Study of Protein Hydrodynamics with Light Scattering: Size and Charge of Lysozyme

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Effective determination of the size and charge status of biological macromolecules like proteins plays a crucial role in pharmaceutical, clinical and other biotechnological applications. In the present study, dynamic light scattering (DLS) and electrophoretic light scattering (ELS) are employed to characterize the size and charge of Lysozyme, a common protein. It is shown that the sensitivity and precision of these techniques are well suitable to discern the subtle effects of buffer solution conditions on the protein size and charge.

Introduction

Proteins are not only important nutrient source for human society, but also play a crucial role in devising various protein-based therapies for many terminal diseases [1]. Therapeutic proteins are usually administrated by the parenteral route. Their dosage form needs to be formulated either as a solution, a suspension or a reconstituted solid dosage form. Therefore, for protein therapeutic products development, protein hydrodynamic behavior in solutions or suspensions is proven to be much more relevant than its isolated and denatured characteristics revealed via advanced microscopy techniques. This can be manifested as either protein size monitoring for QC purposes of its purification processes or their charge property control for optimized formulation stability. The critical requirements for these applications often include, but not limited to, non-invasive (in-situ) nature of measurement techniques, easy-of-use and rapidness of tests.

Laser light scattering techniques satisfy these requirements and provide vital access to the hydrodynamic characteristics of biological macromolecules in their native application environments [2]. Their diffusive displacement ("Brownian" motion) can be quantified as diffusion coefficient with a dynamic light scattering measurement. In turn, their hydrodynamic radius can be calculated. When placed under an electric filed, the charged proteins undergo a uniform translational movement, the so-called electrophoretic motion or electrophoresis. It results in a Doppler phase/frequency shift of the light signal scattered from the moving proteins, which can be quantitatively evaluated with a carefully designed electrophoretic light scattering experiment. From the measured phase/frequency shift, the charge status of proteins can be extracted.

Lysozyme (extracted from hen egg white) is a 14.3 kDa enzyme protein, with a hydrodynamic radius of ~ 2 nm. It is used in the present study as an example to demonstrate the value of light scattering techniques in determining the protein hydrodynamic characteristics: size and charge status. The practical measurement precision and efficiency of Brookhaven's dynamic light scattering (DLS) and phase analysis light scattering

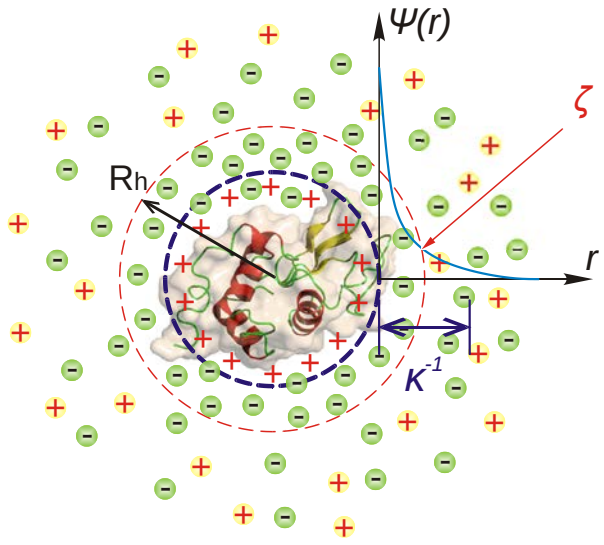

Figure 1. Lysozyme in a buffer solution (schematic): macro-ion, Debye-Hückel cloud and electrostatic double-layer (EDL).
R_h: hydrodynamic radius;
κ^{-1}: Debye length;
$\Psi(r)$: electrostatic potential;
ζ: zeta potential.

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(PALS) measurement capabilities are evaluated. The effect of buffer solution in terms of its pH value and ionic strength is examined.

