Protein Diffusion Coefficients Determined by Macroscopic-Gradient Rayleigh Interferometry and Dynamic Light Scattering

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Dynamic light scattering (DLS) is extensively used for measuring macromolecule diffusion coefficients. Contrary to classical techniques based on macroscopic concentration gradients, DLS probes microscopic fluctuations in concentration. DLS accuracy and its concordance with macroscopic-gradient techniques remains an outstanding important issue. We measured lysozyme diffusion coefficients in aqueous salt using both DLS and Rayleigh interferometry, a highly accurate macroscopic-gradient technique. The precision of our results is unprecedented. We find that our DLS values were systematically 2% higher than interferometry values. We believe that our interferometric mesurements have produced the most accurate diffusion data ever reported for a protein, providing a new standard for quality control of DLS measurements. Furthermore, by interferometry, we have determined the whole diffusion coefficient matrix required for rigorously describing lysozyme−salt coupled diffusion. For the first time, we experimentally demonstrate that DLS does not provide the protein diffusion coefficient but one eigenvalue of the diffusion coefficient matrix.

Introduction

Mutual diffusion coefficients characterize the motion of molecules in the presence of concentration gradients. For a binary solution, the diffusion coefficient, D, is defined by Fick's first law

$$-J = D\nabla C \tag{1}$$

where J is the flux of the solute due to its concentration gradient ∇C.[1,2]

Diffusion coefficients are important for understanding and modeling kinetics of phase transitions, chemical reactions, centrifugation, dialysis, controlled release of molecules, and so forth.[2] They are also used for the determination of particle size through the Stokes−Einstein equation[3] and molecular interactions through the dependence of D on concentration.[4] This information is very valuable for assessing the conditions of molecular association and monitoring aggregation in solution.[5−7]

Dynamic light scattering (DLS), also known as quasi-elastic light scattering or photon correlation spectroscopy, is a versatile optical method used for measuring mutual diffusion coefficients of macromolecules in a fluid.[5,6,8] This technique probes relaxation times of microscopic concentration fluctuations in solution. Compared to other methods, it has the great advantage of requiring very small samples and short experimental times. Hence, many DLS instruments are commercially available for routine measurements of diffusion coefficients.[6] Moreover, the demand for DLS instruments has grown significantly over the past few years because of their common use in pharmaceutics and biotechnology (www.drugresearcher.com). Most of the macromolecules of interest in these fields are proteins, viruses, micelles, liposomes, and other nanoparticles.[5−12]

Contrary to DLS, classical techniques for measuring mutual diffusion coefficients, such as the diaphragm cell, Taylor dispersion, and Rayleigh/Gouy interferometry, are based on monitoring macroscopic concentration gradients established by the experimentalist.[1] Typically, this is achieved by interfacing two solutions with slightly different compositions. Classical techniques, compared to DLS, are more directly connected to eq 1, thereby offering superior accuracy in the determination of mutual diffusion coefficients.[1] They can also be used to determine the whole diffusion coefficient matrix of a multicomponent system (see Theory), which is another important advantage with respect to DLS.[1] A direct comparison between DLS and classical techniques provides a means to reveal the actual accuracy of DLS diffusion coefficients and validate or refine DLS theories.

Here, we report a solid experimental comparison between the DLS method and a very accurate macroscopic-gradient technique. We determine ternary diffusion coefficients using the high-precision Gosting diffusiometer[13] operated in its Rayleigh interferometric mode.[1,14] The accuracy of the measured diffusion coefficients is

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known to be ~0.1% and is superior compared to other macroscopic-gradient techniques such as Taylor dispersion and the diaphragm cell.[1,2,14] The chosen system for our comparison is the ternary system: lysozyme (**1**)−sodium chloride (**2**)−water at pH 4.5 and 25 °C. Lysozyme is a stable protein that is commercially available at high purity.

