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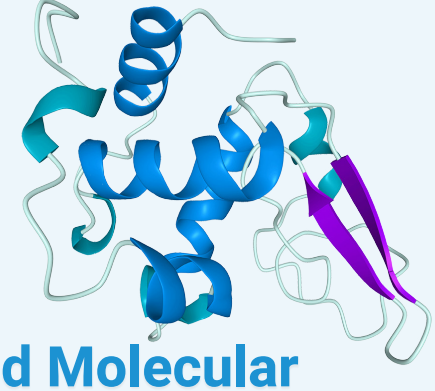
APPLICATION NOTE



Investigating Size, Zeta Potential, and Molecular Weight and Evaluating Stability of BSA Solution

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Introduction

Proteins composed of amino acids are essential macromolecules that play vital roles in all kinds of activities in living organisms. Specifically, amino acids are held together by peptide bonds to form polypeptides that are further folded into particular tertiary conformations. The conformation of protein will change when exposed to different environments, changing the properties of proteins. For example, when dispersed in an aqueous environment, protein's structure and biological activity depend on solvent composition, ions concentration and also temperature. Proteins are studied and applied in various fields such as pharmaceuticals, biological sciences, and food and beverage, etc.

Bovine serum albumin (BSA) is a frequently used protein, which is purified from cows' blood. Because of its abundance source, low price, stable and sphere-like structure, BSA is widely used in protein research and industries. In this application note, the BeNano 90 Zeta (Bettersize Instruments Ltd.) was used to characterize the size, molecular weight, and zeta potential of BSA aqueous solutions in order to investigate the dependence of protein structure and molecular interaction on the dispersant environment.

Instrumentation

Employed with the dynamic light scattering (DLS), static light scattering (SLS), and electrophoretic light scattering (ELS) technologies, the BeNano 90 Zeta was used for the size, molecular weight, and zeta potential measurements.

A solid-state laser beam with a wavelength of 671 nm and a power of 50 mW was used to illuminate the sample. An avalanche photodiode (APD) detector coupled with fiber is used to collect scattered light signals from 12° for zeta potential measurement and 90° for size and molecular weight measurements, respectively.

Theory

Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a sizing technology measuring the diffusion behavior of the particles dispersed in liquids. When nanoparticles are suspended in the liquid medium, the continuous random movement of the nanoparticles are defined as the Brownian motions, whose speed related to the sizes of particles. When a laser beam illuminates the nanoparticles in suspensions, the intensity of scattered light fluctuates due to the Brownian motion of particles. The intensity of the scattered light is then detected by APD and converted to a correlation function using the correlator. By analyzing the correlation function, the diffusion coefficient (D) that describes the speed of Brownian motion is thereby calculated. The hydrodynamic diameter (D_H) is obtained using the Stokes-Einstein equation:

$$D = \frac{k_B T}{3\pi\eta D_H}$$

where k_B is the Boltzmann's constant, T is the temperature, and η is the viscosity of the solvent. Ideally, the diffusion

coefficient (D) in the Stokes-Einstein equation is specified for self-diffusion in a dilute concentration range, which means there is no particle-particle interaction in the suspension. Based on this, the diffusion coefficient could be denoted as D_0. However, as the concentration of the suspension system increases, the particle-particle interaction becomes stronger and therefore could no longer be neglected. For high-concentration samples, particles' diffusion motions also depend on concentrations and can be expressed as:

$$D_{app} = D_0(1 + k_D c)$$

where D_{app} is the apparent diffusion coefficient obtained from DLS, c is particle concentration, and k_D is the interaction parameter, which is related to the second virial coefficient as:

$$k_D = 2B_{22}M_w - k_f - 2v$$

where B_{22} is the second osmotic virial coefficient, M_w is the molecular weight of the sample, k_f is the first term in the expansion of friction coefficient, and v is the specific volume. The interaction parameter describes the dependence of the apparent diffusion coefficient on concentration. It is also often used to characterize the stability of suspensions. Typically, a system with a larger k_D means stronger repulsive forces among particles, which leads to a more stable state that is less likely to form aggregates.

