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An Evaluation of Protein-Ligand Interactions Through Diffusion Coefficients Determined via Taylor Dispersion Analysis

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by

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As always, “It’s a great day to be a highlander”

ABSTRACT OF THE THESIS

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Previous methods to determine equilibrium binding constants of proteins interacting with ligands include isothermal titration calorimetry, surface plasma resonance and fluorescence polarization. In this work, diffusion coefficients determined through Taylor dispersion analysis are used to obtain equilibrium binding constants and complex diffusion coefficients of ligands interacting with proteins. An equation developed by Jensen et al 2010 is used to fit a plot of measured diffusion coefficients versus ligand concentration. The best fit of this equation provides the equilibrium binding constant and the diffusion coefficient of the ligand interacting with the protein.

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Chapter 1 – Introduction

Introduction

Protein-ligand interactions are a fundamental process in all biological systems ranging from regulating cellular metabolism through signal transduction to the development of therapeutic drugs (Myers et al 1987; Huang et al 2010). Because of its significance in biological systems, it is important to study binding properties of protein-ligand interactions. Current methods such as circular dichroism, nuclear magnetic resonance (NMR), uv-vis absorption, isothermal titration calorimetry (ITC), Fourier transform infrared spectroscopy (FTIR) and surface plasma resonance (SPR) are used to determine binding properties. In this thesis, binding properties of protein-ligand interactions will be studied using a unique method involving diffusion coefficients.

Objective of Thesis

The objective of this thesis is to investigate protein-ligand interactions through the use of diffusion coefficients via Taylor dispersion analysis. Taylor dispersion is a method for obtaining diffusion coefficients by injecting a sample into a parabolic carrier stream. In this phenomenon, as the injected sample travels with the carrier stream, it will spread and disperse axially by convection and radially by diffusion. From the dispersed concentration profile, regression techniques are used to obtain the diffusion coefficient. The theory of Taylor dispersion will be elaborated on in greater detail in chapter two.

The use of diffusion coefficients through Taylor dispersion to extract out important parameters between interacting species such as the equilibrium binding

constant and the diffusion coefficient of two species in complex is a relatively new process. Jensen and Østergaard published a paper in early 2010 (Jensen et al 2010) in which they used diffusion coefficients experimentally determined through Taylor dispersion to quantify equilibrium binding constants and complex diffusion coefficients of β-cyclodextrin interacting with α-naphthol and naproxen. For obtaining these parameters, Jensen and Østergaard were able to regress out the binding constants and the complex diffusion coefficients for α-naphthol and naproxen by fitting a plot of the measured diffusion coefficient versus concentration of β-cyclodextrin. In the fitting equation (1.1) developed by Jensen and Østergaard (Jensen et al 2010) and rewritten in terms of diffusion coefficients, D is the measured diffusion coefficient, D_A is the diffusion coefficient of the analyte, D_{AL} is the diffusion coefficient of the analyte-ligand complex, K is the equilibrium binding constant and L is the concentration of the ligand. In this equation, K and D_{AL} are fitting parameters.

$$D = \frac{D_A + D_{AL}K[L]}{1 + K[L]} \qquad 1.1$$

In another publication, later in 2010, (Bielejewska et al 2010) extended the work of Jensen and Østergaard by using Taylor dispersion to the find the equilibrium binding constants and complex diffusion coefficients of BSA interacting with various ligands at different pH values.

In this work, the focus is to further the validity of this relatively new method for determining binding constants by studying the interactions of different proteins and

ligands. For this work, the proteins of interest are bovine serum albumin (BSA), lysozyme and α-lactalbumin. The ligands of interest are 1-anilinonaphthalene-8-sulfonate (ANS) and ascorbic acid. This thesis is comprised of two specific aims:

1) Establish a Taylor dispersion apparatus for quick and accurate diffusion measurements of proteins, ligands and protein-ligand complexes.
2) Determine equilibrium binding constants of protein-ligand interactions through the use of diffusion coefficients via Taylor dispersion analysis.

Background of ANS Binding to Proteins

In this thesis, the interactions of three different proteins with ANS will be examined. The ligand, ANS, is advantageous to use in binding experiments because it becomes fluorescent when it binds to hydrophobic patches on proteins (Matulis et al 1999; Slavik 1982). The binding in the hydrophobic regions of proteins occurs at the anilinonaphthalene region of ANS (Matulis et al 1999; Slavik 1982). The two important factors that contribute to the ANS fluorescence when bound is that there must not be water present and the geometry of the phenyl ring connected to the anilinonaphthalene region (Matulis et al 1999; Slavik 1982). Water and other low viscous solutions quench the fluorescence because the relaxation time after excitation occurs quickly before emission of a fluorescent photon even happens (Slavik 1982). The conformational hindrance of the phenyl ring also contributes to the lifetime of the fluorescence (Kirk et al 1996). ANS also binds to cations on the protein through its negatively charged sulfonate group (Matulis et al 1998; Matulis et al 1998). That being said, ANS binding is

dependent on pH, which dictates protein cationic charge (Matulis et al 1998; Matulis et al 1998). Changes in pH also allow for proteins to partially unfold and conform to a molten globule state thus revealing other hydrophobic regions (Ray et al 2001).

The first protein to be looked at is BSA, which is a large protein with a molecular weight of around 66.5 kDa and makes up about 60% of the serum (Togashi et al 2008). BSA is a transport protein, responsible for moving things such as fatty acids and metabolites (Togashi et al 2008). BSA is useful protein to study not only because of its abundance, but because it is 76% homologous to human serum albumin (HSA). The key difference between BSA and HSA is that BSA contains two tryptophan amino acids whereas HSA only has one (Togashi et al 2008). In terms of ANS binding, BSA has five hydrophobic binding sites in the pH range of 5 to 7 (Daniel et al 1966) and 100 cationic binding sites and 12 hydrophobic patches at pH 2.5 (Matulis et al 1998; Matulis et al 1999).

Another protein that will be used is lysozyme from hen egg white. Lysozyme is considerably smaller than BSA having an approximate molecular weight of 14.3 kDa and serves an important function of breaking down bacterial cell walls in biological systems. Lysozyme is unique in that it is fairly compact because of four disulfide bonds that prevent the protein from expanding even at low pH values (Ray et al 2001). However, reduction of these disulfide bonds allows the protein to be partially folded thus allowing for more ANS binding in hydrophobic regions (Ray et al 2001; Ramen et al 1996). In its oxidized (non-reduced) state, Lysozyme does have a cationic charge of 19 at pH 2.0, thus allowing for some ANS binding (Matulis et al 1998).

The last protein to be studied is α-lactalbumin (14.1 kDa) which is structurally similar to lysozyme even including four disulfide links (Permyakov et al 2000; Ray et al 2001; Kuwajima 1996). α-lactalbumin is found in mammalian milk and is involved in modifying galactosyltransferase which leads to the production of lactose (Kuwajima 1996). Unlike lysozyme, oxidized (native) α-lactalbumin forms a molten globule conformation at low pH values and has been shown to have four hydrophobic regions for ANS binding (Permyakov et al 2000; Ray et al 2001). In addition at a pH of 2.5, there are 20 possible cationic binding sites as well.

Background of Ascorbic Acid Binding to BSA

Another key feature of this thesis is to investigate a different ligand binding to a protein. For this scenario, ascorbic acid, commonly known as vitamin C will be used as the ligand and will interact with BSA. It has been found through ITC, uv-vis spectroscopy and FTIR that ascorbic acid binds to BSA non-specifically at multiple sites on the surface through hydrogen bonding, hydrophilic interactions and van der Waals forces (Li et al 2014; Nafisi et al 2011). However to the best of our knowledge, no one has explored ascorbic acid binding to BSA through Taylor dispersion analysis.

Chapter 2 – Theory of Taylor Dispersion

Introduction

In 1911, Albert Griffiths was the first person to observe the dispersion process when he experimented with weak concentrations of fluorescein dye injected into a capillary tube carrying water (Griffiths 1911). Forty-two years later, Griffith's initial findings lead British scientist Sir Geoffrey Taylor to develop a mathematical reasoning of the dispersion process (Taylor 1953).

In a typical Taylor dispersion experiment, a carrier solution flows laminarly in a narrow capillary tube with a parabolic velocity profile given in eq. 2.1.

$$v = 2\bar{v}\left[1 - \left(\frac{r}{R}\right)^2\right] \tag{2.1}$$

Once a fully developed flow is created, a bolus injection of some species is made into the carrier stream. The injected species is subjected to convective transport axially and diffusive transport radially as it disperses downstream. As the species disperses, particles in the fast flowing regions near the front will diffuse outward while particles in the slower flowing regions near the rear will diffuse inward. After an adequate amount of residence time the species is fully dispersed and travels with the average velocity of the carrier solution. The concentration profile of the dispersed species may be measured downstream, generally with a UV-Vis or fluorescent spectrometer. In most binary Taylor Dispersion experiments, the concentration profile configures to a Gaussian curve.

Diffusion Coefficient

The diffusion coefficient in one dimension is defined by Crank "as the rate of transfer of the diffusing substance across unit area of a section, divided by the space gradient of concentration at the section" (Crank 1975). The diffusion coefficient was first introduced in Fick's law (1855) which describes the mass flux J as the diffusion coefficient, D, multiplied by the concentration gradient ∇C (Eq. 2.2). The diffusion coefficient has units of length squared per time.

$$J = -D\nabla C \tag{2.2}$$

Dispersion Coefficient

The dispersion coefficient originally derived by Taylor (Taylor 1953) and also derived by Cussler (Cussler 1997), describes the spreading process of the species, and is inversely proportional to the diffusion coefficient. Thus, a large value for the dispersion coefficient means that there is slower diffusion whereas a small value represents higher diffusion. The dispersion coefficient may be derived by starting with the governing convective-diffusion equation (Eq. 2.3).

$$\frac{\partial c}{\partial t} = \nabla \cdot (\mathrm{D}\nabla \mathrm{c}) - \nabla \cdot (\vec{v}c) + R \tag{2.3}$$

Using the cylindrical coordinate system and accounting for concentration changes in the radial (r) and axial (z) directions, the convective-diffusion equation reduces down to Eq. 2.4 after substituting in the parabolic velocity profile.

$$\frac{\partial c}{\partial t} = \frac{D}{r}\frac{\partial}{\partial r}r\frac{\partial c}{\partial r} - 2\bar{v}\left[1 - \left(\frac{r}{R}\right)^2\right]\frac{\partial c}{\partial z} \tag{2.4}$$

The boundary conditions for this equation are:

$$t = 0 \qquad z = 0 \qquad c = \left(\frac{M}{\pi R^2}\right)\delta(z) \tag{2.5}$$

$$t > 0 \qquad r = R \qquad \frac{\partial c}{\partial r} = 0 \tag{2.6}$$

$$t > 0 \qquad r = 0 \qquad \frac{\partial c}{\partial r} = 0 \tag{2.7}$$

Then using dimensionless analysis, we may define dimensionless parameters as:

$$\eta = \frac{r}{R} \rightarrow \partial r = R\partial\eta \tag{2.8}$$

$$\zeta = \frac{z - \bar{v}t}{R} \rightarrow \partial z = (R + \bar{v}t)\partial\zeta \tag{2.9}$$

and substitute the parameters into Eq. 2.4.

$$\frac{D}{\eta}\frac{\partial}{\partial\eta}\left(\eta\frac{\partial c}{\partial\eta}\right) = 2\bar{v}R\left(\frac{1}{2} - \eta^2\right)\frac{\partial c}{\partial\zeta} \tag{2.10}$$

After integrating Eq. 2.10 twice with respect to η, Eq. 2.11 is created where A is a constant of integration.

$$\frac{\partial c}{\partial\eta} = \frac{2\bar{v}R}{D}\left(\frac{1}{4}\eta - \frac{1}{4}\eta^3\right)\frac{\partial c}{\partial\zeta} + A \tag{2.11}$$

Applying boundary condition Eq. 2.7

$$r = 0 \qquad \eta = 0 \qquad \therefore A = 0 \tag{2.12}$$

$$\frac{\partial c}{\partial\eta} = \frac{2\bar{v}R}{D}\left(\frac{1}{4}\eta - \frac{1}{4}\eta^3\right)\frac{\partial c}{\partial\zeta} \tag{2.13}$$

Further integration leads to Eq. 2.14 where B is another constant of integration.

