

Characterizing Protein-Protein Interactions Via Static Light Scattering: Inhibition Kinetics and Dissociation

Quantification of the kinetic rates of protein association, dissociation, and aggregation is an essential factor in understanding and ultimately manipulating these phenomena. A variety of measurement techniques are commonly applied, yet a given system may or may not be amenable to monitoring via any of these detection methods. Standard enzymatic spectrofluorescence or radiometric assays require labeling the molecules of interest, bringing into question the effect of the modification on the reaction rates. Selection of an appropriate substrate also holds the potential for confounding the measurement. Biophysical assays such as surface plasmon resonance (SPR) require receptor immobilization on a surface, again with the potential for modifying the reaction relative to a true free-solution measurement. More generally, these techniques provide only an indirect measure of the actual physical phenomenon of interest. For example, while fluorescence may be well-correlated to complex formation, the fluorescence signal itself is not a direct measure of an inherent property of the complex.

CG-MALS and TD-MALS

Multiangle, static light scattering (MALS) is a widespread technology for characterizing macromolecules in solution, providing an absolute measure of molar mass and size. Typically, MALS detectors are coupled to a separation technique such as size exclusion chromatography (SEC-MALS) or field flow fractionation (FFF-MALS) for determining the distribution of molecules in solution by molar mass, without reference to standards. A more recently developed technique is automated composition gradient, or CG-MALS, wherein equilibrium macromolecular interactions are quantified by measuring the light scattering signal over a series of compositions. As described previously,¹ CG-MALS can determine equilibrium association constants and stoichiometries of reversibly associating protein complexes, both homo- and heteroassociations. CG-MALS measurements do not require fluorescent or radioactive tagging, additional substrates, or immobilization, and thus access free-solution properties of unmodified samples in almost any solution. Some of the more promising applications of CG-MALS in the pharmaceutical industry include antibody/antigen binding, small-molecule inhibition, liposome uptake of proteins, and antibody formulations. Fundamental studies of chaperon proteins, enzyme/inhibitor interactions, binding of protein-surfactant or DNA-protein systems, and so forth will also benefit from this technique.

An essential advantage of static light scattering over some other methods is its ability to determine the molar masses of the complexes and constituents directly from the measured signals. Analysis of the CG-MALS data yields the stoichiometry of complexes formed, as well as the association constants.

A natural extension of CG-MALS is time-dependent static light scattering, or TD-MALS, wherein the intensity of light scattered

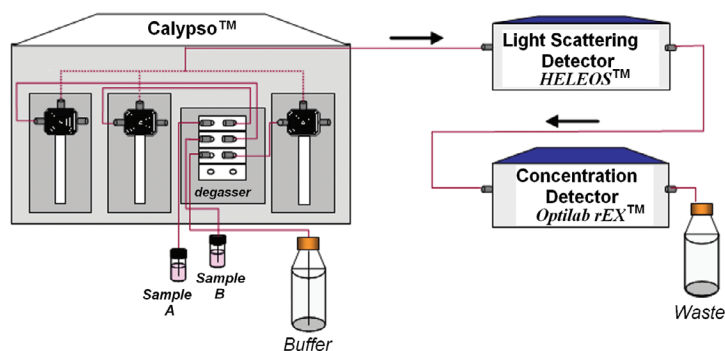


Figure 1 Schematic TD-MALS setup, including the Calypso triple-syringe pump accessory, DAWN-HELEOS static light scattering detector, and Optilab rEX differential refractometer.

by a solution is monitored over time, after a rapid step of preparation and delivery to the detectors. While not new, the application of static light scattering to protein-protein interaction kinetics has been fairly limited. Some of the systems studied via stopped-flow static light scattering include dimer-tetramer equilibration of human hemoglobin upon rapid pH changes,² ribosome dissociation and subunit association,³ viral matrix protein binding to host nucleocapsids,⁴ DNA/poly-L-lysine polyplex formation,⁵ and fibrinogen formation.⁶ The potential applications of TD-MALS include not only fundamental molecular biology but also investigation of reversible and irreversible aggregation of therapeutic proteins in formulation buffers.