Electrophoretic Light Scattering (ELS)

In an ELS experiment, a laser beam irradiates the sample, where the scattered light is detected at a forward angle of 12°. The sample solution or suspension is subjected to an electric field applied to both ends of the sample cell, resulting in the electrophoretic movement of the charged particles. As a consequence, the scattered light experiences a frequency shift compared to the incident light due to the Doppler effect. The scattered light signals with a frequency shift are converted to phase shift via PALS analysis. By the phase plot, the velocity of electrophoretic movement per unit electric field, which is denoted as the electrophoretic mobility μ, is obtained. Through Henry's equation, one can relate the electrophoretic mobility μ and its zeta potential ζ as follow:

$$\mu = \frac{2\varepsilon_r \varepsilon_0 \zeta}{3\eta} f(K\alpha)$$

where ε_0 is the solvent dielectric constant in vacuum, ε_r is the relative dielectric constant, η is the solvent viscosity, $f(K_\alpha)$ is the Henry function, K is the reciprocal Debye length, α is the particle radius, and K_α refers to the ratio between the thickness of the double layer and the particle radius. Zeta potential is a key indicator of the stability of a particle suspension system. With a high zeta potential, the repulsive force between particles is strong and the system tends to be stable.

Static Light Scattering (SLS)

In an SLS experiment, the scattering intensity of macromolecules in the solution is detected and analyzed to obtain their molecular weights. Typical samples include polymers and proteins. When a vertically polarized laser beam irradiates onto the sample, the macromolecules scatter light in all directions, and according to the Rayleigh equation, the molecular weight and the scattering intensity at a certain angle is related by:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w} + 2A_2 c$$

Where c is the sample concentration, θ is the detection angle, R_θ is the Rayleigh ratio that is used to characterize the intensity ratio between the scattered light and the incident light at angle θ, M_w is sample's weight-average molecular weight, and A_2 is the second light scattering virial coefficient. K is constant and can be expressed as:

$$K = \frac{4\pi^2}{\lambda_o^4 N_A} \cdot \left(n_o \frac{dn}{dc}\right)^2$$

where λ_0, N_A, n_0 and dn/dc are the wavelength of the incident laser in vacuum, Avogadro's number, the refractive index of the solvent, and the refractive index increment of the suspension with respect to concentration under constant temperature, respectively.

In the BeNano 90 Zeta, the scattering intensity is detected at 90°. The Debye plot is then constructed by plotting and linearly fitting the K_c/R_θ values versus the concentration profiles of the samples. The slope of the linear regression equation is used to calculate the second virial coefficient A_2, while the intercept yields the reciprocal of molecular weight.

Experimental

Sample Preparation

To investigate the relationship between the solution environments and BSA structure, the dispersant and the concentration were the two variables set up in this experiment. Specifically, BSA solutions with concentrations of 0.5, 1.0, 2, 3.5, and 5 mg/mL were prepared using 20 mM NaCl, 100 mM NaCl and 10 mM PBS buffer with pH of 7 as dispersant, respectively. All solutions were thoroughly mixed using a magnetic stirrer and filtered with 0.22 μm polyethersulfone (PES) filters to remove dust or aggregates. All measurements were performed at 25 °C. Each sample was measured three times to check the repeatability of the results.

Particle Size Measurement

BSA solutions with different concentrations were filtered and transferred into polystyrene (PS) cells for particle size measurement.

Zeta Potential Measurement

Zeta potential measurement were performed by transferring around 0.75 mL of BSA soution in three dispersants into the folded capillary cell. The concentration of the samples is 5 mg/mL.

Molecular Weight Measurement

In the static light scattering experiment, the scattering intensities of samples at different concentrations were detected. A toluene standard with a known Rayleigh ratio and the refractive index was prepared and used for reference. The BeNano 90 Zeta measured the dark count rate, scattering intensity of standard toluene sample, scattering intensities of the dispersant, and scattering intensities of samples at different concentrations. Subtracting the dark count rate and scattering intensity of dispersant yields the absolute scattering intensities of the solutes, which can be further used to calculate the Rayleigh ratio of samples at different concentrations. Finally, the Debye plot is constructed by plotting the Rayleigh ratio versus samples concentrations.

