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# DYNAMIC LIGHT SCATTERING: A USEFUL OPTICAL METHOD TO PROBE COMMON-ION EFFECTS IN PROTEIN-SALT AQUEOUS SOLUTIONS

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We report measurements of protein diffusion coefficients for lysozyme aqueous solutions using dynamic light scattering (DLS). DLS measurements were performed on the buffer-free lysozyme-NaCl-water and lysozyme-$Na_2SO_4$-water ternary systems at pH 4.5 and 25 ºC. The dependence of lysozyme diffusion coefficients as a function of salt concentration is analyzed. We find that the behavior of the protein diffusion coefficient in the presence of $Na_2SO_4$ is significantly different from that in the presence of NaCl. Our DLS measurements show that the common-ion effect plays an important role in the case of lysozyme-NaCl solutions but not in the case of lysozyme-$Na_2SO_4$ solutions. Therefore DLS is a useful optical method that can be used to probe the presence of common-ion effects in protein-salt aqueous solutions.

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## 1. Introduction

Light-scattering techniques are versatile optical methods for characterizing physicochemical properties of macromolecular solutions [1,2]. For this reason, they are among the most important tools for studying the properties of protein aqueous solutions. One important application of light scattering is the determination of the second virial coefficient, $B$, of protein solutions [1]. This quantity is directly related to the solvent-mediated protein-protein interaction energy. If protein-protein interactions are attractive, the value of $B$ is negative. Since the presence of protein-protein attraction is a necessary condition for protein crystallization, the second virial coefficient is a powerful tool for finding crystallization conditions by changing the concentration of additives in solution [1].

Salt additives are often employed for protein crystallization. It has been found that the value of $B$ decreases as the salt concentration increases. This is consistent with a corresponding increase of protein-protein attraction, which ultimately leads to protein precipitation [3]. One limitation of the second virial coefficient is that the obtained values of $B$ lack a precision. Consequently, the dependence of $B$ on salt concentration is not well characterized. This is required for a better understanding of the effect of salt on protein solutions.

Dynamic Light Scattering (DLS), also known as photon correlation spectroscopy, is a light scattering technique that probes the temporal fluctuations of the light scattered by a sample [2,4]. This dynamic information can be used to obtain the protein diffusion coefficient, $D_{DLS}$. An important advantage of $D_{DLS}$ compared to $B$ is that its precision is relatively high. We observe that the typical

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error of $B$ values is about 10%, whereas the typical error of $D_{DLS}$ is about 1% or better. Thus the dependence of $D_{DLS}$ on salt concentration is well defined. DLS, compared to other techniques used for diffusion measurements [5], has the practical advantages of requiring short experimental times and small protein samples [2].

In this paper, we first briefly describe the DLS optical method. We then report the values of $D_{DLS}$ obtained for buffer-free lysozyme aqueous solutions at pH 4.5 and 25 $^{o}$C in the presence of NaCl and $Na_2SO_4$. DLS measurements were performed at constant lysozyme concentration, $C_1$ =0.6 mM and at several salt concentrations, $C_2$. The comparison between the behavior of lysozyme diffusion coefficient, $D_{DLS}(C_2)$, in the presence of two different salts, allow us to understand how the effect of the salt on $D_{DLS}$ depends on the nature of the anion. Our results may provide guidance on the understanding of the effect of salt on protein precipitation.

## 2. DLS theory

A DLS apparatus measures the temporal fluctuations in light intensity. In our DLS setup shown in Fig. 1, light coming from a laser is scattered by a sample and is collected at a given angle, $\theta$ (usually 90º) by an avalanche photodiode detector [2]. The scattering angle defines the direction of the scattering vector:

$$q = (4\pi n/\lambda_0)\sin(\theta/2), \quad (1)$$

where $\lambda_0$ is the wavelength of light in vacuum and $n$ is the refractive index of the sample. The most important aspect of the experiment is the determination of the normalized autocorrelation function:

$$g^{(1)}(\tau) = \langle E(t)E^*(t+\tau)\rangle / \langle E(t)E^*(t)\rangle \quad (2)$$

associated with random temporal fluctuations of the scattered electrical field, $E(t)$. In the usually employed homodyne mode, this is obtained by measuring the temporal fluctuations of light intensity $I(t)$ at the scattering angle. These fluctuations are then analyzed by a correlator, which gives the intensity autocorrelation function:

