TITLE:

Study of Protein Dynamics via Neutron Spin Echo Spectroscopy

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SUMMARY:

The present protocol describes methods for investigating the structure and the dynamics of two model proteins, having an important role in human health. The technique combines bench-top biophysical characterization with Neutron Spin Echo spectroscopy (NSE) to access the dynamics at time-and-length scales relevant for protein interdomain motions.

ABSTRACT:

Most human body proteins' activity and functionality are related to configurational changes of entire subdomains within the protein crystal structure. The crystal structures build the basis for any calculation that describes the structure or the dynamics of a protein, most of the time with strong geometrical restrictions. However, these restrictions from the crystal structure are not present in the solution. The structure of the proteins in the solution may differ from the crystal due to rearrangements of loops or subdomains on the pico-to-nanosecond time scale, i.e., the internal protein dynamics time regime. The present work describes how slow motions on several tens-of-nanosecond timescales can be accessed employing neutron scattering. In particular, the dynamical characterization of two major human proteins, an intrinsically disordered protein that lacks a well-defined secondary structure and a classical antibody protein, is addressed by Neutron Spin Echo spectroscopy (NSE) combined with a wide range of laboratory characterization methods. Further insights into protein domain dynamics were achieved using mathematical modeling to describe the experimental neutron data to determine the crossover between combined diffusive and internal protein motions. The extraction of the internal dynamic contribution to the intermediate scattering function obtained from NSE, including the timescale of the various motions, allows new insights into the softness of proteins and the mechanical properties of single proteins in their nearly natural environment in the crowded protein solution.

INTRODUCTION:

Probing dynamics of soft matter with neutrons

Investigating the dynamical properties of proteins and peptides is a major part of biophysical

research, and many well-developed methods exist today to access a wide range of energy landscapes[1]. Relating the experimentally revealed dynamics of the proteins to their biological function is a far more difficult task requiring complex mathematical models and computer-aided dynamics simulations. The importance of neutron spectroscopy for the analysis of proteins motions has been emphasized in several well-received and widely recognized studies[1–5]. Before exploring the diverse energy landscape of internal protein dynamics, a short overview of the dynamical processes in soft matter and how neutrons can access them is required.

The sensitivity of neutrons to isotopic configuration and the type of interactions they display with soft matter makes neutron scattering one of the most versatile investigation technique[6]. There is a broad spectrum of correlation length scales and correlation times that neutrons can access: from nuclear excitations and atomic vibrations to collective motions and slow relaxation processes like isotropic rotations and diffusive motions. When investigating the scattered neutrons for their energy transfer, three main interactions can be distinguished: the elastic scattering where there is no energy exchange between incoming neutron and particle in the sample, the inelastic scattering with large, quantifiable energy exchange between neutron and particle, and the peculiar case of quasi-elastic scattering that designates a very small energy transfer compared to the incident neutron energy[1, 7]. These interactions give precise information about the material investigated and form the theoretical basis of a wide variety of neutron scattering techniques.

In elastic scattering, the detector records the directions of the neutrons as a diffraction pattern that shows the position of the sample atoms relative to one another. Information about the correlations of atomic positions is acquired, i.e., integrated intensity $S(Q)$ concerning the momentum transfer Q, that pertains to structural information alone. This principle forms the basis of neutron diffraction[8].

Complexity arises when the energy transfer is no longer zero due to excitations and internal fluctuations in the sample material. This forms the basis of neutron spectroscopy, where the scattered neutrons are investigated as a function of both the energy transfer E and the momentum transfer Q. Dynamical and structural information is obtained. Neutron spectroscopy measures the same integrated intensity $S(Q)$ for energy transfer, i.e., velocity change of the neutrons due to sample scattering, $S(Q, \omega) = S(Q, E)$, also referred to as the dynamic structure factor[9].

Calculating the scattering from a material is more adequate to use the pair correlation function[7, 10]. In the diffraction case, the static pair correlation function $G(r)$ gives the probability of finding the center of a particle at a given distance r from the center of another particle. The spectroscopy generalizes the static pair correlation function and includes energy/frequency/time into the scattering equation. The pair correlation function $G(r)$ becomes a function of time $G(r, t)$ that may be decomposed into a distinct atom pair correlation function $G_D(r, t)$, and a self-correlation function $G_S(r, t)$. These describe two types of correlations: pair-correlated motions of atoms that govern the coherent scattering and self-correlation that govern the incoherent scattering[10].