Results and Discussion

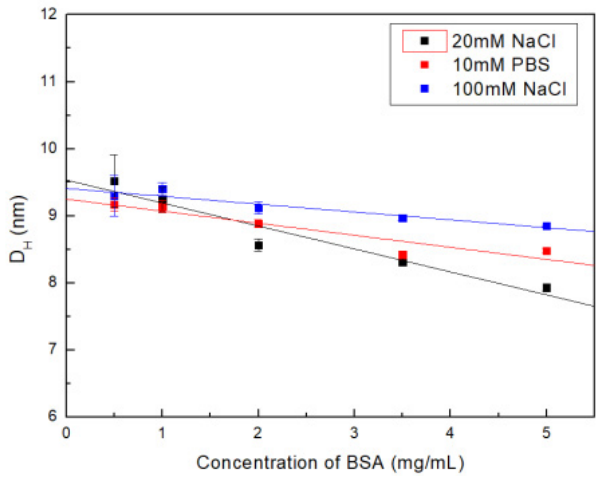

Figure 1. Dependence of BSA size in different dispersants on concentration

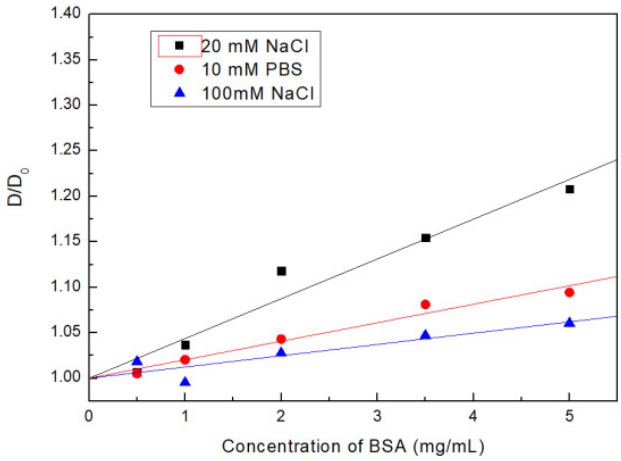

Figure 2. BSA diffusion coefficients versus concentration curves at different concentrations

As can be seen from Figure 1, the sizes of BSA in all three dispersants decreased as the concentrations increased. These solid lines are linear fitting for the data. From the slopes of the fitting curve, it suggests that the order of the size decrease is 20 mM NaCl > 10 mM PBS > 100 mM NaCl. There are two possible contributions to the change of the size with concentration.

The first one is sample concentration. Due to the presence of the functional groups in proteins, the surfaces of BSA were charged when dispersed in the liquid medium. As BSA concentrations increased, the average distance among the BSA particles decreased, and the electrostatic repulsion increased. Consequently, the Brownian motions speeded up and the diffusion coefficient D increased, as shown in Figure 2. According to the Stokes-Einstein equation:

$$D = \frac{k_B T}{3\pi\eta D_H}$$

the hydrodynamic diameter D_H decreased as the diffusion coefficient D increased, which explains the trends that the particle sizes of BSA decreased as concentration increased.

The second one is the ionic strength of dispersants. The ions in dispersants may shield the potential on the particle, and therefore decrease the electrostatic repulsion. Moreover, the shielding effects got stronger as the ionic strength increased. Therefore, in dispersants that had higher ionic strength, or in other words, dissociated more ions (i.e., 100 mM NaCl), the sample concentration effected the particle sizes less. This explains the trends that the particle sizes decreased less dramatically in the 100 mM NaCl dispersant.