$$c = \frac{\bar{v}R}{4D}\frac{\partial c}{\partial\zeta}\Big|_{\eta=0}\left(\eta^2 - \frac{1}{2}\eta^4\right) + B \tag{2.14}$$

This expression gives the concentration at a local point, however for this problem we are concerned with the average concentration across the capillary tube. The average concentration can be expressed as Eq 2.15.

$$\bar{c}(z) = \frac{1}{\pi R^2}\int_0^R 2\pi r c(r,z)dr \tag{2.15}$$

After plugging in for concentration

$$\bar{c}(\zeta) = \frac{1}{\pi R^2}\int_0^1 2\pi R\eta\left[\frac{\bar{v}R}{4D}\frac{\partial c}{\partial \zeta}\left(\eta^2 - \frac{1}{2}\eta^4\right) + B\right]\partial\eta \tag{2.16}$$

Integration and evaluation at the limits of integration lead to

$$\bar{c}(\zeta) = \frac{\bar{v}R^2}{12D}\frac{\partial c}{\partial \zeta} + B \tag{2.17}$$

Then solving for B

$$B = \bar{c}(\zeta) - \frac{\bar{v}R^2}{12D}\frac{\partial c}{\partial \zeta} \tag{2.18}$$

Plugging B back into Eq. 2.16 gives

$$\bar{c} = \bar{c}(\zeta) + \frac{\bar{v}R^2}{4D}\frac{\partial c}{\partial \zeta}\left[-\frac{1}{3} + \eta^2 - \frac{1}{2}\eta^4\right] \tag{2.19}$$

The average flux across the system may then be described as

$$J = \frac{1}{\pi R^2}\int_0^R 2\pi r\bar{c}\bar{v}dr \tag{2.20}$$

After integration, the resultant flux is

$$J = -\frac{(\bar{v}R)^2}{48D}\frac{\partial \bar{c}}{\partial \zeta} \tag{2.21}$$

Because mass is conserved throughout the dispersion process, the continuity equation holds giving the final result as

$$\frac{\partial \bar{c}}{\partial t} = \frac{(\bar{v}R)^2}{48D}\frac{\partial^2 \bar{c}}{\partial \zeta^2} \tag{2.22}$$

Where the dispersion coefficient E is represented by

$$E = \frac{(\bar{v}R)^2}{48D} \tag{2.23}$$

In Taylor's work, he examined the dispersion process under a high Péclet number, however Rutherford Aris wrote a paper in 1956 (Aris 1956) that expanded Taylor's works and considered dispersion in systems where convection was lower. This lead Aris to use moment's analysis to derive a new dispersion coefficient that takes in account axial diffusion (Aris 1956).

$$E = D + \frac{(\bar{v}R)^2}{48D} \tag{2.24}$$

Experimental Criteria

In addition to the theoretical understanding of Taylor dispersion, practical considerations for a Taylor dispersion experiment were developed most notably by Leaist and Alizadeh. Alizadeh et al 1980 found that the laminar flow needs to be bound by:

$$700\frac{D}{r} < v < \frac{DL}{0.6r^2} \tag{2.25}$$

In this parameter, D is the diffusion coefficient, r is the radius and of the capillary tube and L is the length of the tube.

Alizadeh et al 1980 also provided an expression for the concentration profile at the point of detection for a binary system as:

$$\Delta c(t) = c(t) - \bar{c} = \frac{2\delta}{\pi r^3 v}\left(\frac{3D}{\pi t_r}\right)^{\frac{1}{2}} \exp\left[-\frac{12Dv(t-t_r)^2}{r^2 L}\right] \tag{2.26}$$

where $\bar{c}$ is the average concentration of the carrier stream, t_r is the residence time $(t_r = \frac{L}{v})$ and δ is the excess moles in the injection solution. $\delta = V(c - \bar{c})$, where c is the concentration in the injection and V is the volume of the injection solution.

Using the concentration profile expression developed by Alizadeh et al 1980, Derek Leaist created an equation to fit the raw data from a Taylor dispersion experiment (Leaist et al 1992).

$$v(t) = v_\infty + v_1 t + v_{max}\sqrt{\frac{t_R}{t}} \exp\left[-\frac{12D(t-t_r)^2}{r^2 t}\right] \tag{2.27}$$

In this equation, $v(t)$ is the output voltage as a function of time, $v_\infty$and v_1 account for drifts in the baseline, v_{max} is the peak voltage, t_r is the residence time, r is the radius of the tube and D is the diffusion coefficient. $v_\infty, v_1, v_{max}, t_r \; and \; D$ are regression parameters. Callendar and Leaist and later developed an easier model to extract out diffusion coefficients using the peak width of the voltage output (Callendar et al 2006).

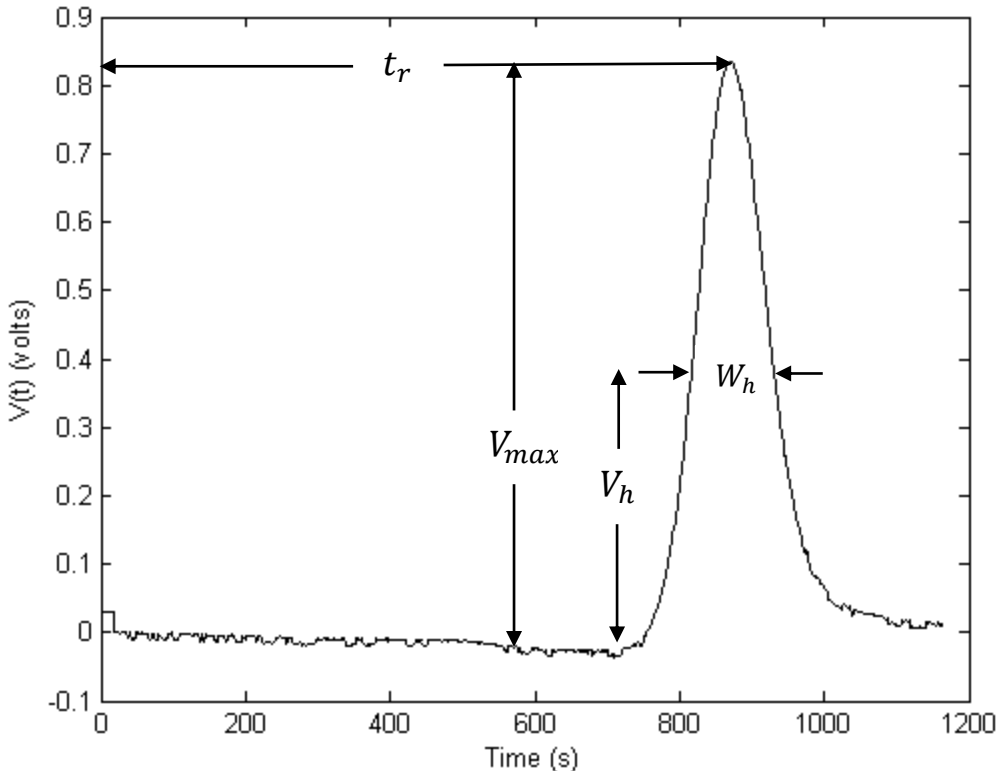

Figure 2.1 Another method for determining the diffusion coefficient from a typical Taylor dispersion response. This figure was adapted by Callendar et al 2006.

$$h = \frac{V_h}{V_{max}} \tag{2.28}$$

$$D = -\frac{lnh}{3}\frac{r^2 t_r}{W_h^2} \tag{2.29}$$

In this equation h is the ratio of V_h over V_{max}, r is the radius of the capillary tube, t_r is the residence time and W_h is the width of the peak.

Other experimental criteria for a Taylor dispersion experiment include limits on the volume of the injection sample relative to the total volume of the capillary tube (Evans et al 1965).

$$\frac{V_{capillary}}{V_{injection}} > 100 \tag{2.30}$$

Chapter 3 – Experimental Methods

Apparatus

The Taylor dispersion apparatus consists of four main components: 1) a high pressure syringe pump, 2) a low volume injection valve, 3) a uv-vis detector, 4) and a personal computer for data collection. In this setup, the syringe pump (ISCO®, model: 100DM, Lincoln, NE) is used to pump the carrier solution. A HPLC pump should suffice should a high pressure syringe pump not be available. Flexible fused silica capillary tubing (ID = 0.01 cm, Length = 975 cm) (Polymicro Technologies™, model: TSU100375, Phoenix, AZ) with a deep-uv coating allowing 90% transmission at 214nm was used for the carrier solution and sample. The capillary tubing was positioned in a coil with a diameter of 24.13cm. 0.52μL injections of sample were made using a 2 position, 6-port valve (Rheodyne®, model: MXP9900-000, Rohnert Park, CA). The concentration profile at the output was measured using a uv-vis detector (ISCO®, model 3850, Lincoln, NE) as part of a capillary electrophoresis instrument. The voltage signal was collected on a personal computer with LabView® (National Instruments, version 2013, Austin, TX), with a sample frequency of one data point per second.

Spectroscopy Measurements

Spectroscopy measurements of BSA, lysozyme, α-lactalbumin, ANS and ascorbic acid were made to determine the wavelengths at which each species absorbs at. Each sample was measured at a concentration of 1.5×10^{-6} M. Full spectrums of each species

was collected from 250nm to 800nm using a uv-vis spectrometer (Agilent, model Cary 50 BIO, Santa Clara, CA).

Operating Conditions

The operating flow rate was 0.006 ml/min for the protein-ligand experiments and was 0.06 ml/min for the binary ligand measurements. These flow rates were chosen based on the limits presented by (Alizadeh et al 1980). For the protein-ligand interaction experiments, the detector was set to collect at 355nm with an integration time of 0.8 seconds. For the binary experiments with the proteins and ligands the detector was set to collect at 280nm with an integration time of 0.8 seconds. The baseline was zeroed before each run. All experiments were conducted at room temperature.

Protein and Ligand Samples

Fresh protein samples of BSA (RPI, Lot# 33055), lysozyme (Sigma, Lot# SLBG8654V) and α-lactalbumin (Sigma, Lot# 012M7000V) were made prior to each experiment. The protein concentration for each experiment was 4.51×10^{-5} M and was made in a 0.15 M NaCl (RPI, Lot# 29800) solution. The pH for BSA was adjusted to 7.4 and 2.5, lysozyme was adjusted to pH 7.0 and α-lactalbumin was adjusted to pH 2.5. The pH adjustments were made with either 1 M NaOH or HCl.

A stock solution of 3.10×10^{-3} M was made by dissolving ANS (Sigma, Lot# SLBB6154V) in nanopure water. The concentration of the stock solution was verified with the extinction coefficient for ANS ($\varepsilon_{350nm} = 4.95 \times 10^{3}$ M^{-1} cm^{-1}; Haskard et al 1998).

ANS solutions were made by diluting the stock solution to concentrations ranging from $0.15x10^{-3}$ M to $1.5x10^{-3}$ M. The pH was adjust to 2.5 for each solution.

The ascorbic acid (Sigma, Lot# 031M0164V) solution was prepared by dissolving L-ascorbic acid in nanopure water, making a stock solution with a concentration of $3.10x10^{-3}$ M at pH 7.4. The concentration of the stock solution was verified with the extinction coefficient for ascorbic acid ($\varepsilon_{265nm} = 1.45x10^{4}$ M^{-1} cm^{-1}; Buettner 1988). Ascorbic acid samples were prepared by diluting the stock solution to concentrations ranging from $0.15x10^{-3}$ M to $1.5x10^{-3}$ M. All solutions were filter with a 0.22µm filter before use.

Fluorescence Measurements

Fluorescence measurements were made for ANS bound to the three different proteins. For this, the fluid containing both the carrier protein and the ANS injection were collected in a clean vial for each concentration of ANS. The fluid was collected for three trials over the period of one hour. The total volume in each vial was approximately 200µL. Of the 200µL, only 150µL were used in the fluorescent measurements. Each sample was place in a separate well of a 96-well plate. The fluorescence was measured using a plate reader (Tecan, model F200, San Jose, CA). The excitation was set at 340nm with a bandwidth of ± 20nm and the emission was set at 485nm with a bandwidth of ± 20nm. Fluorescence measurements were also taken of ANS in the absence of the protein and the protein in absence of ANS.