Coherent scattering is the scattering from "the average" and depends on the relative phase of the scattered waves. In the small-angle scattering regime, the scattered neutron waves from different scattering centers (different atoms) interfere constructively (have similar phases), and the collective motion of the atoms is observed with strong intensity enhancement. Coherent scattering essentially describes the scattering of a single neutron from all the nuclei in the sample[10].

When no constructive interference occurs between the scattered neutron waves from different centers, a single atom is followed in time, and the self-correlation between the position of the atom at time $t = 0$ and the same atom at time t is observed. Thus, the information on the relative positions of atoms is lost, and the focus is only on local fluctuations. Scattering from local fluctuations governs incoherent scattering. Incoherent scattering is isotropic, contributes to the background signal, and degrades the signal-to-noise[10, 11].

Combining all of the above, we distinguish four major neutron scattering processes[10]: (1) Elastic Coherent (measures the correlations of atomic positions), (2) Inelastic Coherent (measures collective motions of atoms), (3) Elastic Incoherent (contributes to the background, reduces scattering intensity by Debye-Waller Factor (DWF) and measures Elastic Incoherent Structure Factor (EISF), describing the geometry of diffusive motions in confined geometry, and (4) Inelastic Incoherent (measures single atom dynamics and self-correlation).

Dynamics processes that neutrons can access in biology range from damping of low frequency atomic and molecular vibrations, the interaction of solvent molecules with bio-surfaces, diffusion processes in the hydration layer of macromolecules and confined geometry, to short-range translational, rotational, and tumbling diffusive motions and protein domains and allosteric motions[1]. The wide diversity of neutron methods and instruments to measure protein dynamics is based on how the achromatization of the incident or outgoing neutron beam is achieved and how the energy analysis of the scattered neutrons is performed. From triple-axis to time-of-flight, backscattering, and spin-echo spectrometers, one can explore dynamical processes with characteristic times between 10^{-14}s and 10^{-6}s (femtoseconds-to-microseconds)[12].

Oak Ridge National Laboratory, with its two renowned neutron sources, Spallation Neutron Source - SNS[13] and High Isotope Flux Reactor - HFIR[14], has one of the best suite of spectrometers to investigate dynamics in bio-materials. Some of the most eloquent examples include the use of Cold Neutron Chopper Spectrometer (CNCS) at SNS[15] to investigate the dynamical perturbation of hydration water around green fluorescent protein in solution[16] or the sub-picosecond collective vibrations of several proteins[17]. A recurring problem of inelastic neutron scattering investigations is that some biological processes are too slow to be observed. Without extreme setups that lead to a huge loss of neutron intensity, time-of-flight spectrometers are limited to 10 μeV energy resolution, corresponding to a maximum time scale of ~ 200 ps[10, 11]. This is not sufficient to observe large-scale motions in proteins. Therefore, instruments with higher energy resolution like the backscattering spectrometers are often needed. Combining the time-of-flight and backscattering techniques proved powerful to investigate the change in internal dynamics of Cytochrome P450cam (CYP101), an enzyme that catalyzes the hydroxylation camphor[18].

Microscopic diffusivity measured by the backscattering spectrometer at SNS–BASIS[19] was surprisingly well defined and could be separated into diffusivity of water (hydration, cytoplasmic, and bulk-like water) and diffusivity of cell constituents in planarian flatworms, the first living animal to be studied by neutron scattering[20]. Backscattering is a high-resolution spectroscopic technique but is also limited to several μeV = several nanoseconds, while the slow dynamics in biomaterials also manifest as the survival time of correlation between atomic position or spin orientations, e.g., relaxation processes, which regularly happen in the time range of ten-to-hundreds of nanoseconds.

