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PROTEIN BINDING STUDY OF ANTIHYPERTENSIVE DRUG BY UV SPECTROPHOTOMETRY

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ABSTRACT:

From a pharmaceutical standpoint, research on protein–protein interactions (PPIs) has grown in significance, particularly in high-concentration solutions. However, the majority of analytical techniques for researching protein interactions depend on finding nonideality in solutions that are moderately diluted (less than 50 mg/mL). Here, we describe the use of variablepathlength ultraviolet (UV)–visible absorption spectroscopy on a number of typical proteins to investigate and clarify such interactions over a broad concentration range (5–240 mg/mL). Delta absorbance (Abs), the difference between the measured absorbance and the corresponding theoretical absorbance (calculated from gravimetric dilution), was used in this study to track the change in UV absorption, also known as the extinction coefficient, over a wide range of protein concentrations. It was discovered that for three model proteins (bovine serum albumin, lysozyme, and monoclonal antibody), the Abs increased with protein content after being compensated for light scattering. We looked at the relationship between Abs readings and viscosity as a function of protein content because PPIs affect solution viscosity. Though to varying degrees for various proteins, the magnitude of Abs and solution viscosity followed comparable trends with increasing protein content. These findings bolster the application of such Abs measurements as a substitute method for tracking and assessing interactions in protein solutions at high concentrations.

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Keywords: viscosity, UV/Vis spectroscopy, light scattering, protein, high concentration, interaction

1. INTRODUCTION

A variety of biological processes are governed by macromolecular complexes and their interactions.1 A plethora of qualitative and quantitative techniques have been developed and validated to understand the stoichiometry and strength of intermolecular interactions.2–13 These interactions have generated significant interest in the context of better understanding the folding,14 solubility,15 osmolarity,16 crystallization,17–19 colloidal behavior,20 self-association,21 viscosity,22–24 and stability25–27 of proteins and other macromolecular systems. With the appearance of an increasing number of highconcentration protein therapeutic drugs [e.g., monoclonal

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antibodies (mAbs)], pharmaceutical challenges such as storage stability (conformational instability and aggregation), solution viscosity, and process optimization have also arisen.

A number of the analytical techniques, such as light scattering, membrane osmometry, sedimentation equilibrium, and self-interaction chromatography, used to study protein–protein interactions (PPIs) determine the second virial coefficient (B22), a thermodynamic parameter used to characterize nonideality of solutions. B22 has traditionally been used as a guide to understand phenomena such as solubility,15 crystallization,19 and self-association.21 Limited experimental data that measure nonideality effects in highly concentrated (>50 mg/mL or volume fraction >0.1) protein solutions or that employ nonhydrodynamic approaches are currently available. Experimental limitations of currently available methods such as low throughput, high protein requirements, increased viscosity, or the need for prior immobilization of proteins encourage the development of complementary analytical technologies to better understand PPIs at high concentrations.

In this work, we present one such new approach which employs a variable-pathlength ultraviolet (UV)–visible spectrophotometer to study interactions over a wide range of concentrations for three model proteins: bovine serum albumin (BSA), lysozyme, and a mAb (IgG2). We determine a unique parameter referred to as delta absorbance (Abs), which is defined as the difference between the measured absorbance of proteins in solution at different protein concentrations and their corresponding theoretical/calculated absorbance values (determined by gravimetric dilution from a stock protein solution of known concentration). The origin of Abs is hypothesized to be due to potential changes in the optical properties of interacting protein molecules in solution, rearrangement of water molecules around chromophores due to PPIs, and/or light scattering. A similar concept forms the basis of concentration difference spectrophotometry in which association or dissociation (or both) of proteins, such as mammalian hemoglobin, can be evaluated.29 This phenomenon is studied by precisely measuring the concentrations of each monomeric and oligomeric species in solution upon dilution using a variablepathlength cuvette. Numerous other studies30–34 have been performed using either nonassociating and/or self-associating proteins to study the effect of increasing protein concentrations (up to hundreds of milligrams per milliliter) on light scattering intensity. Concentration-dependent Raleigh scattering intensity was found to deviate from both ideal scattering and the scattering values predicted by first-order corrections to nonideality.30,33 This nonlinear dependence of light scattering on protein concentration has been attributed to repulsive interactions (excluded volume effects) and other short-/long-range effects that modulate the intermolecular interactions in globular proteins.30,32 Theoretical models using Rayleigh scattering theory, and subsequent experimental results, have quantitatively determined the magnitude of these

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contributions, which become especially significant at high concentrations. Various models such as simple hard-spheres, adhesive hardspheres, and effective hard-sphere mixture models have been employed to characterize different types of intermolecular interactions such as steric repulsion, short-/long-range interactions, and equilibrium self-association.31–33 The measurement of Abs potentially provides complementary information to aid in the detection and understanding of these PPIs. The unavailability of a convenient analytical technique to collect absorption spectra at high protein concentrations without prior sample handling and dilution, as well as challenges in obtaining a wide range of pathlength cuvettes especially at very short pathlengths, have previously precluded such a study. Since increased solution viscosity is one of the most immediate consequences of high-concentration protein solutions, we also evaluated the possibility that correlations might exist between Abs and solution viscosity as a function of protein concentration.

