Technical Article

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Characterizing Protein–Protein Interactions via Static Light Scattering: Nonspecific Interactions

The quantitative characterization of nonspecific protein–protein interactions is essential in understanding basic biomolecular function as well as the development of new biotechnology products. Virial coefficients indicate the overall attraction or repulsion between molecules, providing a general measure of the intermolecular potential as mediated by the solvent. In biotechnology applications, fine-tuning self-virial coefficients A_2 (SVC) and cross-virial coefficients A_{11} (CVC) by adjusting pH, ionic strength, and concentrations of various excipients in the buffer can help set optimal conditions for stability, purification, and crystallization. For example, George and Wilson[1] have shown that crystallization typically occurs within a small range of A_2 values corresponding to a weak attraction. Luisi et al.[2] have demonstrated the correlation between A_2 (measured at low concentrations) and opalescence (occurring at high concentration). Ho et al.[3] found a strong correlation between A_2 and aggregation during renaturation. All of these point to the importance of measuring and interpreting virial coefficients for protein characterization.

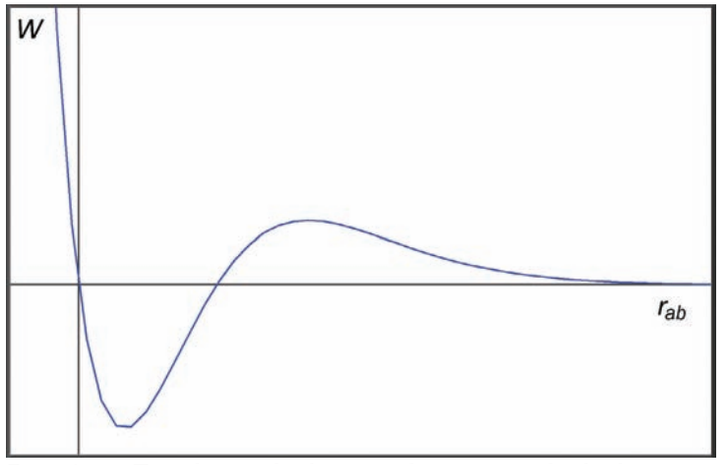

Figure 1 Typical intermolecular potential versus intermolecular distance for proteins in solution. High potential left of the vertical line corresponds to the hard-core repulsion.

Understanding virial coefficients

The information captured in A_2 may be appreciated by examining the origins of the virial expansion for osmotic pressure. In the absence of intermolecular interactions, the behavior of molecules in solution may be described by the ideal gas law: $p = k_BTc/M$, where p is the pressure, k_BT the Boltzmann factor, c the weight concentration of molecules, and M their molar mass. Real molecules interact via intermolecular potentials such as the one presented in *Figure 1*, consisting of a hard-sphere repulsion due to the molecular core, together with short- and long-range interactions due to net charge, dipoles, van der Waals potentials, etc. The interaction is modified by solvent contributions such as ionic screening and hydration forces. The net effect of these interactions on the osmotic pressure under dilute conditions is embodied in the virial expansion: $p = k_BTc(1/M+A_2(T)c+A_3(T)c^2+\ldots)$, which reduces to the ideal gas law as $c\rightarrow 0$. A_n are the "virial coefficients,"with A_2 being the most important. If the net effect of the interaction potential is repulsive, A_2 is positive, and if the net effect is attractive, A_2 is negative.

When characterizing proteins and other molecules for stability, purification, and crystallization, we are usually interested only in the "soft" interactions—the attractive and repulsive forces, excluding the hard-core repulsion that does not affect these processes. The "baseline" value of A_2—the value that would be measured if the only interaction between molecules was that due to the hard core—is readily calculated for a globular protein of mass M as per Eq. (1):

$$A_2^{HC} = \frac{16\pi N_A r^3}{3M^2} \qquad (1)$$

where N_A is Avogadro's number and r is the effective radius. If not known from the molecular structure, a reasonable measure of r is the hydrodynamic radius r_h obtained from dynamic light scattering.

A typical example of A_2^{HC} is that of bovine serum albumin (BSA) monomer: r_h~3.5 nm, M = 66.4 kDa; hence A_2^{HC} ~$1.0 \cdot 10^{-4}$ mol · mL/g^2. In phosphate buffered saline (PBS) at pH 7.2, the measured value of A_2 for BSA is ~$1.4 \cdot 10^{-4}$ mol · mL/g^2, indicating additional repulsion due to the net charge present under these conditions.

