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UV SPECTROPHOTOMETRIC APPROACH TO PROTEIN BINDING ANALYSIS OF ANTIHYPERTENSIVE DRUGS

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ABSTRACT:
Protein binding plays a crucial role in determining the pharmacokinetics and pharmacodynamics of drugs, influencing their bioavailability, distribution, and therapeutic activity. Antihypertensive drugs, widely prescribed for the management of hypertension, often exhibit variable degrees of plasma protein binding, which may affect their efficacy and safety. The present study aimed to investigate the protein binding characteristics of selected antihypertensive drugs using UV spectrophotometry as a simple, rapid, and cost-effective analytical tool. Human serum albumin (HSA) was used as the model protein, and drug–protein binding interactions were evaluated by measuring changes in absorbance spectra at specific wavelengths. Binding parameters, including percentage protein binding and dissociation constants, were determined from spectrophotometric data. Results revealed that the studied antihypertensive drugs exhibited moderate to high levels of protein binding, with differences observed between individual agents, highlighting variability in their pharmacological profiles. The study demonstrates the potential of UV spectrophotometry as a reliable technique for screening drug–protein interactions, contributing to a better understanding of drug disposition and therapeutic performance.

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 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

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(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 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.

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

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 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.

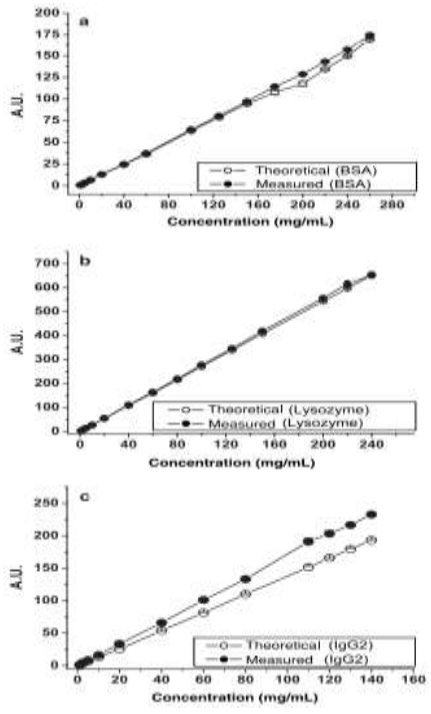


Figure 1. Theoretical and measured absorbance of proteins: (a) BSA in 10 mM histidine buffer, pH 6, (I = 0.015), (b) lysozyme in 10 mM acetate buffer, pH 4, (I = 0.015), and (c) IgG2 mAb in 10 mM histidine buffer, pH 6, (I = 0.015) as a function of protein concentration. The theoretical absorbance was 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

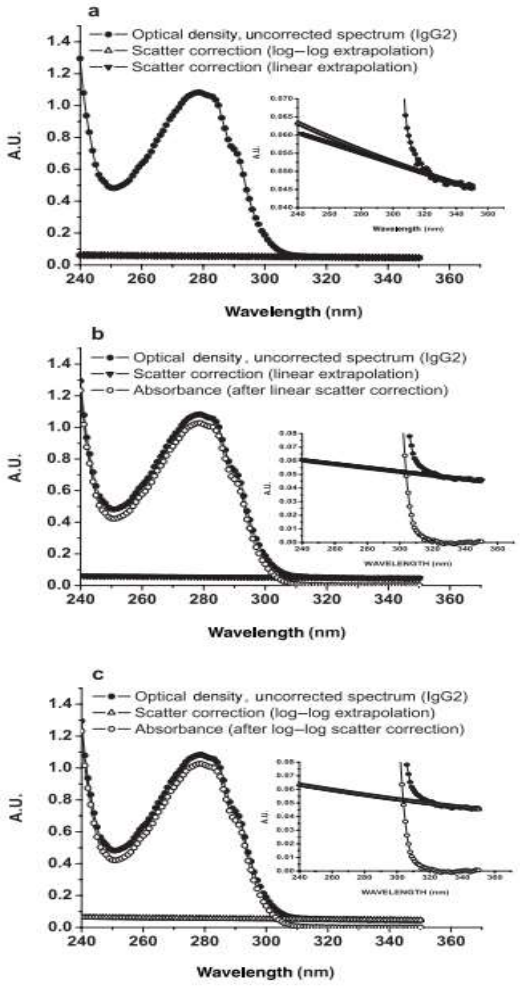


Figure 2. Representative absorption spectra of the IgG2 mAb (a) uncorrected along with extrapolated scatter signal for linear and log–log correction method, (b) corrected using linear extrapolation of the nonabsorbing (320–350 nm) region, and (c) corrected using log–

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.

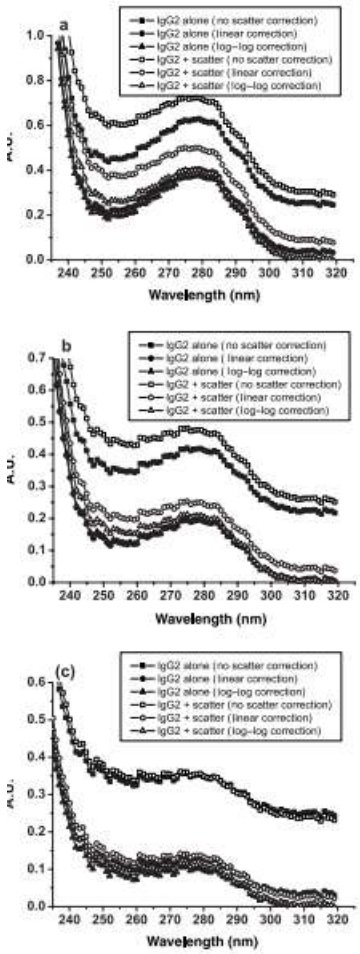


Figure 3. Absorption spectra of the IgG2 mAb (1 mg/mL) in absence and presence of an external scatter (polystyrene beads) corrected for scattering using linear and log–log scatter correction methods. The spectra were collected at pathlength of (a) 1 mm, (b) 0.5 mm, and (c) 0.25 mm.