Theory

A DLS apparatus measures the fluctuations in light intensity. In typical DLS geometry, light coming from a laser is scattered by a sample and is collected at a given angle, θ (usually 90°), by a photodetector. The scattering angle defines the direction of the scattering vector $q = (4\pi n/\lambda_0)\sin(\theta/2)$, where λ_0 is the wavelength of light in vacuum and n is the refractive index of the sample. The key step is the determination of the normalized autocorrelation function $g^{(1)}(\tau) = \langle E(t)E^*(t+\tau)\rangle/\langle E(t)E^*(t)\rangle$ associated with stochastic temporal fluctuations of the scattered electric field, $E(t)$.[4] In the commonly employed homodyne mode,[15] this is achieved by monitoring the temporal fluctuations of light intensity $I(t)$ at the scattering angle. These fluctuations are then processed by a correlator, yielding the intensity autocorrelation function $G^{(2)}(\tau) = \langle I(t)I(t+\tau)\rangle$. The field autocorrelation function, $g^{(1)}(\tau)$, is then extracted from $G^{(2)}(\tau)$ by using the Siegert relation $G^{(2)}(t) = \langle I\rangle^2(1+\gamma|g^{(1)}(t)|^2)$, where $\langle I\rangle$ is the average scattered intensity and γ is an efficiency factor.[6]

In the limit in which particles are small compared to the length of the inverse scattering vector q^{-1}, thermodynamic fluctuation theory and hydrodynamics provide a theoretical framework that relates $g^{(1)}(\tau)$ to diffusion coefficients.[8,16] The main point is that the fluctuations of the scattered electric field depend on concentration fluctuations, which dissipate by diffusion. For a binary solution, the normalized field correlation function is, to excellent approximation,[8,16] given by

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

where D_{DLS} is the mutual diffusion coefficient obtained by DLS.

For a multicomponent solution, the description of its diffusion properties becomes more complex. For instance, in the case of a ternary solution, eq 1 is replaced by the extended Fick's first law[1,17]

$$\begin{aligned} -J_1 &= D_{11}\nabla C_1 + D_{12}\nabla C_2 \\ -J_2 &= D_{21}\nabla C_1 + D_{22}\nabla C_2 \end{aligned} \tag{3}$$

where J_1 and J_2 are the fluxes of the two solutes due to their concentration gradients ∇C_1 and ∇C_2. The main-diffusion coefficients D_{11} and D_{22} determine the flux of the solutes due to their own concentration gradients, whereas the cross-diffusion coefficients D_{12} and D_{21} describe the coupling between solute fluxes in solution.

Pusey et al.[18] developed a DLS theory that related $g^{(1)}(\tau)$ to multicomponent diffusion. For a ternary system, their results can be summarized by the following relation

$$g^{(1)}(\tau) = I_1 \exp(-q^2\Lambda_1\tau) + I_2 \exp(-q^2\Lambda_2\tau) \tag{4}$$

where Λ_1 and Λ_2 are the two eigenvalues of the matrix of the four diffusion coefficients and I_1 and I_2 represent, approximately, the normalized scattering contributions of the two solutes.[18,19] Clearly, the eigenvalues of the diffusion coefficient matrix are the only quantities that can be determined by DLS. If cross-diffusion coefficients are negligible, then Λ_1 and Λ_2 become equal to D_{11} and D_{22}, respectively.

It is important to remark that DLS sensitivity is best achieved in solutions containing macromolecules because they can appreciably scatter light.[5] Thus, a reliable comparison with classical methods must be performed on macromolecular solutions. However, even in the most favorable cases, complications arise because of both the polydispersity of macromolecules[18] and the presence of additives.[19] For instance, even biologically relevant aqueous solutions of monodisperse proteins are multicomponent because they also contain small inorganic salts and buffer components. Nonetheless, because the scattering contribution of these additives is negligible, DLS results are interpreted according to **2**, and the extracted value of D_{DLS} is assumed to be the diffusion coefficient of the protein. Clearly, this is an approximation because cross-diffusion coefficients may not be negligible.[13,19,20] This implies that D_{DLS} must be equal to one of the eigenvalues (the smallest) of the diffusion coefficient matrix as indicated by eq 4. Hence, the multicomponent nature of macromolecular systems poses another accuracy limitation for DLS results.

To our knowledge, Leaist and Hao[19] reported the only reliable comparison of DLS with macroscopic-gradient techniques. Using the Taylor dispersion method,[1] they measured the four diffusion coefficients of the ternary system sodium dodecyl sulfate (**1**)−sodium chloride (**2**)−water and compared his results with those obtained by Corti and Degiorgio using DLS.[21] Because the contribution of NaCl to scattering is negligible, the second exponential term in eq 4 can be ignored. Thus, DLS results can be analyzed according to eq 2, where D_{DLS} should coincide with the eigenvalue, Λ_1. Leaist found that the diffusion coefficient of sodium dodecyl sulfate, D_{11}, was 7−14% lower than Λ_1 computed from the diffusion coefficient matrix.[19] In addition, D_{11}, Λ_1, and D_{DLS} of Corti and Degiorgio were plotted against surfactant concentration. An examination of this plot shows that the concentration dependences of Λ_1 and D_{DLS} were appreciably different. It is likely that the presence of the monomer−micelle equilibrium in solution complicates the comparison between these two techniques. In summary, the concordance between DLS and macroscopic-gradient techniques remains an outstanding intriguing issue.

