Light Scattering

Light scattering methods can provide information about the native molecular weight, oligomeric composition, and gross conformation of a protein in solution. These methods are particularly well suited for studying large oligomeric systems or glycoproteins and can be used to characterize much larger structures involving protein such as viruses and even bacterial spores (Harding, 1997). Light scattering techniques are not so well suited for characterization of smaller protein systems (where the molecular weight, M, is <30,000 Da); in these cases other methods such as analytical ultracentrifugation (*UNIT 7.5*) and solution X-ray scattering are more suitable. All light scattering measurements on solutions of proteins and protein assemblies are based on the principle of analyzing the intensity of light scattered by the solution (Fig. 7.8.1), either in terms of the time-averaged intensity ("classical" or "static" light scattering) or intensity fluctuations with time ("dynamic" or "quasielastic" light scattering) at a given angle or series of angles.

There are three types of "static" light scattering experiment:

1. Turbidimetry, which is simple but gives only crude molecular-weight estimates for large assemblies;
2. Low-angle light scattering, which is also simple and gives molecular-weight and molecular-weight-distribution information;
3. Multiangle light scattering, which gives more reliable molecular-weight and molecular-weight-distribution information and, for proteins of molecular weight at least ~50,000 Da, solution conformation information.

There are also three types of "dynamic" light scattering measurements:

1. Fixed-angle (90°-angle) measurements, which are simple and give an estimate for the translational diffusion coefficient and an idea of sample polydispersity for approximately globular macromolecules;
2. Variable-angle measurements, which are less simple, give more reliable estimates for translational diffusion coefficient and sample polydispersity, and can in some circumstances yield an estimate for rotational diffusion coefficients;

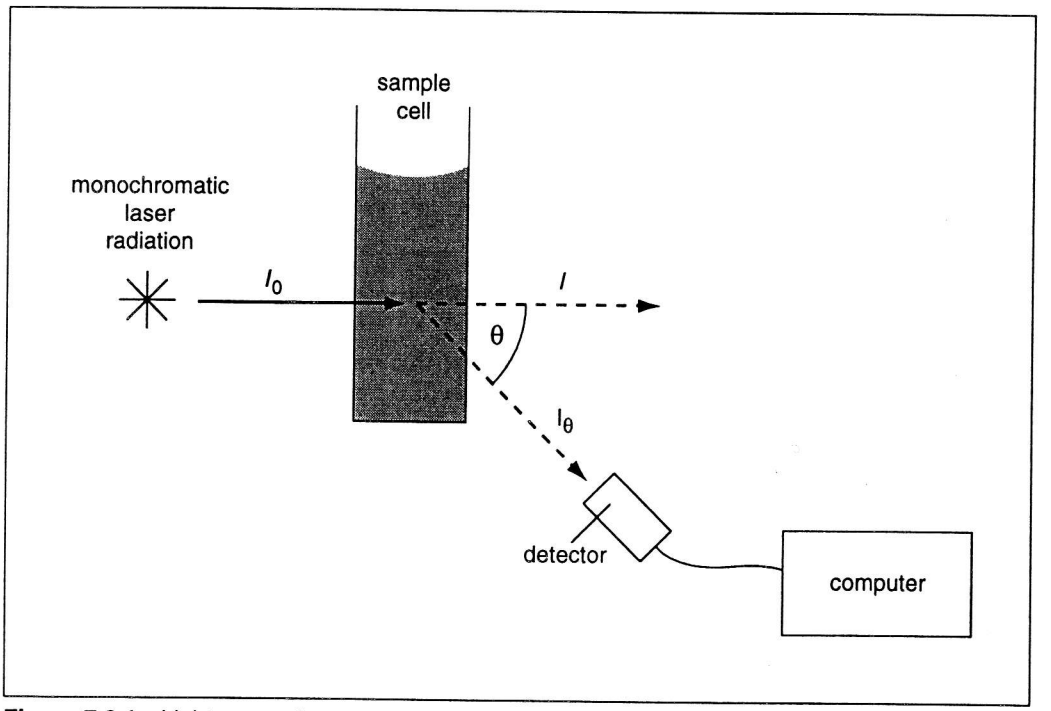

Figure 7.8.1 Light scattering studies on biomolecular solutions involve consideration of the relationship between the time-averaged scattered light intensity, $I(\theta)$, with the incident intensity, I_0, and angle of detection, θ ("static light scattering") or of the rapid fluctuations of the scattered intensity, $I(\theta)$, with time, τ ("dynamic light scattering"). Turbidimetry involves static light scattering measurements on the relation between I_0 and transmitted light intensity, I (at zero angle) alone.