Neutron Spin Echo spectroscopy (NSE) is the only neutron scattering technique to reach such high resolution. Unlike other neutron techniques, NSE doesn't require achromatization of the beam since it uses the quantum mechanical phase of the neutrons, their magnetic moments. The manipulation of magnetic moments allows using a broad neutron beam wavelength distribution, while the technique is sensitive to very small neutrons velocity changes in the order of 10^{-4}. NSE has been successfully used to investigate the slow dynamics of proteins in solution for many proteins. Among these many pioneer studies, we acknowledge the study of the segmental flexibility of pig immunoglobulin[21]; the coupled domain motions in Taq polymerase[22]; the domain motions in the tetramer of yeast alcohol dehydrogenase[23]; the change of conformation in phosphoglycerate kinase upon substrate binding[3]; the activation of domain motions and the dynamic propagation of allosteric signals in the Na^+/H^+ exchange regulatory cofactor 1 (NHERF1) protein[4, 24, 25]; the dynamics of a compact state of mercuric ion reductase[26] and the diffusion of hemoglobin in red blood cells[27]. Two more recent studies in protein dynamics exposed the flexibility of human antibody Immunoglobulin G (IgG) as an entropic spring[28] and the characteristics of solvent contribution to the dynamics of intrinsically disordered Myelin Basic Protein (MBP)[5].

The present article explains the basic principles of NSE, the multiple preparatory methods recommended for a thorough protein dynamics investigation, as well as the methodology and the experimental protocol for NSE data acquisition at the NSE spectrometer at SNS, SNS-NSE. The protocol characterizes two proteins: IgG, a regular human antibody protein, and the intrinsically disordered protein MBP. The biophysical implications, the research relevance of the examples, and the limitations of the technique will be discussed briefly.

NSE spectroscopy, the method for slow dynamics measurements

Neutron Spin Echo (NSE) is a polarized technique that uses neutrons time-of-flight to measure the exchange of energy (loss of polarization) due to the quasi-elastic interaction between neutrons and atoms in a sample. At the core of NSE spectroscopy lay two basic principles: (a) the ability of the neutron spin to precess in the magnetic field with a frequency proportional to magnetic strength $\vec{B}$, namely, the Larmor frequency[29] and (b) the spin-echo or Hann echo representing the manipulation and refocusing of the polarization signal applying a series of radiofrequency pulses[30].

The basics of the NSE process can be summarized in a few simple steps using **Figure 1**[6, 11]: (1) The

neutron beam produced by the source (position 1) is polarized (position 2), guided and transported (position 3), and arrives at the entrance of the NSE spectrometer where gets rotated by 90° by the first pi-half flipper (position 4). (2) The polarized beam, e.g., neutron magnetic moments, become perpendicular to the first magnet's magnetic field lines (first precession zone, position 5) and starts to precess. (3) At the end of the magnet, neutron spins accumulate a certain precession angle proportional with the magnetic field strength and the time-of-flight spent inside (basically inversely proportional to the neutron velocity). The individual neutrons velocities are encoded within their precession angle at the end of the first precession zone. (4) Close to the sample position, the pi-flipper (position 6) reverses the orientation of the spin with 180°, changing the sign of the precession angle. (5) The neutrons interact with the sample's molecules (position 7) and get scattered. (6) The scattered neutrons enter and precess in the second precession zone (position 8) but become reversed-oriented. (7) Another pi-half flipper (position 9) is used to rotate the orientation of the spin from perpendicular to the horizontal direction. This will stop the precession, translating the precession angle φ into polarization proportional to $\cos(\varphi)$. (8) The analyzer (position 10) selects the neutrons based on one orientation. If the interaction with the sample is elastic, the neutron's velocity will not change. Neutrons will spend an identical amount of time flying in the first and second precession zones, and the accumulated precession angles are fully recovered. The full polarization is restored on the detector (position 11) as an echo of the original polarization, i.e., spin-echo. (9) However, in NSE, the scattering is quasi-elastic, so a small energy exchange between neutrons and sample molecules leads to different neutron velocities after scattering by the sample. Due to different velocities, the neutrons will spend an additional time flying through the second precession zone and will not have properly recovered their precession angle. A partial polarization is retrieved on the detector, and the loss of polarization due to spin relaxation is proportional to the cos-Fourier-transform of the spectral function $S(Q, \omega)$, the intermediate scattering function $F(Q, t)$. (10) The time parameter of the function $F(Q, t)$ is proportional to the precession magnetic field strength. Scanning the loss of polarization as a function of magnetic field strength yields, therefore, a relaxation function that depends on the dynamical processes within the sample.