The variable-pathlength (0.01–15 mm) tool that serves as a cuvette (the SoloVPE; C. Technologies Inc., Bridgewater, New Jersey) used in these studies employs the principle of slope spectroscopyTM to reliably measure low and high protein concentrations without dilution using a coupled Varian Cary 50TM UV– visible spectrophotometer. This instrument is able to record and generate absorbance versus pathlength linear plots using its variable pathlength capability. The slope determined from absorbance versus pathlength relationships is further used to determine precise protein concentrations using the known extinction coefficients of proteins under investigation. This variable pathlength spectrophotometer is employed in the current studies to detect potential change(s) in optical properties of individual and/or interacting molecules for a wide range of protein concentrations. The absorbance values were computed using the Beer–Lambert law with experimentally determined protein concentrations and known values of extinction coefficients for BSA, lysozyme, and IgG2. The theoretical absorbance was calculated after gravimetric dilution of a protein stock solution of known concentration. The calculation of theoretical absorbance was appropriately corrected for changes in density with protein concentration, which is especially significant at higher protein concentrations. The density measurements were performed at 20◦C using a DMA-5000 high-precision densitometer (Anton Paar, Graz, Austria) with a precision of 1 × 10−6 g/cm3 and 0.001◦C. This new analytical technique is simple, nondestructive, and requires only small volumes (10– 150 :L, which can be recovered for other studies) of protein solution. It can potentially provide a simple and unique measure to study intermolecular interactions for a wide range of protein concentrations.

2. EXPERIMENTAL

Materials

Bovine serum albumin and lysozyme (chicken egg white) were obtained from Sigma –Aldrich (St. Louis, MO). All chemicals and buffer components were purchased from Sigma–Aldrich. The mAb

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(IgG2) was procured from a commercial source. The chemicals and protein samples were used without further processing or purification.

Sample Preparation

Protein samples were extensively dialyzed into their respective pH-adjusted buffers and filtered through 0.22 :m Millipore filters (Fischer Scientific, Pittsburgh, PA) prior to use. Stock solutions of BSA (250 mg/mL) and IgG2 (150 mg/mL) were prepared in 10 mM histidine buffer (pH 6) with NaCl to produce a final ionic strength of 0.015. Lysozyme stock solution (240 mg/mL) was made by dissolving an appropriate amount of protein in 10 mM acetate buffer, pH 4, with NaCl to an ionic strength of 0.015. The ionic strength was kept low to minimize screening of electrostatic interactions. The pH of the final buffer solutions was determined after dialysis and found to be ±0.05 units. The concentrations of the stock solutions were measured by the traditional dilution method and the absorbance was determined using a NanoDrop 2000 spectrophotometer (NanoDrop Products, Wilmington, DE) and reconfirmed by an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies, Santa Clara, CA). The series of solutions of varying protein concentrations were then prepared by gravimetric dilution of the stock solution, and the theoretical absorbance was calculated using the extinction coefficients for BSA [0.66 mL/(mg cm)],35 lysozyme [2.72 mL/(mg cm)],36 and IgG2 [1.45 mL/(mg cm)].

Optical Density/Absorbance Measurements Using a Variable-Pathlength Spectrophotometer

The SoloVPE (C. Technologies Inc.) takes advantage of its capability to change the optical pathlength (Eq. 1) (which is held constant in traditional spectrophotometers using fixed-pathlength cuvettes) and the linear Beer–Lambert law to measure concentrations of solutions at higher concentrations than fixedpathlength spectrophotometers. The Beer–Lambert law is expressed as:

$$A = alc \qquad (1)$$

where "A" is the measured absorbance, "a" is the molar absorption coefficient, "l" is the pathlength, and "c" is the sample concentration. Thus,

$$A/l = ac \qquad (2)$$

For absorbance versus pathlength measurements,
where "m" is the slope and "b" is the y-intercept.

$$A/l\alpha m \qquad (3)$$

This dimensional equality allows direct replacement of the A/l term in Eq. 2 with the slope term (m) in Eq. 3,

$$m = ac \qquad (4)$$

where the concentration is:

$$c = m/a \text{ (if "a" is known)} \qquad (5)$$

and the molar absorptivity is:

$$a = m/c \text{ (if "c" is known)} \qquad (6)$$

These measurements are acquired by capturing the light passing from a Varian Cary 50TM UV–visible spectrophotometer through the sample solution onto a detector through an optical fiber (FibretteTM). The FibretteTM (C. Technologies Inc.) can be moved up and down relative to the bottom of the sample