Contributed by Stephen E. Harding and Kornelia Jumel
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3. Electrophoretic light scattering measurements ("ELS"), popularly used for studying colloid solubility.

STATIC LIGHT SCATTERING ANALYSIS OF PROTEIN SOLUTIONS

Basic Theory

The basic equation for the angular dependence of light scattered from a solution of proteins or protein assemblies is the Debye-Zimm relation (Zimm, 1948), shown in Equation 7.8.1,

$$\frac{Kc}{R_\theta} = \frac{1}{MP(\theta)}(1 + 2A_2c + \ldots)$$

Equation 7.8.1

In this equation, A_2 (ml mol/g^2) is the thermodynamic (2nd virial) nonideality coefficient. R_θ is the Rayleigh excess ratio (the ratio of the intensity, I_θ, of excess light scattered compared to pure solvent) at an angle θ to that of the incident light intensity, I_0 (a correction term is necessary if unpolarized light is used but not necessary if lasers are used). K is an experimental constant dependent on the square of the solvent refractive index, the square of the refractive index increment (*dn/dc* in ml/g), and the inverse fourth power of the incident wavelength, λ (cm). M is the molecular weight in Da, c is the solute concentration (g/ml), and $P(\theta)$ is the form factor. Equation 7.8.1 is valid if the proteins/protein assemblies satisfy the Rayleigh-Gans-Debye criteria illustrated in Equation 7.8.2:

$$\left|\frac{n}{n_0} - 1\right| << 1$$

and

$$\left(\frac{4\pi n_0}{\lambda_0}\right) d \left|\frac{n}{n_0} - 1\right| << 1$$

Equation 7.8.2

where n is the refractive index of the solution, n_0 is the refractive index of the solvent, λ_0 is the incident wavelength (in vacuo), and d is the maximum dimension of the particle. The form factor $P(\theta)$ can also be given to a good approximation by Equation 7.8.3,

$$\frac{1}{P(\theta)} \approx \left[\frac{1 + 16\pi R_\theta^2}{3\lambda^2} \sin^2\left(\frac{\theta}{2}\right)\right]\left(\frac{1}{M} + 2A_2c\right)$$

Equation 7.8.3

R_g is extensively referred to as the "radius of gyration" of the macromolecule and c is the solute concentration (g/ml). If the solute is heterogeneous, M (g/mol = Da) will be a weight average, M_W, and R_g a z-average. Equation 7.8.1 is generally a good representation for particles whose maximum dimension is between $\lambda/20$ and λ. Since λ is typically ~600 nm, this covers all proteins and protein assemblies up to the sizes of filamentous viruses. For larger particles, much more complex representations are necessary.

For particles of dimension $<\lambda/20$ ($M <$ 50,000 Da) the angular term in Equation 7.8.1 is small. No angular-dependence measurements are necessary to obtain M (although R_g cannot be obtained; if this is needed then X-ray or neutron solution scattering measurements need to be employed instead). For particles of dimension $>\lambda/20$, a double extrapolation to zero angle and zero concentration is necessary. This is usually performed on a grid-like plot referred to as a "Zimm plot" or via measurement at a single angle assumed small enough so that $\sin^2(\theta/2) \sim 0$. Other methods of representing the data have been in terms of "disymmetry": $z(\theta)$ (defined as the ratio of the scattering intensity at an angle θ, typically 45°, to that at 180° − θ) versus θ plots. Both $z(\theta)$ and R_g provide useful guides to the conformation of a macromolecule, with $z(\theta)$ being more popular with linear DNA molecules and R_g being more suited for descriptions of protein conformation. Other useful representations for nonprotein systems are also available (see Burchard, 1992).

For fairly rigid protein systems, R_g can be used directly to model gross conformation—either as an additional parameter to the diffusion coefficient (see Dynamic Light Scattering Analysis) and other hydrodynamic parameters for representing the structure in solution of complex protein systems in terms of bead modeling (Garcia de la Torre et al., 1997)—or as a parameter, after combination with the second virial coefficient A_2 and a parameter from solution viscometry known as the intrinsic viscosity, for representing the triaxial structure of a protein (Harding et al., 1997).