[Place **Figure 1** here]

PROTOCOL:

The present work characterizes two proteins: a regular human antibody protein, Immunoglobulin G (IgG), and the intrinsically disordered Myelin Basic Protein (MBP). The lyophilized form of the proteins was obtained from commercial sources (see **Table of Materials**).

1. Protein sample preparation

1.1. Prepare a 50 mM sodium phosphate + 0.1 M NaCl buffer by weighing and dissolving the respective solid reagents in heavy water (D_2O) (see **Table of Materials**). This is the deuterated buffer solvent for IgG.

1.1.1. Adjust the pH of the buffer solution to 6.6.

1.2. Prepare a 20 mM sodium phosphate + 6 M Urea buffer by weighing and dissolving the respective solid components in heavy water (D_2O). This is the deuterated buffer solvent for MBP.

1.2.1. Adjust the pH of the buffer solution to 4.7.

1.3. Filter the buffer solvents using 0.2 μm pore size filters (see **Table of Materials**).

1.4. Weigh and dissolve the purified protein lyophilized powders in the deuterated solvents for the respective proteins (steps 1.1–1.2) at high protein concentration (~50 mg/mL).

1.5. Load the protein solution in dialyze baskets with dialysis membranes of 3.5K MWCO and dyalize against the filtered buffer for 24 h at 10°C for MBP and 25°C for IgG (see **Table of Materials**) by slightly shaking the tubes to create a diffusion gradient.

1.6. Dilute the protein solution using the dialysis buffer in a series of concentrations as follows: 1, 2, 5, 10 and 50 mg/mL.

1.7. Determine the exact concentrations using a Nanodrop spectrophotometer (see **Table of Materials**).

2. Preliminary sample characterization by Dynamic Light Scattering (DLS)

2.1. Load 80 μL from each protein solution from the concentration series prepared above (step 1.6) into the DLS disposable cell (see **Table of Materials**) and determine the diffusion coefficients, averaging over ten acquisitions.

2.2. Plot the translational diffusion coefficients as a function of protein concentration and extrapolate to zero concentration.

2.3. Load each protein solution from the concentration series into capillary tubes of a viscometer (see **Table of Materials**) and measure the dynamic viscosity.

2.4. Plot the dynamic viscosities measured as a function of protein concentration and extrapolate to zero concentration.

NOTE: The extrapolation of DLS diffusion to zero concentration yields the value of translational diffusion for one single protein. The extrapolation of dynamic viscosity to zero concentration must yield the dynamic viscosity value measured experimentally for the buffer solution.

3. Collection of small-angle scattering (neutron or x-ray), SANS and/or SAXS spectra

3.1. Measure SANS and/or SAXS (see **Table of Materials**) on four protein concentrations, preferably: 2 mg/mL, 5 mg/mL, 10 mg/mL, and 50 mg/mL.

3.2. Normalize SANS and SAXS spectra by protein concentration.

3.3. Fit protein form factor *P*(Q) to the SANS and SAXS spectra using ensemble optimization[31] and/or SasView[32] software.

3.4. Calculate structure factor *S*(Q,c) by dividing SANS and SAXS signal with *P*(Q) for each concentration.

NOTE: Readers interested in how to measure and interpret small-angle scattering data as support for NSE measurements are encouraged to thoroughly consult References[17,24,28–30], and the reference article[33].

4. Measurement of Neutron Spin Echo (NSE)

4.1. Setup for the experiment and mount the sample following the steps below.

4.1.1. Select the thickness of the cell for sample loading based on the concentration of the protein solution, the temperature needed for measurement, and the amount of solution available.

NOTE: The present study used top loader transparent quartz containers of 40 mm x 30 mm x 4 mm.

4.1.2. Clean the cell repeatedly, alternating between phosphate-free dish detergent, deionized water, and 70% ethanol(see **Table of Materials**).

4.1.3. Dry the cell in the convection oven; do not exceed 80 °C for the quartz cells.

4.1.4. Load 4 ml of protein solution in the cell, and close with caps. Use wax film or any sealant (see **Table of Materials**) to seal the sample cells.