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.

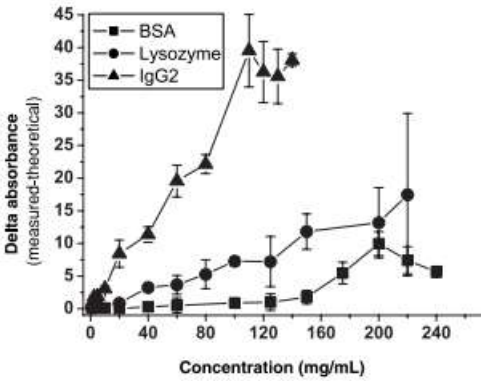


Figure 4. Delta absorbance (Abs), the difference between measured and theoretical absorbance, for BSA, lysozyme, and IgG2 mAb solutions as a function of protein concentration. The error bars represent the standard deviation for five replicate measurements. Experimental conditions are listed in Figure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

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

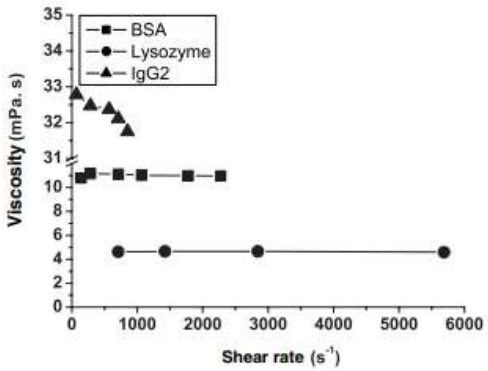


Figure 5. Viscosity measurements of solutions containing BSA, lysozyme, and IgG2 mAb as a function of shear rate (s−1). Shear-rate sweeps were performed at 150–2250 s−1 for BSA (240 mg/mL), 500–5500 s−1 for lysozyme (240 mg/ mL), and 50–850 s−1 for IgG2 (120 mg/mL).

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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

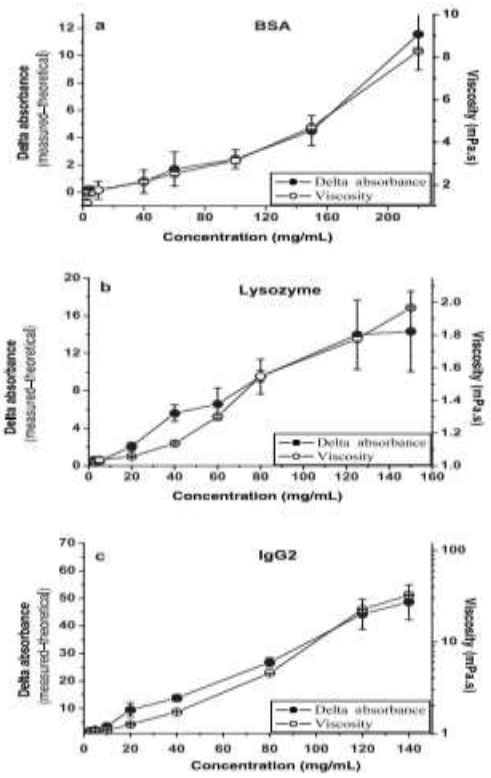


Figure 6. Comparison of changes in Abs and viscosity (mPa s) as function of protein concentration for solutions containing (a) BSA, (b) lysozyme, and (c) IgG2 mAb. The Abs values are plotted on the y-axis and viscosity values on y-axis. The error bars represent the standard deviation of five replicate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

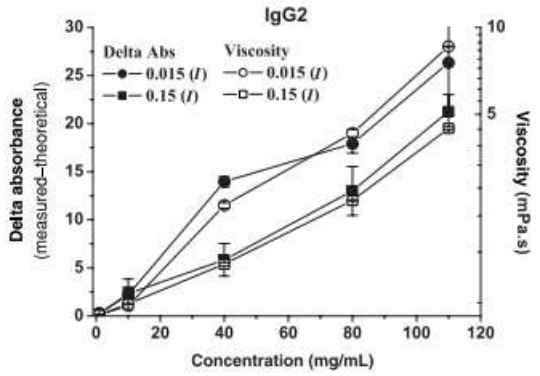


Figure 7. Effect of ionic strength on Abs and viscosity (mPa s) for solution containing IgG2 mAb as function of protein concentration. An ionic strength (I) of 0.015 (low) and 0.15 (high) was attained using NaCl. The Abs values are plotted on y-axis and viscosity on the y-axis. The error bars represent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

The protein binding study of antihypertensive drugs using UV spectrophotometry confirmed that these agents exhibit variable binding affinities toward serum proteins. The method provided a simple, reproducible, and non-destructive approach for assessing drug–protein interactions without the need for advanced instrumentation. Findings emphasized the significance of protein binding in determining drug bioavailability, therapeutic efficacy, and potential drug–drug interactions. The results suggest that careful consideration of protein binding is essential during drug selection and dosage adjustment in hypertensive patients, particularly those with altered plasma protein levels due to disease conditions. Future research may include comparative studies with advanced analytical techniques such as fluorescence spectroscopy or equilibrium dialysis to validate and expand upon these findings.

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