The principal useful parameters to be derived from static light scattering measurements

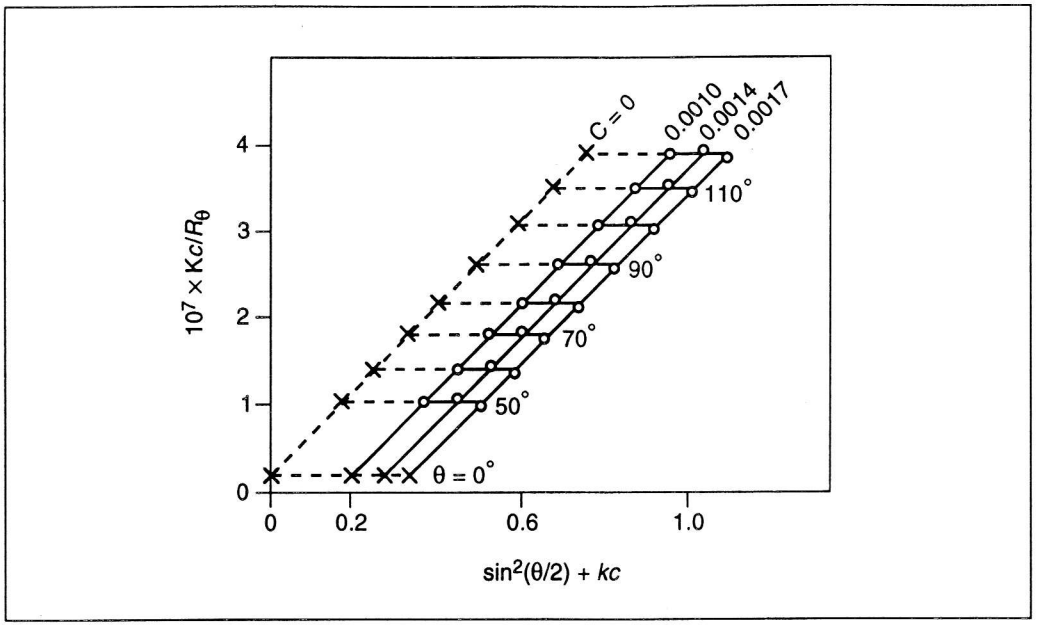

Figure 7.8.2 Zimm biaxial plot for a (diptheria) toxin-antibody aggregate. The arbitrary scaling constant, k, equals 200 ml/g. The molecular weight (M_w) as calculated from the (reciprocal) common intercept of the $c = 0$ and θ = lines is equal to ~78×10^6 Da. Data from Johnson and Ottewill (1954) and Johnson (1993).

are thus M, R_g, and to a lesser extent A_2. M can be obtained to a reasonable accuracy (usually to within 5%). The extraction of R_g is more difficult, requiring considerable care in the form of the angular extrapolation, and becomes even more difficult as the lower limit of $\lambda/20$ is approached. The use of light scattering photometers incorporating a flow cell that can be linked directly on-line to size-exclusion chromatography columns is becoming increasingly popular, particularly for the characterization of polydisperse systems (the hallmark, for example, of many glycoproteins), and as an on-line sample clarification system.

Turbidimetry

Turbidimetry involves the measurement of the total loss of intensity by a solution through scattering, summed over the entire angular intensity envelope and compared with the intensity of the incident radiation. It can be used to measure the molecular weights of protein assemblies of $M > 10^5$ Da (Bahls and Bloomfield, 1977). It is a type of measurement that can be performed on a good-quality spectrophotometer (i.e., one whose detector does not accept appreciable amounts of scattered light). Measurements have to be made at wavelengths away from the influence of absorption maxima.