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vessel, thus precisely and accurately controlling the pathlength (the distance between the lower tip of the FibretteTM and the bottom of the sample vessel). The light passing through the solution is monitored by a detector system housed below the sample vessel. The pathlength range achievable by this assembly is from 0.01a mm (10 :m) to 15 mm (1.5 cm) at pathlength intervals of 0.005 mm (5 :m). Absorption spectra (or individual wavelength-specific absorbance measurements) can be collected for a range of predetermined pathlengths, and absorbance versus pathlength plots are then created and analyzed by linear regression analysis. The slope of this absorbance versus pathlength plots at a specific wavelength of interest (e.g., 280 nm for proteins) can be used to determine the concentration of protein in solution using Eq. 5. The smallest accessible pathlength of 10 :m allows this technology to conveniently measure protein concentrations of hundreds of milligrams per milliliter without dilution. The pathlength accuracy and concentration linearity of the SoloVPE (C. Technologies Inc.) were confirmed by using a proprietary dye (CHEM013 VPE 1-mm standard, lot #C141923) over a pathlength range of 0.01–1 mm.

The "Quick Slope" software option provided by the manufacturer (C. Technologies Inc.) is a rapid method for measuring protein concentration without dilution or need to optimize data collection parameters. For the more systematic application of studying physical phenomena such as intermolecular interactions, however, the "Setup" mode was used. The "Setup" option allows the user to tailor the experimental and data collection parameters, which may be required especially at higher protein concentrations. Absorbance measurements were acquired at room temperature for a series of pathlengths spanning 10 :m to 3 mm by tailoring the data collection parameters in the "Setup" mode of the software for BSA, lysozyme, and IgG2. The step size for pathlength scanning was selected to obtain a maximum number of points (>5) to obtain a coefficient of determination (r2) ≥ 0.999 for absorbance versus pathlength plots. To maintain the linear range of absorbance, an absorbance threshold of 1.0 (A280 = 1) was sufficient to derive the desired number of data points. For only a few cases, the absorbance threshold was increased to above 1.0. This was required for lysozyme at higher protein concentrations due to the protein's relatively high extinction coefficient.

The measured absorbance values for proteins were corrected for scattering contributions (onewavelength or two-wavelength corrections) at each of the pathlengths tested. The one-wavelength scattering correction subtracts the absorbance value at one specific wavelength in a nonabsorbing region (e.g., 350 nm) from the optical density spectrum through the absorbing region. The two-wavelength scattering option in the software corrects for scattering contribution by linear extrapolation of the nonabsorbing region of the spectrum from 320 to 350 nm (these wavelengths can be selected by the user) through the absorbing region and subtracting the extrapolated

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scattering component to obtain protein-specific absorbance values. These corrections are discussed in more detail later in the text.

Viscosity Measurements

The viscosity of the protein samples was measured using mVROC, a Viscometer/Rheometer-on-a-Chip (RheoSense Inc.), at 25 ± 0.1◦C after equilibration for 5 min. The mVROC (RheoSense Inc.) determines shear-rate-dependent viscosity of protein solutions by measuring the pressure drop of the solution when the liquid flows through a rectangular glass slit containing a monolithic Si pressure sensor array placed at different positions from the entrance. The pressure drop as a function of the position of the pressure sensor is used to compute wall shear stress (τ) to determine the viscosity of Newtonian solutions. The viscosity of non-Newtonian solutions can also be determined using appropriate corrections available in the software. The instrument can determine the viscosity of the sample at either a fixed shear rate (single-point measurement) or perform shear-rate sweeps (multipoint measurements).38 We performed shear-rate sweeps for BSA (240 mg/mL; shear rate: 150–2250 s−1), lysozyme (240 mg/mL; shear rate: 500–5500 s−1), and IgG2 (120 mg/mL; shear rate: 50–850 s−1) to determine the Newtonian and/or non-Newtonian behavior of these protein solutions at the highest concentration used in these studies. The measurement was made for 20 s with a wait time of 3 s before each shearrate determination. The shear-rate range used in the study was optimized on the basis of acceptable criteria (i.e., 5% < x)

Dynamic Light Scattering

Dynamic light scattering (DLS) was used to evaluate IgG2 self-association as a function of protein concentration. The DLS measurements were carried out at 20◦C using a DynaProTM Plate reader DLS system from Wyatt Technology (Santa Barbara, California) with a data acquisition time of 30 s and an average of 10 acquisitions per measurement.

3. RESULTS AND DISCUSSION

Optical Density Measurements and Scattering Correction

Figure 1 shows plots of the theoretical and measured absorbance versus protein concentration for BSA (a), lysozyme (b), and IgG2 (c). The theoretical absorbance was calculated from the gravimetric dilution of protein stock solution at known concentrations, whereas the measured absorbance at each concentration point was determined using the measured protein concentration [without further dilution by SoloVPE (C. Technologies Inc.)] and the extinction coefficient of the protein. The measured absorbance values for all the three proteins show a positive deviation from the theoretical values, the magnitude of which increases as the protein concentration rises. The deviation from linearity observed in the theoretical plot for BSA is due to small dilution errors at higher concentrations. The measured absorbance values were corrected for light scattering contributions using the two-wavelength scatter correction method available in the instrument software.

