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Short-time dynamics of proteins in solutions studied by neutron spin echo

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Abstract

Dynamics of proteins in solutions include translational, rotational, and internal motions that are linked to different protein properties. Because most proteins are small with the sizes of just a few nanometers, the timescale for their short-time dynamics usually ranges from a few nanoseconds to a few hundreds of nanoseconds, during which a protein usually does not rotate too much. Protein short-time dynamics has been shown to be useful to study liquid theories, protein cluster formation, gelation transitions of concentrated protein systems, and protein internal motions. Neutron spin echo, which is able to measure protein motions with the right correlation time at the appropriate length scale, is ideally suitable to study the short-time dynamics of proteins in solutions. Here, we review recent activities of using neutron spin echo to study the protein short-time motions. Despite all progresses, there are still both theoretical and experimental challenges to exploit the full capability of neutron spin echo to study protein dynamics.

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Protein, Neutron spin echo, Internal motions, Short-time dynamics.

Introduction

Proteins are biomolecules commonly found in biological systems and considered workhorses for cells. Certain types of proteins, such as monoclonal antibodies, have been widely used in modern cancer treatment drugs [1]. In fact, the monoclonal antibody (mAb)—based therapeutic drug is the fastest growing sector of the pharmaceutical industry. Because of the important roles proteins play in many systems, it is extremely important to have a full understanding of their structures and dynamics.

The dynamics of proteins in solutions covers a wide range of timescales, from the picosecond level for the internal vibration to more than a few seconds for slow protein motions in porous materials [2,3]. Among different dynamic properties, there are recent interests in studying the short-time dynamics of proteins in solutions [4]. In colloidal science, a characteristic time for the translational diffusion, τ, is defined as $\frac{(\frac{\sigma}{2})^2}{D_0}$, where D_0 is the free diffusion coefficient and σ is the diameter of a protein [5]. (Sometimes, the time needed for a protein to diffuse through a distance of its own diameter, t_D, is used to define this characteristic time as follows: $t_D = \frac{2}{3}\tau$.) For any dynamics with relaxation time much shorter than τ, it is considered short-time dynamics. Note that the first-order rotational relaxation time is also comparable with τ. Thus, for particles at the short time limit, they will not rotate too much. This is quite different from the long-time dynamics of proteins studied by dynamic light scattering (DLS) and nuclear magnetic resonance, for which experimental results are typically averaged values over time as proteins can rotate many times during a measurement. Because typical sizes of proteins are of just a few nanometers, τ ranges from a few tens of nanoseconds to a few hundreds of nanoseconds depending on the protein size. In addition, the domain motions of some proteins are shown to be around a few nanoseconds too. Thus, short-time dynamics include translational, rotational, and internal domain motions.

To investigate the short-time dynamics of proteins, measurement tools are needed to probe the timescale at the nanosecond level. Neutron spin echo (NSE) has been shown to be one of the most powerful tools to study the short-time dynamics of proteins [4,6–11]. NSE can access the timescale from about a few picoseconds to a few hundreds of nanoseconds [9,12]. In addition, it can simultaneously measure the structure at the length scale from a few angstroms to a couple of hundreds of angstroms that covers the size range of many proteins. Its capability of associating certain

dynamics with the appropriate length scale of protein structures makes it very powerful to understand the physical origins of certain dynamics. Thus, NSE has been used widely in the past decade to study protein dynamics both at low and high protein concentrations.

Note that NSE has been widely used to study many other systems too, such as microemulsions, lipid vesicles, polymer nanocomposites, wormlike micelles, and dendrimers [9,13–17]. Here, we only focus on the recent activities of studying protein dynamics in aqueous solutions using NSE. Special attention is paid to explain the role of NSE in determining certain types of dynamics. Personal opinions of the challenges for different types of experiments are discussed too. Because the interpretation of NSE signals is not trivial, the theories used in the literature are also discussed and commented.

NSE instrument and theories

NSE uses the fact that the neutron has a spin that can rotate in a magnetic field. The working principles of a prototypical NSE instrument are briefly introduced here. Details of the NSE instrument can be found in other articles and reviews [9,12]. A prototypical NSE instrument has two main magnets. A sample is positioned in between these two magnets. Neutrons from a source are first polarized so that all neutron spins point into one direction. When these neutrons enter the first magnet, the neutron spin rotates while passing through it. When a neutron exits from the first magnet, its spin gains a rotational angle depending on the strength of the magnet's field and the time a neutron stays. Then, it hits a sample. If this neutron does not lose or gain energy when interacting with the sample, it does not change its velocity when it enters the second magnet. The design of the second magnet is to let the spin rotate in the opposite direction with the same magnetic field strength. Because the path length of the neutron in the magnet is typically designed to be similar, the flying time of a neutron is then the same as that in the first magnet's field if the velocity of a neutron does not change after interacting with the sample. As a result, at the time a neutron flies out of the second magnet, the net angle it rotates is zero. However, if a neutron loses energy or gains energy, the velocity of a neutron changes after interacting with a sample. As a result, the time a neutron stays in the second magnet is different from that in the first magnet. Therefore, the net rotating angles are not zero anymore. Because of this, the scattered neutron intensity obtained after passing through analyzers is affected by the energy gain or loss of a neutron when interacting with a sample, which is related with the dynamics of a sample. The aforementioned information is a highly simplified description of how a prototypical NSE instrument works. Modern NSE instruments are more complicated than what are described here. However, the physical principles are the same.

For an instrument user, three important parameters for a NSE instrument are the neutron flux on a sample, the range of the Fourier time it can reach, and the scattering wave vector, Q, it can cover. The neutron flux determines how long a measurement needs to be. The Fourier time and the scattering wave vector decide the type of the dynamics a NSE instrument can study. The Fourier time is essentially the correlation time of the intermediate scattering function of a sample that a NSE instrument probes. The Fourier time for the IN15 at the Institut Laue-Langevin ranges from about 1 ps to more than 600 ns, with the Q range from about 0.02 Å^{-1} to about 1 Å^{-1}. With a sample with very strong scattering, it is possible to extend the measurable correlation time up to about one microsecond. The NSE instrument at the NIST Center for Neutron Research probes the correlation time of a system from a few picoseconds to almost 300 ns, with the Q range from about 0.03 Å^{-1} to 1.8 Å^{-1}. The Fourier time of a NSE instrument is equal to $\frac{\gamma_L m^2 \lambda^3 \int BdL}{2\pi h^2}$, where λ is the neutron wavelength, m is the neutron mass, γ_L is the neutron gyromagnetic ratio ($\gamma_L = 1.832 \times 10^8\ rad\ /s/T$), B is the strength of the magnet field, $\int BdL$ is the magnetic field integral, and h is the Planck constant. Therefore, changing the wavelength is a very efficient way to change the Fourier time as increasing the neutron wavelength by a factor of two can increase the accessible Fourier time by a factor of eight.