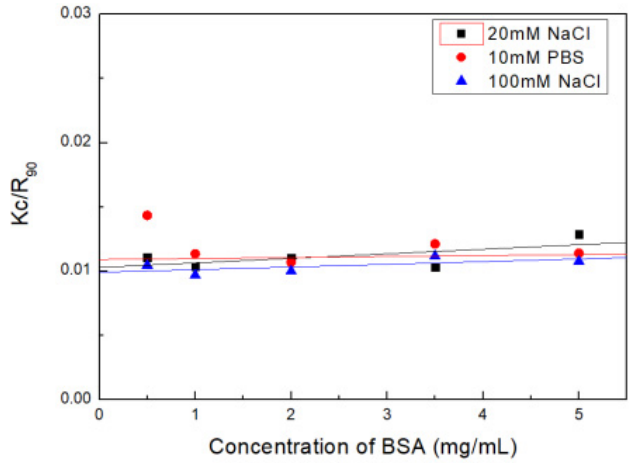

Figure 3. Debye plot of BSA solutions in different dispersants

Figure 3 shows the Debye plot of BSA constructed by plotting and linearly fitting the Kc/R_{90} values versus the concentration profiles of the BSA. The molecular weight M_w can be provided by the intercept of the linear fit of the plot. And the second virial coefficient A_2 relates to the slope of the linear regression equation. M_w is listed in Table 1, and A_2 is in Table 3.

Table 1. Molecular weight M_w of BSA in different dispersants

Dispersant	M_w (kDa)
20 mM NaCl	96.15
10 mM PBS	91.74
100 mM NaCl	101.01

As can be seen in Table 1, M_w in three dispersants ranges from 91.74 kDa to 101.01 kDa. According to our knowledge, the BSA monomer is 66.5 kDa. When BSA is dissolved in solutions, a few monomers will form oligomers and aggregates. For instance, a BSA dimer consists of two monomers and thereby has the M_w of 132 kDa, and a trimer has the M_w of around 200 kDa. As a result, the average molecular weight of BSA measured is higher than that of monomer due to the presence of oligomers and aggregates.