$$G^{(2)}(\tau) = \langle I(t)I(t+\tau)\rangle. \quad (3)$$

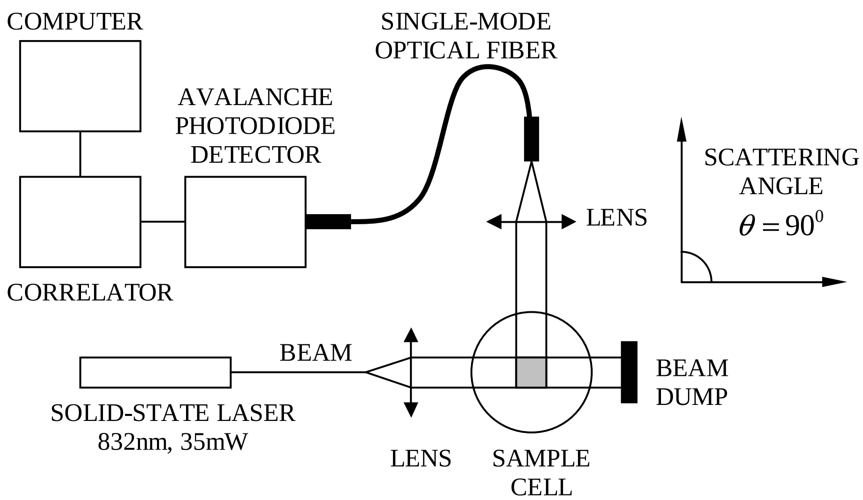


Fig. 1. Scheme of the DLS apparatus used for measurements of lysozyme diffusion coefficients.

The electric-field autocorrelation function, $g^{(1)}(\tau)$, is then obtained from $G^{(2)}(\tau)$ by employing the Siegert relation:

$$G^{(2)}(t) = \langle I \rangle^2 \, (1+\gamma \,|\, g^{(1)}(t)\,|^2), \tag{4}$$

where $\langle I \rangle$ is the average scattered intensity and $\gamma$ is the coherence coefficient ($\gamma$<1) [2,4].

In the limit that particles are small compared to the length of the inverse scattering vector $q^{-1}$, hydrodynamics and thermodynamic fluctuation theory can be used to relate $g^{(1)}(\tau)$ to the particle diffusion coefficient [4,6]. Since the fluctuations of the scattered electric field are related to concentration fluctuations, they will be related to the mobility of the particles, i.e. to their diffusivity. For a binary system, Fick's first law defines the diffusion coefficient, $D_1$ of the solute component "1":

$$-J_1 = D_1 \nabla C_1 \tag{5}$$

where $J_1$ is the flux of the solute due to its concentration gradients $\nabla C_1$. The normalized field correlation function is given by

$$g^{(1)}(\tau) = \exp(-q^2 D_{DLS}\, \tau) \tag{6}$$

where $D_{DLS}$ is the mutual diffusion coefficient obtained by DLS [4].

For a ternary system (with two solutes), Eq. 5 is replaced by the extended Fick's first law:

$$\begin{aligned} -J_1 &= D_{11}\nabla C_1 + D_{12}\nabla C_2 \\ -J_2 &= D_{21}\nabla C_1 + D_{22}\nabla C_2 \end{aligned} \tag{7}$$

where $J_1$ and $J_2$ are the fluxes of the two solutes due to their concentration gradients $\nabla C_1$ and $\nabla C_2$ [7,8]. The maindiffusion coefficients $D_{11}$ and $D_{22}$ characterize the flux of the solutes due to their own concentration gradients, while the cross-diffusion coefficients $D_{12}$ and $D_{21}$ describe the coupling between solute fluxes in solution. Interestingly, the normalized field correlation function for a ternary system is given by the following relation [9]:

$$g^{(1)}(\tau) = f_+ \exp(-q^2 L_+ \tau) + f_- \exp(-q^2 L_- \tau) \tag{8}$$

where $L_+$ and $L_-$ are the two eigenvalues of the diffusion coefficient matrix:

$$L_+ = \left[ \frac{1}{2} \left( \frac{D_{22} + D_{11} + \sqrt{\theta}}{D_{11}D_{22} - D_{12}D_{21}} \right) \right]^{-1} \tag{9a}$$