The NSE instrument is a relatively complicated neutron instrument. All NSE instruments are built by large neutron facilities. Even at these national facilities, the neutron flux is still very limited. Therefore, the measurement for each sample on a NSE instrument may take a long time. It is not uncommon to measure one sample for a few hours at one experimental condition. To maximize the scattering intensity, NSE instruments usually have a large beam size and require a relatively large amount of sample volume. (It usually needs more than 1 mL for one sample.) In addition, protein concentration needs to be reasonably large so that the coherent scattering intensity of proteins can be significantly larger than the background signal. Protein concentrations used so far are mostly higher than 5 mg/mL. And higher concentrations (>10 mg/mL) are preferred in many instruments. Currently, NSE instruments can be accessed at major neutron facilities, such as the Institut Laue-Langevin (France), the NIST Center for Neutron Research (USA), SNS (USA), FRM-II (Germany), and J-PARC and JRR-III (Japan).

NSE and DLS

Often, NSE is compared with DLS as both are scattering techniques that can measure the full-intermediate scattering function. There are other scattering techniques optimized to measure the self-intermediate scattering function. In the rest of the article, the

intermediate scattering function always means the full-intermediate scattering function unless explicitly mentioned otherwise. The physical principles of NSE and DLS are quite different. NSE directly measures the intermediate scattering function of a system, $I(Q,t)$, where $Q = \frac{4\pi}{\lambda}\sin\left(\frac{\theta}{2}\right)$ is the magnitude of the scattering wave vector (or wave vector transfer), t is the correlation time [9,18], and θ is the scattering angle. Because the Fourier time of a NSE instrument is the correlation time of the intermediate scattering function of a sample it measures, we use the correlation time and Fourier time interchangeably for NSE experiments in the rest of the article. Unlike NSE, DLS obtains the intermediate scattering function indirectly by measuring the intensity correlation function, which is typically called $g_2(Q,t)$. $g_2(Q,t)$ can be linked to the intermediate scattering function, $I(Q,t)$, by the Siegert relation [19]. Although the assumptions for the Siegert relation are satisfied for most cases, there are some cases in which those assumptions may break down [20,21].

The ranges of accessible Q and t are very different for NSE and DLS although both can measure the intermediate scattering function. Typically, the correlation time of a commercial DLS instrument ranges from about 1 μs to hundreds of seconds. However, the time window for NSE is from a few picoseconds to close to one microsecond. For protein systems, NSE usually measures the short-time dynamics, whereas DLS is more suitable for long-time dynamics of proteins. The Q range for NSE is from about 0.02 $Å^{-1}$ to about 2 $Å^{-1}$, whereas the Q value for DLS is very small ($\approx$0.001 $Å^{-1}$). Thus, for protein systems, DLS is sensitive to the center of mass motions, whereas NSE can access both internal and rotational motions in addition to the center of mass translational motions.

Intermediate scattering functions of proteins in solutions

The measured signal by NSE instruments is usually dominated by coherent scattering. In fact, most studies using NSE focus on the coherent scattering for soft-matter systems. The reported value from NSE is typically the normalized intermediate scattering function, $\tilde{I}(Q,t) = \frac{I(Q,t)}{I(Q,0)}$ [9,18]. As a result, $\tilde{I}(Q,0) = 1$. For many protein systems, $\tilde{I}(Q,t)$ follows a single exponential function as a function of t [6,11]. Usually, people define the effective diffusion coefficient, $D_{eff}(Q) = -\frac{1}{Q^2}\lim_{t\to 0}\frac{\partial}{\partial t}\ln(\tilde{I}(Q,t))$ as $\tilde{I}(Q,t) = e^{-Q^2 D_{eff}(Q)t}$ [18]. Different theories have been proposed to analyze either $\tilde{I}(Q,t)$ or $D_{eff}(Q)$ to extract either structure or dynamic information.

To rigorously evaluate $\tilde{I}(Q,t)$ and $D_{eff}(Q)$ at a wide range of protein concentration, Liu [18] proposed the dynamic decoupling approximation, which is shown as follows:

$$\tilde{I}(Q,t) = \frac{1}{1+\beta(Q)(S(Q)-1)}\left(S_{self}(Q,t) + \beta(Q)\left(S(Q,t) - < e^{-Q^2 D_s(\vec{Q})t} >_\theta\right)\right)$$

where $\beta(Q) = |< F(\vec{Q},0) >_\theta|^2/P(Q)$, $S_{self}(Q,t) = \frac{<F(\vec{Q},0)F^*(\vec{Q},t)e^{-Q^2 D_s(\vec{Q})t}>_\theta}{P(Q)}$, $S(Q,t)$ is the intermediate scattering function for the center of mass of all proteins in solutions, and $S(Q)$ is the interparticle structure factor. $F(\vec{Q},t)$ is the Fourier transformation of the scattering length of an object at time t, and $P(Q)$ is the form factor. $< >_\theta$ indicates the angular average. $D_s(\vec{Q})$ is the one-dimensional diffusion coefficient of the center of mass of a protein along the direction of $\vec{Q}$. For dilute protein samples, $\tilde{I}(Q,t) = S_{self}(Q,t)$.

Based on the dynamic decoupling approximation, the effective diffusion coefficient, $D_{eff}(Q)$, can be calculated as follows:

$$D_{eff}(Q) = \frac{1}{1+\beta(Q)(S(Q)-1)}\left(D_{self,eff} + \beta(Q)(S(Q)D_T(Q) - D_s(Q))\right)$$

where $D_T(Q)$ is the collective diffusion coefficient of the center of mass of proteins, $D_s(Q)$ is the angular averaged self-diffusion coefficient of the center of mass of proteins, and

$$D_{self,eff} = D_{T,self} + D_{Intra,eff}$$

where $D_{T,self} = \frac{<F(\vec{Q},0)F^*(\vec{Q},0)D_s(\vec{Q})>_\theta}{P(Q)}$ is related to the translational motion of the center of mass of individual proteins and $D_{Intra,eff}$ contains the information of both the rotational and internal motions. The detailed definitions of different terms are discussed in the study by Liu [18]. For dilute protein samples, $D_{eff}(Q) = D_{self,eff}$. For the coherent scattering measured by NSE, it is straightforward to show based on the dynamic decoupling approximation that there is no contribution to $D_{self,eff}$ by rotational motions for perfect spherical isotropic particles [18]. However, for the incoherent scattering signals that can be measured by other quasi-elastic neutron scattering techniques, the contribution by rotational diffusion cannot be ignored even for spherical isotropic particles [22].

