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Automated measurement of the static light scattering of macromolecular solutions over a broad range of concentration

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Abstract

A method and apparatus for automated measurement of the concentration dependence of static light scattering of protein solutions over a broad range of concentration is described. The gradient of protein concentration is created by successive dilutions of an initially concentrated solution contained within the scattering measurement cell, which is maintained at constant total volume. The method is validated by measurement of the concentration dependence of light scattering of bovine serum albumin, ovalbumin, and ovomucoid at concentrations up to 130 g/L. The experimentally obtained concentration dependence of scattering obtained from all three proteins is quantitatively consistent with the assumption that no significant self-association occurs over the measured range of concentration.

Keywords

Rayleigh scattering; non-ideal solutions; concentrated protein solutions; bovine serum albumin; ovalbumin; ovomucoid

I. Introduction

Characterization of protein interactions in concentrated solution ($>ca$ 30 g/L) is complicated by significant contributions from weak nonspecific protein-protein interactions that lead to significant deviations from thermodynamic ideality [1–3]. Conventional analysis of the concentration dependence of colligative properties of protein solution – osmotic pressure, sedimentation equilibrium and static light scattering – have been limited to first-order deviations from thermodynamic ideality, or so called second virial coefficient corrections [4]. However, such corrections cannot account quantitatively for the behavior of solutions at concentrations exceeding ca 50 g/L [5,6]. A semi-empirical treatment of nonideal behavior referred to as the effective hard particle model [1,7] has proven useful in accounting quantitatively for the concentration dependence of all three colligative properties of protein solutions at concentrations up to several hundred g/L [5,8].

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Although the measurement and analysis of static light scattering of protein solutions has long been a standard method for the characterization of the molar mass and of the self-association of proteins [9], studies of the light scattering of highly concentrated protein solutions [10,11] are quite rare due to technical difficulties associated with sample preparation, conventional techniques of batch measurement, and limitation of conventional analyses to treatment of first-order deviations from thermodynamic ideality. We have recently presented an extension of multicomponent light scattering theory that is applicable in principle to solutions of multiple species of proteins at arbitrary concentration, taking into account both attractive interactions leading to self- and/or hetero-association, and nonspecific repulsive interactions leading to thermodynamically nonideal behavior [5]. In order to test the applicability of this theory to real solutions of real proteins we have developed a novel method for automatically and rapidly performing a measurement of the concentration dependence of light scattering over an extremely broad range of concentrations. The purpose of the present report is to describe this method in detail and to validate it by means of measurements performed upon single non-associating proteins whose properties are documented in the literature. A second report, currently in preparation, will present results and analysis of data obtained from more complex solutions containing protein mixtures and self-associating protein.

II. Materials and methods

Materials

Monomeric bovine serum albumin (BSA) was obtained from Sigma-Aldrich (A1900). Ovalbumin from egg white and ovomucoid were obtained from Worthington Biochemical Corporation (LS003048 and LS003087, respectively). Before use, proteins were dialyzed against phosphate buffer, 0.05 M Phosphate, 0.15 M NaCl at pH 7.2. Dialysis for buffer exchange was performed against excess solvent overnight using Pierce 10000 MWCO Slide-A-Lyzer Dialysis cassettes. To prepare concentrated protein solutions, proteins were dissolved at low concentration (< 30 g/l), dialyzed, and then concentrated using Centricon filter devices (Millipore, Ultracel YM-10 membrane - 10,000 NMWL) up to different final concentrations, determined from the absorbance at 280 nm using the following values of $A(280 \text{ nm}, 1 \text{ g/L})$: BSA, 0.65; ovalbumin, 0.75; and ovomucoid, 0.41 [13]. Buffer and protein were prefiltered through 0.02- μm Whatman Anotop filters. Immediately prior to measurement of light scattering, protein solutions were centrifuged at 80,000 g for 30 min to remove residual particulates and microscopic bubbles, and then transferred via pipet to the light scattering cuvette described below.

Methods

A Wyatt MiniDAWN Tristar light scattering detector (Wyatt Technology Corp., Santa Barbara, CA) was modified as follows. A Variomag Mini cuvette stirrer (Variomag-USA, Daytona Beach, FL) was mounted in the base of the MiniDAWN read head and connected to a Variomag Mini 20 P controller. Wratten neutral density filters of the appropriate optical density (typically 1 or 1.5 OD) were placed over the photodiode scattering detectors to attenuate light scattered from highly concentrated protein solution, and the instrument was recalibrated using a freshly prepared dilute solution of monomeric BSA (MW 70,000). The light scattering flow cell provided as standard equipment with the MiniDAWN detector was replaced by a square cuvette holder, supplied as part of the Wyatt Micro-batch accessory. A square fluorescence cuvette (internal cross section 10 mm $\times$ 10 mm) containing 2 ml of a protein solution of predetermined concentration and an 8 mm long magnetic stirring bar is inserted into the cuvette holder. The cuvette is connected to a liquid handling system shown schematically in Figure 1, consisting of a Hamilton Microlab 541C syringe pump (Hamilton Co., Reno NV) with dual 1000 μl syringes under computer control. The cuvette is then stoppered with a teflon cap containing

two 21 gauge stainless steel tubes for addition of buffer and removal of solution, and an opening for a IT-18 thermocouple probe (Physitemp Instruments, Clifton, NJ).