$$L_- = \left[ \frac{1}{2} \left( \frac{D_{22} + D_{11} - \sqrt{\theta}}{D_{11}D_{22} - D_{12}D_{21}} \right) \right]^{-1} \tag{9b}$$

where: $\theta = (D_{22} - D_{11})^2 + 4D_{12}D_{21}$. The pre-exponential coefficients: $f_+$ and $f_-$ represent, approximately, the normalized contributions of the two solutes to the scattered intensity. If solute "1" is a protein (high molecular weight solute) and solute "2" is an inorganic salt (low molecular weight solute), then $f_+ >> f_-$ and we can write [10, 11]:

$$g^{(1)}(\tau) = \exp(-q^2 L_+ \tau) \tag{10}$$

If cross-diffusion coefficients are not very large:

$$L_{+} \approx D_{11} \tag{11}$$

For protein-salt aqueous ternary solutions, DLS results are analyzed according to Eq. 6. According to Eq. 10 and Eq. 11, this implies that $D_{DLS} = L_{+} \approx D_{11}$.

## 3. Experimental section

### 3.1 Materials

All the materials, solution preparation procedures, apparatus and density measurement procedures are described in details in [8]. In brief, we used hen egg-white lysozyme purchased from Seikagaku America without further purification. This supplier provides lysozyme at the highest purity. Analytical reagents: NaCl and $Na_2SO_4$ were purchased from Mallinckrodt and used without further purification. The molar mass of 14307 g/mol was used for lysozyme; the molar masses of 58.44 g/mol and 142.04 g/mol were respectively used for NaCl and $Na_2SO_4$.

### 3.2 Preparation of solutions

All weight measurements were performed with a Mettler Toledo AT400 electrobalance. Measurements of density were used to determine molar concentrations. In the case of lysozyme-NaCl-water solutions, precise masses of dried NaCl were added to flasks containing previously weighed masses of lysozyme stock solutions. In the case of lysozyme-$Na_2SO_4$-water solutions, precise masses of $Na_2SO_4$ stock solutions were added to the flasks instead of dried $Na_2SO_4$. The pH was adjusted to 4.50 using small amounts of a HCl stock solution (pH~1) in the case of NaCl and small amounts of a $H_2SO_4$ stock solution (pH~1) in the case of $Na_2SO_4$. The solutions were then diluted to their final volumes. The final solution pH was re-measured to confirm its value of 4.50.

### 3.3 Measurements of pH

The pH measurements were made using a Corning model 130 pH meter with an Orion model 8102 combination ROSS pH electrode. The meter was calibrated with standard pH 7.00 and pH 4.00 buffers.

### 3.4. Density measurements

All density measurements were made with a Mettler-Paar DMA40 density meter. By time averaging the output, a precision of 0.00001 g/cm$^3$ or better could be achieved. The temperature of the vibrating tube in the density meter was controlled with water from a large well-regulated water bath whose temperature was $25.00 \pm 0.01$ ºC.

### 3.5 DLS apparatus

We measured the dynamic light scattering coefficients, $D_{DLS}$, using a Protein Solution DynaPro-801 TC Molecular Sizing Instrument. A miniature solid state LASER (25 mW power) with $\lambda$=832 nm was employed. A monomodal optical fiber was employed to collect the TEM (transverse electromagnetic mode) or true Gaussian light. A Peltier temperature control module was used for controlling the temperature at 25°C in the optics block. Lysozyme solutions were injected through a Whatman "Anotop 10" $0.02\,\mu$m filter. Spectrophotometric determination of the sample protein concentration before and after filtering does not indicate relevant concentration changes due to the adsorption of the macromolecules on the filter. The monomodal mode in the Protein Solution-Dynamics V4.0 software was used in the analysis. The experiments performed on ternary lysozyme-$Na_2SO_4$-water solutions are in excellent agreement with the assumed Eq. 4 and Eq. 6. The values of $q^2$ were calculated from refractive-index values available for the binary salt-water systems at 25 ºC.

The contribution of lysozyme to the refractive index is small at $C_1$=0.6 mM. The dependence of water refractive index on light wavelength was used to calculate the refractive index of the ternary solutions.

