An Application of Ultraviolet Spectroscopy to Study Interactions in Proteins Solutions at High Concentrations

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ABSTRACT: Studies of protein–protein interactions (PPIs), especially in high-concentration solutions, have become increasingly important from a pharmaceutical perspective. Analytical methods used to study protein interactions, however, rely primarily on the detection of nonideality in relatively dilute (<50 mg/mL) solutions. We present here an application of variable-pathlength ultraviolet (UV)–visible absorption spectroscopy to examine and better understand such interactions over a wide concentration range (5–240 mg/mL) using several representative proteins. In this study, the change in UV absorption (or extinction coefficient) was monitored by determining delta absorbance (ΔAbs), the difference between the measured absorbance and the corresponding theoretical absorbance (calculated from gravimetric dilution), over a wide range of protein concentrations. The ΔAbs, corrected for light scattering, was found to increase with protein concentration for three model proteins (bovine serum albumin, lysozyme, and monoclonal antibody). Because PPIs influence solution viscosity, we studied the correlation between ΔAbs measurements and viscosity as a function of protein concentration. The magnitude of ΔAbs and solution viscosity followed similar trends with increasing protein concentration, albeit to different extents for different proteins. These data support the use of such ΔAbs measurements as an alternative approach to monitor and evaluate interactions in protein solutions at high concentration. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:3051–3061, 2012

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

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 stability[25–27] of proteins and other macromolecular systems. With the appearance of an increasing number of high-concentration 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.[28]

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 (B_{22}), a thermodynamic parameter used to characterize nonideality of solutions. B_{22} 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.

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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 (ΔAbs), 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 ΔAbs 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 variable-pathlength cuvette. Numerous other studies[30–34] 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 hard-spheres, 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 ΔAbs 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 ΔAbs 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 spectroscopy$^{\text{TM}}$ to reliably measure low and high protein concentrations without dilution using a coupled Varian Cary 50$^{\text{TM}}$ 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/cm^3 and 0.001°C. This new analytical technique is simple, nondestructive, and requires only small volumes (10–150 μL, 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.

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.

Sample Preparation

Protein samples were extensively dialyzed into their respective pH-adjusted buffers and filtered through 0.22 μm Millipore filters (Fischer Scientific,