To determine the suitability of this method to appropriately correct for scattering, we

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compared (Fig. 2) the two-wavelength scatter correction method (linear extrapolation, Fig. 2b) with the standard multiwavelength method,39 which employs a log–log extrapolation (Fig. 2c) using a 120 mg/mL IgG2 solution. This concentration of IgG2 was chosen because it showed the highest deviation among the samples. The variability in the concentration of IgG2 solution obtained at 280 nm and 0.05 mm pathlength (Abs ~1.0) was found to be less than 0.5%. This variability in the measured concentration values was even smaller at lower concentrations. Furthermore, the intersample variability (~2.5% standard deviation) found by analysis of five replicate measurements was higher than the variability.

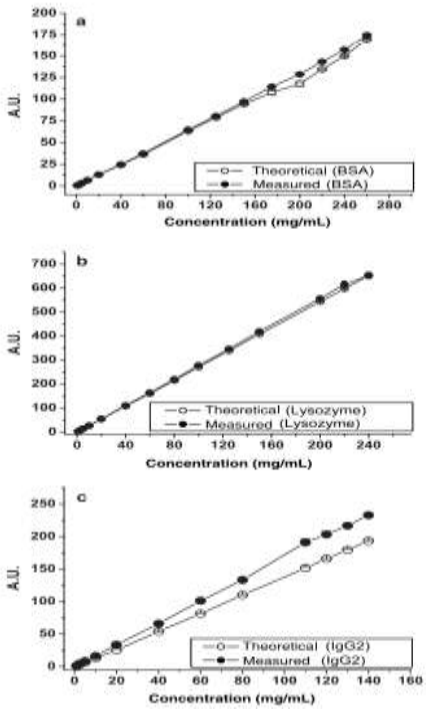

Figure 1. Theoretical and measured absorbance of proteins: (a) BSA in 10 mM histidine buffer, pH 6, (I = 0.015), (b) lysozyme in 10 mM acetate buffer, pH 4, (I = 0.015), and (c) IgG2 mAb in 10 mM histidine buffer, pH 6, (I = 0.015) as a function of protein concentration. The theoretical absorbance was calculated from the gravimetric dilution of protein stock solutions of known concentration. The measured absorbance at each concentration was determined at room temperature using the extinction coefficient of the protein and the measured protein concentration, respectively. The measured concentration was appropriately corrected for light scattering as described in the text. The line connecting the data points is for visual aid only. When error bars (representing standard deviation of five replicate measurements) cannot be seen, they are encompassed within the individual symbols

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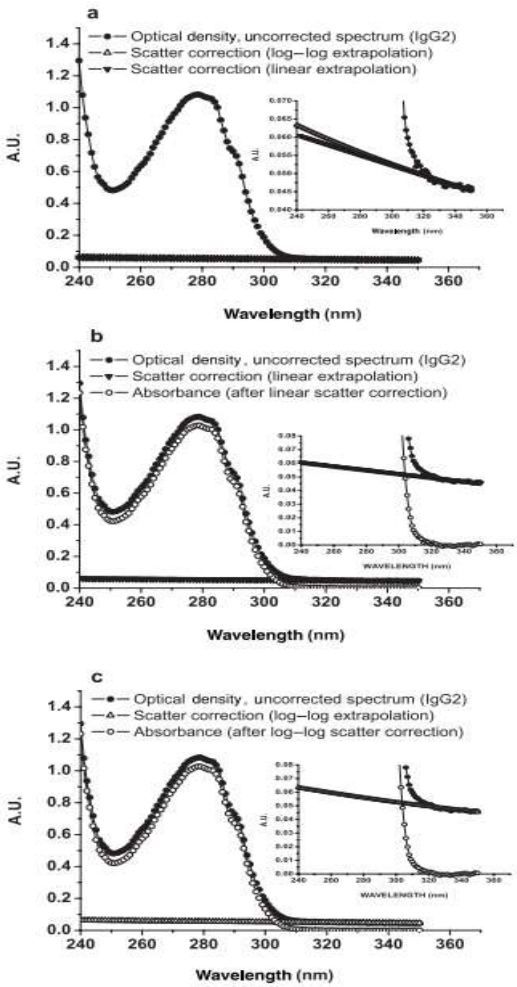

Figure 2. Representative absorption spectra of the IgG2 mAb (a) uncorrected along with extrapolated scatter signal for linear and log–log correction method, (b) corrected using linear extrapolation of the nonabsorbing (320–350 nm) region, and (c) corrected using log–log extrapolation of nonabsorbing (320–350 nm) region. Protein sample contained IgG2 (10 mM histidine buffer, pH 6, I = 0.015) at 120 mg/ mL and measurements employed a pathlength of 50 :m.