Experimental Section

Materials and Solution Preparation. Hen egg-white lysozyme (14 307 g mol^{-1}), recrystallized six times and lyophilized, was purchased from Seikagaku America and used without further purification. This supplier provides lysozyme at the highest purity.[22] Deionized water was distilled and then passed through a four-stage Millipore filter system to provide high-purity water

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for all of the experiments. Mallinckrodt AR NaCl was dried by heating at 450 °C for about 7 h and used without further purification. The purity of the NaCl was listed as 99.9% by the supplier. Its molecular mass was taken to be 58.443 g mol^{-1}. Mallinckrodt reagent HCl (~12 M) was diluted to about 0.063 M (pH ~1.2) and used to adjust the pH of solutions to 4.50. Measurements of pH were made using a Corning model 130 pH meter with an Orion model 8102 combination ROSS pH electrode. All solutions were prepared by weight using a Mettler-Toledo AT400 analytical balance. Molar concentrations were obtained from the density of solutions. All density measurements were made with a Mettler-Paar DMA40 density meter, thermostated with water from a large, well-regulated (±0.01 °C) water bath. This instrument is interfaced to a computer for time averaging and gives a precision of $\pm 2 \times 10^{-5}$ g cm^{-3} or better. More experimental details on solution preparation are reported in ref 13.

Macroscopic-Gradient Rayleigh Interferometry. All macroscopic-gradient diffusion measurements were made with the high-precision Gosting diffusiometer operated in its Rayleigh interferometric optical mode.[13,14] This interferometer consists of a ~9 m optical bench (5000 kg) with vibration isolation and several components. The light source for generating the Rayleigh interference pattern is a He−Ne Uniphase laser (543.5 nm, 5 mW). The lens components are the main lens (two-element air-spaced achromat, focal length 145 cm) and the cylinder lens (two plano-convex lenses, focal length 68 cm). A cell holder is located between these two lens components inside a water bath. The temperature of the bath was regulated at 25.00 °C with a model PTC-41 Tronac temperature controller to a precision of ±0.001 °C. The cell holder has the function to support a Tiselius cell (where macroscopic-gradient diffusion occurs) and a mask, which consists of a double window. Here the laser beam is split into two parts: one going through the diffusion channel of the Tiselius cell and one passing through the water bath (reference channel). The cylinder lens focuses the diffusion channel at the detector, where the Rayleigh interference pattern is observed. Data from the Rayleigh interference patterns were collected with a linear CCD array (6000 pixels, 10 μm × 10 μm pixels) mounted vertically on a precision stage. The stage with this vertical array was stepped horizontally through the 2D interference pattern to collect the data necessary to calculate the diffusion coefficients. Horizontal positions were obtained with an optical encoder with ±0.5 μm accuracy. Data acquisition was controlled via computer, which also performed the subsequent data reduction. The magnification factor is measured using a precision ruled quartz scale (100 lines/cm, accuracy 0.25 μm; Photo Sciences Inc., Torrance, CA). In brief, a typical diffusion experiment using the Gosting diffusiometer starts from preparing a sharp boundary (using a peristaltic pump) between two uniform solutions of slightly different solute concentrations located inside a vertical channel. The diffusion coefficients are reported at the average composition. The precision of measurement appears to be better than ±0.1% for the binary diffusion coefficients and for the main-term diffusion coefficients (of eq 3) of the ternary diffusion experiments.

The refractive index profile inside the channel is described by the function $f = 2(n - \bar{n})/\Delta n$, where n is the refractive index at a given position inside the channel and $\bar{n}$ and Δn are respectively the average and the difference in the refractive index between the two solutions. The quantity f is obtained by locating the fringe position of the resulting Rayleigh interference pattern. For a ternary system, f is related to the diffusion coefficient matrix by

$$f(y) = \Gamma_1 \mathrm{erf}\left(\frac{y}{\Lambda_1^{1/2}}\right) + \Gamma_2 \mathrm{erf}\left(\frac{y}{\Lambda_2^{1/2}}\right)$$

where $y = (1/2M)x/t^{1/2}$, x is the position inside the vertical channel, t is the time, and M is a lens magnification factor. The quantities Γ_1 and Γ_2 are normalized refractive index contributions. By performing experiments with different concentration gradients, the four diffusion coefficients are obtained. More details on Raileigh interferometry and the Gosting diffusiometer can be found in refs 13 and 14.

