IN SITU DYNAMIC LIGHT SCATTERING COMPLEMENTING NEUTRON SPIN ECHO MEASUREMENTS ON PROTEIN SAMPLES

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Abstract - Monitoring the state of the sample on the minute-time scale is crucial in case of sensitive soft matter or biological samples, given that neutron spin echo measurements take up to several days. Moreover, there is no method to interpret the normalized intermediate scattering function obtained by neutron spin echo measurements if relevant sample properties change during the measurement. Dynamic light scattering provides information on the diffusion constant of particles in solution (biological macromolecules like proteins, protein aggregates, polymer particles, etc.) with average hydrodynamic radii in a broad range from a few nanometers up to several microns. This information can be obtained within a few minutes and it offers a good overview of the current sample state. Details on the novel *in situ* dynamic light scattering set-up with one fixed scattering angle and first results obtained on a molten globule state of apo-myoglobin are presented.

Keywords: bio-polymers, spin echo spectroscopy, *in situ* dynamic light scattering, aggregation.

INTRODUCTION

Neutron Spin Echo Spectroscopy (NSE) has been used successfully to elucidate polymer dynamics [1]. In the recent years, this method has been also used to investigate the dynamics of biological macromolecules in solution [2], since it can follow their equilibrium dynamics without the need to use spin labelling [3], or attach or incorporate additional labels onto the molecule of interest [4] [5]. However, the pitfall of the spin echo technique is the need for fairly high concentrations. Often this concentration exceeds 3 weight % for the biopolymers in solution. At these high concentrations, aggregation can occur and then the spin echo data is unreliable. Due to the long duration and the nature of the spin echo measurement, this influence cannot be deduced from the data itself while measuring. In case of some delicate

protein samples, there is a need to monitor the oligomeric state or aggregation of the sample by additional methods. Dynamic light scattering (DLS) is an ideal tool for such additional control measurements.

Also known as photon-correlation spectroscopy or quasielastic light scattering, this technique is based on the temporal analysis of the intensity fluctuations of the scattered light caused by the Brownian motion of particles (protein molecules, aggregates, polymer particles etc.) in solution. It primarily determines diffusion coefficients. Under the assumption that the particles are spheres, the translational diffusion coefficient is determined and the hydrodynamic radii of those hypothetical spheres can be calculated. This technique allows the identification of particles with average hydrodynamic radii in a broad range from a few nanometers up to several microns. If several particles are present in the solution, they can be distinguished if they are at least one order of magnitude different in their diffusion constant. With the set-up presented here, reasonable DLS data can be acquired every 5 minutes. Monitoring the aggregation state and the sample composition on this minute-time scale is crucial in case of biological samples, which are sensitive to slight changes of temperature, pH of the solvent etc.

Moreover, the DLS data can be used to complement the interpretation of the spin echo data. The normalized intermediate scattering function I(q,t)/I(q,0) obtained in the neutron experiment offers information on the overall dynamics of polymer and protein systems, i.e. translational diffusion, rotational diffusion and internal dynamics. Only with further modelling, the separate contribution of each of these processes can be quantified. Since DLS accesses a q value (laser wavelength 632.8 nm, scattering angle of 130° and refractive index 1.3 for water) of 0.0023 $Å^{-1}$, the intermediate scattering function, also known as the first-order correlation function $g^{I}(\tau)$ (if no further structure factor is considered), mainly includes the translational diffusion coefficient.

METHODS

NSE Data Collection and processing

The NSE data were acquired at the Jülich Neutron Spin Echo instrument J-NSE Phoenix [6] at the Heinz-Maier-Leibnitz Zentrum (MLZ) in Garching, Germany. The instrument covers a Q-range of 0.03-1.0 $Å^{-1}$, reaching Fourier Times up to 250 ns using 12 and 8 Å neutrons. In the experiments presented here, a Q-range of 0.03-0.15 $Å^{-1}$ was explored, which was possible