NOTE: In the present study, 4.8 mL of solution at ~50 mg/mL was used to obtain sufficient scattering intensity.

4.1.5. Load 4 ml of dialysis buffer in an identical container as the protein sample and seal.

4.1.6. Transport samples to the beamline, close the shutter and enter the spectrometer enclosure cave area[34].

4.1.7. Mount the sample cell on the Aluminum sample holder by tightening the screws and the holding plates (**Figure 2**, left panel).

NOTE: One can mount two sample cells at the time, given that the same measurement protocol

is needed for all samples.

4.1.8. Mount the Graphite sample and/or Al_2O_3 powder sample loaded in an identical container as the protein sample. These are standards provided by the SNS-NSE beamline support.

4.1.9. Place the sample holder by gently sliding it into the can of the Temperature Forcing System (TFS, see **Table of Materials**).

NOTE: TFS is the most used sample environment at SNS-NSE and pumps dry air in the sample canister to achieve the desired temperature (**Figure 2**, middle and right panel).

4.1.10. Close the TFS lid and set the temperature to the desired value by accessing the interactive screen of the TFS.

4.1.11. Mount the Neutron Camera (see **Table of Materials**) for the alignment of the samples to the beam.

4.1.12. Sweep the instrument enclosure, evacuate, close doors, and open beam shutter.

NOTE: Sample cells are provided by the SNS-NSE beamline support. For available sample cells and sample environment library, please refer to the SNS-NSE beamline web page[7,32].

[Place **Figure 2** here]

4.2. Collect the NSE data following the steps below.

4.2.1. Align the sample in the neutron beam using the neutron camera and the four independent sample apertures.

4.2.2. Open the SNS-NSE data collection software[36] and collect sample statistics by running diffraction scans for the desired scattering angles and wavelength.

4.2.3. Setup the measurement protocols based on the statistics collected for each sample by editing the measurement macros provided to you by the assisting Instrument Scientist.

4.2.4. Start scanning by typing the protocol name at the command prompter and acquire echoes for the sample.

4.2.5. Start scanning and acquire echoes also for the elastic reference and the buffer solvent. You will need to perform intermittent beam shutter operation for the sample change.

NOTE: Elastic reference, i.e., resolution, and buffer solvent, with intermittent beam shutter operation for the desired samples change.

5. NSE data reduction.

NOTE: SNS-NSE is equipped with a dedicated software named DrSpine (Data reduction for Spin-echo)[34,35] available on the ORNL Neutron Sciences Remote Analysis Cluster, a Quick User Guide, and built-in help support.

5.1. Log into Neutron Sciences Remote Analysis Cluster (see **Table of Materials**) with the ORNL user credentials, and press the **Launch Session** button.

5.2. Setup *the data reduction software* following the steps below:

5.2.1. In the user directory open a terminal window and type: **source/SNS/software/nse/etc/setup_nse.sh**.

5.2.2. Next type: **drspine_create_env.sh**.

5.3. Create a folder for the data reduction in the home directory and copy the provided scripts and macros from the shared directory.

5.4. Edit, rename, and save accordingly the reduction macro provided.

5.5. Type **drspine** at the command prompter and press **enter** to start the software reduction environment.

5.6. Type "the name of the reduction macro" edited at step 5.4 in the command prompter within the software environment and press **Enter**.

6. NSE data fitting

6.1. Edit the python script "stapler-drspine.py", provided by the assisting Instrument Scientist, with the names of the reduced file data.

6.2. Edit the function to fit from the library provided.

6.3. Type the name of the edited script "stapler-drspine.py" at the command prompter and press **Enter** to read, fit, and plot reduced NSE data.

NOTE: The Instrument Scientist will provide a template for the reduction macro and the "stapler-drspine.py" python script that can read and fit NSE reduced data. The final reduced NSE data are in ASCII format and can be read by various preferred software.