Automation

In the TD-MALS method, a stopped-flow measurement is performed over a series of conditions. A TD-MALS procedure consists of preparing a series of solutions, delivering each in turn to both MALS and concentration detectors over a time scale that is short in comparison to the relaxation time, acquiring the data, and determining the reaction rate under each condition. The total time span of the experiment may be as short as a few minutes or as long as many hours. Depending on the number of desired conditions and the required data acquisition time, the experiment may become tedious, time-consuming, and prone to operator error. The Calypso™ system (Wyatt Technology Corp., Santa Barbara, CA) overcomes the difficulties of manual TD-MALS by automating the entire procedure, integrating sample preparation and delivery with data acquisition and analysis in one comprehensive package. The system works in conjunction with a Wyatt MALS detector, such as the DAWN®-HELEOS™ or miniDAWN®-TREOS™, and an optional on-line concentration detector such as the Wyatt Optilab® rEX™ differential refractometer or a third-party UV absorption detector, to provide a complete TD-MALS setup (see Figure 1).

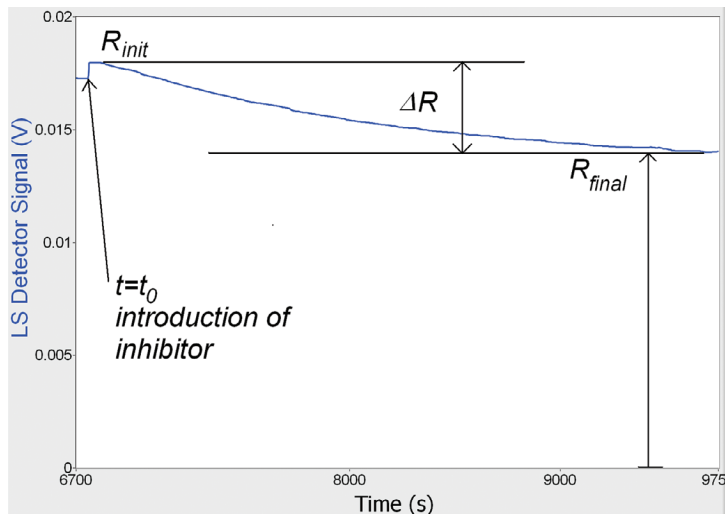


Figure 2 Time-dependent behavior of light scattering signal from monomer-dimer-inhibitor solution. Upon introduction of the inhibitor molecule, the signal decreases exponentially from R_{init} to R_{final} with time constant τ .

Inhibition kinetics

This paper demonstrates the use of an automated TD-MALS system for characterizing the kinetics of dissociation of a protein complex. Alpha-chymotrypsin is known to reversibly self-associate at low pH, forming a monomer-dimer equilibrium.⁷ 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) is an irreversible serine protease inhibitor that binds to the chymotrypsin active site,⁸ which is also the binding site for self-association. Upon adding AEBSF to a chymotrypsin solution, the inhibitor will gradually diffuse into the dimers and bind to the active site, causing the dimers to break up into monomers. If the inhibitor molarity is much larger than that of the chymotrypsin, the drop in dimer concentration follows an exponential time dependence, until all of the chymotrypsin dimers have dissociated. The accompanying decrease in the weight-averaged molar mass of the chymotrypsin solution is reflected in the TD-MALS signal, as shown in Figure 2.

For a single species in solution under dilute (“ideal”) conditions, the light scattering signal $R(t)$ is proportional to the product of molar mass M and weight concentration c :

$$\frac{R}{K} = Mc \left(\frac{dn}{dc} \right)^2$$

where K is a function of system parameters such as wavelength and solvent refractive index, and dn/dc is the sample refractive increment. If more than one species is present, then the total light scattering is just the sum of contributions from each species. The small molar mass of AEBSF relative to chymotrypsin ensures that the light scattering signal directly reflects the monomer-dimer balance, regardless of a small fraction of the inhibitor (which is present in a large molar excess) binding to the chymotrypsin.

Figure 2 describes the evolution of the light scattering intensity in an inhibition experiment. Initially, the monomer and dimer forms are in thermodynamic equilibrium; upon introducing the protein mixed with inhibitor at time $t = t_0$, the signal at first

increases slightly due to the small inhibitor scattering intensity, then follows the exponential decrease in dimer concentration, as described in Eq. (1):

$$\frac{R(t)}{K} = M \left(c^{tot} + c^{D,0} e^{-(t-t_0)/\tau} \right) \left(\frac{dn}{dc^p} \right)^2 + mc^I \left(\frac{dn}{dc^I} \right)^2 \quad (1)$$

where M and m are the molar masses of monomeric chymotrypsin and the inhibitor, respectively; c^{tot} and c^I are the total chymotrypsin and inhibitor concentrations; $c^{D,0}$ is the equilibrium concentration of dimer; and dn/dc^I and dn/dc^p are the protein and inhibitor refractive increments. Note that the difference between initial and final LS values reflects the initial dimer concentration $c^{D,0}$:

$$\frac{\Delta R}{K} = \frac{R_{init} - R_{final}}{K} = Mc^{D,0} \left(\frac{dn}{dc^p} \right)^2 \quad (2)$$