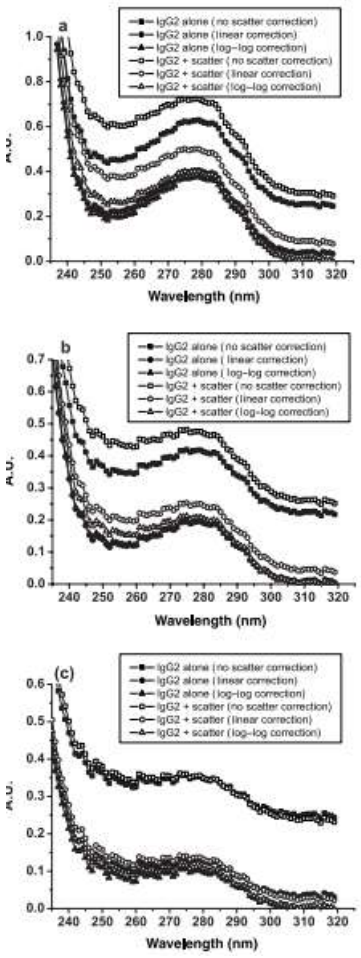

Figure 3. Absorption spectra of the IgG2 mAb (1 mg/mL) in absence and presence of an external scatter (polystyrene beads) corrected for scattering using linear and log–log scatter correction methods. The spectra were collected at pathlength of (a) 1 mm, (b) 0.5 mm, and (c) 0.25 mm.

the linear and log–log correction methods were able to correct for scattering in the IgG2 solution (1 mg/ mL) with no added scatter (the nonscattering control). At 1 mm pathlength, the log–log scatter correction method, however, was more efficient in retrieving the spectrum corresponding to the nonscattering control from the protein solutions containing an external scatterer. In contrast, as the pathlength of absorbance measurement

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was lowered (for instance, to 0.25 mm; Fig. 3c), both the linear and log–log correction methods were equally effective in correcting for light scattering. Since a majority of absorbance measurements at high protein concentrations were made at pathlengths below 0.25mm, the choice of the light scattering correction method should not influence the measurement of intermolecular interactions described in these studies.

Light scattering intensity generally increases with increases in protein concentration. An effective hard particle model based on Raleigh scattering theory for single- and multicomponent systems can accurately describe this concentration dependence of light scattering for single nonassociating, nonassociating mixture, and self-associating proteins.30–33 Thus, the log–log extrapolation (or the two-wavelength scatter correction) method described above should, to a first approximation, account for the concentrationdependent increase in Raleigh scattering intensity for the different protein systems. The small differences in the measured and calculated absorbance values may therefore qualitatively represent some form of weak interactions at both low and high protein concentrations. The magnitude of these deviations, which is a function of protein concentration, suggests that the variable-pathlength spectrophotometer is sensitive enough to detect subtle spectral changes arising from the interactions between protein molecules as a function of protein concentration (see below).

The characteristics and behavior of proteins in aqueous solutions are governed by both long- and short-range interactions between protein molecules as well as their interactions with solvent and other cosolute molecules.40 These interactions are known to be a function of protein concentration with long-range repulsive charge–charge interactions dominating at low protein concentrations, whereas short-range interactions such as van der Waals attraction and dipole–dipole interactions significant at higher concentrations. These interactions along with excluded volume effects are known to increase the probability of PPIs,27,41,42 especially at high protein concentrations. A variety of analytical techniques such as light scattering [static (SLS) and dynamic (DLS)] and analytical ultracentrifugation are used to study PPIs by measuring either the B22 (SLS) or an interaction parameter (kD) (DLS).

It has been pointed out42 that the interparticle distance between mAb molecules is reduced from 22 to 12 nm as the protein concentration increases from 20 to 120 mg/mL. Similarly, concentrated protein solutions with fractional volume occupancy above 0.1 increase the propensity for PPIs.43 The basis of the current work is the hypothesis that these PPIs might affect the optical characteristics of interacting protein molecules and/or cause fluctuations in hydrating water molecules around one or more aromatic amino acid residues. This may cause either an increase or decrease in the measured UV absorbance (extinction coefficient) compared with that of the theoretical absorbance, which assumes a linear relationship between absorbance and protein concentration. The theoretical

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absorbance was computed from gravimetric dilution of protein stock solution of known concentration using the Beer–Lambert law and is highly accurate with an error less than 1.5%. The capability of the variable-pathlength technology to measure a wide range of protein concentrations without dilution enabled the accurate direct measurement of absorbance at both low and high protein concentrations, which in turn could potentially detect change(s) in optical characteristics of interacting macromolecules.

