

Calypso II

Absolute characterization of macromolecular interactions



WYATT
TECHNOLOGY

The Calypso® II

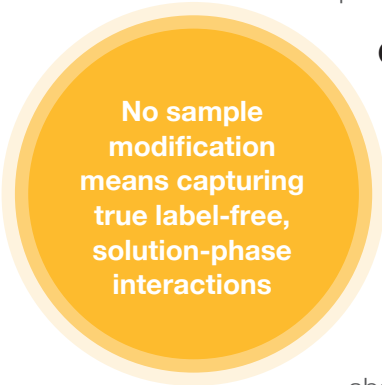
Illuminate protein-protein association and aggregation with CG-MALS

Macromolecular interactions play key roles in a host of scientific and biotechnological studies—from basic biomolecular research to protein therapeutic R&D—and the Wyatt Calypso is a versatile, unique tool for characterizing a wide range of interactions.

Utilizing a novel twist on a common technique—static light scattering—simple and repeatable, automated measurements of macromolecular interactions without tagging, immobilization or other sample modifications are now available to every analytical biochemist and biophysicist.

The Power of CG-MALS

Composition Gradient Multi-Angle static Light Scattering (CG-MALS) is one of the few analytical techniques that can characterize both specific and nonspecific solute-solute interactions over a wide range of interaction strengths. Depending on the molar masses and buffer conditions, concentrations from less than 1 µg/mL to over 100 mg/mL can be probed with the Calypso system.



No sample
modification
means capturing
true label-free,
solution-phase
interactions

Calypso's unique software analyzes:

- Self- and hetero-association
- Nonspecific interactions, both attractive and repulsive
- Reversible and irreversible kinetics of aggregation and dissociation

A comprehensive variety of interaction parameters may be determined from CG-MALS signals, such as virial coefficients (A_2), equilibrium dissociation constants (K_d), stoichiometry and reaction rates. The absence of columns and surfaces plus the inherently large dynamic range of Wyatt MALS detectors means a large range of concentration and, hence, of measurable interaction strengths.

Calypso's robust system enables biomolecular scientists to:

- Validate drug efficacy requiring strong, specific interactions between biopharmaceuticals and targets
- Optimize weak repulsion between macromolecules to ensure solubility and stability of a formulation
- Study cooperative and competitive allostery of compounds exhibiting multiple binding sites
- Fine-tune buffer conditions to minimize solution viscosity due to self-attractive forces

CG-MALS and Calypso

Designed with maximum automation, flexibility and ease-of-use in mind, the Calypso system provides hands-off operation for excellent repeatability and unattended runs.

The end result is enhanced productivity, whether you are investigating complex homo/hetero-associations or require an extensive series of A_2 measurements under varying buffer conditions.

Calypso is an automated, repeatable means of investigating sample interactions

The Calypso system consists of hardware and software for carrying out CG-MALS measurements, working in conjunction with any Wyatt MALS instrument, such as the DAWN® or miniDAWN®, and an optional concentration detector, such as the Optilab® Differential Refractometer or a third-party UV/Vis absorption spectrometer.

The Calypso II hardware generates and delivers accurate sample composition gradients to the detectors. The Calypso II comprises a set of three computer-controlled syringe pumps together with associated degassers, filters, mixers and valves. Microstepper syringe pumps provide pulse-free delivery and precise mixing ratios.

The CALYPSO™ software integrates all aspects of CG-MALS: Controlling the pumps, synchronizing injections with data acquisition, storing and analyzing the molecular interaction data and reporting the results.

Compared to manual batch measurements, sample preparation times are significantly reduced since only a single stock solution of each sample type is prepared manually. The Calypso performs all further dilutions and mixing, minimizing human error in the preparation of composition series, resulting in improved reproducibility and consistency of experimental results. Optional external modules like the Orbit™ valve further enhance operations with minimal user intervention.