For dilute concentrations, Bu et al. [11] proposed a method to calculate $D_{self,eff}(Q)$ for rigid proteins at

dilute protein solutions. They further suggested a method to model the internal domain motion. Later, to study the internal motions of proteins in a concentrated solution, Biehl et al. [23] attempted to propose a way to directly model $\tilde{I}(Q,t)$. This model is different from the dynamic decoupling approximation. Although theoretically, the dynamic decoupling approximation should provide more accurate theoretical predictions at this concentration range [18], future experimental works using dynamic decoupling approximation are still needed to test how much it may improve the theoretical predictions. Note that both $D_{T,self}$ and $D_{Intra,eff}$ can be a strong function of Q. The free diffusion coefficient, D_0, measured by DLS is $D_{self,eff}(Q = 0)$. The experimentally measured $D_{self,eff}(Q)$ is typically larger than D_0 [11].

If all proteins are globular proteins without internal motions, the equations for the dynamic decoupling approximation reduce to $\tilde{I}(Q,t) = S(Q,t)$ and $D_{eff}(Q) = D_T(Q)$. At the short time limit, $D_T(Q) = D_0\frac{H(Q)}{S(Q)}$ [5], where $H(Q)$ is the hydrodynamic function. Thus, for globular proteins, NSE only measures the center of mass motions that can be calculated by theories. These simplified equations are widely used for NSE experiments using globular proteins [6,7]. Note that at the high Q region, $D_T(Q)$ approaches the self-diffusion coefficient, D_s.

Applications of NSE to study proteins in solutions

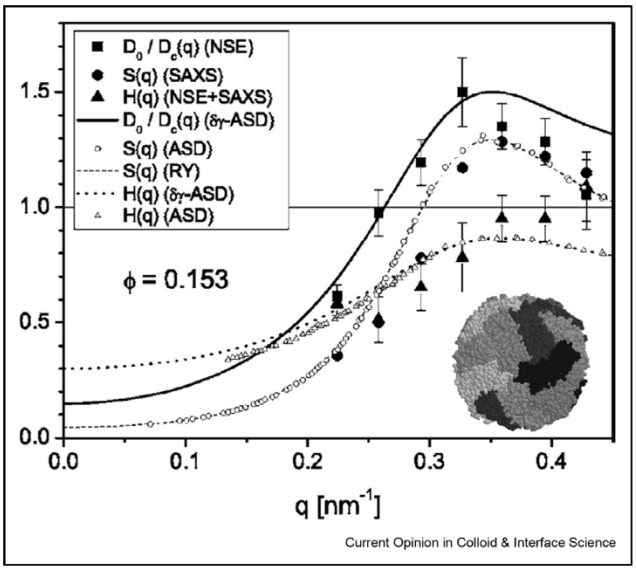
In the past ten years, the focus of using NSE to study protein solutions has been on either the translational or internal motions. It has been shown that the information of translational motions is important to examine liquid theories [24–26], investigate the cluster formation [4,6,7,27–29], and probe local motions in glass states [8,28,30] and protein domain motions [11,31–34]. The slow domain motions at nanosecond time windows have been very difficult to be measured by other techniques. In this regard, NSE has a unique capability to study the slow domain motion at this time window. And the community has seen the increased research effort to study internal protein motions by NSE [35].

Examining liquid theories with concentrated protein solutions

The short-time dynamics, such as diffusion coefficients and hydrodynamic functions, can be calculated by liquid theories [5]. Therefore, using spherical proteins as model systems, these results measured by NSE can be used to examine the accuracy of theoretical predictions.

Häußler and Farago [10], Gapinski et al [24], and Häußler [36,37] measured the short-time dynamics of the apoferritin protein for the concentration up to about 259 mg/mL. Apoferritin has a spherical shape with the radius around 6 nm. The schematic view of an apoferritin protein is shown in Figure. 1. Gapinski et al [24] and Häußler et al. [38] examined the structure factor obtained from SAXS and concluded that the protein–protein interaction between apoferritins can be modeled by a hard sphere with a screened coulomb repulsion. As shown in Figure. 1, using the experimentally obtained potential parameters, they calculated the hydrodynamic function, $H(Q)$, with the revised $\delta\gamma$ scheme based on simulations, and found a qualitative agreement between the experimental and theoretical results [24]. This indicates that the $\delta\gamma$ scheme not only works properly for hard-sphere systems but also remains to be qualitatively useful for studying the hard-sphere system with a repulsive potential.

Figure 1



Comparison between theoretical and experimental values for short-time diffusion coefficient, interparticle structure factor, and the hydrodynamic function for apoferritin proteins in solutions. A schematic view of an apoferritin protein is shown in the figure too (Reprinted from Gapinski et al [24] with the permission of AIP Publishing). NSE, neutron spin echo ; SAXS, small angle x-ray scattering; ASD, accelerated Stokesian dynamics simuation.

Doster and Longeville [25], Häußler [37], and Longeville et al [39] studied concentrated myoglobin and hemoglobin protein solutions using NSE. By studying the Q dependence of $D_{eff}(Q)$, it is found that the effective diffusion coefficient change from the collective diffusion coefficient to the self-diffusion coefficient when Q increases [39]. This is not surprising. People have used a similar method to study the self-diffusion coefficient using DLS for large colloidal particles [40]. This means that NSE can be a powerful tool to measure the self-diffusion coefficient without labeling a protein. Using this method, Doster et al. [25] measured the self-diffusion coefficient of hemoglobin as a function of protein volume fraction up to about 330 mg/mL. It is

found that the short-time self-diffusion coefficient decays faster as a function of volume fraction than that for a pure hard-sphere system when the protein volume fraction is calculated using the dry protein density. It is shown that considering the hydration volume on protein surfaces can make the agreement better between the experimental and theoretical values calculated with theories of hard-sphere systems. They concluded that the reduced mobility of the concentrated hemoglobin solutions is mainly caused by the hydrodynamic effect rather than the direct interaction between proteins.