REPRESENTATIVE RESULTS:

IgG protein from human serum and bovine MBP proteins were reconstituted at high concentrations (~50 mg/mL) in D_2O-base buffers. Since proteins are dissolved in high

concentrations, the solutions obtained are crowded proteins solutions. The dynamics investigated by NSE will suffer from the crowded environment the proteins reside in (structure factor interactions and hydrodynamics effects)[5, 28, 39]. The Dynamic Light Scattering (DLS) on a concentration series for each protein is performed to account for crowding effects. The extrapolation to zero concentration of the translational diffusion coefficients measured by DLS yields the value of translational diffusion for a single protein. The dynamic viscosity as a function of protein concentration must also be measured prior to NSE to account for the hydrodynamic interactions. The extrapolation to zero concentration of the dynamic viscosities as a function of concentration needs to yield the value one can experimentally measure for the buffer solution for each protein sample prepared.

The shape and the structure of proteins in concentrated solution were assessed prior to any NSE experiment to gain insights on the protein form factor and the strength of the structure factor that might influence how the proteins move in solution. Small Angle Scattering is the method of choice for these investigations. In the presented study, the structural conformation of IgG protein in solution was observed by Small Angle X-ray Scattering (SAXS), and the structure of MBP was assessed by Small-Angle Neutron Scattering (SANS). The scattering intensity (Q) measured (steps 3.3–3.4) is proportional to the product between form factor *P*(Q) and structure factor *S*(Q,c) weighted by the number of particles[5, 28, 39] (Equation 1):

$$I(Q) = N \cdot P(Q) \cdot S(Q, c)$$

SAXS was measured on a Kratky-type SAXS instrument[40], with an X-ray wavelength of 0.154 nm for IgG, and SANS was measure[41]d for MBP for a neutron wavelength of 4.5 Å. The experimental small-angle intensities *I*(Q) are background and solvent corrected, scaled by solution concentration, and the form factor is calculated using free, available software packages like Ensemble modeling-EOM and SasView[24,25,29,38–40]. Further, the structure factor for each protein was obtained from Equation 1.

For the present study, the NSE experiments were performed at two spin-echo spectrometers: the SNS-NSE instrument[34] in **Figure 1** and the Phoenix-J-NSE instrument[44]. Incident wavelengths between 8Å–12Å with Fourier times between 0.1 ns ≤ t_{max} ≤ 130 ns for several *Q* measurements between 0.05Å^{-1}–0.2Å^{-1} are measured. The difference between the two spectrometers is a vital point in the understanding of NSE science and represents both the old concept of a so-called "classic spin echo" for the static neutron source of a reactor and the new "time-of-flight concept" for the pulsating neutron source. The Phoenix-J-NSE spectrometer is a classic type NSE where a velocity selector selects a particular wavelength. To allow variations in the neutron velocities a ±10% wavelength bandwidth is introduced. Despite the very narrow energy band used for a single scan, the situation of the Phoenix-J-NSE spectrometer at a high flux reactor source ensures the covering of space-vector and correlations time range in relatively short measurement time. The SNS-NSE spectrometer is the new-generation, ultrahigh resolution, choppers neutron spectrometer, with a wavelength span of 2 Å < λ < 14 Å and uses a simultaneous wavelength bandwidth of 2.4 Å–3.6 Å, depending on the position of the spectrometer. Due to this wide bandwidth, high data collection efficiency is achieved allowing nearly gapless coverage of a broad wave-vector-time-range with only few scattering angle settings. The selection of wavelength

band at SNS-NSE is made by a chopper system consisting of four choppers. Both NSE spectrometers discussed here have precession fields based on superconducting technology with high-magnetic-field homogeneity, novel state-of-the-art field correction elements for stray field corrections and novel polarizing benders[41,42].

The NSE spectra were measured at 10 °C for MBP protein and 25 °C for IgG1 protein. The coherent intermediate scattering intensity /(Q, t)/*I*(Q, t=0) measured by NSE is the contributive result from all the dynamic processes in the sample that happen within the time scale investigated. This contains the internal protein dynamics, the overall translational diffusion, the rotational and tumbling diffusion, and the segmental motions within the protein molecule itself. A simplified model assumes that the internal dynamics and the translational and rotational diffusions are totally decoupled[5, 45–48]and can be characterized separately. For the single-particle, the coherent scattering function can be written as the product[23, 48] (Equation 2):