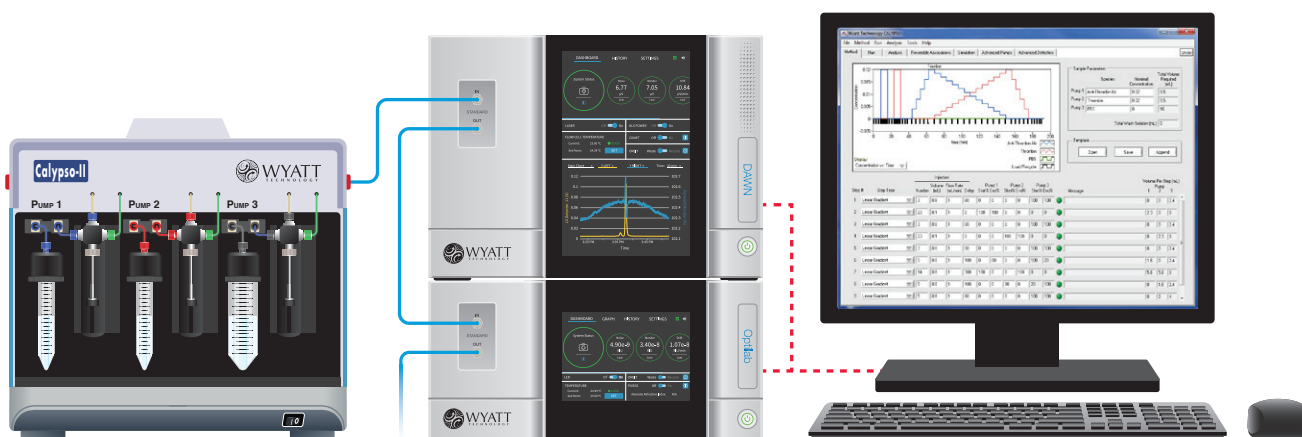


Figure 1 Calypso II connected in series to light scattering and concentration detectors, orchestrated by the CALYPSO software.



Streamline Measurements

SPECIFIC INTERACTIONS

- Reversible self- and hetero-association
- Equilibrium dissociation constant K_d (pM-mM)
- True solution-phase stoichiometry

NON-SPECIFIC INTERACTIONS

- Self-virial coefficients A_2, A_3
- Cross-virial coefficients

KINETICS

- Aggregation, association or dissociation reaction times from seconds to hours

MOLECULAR SIZES

- Weight-averaged molar mass M_w
- Root mean square radius R_g (a.k.a. radius of gyration)

REFRACTIVE INDEX

- Differential refractive index increment, dn/dc

Macromolecular Interactions

CG-MALS quantifies interaction strengths spanning orders of magnitude

Macromolecular interactions are measured with unfractionated samples or “batch” measurements, via the technique known as Composition Gradient MALS (CG-MALS).

The CG-MALS technique entails automatically preparing and injecting into a light scattering detector a series of compositions or concentrations of a macromolecular solution. After each injection the flow stops to permit the reaction to reach equilibrium. The apparent weight-average molar mass, $M_{w,app}$, is determined for each step in the gradient by analyzing light scattering and concentration data. Significant interactions between macromolecules manifest as changes in $M_{w,app}$ vs. composition—decreasing with repulsive interactions and increasing with attractive interactions. Depending on the application, each composition gradient can comprise dilutions of a single species or different ratios of two or even three species mixed together.

A rich assortment of interaction models is available for data analysis. The virial expansion and effective hard sphere approximation are best suited for nonspecific interactions. Self-association may be represented as equilibrium between monomers and one or more larger oligomers. Hetero-association models include standard monovalent and multivalent interactions, as well as more complex stoichiometries—combinations of self- and hetero-association and even metacomplex formation.