Dynamic Light Scattering. DLS measurements were performed by using a Protein Solutions DynaPro-801TC at the 90° scattering angle. The light source was a solid-state laser (35 mW) with $\lambda_0 = 832.0$ nm. Solutions were filtered through 0.02 μm filters (Whatman Anatop 10). DLS and interferometric experiments were performed at the same time. This eliminated problems associated with differences in protein samples, solution preparation, and minor degradation processes. That the measurements were not corrupted by the retention of protein on the filter was established by measuring DLS for solutions that had been passed through two sequential filters. DLS measurements agreed with those for single-pass filtration to within 0.3%, which is approximately the measurement error.

The Gaussian monomodal mode (Protein Solutions Dynamics V4.0) was used in the analysis. The polydispersity coefficients were small, indicating that eq 2 applies to our system. All baseline values were 1.000 ± 0.001. Values of the solution refractive index, which are used to determine q^2 in eq 2, were first determined at 543.5 nm from Rayleigh interferometry and then corrected to 832.0 nm using the known wavelength dependence of the water refractive index. The obtained values of q^2 are $(2.019-2.021) \times 10^{10}$ cm^{-2} at $C_2 = 0.25$ M, $(2.027-2.029) \times 10^{10}$ cm^{-2} at $C_2 = 0.50$ M, $(2.031-2.032) \times 10^{10}$ cm^{-2} at $C_2 = 0.65$ M, and $(2.038-2.039) \times 10^{10}$ cm^{-2} at $C_2 = 0.90$ M. Each reported D_{DLS} value was obtained by averaging at least 50 results.

Results and Discussion

In Table 1, we report our results on D_{DLS}, D_{11}, and Λ_1 as a function of protein concentration, C_1, and for four NaCl concentrations: $C_2 = 0.25$, 0.50, 0.65, and 0.90 M. These four sets of data were fitted according to the linear relation $D_p^0 - kC_1$. For completeness, we also describe our results according to the linear relation $D_p^0(1 - \alpha C_1)$, where $\alpha = k/D_p^0$. The obtained values of D_p^0, k, and α are reported in Table 2. At $C_1 = 0$, D_{11} and Λ_1, both obtained from Rayleigh interferometry, yielded the same values of D_p^0 as expected. These values represent the tracer diffusion coefficient[1] of the protein as a function of salt concentration. However, the values of k and α obtained for the protein diffusion coefficient, D_{11}, were 10−20% lower than those obtained for the eigenvalue, Λ_1. This difference is related to the coupling between solute fluxes in solution.

We find that the D_p^0 values obtained from DLS were ~2% higher than those obtained from Rayleigh interferometry at all four NaCl concentrations. However, remarkable agreement in k and α (1−4%) was found between Λ_1 and D_{DLS}. In three cases, this agreement was nearly perfect (Table 2). The behavior of D_{DLS}, D_{11}, and Λ_1 as a function of protein concentrations at all four NaCl concentrations is illustrated in Figure 1. That the behavior of $D_{DLS}(C_1)$ closely matches that of $\Lambda_1(C_1)$ is convincing experimental evidence that D_{DLS} can be identified with one of the eigenvalues of the diffusion coefficient matrix. The slope of the diffusion coefficient is commonly used to characterize macromolecular interactions in solution by employing microscopic models.[4,21,23,25,26] In most of them, the explicit effect of small salt ions is ignored. Thus, the reported difference between D_{11} and Λ_1 will provide the means to appraise the degree of approximation employed in these models.