Porcar et al [7], Liu et al [6], Cardinaux et al [30], and Godfrin et al [8,28] studied the short-time dynamics of lysozyme proteins by NSE. Different from the aforementioned protein systems, the interaction between lysozyme has both a short-range attraction and long-range repulsion [6]. (Readers with interests in colloidal systems with both a short-range attraction and long-range repulsion can read a recent review article on this topic and the references therein [41].) The lysozyme radius is about 1.7 nm, much smaller than hemoglobin and mAb. The interaction potential between lysozyme proteins and the short-time self-diffusion coefficients has been reported [28]. Riest et al. [26] calculated the short-time self-diffusion coefficient and the hydrodynamic function of lysozyme solutions at low salinity conditions using the hybrid Beenakker-Mazur pairwise additivity scheme and compared the results with the experimental values. Very interestingly, there is a quantitative agreement between the theoretical and experimental results at the relatively low lysozyme concentrations. However, at larger concentrations, the calculated theoretical values of the hydrodynamic function tend to be always higher than the experimental results. The discrepancy was attributed to the no-spherical shape of lysozyme and potentially patchy interactions between proteins. It is possible that the formation of dynamic clusters at large protein concentration could contribute to this discrepancy too.

The estimation of the hydrodynamic function is quite important to understand the short-time dynamics of concentrated protein solutions. Combining both NSE and small angle neutron scattering (SANS)/SAXS, the hydrodynamic functions can be measured and checked against the existing theories. And most theories can only calculate the hydrodynamic effect for spherical systems. However, many proteins are nonspherical. Therefore, computer simulations can be very useful to evaluate the hydrodynamic effect and study the short-time dynamics [42,43]. Wang et al. [42] studied mAb solutions by incorporating the hydrodynamic effect and evaluated the self-diffusion coefficients as a function of concentration.

It should be noted that for the aforementioned studies, the internal motions are ignored as these proteins are not known to have strong internal motions within the studied time window. In addition, because these proteins are quite spherical, the rotational motion can be ignored as NSE typically measures the coherent scattering. Owing to the correlation time a NSE instrument can reach, the self-diffusion coefficient is only the short-time diffusion coefficient for large proteins. For small proteins, such as lysozyme, it may be able to reach the correlation time longer than τ.

Other quasi-elastic neutron scattering techniques, such as neutron backscattering, can also measure the short-time diffusion coefficients. For example, the self-diffusion coefficients of concentrated bovine serum albumin protein solutions have been studied by neutron backscattering technique [44]. The accessible dynamics window of a backscattering instrument is typically less than ten nanoseconds. Note that the studies using neutron backscattering technique usually focus on the incoherent neutron scattering. The incoherent neutron scattering is determined by self-motion of atoms that is related to self-intermediate scattering function. In contrast, the coherent scattering techniques, such as DLS and NSE, probe collective motions that are related to the full-intermediate scattering function.

Cluster formation in concentrated protein solutions studied by NSE

Proteins can form tightly bounded clusters in solutions. Because of the increased size of clusters, its short-time diffusion coefficient is affected. Hence, there are many studies in the past decade using NSE to study protein cluster formations.

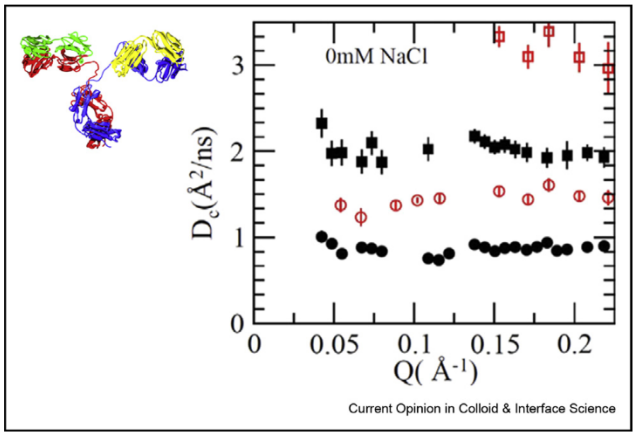
For concentrated protein solutions, they may form dynamic clusters when the concentration increases [4,6–8]. The dynamic clusters are also called reversible clusters, reversible associations in some studies [41,45]. One of the key features of dynamic clusters is that they are concentration dependent [7,41]. At dilute conditions, these proteins stay mostly as monomers and only associate with each other at high concentrations. And there is typically no sharp transition from monomer-dominating states to cluster-dominating states [27]. In general, the attraction that drives the cluster formation is relatively weak. This makes it very difficult to study the dynamic cluster formation as one can only investigate it in concentrated solutions. Sometimes, the structure measurements using SANS/SAXS experiments may not have a clear indication of the dynamic cluster formation [6]. This is not surprising as cluster formation is intrinsically linked to the dynamic properties of proteins [6,7,27]. And because DLS can only measure the collective diffusion coefficient, it is difficult to infer the dynamic cluster formation from the DLS results alone.

Porcar et al [7], Liu et al [6], and Falus et al [27] proposed that the dynamic cluster formation can be

determined by measuring the self-diffusion coefficients of concentrated protein solutions. As mentioned before, NSE is able to measure the self-diffusion coefficient at relatively large Q values without labeling proteins. The self-diffusion coefficients of lysozyme samples at low salt conditions were measured as a function of concentration [7]. By approximately using the hydrodynamic function of hard-sphere systems, the hydrodynamic radius of dynamic clusters in concentrated lysozyme solutions is approximately obtained as a function of concentration [7]. Using the results of the temperature dependence of the lysozyme solution at about 10% mass fraction with NSE, it has been concluded that the dynamic cluster formation should happen at higher protein concentrations than 10% mass fraction [27]. Note that although computer simulations find cluster formation at much smaller concentrations, those clusters may have a very short lifetime and could thus be mostly transient clusters [8].

Using a similar method, Yearley et al. [4] studied the reversible cluster formation in mAb solutions. Unlike lysozyme, mAbs are highly anisotropic, whose schematic picture is shown in Figure. 2. Based on the dynamic decoupling approximation, $\beta(Q)$ decays to zero very quickly at relatively large Q values [46]. As a result, the diffusion coefficients at large Q are essentially $D_{self,eff}(Q)$ that is only related to the self-diffusion coefficient. The self-diffusion coefficients for two different mAbs are measured by Yearley et al. [4] Figure. 2 shows the diffusion coefficients of these mAbs at different concentrations. Because one mAb is known to form reversible clusters and another one does not, the formation of small clusters is confirmed by studying the ratio of the two self-diffusion coefficients. And the formation of small clusters is shown to be correlated with the drastic increase of the viscosity for one mAb at high concentrations [46]. In general, the reversible cluster formation has strong impact on the viscosity of protein solutions [4,8,47].

Figure 2



The effective diffusion coefficients measured by NSE are shown for two different monoclonal antibody proteins at different concentrations. A schematic view of a monoclonal antibody is also shown in the figure (Reprinted from Yearley et al [4] with the permission from ELSEVIER). NSE, neutron spin echo.