Standard first-order kinetics analysis may be applied to the reaction rates, allowing for a reaction of the form:



where D indicates the dimer, M the monomer, and I the inhibitor molecules. $M \bullet I$ is a reversibly associated intermediate state, whereas MI is an irreversibly bound final state. The result of this analysis for the dependence of the light scattering decay time τ on inhibitor and protein concentrations is presented in Eq. (3):

$$\tau = \frac{c^{tot}}{c^{tot} - c^{D,0}} \frac{k_M m}{k_{+2} c^I} + \frac{1}{k_{+2}} \quad (3)$$

In Eq. (3), $k_M = (k_{-1} + k_{+2})/(k_{+1})$ is the Michaelis constant, a standard parameter of interaction kinetics. Fitting the dependence of reaction time constants on c^I and to Eq. (3) yields k_M and k_{+2} .

In addition to kinetic parameters, the same measurement may also provide the equilibrium dissociation constant K_d via the ratio of dimer-to-monomer concentration in equilibrium, calculated with the help of Eq. (2). However, this analysis is not generally suitable for calculating K_d if other factors are present, e.g., some unknown fraction of the stock solution is incompetent to associate, or if the association stoichiometry is not known and differs from monomer-dimer. K_d may be determined rigorously via the CG-MALS technique, as described by Attri and Minton,⁹ which consists of analyzing the equilibrium light scattering signals of a series of chymotrypsin concentration steps (with no inhibitor).

Method

A stock solution of chymotrypsin (Worthington Biochemical Corp., Lakewood, NJ) was prepared in a 100 mM NaCl citrate buffer at pH 3.8 and dialyzed against the buffer by means of a Sephadex desalting column (HiPrep, GE Healthcare BioSciences, Uppsala, Sweden). AEBSF (Sigma-Aldrich, Milwaukee, WI) was prepared in the same buffer. Both solutions were filtered to 0.02 μm via an Anotop syringe-tip filter (Whatman, Kent, U.K.).

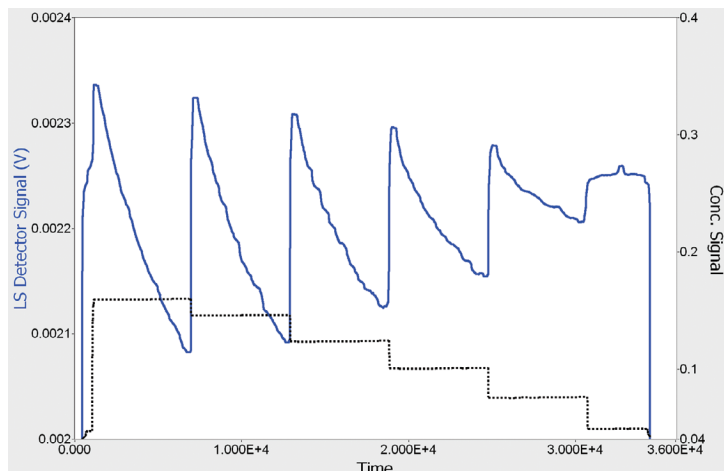


Figure 3 TD-MALS run, single chymotrypsin concentration, and multiple AEBSF concentrations, indicating change in reaction rate with inhibitor concentration. Solid blue plot: light scattering detector signal; dotted black plot: dRI signal. The final step corresponds to chymotrypsin with no inhibitor; hence the signal does not exhibit kinetics.

The experimental setup consists of the Calypso triple-syringe pump accessory, a HELEOS multiangle static light scattering detector, and an Optilab rEX differential refractometer. The Calypso pump accessory aspirates the required quantity of each solution (chymotrypsin, AEBSF solution, and pure buffer) in three computer-controlled syringe pumps. After delivering the desired composition to the detectors via a static mixer, the flow is stopped and the data acquired. An entire method consisting of multiple concentrations of protein and/or inhibitor may be programmed in the Calypso software for unattended operation. In this measurement, data were acquired for 5000 sec at each condition; the stock solution of sample was refrigerated during the course of the rather long run to prevent degradation.