## 4. Results and discussion

In Table 1, we report the values of $D_{DLS}$ as a function of $C_2$ for both NaCl and $Na_2SO_4$ and at constant protein concentration: $C_1$=0.6 mM. In the same table, we include the values of $q^2$ used for the determination of $D_{DLS}$ from Eq. 6. From Fig. 2, one sees that $D_{DLS}$ significantly decreases as the salt concentration increases for both salt cases. The observed change for lysozyme in aqueous $Na_2SO_4$ is significantly larger than in aqueous $Na_2SO_4$.

Table 1.

| $C_2$ (NaCl) (M) | $q^2$ (NaCl) ($10^{10}$ cm$^{-2}$) | $D_{DLS}$ (NaCl) ($10^{-5}$ cm$^2$ s$^{-1}$) | $C_2$ ($Na_2SO_4$) (M) | $q^2$ ($Na_2SO_4$) ($10^{10}$ cm$^{-2}$) | $D_{DLS}$ ($Na_2SO_4$) ($10^{-5}$ cm$^2$ s$^{-1}$) |
|---|---|---|---|---|---|
| 0.25 | 2.020 | 0.1273 | 0.10 | 2.020 | 0.1202 |
| 0.50 | 2.027 | 0.1203 | 0.25 | 2.029 | 0.1119 |
| 0.65 | 2.031 | 0.1170 | 0.50 | 2.044 | 0.1002 |
| 0.90 | 2.039 | 0.1122 | 0.65 | 2.053 | 0.0926 |
| | | | 0.80 | 2.061 | 0.0851 |

In order to analyze our results, we need to consider the effect of solution viscosity on lysozyme mobility, $D_p$. The effect of viscosity is taken into account by the Stokes-Einstein equation [12]:

$$D_{\mathrm{p}} = \frac{k_B T}{6\pi\eta R_{\mathrm{h}}^{\mathrm{e}}}, \tag{12}$$

where $k_B$ is the Boltzmann constant, $T$ the absolute temperature and $\eta$ is the viscosity of the fluid surrounding the protein. In our case, this fluid is the binary salt-water solution. According to Eq. 12, we can remove the effect of viscosity on protein mobility if we multiply the $D_{DLS}$ values by the relative viscosity coefficient, $\eta_r$, of the binary salt-water solutions. We have used the viscosity values from [13] for NaCl, and from [14] for $Na_2SO_4$. In Table 2, we report the values of $\eta_r$ and $D_{DLS}\eta_r$. From this table, we can see that the effect of $Na_2SO_4$ on solution viscosity is significantly larger than that of NaCl. This is qualitatively consistent with the observed difference between the two salts, shown in Fig. 2.

Table 2.

| $C_2$ (NaCl) (M) | $\eta_r$ (NaCl) | $D_{DLS}\,\eta_r$ (NaCl) ($10^{-5}$ cm$^2$ s$^{-1}$) | $C_2$ ($Na_2SO_4$) (M) | $\eta_r$ ($Na_2SO_4$) | $D_{DLS}\,\eta_r$ ($Na_2SO_4$) ($10^{-5}$ cm$^2$ s$^{-1}$) |
|---|---|---|---|---|---|
| 0.25 | 1.023 | 0.1303 | 0.10 | 1.047 | 0.1257 |
| 0.50 | 1.046 | 0.1259 | 0.25 | 1.115 | 0.1247 |
| 0.65 | 1.060 | 0.1240 | 0.50 | 1.233 | 0.1235 |
| 0.90 | 1.085 | 0.1218 | 0.65 | 1.310 | 0.1212 |
| | | | 0.80 | 1.397 | 0.1188 |

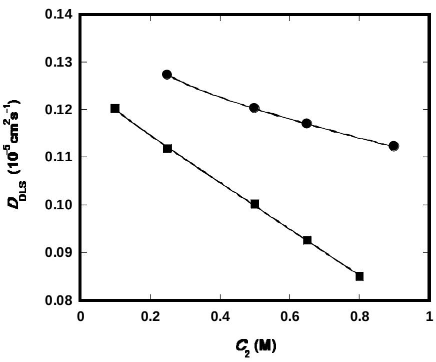


Fig. 2. Lysozyme diffusion coefficient, $D_{DLS}$, as a function of salt concentration (●, NaCl; ■, $Na_2SO_4$) at 25 ºC, pH 4.5 and constant protein concentration: 0.6 mM. The solid curves are smoothed curves through the points.