Abs Measurements

Figure 4 represents these deviations in the form of Abs, which is the difference in measured and theoretical absorbance, for the three proteins. The average ratio of Abs to total absorbance was found to be approximately 4% for BSA (5–240 mg/mL), approximately 3% for lysozyme (5–220 mg/mL), and approximately 17% for IgG2 (5–140 mg/mL). The magnitude of Abs should represent a measure of weak association arising from the various types of interactions undergone by proteins under conditions of increasing thermodynamic activity.

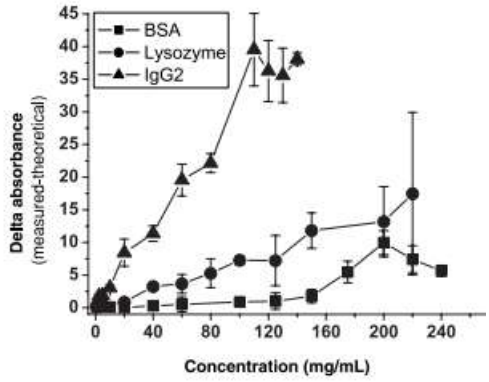

Figure 4. Delta absorbance (Abs), the difference between measured and theoretical absorbance, for BSA, lysozyme, and IgG2 mAb solutions as a function of protein concentration. The error bars represent the standard deviation for five replicate measurements. Experimental conditions are listed in Figure 1.

equilibria, reported repulsive short-range interactions, and no significant self-association (or attractive intermolecular interactions) up to approximately 100 mg/mL for BSA, which should further explain the lower magnitude of Abs observed in dilute BSA solutions. The magnitude of Abs, however, clearly increases at higher BSA and lysozyme concentrations (>100 mg/mL), suggesting that, at these concentrations, PPIs may result in nonideal solution behavior of these two proteins. Furthermore, self-interaction chromatographic studies of a peptide45 and light scattering measurements using model proteins32,33 suggest attractive interactions to be predominant at higher (>100 mg/mL) concentrations. Similarly, the increase in magnitude of Abs values observed at high (>100 mg/mL) BSA and lysozyme concentrations may therefore represent increase in the extent of attractive interactions between protein molecules Various symmetric potentials such as hard-sphere or excluded-volume effects, van der Waals dispersion, charge–charge repulsion, attractive interactions due to presence of salts, square-well interactions representing specific self-association, and dipole interactions potentially contribute to interactions between globular proteins and affect the center-to-center distance “r”46 between molecules in solution. The

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interplay of these contributions will govern the overall interactions of a solute in solution and therefore may influence an experimentally measured parameter such as Abs in any number of different ways. The repulsive charge–charge interactions, known to be predominant at low protein and low salt concentrations, might increase the interparticle distance "r" and thus fail to affect optical properties such that he measured absorbance of the molecules in solution does not deviate significantly from the theoretical absorbance. This would explain the low magnitude of Abs at low BSA and lysozyme concentrations. At high BSA and lysozyme concentrations, the van der Waals and dipolar potentials (among others) could have a larger contribution to the sum of potentials of the mean force (W22) between interacting particles, thus lowering the interparticle distance. This lowering of 'r' may significantly perturb the spectral properties of interacting molecules compared to individual non-interacting molecules and therefore increase the magnitude of Abs at higher concentration.

In the case of the IgG2 (Figs. 1c and 4), however, the Abs values deviate from theoretical absorbance values even at the lower protein concentrations of approximately 20 mg/mL, and the magnitude of the effect increases markedly at above 40 mg/mL. The magnitude of Abs reached a plateau at above 100 mg/ mL. An increase in Abs at lower (~20 mg/mL) IgG2 concentration could potentially be due to increases in the square–well interaction and account for weak selfassociation. Jimenez et al.47 not only reported weak self-association of IgG2 molecules at approximately 30 mg/mL for the antibody of their study, but also suggested formation of approximately 33% trimers at 100 mg/mL and approximately 50% at 200 mg/ mL because of predominant attractive interactions. A recent work33 provides a detailed account of such interactions, using different hard-sphere models and light scattering data, successfully accounting for the self-association of mAbs. The steep increase in Abs at IgG2 concentration greater than approximately 100 mg/mL (Fig. 4) may thus be a consequence of the formation of higher order reversible oligomeric species in solution. In addition, the markedly different behavior of Abs for each of the model proteins as a function of concentration argues strongly that the apparent observed changes in UV absorbance or extinction coefficient are not due to an instrumental artifact (Fig. 4).

Nonetheless, orthogonal methods to study these types of interactions are needed to validate these possibilities and to correlate the change in magnitude of Abs with the physical phenomenon occurring for IgG2. DLS was therefore employed to see whether IgG2 self-association could be detected as the protein concentration was raised. It was found that the average diameter of the mAb increased from 9.8 ± 0.1 to 10.9 ± 0.03 nm (n = 3) as the protein concentration was increased from 0.5 to 10 mg/mL. Measurements could not be performed above 10 mg/mL due to multiple scattering (data not illustrated). These results suggest association of the IgG2, and this effect may thus be responsible for the observed changes in Abs even at lower concentrations.