$$F(Q, t) = F_{trans}(Q, t) \cdot F_{rot}(Q, t) \cdot F_{int}(Q, t)$$

where the translational diffusion term for a single rigid protein is an exponential function of the translational diffusion coefficient D_T (Equation 3)[23, 48]:

$$F_{trans}(Q, t) = exp(-Q^2 \cdot D_T \cdot t)$$

For large concentrations of proteins, the translational diffusion coefficient D_T is influenced by protein-protein interactions quantified by the structure factor $S(Q)$ and by hydrodynamic interactions described by the hydrodynamic function $H_T(Q)$ as (Equation 4)[23, 48]:

$$D_T(Q) = D_{T0} \cdot H_T(Q, c) / S(Q, c)$$

In Equation 4, D_{T0} is the extrapolated diffusion value to zero concentration from DLS measurements (step 2.2). H_T is calculated as $H_T = 1 - c \cdot [\eta]$ with c = protein concentration, $[\eta]$ = intrinsic viscosity calculated by $[\eta] = (\eta - \eta_0) / \eta_0 \cdot c$, and η_0 is the measured dynamic viscosity (steps 2.3–2.4). The structure factor $S(Q, c)$ was obtained from SAXS measurements for the IgG study and SANS measurements for the MBP protein.

Figure 3 displays examples of intermediate scattering functions $I(Q, t)/I(Q, t=0)$ as measured by NSE for IgG and MBP proteins. A clear deviation from a simple diffusion-like relaxation process was observed on the short Fourier time scale < 25 ns, indicating the accessibility of protein internal dynamics by NSE and the need for a more complex model to describe these dynamical processes observed.

[Place **Figure 3** here]

Therefore, the scattering functions are fitted by atomic modeling of both proteins using models developed by R. Biehl described in references[18,25,38,43,44,46]. Intermediate scattering function is calculated by coarse-graining to a couple of hundreds of individual grains, as opposed to all-atom simulation, to reduce computational load and time, using the MMTK[49] software library to access atomic coordinates and to implement trans-rotational motions on proteins domains and fragments. The results of the intermediate scattering function calculation for both proteins are

in excellent agreement with the experimental NSE data and proved that slower dynamics observed at long Fourier times is attributed to the overall translational and rotational diffusion processes, while the fast dynamics observed at a short-time scale is attributed to dynamics of protein domains.

FIGURE LEGENDS:

Figure 1: The NSE spectrometer at SNS (SNS-NSE) photo and neutron fly path schematic with the most important functional components. From right to left: 1 = neutron source; 2 = choppers-bender-polarizer-secondary shutter system; 3 = beam transport guides; 4 = pi/2 flipper for first 90° spin-turn; 5 = first precession zone; 6 = pi flipper for 180° spin-turn; 7 = sample area and sample environment (here the cryo-furnace is shown); 8 = second precession zone; 9 = pi/2 flipper for second 90° spin-turn; 10 = analyzer; 11 = detector. (Note that portions of 3 as well as 2 and 1 are situated behind the blue wall inside shielding; the choppers are replaced by a velocity selector at reactor based NSE).

Figure 2: NSE measurement system. Left panel: protein solution samples in quartz container mounted with screws and plates on the Al (aluminum) sample holder. The Al sample holder offers the possibility to mount two samples simultaneously inside the sample environment. Middle panel: Temperature Forcing System (TFS) sample can mount at the sample stage; neutron beam window is on the right while neutron camera used for alignment is visible on the left side. Right panel: placing the sample holder with two samples into the sample can work with Si(silicon) windows.

Figure 3: NSE relaxation spectra for IgG and MBP proteins. The IgG data are shown here fitted just by a single-exponential function to reveal the deviation from diffusion at shorter Fourier times. The deviation was observed for both proteins. In contrast, the MBP data are shown here in the full-relaxation range fitted by the model developed by R. Biehl[45]. The model implies coarse-graining and enables simultaneous fit of short, intermediate, and long Fourier time regimes.

DISCUSSIONS:

NSE spectroscopy delivers a unique and detailed view of the dynamics of proteins, which any other spectroscopic technique cannot resolve. Measurements over an extended time scale provide observations on both proteins' translational and rotational diffusion presented here. The segmental dynamics and other internal oscillations reveal themselves as a strong decay of the coherent scattering function $S(Q, t)$ at a short time scale and are well separated from the overall diffusional relaxation processes. The main limitations of the NSE technique are the extended measurement time and the large sample volumes needed to acquire a good statistical signal. This can pose a challenge to measure relevant macromolecular systems that exhibit low concentrations of the mobile species and reduced lifetime.