In Fig. 3, we plot $D_{DLS}\eta_r$ as a function of salt concentration $C_2$. We can see that $D_{DLS}\eta_r$ still decreases as the salt concentration increases, though the decrease of $D_{DLS}\eta_r$ is significantly smaller than that of $D_{DLS}$ for both salt cases. Interestingly, we find that the behavior of $D_{DLS}\eta_r$ $(C_2)$ for $Na_2SO_4$ is qualitatively different from that for NaCl. In the case of NaCl, the decrease of $D_{DLS}\eta_r$ is larger at low salt concentrations, whereas, for $Na_2SO_4$, it is larger at high salt concentration. This significant difference suggests that protein-protein interactions in the presence of $Na_2SO_4$ are significantly different from those in the presence of NaCl.

We remark that lysozyme is positively charged at pH 4.5. At this pH, the charge value obtained by titration is 11 [15]. However some counterions may bind to lysozyme, thereby reducing the charge value. It is expected that the binding properties of $Cl^-$ to be different from those of $SO_4^{2-}$. Precision ternary diffusion measurements have been used to determine the dependence of the protein chemical potential on salt concentration [16]. These thermodynamic data can be used to estimate lysozyme effective charge, $z$, in the presence of NaCl [16] and $Na_2SO_4$ [17] at pH 4.5. From these thermodynamic data, we obtain [16] $z \approx 9$ for NaCl and $z \approx 7$ for $Na_2SO_4$. This implies that there are respectively $\approx 2$ chloride anions and $\approx 2$ sulfate anions bound to lysozyme.

That lysozyme is positively charged implies that the common-ion effect may play an important role on both thermodynamic and diffusion properties of lysozyme [16]. Hence, the lysozyme neutral component consists of macro-cations: $P^{z+}$ and free anions (i.e. $Cl^-$ or $SO_4^{2-}$). These free anions, which also belong to the salt component, are responsible for the common-ion effect.

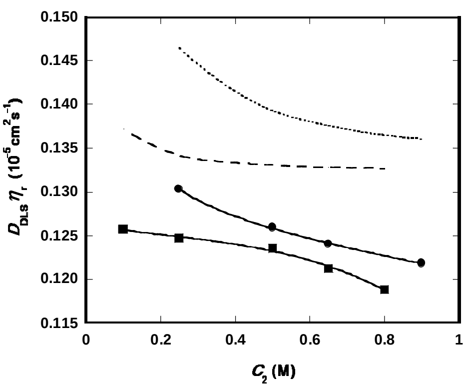


Fig. 3. Viscosity-corrected diffusion coefficients, $\eta_r D_{DLS}$, as a function of salt concentration (●, NaCl; ■, $Na_2SO_4$). The solid curves are smoothed curves through the points. The dashed curves (····, NaCl; – –, $Na_2SO_4$) are the Nernst-Hartley predictions.

In the case of diffusion, a concentration gradient of the protein component will give rise to a gradient of anions. These free anions will exert an electrostatic dragging on the slower positively-charged lysozyme in order to preserve the local electro-neutrality of the solution [16]. In the limit of ideal-dilute solutions, Nernst-Hartley equations [7] describe the diffusion properties of electrolyte systems. In the case of NaCl, we obtain: $C_p = C_1$, $C_{Na^+} = C_2$, $C_{Cl^-} = z\,C_1 + C_2$ and

$$D_{11} = D_p \left( 1 + z^2 \frac{D_{Cl^-} - D_p}{z^2 C_p D_p + C_{Na^+} D_{Na^+} + C_{Cl^-} D_{Cl^-}} C_p \right) \tag{13a}$$

In the case of $Na_2SO_4$, we obtain: $C_p = C_1$, $C_{Na^+} = 2C_2$, $C_{SO_4^{2-}} = (z/2)\,C_1 + C_2$ and

$$D_{11} = D_p \left( 1 + z^2 \frac{D_{SO_4^{2-}} - D_p}{z^2 C_p D_p + C_{Na^+} D_{Na^+} + 4C_{SO_4^{2-}} D_{SO_4^{2-}}} C_p \right) \tag{13b}$$

In Eq.13a,b, $D_{Na^+}$, $D_{Cl^-}$ and $D_{SO_4^{2-}}$ are the tracer diffusion coefficients of individual ions.