An automated concentration gradient experiment proceeds under computer control as follows. (1) The magnetic stirrer is turned on and a volume of filtered buffer calculated to dilute the solution by a user-selected fraction is added to the solution using the left-hand syringe of the Hamilton pump. Buffer added passes through a Whatman Anotop 0.1 μm filter prior to entering the scattering cell. After mixing is complete (typically within a few seconds) the stirrer is turned off. (2) The 90° scattering intensity is read for a period of time sufficient to obtain a precise average value, typically three to four minutes. (3) A volume of solution identical to that added in step 1 is removed using the right-hand syringe of the Hamilton pump to restore the initial volume of fluid in the cuvette. Steps (1) through (3) are repeated until near-baseline levels of scattering are obtained. The cuvette is then automatically rinsed several times with large volumes of buffer and the baseline scattering intensity is measured precisely. A typical dilution run requires approximately one hour. In Fig. 2 the relative intensity of 690-nm light scattered at 90° recorded in a typical experiment is plotted as function of time.

Since the Wyatt MiniDAWN detector is not equipped with temperature control, the temperature of solution in the cuvette is measured continuously by means of the aforementioned thermocouple probe connected to a Physitemp BAT-12 thermocouple thermometer. Scattering and temperature data are recorded together using Wyatt ASTRA software (version 4.9.08), and it is verified that the temperature remains essentially constant (26 ± 1 degree C) throughout the dilution experiment. Although the temperature of the unstirred solution rises slowly under the influence of laser illumination, it is restored to near-room temperature upon dilution and mixing with buffer.

As described previously [14], excess scattering intensity is converted to values of the Rayleigh ratio R normalized to units of the optical constant K_o , given by [5]

$$K_o = \frac{4\pi^2 \tilde{n}_0^2 (d\tilde{n}/dw)^2}{\lambda_0^4 N_A} \quad (1)$$

Where $\tilde{n}$ denotes the refractive index of solution and $\tilde{n}_0$ that of solvent, w the w/v concentration of scattering solute, λ_0 the wavelength in vacuum of scattered light (690 nm in the present experiments) and N_A Avogadro's number. The value of $d\tilde{n}/dw$ is taken as 0.185 cm^3/g for all three proteins [15]. The protein concentration at each dilution step is calculated from the prior concentration (or initial concentration) and present dilution factor. Since the determination of concentration calculated in this fashion is subject to compound error with increasing number of dilution steps, we have verified the accuracy of our calculated concentrations in two ways. (1) The concentration is checked by spectrophotometric measurement of protein concentration after a large number of dilution steps. (2) The scattering of a protein solution of spectrophotometrically determined concentration is compared to that of a solution whose nominally near-identical concentration is calculated following a substantial number (*ca.* 10) dilution steps. Raw data are processed using user-written scripts written in MATLAB (Mathworks, Natick MA) to create secondary data sets consisting of tables of the Rayleigh ratio normalized to the optical constant K_o , as a function of protein concentration.¹

III. Results

The concentration dependence of light scattering of three proteins, BSA, ovalbumin and ovomucoid, at concentrations up to ca 130 g/L is plotted in Figure 3. The concentration

¹Scripts available upon request.

dependence for each protein was measured several times, starting at different loading concentrations. Each measurement is plotted with a different symbol type. The superimposability of these multiple determinations is a measure of the accuracy with which we can calculate the concentration of a sample after multiple dilution steps. We have fitted to the data for each protein the following relation for the concentration dependence of light scattering of a single non-associating species at arbitrary concentration [5]:

$$\frac{R}{K_o} = \left(\frac{\tilde{n}}{\tilde{n}_0} \right)^2 \frac{Mw}{1 + w \frac{d \ln \gamma}{dw}} \quad (2)$$

where M denotes the molar mass and γ the thermodynamic activity coefficient of the scattering solute. The dependence of solution refractive index upon protein concentration is given by [16]

$$\tilde{n} = \tilde{n}_0 + \frac{d\tilde{n}}{dw} w \quad (3)$$

The concentration dependence of the logarithm of the activity coefficient is obtained by modeling the protein molecule as an effective hard spherical particle [7]:

$$\frac{d \ln \gamma}{dw} = 8\phi + 30\phi^2 + 73.4\phi^3 + 141.2\phi^4 + 238.5\phi^5 + \dots \quad (4)$$

where ϕ denotes the fraction of volume occupied by protein, given by

$$\phi = v_{\text{eff}} w \quad (5)$$

and v_{eff} is the specific volume of the effective hard spherical particle². The infinite power series indicated in equation (4) is truncated after five terms since test calculations indicated convergence over the measured range of concentration. According to this model the contribution of repulsive nonideal interactions to the light scattering at all protein concentrations is determined by the value of the single parameter v_{eff} [17]. The best fit of equation (1) to equation (5) to each of the protein data sets is plotted together with the data in Figure 3, and the values and uncertainties of the best-fit values of M and v_{eff} for each protein given in the figure caption.

