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Characterizing Protein–Protein Interactions Via Static Light Scattering: Reversible Heteroassociation

The quantitative characterization of reversible protein–protein interactions is fundamental to the elucidation of basic biological function as well as to the development of new medical products. Attri and Minton[1] and Kameyama and Minton[2] recently demonstrated a novel technique for quantifying reversible association that overcomes many of the disadvantages of other methods. Composition gradient-multiangle light scattering (CG-MALS) employs static light scattering (SLS) to determine stoichiometry and equilibrium association constants of self- and heteroassociations. This is accomplished without recourse to sample modifications such as fluorescent tagging or surface immobilization, and with no restrictions on buffer composition, the experiment may be carried out in native solution or the desired formulation buffer.

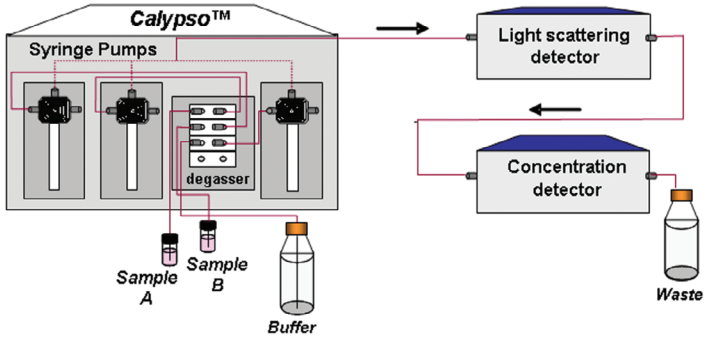

Figure 1 CG-MALS experimental setup utilizing the Calypso SP3 sample preparation and delivery hardware, a static light scattering detector, and a concentration detector. Various components (filters, mixers, etc.) not shown.

The CG-MALS technique

The key to understanding how CG-MALS operates lies in the basic premise of SLS: The intensity of light scattered from macromolecules in solution is proportional to the product of the concentration, c, and the weight-averaged molar mass, M_w. As protein complexes form, M_w increases; for example, were all the molecules present to dimerize, the scattered intensity would double. In a reversible association, the ratio of protein complexes to protein monomers reaches an equilibrium value that depends on the initial concentration of each species, as well as on the buffer conditions. Under changing compositions and concentrations, different ratios will lead to differing values of M_w. By analyzing a series of SLS measurements taken under various compositions and concentrations, it is possible to determine which complexes are being formed, and their respective binding affinities in the form of K_a, the association constant, or $K_d = 1/K_a$, the dissociation constant. With current instrumentation, CG-MALS accesses protein dissociation constants in the range of 100 picomolars to millimolars.

Some of the other techniques commonly used in characterizing reversible associations are nuclear magnetic resonance (NMR), fluorescence quenching (FQ), sedimentation equilibrium (SE) by analytical ultracentrifuge, isothermal titration calorimetry (ITC), and surface plasmon resonance spectroscopy (SPR). Neither FQ, ITC, or SPR can clearly establish stoichiometry, especially in the case of the simultaneous presence of multiple complexes. SPR requires immobilization of one of the constituent species on a substrate, raising questions as to the validity of the measurement with respect to molecules normally in solution. FQ requires fluorescent tagging of the molecules, possibly modifying their charge state and other properties. Both NMR and SE (which has generally been considered the "gold standard" for characterizing reversible associations) require expensive equipment and rather long measurements, several days in the case of SE.

Automation

A CG-MALS procedure consists of preparing solutions of each of the required compositions, delivering each to an SLS instrument and a concentration detector, recording the scattered intensity and concentration values, and performing a nonlinear least-squares fit of the data to each of the association models to be tested against the experimental data. For rich interactions, the number of individual compositions needed to clearly discriminate between models and obtain a reliable set of association constants may number in the dozens. Manual preparation and measurement procedures would clearly be tedious, time consuming, and prone to operator error. The Calypso™ system (**Wyatt Technology Corp.**, Goleta, CA) overcomes the difficulties of manual CG-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 on-line concentration detector such as the **Wyatt** Optilab rEX differential refractometer or a third-party UV absorption detector, to provide a complete CG-MALS setup (see *Figure 1*).