Data Processing

The raw data was fitted to equation 2.27 (from chapter 2) in Table Curve 2D (Systat Software,version 5.01, San Jose, CA) to extract out the diffusion coefficient. In Table Curve 2D, the regression procedure follows the Levenberg-Marquartdt algorithm and minimizes the error using least squares. The algorithm in most cases iterates through five to ten times before a best fit is achieved. Extra data points in the baseline leading up to and after the peak were removed before regression. The diffusion coefficients were then plotted versus concentration of the ligand which was fitted to equation 1.1 to extract out the equilibrium binding constant and the diffusion coefficient of the ligand bound to the protein.

Chapter 4 – Results and Discussion

Accuracy of Diffusion Coefficient Measurements

The precision of the diffusion coefficient measurements from the Taylor dispersion apparatus was confirmed by looking at the diffusion coefficient of BSA at pH 7.4. BSA was chosen to validate the system because its diffusion coefficient has been widely published in literature. The diffusion coefficient for BSA can vary, ranging anywhere from 4.3×10^{-7} cm^2/s to 9.5×10^{-7} cm^2/s depending on pH and ionic strength (Meechai et al 1999; Bielejewska et al 2010; Raj et al 1974). For our system, the average diffusion coefficient of BSA at pH 7.4 at an ionic strength of 0.15M for three trials is 4.51×10^{-7} cm^2/s. This certifies that the Taylor dispersion apparatus is providing accurate measurements for diffusion coefficients.

Spectroscopy Measurements

Absorbance measurements were made for each protein and ligand to see at which wavelengths they could be detected at. This was done so we would know at which wavelength to collect the concentration distribution in the Taylor dispersion experiments. For BSA, lysozyme and α-lactalbumin, the peak absorbance was around 280nm. Absorbance in proteins at 280nm is generally from amino acids with aromatic rings such as tyrosine and tryptophan (Anthis et al 2013). ANS absorbs at two wavelengths, 280nm and 355nm. The peak at 280nm has an optical density much higher than the peak at 355nm. Ascorbic acid has an absorbance at 280nm. The spectra of each protein and ligand may be seen in figures 4.1-4.5.

Single Species Diffusion Coefficients

The diffusion coefficient for each protein and ligand was measured independently. For the protein readings, the carrier solution had a concentration of $4.51x10^{-5}$ M of the protein of interest and the concentration of the injection was $0.5x10^{-3}$ M. The diffusion coefficients of the proteins were taken at different pH values. Lysozyme was taken at pH 7.0, α-lactalbumin was taken at pH 2.5 and BSA was taken at pH 2.5 and 7.4. After three trials for each protein the average diffusion coefficient for lysozyme is $6.87x10^{-7}$ cm^2/s, α-lactalbumin is $6.93x10^{-7}$ cm^2/s, BSA pH 2.5 is $4.13x10^{-7}$ cm^2/s and BSA pH 7.4 is $4.51x10^{-7}$ cm^2/s. Literature suggests that the diffusion coefficient for BSA at pH 2.5 is lower than at pH 7.4. This is because at pH 2.5, BSA is in an acid expanded conformation (Matulis et al 1999). In this conformation, positive charges from titratable side chains cause repulsion, thus making BSA a more bulky molecule. The diffusion coefficients of the ligands were obtained in a similar fashion as the proteins. The concentration of the carrier stream was $4.51x10^{-5}$ M of the ligand of interest and the injection concentration was $0.5x10^{-3}$ M. Both ligands were collected at 280nm. The average (n=3) diffusion coefficient for ascorbic acid came out to be $6.26x10^{-6}$ cm^2/s. For ANS, the average (n=3) diffusion coefficient is $4.66x10^{-6}$ cm^2/s. After an elaborate literature search, a diffusion coefficient for ANS could not be found. However, given its size (ANS molecular weight is 299 g/mol), the diffusion coefficient of ANS is comparable to other species with similar molecular weights such as warfarin whose molecular weight is 308 g/mol and its diffusion coefficient is $4.75x10^{-6}$ cm^2/s (Bielejewska et al 2010). In addition, a diffusion coefficient for ANS was calculated to be

4.54 x10^{-6} cm^2/s based on its molecular weight (Evans et al 2013). Overall, the diffusion coefficients obtained here are in a good agreement with the literature values. All of the diffusion coefficients for each trial and their value in literature may be found in table 4.1. A typical output response of a Taylor dispersion experiment for each protein and ligand may be found in figures 4.6-4.11.

Protein Binding to Ligand

To study the interactions between protein and ligand, the carrier stream was a constant concentration (4.51x10^{-5} M) of the protein and seven to eight different concentrations (0.05x10^{-3} M to 1.5x10^{-3} M) of ligand were injected. Three trials were conducted for each concentration. As the concentrations of the ligand increased, the diffusion coefficients would decrease exponentially until it eventually reached a constant value. The typical response for the proteins of interest interacting with its ligand in a Taylor dispersion experiment may be found in figures 4.12-4.14. The equilibrium binding constant and the diffusion coefficient of the ligand interaction with the protein is obtained by fitting the measured diffusion coefficients versus concentration of ligand (figures 4.15-4.17) to equation 4.1 (Jensen et al 2010; Bielejewska et al 2010).

$$D = \frac{D_{Ligand} + D_{Protein-Ligand} K_{eq}[L]}{1 + K_{eq}[L]} \tag{4.1}$$

For BSA at pH 2.5 interacting with ANS the equilibrium constant determined from the diffusion coefficients is 4.00 (± 0.55) $x10^4$ M^{-1}. This value is in good agreement with the literature equilibrium constant ($2.00x10^5$; Moller et al 2002) for BSA-ANS at pH 3.0. The diffusion coefficient of ANS bound to BSA was determined to be 8.31 (± 0.55) $x10^{-7}$ cm^2/s. This is in good agreement with warfarin (similar molecular weight as ANS) bound to BSA which has a complex diffusion coefficient of $1.47x10^{-6}$ cm^2/s (Bielejewska et al 2010). Logically, it makes sense too that the diffusion coefficient for the complex species is smaller than free ligand because of its increased size. Lysozyme pH 7.0 interacting with ANS has an equilibrium constant of 3.69 (± 0.49) $x10^3$ M^{-1} and a complex diffusion coefficient of 4.65 (± 1.62) $x10^{-7}$ cm^2/s. As discussed in chapter one, oxidized lysozyme doesn't have any hydrophobic binding regions for ANS however at pH 7.0 it does have a positive cationic charge, contributing to the ANS binding through the sulfonate group. The binding constant and complex diffusion coefficient for α-lactalbumin at pH 2.5 is 5.23 (± 0.83) $x10^3$ M^{-1} and 7.39 (± 2.86) $x10^{-7}$ cm^2/s respectfully. In comparing the binding constants of lysozyme-ANS and α-lactalbumin-ANS (both proteins have a molecular weight around 14 kDa), the data makes sense because α-lactalbumin conforms to a molten globule state at low pH thus allowing more sites for ANS binding as discussed in chapter one. In addition at low pH, α-lactalbumin has a higher cationic charge allowing for more binding which is revealed in the binding constant being larger and for lysozyme-ANS. Table 4.2 provides a summary of binding constants and the complex diffusion coefficients.

The other ligand that was studied in this work was ascorbic acid interacting with a BSA (Figure 4.18). The pH of BSA and ascorbic acid was 7.4. The obtained binding constant for ascorbic acid bound to BSA is 3.36 (± 0.61) $x10^3$ M^{-1} and the complex diffusion coefficient is 1.49 (± 0.26) $x10^{-6}$ cm^2/s (Figure 4.19). The binding constant for BSA-ascorbic acid at pH 7.2 in literature is $1.57x10^4$ M^{-1} (Nafisi et al 2011), which is comparable to the value we obtain using Taylor dispersion analysis.

Fluorescence Measurements

With knowledge that ANS becomes fluorescent when it binds to hydrophobic patches on proteins (Matulis et al 1999; Matulis et al 1998) fluorescence readings were conducted for BSA-ANS, lysozyme-ANS and α-lactalbumin-ANS. These fluorescence measurements were done to further validate that binding is occurring in our system. In the fluorescence readings we see that there is increased fluorescence in BSA and α-lactalbumin with increasing concentration of ANS. For lysozyme, since we used oxidized form, there are no fluorescent binding sites which is proven true in the fluorescence intensity. Also, readings of ANS and the proteins were taken independently to confirm that there is no fluorescence in the unbound state. The fluorescence data is found in figures 4.20-4.22.

Table 4.1 The binary diffusion coefficients of the proteins and ligands. The diffusion coefficient is presented as the average of three trials

Species	Diffusion Coefficient D ($x10^7$ cm^2/s)	Literature Diffusion Coefficient D ($x10^7$ cm^2/s)	Reference
BSA pH 2.5	4.13 (± 0.09)	4.3 – 9.5	Meechai et al 1999 Bielejewska et al 2010 Raj et al 1974
BSA pH 7.4	4.51 (± 0.20)	4.3 – 9.5	Meechai et al 1999 Bielejewska et al 2010 Raj et al 1974
Lysozyme pH 7.0	6.87 (± 0.36)	5.4	Tomme et al 2005
α-lactalbumin pH 2.5	6.93 (± 0.34)	10.6	Engel et al 2002
ANS pH 2.5	46.6 (± 3.20)	45.4	Evans et al 2013
Ascorbic Acid pH 7.4	62.6 (± 3.71)	53.2	Robinson et al 1990

Table 4.2 A summary of the diffusion coefficients and the equilibrium binding constants of the ligand bound to the protein (complex)

Species	Complex Diffusion Coefficient D ($x10^7$ cm^2/s)	K_{eq} K_{eq} ($x10^{-4}$ M^{-1})	Literature K_{eq} K_{eq} ($x10^{-5}$ M^{-1})	Reference
BSA-ANS	8.31 (± 0.55)	4.00 (± 0.55)	2.0	Moller et al 2002
Lysozyme-ANS	4.65 (± 1.62)	0.37 (± 0.05)	N/A	N/A
α-lactalbumin-ANS	7.39 (± 2.86)	0.52 (± 0.08)	N/A	N/A
BSA-Ascorbic Acid	14.9 (± 2.60)	0.34 (± 0.06)	0.16	Nafisi et al 2011

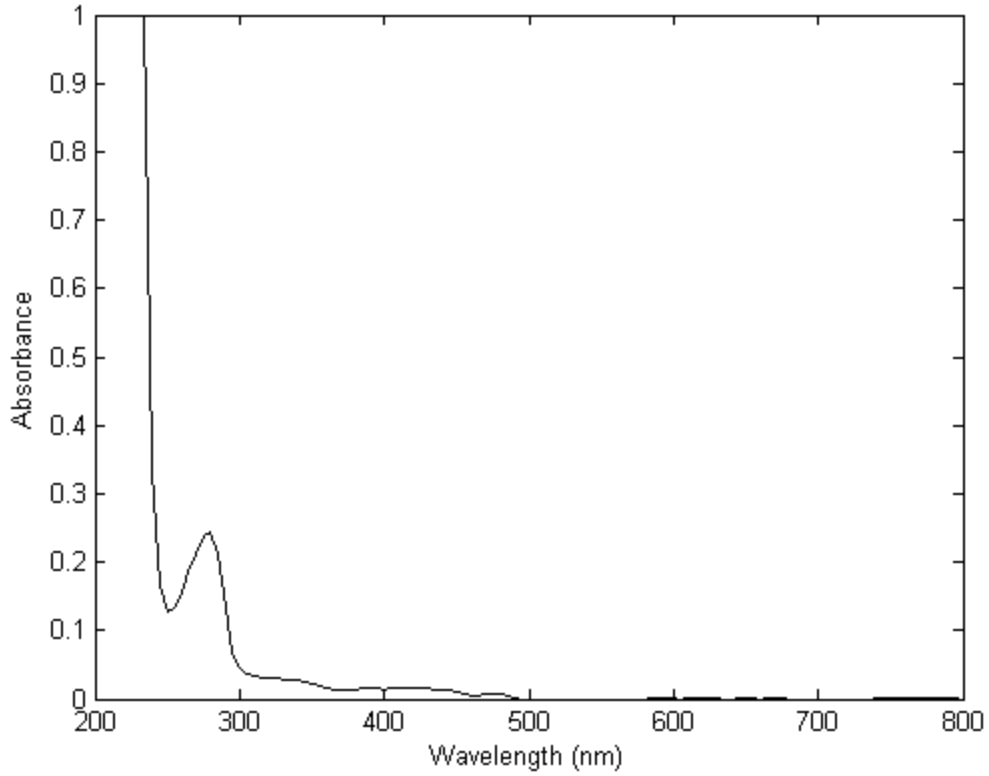

Figure 4.1 The absorbance spectrum of BSA at a concentration of $5x10^{-6}$ M. BSA absorbs at 280nm.