SELF INTERACTIONS

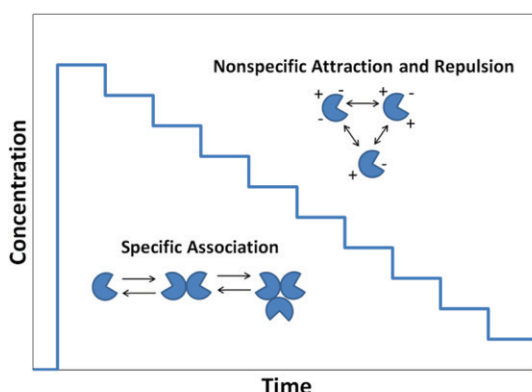


Figure 2
The light scattering signal from a single-species concentration gradient determines molecular weight and interactions:

Affinity (K_d) and stoichiometry of oligomerization

Virial coefficients (A_2) for nonspecific attraction and repulsion

BUFFER CONDITIONS

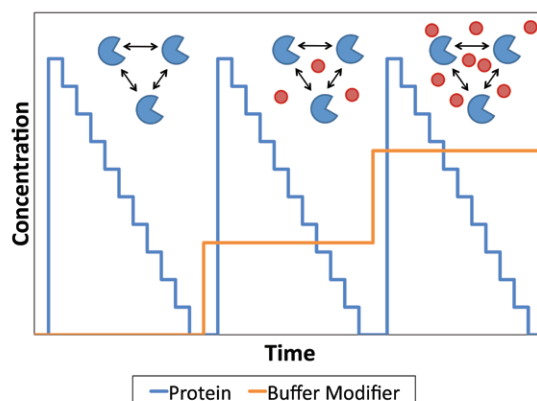


Figure 3
Macromolecular interactions are highly influenced by solvent conditions. The Calypso II fully automates repeat experiments to probe the effects of pH, ionicity, and concentrations of other excipients.

HETERO-ASSOCIATIONS

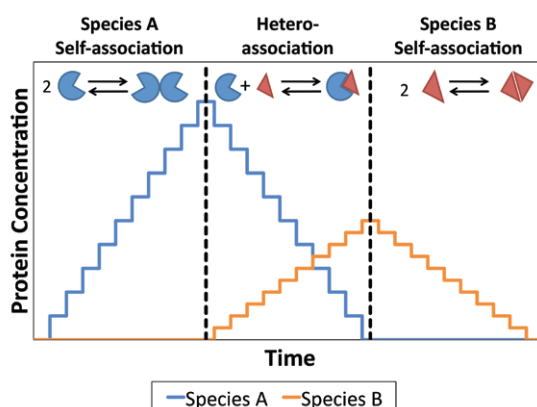


Figure 4
Identifying the magnitude and stoichiometry of interactions between two binding partners—entirely in solution—has never been easier, with a full suite of models to analyze multiple stoichiometries and high order complex formation!

How CG-MALS Works

Self-association and Hetero-association

Reversible binding phenomena—involving molecules of the same or different species—are governed by thermodynamic equilibrium between free monomers and complexes, described in terms of equilibrium dissociation constants K_d . Specific complexes form at different concentration ranges and, in the case of hetero-association, different ratios of species A and B. The presence of associated complexes manifests through changes in the solution's weight-average molar mass, M_w , which is reflected in turn by the excess Rayleigh ratio R_θ —the fraction of incident light scattered by the sample.

In the case of an ideal, dilute solution, the total excess light scattering measured is just the sum of intensity from each species present:

$$\frac{R_\theta}{K^*} = M_A c_A + M_B c_B + \sum M_i c_i$$

The concentration of each species c_i is dependent on $K_{d,i}$ and the monomer concentrations c_A and c_B . The Calypso software fits user-selected or defined association models to MALS data from a series of compositions in order to solve for both the affinity, in terms of association constants, and stoichiometry. CG-MALS clearly distinguishes between different stoichiometries with the same stoichiometric ratio, e.g. 1:1 vs. 2:2.

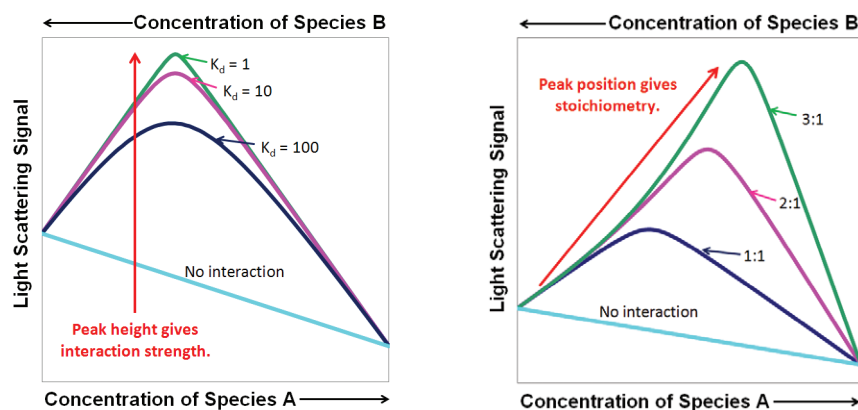


Figure 5 Measuring specific interactions by light scattering allows for the calculation of both the interaction strength (K_d) and complex stoichiometry.