by tilting the second instrument arm to an angle of up to 30°. A graphite powder sample was measured as a scattering reference, followed by the protein sample and the buffer solution.
A sample holder designed to cover the temperature range 0-200°C was used. It is made of polyimide and thermally decoupled from the neutron instrument components through a ceramic plate. The temperature is set using a dry airstream from a temperature forcing system ThermoJet (SP Scientific) boosted from the lower side of the holder and released through a chimney-like pipe on the upper side of the holder (see Fig. 2). The sample holder can be moved up and down in the neutron beam to switch between the two sample positions. It has an internal aluminum frame that can be taken out, on which two sample cells can be mounted (using non-magnetic small plates and screws). 4 cm wide quartz cells (Hellma GmbH & Co. KG, Germany) with a neutron path length of 2 and 4 mm can be used. To allow both the neutrons and the light beam to reach the quartz cells, but still offer the temperature stability, the sample holder has two quartz windows on each side.
For the presented measurements, the temperature of the sample cells was 10° C. Because of the double quartz windows on each side, there was no sign of condensation.

Protein Sample

The sample of interest for the measurements presented here is a 3 weight% apo-myoglobin (molten globule) in D_2O solution. The protein sample is obtained by removing the heme-group of horse-heart myoglobin (Sigma-Aldrich/Merck KGaA, Germany) and adding deuterium chloride (Sigma-Aldrich/Merck KGaA, Germany) until the solution pH read-out is 3.6 (Methrom, Mettler Toledo, Switzerland). After the aggregates are removed using syringe filters (20 nm, Whatman Anotop, Sigma-Aldrich/Merck KGaA, Germany), the solution is brought to the final concentration by centrifugation (Vivaspin 3,000 MWCO, Sartorius, Germany; 3k30, Sigma Laborzentrifugen). The protein concentration is determined by UV/VIS spectroscopy (Cary UV/VIS 100, Agilent Technologies Inc, USA). Because the protein solution is in a non-native state and at high concentration, it is subject to aggregation.

DLS Instrumentation

The DLS set-up consists of five major components (see Fig. 1): light source (laser box), optics defining the scattering geometry (the temperature controlled sample holder with optics), single photon detector, correlator and computer. The optics defining the scattering

geometry are placed at the sample position, whereas the other components are mobile and connected through optical fibers.

In detail, the light from a 20 mW He-Ne laser (1145/P, JDS Uniphase) at a wavelength of 632.8 nm is coupled into a fiber (SMC 630-4.1-NA012-3-APC-Ti-0PC-0-500, Schäfter + Kirchhoff GmbH, Germany) and guided to the neutron instrument. A collimator (60 FC-A6.2S-02-Ti, Schäfter + Kirchhoff GmbH, Germany) and a silver mirror focus the light from the fiber into the center of the quartz cell, which was previously aligned in the center of the neutron beam. The light scattered under the angle θ_s (in the present case θ_s =130°) is then mirrored into another collimator that is linked to a second fiber. The optics are mounted using non-magnetic optic holders (Liop-Tec Germany) placed on the sample holder's platform.

The scattering angle θ_s is determined by considering the refractions according to the formula

$$\theta_s = 180° - arcsin(\frac{n_{air}}{n_{liq}} sin\theta_i) - arcsin(\frac{n_{air}}{n_{liq}} sin\theta_f)$$

where n_{air} and n_{liq} are the refractive indices of air and the liquid sample, respectively and θ_i and θ_f are the angles of the incident and refracted beam outside the cell, measured manually.

To cross-validate the scattering angle, polystyrene beads of known size can be measured in a different quartz cell. This size can be converted into a diffusion constant and from this the scattering angle after the alignment can be determined and compared with the calculated value.

The alignment of the DLS-set-up takes about half an hour and it can be performed independently of the alignment of the sample holder in the neutron beam. Thus, the *in situ* set-up does not consume expensive spin echo beam time.

The scattered photons arrive at an APD detector (Excelitas Technologies Corp. SPCM-AQRH-14-FC). The recorded photons are auto-correlated in their arrival time with an ALV-7004 Multiple Tau Digital Correlator (ALV-GmbH, Germany). A computer displays the measured normalized intensity-intensity auto-correlation function $g^{II}(\tau)$. The recorded intensities and $g^{II}(\tau)$ are saved for further analysis.