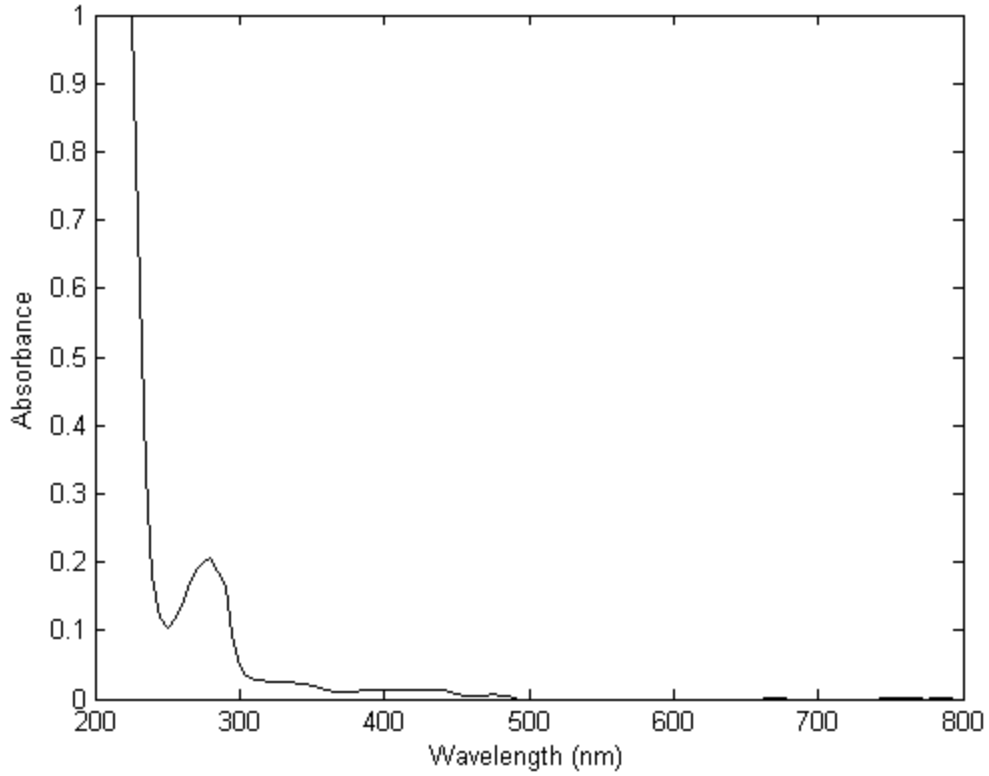

Figure 4.2 The absorbance spectrum of lysozyme at a concentration of $5x10^{-6}$ M. Lysozyme absorbs at 280nm.

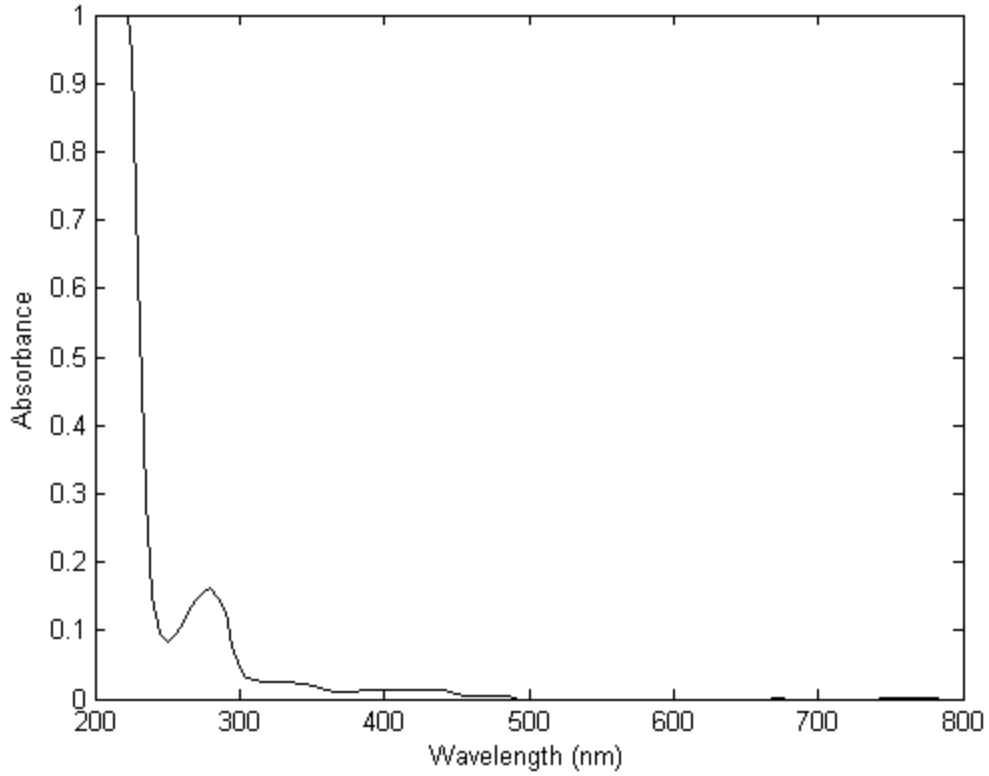

Figure 4.3 The absorbance spectrum of α-lactalbumin at a concentration of $5x10^{-6}$ M. α-lactalbumin absorbs at 280nm.

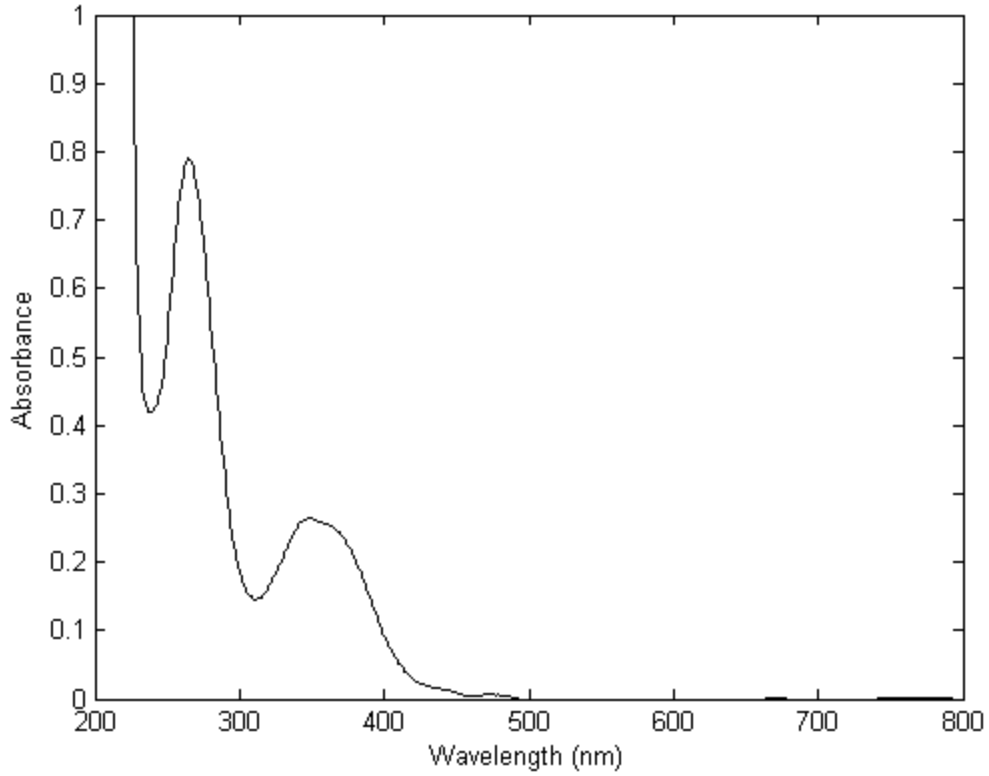

Figure 4.4 The absorbance spectrum of ANS at a concentration of $5x10^{-5}$ M. ANS absorbs at 280nm and at 355nm.

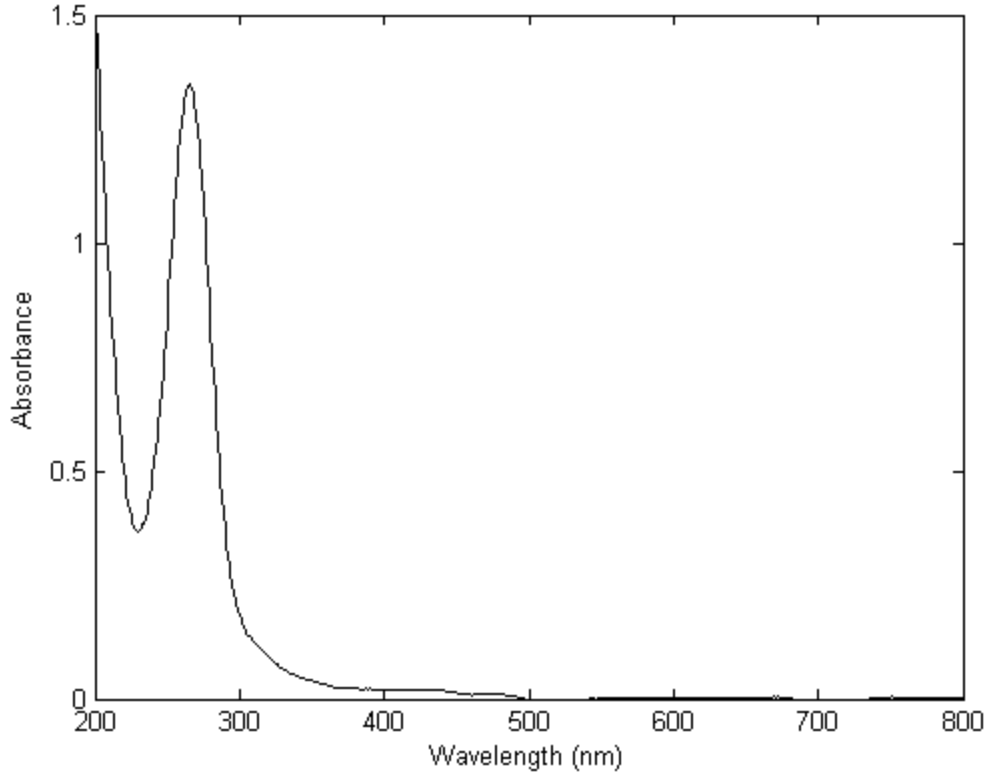

Figure 4.5 The absorbance spectrum of ascorbic acid at a concentration of 1.5×10^{-3} M. Ascorbic acid absorbs at 280nm.

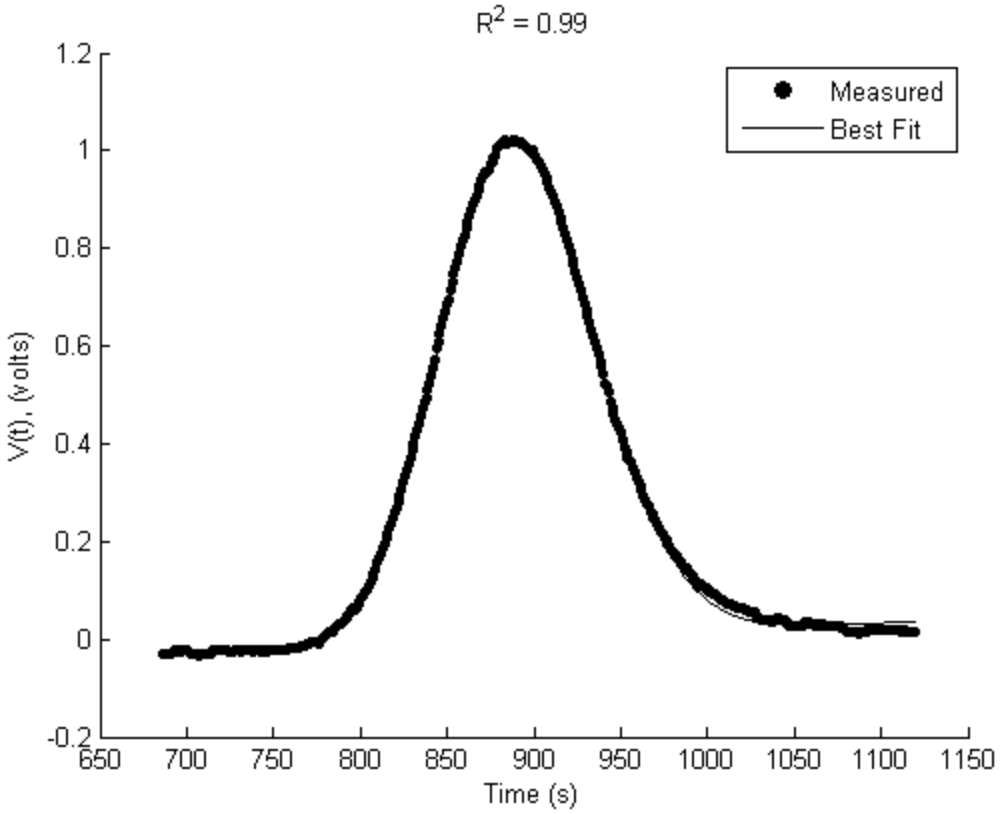

Figure 4.6 A typical output response from a Taylor dispersion experiment for BSA at pH 7.4. In this experiment the carrier solution was 4.51×10^{-5} M BSA at pH 7.4 in 0.15M NaCl and the injection was 0.5×10^{-3} M BSA at pH 7.4 in 0.15M NaCl.