Nonspecific Interactions

In the limit of low concentrations, the fundamental relationship linking the intensity of scattered light and the molecular properties of a single non-associating species is:

$$\frac{R_\theta}{K^*c} = \frac{M}{1 + 2A_2Mc + 3A_3Mc^2 + \dots}$$

R_θ is the excess Rayleigh ratio extrapolated to zero scattering angle,

K^* is an optical parameter that depends on system constants such as the laser wavelength and solvent refractive index,

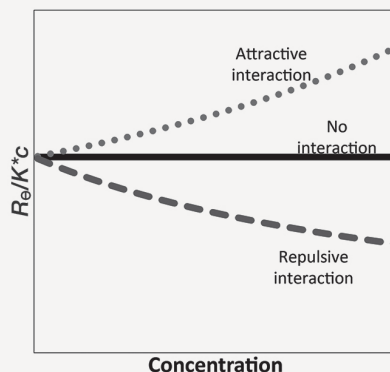
c is the sample concentration (g/L),

M is the weight-average molar mass (g/mol),

A_2 is the second virial coefficient,

A_3 is the third virial coefficient

A plot of R_θ/K^*c vs. c yields a curve whose intercept gives M and whose slope at low concentrations gives A_2 , an indicator of non-specific interactions between molecules, as mediated by the solvent. The figure below shows qualitatively the effect of attractive and repulsive interactions on the concentration dependence of light scattering. At higher concentrations the curve may be fit to yield A_3 as well.



Applications

From high-affinity protein-protein binding to high-concentration formulation characterization, the Wyatt Calypso II does it all!

Antibody-antigen Interactions

In biopharmaceutical development, desirable drug-target interactions typically exhibit strong affinity and high specificity. Standard antibody-target complexes bind with a 1:1 or 1:2 stoichiometry, but other stoichiometries are possible when one or both molecules self-associate. Many interaction assays cannot identify conclusively the true stoichiometries present in solution. In this example (shown in Figure 6 & 7) of a commercial drug product, CG-MALS—automated by Calypso—determined the affinity and stoichiometry, without labeling or immobilization, for an antibody-antigen pair.

The dramatic increase in light scattering signal observed during the hetero-association portion of the experiment, despite a nearly constant overall protein concentration, is a direct indicator of antibody-antigen binding.

Using concentration gradients of 5-50 µg/mL for each protein, the expected bivalent association was confirmed; other stoichiometric models just do not fit the data. A calculated equilibrium dissociation constant K_d of 2 nM per binding site matched previous analysis by an orthogonal technique, surface plasmon resonance (SPR).

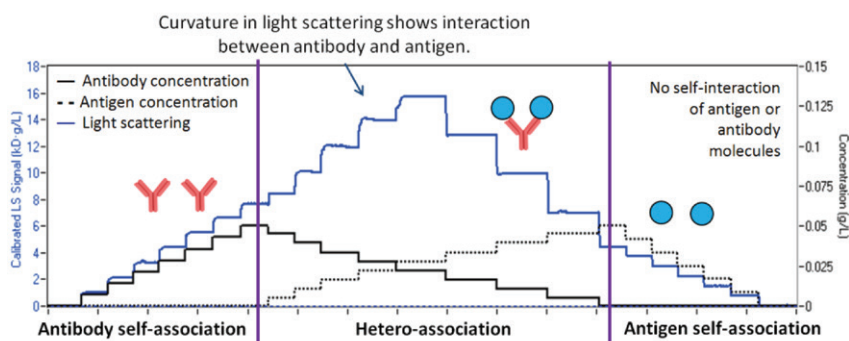


Figure 6 Light scattering and concentration data for antibody-antigen interaction, utilizing the method of Figure 4 to identify possible self- and hetero-association.