In our experiments, the molar concentration of lysozyme ($C_1$= 0.0006 M) is significantly lower than that of both NaCl and $Na_2SO_4$ ($C_2 \geq 0.1$ M). We can therefore consider the limit: $C_1 << C_2$. Applying this limit to Eq.13a,b and including the viscosity correction, we obtain:

$$D_{DLS}\,\eta_r \approx D_p \left( 1 + z^2 \frac{D_{Cl^-} - D_p}{D_{Na^+} + D_{Cl^-}} \frac{C_1}{C_2} \right) \tag{14a}$$

for NaCl, and

$$D_{DLS}\,\eta_r \approx D_p \left( 1 + \frac{z^2}{2} \frac{D_{SO_4^{2-}} - D_p}{D_{Na^+} + 2D_{SO_4^{2-}}} \frac{C_1}{C_2} \right) \tag{14b}$$

for $Na_2SO_4$.

Nernst-Hartley equations are rigorously valid only at very low concentrations. At relatively high concentrations, they are still important for qualitatively describing the experimental behavior of the protein diffusion coefficients. According to Eq. 14 a,b, a significant decrease of $D_{DLS}\eta_r\,(C_2)$ is expected only at low salt concentrations. This is qualitatively consistent with the behavior observed in the case of NaCl (see Fig. 3).

To further analyze our results, we estimate the dragging effect on lysozyme. We can write Eq.14a,b in the following more general way:

$$D_{DLS}\,\eta_r \approx D_p^0 \left( 1 + \alpha \frac{C_1}{C_2} \right) \tag{15}$$

To determine $\alpha$, we consider the following values of the tracer diffusion coefficients: $D_p$ =0.132 × $10^{-5}$ $cm^2s^{-1}$, [9,18] $D_{Na^+}$ =1.33 × $10^{-5}$ $cm^2s^{-1}$ [19], $D_{SO_4^{2-}}$ =1.06 × $10^{-5}$ $cm^2s^{-1}$ [19] and $D_{Cl^-}$ =2.03 × $10^{-5}$ $cm^2s^{-1}$ [19]; we use $z$ =9 in the case of NaCl and $z$ =7 in the case of $Na_2SO_4$. We find that $\alpha \approx 46$ in the case of NaCl and $\alpha \approx 6.6$ in the case of $Na_2SO_4$. This implies that the effect of electrostatic dragging in the case of NaCl is about seven times larger than that in the case of $Na_2SO_4$.

In Fig. 3, we report the $D_{DLS}\eta_r$ values (dashed curves) predicted using Eq.15. From the figure, we can see that, although quantitative prediction of $D_{DLS}\eta_r(C_2)$ cannot be obtained, the experimental difference between the two salt cases is in good agreement with the difference predicted by Eq. 14 a,b. We, therefore, conclude that the electrostatic dragging effect is significant only in the case of NaCl.

We note that the experimental $D_{DLS}\eta_r(C_2)$ decreases at high salt concentrations, whereas the plots of Eq. 15 become nearly constant. This difference can be related to the presence of specific protein-protein attractive interactions, which lead to a further decrease of the lysozyme diffusion coefficient [20]. Our results show that the effect of NaCl on lysozyme diffusion coefficient is mainly related to the common-ion effect within the experimental range of salt concentrations. On the other hand, the effect of $Na_2SO_4$ on lysozyme diffusion coefficient appears to be related to more specific salt-mediated protein-protein interactions.

## 5. Conclusion

We have shown that the dependence of the viscosity-corrected diffusion coefficient of lysozyme is very sensitive to the type of salt anion. We have found that the electrostatic dragging effect plays an important role in defining the behavior of lysozyme diffusion coefficient as a function of NaCl concentration. We also find that the electrostatic dragging effect is small for lysozyme in the presence of $Na_2SO_4$. This implies that the common-ion effect is important only in the case of NaCl. On the other hand, specific salt-mediated interactions between protein molecules are responsible for the observed behavior of lysozyme diffusion coefficient as a function of $Na_2SO_4$ concentration. Our results suggest that the common-ion effect plays an important role in the thermodynamic behavior of lysozyme-NaCl solutions but not in the thermodynamic behavior of lysozyme- $Na_2SO_4$ solutions. In conclusion, DLS is a useful optical method that can be used to probe the presence of common-ion effects in protein-salt aqueous solutions.

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