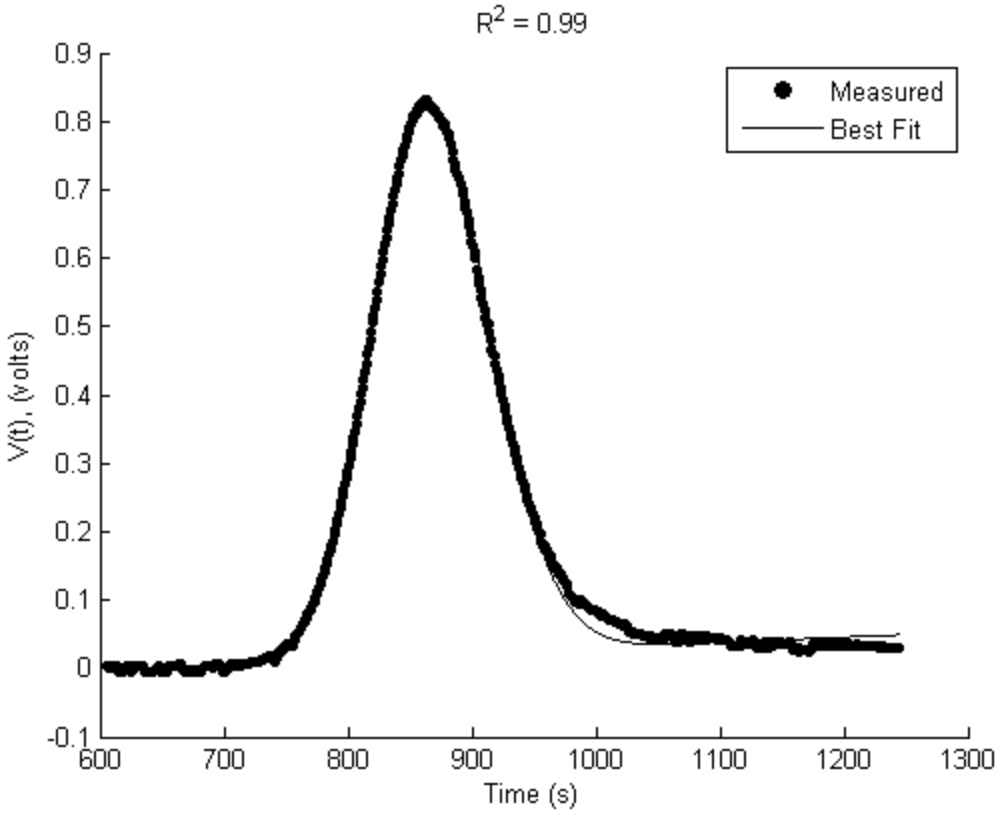

Figure 4.7 A typical output response from a Taylor dispersion experiment for BSA at pH 2.5. In this experiment the carrier solution was 4.51×10^{-5} M BSA at pH 2.5 in 0.15M NaCl and the injection was 0.5×10^{-3} M BSA at pH 2.5 in 0.15M NaCl.

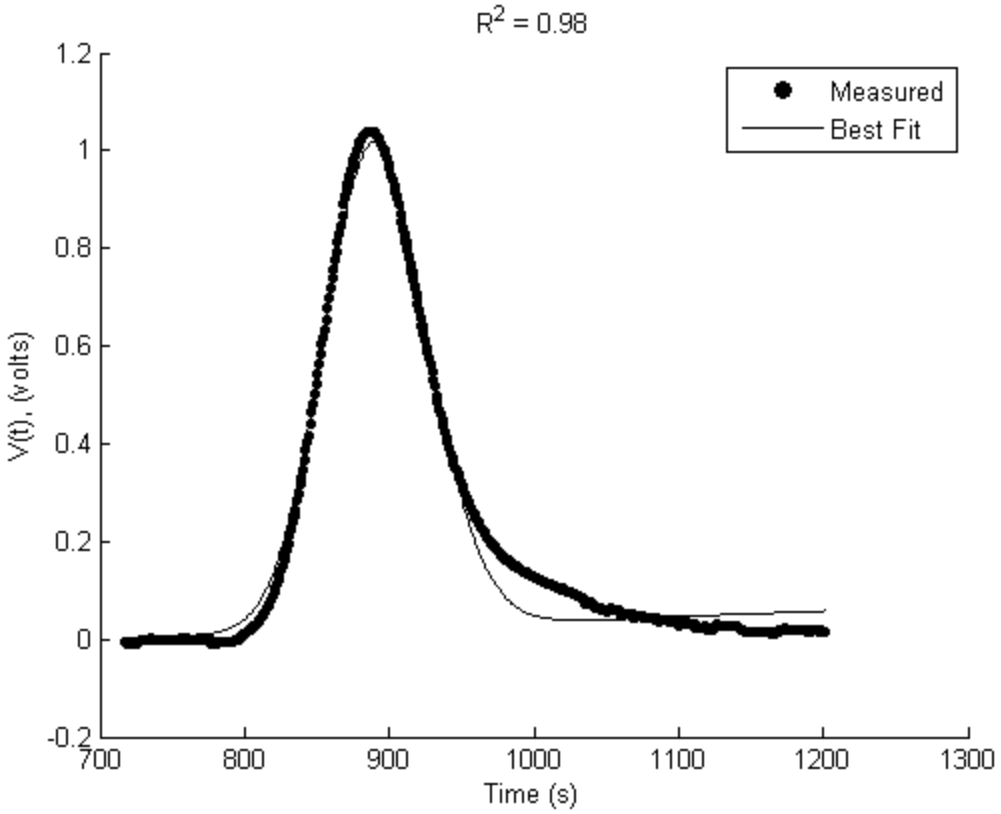

Figure 4.8 A typical output response from a Taylor dispersion experiment for lysozyme at pH 7.0. In this experiment the carrier solution was 4.51×10^{-5} M lysozyme at pH 7.0 in 0.15M NaCl and the injection was 0.5×10^{-3} M lysozyme at pH 7.0 in 0.15M NaCl.

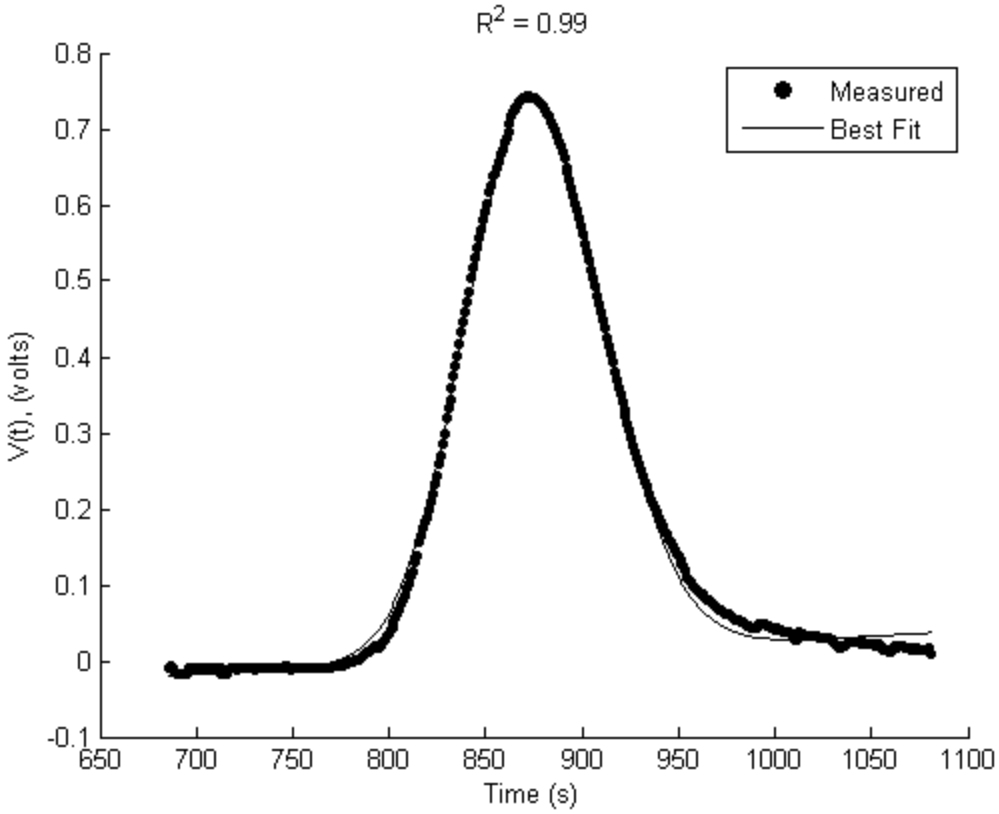

Figure 4.9 A typical output response from a Taylor dispersion experiment for α-lactalbumin at pH 2.5. In this experiment the carrier solution was 4.51×10^{-5} M α-lactalbumin at pH 2.5 in 0.15M NaCl and the injection was 0.5×10^{-3} M α-lactalbumin at pH 2.5 in 0.15M NaCl.

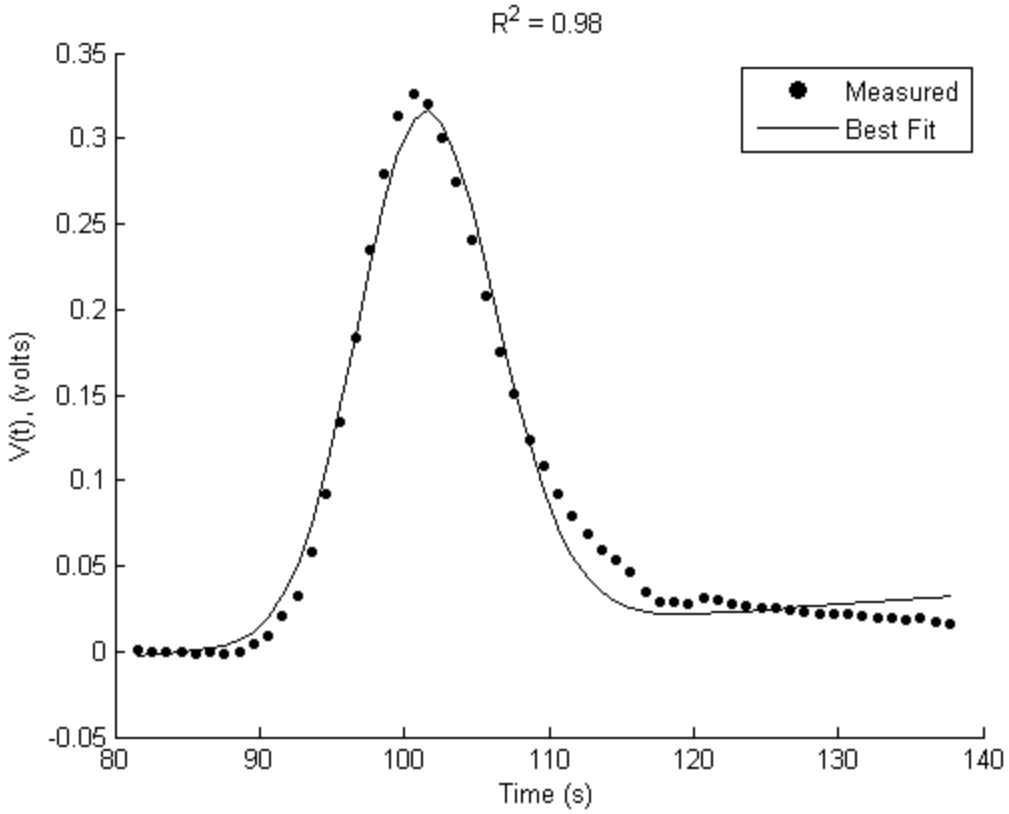

Figure 4.10 A typical output response from a Taylor dispersion experiment for ANS at pH 2.5. In this experiment the carrier solution was 4.51×10^{-5} M ANS at pH 2.5 in nanopure water and the injection was 0.5×10^{-3} M BSA at pH 2.5 in nanopure water.