It should be noted that owing to the time and length scale a NSE instrument can access, NSE probes only tightly bounded small clusters. For loosely bound clusters, it may not be sensitive to it. Godfrin et al. [48] used NSE to investigate the tightly bounded small clusters but used rheometers to study the large loosely bounded clusters. With these combined studies, they pointed out that the hierarchical cluster formation plays an important role in determining the viscosity of protein solutions.

Although computer simulations have been used to study cluster formation, a recent article pointed out that the clusters determined by many computer simulations are geometrically connected clusters (GCCs), which may have different dynamic properties depending on the binding strength [8,41]. The GCCs cannot be experimentally measured for protein systems. Many GCCs may have different dynamic properties and belong to different types of clusters, such as transient clusters, dynamic clusters, or permanent clusters [6–8,41]. The properties of transient, dynamics, and permanent clusters are discussed in details in the studies by Liu et al [6] and Porcar et al [7].

Permanent cluster formation has been also studied by NSE. Erlkamp et al. [29] studied cluster formation in insulin solutions. By controlling solvent conditions and temperatures, the formation of amyloid fibrils can be controlled. It is shown that the increase of the fibril formation is associated with the decrease of the self-diffusion coefficient at the short time limit.

To study the cluster formation, most works focus on the high Q values of $D_{eff}(Q)$, at which the results are essentially self-diffusion coefficients. The results of $D_{eff}(Q)$ at low Q values are only discussed qualitatively in some works. When clusters form in solutions, it is reasonable to expect that $D_{eff}(Q)$ at low Q should have some features associated with the cluster formation. However, there have been no theories and models that can offer quantitative analysis of low Q data. It is thus desirable for future works to look into this problem.

Studying the mean square displacement of concentrated solutions

NSE has been used to measure the mean square displacements of concentrated protein solutions [8,28]. Note that DLS has been used in a similar way to study systems of very large spherical colloids [40]. For globular protein solutions at relatively large Q values, $\tilde{I}(Q, t) \approx e^{-\frac{1}{6}Q^2\Delta r(t)^2}$, where $\Delta r(t)^2$ is the mean square displacement of a particle in three dimension and $\Delta r(t)$ is

a function of time. As long as the particle displacement follows the Gaussian distribution, the aforementioned equation is valid. Because it is challenging to image individual proteins directly to obtain their three-dimensional coordinates, obtaining mean square displacements as a function of time is difficult using optical tools. NSE and nuclear magnetic resonance are useful tools to measure the mean square displacements.

Godfrin et al. [28] used this method to study the mean square displacement of lysozyme proteins at a wide range of protein concentrations and compared their results with the estimated mean square displacements at the long time limit. Figure. 3 shows the normalized mean square displacements measured by NSE (solid symbols) and mean square displacements at the long time limit (open symbols). At relative low concentrations and high temperatures, the mean square displacements at the short time and long time limit agree with each other, indicating that the diffusion coefficient is about the same at both the short time and long time limits. However, at very high concentrations, there is a discontinuity of the mean square displacement (MSD) between the short time and long time limits. Interestingly, the normalized self-diffusion coefficients for some lysozyme solutions are as small as many large colloidal systems in glass states. Therefore, these lysozyme samples are believed in localized glassy states.

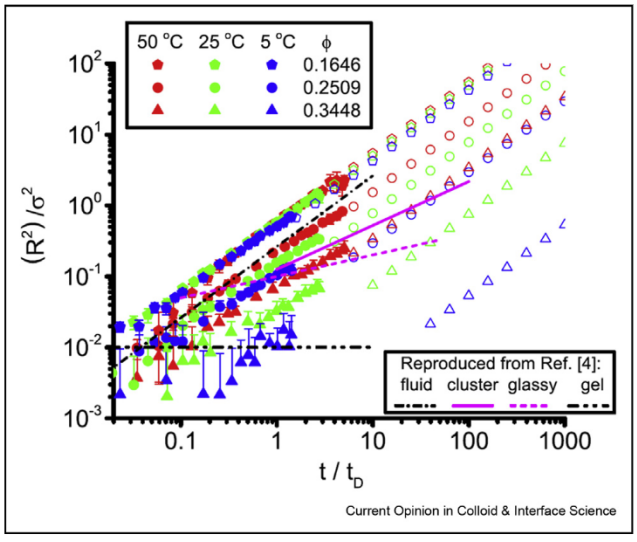
Mean square displacement is an important dynamic property of any colloidal systems. Although obtaining short-time mean square displacements using NSE has only been used for concentrated protein solutions, it may be also useful for proteins diffusing in a complicated matrix, such as porous media. In addition, there are strong interests in studying the effect of crowding agents on the dynamic properties of protein systems [49]. This method may thus be useful for studying the dynamic change of proteins in crowded environments.

Studying slow domain motions of flexible proteins

In the past ten years, there is increasing interest in studying the internal motions of flexible proteins using NSE. As mentioned before, NSE measures the intermediate scattering functions that include translational, rotational, and internal motions. Protein internal motions have a wide dynamic range from picoseconds to at least nanoseconds [3,11]. NSE has so far been used to understand the internal motions of proteins at the nanosecond timescale. By varying the scattering wave vector, Q, NSE can study the internal dynamics at a different length scale, from which the structural origin of internal motions could be identified.

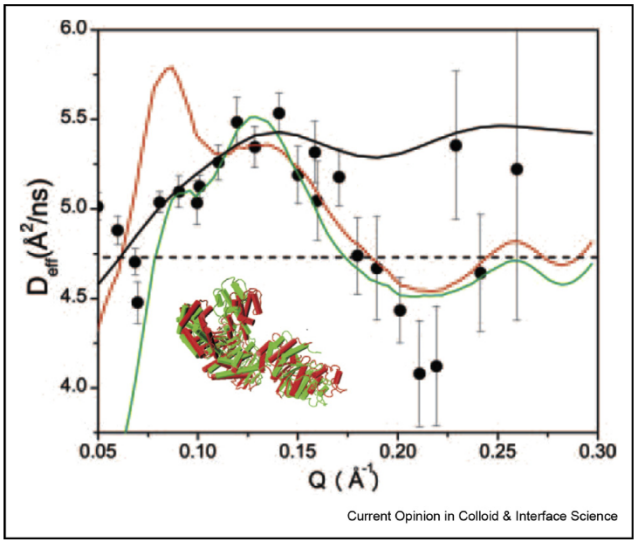
The early study of internal motions of proteins using NSE can be at least traced back to 1985. Alpert et al. [50] studied the dynamics of pig immunoglobulin G using NSE and concluded that the studied proteins are not rigid in solutions and the arms of the protein should wobble around the hinge region.