Low-Angle Light Scattering (LALLS)

As θ approaches 0, Equation 7.8.1 becomes Equation 7.8.4:

$$\frac{Kc}{R_\theta} = \frac{1}{M} + 2A_2c$$

Equation 7.8.4

Low-angle light scattering photometers permit the measurement of Kc/R_θ at one fixed, small scattering angle θ (usually <8°) at which Equation 7.8.4 is taken to be valid (see Jumel et al., 1992). Although the angle used is assumed low enough that no angular correction of the scattering data is required, an extrapolation to zero concentration of Kc/R_θ may be necessary. The method can thus provide values for M and A_2 of a system, but not for R_g since no record is made of the angular dependence of Kc/R_θ. At low concentration, and especially when the light scattering detector is on-line to size-exclusion chromatography columns, the further approximation that $2A_2c \sim 0$ can be made, and hence M is simply $\sim R_\theta/Kc$. Since lasers are now routinely used as the light source, this technique takes the popular acronym of "LALLS" (low-angle laser light scattering).

Multiangle Light Scattering (MALLS)

Multiangle light scattering measurements are based on the full Debye-Zimm equation (Equation 7.8.1). Performing measurements at multiple angles permits extrapolation of the ratio Kc/R_θ to zero $\sin^2(\theta/2)$, which, together with an extrapolation to zero concentration,

forms the basis of the Zimm plot (Fig. 7.8.2), a method that can yield M, A_2 and R_g.

Values for A_2 returned are typically between 10^{-5} ml mol/g^2 (for globular proteins) and 10^{-3} ml mol/g^2 (for large glycoconjugates). A warning is in order here—a common misconception is to ignore these values as small numbers. In actuality, it is the product $2 \times A_2 \times M \times c$ that manifests the influence of nonideality. The expression $1/(1 + 2A_2Mc)$ represents the factor by which an "apparent" molecular weight measured at a finite concentration c underestimates the true or "ideal" value (Tanford, 1961). An opposing effect to that of thermodynamic nonideality is that of reversible interaction phenomena (as represented for example by a molar dissociation constant, K_d), and in some cases ("pseudoideality") the two effects approximately cancel.

As with LALLS, lasers are now routinely used as the light source, and hence the acronym "MALLS" (multiangle laser light scattering) has now been adopted. Besides the potential for extracting R_g, a more important advantage of MALLS over LALLS is that the angular extrapolation permits identification and avoidance of any spurious results at the lowest angles. Plots of Kc/R_θ are only linear over a limited range of angles. For globular proteins of $M <$ 50,000 Da (corresponding to a maximum dimension $\sim\lambda/20$), the angular dependence of K_c/R_θ will be negligible, permitting a more accurate determination of M but of course excluding the possibility of measuring R_g. As with LALLS, at low concentration, the further approximation that $2A_2c \sim 0$ can be made; hence Equation 7.8.1 reduces to

$$\frac{Kc}{R_\theta} = \frac{1}{MP(\theta)}$$

Equation 7.8.5

and where there is no angular dependence, this reduces further to $M \sim R_\theta/Kc$, as noted above.

Limitations of Static Light Scattering Methods

The principal limitation for all these "static" light scattering measurements is the need for sample clarification to avoid dust and supramolecular aggregates (see Clarification of Solutions and Scattering Cells). This is especially serious if solutions of proteins of M <50,000 Da are being studied, and also if LALLS is used, since large-particle contamination effects become disproportionally larger at low angles and the LALLS instruments provide no angular check for this. Another limitation is that a separate precise measurement of the refractive index increment dn/dc is required, preferably at the same wavelength used in the light scattering photometer. With MALLS, R_g cannot be measured as noted above for particles of molecular weight <50,000 Da. For very large macromolecular assemblies (maximum dimension $>\lambda/2$), the reliability of the theory on which Equation 7.8.1 is based (known as Rayleigh-Gans-Debye theory) becomes doubtful.

SEC-LALLS and SEC-MALLS

A revolutionary development has been the coupling of LALLS or MALLS photometers to size-exclusion chromatography (SEC) systems (Jumel et al., 1992; Wyatt, 1992). This is made possible by replacing the standard light scattering cuvette with a flow cell that can be coupled downstream from an HPLC pump and size-exclusion chromatography column(s), and upstream from a (UV-absorption or refractive index) concentration detector (Fig. 7.8.3). This has the double advantage of providing an online clarification system to remove supramolecular contamination and allowing fractionation of polydisperse materials prior to light scattering analysis. However, care must still be taken, particularly against the spurious shedding of column material. The coupled SEC systems are referred to as "SEC-LALLS" or "SEC-MALLS."