To protect the detection components, a filter wheel with neutral density filters (ALV GmbH, Germany) is placed before coupling the laser light into the fiber. Based on the photon counts detected for each sample, the filter wheel adjusts to the optimum neutral density filter for each measurement.

DLS Data collection and processing

The DLS experiment is initiated after the neutron measurement is started, controlled by the instrument control software. A typical DLS measurement takes 5 minutes. Therefore, over the time span of typically 20 hours of the neutron measurement on the very same sample, 220 DLS intensity autocorrelation curves can be measured. The DLS data are recorded in parallel and saved on a computer with a time stamp.

Data processing is performed post-experiment, by obtaining the electrical field first-order correlation function $g^{I}(\tau)$ from the recorded $g^{II}(\tau)$, according to the Siegert relation. $g^{I}(\tau)$ is modeled using a python routine based on the CONTIN algorithm [7]. To apply the algorithm, 100 points were chosen to discretize the delay time τ axis between 10^{-2} and 10^{6} μs. The chosen regularization parameter was α=1.

RESULTS

In the presented *in situ* DLS measurement (see Fig. 4), one can attribute the intensity of the first-order correlation function $g^{I}(\tau)$ as following: 9% of the amplitude of the $g^{I}(\tau)$ function as integrated from the CONTIN curve (Fig. 4B) stem from the apo-myoglobin monomer (with a decay time constant distribution centred at 0.01 ms, corresponding to an average hydrodynamic radius r_H of 1.4 nm), 87% from aggregates (nominal average r_H of 200 nm). Less than 4% of the amplitude of the autocorrelation function can be attributed either to large-sized aggregates (average r_H of 2 μm) or can be assumed to be due to noise caused by mechanical vibrations, for example caused due to vacuum pumps at the neutron instrument. Under the following assumptions, the intensity distribution can be converted into a number density distribution: all particles are spherical, homogeneous and their refractive index is known. According to the Rayleigh approximation, the intensity of the electrical field *I* is proportional to the square of the scattered electric field E^2. *E* is proportional to the particle polarizability α, which is proportional to the particle volume V. Therefore, the intensity is proportional to the sixth power of the particle radius ($I \propto r^6$) [8]. The amplitude percentages of the $g^{I}(\tau)$ function correspond to a number density of 99% monomer and less than 1% aggregates (see inset Fig. 4).

Since the measured auto-correlation curves at the beginning of the neutron measurement, during and at the end nicely overlap (see Fig. 4 presenting the measurements performed at 4, 10 and 18 h after starting the neutron experiement), the aggregation state of the sample did not change significantly during the neutron beamtime. This is also visible in the number density distributions at the different times that show no significant contributions from slow diffusing aggregates apart from the diffusion of the monomer.

CONCLUSION

Hereby, a novel set-up capable of performing *in situ* dynamic light scattering measurements on a liquid sample investigated in a quartz cell by neutron spin echo spectroscopy is presented. The sample holder is able to set temperatures at the sample position between 0 and 200 °C. The sample holder has two positions, one for the sample that is subject to DLS monitoring and another one for a buffer or reference sample cell. Data from a 3% solution of a partially unfolded state of apo-myoglobin, which is known to aggregate in time, are presented. The DLS results indicate that the aggregation state of the sample was almost constant during the spin echo measurement rendering the latter useful for further data interpretation. The spin echo data will be presented in an upcoming publication.

The presented *in situ* DLS set-up is well capable of detecting aggregates. In case aggregates of relevant sizes are found, the neutron users can consider replacing the sample with a fresh one. This set-up is now routinely available for user operation at the Jülich Neutron Spin Echo spectrometer (J-NSE Phoenix). Further improvement of the instrument control software will send the DLS data to the user every two hours or issue a warning when a certain aggregation peak sets in.

In a future set-up, the transmitted light beam can be detected and used to carefully control the state of thermo-responsive polymers [9]. In such cases, tenths of a degree Celsius make a difference to which state is investigated (swollen or collapsed). Due to the limitations of a thermometer being close enough to the sample, only an optical turbidity measurement can tell which state the sample is currently in.