Data analysis

It is only necessary to vary either the inhibitor concentration or the protein concentration in order to fit Eq. (3). In the current study, the concentration of AEBSF was varied from 0 to 5 mM in ~ 1 mM steps at a fixed chymotrypsin concentration. The data are shown in Figure 3. The dotted black plot corresponds to the differential refractive index (dRI) signal, primarily reflecting the inhibitor concentration, while the solid blue line is the light scattering signal (LS). At each injection, the LS data are fit by the Calypso software in order to determine the decay time constant τ and amplitude ΔR of the light scattering exponential decay.

The dependence of the reaction time on $[(c^{tot})/(c^{tot} - c^{D,0})] \cdot [(m/c^I)]$ is plotted in Figure 4. Since the y-intercept is too small to estimate accurately, only the result for the slope is reported: $k_M/k_{+2} = 15.7 \text{ M}\cdot\text{sec}$. This system has been studied at a neutral pH where chymotrypsin does not self-associate⁸ (hence, at neutral pH, inhibitor kinetics of the homoassociation may not be studied via TD-MALS), but the authors have not found published results corresponding to low pH for comparison. An estimate of

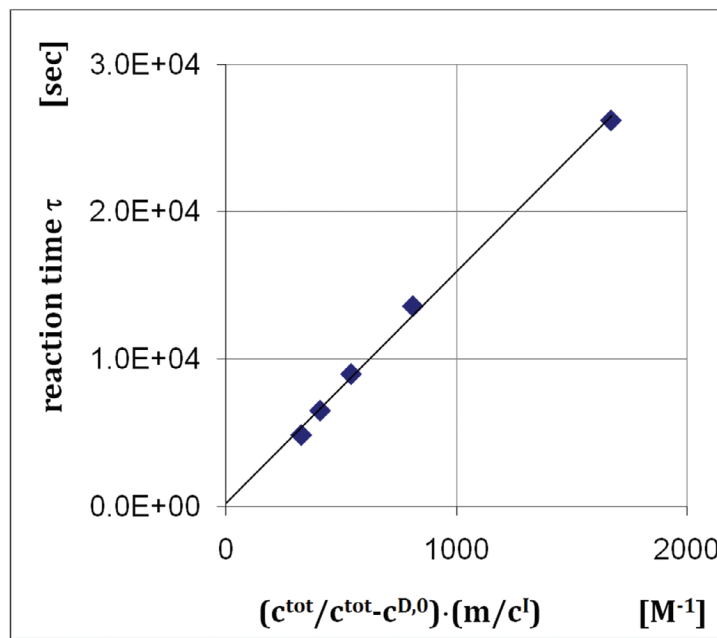


Figure 4 Dependence of reaction time τ on the concentrations of inhibitor, total protein, and equilibrium monomeric protein. The slope and intercept indicate standard kinetic parameters k_M and k_{+2} .

the affinity for protein self-association yields $K_d \sim 18 \mu\text{M}$, which is comparable to the value determined by equilibrium CG-MALS analysis of $25 \mu\text{M}$, and $14\text{--}20 \mu\text{M}$ by enzymatic methods⁷ at similar buffer conditions.

Summary

TD-MALS is a powerful but underutilized technology for kinetic studies of protein–protein and protein–inhibitor interactions in solution without labeling, immobilization, added substrates, or other sample modification. With highly sensitive detectors such as the DAWN-HELEOS, the Calypso system can accommodate a wide range of kinetic phenomena including k_{on} rates* to $10^7 \text{ M}^{-1}\text{sec}^{-1}$ and k_{off} rates to $\sim 1 \text{ sec}^{-1}$. In addition to inhibition kinetics, the authors have demonstrated antibody/antigen association kinetics¹⁰ analysis and aggregation of heteroassociating complexes to form metacomplexes. Equilibrium association constants derived from the kinetics data may be compared with the more rigorous composition-gradient light scattering experiments using the same instrumentation.

*Maximum k_{on} given for 100-kDa proteins with $K_d < 10 \text{ nM}$; the upper limit is proportional to molar mass and will also depend on the dissociation constant K_d .

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Dr. Some is Principal Scientist, and Ms. Hanlon is an R&D Engineer, **Wyatt Technology Corp.**, 6300 Hollister Ave., Santa Barbara, CA 93117, U.S.A.; tel.: 805-681-9009; fax: 805-681-0123; e-mail: dsome@wyatt.com.