Samples for Analysis in Static Light Scattering

Solutions should be dialyzed against an appropriate buffer of defined pH and ionic strength, I. Normally an ionic strength of at least 50 mM is needed to suppress the contribution of molecular charge on the protein to the nonideality coefficient (A_2 in Equation 7.8.1) although, of course, the I chosen will obviously depend on its effect on the protein.

The concentration of protein required depends on: (1) its molecular weight, since the scattering signal is approximately proportional to the product of concentration and molecular weight; (2) the output of the laser; and (3) the clarity of the solutions, since substantial material may be lost on filtering or from the column in SEC-LALLS or SEC-MALLS. For scrupulously clean solutions, a 5-mW laser for a loading concentration of ~3-mg/ml is sufficient for proteins of molecular weight ~40,000 Da. For smaller proteins, a proportionally higher con-

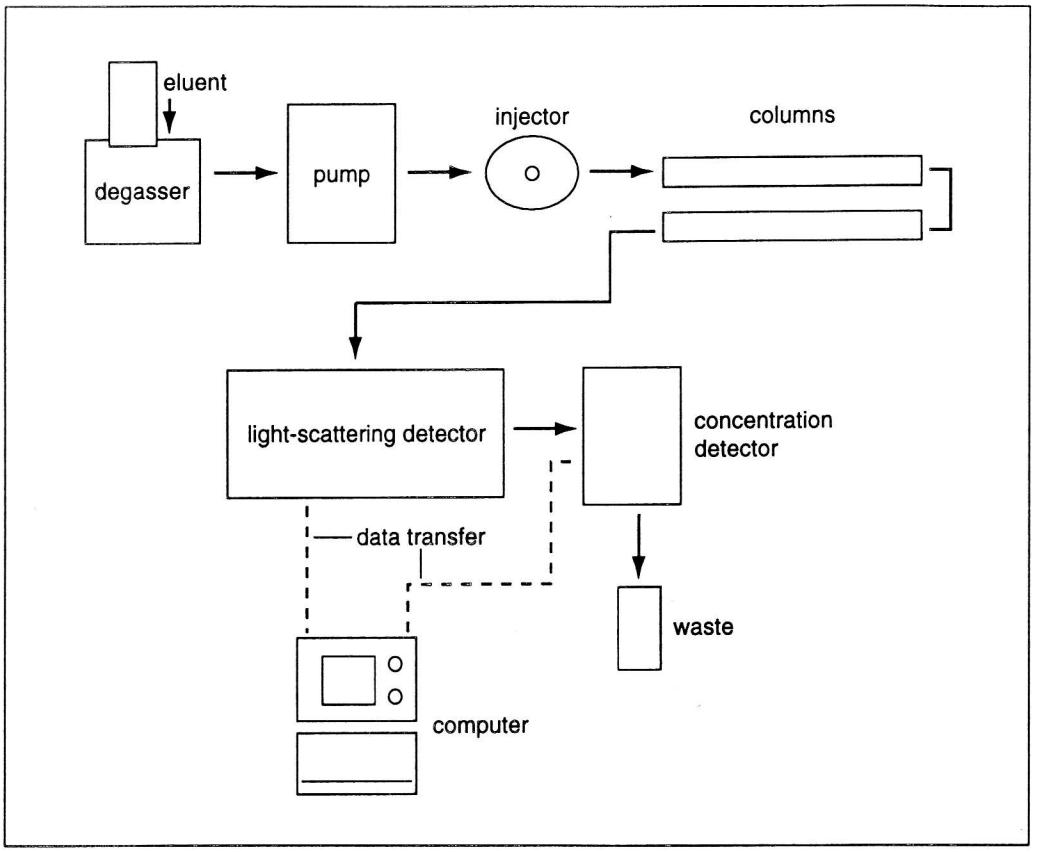

Figure 7.8.3 Experimental setup for SEC-MALLS (see Wyatt, 1992).

centration and/or higher laser power is required.

With regard to volume requirements, if a flow cell arrangement is used (linked, e.g., to an SEC system), loading volumes can be as low as 0.1 ml. For use with scintillation vials (which are nowadays used instead of fluorimeter-type cuvettes), ≥3 ml of solution is required. Square cuvettes, of course, prevent measurements in the angular part of the scattering envelope near the corners of the cuvette; if cylindrical cuvettes are employed, then small diameters (<2 cm) are to be avoided because of extraneous scattering or reflections from the glass walls, although large-diameter cuvettes can be expensive in terms of quantity of solution required. Flow cells are now preferred, and a typical experimental set up would have MALLS coupled to an SEC, but with a separate injection port (via a filter or guard column) if the SEC separation is not needed.