Internal dynamics of IgG

IgG protein has a Y-shape structure specific to antibodies that can be approximated by three large fragments connected by flexible linkers. A mathematical model of three sizable particles residing

in harmonic potential was assumed for this kind of structure. The linkers were approximated by elastic springs that give a fixed equilibrium position but allow fluctuations as a form of Brownian motion. The three degrees of freedom are permitted for each fragment around the relative equilibrium. [28]The internal dynamics represented by $F_{int}(Q, t)$ in Equation 2 was described by the Ornstein-Uhlenbeck process[40,41]. The model allows calculation of mean square displacement of the fragments in the potential dip, the timescale of different motions, friction, and force constant that occur. The amplitude of the internal motion was approximated by Normal Modes analysis considering different degrees of motion for each fragment. An extensive description of the model and resulting mathematical parameters is out of the scope here but can be found in detail in Reference[28]. In this case study of IgG, a clear signature of the internal dynamics on the timescale of several nanoseconds was observed. The calculated spring constant of the linker appears to be realistically compared to an entropic spring of similar length. The observed effective friction appears to be close to the friction of a free unbound IgG fragment. The pre-existing equilibrium hypothesis is validated with assumed coexisting "open" or "closed" configurations of IgGs that are in equilibrium exhibiting different binding sites and binding specificity[28]. This information may be relevant to understanding the mechanics of antibodies and if that mechanical behavior can be used to improve biocompatibility and bioavailability.

Internal dynamics of MBP

MBP structure has a compact globular core with flexible random coil ends that allow strong stretching and bending motions. MBP dynamics were interpreted using flexible polymers models, with and without internal friction added[25,38]. As in the case of IgG, the observations of the internal dynamic on MBP can be reconciled within the theory of Brownian motion, where a larger amplitude of motion results in longer relaxation time with influences from the internal friction within the protein chain. The increased relaxation time with a larger amplitude of motions but smaller friction can be described using the same Ornstein-Uhlenbeck process[48,49] of Brownian motion in a harmonic potential. Internal motions with large amplitudes but slow relaxation times become active upon full denaturation of MBP from its native state, by reducing the restoring forces of chain configuration (dihedral potentials) and by smoothing the local energy barriers. Detailed descriptions of the MBP internal dynamics in the native and denatured state can be further found in References[25,38]. In the study of MBP, the investigation by NSE revealed a dynamical behavior pointing toward intermediary compactness of the protein between globular proteins and random coil polymers. The significant contribution of internal protein dynamics with a relaxation rate of several nanoseconds was governed by low-frequency collective stretching and bending motions.[5] Description of native MBP dynamics by models from polymer theory breakdown due to a large value of the protein internal friction was provided. In denatured MBP, the internal friction within the protein chain is reduced, and the polymer chain character of the relaxation spectrum prevails. The high flexibility of the structural ensemble motions might help increase the accessible protein surface, which facilitates the interaction with different binding partners.

Dynamical models

While Neutron Spin Echo (NSE) as a technique is experimentally unique in assessing low-frequency harmonic motions in biological macromolecules and molecular subunits, the analysis

of the intermediate scattering function requires a comparison with neutron spectra derived from various mathematical models. The model presented here and developed by Biehl et al.[28] for concentrated protein solutions uses an elastic network approximation in which the intermediate scattering function is a convolution of proper modular functions, and Brownian oscillators represent the internal dynamics. This is one of the very few available models to analyze the segmental dynamics of proteins. Another well-established model for dilute protein solutions is the model proposed by Bu et al.[52], a robust model that uses statistical mechanics and doesn't require complex fits or multiple parameters. A similar approach based on dynamic decoupling approximation can also be applied for dilute systems to characterize the effective diffusion as a sum of self-translational and internal motions[53]. Comprehensive reporting on these models and their limitations can be found in several of our references. We strongly recommend J. Fitter et al.,[1] and Yun Liu et al.[54].

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