Characterizing Protein–Protein Interactions Using Composition Gradient Multi-angle Light Scattering

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Light scattering, including dynamic and static versions, is the most versatile technology for obtaining information about macromolecules and their properties in solution. Composition gradient multi-angle light scattering (CG-MALS) is a relatively new variant of static light scattering technology, particularly powerful for characterizing macromolecular interactions without labelling or immobilization. This article reviews the principles of static light scattering and discusses how the new CG-MALS technology complements conventional MALS separation methods to achieve full characterization of proteins and their interactions.

Scattered Light Intensity

When laser light impinges on a macromolecule, the oscillating electric field of the light induces an oscillating dipole within it. Positive and negative charges isolate in opposite directions. These charges include all the protons and electrons in the molecule and not just the net charges, which may be present as a result of interactions with the solvent. The oscillating dipole re-radiates light in all directions; the so-called scattered light.

The amplitude of the scattered light field depends on the magnitude of the dipole induced in the macromolecule, which, in turn, depends on the total number of electrons and protons and their polarizability relative to that of the surrounding solvent. This dependence can be conveniently expressed as the product of molar mass and the refractive increment dn/dc; the refractive increment may be measured readily by means of a differential refractometer and is nearly a constant for most proteins in aqueous solutions. For an ensemble of molecules, the intensity of the scattered light is proportional to the weight-averaged molar mass, the square of the refractive increment, and the concentration. Additionally, the angular dependence of the scattering carries information about the size of the macromolecule, its root-mean-square radius (rms), though most monomeric proteins are too small to obtain a reliable measure of size in this manner.

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Static Light Scattering

In a typical static light scattering experiment, a well collimated, single frequency polarized light beam is issued to illuminate a solution containing a suspension of macromolecules of interest. The intensity of the scattered light is measured as a function of angle and concentration, generating important information about macromolecules, including the molar mass, the rms radius and the second virial coefficient (A_2). For certain classes of particles, static light scattering may also be combined with dynamic light scattering to yield the size, shape and structure while also allowing scientists to monitor the presence and formation of aggregates.

Conventional MALS Separation Techniques

Size exclusion chromatography MALS (SEC MALS) and field flow fractionation MALS (FFF MALS) are the most common MALS applications. By separating the molecules, the light scattering provides an absolute measure of the molar masses of each species and defines their distribution in solution.

Separation techniques make it possible to investigate molecular complexes if they are irreversible or bound tightly enough to survive the shear and the dilution of a separation column. For such systems, SEC MALS and FFF MALS facilitate conjugate analysis, which combines information from three detectors (MALS, UV, absorption and differential refractometer) and enables analysts to determine the stoichiometry of complexes of two different molecules.

Nevertheless, there are certain limitations associated with SEC-MALS and FFF-MALS when studying interactions. As the molecules become separated in the course of dilution and fractionation, they no longer interact. Additionally, upon reaching the detectors the molecules are often not in equilibrium, neither are they in a well-defined kinetic state. Composition gradient MALS (CG-MALS) complements traditional MALS separation techniques, keeping the molecules in close proximity to probe their interactions.

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Composition Gradient MALS (CG-MALS)

CG-MALS employs a series of unfractionated samples of different composition or concentration to characterize macromolecular interactions such as reversible self- and hetero-association, reaction rates and affinities of irreversible aggregation or non-specific interactions as quantified by virial coefficients. No special modifications, such as sample tagging or immobilization procedures, are necessary. Samples are entirely in solution.

A typical CG-MALS system is comprised of a MALS detector and a concentration detector fed by a sample preparation accessory featuring three syringe pumps, each of which is plumbed to a sample vial or a buffer reservoir. For each composition step, the necessary amount of sample and buffer are loaded, delivered through the three pumps and mixed on-the-fly. The actual composition is controlled by varying the flow-rate ratio between the pumps. The final solution is directed to the detectors for measurement.

There are certain sample requirements that need to be fulfilled to perform a successful CG-MALS experiment. Samples must be well purified because everything in solution larger than ~ 1 KDa scatters light. Moreover, samples should be well dialysed with respect to a common solvent, particularly when relying on a refractive index concentration detector. Samples must also be well filtered. Thorough filtration is critical given that light scattering is particularly sensitive to dust and other large particles. It is also important for samples to be bubble-free because bubbles scatter light much like large particles. Debubbling can be achieved via centrifugation or vacuum in case an in-line degasser is not used. Well-prepared samples assure accurate and reliable results.

A Wealth of Benefits

CG-MALS is a batch technique that does not involve the separation of molecules. Instead, molecules are kept together in the same volume so as to observe their interactions; light scattering measurements generate the weight-averaged molar mass of the solution. When complexes form, the weight-averaged molar mass increases, whereas repulsion between molecules causes the apparent weight-averaged molar mass to decrease. Analysis of the variation in apparent weight-averaged molar mass with composition results in association constants, binding affinities, stoichiometry and active fractions. In addition to the tightly bound complexes that can be studied using a separation technique, batch measurements enable users to study a wide range of weak or strong associations.