Generally, a discrepancy in D_p^0 between DLS and Rayleigh interferometry can be related to several physicochemical factors such as polydispersity, self-association, molecular size, and anisotropy. However, lysozyme is monodisperse and does not self-associate in solution (at pH 4.5). This is a clear advantage with respect to other macromolecules such as polymers and micelles. Furthermore, the radius of lysozyme (~2 nm)[23] is significantly smaller than our experimental q^{-1} (~70 nm), meaning

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Table 1. Values of D_{DLS}, D_{11}, and Λ_1

C_1 (mg/cm^3)	D_{DLS} (10^{-5}cm^{2}s^{-1})	D_{11} (10^{-5}cm^{2}s^{-1})	Λ_1 (10^{-5}cm^2 s^{-1})	C_1 (mg/cm^3)	D_{DLS} (10^{-5}cm^{2}s^{-1})	D_{11} (10^{-5}cm^{2}s^{-1})	Λ_1 (10^{-5}cm^2 s^{-1})
(a) $C_2 = 0.25$ M				(c) $C_2 = 0.65$ M			
7.15	0.1279			6.44	0.1197		
9.30	0.1272			7.15	0.1188		
11.45	0.1262			8.58	0.1170		
17.17	0.1235			9.30	0.1159		
24.32	0.1200			11.45	0.1137		
35.77	0.1155			14.31	0.1106		
38.63	0.1143			17.17	0.1076		
4.29		0.1272	0.1266	18.60	0.1062		
6.44		0.1264	0.1256	20.03	0.1049		
8.58		0.1254	0.1241	21.46	0.1034		
10.01		0.1251	0.1237	24.32	0.1006		
14.31		0.1237	0.1219	4.29		0.1194	0.1187
21.46		0.1211	0.1186	6.44		0.1174	0.1165
35.77		0.1162	0.1121	8.58		0.1147	0.1136
				11.45		0.1126	0.1111
(b) $C_2 = 0.50$ M				14.31		0.1099	0.1082
7.15	0.1223			17.17		0.1073	0.1052
8.58	0.1198			21.46		0.1036	0.1007
14.31	0.1143						
18.60	0.1104			(d) $C_2 = 0.50$ M			
21.46	0.1083			6.44	0.1152		
24.32	0.1053			7.15	0.1144		
38.63	0.0949			9.30	0.1109		
4.29		0.1221	0.1216	11.45	0.1082		
6.44		0.1206	0.1196	14.31	0.1046		
8.58		0.1182	0.1170	17.17	0.1007		
14.31		0.1140	0.1123	4.29		0.1158	0.1152
21.46		0.1084	0.1059	6.44		0.1131	0.1121
35.77		0.0986	0.0947	8.58		0.1102	0.1089
				14.31		0.1039	0.1018

Table 2. Values of D_p^0, k, and α for D_{DLS}, D_{11}, and Λ_1[a]

C_2 (M)	0.25	0.50	0.65	0.90
D_{DLS}				
D_p^0 (10^{-5} cm^2 s^{-1})	0.1311 ± 0.0002	0.1271 ± 0.0008	0.1261 ± 0.0002	0.1238 ± 0.0003
k (10^{-5} cm^5 s^{-1} g^{-1})	0.438 ± 0.007	0.86 ± 0.04	1.06 ± 0.02	1.35 ± 0.03
α (cm^3 g^{-1})	3.34 ± 0.05	6.8 ± 0.3	8.4 ± 0.1	10.9 ± 0.2
D_{11}				
D_p^0 (10^{-5} cm^2 s^{-1})	0.1286 ± 0.0001	0.1250 ± 0.0003	0.1231 ± 0.0002	0.1207 ± 0.0004
k (10^{-5} cm^5 s^{-1} g^{-1})	0.347 ± 0.004	0.75 ± 0.02	0.92 ± 0.02	1.18 ± 0.04
α (cm^3 g^{-1})	2.70 ± 0.03	6.0 ± 0.1	7.5 ± 0.2	9.8 ± 0.3
Λ_1				
D_p^0 (10^{-5} cm^2 s^{-1})	0.1284 ± 0.0001	0.1248 ± 0.0004	0.1230 ± 0.0002	0.1207 ± 0.0004
k (10^{-5} cm^5 s^{-1} g^{-1})	0.456 ± 0.007	0.85 ± 0.02	1.04 ± 0.02	1.33 ± 0.04
α (cm^3 g^{-1})	3.55 ± 0.05	6.8 ± 0.2	8.5 ± 0.1	11.0 ± 0.4

[a] The listed errors are standard deviations.

that DLS should yield mutual diffusion coefficients.[8] Lysozyme anisotropy is also small,[23] and effects due to the rotational motion of the protein can be completely ignored at this scattering vector.[24] It is likely that the higher value of D_p^0 obtained from DLS measurements is related to instrumental issues such as sample local heating caused by the laser or imperfect cell alignment.[27] However, it is very difficult to assign this small discrepancy unambiguously.