Clarification of Solutions and Scattering Cells

For molecular weights <200,000 Da, the most serious experimental problem has been that of clarification. All traces of dust and supramolecular aggregates have to be removed since experiments on incompletely purified material are "not useful" (Johnson, 1993), and clarification can lead to a significant loss of material. Contaminating particles can be removed by ultracentrifugation and ultrafiltration.

If scintillation vials are used, these too must be scrupulously clean. They should be cleaned and rinsed in double-distilled water, then dried and covered with aluminum foil to prevent any dust from entering the vials. It is also extremely important that there are no scratches or fingerprints or other marks on the outside of the vial. Sample preparation for batch work should, if possible, be carried out in a flow cabinet to prevent any dust from entering the solutions.

If a flow-cell device is used for clarification (Sanders and Cannell, 1980), this too will frequently need to be removed and cleaned using detergent, acetic acid, and a final necessary rinse with ultrafiltered water. An acetone reflux may also be occasionally necessary. When attached to an SEC column, prior to solution injection and after all the solution has been eluted, the solvent eluant must be monitored for any shedding from the column material. When the solution is injected, this needs to be done via a Millipore filter of appropriate size.

Calibration (LALLS and MALLS)

The quantities directly measured by a light scattering photometer are voltages and not light scattering intensities. For this reason, the instrument has to be "calibrated"—usually with a strong Rayleigh scatterer with a known Rayleigh ratio such as toluene (Stacey, 1956; Johnson and McKenzie, 1977). Measurement of the scattered and incident light intensities (I_θ and I_0, respectively) then facilitates calculation of the calibration constant, which is then used to calculate Rayleigh ratios for sample solutions from the output of the instrument. Calibration does *not* mean that the light scattering measurements have to be made relative to protein standards as in calibrated gel chromatographic methods. A typical calibration procedure would include the following steps.

1. Switch on the light scattering detector and laser at least 1 hr before measurements are made.
2. Make sure that the sample cell is scrupulously clean and the calibrating solvent (in most cases toluene as it is the strongest Rayleigh scatterer with a known R_θ of $1.406 \times 10^{-5}\text{cm}^{-1}$ at a wavelength of 633 nm) is HPLC grade.
3. Inject the solvent into the cell via a membrane filter (preferably ≤0.2 μm pore size).
4. Measure the scattered light intensity and calculate the instrument calibration constant by means of the dedicated software (e.g., ASTRA, Wyatt Technology), using the known Rayleigh ratio of the solvent.

Normalization (MALLS)

For simultaneous multiangle detection in MALLS photometers of the type now commonly available, the detectors have to be "normalized" to allow for the different scattering volumes as a function of angle and the differing responses of the detectors. This is normally achieved using a solution of molecules that scatter light isotropically (i.e., with equal intensity in all directions)—as is the case when the diameter of the sample molecule is less than ~λ/20 of the incident wavelength. The best molecules of this kind for use in a chromatography system are either a polystyrene standard in toluene or tetrahydrofuran (THF) with molecular weight of ~30,000 Da or a pullulan or dextran standard of 20,000 to 30,000 Da in aqueous solution; the R_g of these molecules is ~5 nm. In the batch mode—i.e., with the sample in a vial–10 mg/ml solutions of 4000-Da polystyrene in toluene or THF, or 5000 Da pullulan in water work well; these standards have R_g's of ~2 nm.

The procedures described below are typical normalizations for the MALLS instruments distributed by Wyatt Technology.

Normalization for chromatography

Inject a known amount of sample into the system and collect data using the ASTRA software. After the run has finished, set baselines and peak limits. For normalization, it is necessary to set very narrow, symmetrical limits at the top of the peak. Enter the R_g value for the standard used in the Normalize menu, click "normalize," and the normalization coefficients will be displayed on the screen. This procedure only needs to be repeated if the solvent is changed or the flow cell has been cleaned.