Analogous to the self-virial coefficient, the cross-virial coefficient A_{11} measures the nonspecific interactions between cosolutes. The hard-sphere value of A_{11} may be calculated from the radii and masses of the two molecules as per Eq. (1a):

$$A_{11}^{HC} = \frac{2\pi N_A(r_1+r_2)^3}{3M_1M_2} \qquad (1a)$$

Additional repulsion will increase A_{11}, while attraction will reduce A_{11}, going to negative values for relatively strong attraction.

Measuring virial coefficients

The most generally applicable, rapid, and nondestructive means of determining A_2 and A_{11} of molecules in solution is composition gradient-multiangle static light scattering (CG-MALS). The CG-MALS technique was previously introduced in this journal as a means of characterizing specific heterointeractions of reversibly associating proteins.[4] CG-MALS has few limitations on solvents, and may be used with general formulation buffers or under native solution conditions.

The virial equation of light scattering for a single species in dilute solution is:

$$R(c,\theta) = K\left(\frac{dn}{dc}\right)^2 \frac{McP(r_g,\theta)}{1 + 2A_2McP(r_g,\theta) + \ldots} \qquad (2)$$

where $R(c,\theta)$ is the excess Raleigh ratio, effectively the light scattering intensity; K is a constant that depends on the illumination wavelength and solvent refractive index; dn/dc is the sample refractive index increment, a readily measurable quantity; and $P(r_g,\theta)$ represents the angular dependence of the scattered light on r_g, the radius of gyration of the sample. For most proteins and other molecules smaller than ~25 nm in diameter, the value of $P(\theta)$ is very nearly 1, but for larger particles, e.g., liposomes, the angular dependence of the scattered light must be taken into account. Eq. (2) is often written as:

$$\frac{Kc}{R(c,\theta)}\left(\frac{dn}{dc}\right)^2 = \left(\frac{1}{MP(r_g,\theta)} + 2A_2c + \ldots\right) \qquad (2a)$$

Determination of SVC via the CG-MALS technique consists of measuring $R(c,\theta)$ over several values of c, and fitting the results to Eq. (2a).

Likewise, the CVC may be determined by measuring $R(c,\theta)$ at a series of compositions of the two cosolutes A and B—changing the ratio c^A:c^B and/or total concentration—and fitting the results to Eq. (3):

$$\frac{R(c,\theta)}{K} = \left(\frac{dn}{dc^A}\right)^2 \frac{M^A c^A P(r_g^A,\theta)}{1 + 2A_2{}^A M^A c^A P(r_g^A,\theta)} + \left(\frac{dn}{dc^B}\right)^2 \frac{M^B c^B P(r_g^B,\theta)}{1 + 2A_2{}^B M^B c^B P(r_g^B,\theta)} - 4\left(\frac{dn}{dc^A}\right)\left(\frac{dn}{dc^B}\right) A_{11} M^A M^B c^A c^B P(r_g^A,\theta) P(r_g^B,\theta) + \ldots \qquad (3)$$

The deviations from ideality due to SVC and CVC are typically small effects. At moderate sample concentrations of a few mg/mL, SVC and CVC contributions might change the scattered intensity by only a few percent. Precise measurements of light scattering intensities, which are critical to determining virial coefficients with good accuracy, require a stable environment that may only be obtained with a quality MALS flow cell as well as highly linear photodiodes. SVC or CVC measurements with cuvettes or scintillation vials are inadvisable, since these often give different readings each time they are reinserted into the instrument. Avalanche photodiodes and UV absorbance detectors are typically nonlinear in the required concentration regime and will produce biased data. The optimal concentration detector for SVC and CVC measurements is an on-line differential refractometer.

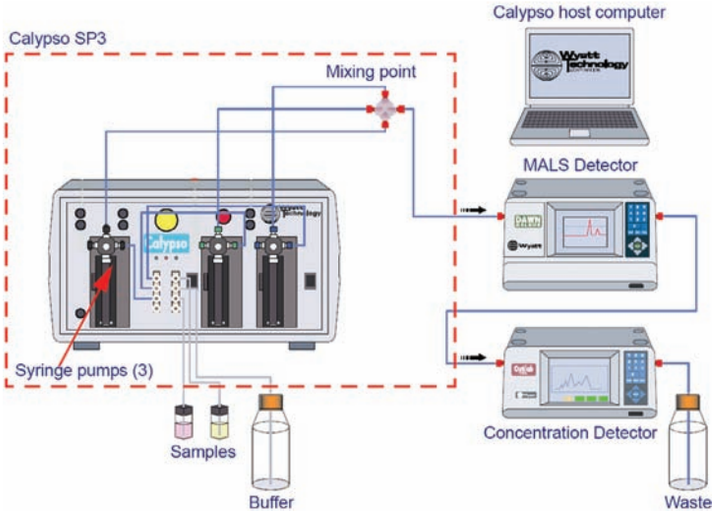

Figure 2 CG-MALS experimental setup utilizing the Calypso SP3 sample preparation and delivery hardware, a static light scattering detector, and a concentration detector.