ACKNOWLEDGMENTS

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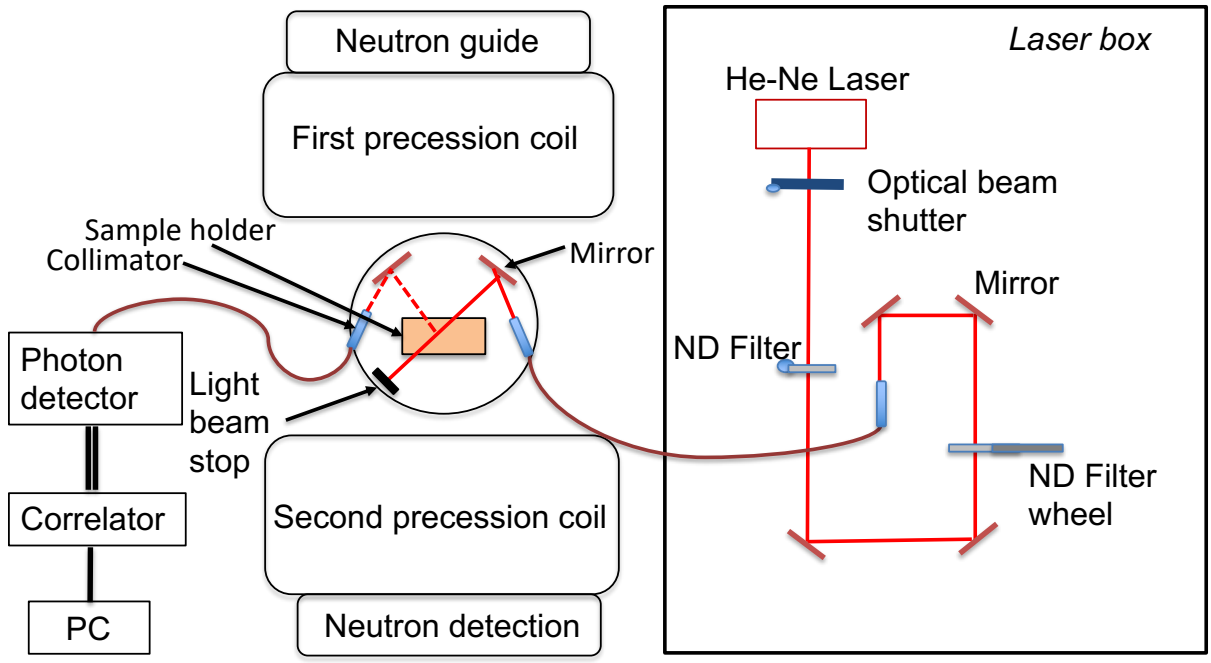

Fig. 1. Schematic view of the *in situ* DLS set-up. A photo of the sample holder and the surrounding optics can be seen in Fig. 2.

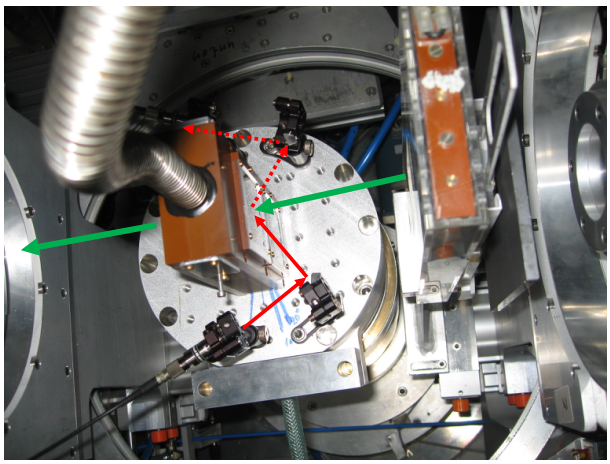

Fig. 2. A bird view of the high temperature sample-holder at the J-NSE Phoenix. The sample holder can be moved in the neutron beam (green arrow) on the vertical plane: on the upper position, the sample is placed, whereas on the lower position, the buffer solution.