Normalization for batch collection

Remove the flow cell from the read head and assemble alignment rings and flow-to-batch conversion plate in read head as described in the manual. First, place a scintillation vial containing the filtered solvent into the aperture and observe the scattering intensities. Once these have stabilized, begin data collection, which will stop after a preset volume. Save the data. Next, insert the vial containing the sample for normalization measurement and again wait for signals to stabilize. Append the file containing the values for the solvent. After collection has stopped, measure the scattering for the solvent again. Set a baseline and peak limits that cover most of the plateau corresponding to the normalization standard. Go to the Normalization menu and enter the concentration and R_g of the normalization standard. Click "normalize" and the normalization coefficients will appear on the screen.

Evaluation of Molecular Weight (LALLS)

After measurement of R_θ, the molecular weight, M, can be calculated from this value, the concentration, the experimental constant K, and a subsequent extrapolation to $c = 0$ (Equation 7.8.4) if the nonideality term A_2c is significant. The concentration, c (from, e.g., UV absorbance or refractometry) should be known as accurately as possible.

Evaluation of Molecular Weight and Radius of Gyration (MALLS).

If the nonideality term A_2c (and of course any higher-order terms) is insignificant (i.e., if the measurements can be performed at low enough concentration), then only an extrapola-

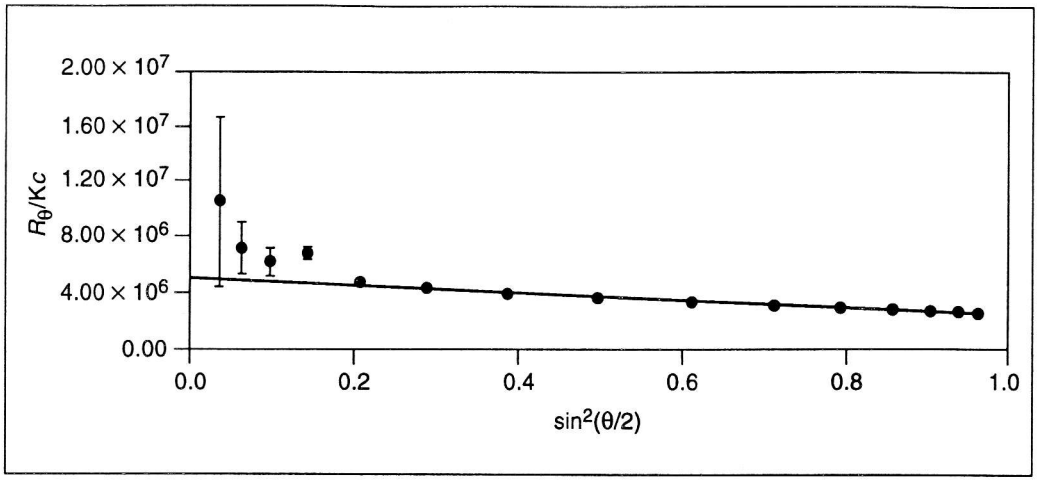

Figure 7.8.4 Debye plot (angles from 21.7° to 58.3°) for a heavily glycosylated protein system (pig gastric mucin glycoprotein). A first-order (linear) fit is shown. This figure illustrates the need for caution when interpreting static light-scattering data: the upward curvature at the lower angles for a simple protein system (or downward curvature in a Kc/R_θ versus $\sin^2(\theta/2)$ plot) would normally be ascribed to supramolecular contamination, and the data points would be ignored or given low weighting. However, for a genuinely polydisperse system, the lower-angle measurements contain proportionally higher information about the higher-molecular-weight species in a distribution, and removal of the low-angle data and/or choice of a first-order fit could bias the distribution towards the low-molecular-weight end.

tion to zero angle and not zero concentration is necessary (see Equation 7.8.5). Sometimes the inverse form of Equation 7.8.4 is used, known as a "Debye plot" of R_θ/Kc versus $\sin^2(\theta/2)$ (Wyatt, 1992). Although this invokes a further approximation, the ordinate axis does correspond to an apparent molecular-weight axis (Fig. 7.8.4). It must be noted that Figure 7.8.4 is a rather extreme example for a highly glycosylated and polydisperse system. Figure 7.8.4 also shows that care must be taken in choosing the appropriate fit, and that there is a proportionally larger effect at low angles of supramolecular contaminants. The order of the fit chosen (usually linear or quadratic) has a more dramatic effect on the (limiting) slope of such plots and hence on the value for R_g returned. This feature needs to be borne in mind when conclusions on macromolecular conformation based on the relationship between M and R_g are being drawn, particularly using the SEC-MALLS devices considered below.