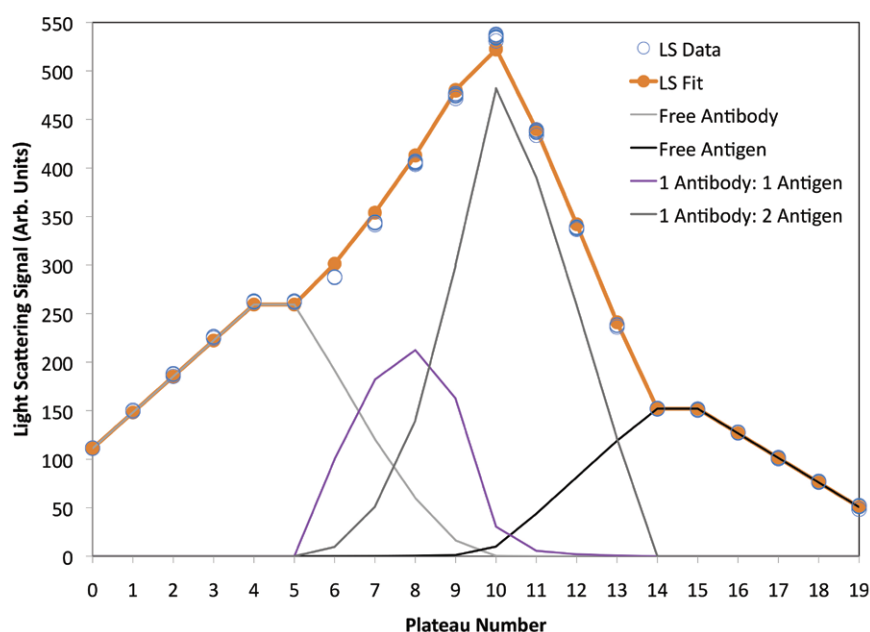


Figure 7 Best fit of light scattering data for hetero-association gradient in Figure 6, showing the contribution of each species to the overall light scattering signal.

Nonspecific Interactions vs. pH

At intermediate concentrations above ~ 1 mg/mL, all macromolecules will exhibit some form of nonspecific interaction. Understanding how these interactions vary with buffer conditions is critical to optimizing buffer formulation, purification and protein crystallization.

Concentration gradients of a protein in buffer solution were automated to produce a series of pH states by programmatically titrating a neutral buffer and sample stock solution with a low-pH buffer. Around the isoelectric point (pI), the charge of the protein approaches neutral, and electrostatic repulsion is at a minimum. This is evidenced by a minimum in the repulsive second virial coefficient, A_2 . Referencing these A_2 values to the hard-core repulsion—the excluded-volume A_2 is $9 \cdot 10^{-5}$ mol \cdot mL/g 2 —indicates net “soft” forces are attractive in the pH range 3 to 6.

Analysis of Protein Formulation at High Concentration

Macromolecules that appear relatively benign at low concentrations can behave unpredictably at high protein concentrations, where weak attractive forces—insignificant at low concentrations—lead to aggregation, poor stability and high viscosity. Especially challenging are therapeutic antibodies, often formulated at 100 to 200 mg/mL, especially for non-intravenous delivery. Such systems must be characterized under true, not dilute, conditions in order to fully elucidate the final product.

For well-formulated proteins, repulsive interactions dominate, manifesting as a highly sub-linear relationship between light scattering and concentration. Figure 9 plots the light scattering and concentration data for a stable solution of bovine serum albumin (BSA) at pH 7, up to 100 mg/mL. Even at the maximal concentration there is no evidence of aggregation.

In contrast, specific self-association of chymotrypsin evolves with concentration, as shown in Figure 10. At concentrations below 10 mg/mL, monomer-dimer equilibrium dominates the interaction landscape. At higher concentrations, pentamers form, and the dimer concentration decreases (C. Fernández & A. P. Minton, *Biophys. J.*, 96, 1992-1998 (2009)).