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Why should the absorbance (or extinction coefficient) change at higher protein concentration? Aromatic residues are known to be dispersed throughout the structure of most proteins. While Phe residues are typically buried, both Trp and Tyr side chains are often at least partially accessible to solvent. Furthermore, the dynamic nature of protein structure is known to permit significant solvent penetration into protein interiors and potentially increase the hydration of these aromatic residues. It seems probable that this phenomenon would be enhanced at higher protein concentrations due to a corresponding increase in the thermodynamic activity of either the hydrating or bulk water. Whichever is the case, small changes in absorbance are not necessarily unexpected at higher protein concentrations.

Three other potential sources of the observed deviation from the theoretical absorbance values at higher protein concentration are absorption flattening, opalescence, and constructive interference. Absorption flattening arises from shadowing of one particle by another and is accompanied by red shifts in the absorption spectrum.48 This effect should become more pronounced as the pathlength is increased. Since neither of these phenomena is seen at the pathlengths used for concentration measurement, it is unlikely to be the source of the deviations manifested by positive Abs. Opalescence is a form of microphase separation and is characterized by a unique shimmering appearance of solutions.49,50 This optical effect was not observed by visual examination for the three model proteins, even at the highest protein concentrations examined. A third possibility is the constructive interference that is observed when a low degree of periodic order is present in liquids containing large solutes as has been observed in the mammalian lens.51–53 Since this phenomenon is known to produce increases in the transmittance of light, such a phenomenon cannot be responsible for the deviations in absorbance observed in the present study. We cannot, however, entirely exclude a contribution by light scattering to these deviations, which is not adequately compensated for by the methods employed. Light scattering effects would appear, however, to provide only a small contribution to the spectral changes as described above. The positive Abs values observed in this study at low and high protein concentrations can therefore be taken as a reflection of the complex interplay of intermolecular interactions in solution.

Viscosity Measurements and Correlation with Abs

Protein–protein interactions in aqueous solution are known to have a direct effect on viscosity, especially with significant self-association at higher protein concentrations.40,54 We therefore studied the

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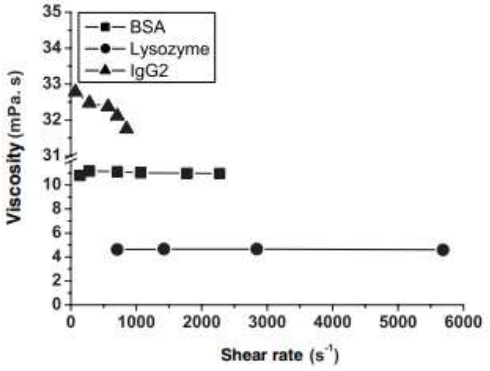

Figure 5. Viscosity measurements of solutions containing BSA, lysozyme, and IgG2 mAb as a function of shear rate (s−1). Shear-rate sweeps were performed at 150–2250 s−1 for BSA (240 mg/mL), 500–5500 s−1 for lysozyme (240 mg/ mL), and 50–850 s−1 for IgG2 (120 mg/mL).

effect of protein concentration on the viscosity, and compared the changes in magnitude of Abs to see whether they correlated with changes in viscosity as a function of BSA, lysozyme, and IgG2 concentrations. Figure 5 illustrates the effect of shear rate (or flow rate) on the viscosity of BSA, lysozyme, and IgG2 at the highest concentrations tested. It is well known that dilute protein solutions behave like Newtonian fluids in which the viscosity of the solution is independent of shear rate. In contrast, non-Newtonian (viscosity-dependent on shear rate) behavior is often observed at higher protein concentrations. We therefore tested the effect of shear rate on viscosity of solutions containing 240 mg/mL BSA and lysozyme, and 120 mg/mL IgG2. The shear rates employed in this experiment were selected on the basis of a predetermined criterion (i.e., 5% < x < 90%) of the maximum limit of the instrument to monitor pressure. BSA and lysozyme were found to behave like Newtonian fluids under the conditions tested in which the viscosity of the solution did not markedly change with shear rate. IgG2 viscosity, however, was found to undergo shear thinning upon an increase in shear rate, suggesting non-Newtonian behavior (Fig. 5).