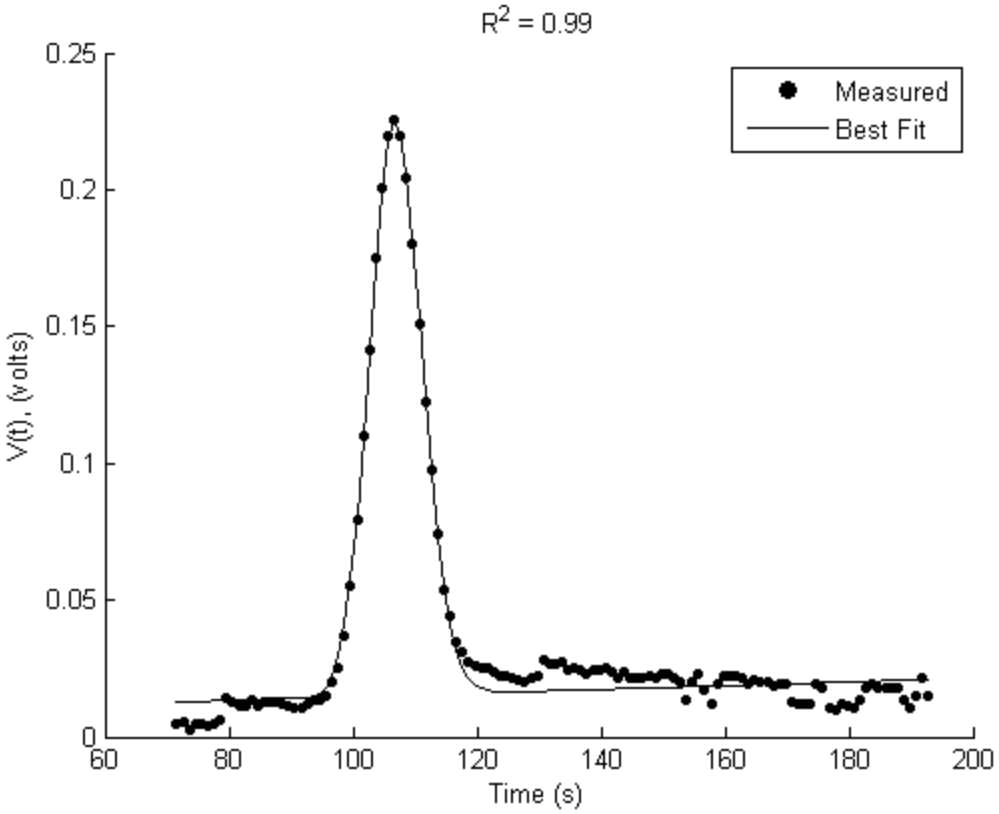

Figure 4.11 A typical output response from a Taylor dispersion experiment for ascorbic acid at pH 7.4. In this experiment the carrier solution was 4.51×10^{-5} M ascorbic acid at pH 7.4 in nanopure water and the injection was 0.5×10^{-3} M ascorbic acid at pH 7.4 in nanopure water.

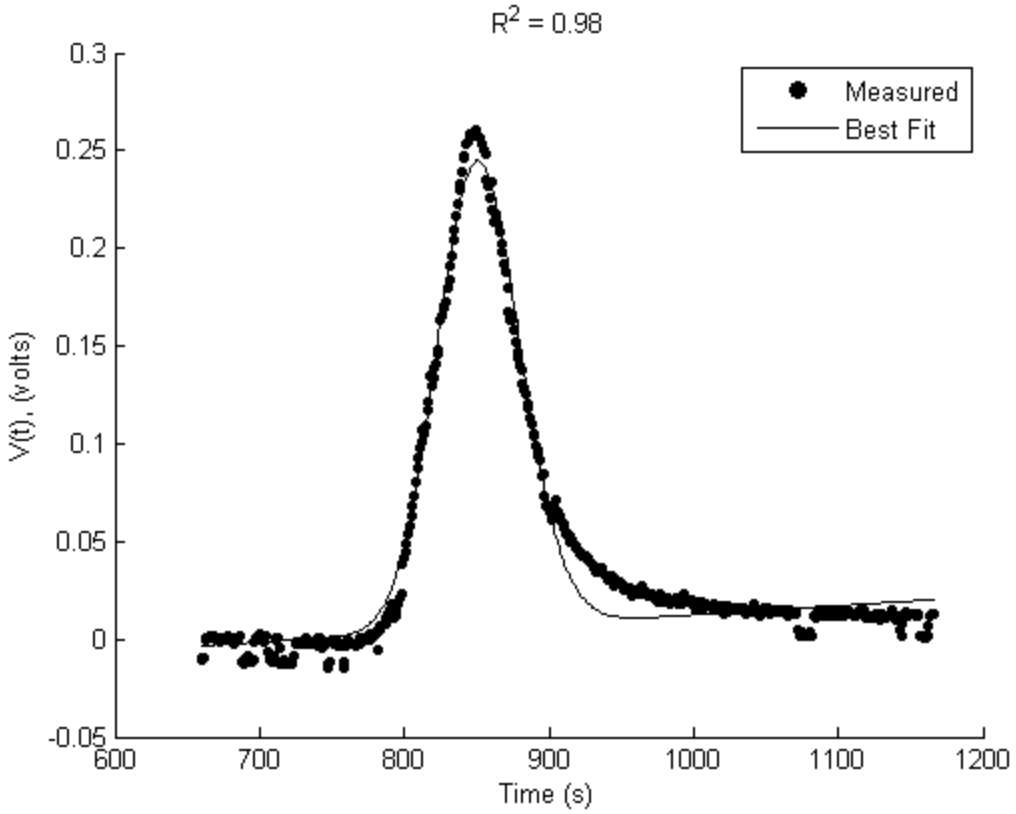

Figure 4.12 A typical output response from a Taylor dispersion experiment for BSA at pH 2.5 interacting with ANS. In this experiment the carrier solution was 4.51×10^{-5} M BSA at pH 2.5 and the injection was 0.45×10^{-3} M ANS.

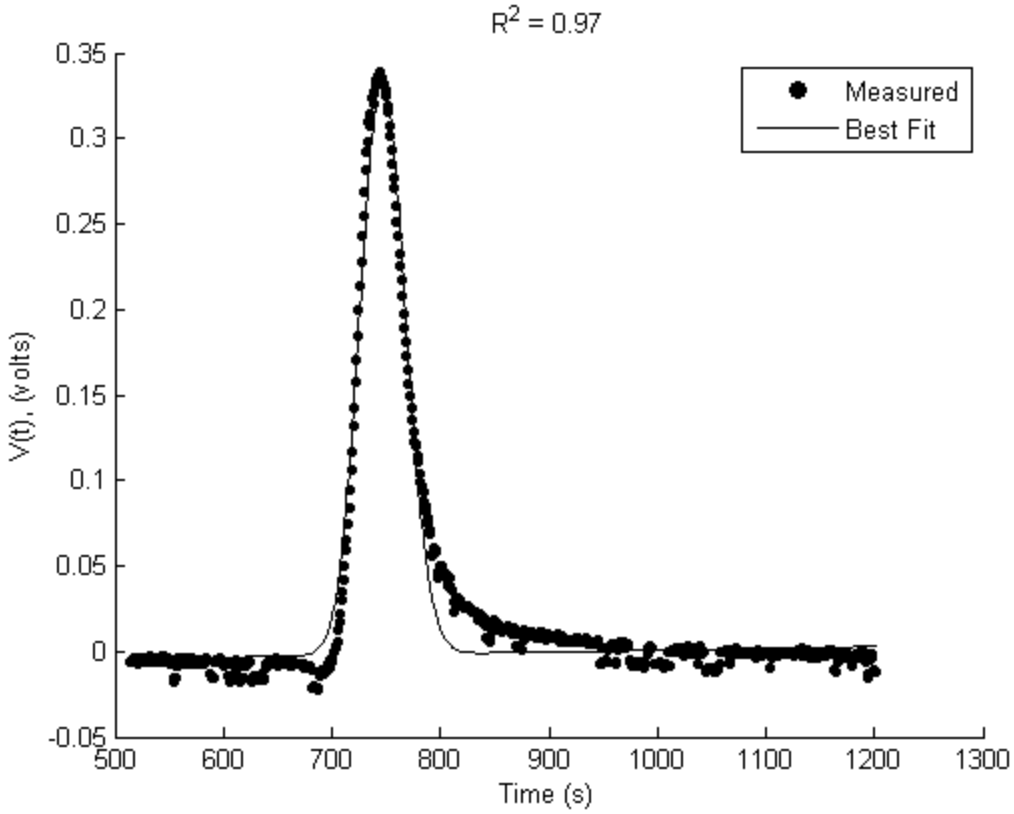

Figure 4.13 A typical output response from a Taylor dispersion experiment for lysozyme at pH 7.0 interacting with ANS. In this experiment the carrier solution was 4.51×10^{-5} M lysozyme at pH 7.0 and the injection was 0.45×10^{-3} M ANS.

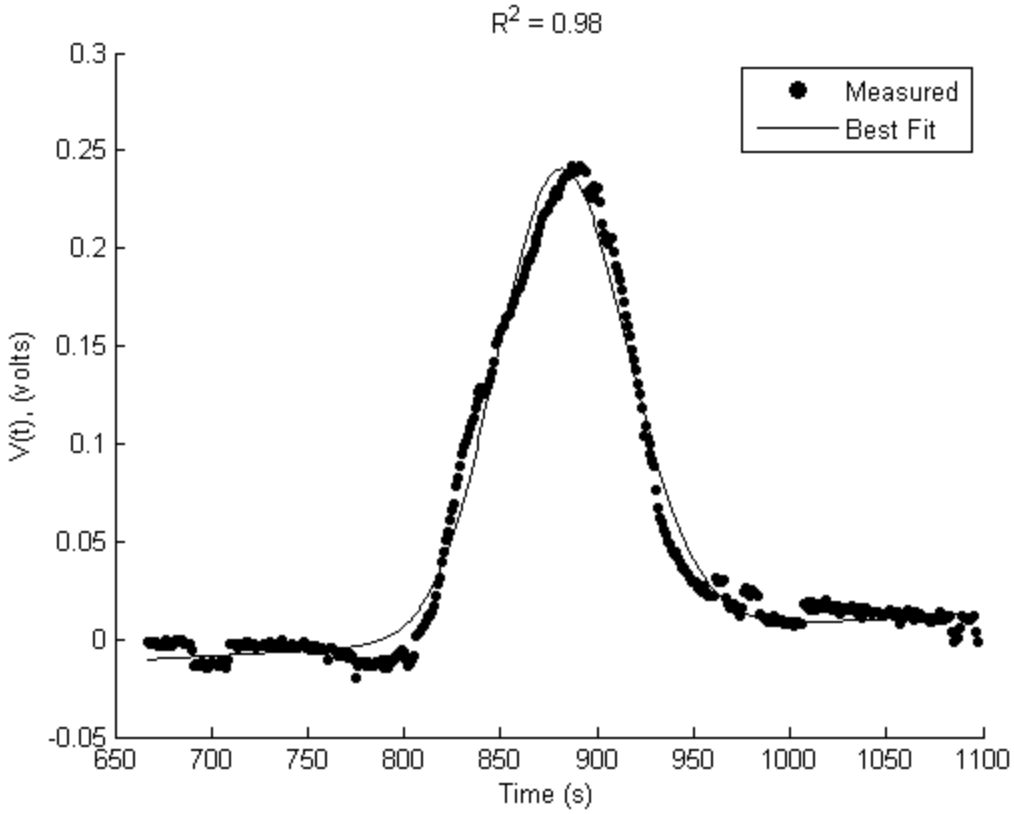

Figure 4.14 A typical output response from a Taylor dispersion experiment for α-lactalbumin at pH 2.5 interacting with ANS. In this experiment the carrier solution was 4.51×10^{-5} M α-lactalbumin at pH 2.5 and the injection was 0.45×10^{-3} M ANS.