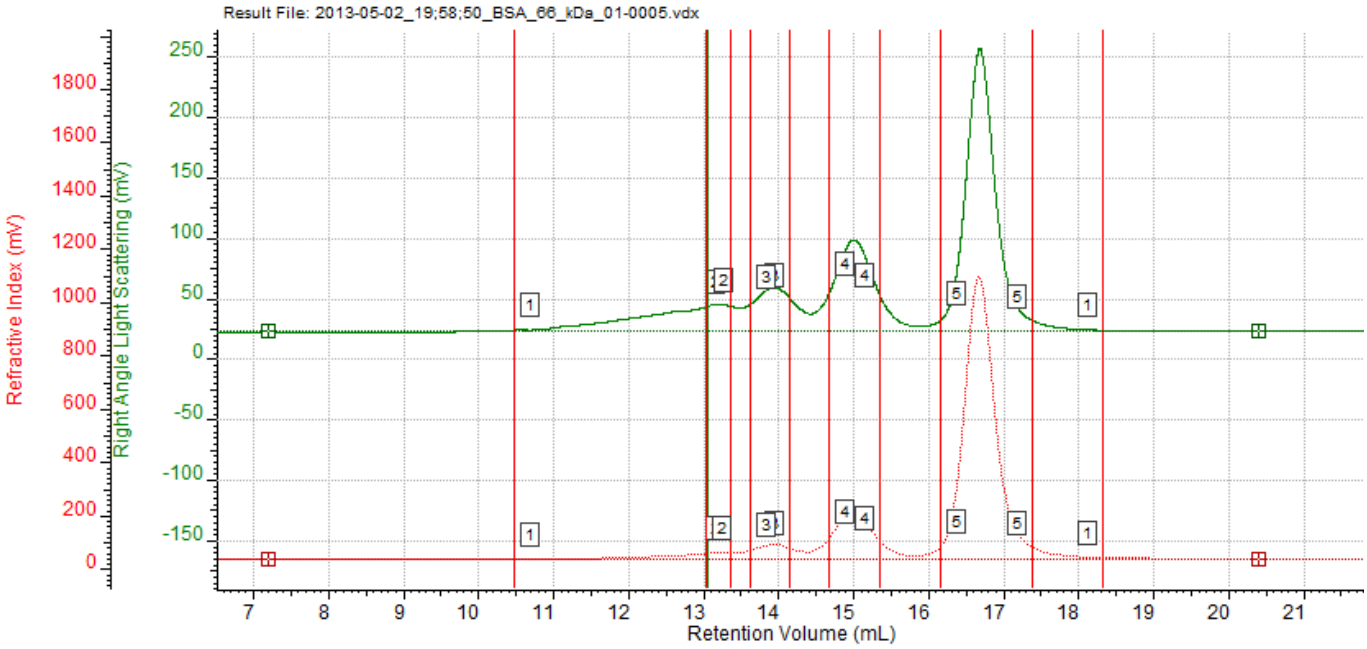

Figure 4. Gel permeation chromatography (GPC) chromatogram of BSA dispersed in PBS buffer

Table 2. Molecular weight values corresponding to different integration regions of GPC

Peak	1	2	3	4	5
M_w (kDa)	97.17	255.51	196.43	131.06	66.78

Table 3. Interaction parameters k_D, zeta potential, and second virial coefficient A_2 of BSA solutions in different dispersants

Dispersant	k_D (mL/mg)	Zeta Potential (mV)	A_2 (mL*mol/g^2)
20 mM NaCl	0.04373	-12.45	$1.5 * 10^{-7}$
10 mM PBS	0.02035	-9.23	$4.0 * 10^{-8}$
100 mM NaCl	0.01241	-8.45	$1.0 * 10^{-7}$

Figure 4 displays the chromatogram of BSA, using PBS buffer as the mobile phase, provided by a gel permeation chromatography (GPC) system which is equipped with a light scattering detector and a differential refractive index detector. There are multiple elution constituents in the chromatogram. Integration region 2-5 corresponds to each individual peak 2-5 respectively. From Table 2 where the Mw obtained by integration are listed, we can tell that peak 5 is a monomer peak because it has the M_w of 66.78 kDa. Likewise, peak 4 is a dimer peak with the M_w of 132.06 kDa, peak 3 is a trimer peak with the M_w of 196.43 kDa, and peak 2 is a tetramer peak with the M_w of 255.51 kDa. With integration region 1 being from peak start to peak end, the average M_w of whole BSA by GPC is measured to be 97.17 kDa, which is in excellent agreement with the average M_w obtained by the BeNano 90 Zeta.

Protein stability has always been a significant concern for users. With DLS, ELS, and SLS technologies, the protein stability can be speculated by measuring either interaction parameter k_D, or zeta potential, or second virial coefficient A_2 correspondingly. The larger the values of k_D, A_2, and the absolute value of zeta potential, the stronger the interaction force and consequently the more stable the protein.

With respect to the values of k_D and zeta potential in Table 1, the BSA stability in three dispersants is in the order: 20 mM NaCl > 10 mM PBS > 100 mM NaCl. Depending solely upon A_2, the speculated stability is, though, in the order: 20 mM NaCl> 100 mM NaCl > 10 mM PBS. Given the complexity of SLS measurement as well as its relatively high deviation, A_2 is less reliable than k_D and zeta potential when it comes to indicating protein stability. Therefore, a reasonable conclusion according to the above results suggests that the BSA stability in the three dispersants is in such an order: 20 mM NaCl > 10 mM PBS > 100 mM NaCl.

To sum up, the BSA dissolved in 20 mM NaCl has the best stability across three samples. A plausible explanation is that being dissolved in an environment of relatively low salt concentration, proteins have higher potentials and stronger static repulsion, and, eventually, better stability.

Conclusions

Three light scattering technologies, i.e., DLS, ELS, and SLS, are incorporated in the BeNano 90 Zeta to enable the measurements of size, zeta potential, and molecular weight, respectively. In this application note, the sizes of BSA in three dispersants are measured, showing the size trend when using different types of dispersants. Then, the molecular weight M_w of BSA is obtained by the BeNano 90 Zeta and shows excellent agreement with the M_w provided by the GPC system. Finally, by utilizing k_D, zeta potential, and A_2, the stabilities of BSA protein in different dispersants were successfully evaluated and sorted.

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