Figure. 3



The mean square displacements of lysozyme solutions at different concentrations are shown for the short time limit (solid symbols). The open symbols are the estimated mean square displacement at the long-time limit. (Reprinted figure with permission from Godfrin et al [28]. Copyright (2015) by the American Physical Society.)

Figure 4



The short-time diffusion coefficients measured by NSE are shown for Taq polymerase. A schematic view of Taq polymerase is also shown in the figure. (Reprinted figure from Bu et al [11]. Copyright (2005) of National Academy of Sciences, USA). NSE, neutron spin echo.

In 2005, Bu et al. [11] studied Taq polymerase in solutions using NSE. The schematic picture of this protein is shown in Figure. 4. They found that the domains of this protein have correlated motions of more than 7 nm. And this correlated motion plays an important role during the DNA synthesis and repair. They proposed a method to calculate the effective diffusion coefficients at the short time limit as a function of Q for rigid proteins. To model the internal motions, they assumed that individual domains are not strongly bounded and can be treated as individual objects at the short time limit. This soft-linker model seems to qualitatively predict the experimental results [11]. Their theoretical calculations together with the experimental results are shown in Figure. 4.

However, the same soft-linker model seems to be insufficient to explain the internal motions of one other protein. Wang et al. [32] studied lysozyme proteins in presence of a cationic azobenzene surfactant (azoTAB). Under UV light, lysozyme with azoTAB takes a more compact structure, whereas under visible light, lysozyme with azoTAB can partially unfold with an expanded conformation. The QQ-dependent diffusion coefficient for the compact lysozyme can be explained by rigid body diffusion. However, for the expanded conformation, there seems to be enhanced dynamics owing to internal motions. The predictions based on the soft-linker model are insufficient to explain the experimental data. By taking a model based on a polymeric system (freely joined model), the prediction seems to agree with the experimental data better [32,51].

Biehl et al. [52] studied the internal motions of the protein, alcohol dehydrogenase, using NSE. They theoretically calculated the effective diffusion coefficient based on the rigid body assumption. And the difference between the experimental data and the rigid body calculation is attributed to the internal motions. The normal-mode analysis method is proposed to understand the data, from which the relative domain motions of alcohol dehydrogenase are inferred.

Relatively concentrated protein solutions have also often been used to study the protein internal motions. However, for concentrated protein solutions, it is very challenging for the data analysis to take into consideration of the concentration-dependent collective diffusions and hydrodynamic effect between proteins. Biehl et al. [23] studied the internal motions of the phosphoglycerate kinase at about 5% protein concentrations. They proposed an approximate model including the collective translational motions and hydrodynamic effect. This approach was also applied to investigate the internal dynamics of an intrinsically disordered protein, myelin basic protein, whose internal motions were reported to be around 8.4 ns. [33]. Recently, the dynamic decoupling approximation is developed and expected to be theoretically more accurate to analyze the data of relatively concentrated protein solutions. It would be interesting to apply this new theory to experimental systems. There are also some other studies on internal protein motions using NSE [31,34]. Because there are already a couple of review articles on this topic, interested readers can check a nice review article (the study by Biehl and Richter [35]) and the references therein.

Overall, the contribution to the effective diffusion coefficient from protein internal motions is relatively small compared with that of the translational and rotational motions based on the reports from the literature. The small contributions by the internal motions require that the experimental data need to be obtained with very good quality. And the model and theory have to be reasonably accurate. This poses a huge challenge to develop appropriate theories/models to analyze $D_{eff}(Q)$ and understand protein internal motions. Besides the importance of developing new theories/models, it is also useful to carefully check existing theories/models to understand their limitations and the consequences of the assumptions built into different theories. Computer simulations are expected to play important roles in understanding the structural origins of measured dynamics. Some works have been attempted to compare the simulation results with the NSE data [53]. More works are encouraged in future to combine the simulation and NSE data to study protein dynamics.

Summaries and perspectives

NSE is a powerful tool to study the translational, rotational, and internal motions of proteins in solution at the short time limit as NSE can access the timescale from a few picoseconds to close to one microsecond. In particular, because NSE can measure the dynamics at different Q values (corresponding to different length scales), it is possible to identify the structure origins of different dynamics by modeling the QQ-dependent effective diffusion coefficients. In the past several decades, NSE has been successfully used to examine liquid theories, investigate cluster formation in concentrated protein solutions, reveal the short-time behaviors of proteins close to glass and gelation transition, and obtain the internal motions of proteins.

Despite these successes, it is still fair to argue that our community has not been using the full capability of NSE to study proteins in solutions. This is partly due to the fact that modeling the dynamics of proteins is very challenging. This is especially true for experimental groups not familiar with neutron scattering techniques because there is a relatively steep learning curve in terms of the data analysis of NSE data. Therefore, more efforts should be devoted to encourage the development of theoretical works and analysis tools. With more and more models available in future, we can expect a significant

change to this. In addition, there are not too many NSE instruments worldwide. And it is usually very competitive to obtain the NSE beam time through user proposals. This shortage of available NSE beam time severely limits the number of experimental groups that can use NSE. Building more NSE instruments will certainly help the community. Given the unique time and length scale NSE can access, it is expected that NSE will play more and more important roles in deciphering the important short-time dynamics of proteins in solutions.

Because the timescale accessible by NSE is well within the timescale that computer simulation can reach, the results from NSE can also be used to gauge the accuracy of force fields used in simulations. Currently, many computer simulations have been already using the structure information obtained by SANS/SAXS to check the algorithms and force fields. Arguably, the dynamic information is, sometimes, more sensitive to the subtle change of force fields. Hence, combining NSE results with other experimental information, we may provide a more comprehensive test to the existing computer simulation packages. On the other hand, computer simulations can provide critical information to help explain the physics origins of dynamics observed by NSE.

Conflict of interest statement

Nothing declared.

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References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Mitragotri S, Burke PA, Langer R: **Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies**. *Nat Rev Drug Discov* 2014, **13**:655–672.

2. Saltzman WM, Langer R: **Transport rates of proteins in porous materials with known microgeometry**. *Biophys J* 1989, **55**: 163–171.

3. Parak FG, Achterhold K: **Protein dynamics on different timescales**. *J Phys Chem Solids* 2005, **66**:2257–2262.

4. ** Yearley EJ, Godfrin PD, Perevozchikova T, Zhang HL, Falus P, Porcar L, Nagao M, Curtis JE, Gawande P, Taing R, Zarraga IE, Wagner NJ, Liu Y: **Observation of small cluster formation in concentrated monoclonal antibody solutions and its implications to solution viscosity**. *Biophys J* 2014, **106**:1763–1770.
Combing SAXS, DLS, and NSE, the formation of dynamic clusters is confirmed and is found to be responsible for the anomlously high viscosity at concentrated solutions for one monoclonal antibody protein. NSE is used to probe the self-diffusion coefficient of proteins at high concentrations.