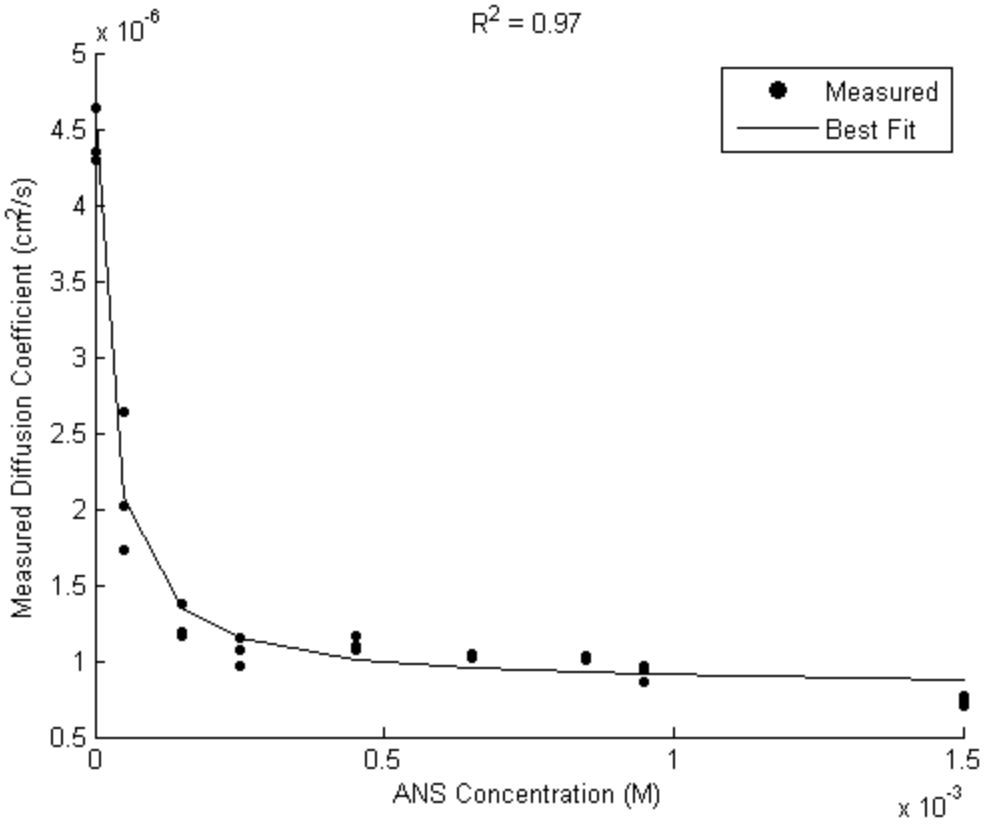

Figure 4.15 The measured diffusion coefficients of BSA interacting with ANS at pH 2.5 versus ANS concentration. The best fit provides the binding constant and the complex diffusion coefficient.

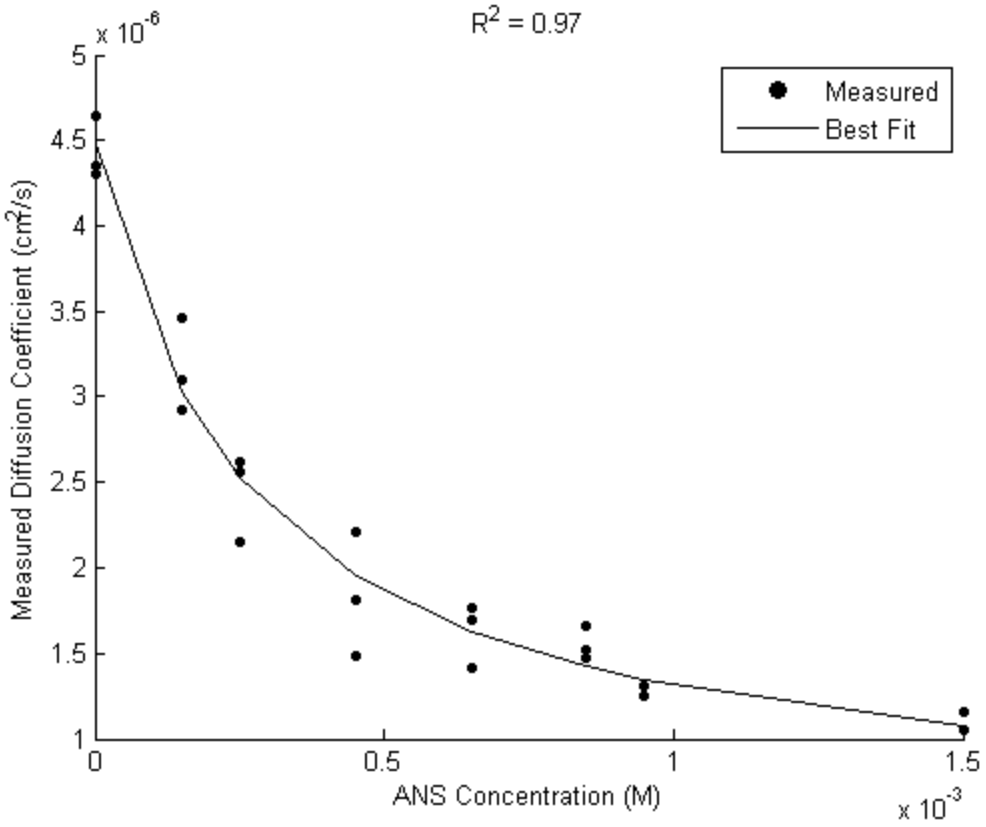

Figure 4.16 The measured diffusion coefficients of lysozyme interacting with ANS at pH 7.0 versus ANS concentration. The best fit provides the binding constant and the complex diffusion coefficient.

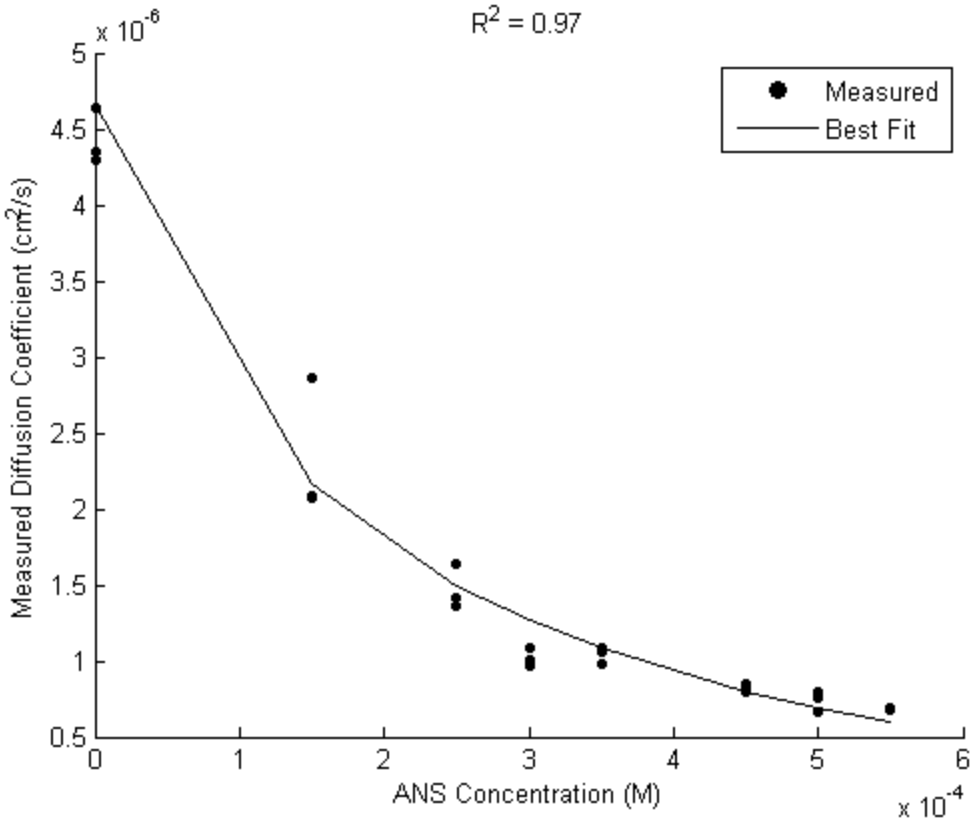

Figure 4.17 The measured diffusion coefficients of α-lactalbumin interacting with ANS at pH 2.5 versus ANS concentration. The best fit provides the binding constant and the complex diffusion coefficient.

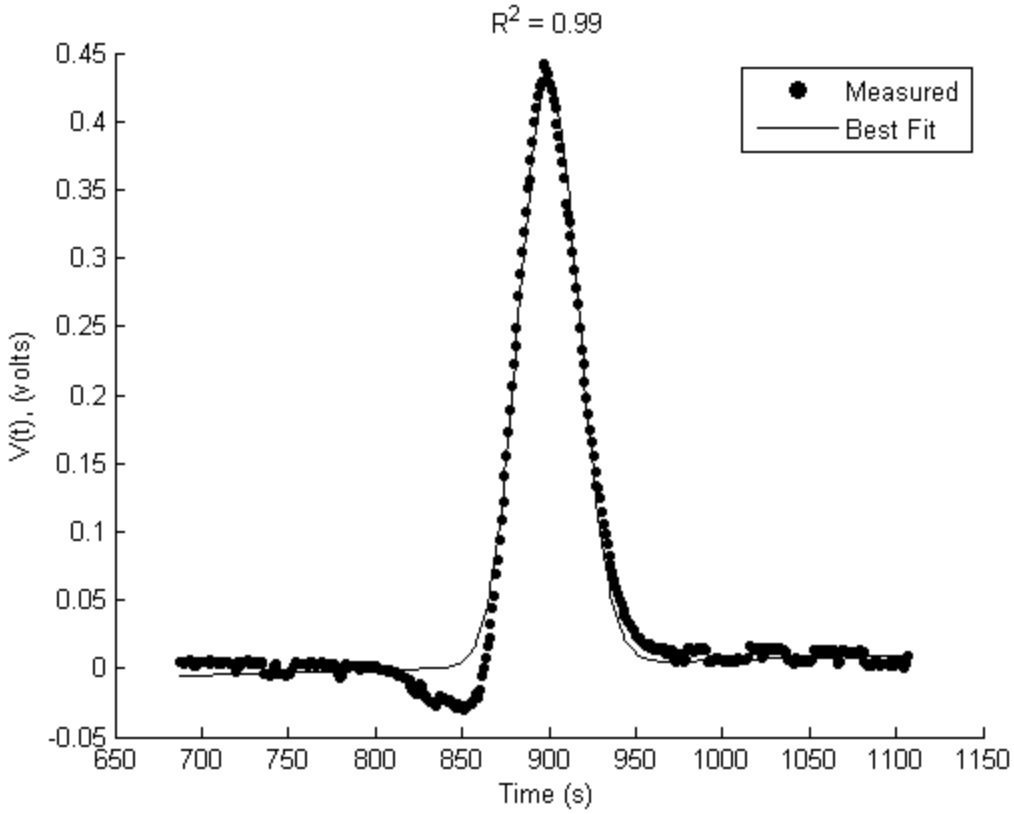

Figure 4.18 A typical output response from a Taylor dispersion experiment for BSA at pH 7.4 interacting with ascorbic acid. In this experiment the carrier solution was 4.51×10^{-5} M BSA at pH 7.4 and the injection was 0.45×10^{-3} M ascorbic acid.