Methods and Terminology

Rooted in proteins primary structure, a protein solution represents a rather complex electrolyte system. For example, in a buffer solution, lysozyme molecules are surrounded with ionic "cloud" [3] (Debye-Hückel cloud, see schematic in Figure 1). They form the so-called electrostatic double layers (EDL) around a protein molecule (macro-ion): Stern layer (adjacent to protein surface) and an (outer) diffuse layer. The loosely defined border between them is called shear plane (red dash-line in Figure 1). The molecules (mostly counter-ions) within Stern layer are tightly associated and moving with lysozyme molecules. In the diffused layer, counter-/co-ion composition and, subsequently, the electrostatic potential $\Psi(r)$ gradually approach electrical neutrality when moving away from the protein surface. The thickness of the EDL is usually characterized with the so-called Debye length (κ^{-1}), a parameter mostly determined by solvent/buffer conditions. There are two important implications stemmed from this well-established EDL model. First, the protein hydrodynamic properties, like size and charge measured with dynamic light scattering technique, should be interpreted as the physical values at the shear plane (see hydrodynamic radius R_h and zeta potential ζ in Figure 1). Secondly, the solvent/buffer conditions determine the dimension and composition of EDL, which in turn affect the hydrodynamic size and charge status of proteins. The effects is particularly pronounced in the cases of small proteins in low and medium salt concentrations, where value of $\kappa \cdot R_h$ is usually near unity. Therefore, accurate measurements of these protein hydrodynamic properties should be able to detect the changes of protein size and charge status due to the solvent/buffer condition variations.

In order to demonstrate this, dynamic light scattering and electrophoretic light scattering experiments are conducted on hen egg white lysozyme (HEWL) obtained commercially. Two dilute lysozyme solutions were prepared from lyophilized lysozyme (Sigma-Aldrich L6876, Lot # LSBG8654V), with lysozyme concentrations of 5 – 10 mg/mL. Sodium acetate (NaOAc) buffered acetic acid (AcOH) buffer solution was used as solvent. The buffer compositions were slightly varied for the two lysozyme solutions. The first sample (Sample-A) was dissolved in a buffer with 15 mM AcOH and 9.5 mM NaOAc, pH = 4.35. Another sample (Sample-B) was prepared in a buffer with 25 mM NaOAc/AcOH (molar-ratio 1:1), pH = 4.60. Lyophilized lysozyme powder was used as received without additional purification and directly dissolved in the buffer solutions. But, in order to ensure the electrostatic equilibrium of proteins in buffer solutions, both samples were dialyzed in their respective buffer for extended period of time before the light scattering measurements.

All light scattering experiments were performed at 25 °C on a NanoBrook Omni from Brookhaven Instruments Corporation. DLS tests were conducted at detection angle of 90° (standard) and 173° (backscatter) to measure protein hydrodynamic radius, while protein charge was examined in phase analysis light scattering (PALS) mode at detection angle of 15° (forward).

Results and Discussion

The microscopic dynamics of proteins are probed in a light scattering experiment. The scattered light autocorrelation decay in DLS is caused by the diffusive mean-displacement of proteins, typically less than a micrometer over a few microseconds. Similarly, the protein electrophoretic velocity of ~ 1 μm/s is usually measured in ELS tests. In order to accurately assess these microscopic physical quantities, sophisticated signal detection, processing and analysis methods have implemented in an advanced light scattering instrument like Brookhaven's NanoBrook series. From application standing-point, the validation of test results should be ensured with good quality of the raw measurement data, which is often overlooked and/or obscured in practice. It is strongly recommended as a good practice for a serious study to examine the raw data quality before delving into the interpretation of test results.

The light scattering measurement raw data shown in Figure 1 are acquired from the lysozyme Sample-A. These are the results accumulated and averaged over 10 minutes of data collecting time. The measurement uncertainty of each data point is quantified with its statistical standard error and displayed as the error bars in the plots. The autocorrelation function of scattered light detected at 173° (backscatter), $C(\tau)=<I(0)\bullet I(\tau)>$, is shown in Figure 2a. The Brownian motion of proteins causes the autocorrelation to decay exponentially as the delay time (τ) increases. The diffusion coefficients of proteins and, in turn, its hydrodynamic radius Rh, are calculated from the decay rate extracted from the decay curve. The small uncertainty (< 1%) at short delay time indicates the good reliability of the measurement results. On the other hand, small noise was detected at longer decay time (baseline noise). It suggests trace amount of impurity remaining in the sample solution, likely from dusts and debris due to less than perfect sample preparation. But, the long delay-time noise can be readily removed with our advanced baseline correction algorithm without significantly affecting the final results. Using cumulant analysis, the hydrodynamic radius of lysozyme in Sample-A is calculated as 2.31±0.04 nm.