AB complex characterization

The first heteroassociation example is a well-characterized enzyme/inhibitor system that forms 1:1 complexes: chymotrypsin (CHTR) and bovine pancreatic trypsin inhibitor (BPTI) at neutral pH. A phosphate buffer was prepared by diluting a commercially available stock (PBS 10× solution [**Thermo Fisher Scientific**, Waltham, MA], 137 mM NaCl after dilution). CHTR and BPTI, obtained from **Sigma-Aldrich** (Milwaukee, WI), were dissolved at nominal concentrations of 0.5 and 0.2 mg/mL, respectively, and then dialyzed against the buffer by means of a Sephadex desalting column (HiPrep, **GE Healthcare BioSciences**, Uppsala, Sweden).

The CG-MALS method consists of a buffer flush to obtain initial baseline values, then a single-component ascending concentration series of CHTR, followed by a crossover composition series of CHTR and BPTI varying from 100% CHTR and 0% BPTI to 0% CHTR and 100% BPTI, a descending concentration series of BPTI, and finally a buffer flush for final baseline. The single-component stepwise gradients serve to characterize the monomer and any self-association that may be present, while the crossover gradient probes the entire range of possible heteroassociation stoichiometries. Calypso's Method table consists of seven program lines (including five Gradient commands, a File Save command, and flushes of all the pumps with wash solution). The Method table is reproduced in *Figure 2*, along with the Timeline graph that assists in method visualization and design.

The system's SP3 hardware accessory features three computer-controlled syringe pumps. Once the two sample vials and buffer reservoir are connected to the pumps via the built-in vacuum degasser, the software automates sample preparation and delivery by combining the output pump streams on-the-fly in a static mixer as they are injected into the detector flow cells. Different compositions are obtained by varying the relative flow rates

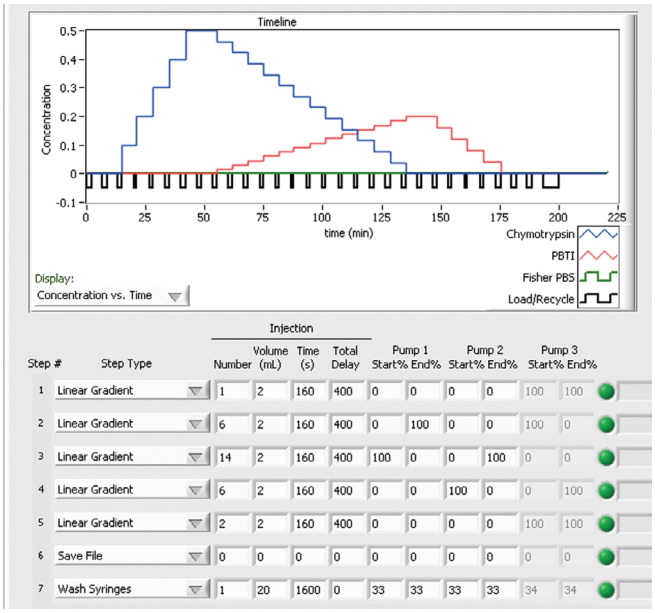

Figure 2 Method design for characterization of self- and heteroassociation of two proteins includes the Method table and the Timeline graph.

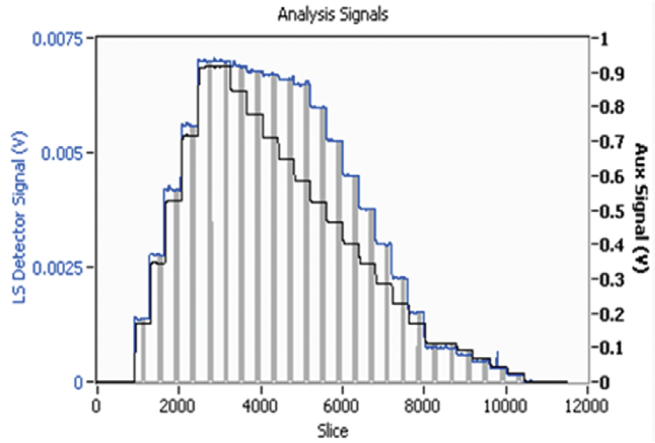

Figure 3 CG-MALS processed data for a heteroassociation experiment: light scattering signal (blue) and concentration signal (black).