Both equilibrium and kinetics can be investigated using CG-MALS. Mixing up the sample, delivering it to the cell and stopping the flow allows for observation of any relaxation effects related to associations or dissociations. The final equilibrium state can be defined and the kinetics properties understood. This is different from SEC-MALS and FFF-MALS or any other separation technique where the state of equilibrium or kinetics is not well defined because as the

sample goes through the column, it is diluting and changing conditions along the way.

A further benefit of CG-MALS is the absence of column or surface interactions, eliminating concerns about extraneous factors that could affect measurements. In fact, it is a completely label-free and immobilization-free technique. Unlike SEC-MALS, there are essentially no limitations on solvents.

For proper quantification, molecules that associate reversibly with tight binding must be studied at low concentrations, while weakly interacting molecules must be studied at high concentrations. CG-MALS instrumentation is quite versatile and can address a wide range of concentrations from picomolars to millimolars so as to characterize a large variety of interactions.

Measuring Protein–Protein Interactions

CG-MALS can be used to efficiently and accurately measure three types of protein–protein interactions; non-specific interactions, specific interactions and kinetics of association or dissociation.

Virial Coefficients as a Measure of Non-specific Interactions

Non-specific interactions affect macromolecular behaviour in terms of solubility, aggregation, crystallization, purification and weak, non-specific associations. Virial coefficients are the quantitative measure of non-specific intermolecular interactions as mediated by the solvent; their variation upon changing the buffer reveals how the interactions between molecules are affected by the solvent.

Virial coefficient analysis can be used both for self-interactions, that is interactions between like molecules and cross-interactions, namely interactions between different types of molecules. Self virial coefficients are determined by analysing the light scattered by a series of concentration steps. At each step, the total light scattering signal is divided by the concentration to obtain the apparent molar mass. An increase in apparent molar mass with concentration indicates a net attractive force, while a decrease indicates an overall repulsion between molecules. The slope of apparent molar mass vs concentration yields the second virial coefficient A_2, which may be referenced to the omnipresent hard-core repulsive effect in order to understand the magnitude of 'soft' interactions because of charge, van der Waals forces and other contributions.

In a similar manner cross virial coefficients are measured by taking two types of molecules, varying their relative concentrations and measuring the scattered light. Cross virial coefficients are particularly useful for optimizing protein purification.

Specific Interactions — Reversible Self- and Hetero-association

Specific interactions such as antibody/antigen association can also be characterized using CG-MALS to determine stoichiometry and equilibrium dissociation constants (K_d). A typical experiment works by taking two types of molecules, mixing them together and allowing them to achieve an equilibrium between reversible complexes and free monomers. Possible equilibrium scenarios include self-association, for example, homodimers and heteroassociation to form AB complexes.

Analysis of CG-MALS reversible association experiments requires fitting the data to association models to yield equilibrium association constants and stoichiometry as well as the molar masses of the individual components. These models may describe simple interactions such as 1:1 binding, or more complicated models that include simultaneous self- and hetero-association, metacomplex formation or progressive self-association. Different complex stoichiometries may be readily distinguished through goodness of fit to the different possible models. An added benefit of the CG-MALS analysis includes estimation of the inactive fraction (i.e., the amount of proteins in the stock solution that do not participate in the association as a result of misfolding, mutation or aggregation).

Kinetics of Association, Dissociation and Aggregation

As a stopped-flow technique CG-MALS can also measure kinetics of association, dissociation and aggregation without radioactive or fluorescent labels by directly measuring the changes in molar mass and size. Typical experiments cover time scales of seconds to hours and may be designed to run through a series of compositions so as to tease out the specific reaction pathways. A recent demonstration of time-dependent CG-MALS calculated the kinetics constants of the inhibition of self-association by a small-molecule protease inhibitor which would otherwise be estimated by enzymatic methods.

Conclusion

Macromolecular interactions such as equilibrium association constants and stoichiometry of reversible self- and hetero-associations, reaction rates, virial coefficients, aggregation and kinetics can be easily characterized using the powerful CG-MALS technique. This vital information complements data on molar mass distributions obtained via SEC MALS or FFF MALS, yet leverages the same underlying detection technology.

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CG-MALS is complementary and orthogonal to other label-free biophysical methods for studying interactions such as surface plasmon resonance or isothermal titration calorimetry.

To find out more about the capabilities of CG-MALS for characterizing protein–protein interactions, please visit www.wyatt.com/Calypso or email info@wyatt.com

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Dr Some obtained his PhD in Physics from Brown University, Providence, Rhode Island, USA where he studied low-dimensional semiconductors by means of ultrafast laser and THz spectroscopy. Post-doctoral appointments at Los Alamos National Lab and Weizmann Institute of Science focused on the integration of lasers with surface probe microscopies. Current research interests include label-free macromolecular interactions, biophotonics and other applications of lasers and electro-optics to real-world problems.

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