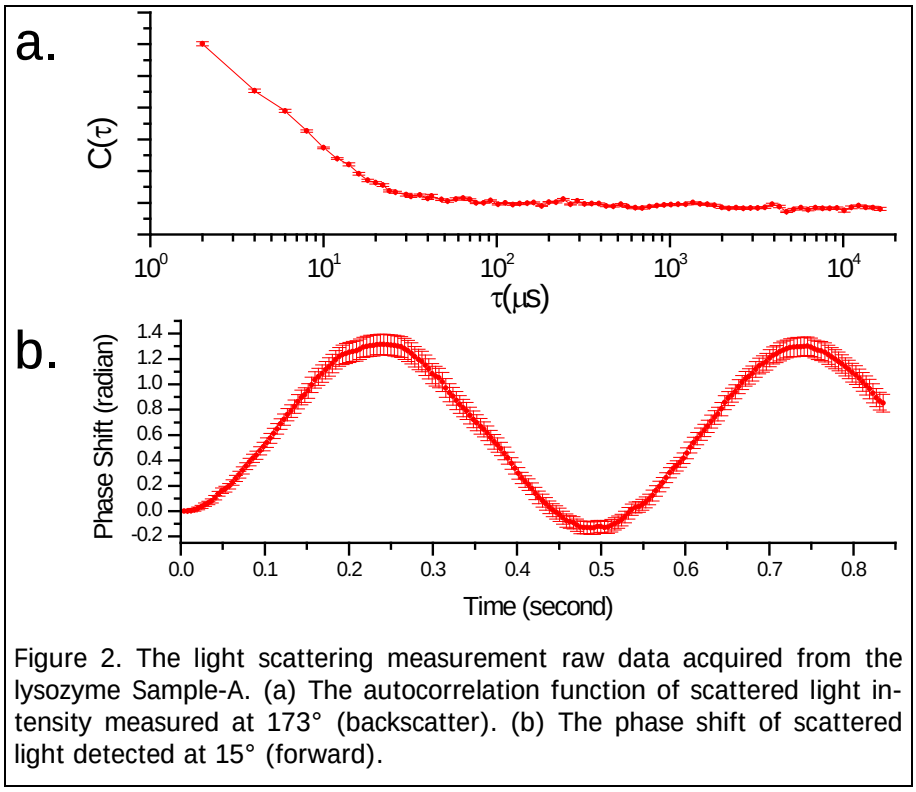

Figure 2. The light scattering measurement raw data acquired from the lysozyme Sample-A. (a) The autocorrelation function of scattered light intensity measured at 173° (backscatter). (b) The phase shift of scattered light detected at 15° (forward).

Due to the delicate nature of proteins, it is undesirable to subject them to relatively high electric voltage. In our electrophoretic light scatter measurements, a sinusoidal AC voltage with amplitude of merely 4 volts is applied between a pair of parallel palladium electrodes (~ 6 mm apart) to induce the electrophoretic motion of proteins. A lysozyme molecule is expected to move at a velocity of less than 1 μm/s under such electric field strength applied, since its net charge is only at order of the fundamental electric charge (1.6×10^{-19} Coulomb). Therefore, phase analysis technique was chosen to take advantage of its ~ 1,000 times higher sensitivity than the conventional frequency analysis of scattered light power spectrum. Additionally, in a Brookhaven's NanoBrook Omni, the phase shift data collection is synchronized with the application of electric field. This allows a valuable feature of averaging phase shift data over multiple electric field cycles, which effectively cancels out the contributions from random protein motions of non-electrophoretic origins and significantly enhances signal-to-noise ratio of phase shift measurements. The raw PALS data of Sample-A is shown in Figure 2b. A sinusoidal trace of phase shift from the scattered light signal clearly demonstrates the effectiveness of our data detection/processing method and validates the quality of the measurement. A close inspection of the PALS data reveals that, despite all the advanced weak-signal detection/processing techniques employed, there are still noticeable uncertainty of individual phase shift data points (~ 10% in term of its standard error) and an overall linear drift remaining (careful comparison between two consecutive peaks or valley points in Figure 2b). These are largely removed with a nonlinear fitting algorithm embedded in our PALS analysis software: $\sin(\omega_e \cdot t) + V_c \cdot t$, where ω_e and V_c are the electric field frequency and the field-independent collective velocity of proteins, respectively. The pure sinusoidal electrophoretic motion of lysozyme is then effectively recovered with much improved accuracy and precision. The steady-state electrophoretic mobility of lysozyme in Sample-A was measured as 0.36±0.02 ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$).