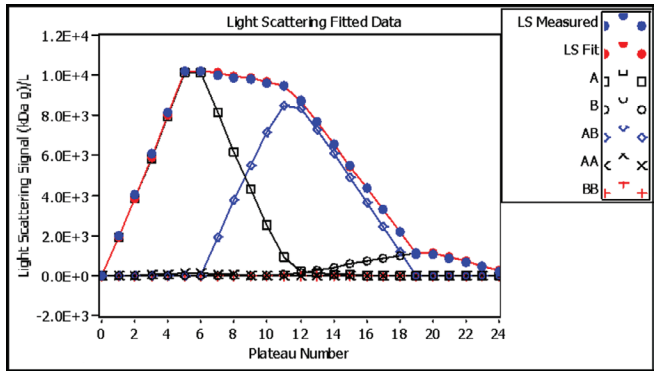

Figure 4 Fit of SLS data to model of self-association and 1:1 complex formation (the fit indicates no self-association). The horizontal axis corresponds to the plateau sequence selected in Figure 3. Full circles: measured and calculated SLS signals. Other symbols: contribution of the various species to the light scattering at each composition. The 1:1 curve reaches a maximum at the point corresponding to 1:1 stoichiometry of the injected samples.

of the pumps. After metering out the predefined volume, flow stops and data may be integrated over any time scale. This stop-flow measurement provides for diffusion and homogenization of sample within the flow cell, as well as observing the kinetics of any reactions that may be occurring. Data acquisition is automatically synchronized to begin and end with the method. The duration of the measurement is typically 0.5–2 hr.

Figure 3 depicts the SLS (blue) and concentration (black) data after preprocessing, which includes baseline subtraction, applying calibration constants and normalization corrections. Each plateau corresponds to the signal from a particular sample composition. The vertical gray bars denote the region of each plateau that has been selected for the modeling analysis. In the course of the semiautomated plateau selection, each data series is assigned to a specific gradient type (single-component or crossover).

Finally, data modeling is carried out. *Figure 4* shows the results of fitting the light scattering and concentration plateau data to a simple AB association, selected from a list of models supported by Calypso's modeling algorithms. The

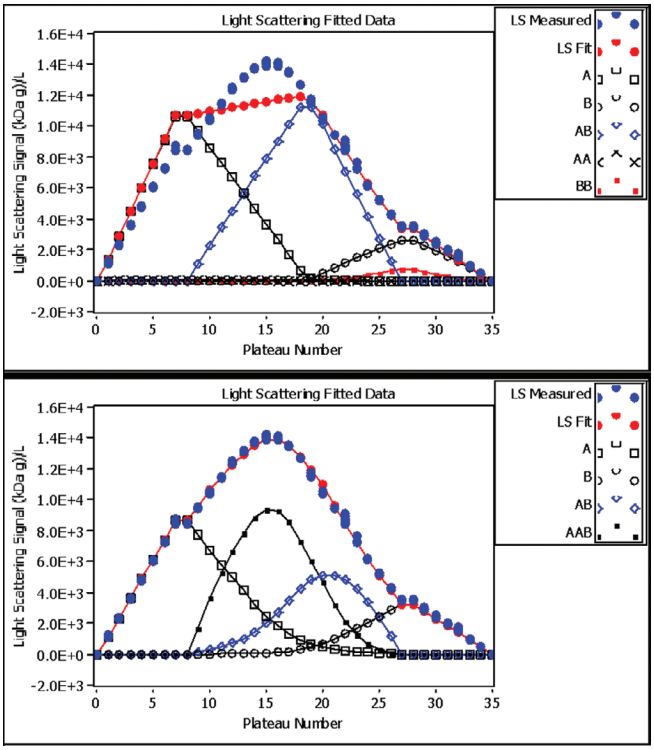

Figure 5 Top: unsuccessful attempt to fit the CG-MALS data for chymotrypsin and soybean trypsin inhibitor, with an (incorrect) model of 1:1 association + self-association. Bottom: fit to a model of two equivalent binding sites on the inhibitor, producing both 1:1 and 2:1 complexes. This result matches the known inhibitor structure.

