy is necessary.

For powder samples of hydrogenated proteins, the freeze-drying procedure was shown to be efficiently completed with a step in a desiccator in presence of P_2O_5 powder to remove as much residual light water as possible[34]. For powder samples, it is also advised to characterize (using atomic force microscopy and X-ray powder diffraction) the powder in its final, hydrated state, to assess the effect of deuteration, freeze-drying, and vapor diffusion on both monomer and fiber states. In particular, freeze-drying can break the fibers, resulting in shortened segments, without affecting the morphology. Also, it has been observed by X-ray power diffraction that slow cooling, instead of flash cooling in liquid nitrogen, could induce the presence of amyloid-like structures (unpublished result).

It is advised to plan the data collection with your local contact prior to the experiment (even if it has been discussed during the proposal writing process). The data collection plan is subject to changes after the first scans, depending on the signal-to-noise ratio that is obtained. For time-resolved experiments in particular, the use of E/IFWS allows for fast data collection – 30 s to 1 min for the elastic line, 4-5 min for a data point at 3 μeV[5] – but the range of accessible energy transfers is inherently limited. Alternatively, a sliding average of QENS data can be used[42]. To this end, the new backscattering and time-of-flight spectroscopy (BATS) option on IN16B offers a higher flux than the classical IN16B with a range of energy transfers up to ± 150 μeV at the cost of lower resolution in energy[11]. Therefore, the BATS option is recommended for time-resolved studies, especially for processes like the amyloid aggregation which takes place over several hours.

$S(q,\omega)$ The scattering function includes all type of motions in the sample and the models described in the above protocol are approximations. In particular for IDP in the liquid state, the large scale motions of the disordered chain can occur over the same length and time scale as the center-of-mass diffusion of the entire protein. Hence, the user should keep in mind that the separation of center-of-mass diffusion and protein internal dynamics is not always straightforward. The fitting procedure can benefit from information on center-of-mass diffusion obtained via complementary methods, such that this parameter can be fixed to obtain a more robust result for internal dynamics.

In addition to neutron scattering, the dynamics of a molecular system can be characterized by nuclear magnetic resonance (NMR), which provides local information on isotope labeled proteins and on a wide range of timescales[43–45]. The method has been used successfully to study amyloid systems[44,46–49], but does not allow to simply study hydration water or to follow in real-time the amyloid aggregation process due to inherent limitation on the system size. Recent developments in electron paramagnetic resonance spectroscopy (EPR) and the EPR-derived method Overhauser dynamic nuclear polarization (ODNP) offer good perspective of combination with neutron scattering. Indeed, though site-directed spin labelling (SDSL), EPR and ODNP can probe protein[50] and hydration dynamics[51], respectively, on the ps-ns timescale around the introduced spin label. These methods were used to study the aggregation of the tau[52,53] protein and will offer great complementarity with neutron scattering that can obtain similar information but averaged over all the sample. Also, infrared spectroscopy can provide dynamical information for high energy motions associated with specific structural patterns, but the complexity of the protein environment (buffer used) can affect data interpretation[54,55]. The neutron backscattering techniques provide a unique and complementary view on protein and hydration water dynamics on the ps-ns timescale along with results from the aforementioned methods. It does not require specific labeling of the sample, the signal quality is not sensitive to the size of the protein and measurements can be done *in vivo* or in highly complex deuterated environment like deuterated bacterial lysate[3,6,7]. As this method provides an ensemble-averaged result, it is well complemented by molecular dynamics simulations to obtain atomic-detail information on the system under study. The simulations can be easily validated by a direct comparison between the experimental dataset and the theoretical QENS spectra computed from the simulation trajectory using software like *mdanse*[56].

In regard to amyloid systems, neutron backscattering has proven useful to characterize the ps-ns protein and water dynamics for various systems and conditions[5,31,57–60]. In particular, neutron backscattering was

used to reveal the correlation between the proportion of the protein sequence involved in the cross-β structure and the amplitude of the water entropy gain upon fibrillation (unpublished results). Furthermore, development of sample environments allow to acquire simultaneously neutron spectra and either dielectric relaxation data[61] or Raman scattering data[62]. Additionally, the incoming neutron flux is expected to be improved for the BATS mode of IN16B in a near future thanks to the use of the so-called variable focusing guide, the geometry of which can be adapted on demand to the instrumental setup used. Pushing further the development of sophisticated sample environment and instrumentation would allow for even more complex experiments in the future, possibly delivering dynamical and structural information at the same time.

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The authors have nothing to disclose.

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