From the measurement raw data described above, the hydrodynamic radius (R_h) of lysozyme molecules is calculated using Stokes-Einstein equation of translational friction force. For lysozyme charge status, based on the balance be-

tween the electric driving force and the translational friction drag, an effective valence value (Z*) is calculated from its steady-state electrophoretic mobility μ_e. The effect of additional drag due to counter-ions moving in opposite direction (the so-called electrophoretic effect) is then corrected with Debye-Hückel-Henry theory [3, 5]. This leads to the so-called Debye-Hückel-Henry valence (Z_{DHH}) that better represents the actual charge status of proteins. The measurement results of the protein size and charge are summarized in 2 charts (Figure 3) for the two samples of lysozyme solutions, along with error-bars to quantify their uncertainties.

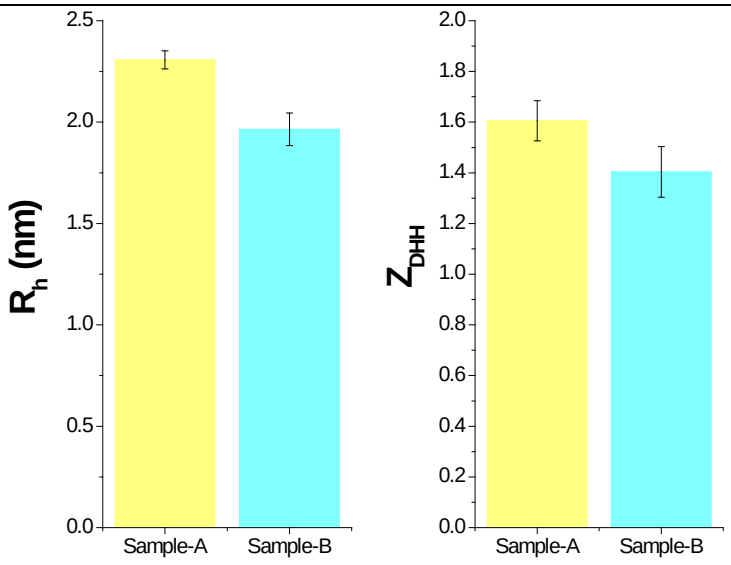

Figure 3. Comparison of the measured size and charge of lysozyme in NaOAc/AcOH buffer solutions with composition as follows.

- Sample-A: 15 mM AcOH and 9.5 mM NaOAc, pH = 4.35
- Sample-B: 25 mM AcOH and 25 mM NaAc, pH = 4.60

The sides-by-side comparison clearly shows the difference between the two samples. At slightly higher molarities of sodium acetate and acetic acid, the lysozyme molecules in Sample-B show a smaller hydrodynamic radius and lower electrostatic charge. The effect is discernable within the certainty of measurement and analysis. It is also meaningful in the context of the well-established EDL theory discussed earlier. Based on their buffer compositions, the Debye length (κ^{-1}) are estimated as 1.36 nm for Sample-A and 0.96 nm for Sample-B, respectively. This suggests that, due to a relatively higher ionic strength, the counter-ion cloud in the diffuse-layer around a lysozyme molecule is more compact in Sample-B than in Sample-A. Consequently, the shear plane of charged lysozyme molecules is expected to be compressed toward lysozyme surface (see the schematic in Figure 1). This results in a decrease in its hydrodynamic radius. At the same time, a more compact counter-ion cloud also increases the effective electrostatic screening of charged lysozyme molecule, which will decrease its effective charge measured from its electrophoretic motion.

Conclusions

It is entirely practical to routinely characterize protein hydrodynamics on a commercial light scattering instrument like Brookhaven's NanoBrook Omni. The size and charge status of proteins can be efficiently extracted from these characteristics by using the well-established theories such as Stokes-Einstein's translational friction and Debye-Hückel-Henry electrophoresis theory. An array of technological innovations on light scattering signal detection, processing and analysis are embedded in a NanoBrook Omni. A superb DLS and ELS measurement performance in terms of sensitivity and precision is warranted with an effective utilization of these technical features. A noticeable benefit is the feasibility of measuring protein size and charge on a single sample of small volume, using only one apparatus.

These have been validated with diluted lysozyme solutions, a challenging case of weak light scatter with small size and low electrostatic charge. It is expected to open up numerous application opportunities. Both academic research and industrial R&D will greatly benefit from this effective and efficient measurement capability.

Acknowledgement

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