5. Nagele G: **On the dynamics and structure of charge-stabilized suspensions**. *Phys Rep-Rev Sect Phys Lett* 1996, **272**:216–372.

6. Liu Y, Porcar L, Chen JH, Chen WR, Falus P, Faraone A, Fratini E, Hong KL, Baglioni P: **Lysozyme protein solution with an intermediate range order structure**. *J Phys Chem B* 2011, **115**:7238–7247.

7. ** Porcar L, Falus P, Chen WR, Faraone A, Fratini E, Hong KL, Baglioni P, Liu Y: **formation of the dynamic clusters in concentrated lysozyme protein solutions**. *J Phys Chem Lett* 2010, **1**:126–129.
SANS and NSE are used to study the dynamic cluster formation in concentrated lysozyme solutions at low salt conditions. Through the measurement of the self-diffusion coefficient as a function of concentration, the approximate hydrodynamic radius of dynamic clusters is estimated as a function of concentration.

8. ** Godfrin PD, Falus P, Porcar L, Hong K, Hudson SD, Wagner NJ, Liu Y: **Dynamic properties of different liquid states in systems with competing interactions studied with lysozyme solutions**. *Soft Matter* 2018, **14**:8570–8579.
Lysosyme is used as a model system to study colloidal systems with a short-range attraction and long-range repulsion. The shor-time dynamics are studied by NSE in dispersed fluid states and random percolated states. The phase diagram of localized glassy states is experimentally identified. The phase boundaries for gel and glass states are estimated.

9. Farago B: **Recent developments and applications of NSE in soft matter**. *Curr Opin Colloid Interface Sci* 2009, **14**:391–395.

10. Haussler W, Farago B: **Diffusive dynamics of ordered solutions of apoferritin near the structure factor peak**. *J Phys Condens Matter* 2003, **15**:S197–S204.

11. ** Bu ZM, Biehl R, Monkenbusch M, Richter D, Callaway DJE: **Coupled protein domain motion in Taq polymerase revealed by neutron spin-echo spectroscopy**. *Proc. Natl. Acad. Sci. U.S.A* 2005, **102**:17646–17651.
NSE is used to study the internal domain motions of Taq polymerase at dilute concentrations. A thearetical model is proposed to calculate the diffusion coefficient as a function of Q for rigid proteins. The authors also proposed a method to evaluate the diffusion coefficients when there are internal domain motions by weakly linked domains. This model with the weakly linked domains predicts the experimental results reasonably well.

12. Mezei F: **Neutron spin-echo - new concept in polarized thermal-neutron techniques**. *Zeitschrift Fur Physik* 1972, **255**:146.

13. Nagao M, Kelley EG, Ashkar R, Bradbury R, Butler PD: **Probing elastic and viscous properties of phospholipid bilayers using neutron spin echo spectroscopy**. *J Phys Chem Lett* 2017, **8**: 4679–4684.

14. Senses E, Ansar SM, Kitchens CL, Mao YM, Narayanan S, Natarajan B, Faraone A: **Small particle driven chain disentanglements in polymer nanocomposites**. *Phys Rev Lett* 2017, **118**:5.

15. Calabrese MA, Wagner NJ: **Detecting branching in wormlike micelles via dynamic scattering methods**. *ACS Macro Lett* 2018, **7**:614–618.

16. Li X, Sanchez-Diaz LE, Wu B, Hamilton WA, Falus P, Porcar L, Liu Y, Do C, Faraone A, Smith GS, Egami T, Chen WR: **Dynamical threshold of diluteness of soft colloids**. *ACS Macro Lett* 2014, **3**:1271–1275.

17. Huang JS, Milner ST, Farago B, Richter D: **Study of dynamics of microemulsion droplets by neutron spin-echo spectroscopy**. *Phys Rev Lett* 1987, **59**:2600–2603.

18. ** Liu Y: **Intermediate scattering function for macromolecules in solutions probed by neutron spin echo**. *Phys Rev E* 2017, **95**: 6.
In order to understand NSE signals of protein solutions with anisotropic shape and/or size dispersity, this paper proposed the dynamic decoupling approximation theory to calculate the intermediate scattering function and the effective diffusion coefficient measured by NSE.

19. Stetefeld J, McKenna SA, Patel TR: **Dynamic light scattering: a practical guide and applications in biomedical sciences**. *Biophys Rev* 2016, **8**:409–427.

20. Gittings AS, Durian DJ: **Gaussian and non-Gaussian speckle fluctuations in the diffusing-wave spectroscopy signal of a coarsening foam**. *Appl Opt* 2006, **45**(10):2199–2204.

21. Schaefer DW, Berne BJ: **Light-scattering from Non-Gaussian concentration fluctuations**. *Phys Rev Lett* 1972, **28**:475.

22. Perez J, Zanotti JM, Durand D: **Evolution of the internal dynamics of two globular proteins from dry powder to solution**. *Biophys J* 1999, **77**:454–469.

23. Biehl R, Monkenbusch M, Richter D: **Exploring internal protein dynamics by neutron spin echo spectroscopy**. *Soft Matter* 2011, **7**:1299–1307.

24. * * Gapinski J, Wilk A, Patkowski A, Haussler W, Banchio AJ, Pecora R, Nagele G: **Diffusion and microstructural properties of solutions of charged nanosized proteins: experiment versus theory**. *J Chem Phys* 2005, **123**:13.
Apoferritin protein is used as a model charged hard sphere system. Collective diffusion coefficients and hydrodynamic functions are calculated based on liquid theories. And the theoretical calculations have reasonable agreement with the experimental results obtained by NSE.

25. * Doster W, Longeville S: **Microscopic diffusion and hydrodynamic interactions of hemoglobin in red blood cells**. *Biophys J* 2007, **93**:1360–1368.
The effective diffusion coefficients of concentrated hemoglobin solutions are studied by NSE. It is found that the reduced mobility is mainly driven by the hydrodynamic effect instead of direct interactions between proteins. The change of self-diffuson coefficient as a function of concentration is also compared with the results from the hard sphere systems.

26. Riest J, Nagele G, Liu Y, Wagner NJ, Godfrin PD: **Short-time dynamics of lysozyme solutions with competing short-range attraction and long-range repulsion: experiment and theory**. *J Chem Phys* 2018, **148**.

27. Falus P, Porcar L, Fratini E, Chen WR, Faraone A, Hong KL, Baglioni P, Liu Y: **Distinguishing the monomer to cluster phase transition in concentrated lysozyme solutions by studying the temperature dependence of the short-time dynamics**. *J Phys Condens Matter* 2012, **24**.