Figure 6 shows a correlation of Abs with viscosity for BSA (a), lysozyme (b), and IgG2 (c) as a function of protein concentration. The change in magnitude of Abs for BSA and lysozyme was found to correlate with increases in viscosity as a function of protein concentration. The change in magnitude of Abs for the IgG2 (Fig. 6c), however, correlated with an exponential increase in viscosity as a function of IgG2 concentration. A similar exponential increase in viscosity was reported earlier for other mAbs.54 The differences in correlation of Abs with viscosity change between

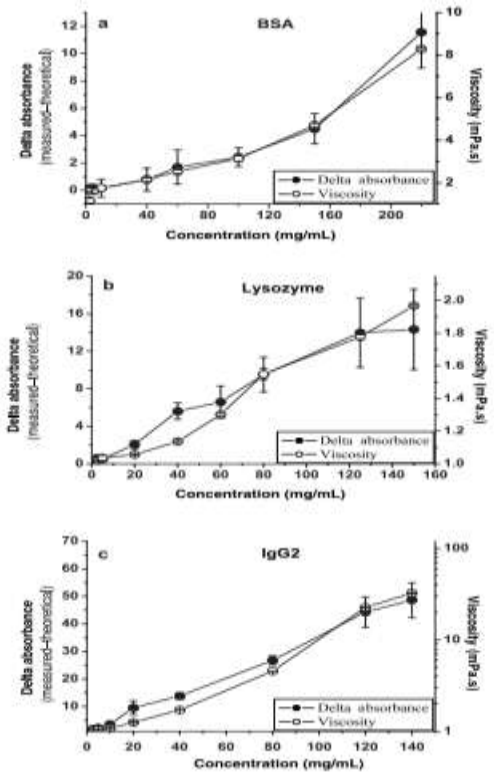

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Figure 6. Comparison of changes in Abs and viscosity (mPa s) as function of protein concentration for solutions containing (a) BSA, (b) lysozyme, and (c) IgG2 mAb. The Abs values are plotted on the y-axis and viscosity values on y-axis. The error bars represent the standard deviation of five replicate measurements.

BSA, lysozyme, and IgG2 are intriguing and require further investigation to better understand the contributions of the different intermolecular forces that dictate solution viscosity.

Because ionic strength is one of the key parameters that affect viscosity, flow behavior, and interactions in proteins solutions,55,56 we studied the effect of varying ionic strength on Abs and the viscosity

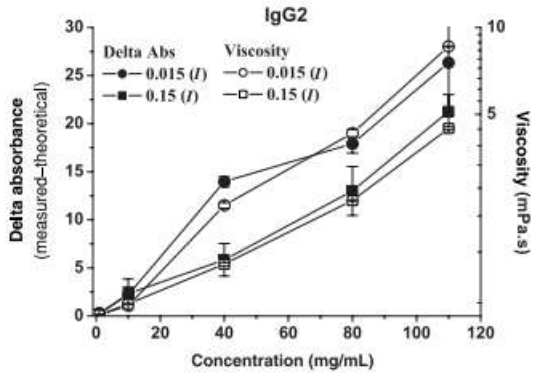

Figure 7. Effect of ionic strength on Abs and viscosity (mPa s) for solution containing IgG2 mAb as function of protein concentration. An ionic strength (I) of 0.015 (low) and 0.15 (high) was attained using NaCl. The Abs values are plotted on y-axis and viscosity on the y-axis. The error bars represent the standard deviation of five replicate measurements.

of IgG2 (Fig. 7). The viscosity of IgG2 solutions was reduced with increases in ionic strength from 0.015 to 0.15. This viscosity mitigating effect was more pronounced at higher protein concentrations (≥40 mg/ mL). A similar effect of ionic strength was observed in terms of the magnitude of experimentally measured Abs value. The Abs was lowered significantly at higher IgG2 concentrations in higher ionic strength solutions. These trends of change in Abs at different IgG2 concentrations correlated well with the changes observed in viscosity in low- and high-ionic-strength solutions. Increases in ionic strength are believed to screen both net attractive (electrostatic component) and net repulsive (excluded volume) interactions in immunoglobulins consistent with both electrostatic and excluded volume effects affecting the properties of high-concentration protein solutions.33 The correlation between solution viscosity and Abs value (measured by UV spectroscopy) may therefore reflect the interplay of the factors responsible for PPIs at higher protein concentrations.

4. Conclusion

A novel method for characterizing the relationship between intermolecular contacts and viscosity in protein solutions with varying concentrations—the degree of which depends on protein concentration—is the determination of Abs values. Abs values were tracked across a broad range of protein concentrations for three model proteins (lysozyme, BSA, and IgG2) with different sizes and molecular weights. For each of the three model proteins at various protein concentrations, there was a correlation between the measured changes in solution viscosity and the observed change in Abs.

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The spectral changes detected by the deviations in the optical characteristics of protein molecules at high concentrations may represent changes in the intermolecular forces governing PPIs, according to the correlation between Abs and solution viscosity for the IgG2 mAb solution, which was observed at different protein concentrations and ionic strengths. The source of the spectrum changes found by UV spectroscopy in this study should be further clarified by a more thorough examination of such PPIs in these circumstances. In any event, this kind of measurement provides a fresh analytical tool for tracking and researching interactions between proteins in concentrated solutions.

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