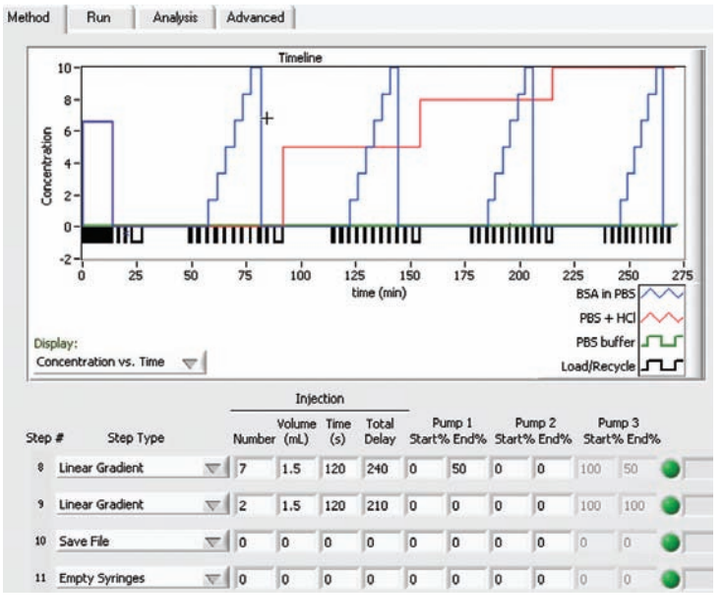

Figure 3 Method design for the characterization of virial coefficients over a range of pH conditions includes the method table and timeline graph.

Automation

A CG-MALS procedure consists of preparing solutions of each of the required compositions, delivering each to a static light scattering (SLS) instrument and a concentration detector, recording the scattered intensity and concentration values, and performing a fit of the data to the appropriate equation. Traditionally, this procedure has been carried out manually, a tedious, time-consuming process 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's SP3 hardware accessory features three computer-controlled syringe pumps linked to sample and buffer reservoirs. The software automates sample preparation and delivery by combining the output pump streams via a static mixer, then injecting into the detectors. Different compositions are obtained by varying the relative flow rates of the pumps. After metering the predefined volume, flow stops and data may be integrated over any time scale. The duration of the measurement is typically 0.5–1 hr per gradient, about 3–5 hr for an entire series characterizing multiple buffer conditions. A complete CG-MALS setup is shown in *Figure 2*.

Solvent variation

The first example examines the dependence of SVC on pH for BSA. Standard phosphate buffers with 50 mM NaCl were prepared at pH 6.7 and at pH 2.0 by addition of HCl. BSA, obtained from **Sigma-Aldrich** (Milwaukee, WI), was prepared at 8 mg/mL in the neutral buffer.

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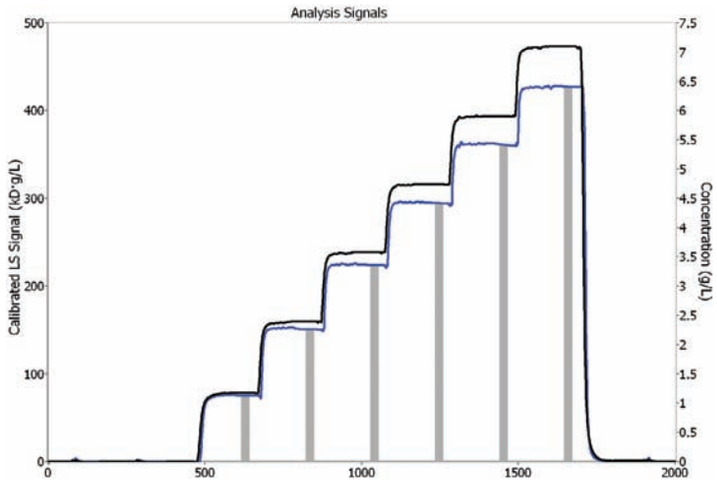

Figure 4 CG-MALS data for measuring A_2: light scattering signal (blue) and concentration signal (black). BSA in PBS, pH 6.7.

A method was programmed in the Calypso software to automate A_2 measurements over a series of pH values by mixing different ratios of the neutral and acidic buffers. In the CG-MALS method, each concentration gradient consists of a buffer flush to obtain initial baseline values, then a concentration series, and finally a buffer flush for final baseline. The sequence is repeated at each pH. Part of Calypso's method table is reproduced in *Figure 3*, along with the timeline graph.