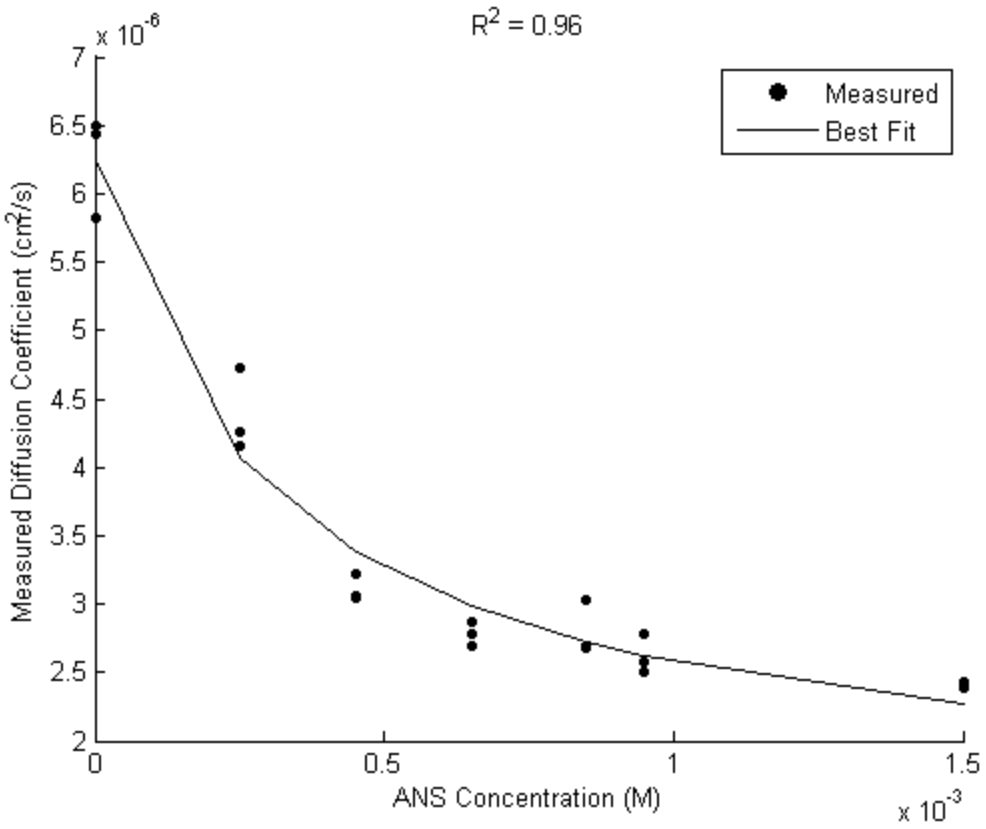

Figure 4.19 The measured diffusion coefficients of BSA interacting with ascorbic acid at pH 7.4 versus ascorbic acid concentration. The best fit provides the binding constant and the complex diffusion coefficient.

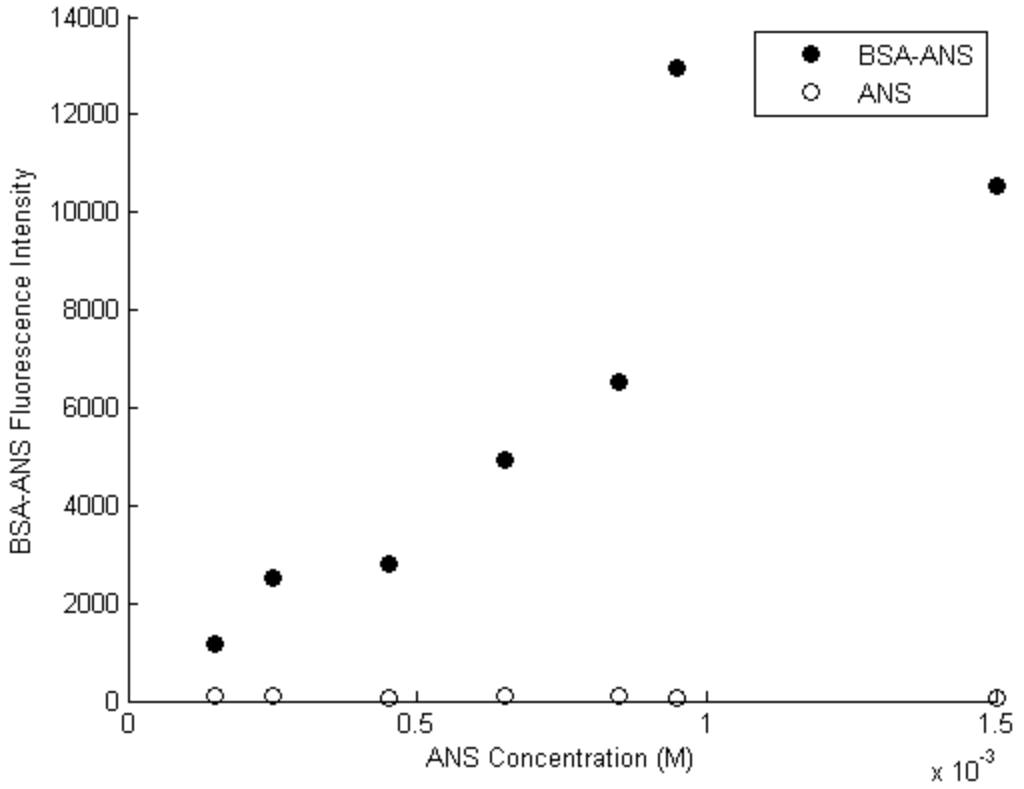

Figure 4.20 A plot showing the fluorescence intensity of the BSA bound to ANS. ANS does not fluoresce in the unbound state as shown above. BSA does not fluoresce in the absence of ANS (BSA fluorescence intensity = 169).

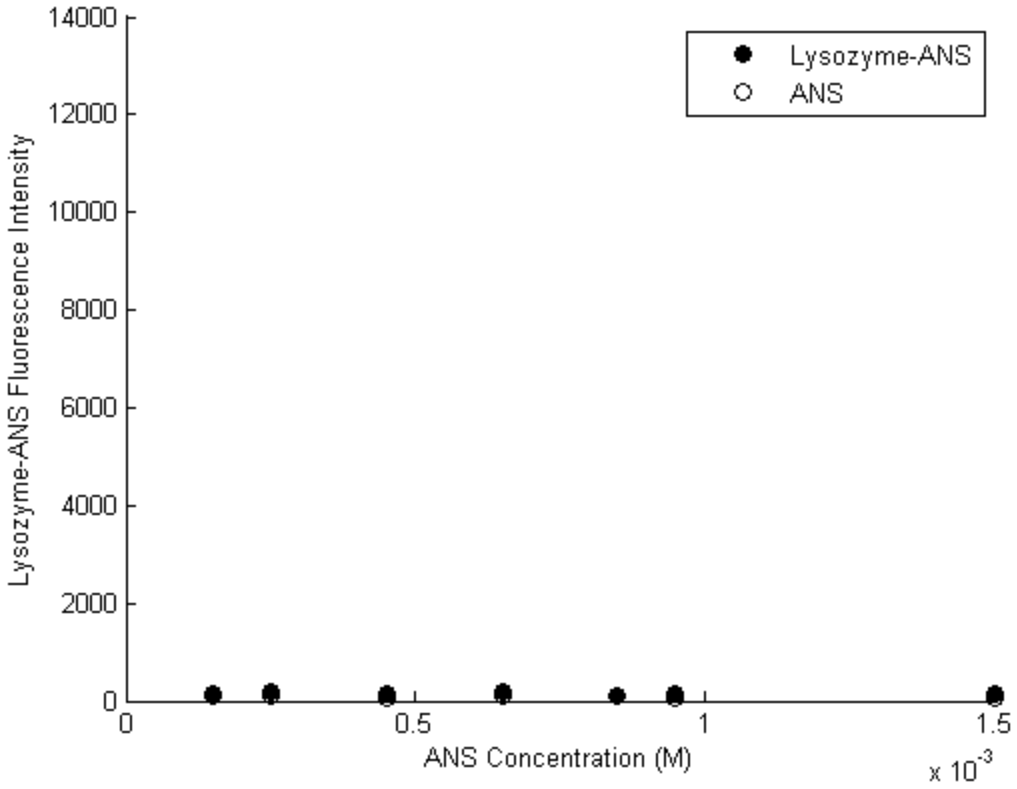

Figure 4.21 A plot showing the fluorescence intensity of the lysozyme bound to ANS. Oxidized lysozyme remains in a tight conformation and does not have any fluorescent binding sites as can be seen above. ANS does not fluoresce in the unbound state as shown above. Lysozyme does not fluoresce in the absence of ANS (Lysozyme fluorescence intensity = 89).

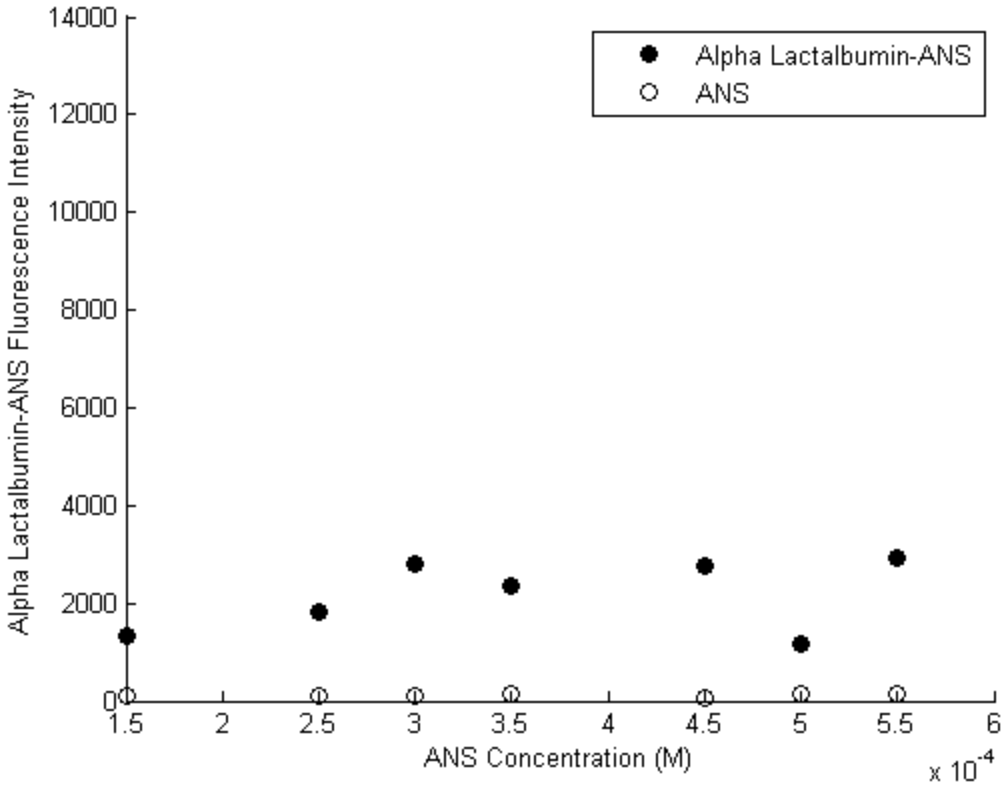

Figure 4.22 A plot showing the fluorescence intensity of the α-lactalbumin bound to ANS. ANS does not fluoresce in the unbound state as shown above. α-lactalbumin does not fluoresce in the absence of ANS (α-lactalbumin fluorescence intensity = 169).

Chapter 5 – Conclusion

In thesis, it has been demonstrated that equilibrium binding constants can be obtained through diffusion coefficients determined through Taylor dispersion analysis. The Taylor dispersion method has been proven to be both accurate and provide quick diffusion measurements. This method is also advantageous because the equipment (syringe pump, uv-vis detector, injection valve) is readily available in most research institutions. In this work, one of the limitations in our dispersion experiments was that the operating flow rate was near the lower limit of the flow criteria. Flow rates at the upper limit could not be achieved in our system because the tubing would eject from the fittings at high pressures. Operating at a lower flow rate did not compromise the results, however diffusion coefficient measurements took about 20 minutes per sample whereas they could have taken only a few minutes at high flow rates. In this work, it was also demonstrated that this method can easily determine the diffusion coefficient of a ligand in complex with a protein.

Lastly, the experiments between ligands and proteins in this work has shown that this method serves a practical application in high throughput screening of binding constants which could be used for drug development. Knowledge of the binding constant between a drug and a protein or receptor is an important parameter in ensuring that the drug binds effectively to its target. For example, some antibiotics when traveling through the blood stream bind to albumin so strongly they lose their activity (Douroumis et al 2012). Knowledge of the binding constant between these two interacting species could help in the development of an antibiotic with a small binding constant to albumin.

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