28. * * Godfrin PD, Hudson SD, Hong K, Porcar L, Falus P, Wagner NJ, Liu Y: **Short-time glassy dynamics in viscous protein solutions with competing interactions**. *Phys Rev Lett* 2015, **115**.
The mean square displacements of concentrated lysozyme protein solutions are obtained using the intermediate scattering funcitons measured by NSE. The extremly slow motions for some protein solutions are observed, from which the localized glassy states are identified. These localized glassy states are driven by the formation of the intermediate range order.

29. Erlkamp M, Grobelny S, Faraone A, Czeslik C, Winter R: **Solvent effects on the dynamics of amyloidogenic insulin revealed by neutron spin echo spectroscopy**. *J Phys Chem B* 2014, **118**:3310–3316.

30. Cardinaux F, Zaccarelli E, Stradner A, Bucciarelli S, Farago B, Egelhaaf SU, Sciortino F, Schurtenberger P: **Cluster-driven dynamical arrest in concentrated lysozyme solutions**. *J Phys Chem B* 2011, **115**:7227–7237.

31. Farago B, Li JQ, Cornilescu G, Callaway DJE, Bu ZM: **Activation of nanoscale Allosteric protein domain motion revealed by neutron spin echo spectroscopy**. *Biophys J* 2010, **99**:3473–3482.

32. Wang SC, Mirarefi P, Faraone A, Lee CT: **Light-controlled protein dynamics observed with neutron spin echo measurements**. *Biochemistry* 2011, **50**:8150–8162.

33. Stadler AM, Stingaciu L, Radulescu A, Holderer O, Monkenbusch M, Biehl R, Richter D: **Internal nanosecond dynamics in the intrinsically disordered myelin basic protein**. *J Am Chem Soc* 2014, **136**:6987–6994.

34. Stingaciu LR, Ivanova O, Ohl M, Biehl R, Richter D: **Fast antibody fragment motion: flexible linkers act as entropic spring**. *Sci Rep* 2016, **6**:13.

35. * * Biehl R, Richter D: **Slow internal protein dynamics in solution**. *J Phys Condens Matter* 2014, **26**:21.
This is a nice review article that discusses many examples using NSE to study the internal protein motions in solutions.

36. Haussler W: **Structure and dynamics in apoferritin solutions with paracrystalline order**. *Chem Phys* 2003, **292**:425–434.

37. Haussler W: **Neutron spin echo studies on ferritin: free-particle diffusion and interacting solutions**. *Eur Biophys J Biophys Lett* 2008, **37**:563–571.

38. Haussler W, Wilk A, Gapinski J, Patkowski A: **Interparticle correlations due to electrostatic interactions: a small angle x-ray and dynamic light scattering study. I. Apoferritin**. *J Chem Phys* 2002, **117**:413–426.

39. Longeville S, Doster W, Kali G: **Myoglobin in crowded solutions: structure and diffusion**. *Chem Phys* 2003, **292**:413–424.

40. van Megen W, Mortensen TC, Williams SR, Muller J: **Measurement of the self-intermediate scattering function of suspensions of hard spherical particles near the glass transition**. *Phys Rev E* 1998, **58**:6073–6085.

41. Liu Y, Xi Y: **Colloidal systems with a short-range attraction and long-range repulsion: phase diagrams, structures, and dynamics**. *Curr Opin Coll Inter Sci* 2019.

42. Wang G, Varga Z, Hofmann J, Zarraga IE, Swan JW: **Structure and relaxation in solutions of monoclonal antibodies**. *J Phys Chem B* 2018, **122**:2867–2880.

43. Nawrocki G, Wang PH, Yu I, Sugita Y, Feig M: **Slow-down in diffusion in crowded protein solutions correlates with transient cluster formation**. *J Phys Chem B* 2017, **121**:11072–11084.

44. Roosen-Runge F, Hennig M, Zhang FJ, Jacobs RMJ, Sztucki M, Schober H, Seydel T, Schreiber F: **Protein self-diffusion in crowded solutions**. *Proc. Natl. Acad. Sci. U.S.A* 2011, **108**:11815–11820.

45. Kanai S, Liu J, Patapoff TW, Shire SJ: **Reversible self-association of a concentrated monoclonal antibody solution mediated by Fab-Fab interaction that impacts solution viscosity**. *J Pharm Sci* 2008, **97**:4219–4227.

46. Yearley EJ, Zarraga IE, Shire SJ, Scherer TM, Gokarn Y, Wagner NJ, Liu Y: **Small-angle neutron scattering characterization of monoclonal antibody conformations and interactions at high concentrations**. *Biophys J* 2013, **105**:720–731.

47. Zhang ZH, Liu Y: **Recent progresses of understanding the viscosity of concentrated protein solutions**. *Current Opinion in Chemical Engineering* 2017, **16**:48–55.

48. Godfrin PD, Zarraga IE, Zarzar J, Porcar L, Falus P, Wagner NJ, Liu Y: **Effect of hierarchical cluster formation on the viscosity of concentrated monoclonal antibody formulations studied by neutron scattering**. *J Phys Chem B* 2016, **120**:278–291.

49. Zorrilla S, Rivas G, Acuna AU, Lillo MP: **Protein self-association in crowded protein solutions: a time-resolved fluorescence polarization study**. *Protein Sci* 2004, **13**:2960–2969.

50. Alpert Y, Cser L, Farago B, Franek F, Mezei F, Ostanevich YM: **Segmental flexibility in pig immunoglobulin-g studies by neutron spin-echo technique**. *Biopolymers* 1985, **24**:1769–1784.

51. Akcasu AZ, Hammouda B, Stockmayer WH, Tanaka G: **1st cumulant for chains with constraints**. *J Chem Phys* 1986, **85**:4734–4743.

52. * * Biehl R, Hoffmann B, Monkenbusch M, Falus P, Preost S, Merkel R, Richter D: **Direct observation of correlated interdomain motion in alcohol dehydrogenase**. *Phys Rev Lett* 2008, **101**:4.
The internal motions of alchohol dehydrogenase is studied by NSE. The normal mode analysis is proposed to analyze the effective diffusion coefficients, from which the structural origin of the internal dynamics of this protein is identified.

53. Smolin N, Biehl R, Kneller GR, Richter D, Smith JC: **Functional domain motions in proteins on the similar to 1-100 ns timescale: comparison of neutron spin-echo spectroscopy of phosphoglycerate kinase with molecular- dynamics simulation**. *Biophys J* 2012, **102**:1108–1117.