Figure 4 depicts the SLS (blue) and concentration (black) signals for a single concentration gradient at pH 6.7. Each plateau corresponds to the signal from a particular sample concentration. The vertical gray bars denote the region of each plateau selected for analysis.

Figure 5 plots the apparent molar mass versus concentration for each plateau of Figure 4 (A_2 is derived from the slope of this plot) and the dependence of A_2 on pH. As the pH approaches the isoelectric point at ~4.6, the charge per molecule decreases, leading to reduced electrostatic repulsion and thus a lower value of A_2. Most of the measured values are below the excluded volume value, indicating overall attraction between molecules. The same procedure may be employed to test the effect of NaCl or other excipients on the solvent dependency of the SVC. For crystallization, the value of SVC should be slightly negative, while for long-term solubility, the optimal SVC would be higher than the hard core value.

Cross-virial coefficients

The second example demonstrates characterization of nonspecific interactions between cosolutes, BSA, and lysozyme. The experimental method incorporates three segments: a concentration gradient of BSA alone, followed by a crossover composition gradient changing the ratio of c^{BSA}:$c^{lysozyme}$, and finally a concentration gradient of lysozyme. The single-component gradients determine the respective SVC values of each protein separately, while the crossover gradient adds the interaction data required for the CVC analysis.

Buffer conditions and BSA preparation were the same as in the previous measurement, while lysozyme, also obtained from **Sigma-Aldrich**, was prepared at 30 mg/mL and similarly dialyzed.

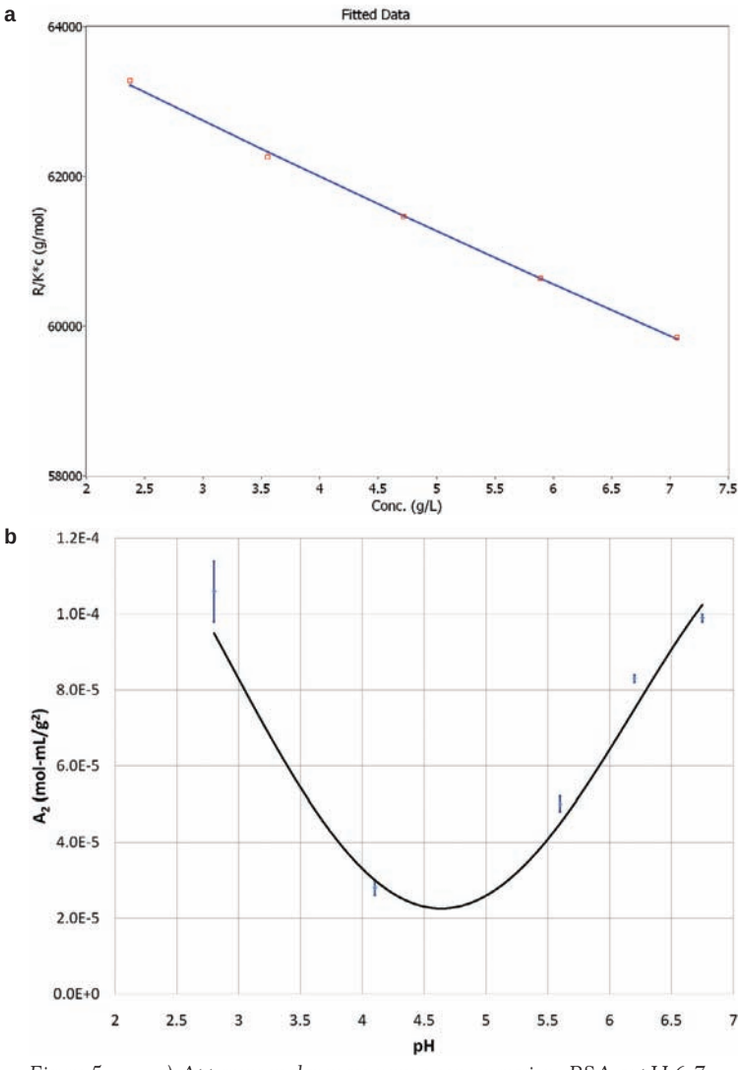

Figure 5 a) Apparent molar mass versus concentration, BSA at pH 6.7, fit to determine A_2. *b)* A_2 *versus pH (the line is a guide to the eye). Minimal* A_2 *occurs at the isoelectric point.*

Figure 6 presents the SLS and concentration signals for the three gradient segments, and the fit of the MALS data to the CVC equation. Analysis of the data produces A_2 values of $+1.23 \cdot 10^{-4}$ mol · mL/g^2 for BSA and $-3.26 \cdot 10^{-4}$ mol · mL/g^2 for lysozyme, while the CVC value is $A_{11} = -3.85 \cdot 10^{-4}$ mol · mL/g^2. In this buffer, BSA molecules repel each other, while lysozyme attracts both lysozyme and BSA.