blue circles correspond to the measured CG-MALS values, while the red circles and line correspond to the fit. The other plots illustrate the light scattering signal contributed by each of the components: A monomer (CHTR), B monomer (BPTI), and the AB complex. It can be seen that the AB signal peaks when the injected sample comprises the stoichiometric ratio of the complex. This is a general feature of CG-MALS that helps distinguish different stoichiometries.

The association constant resulting from this fit is K_a = 1–2 × 10^7 M^{-1}, corresponding to a dissociation constant of K_d = 50–100 nM. This value falls within the range determined elsewhere and by other techniques, i.e., 25–200 nM. Adding other terms to the fitted model, e.g., self-association or AAB complexes, does not change the results since the additional complexes end up with negligible affinities. The entire fitting procedure requires only a few seconds on a moderately fast PC equipped with a Pentium IV processor (**Intel**, Santa Clara, CA).

Characterization of multiple complex stoichiometries

The second example demonstrates characterization of an interacting protein system that exhibits two simultaneous stoichiometries. When chymotrypsin associates with soybean trypsin inhibitor (STI), STI presents two equivalent binding sites to the enzyme, so that both AB and AAB complexes are present. For this measurement, the crossover gradient is divided into finer steps in order to better resolve the two complexes.

The buffer conditions and CHTR preparation were the same as in the previous measurement, while STI, obtained from **Sigma-Aldrich**, was prepared at 0.2 mg/mL and similarly dialyzed. The modeling results are shown in *Figure 5*. The upper graph shows an ill-fated attempt to fit the data to a model that allows for three independent parameters: affinities of the AB, AA, and BB complexes; the lower graph depicts the fit to a model that provides for both AB and AAB complexes, under the assumption of two equivalent binding sites (only one independent parameter, the single binding site affinity). Clearly, the latter produces a perfect fit (K_d = 1 µM, comparing well with the literature values of 0.3–1.2 µM under similar buffer conditions), validating the stoichiometry model. Again, the graphs show not only the measured and fitted total light scattering data, but also the contributions to the MALS signal from each species at each composition. The Calypso software provides plots of the concentrations of each species at each composition step as well as the SLS signals.

These two examples illustrate some common heteroassociations, analyzed with relatively simple modeling. More complicated systems that have been addressed include simultaneous self- and heteroassociation, multiple degrees of self-association, and incompetent protein fractions (some portion of the monomers are misfolded). The Calypso software provides for a large variety of association models as well as simulating experiments in the design stage.

Summary

CG-MALS technology will benefit biophysical characterization laboratories interested in various types of protein–protein associations, including aggregation, enzyme/inhibitor, and antibody–antigen systems. Since many laboratories already employ **Wyatt** MALS detectors for characterizing macromolecular distributions via separation techniques (size exclusion chromatography or field-flow fractionation), Calypso would be an effective add-on for the complementary interaction measurements—the SP3 incorporates a switching valve for sharing the detectors between the two sets of analytical preparation equipment.

References

1. Attri, A.; Minton, A.P. Composition gradient static light scattering (CG-SLS): a new technique for rapid detection and quantitative characterization of reversible macromolecular hetero-associations in solution. *Anal. Biochem.* **2005**, *346*(1), 132–8.
2. Kameyama, K.; Minton, A.P. Rapid quantitative characterization of protein interactions by composition gradient static light scattering. *Biophys. J.* **2006**, 90(6), 2164–9.

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