IV. Discussion

We have introduced an experimental method for automatically and rapidly performing a measurement of the concentration dependence of light scattering over a wide range of concentrations. This method allows the maximum amount of information to be extracted from the concentration dependence of light scattering, due to the acquisition of data at equal logarithmic increments of concentration, so that data obtained at both very low and very high concentration receive equal statistical weight [5].

The method has been validated by measuring the concentration dependence of three proteins, BSA, ovalbumin and ovomucoid. It is shown that the concentration dependence of each may be accounted for quantitatively over a wide concentration range by a simple model, according to which each protein molecule interacts with other protein molecules only via short-ranged repulsive interactions that may be approximated as hard particle interactions, and does not self-associate to any significant extent over the concentration range examined. Molecular weights obtained for all three proteins agree well with literature values [4,18].

²We note that v_{eff} is defined equal to the specific volume of a hard spherical particle whose interparticle interactions best mimic that of the actual protein under a particular set of experimental conditions. When protein molecules interact via repulsive electrostatic interactions the effective hard particle appears larger than the actual molecule [1,7,17].

Our finding that the concentration dependence of scattering for BSA at high concentration may be accounted for by an effective hard sphere model without significant self-association at concentrations below 100 g/L agrees well with the results of earlier measurements of the light scattering, osmotic pressure and sedimentation equilibrium of concentrated BSA solutions [12]. Our finding that the concentration dependence of the scattering of ovalbumin may be accounted for by an effective hard sphere model without significant self-association at concentrations below 130 g/L agrees with a previous interpretation of the concentration dependence of osmotic pressure [19]. However, an earlier measurement of sedimentation equilibrium of concentrated ovalbumin [20] was interpreted as evidence for equilibrium monomer-trimer or monomer-dimer-tetramer self-association. The automated dilution system developed here provides a significant improvement in the experimental precision of measurement over a wide range of concentration, especially at high concentration. Attempts to fit our data to a non-ideal monomer/ trimer or monomer/ dimer/ tetramer model [5] were unsuccessful. Hence the origin of the discrepancy between light scattering and sedimentation equilibrium results remains unresolved. We are unaware of previous characterizations of the colligative properties of ovomucoid at high concentration, although it has been used as an “inert” cosolute at high concentration to study the quantitative effect of macromolecular crowding on protein aggregation [21].

Analytical methods that are used to characterize proteins in solution generally require dilution to solvent conditions that are very different than those in which the protein functions *in vivo* [2,3] or in a biopharmaceutical formulation [22]. In contrast, the apparatus and experimental technique method introduced here provide a relatively inexpensive and moderately high throughput tool for precise quantitative characterization of the state of association of proteins in solution at all concentrations up to the solubility limit. In a following paper we shall describe how data derived from measurements like those described here may be used to detect and quantitatively characterize weak equilibrium self-associations in a highly nonideal solution.

The new method presented here may be compared to the best current automated method for measuring the concentration dependence of light scattering in solutions of proteins and other macromolecules, in which a stock protein solution is automatically mixed with buffer in varying ratios prior to introduction into light scattering and concentration detection flow cells [14,23,24]. The method presented here is easier to implement for very concentrated protein solutions, as no transport of concentrated solution through narrow bore tubing or filters is required. Moreover, no separate concentration flow detector is needed, since the protein solution being measured remains in the measurement cell throughout and the extent of dilution is determined precisely by the high resolution stepping motor-controlled syringe pumps.

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Figure 1.
Schematic diagram of the automated dilution system.

Figure 2.
90° Light scattering (690 nm) of a solution of bovine serum albumin (initial concentration 118 g/L), plotted as a function of elapsed time through a dilution gradient. Each step corresponds to a 20% reduction in protein concentration. Dashed line represents the baseline.

Figure 3.

Experimentally obtained values of normalized scattering intensity (R/K_0) plotted as a function of protein w/v concentration w . Different symbol types represent experiments starting at different initial concentration. Solid curves calculated according to equation (2) – equation (5) with the following best-fit parameter values: BSA, $M = 68,700 \pm 1,600$, $v_{\text{eff}} = 1.81 \pm 0.06$ cm^3/g ; ovalbumin, $M = 45,500 \pm 1,000$, $v_{\text{eff}} = 1.68 \pm 0.05$ cm^3/g ; ovomucoid, $M = 28,100 \pm 800$, $v_{\text{eff}} = 1.65 \pm 0.07$ cm^3/g . Indicated uncertainties correspond to ± 1 standard error of estimate.