One of the practical applications of CVC is purification.[5] In an optimal purification solvent, each molecule would be attracted to like molecules but repelled by different molecules. The purification process may be enhanced by finding a solvent that minimizes SVC values while maximizing CVC.

Summary

These examples illustrate how CG-MALS rapidly and conveniently quantifies nonspecific interactions, useful for improving processes

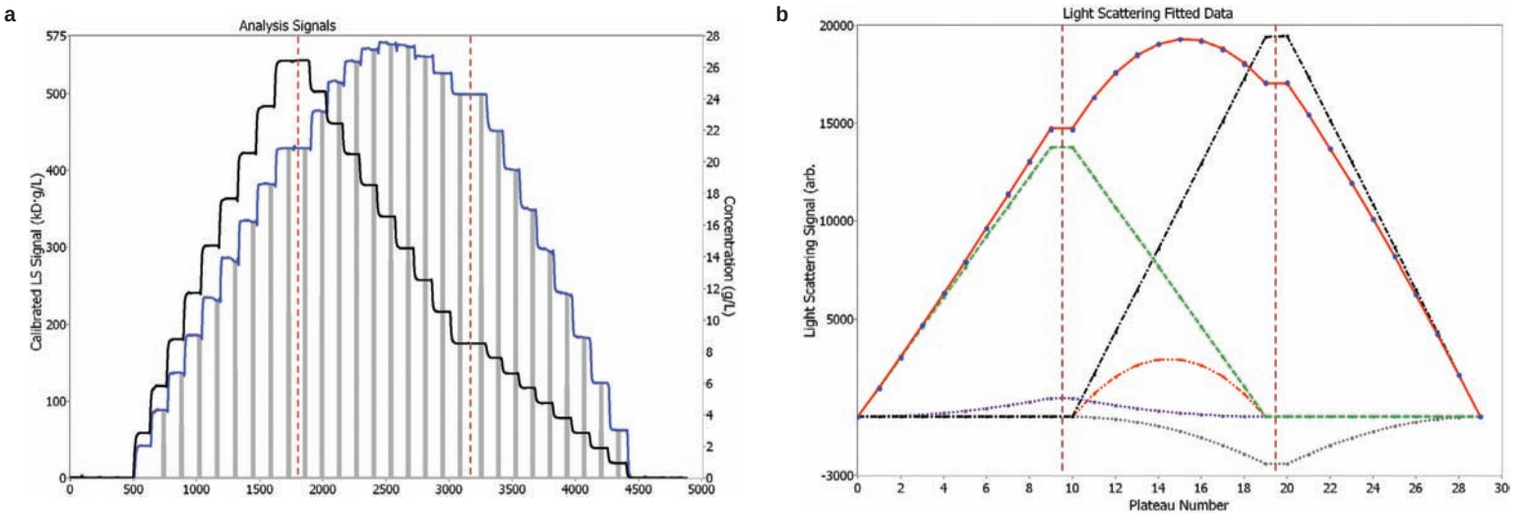

Figure 6 *a) CVC data: SLS (blue) and concentration (black). b) Fit of SLS data to CVC equation,* $A_2^{BSA} = +1.2 \cdot 10^{-4}$; $A_2^{lysozyme} = -3.3 \cdot 10^{-4}$; $A_{11}^{BSA\text{-}lysozyme} = -3.8 \cdot 10^{-4}$ *mol · mL/g*^{2}*. The color plots indicate the light scattering from each component: noninteracting A and B, and interaction terms.*

and formulations of proteins and other molecules or particles of interest in biotechnology. Often monoclonal antibodies and other sophisticated biotechnology products will exhibit complex behavior, including regimes of self-association or aggregation that require different mathematical analyses. Conveniently, CG-MALS produces experimental data compatible with various types of analysis offered by the Calypso software (SVC, CVC, reversible association, and kinetics). For example, a negative SVC could indicate a weak attraction but could also be a harbinger of self-association. It is possible to analyze the same data in terms of both SVC and reversible self-association to dimers and higher oligomers.

CG-MALS technology will benefit biophysical characterization laboratories interested in various types of protein–protein interactions, including nonspecific interactions. 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 a valuable add-on for the complementary interaction measurements.

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