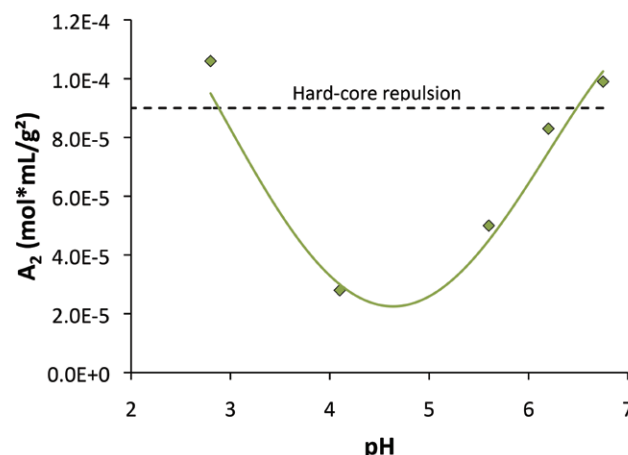


Figure 8 The second virial coefficient for BSA varies as a function of buffer pH, with a minimum at pI .

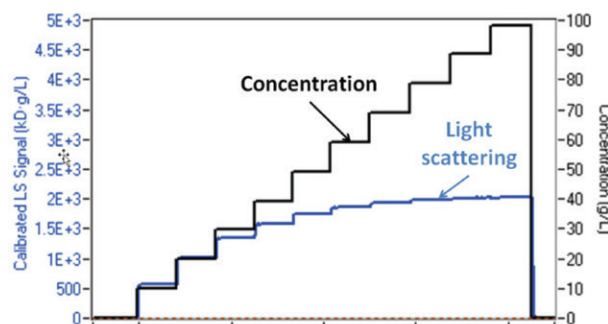


Figure 9 BSA, 10-100 mg/mL. A sub-linear relation between light scattering and concentration indicates strong repulsion between macromolecules.

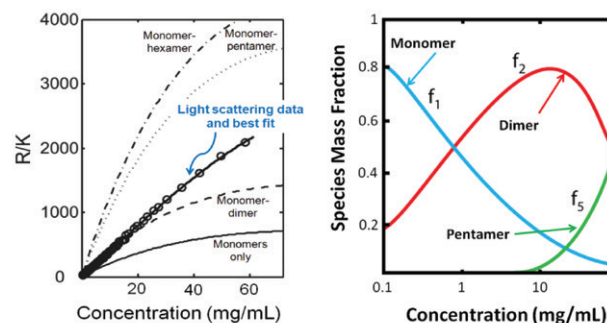


Figure 10 CG-MALS data show that specific oligomers of chymotrypsin appear only at higher protein concentrations.

Left Raw light scattering data and best fit analysis for chymotrypsin self-association; reference lines for incomplete models, as indicated.

Right Concentrations of each species present in best-fit analysis.*

*Reprinted from *The Biophysical Journal*, Vol. 96, C. Fernández & A. P. Minton, “Static Light Scattering From Concentrated Protein Solutions II: Experimental Test of Theory for Protein Mixtures and Weakly Self-Associating Proteins,” 1992-1998, Copyright (2009), with permission from Elsevier.

Comparing CG-MALS to Other Label-free Interaction Methods

Other prevalent biophysical methods for quantifying protein-protein and related macromolecular interactions include surface plasmon resonance (SPR), isothermal titration calorimetry (ITC) and analytical ultracentrifugation sedimentation equilibrium (AUC-SE). As shown in the table below, Calypso provides the broadest range of analyses with no sample modification required:

- No immobilization or labeling, hence no questions about the effect on the interactions
- Range of macromolecular binding affinities equal to or greater than any other technique: pM to mM
- Kinetics of association, dissociation and aggregation:
 $k_{\text{on}} < 10^7 \text{ M}^{-1}\text{s}^{-1}$ (typical for 100 kDa molecule),
 $k_{\text{off}} < \sim 1 \text{ s}^{-1}$
- Allosteric inhibition or cooperativity
- Self- and hetero-association analysis with rich possibilities for association models, e.g., simultaneous self- and hetero-association, aggregation of complexes, multiple binding sites, protein-lipid or protein-carbohydrate complexes, etc.
- Characterize non-specific interactions, as well as binding affinity and stoichiometry of specific complexes
- Account for inactive (non-interacting) sample
- Self- and hetero-association of macromolecules at high concentration
- Share detectors with size exclusion chromatography or field flow fractionation for absolute determination of molar mass distributions
- Add in a WyattQELS™ module for simultaneous measurement of hydrodynamic radius

Comparison of Different Measurement Techniques

	CALYPSO	SPR	ITC	AUC-SE
Sample modification	In solution, label-free	Immobilized on chip surface	In solution, label-free	In solution, label-free
Measurement time	30 minutes to 1 hour	Minutes to hours	1 to 2 hours	Hours to days
Range of K_D	pM to mM. 100 kDa: >100 pM 10 kDa: >10 nM	pM to μM	nM to mM	nM to mM
Self-association	Yes	No	Yes	Yes
Stoichiometry	Any, self+hetero, metacomplexes	1:1; at best 1:n	Stoichiometric ratio only, self+hetero	Any, self+hetero
Non-specific interactions	• Self+cross virial coefficients • Reversible association at high concentration	No	No	Yes
Kinetics	Yes, moderate to slow reaction rates	Yes, but not solution-phase; mass transport limitations	Only very slow	No
Indicates aggregation state	Yes	No	No	Yes
Related separation technique	Yes (SEC-MALS, FFF-MALS)	No	No	Yes (sedimentation velocity)
Additional information	Size: R_g 10 to 500 nm R_h 0.5 to 1,000 nm	None	Thermal capacitance, C_p	Friction coefficient
Manual cell cleaning	No	No, but expensive chips	Yes	Yes
Sample quantity to measure $K_D \sim 100 \text{ nM}$ for 100 kDa protein	100 μg	20 μg	$\sim 100 \mu\text{g}$	$\sim 50 \mu\text{g}$
Challenges	• Particle removal • Molecules <1 kDa	• Mass transport • Immobilization chemistry • Molecular orientation • Regeneration	• Kinetics • Dialysis • Buffer selection	• Sample degradation over course of long measurement • Kinetics • Small molecules

Specifications

Range of Measurement	<p><i>Equilibrium Dissociation Constant, K_d</i>: 100 pM to mM, typical for 100 kDa molecules (actual range varies with molecular weight and association stoichiometry)</p> <p><i>Association Stoichiometry Models</i>: Arbitrary self + hetero-association models, equivalent binding site for self- and hetero-association, aggregation of complexes, incompetent fractions</p>
Repeatability of Measurement	<p>M_w: $\pm 5\%$</p> <p>A_2: $\pm 0.5 \times 10^{-4}$ mol·mL/g² for sample molar mass of 50 to 100 kDa</p> <p>$\log(K_d)$: ± 0.3</p>
Typical Sample Per Analysis	<p>Antibody-receptor interaction: 100 to 200 μg</p> <p>A_2 measurement: 15 to 20 mg</p>
Compatible Detectors	<p>MALS detectors: DAWN or miniDAWN</p> <p>Concentration detectors: Optilab differential refractometer, UV/Vis absorption with analog output or similar</p>
Syringe Volumes	12.5 μ L to 2.5 mL (0.5 mL and 1 mL syringes supplied as standard)
Solvents	Aqueous
Wetted Materials	Borosilicate glass, PEEK, PTFE, polyethylene, alumina, titanium, stainless steel
Auxiliary Inputs/Outputs	Autoinject out, injector valve control, valve control digital and analog I/O for specific applications
Dimensions:	37 cm (W) x 35 cm (H) x 58 cm (D)

* Specifications subject to change without notice.

Warranty All Wyatt instruments are guaranteed against manufacturing defects for 1 year.

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Left to Right

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Dr. Philip J. Wyatt, Chairman of the Board
Clifford D. Wyatt, President

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The Calypso II is just one of the many tools in the Light Scattering Toolkit for Essential Biophysical and Nanoparticle Characterization.

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