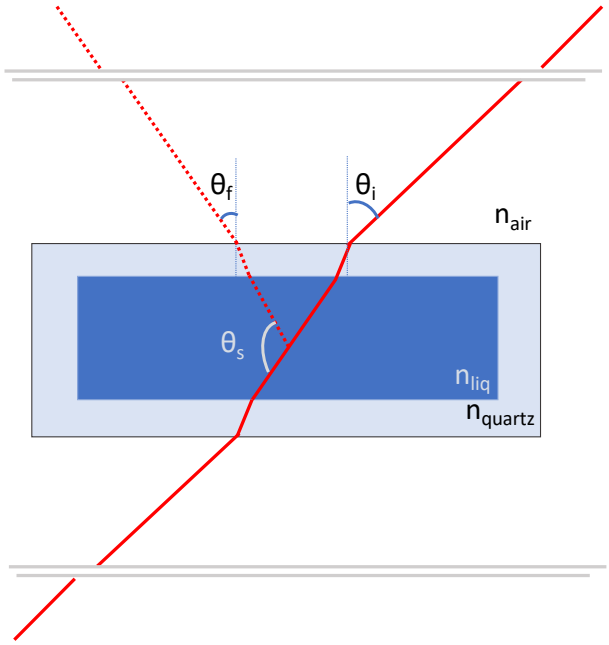

Fig. 3. Relation between the actual scattering angle and the laser beam angles found on the outside of the quartz cell. The double glass windows in front and behind of the cell are indicated with grey lines but ignored in terms of refraction and beam shift due to their small thickness.

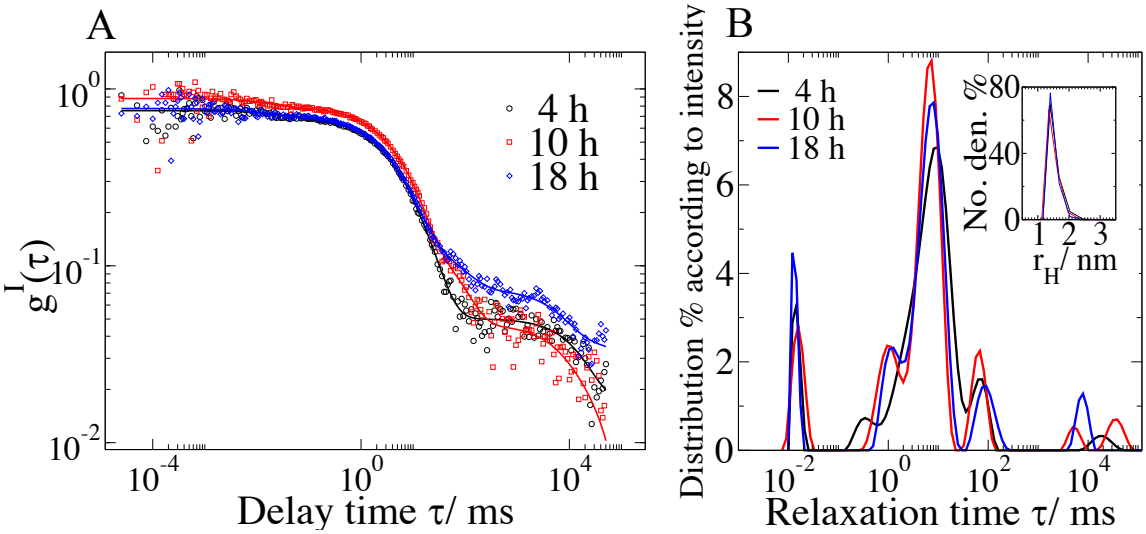

Fig. 4. (A) The electrical field first-order correlation function $g^{I}(\tau)$ obtained from three DLS measurement of 300 seconds duration each on the protein solution analyzed using a CONTIN fit (solid lines). The measurements were taken 4 , 10 and 18 hours after the start of the neutron measurement. (B) The corresponding relaxation time percentage distributions. (B-inset) The derived number density distributions: over 99% of the particles present in solution show a hydrodynamic radius of 1-2 nm and their signal in the measured $g^{I}(\tau)$ function has a relaxation time τ in the range of 0.01 ms.