Hydrodynamic Radius of Lysozyme. It is customary to convert D_p^0 into the equivalent hydrodynamic radius, R_h^e, by applying the Stokes−Einstein relation[3] $D_p^0 = k_B T/6\pi\eta R_h^e$, where k_B is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the fluid surrounding the protein. In this way, temperature and viscosity can be factored out. Most values of the lysozyme hydrodynamic radius, previously obtained by DLS, range from 1.8 to 1.9 nm.[23,25,28] Internal discrepancies were attributed to differences in salt type and experimental setup.[23] We believe that our interferometric mesurements using the Gosting diffusiometer have produced the most accurate set of diffusion data ever reported for a protein, providing a valuable benchmark for other techniques. We convert our interferometric D_p^0 values into equivalent hydrodynamic radii. For each experimental C_2, we use the viscosity values of the corresponding binary NaCl aqueous solution[29] to determine R_h^e. We have found that R_h^e does not depend on C_2 within experimental error, so we report the average value of $R_h^e = 1.863 \pm 0.008$ nm.

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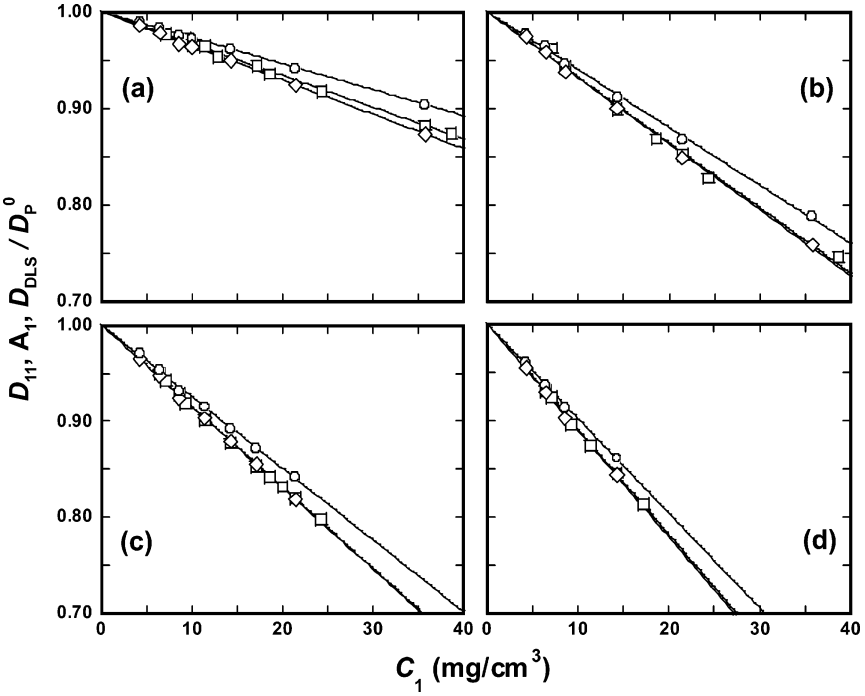

Figure 1. Ratios of the ternary diffusion coefficient, D_{11}/D_p^0 (○), the eigenvalue, Λ_1/D_p^0 (◇), and DLS diffusion coefficient, D_{DLS}/D_p^0 (□), to the corresponding diffusion value at $C_1 = 0$ vs protein concentration, C_1, and at constant NaCl concentrations (a) $C_2 = 0.25$, (b) 0.50, (c) 0.65, and (d) 0.90 M.

This value can be used as a new standard of quality control for commercial DLS instruments.

Summary and Conclusions

We have examined the accuracy of DLS by comparing this technique with Rayleigh interferometry. The former yields diffusion coefficients from microscopic fluctuations, and the latter, from macroscopic gradients. Our results are unique and unprecedented. We have chosen to investigate lysozyme in aqueous NaCl. For this favorable choice, we find an ~2% discrepancy between the two techniques, which can be assigned to DLS instrumental limitations. We have obtained a value for the lysozyme hydrodymic radius that can be used as a reference for DLS measurements. Interestingly, our results unambiguously demonstrate that DLS yields the eigenvalues of the diffusion coefficient matrix. Moreover, our interferometric measurements show that the difference in slope between the protein diffusion coefficient and the smaller eigenvalue ranges from 10 to 20%, which may affect the interpretations of D_{DLS} slopes.

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