If the nonideality term is significant, and an assumed value for A_2 cannot be taken as zero (which can in fact be predicted from the triaxial shape of the protein; Harding et al., 1997)—and charge effects are not significant, then an additional extrapolation to zero concentration is necessary. Both angular and concentration extrapolations are usually done on the same plot, known as a Zimm plot (Fig. 7.8.2), which can yield M from the reciprocal of the common intercept, and estimates for A_2 (from the $\theta = 0$, c extrapolation line) or R_g [from the $c = 0$, $\sin^2(\theta/2)$ extrapolation line]. However it cannot be overstressed that the R_g returned can be very sensitive to the order of extrapolation used. An arbitrary (positive or negative) constant k is used on the abscissa to scale the data points better.

Evaluation of Molecular Weight Distribution (SEC-LALLS) and (SEC-MALLS)

Absolute molecular weight distributions (Fig. 7.8.5) of mixed protein systems or glycosylated protein systems such as mucins, glycoproteins, or glycosaminoglycans may be obtained with either of the above techniques.The sample is injected via the injection valve and separated by the column system. Effluent from the column(s) is monitored by light scattering and concentration detectors. Once the peak area from the chromatogram has been chosen, the concentration and R_θ at each data point within this area are known from light scattering and concentration detectors, respectively, and the molecular weight (M) at each of these points is calculated using Equation 7.8.4 (for SEC/LALLS) or Equation 7.8.5 (for SEC/MALLS). Molecular weight averages for the whole of the peak area may then be calculated. In addition, a so-called "calibration plot" of molecular weight versus elution vol-

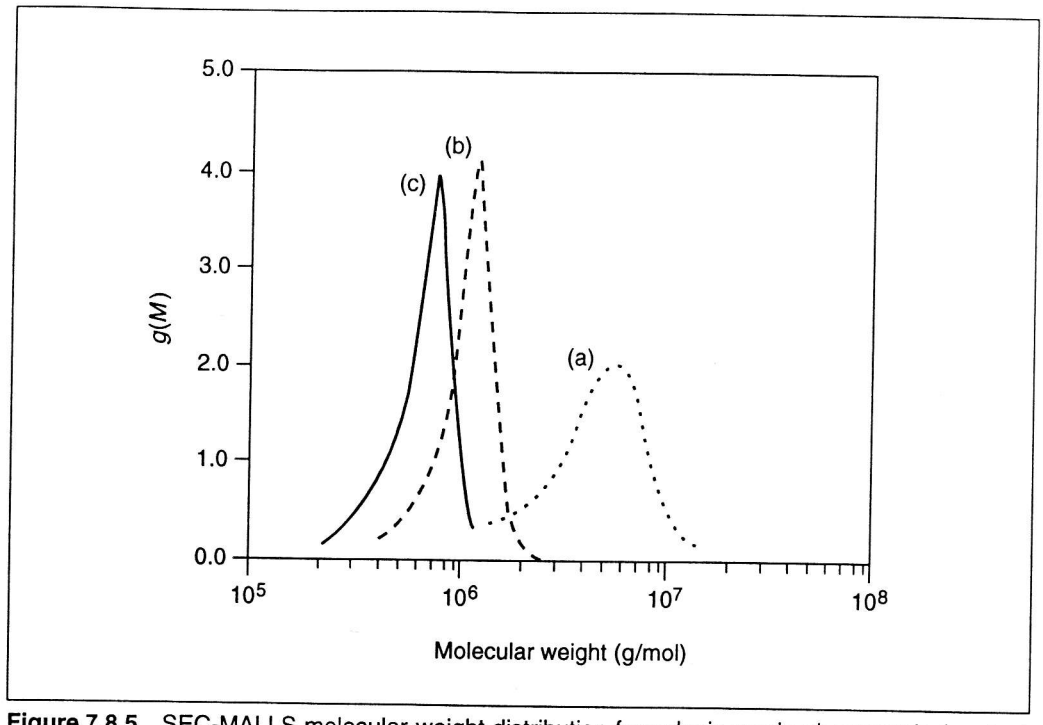

Figure 7.8.5 SEC-MALLS molecular-weight distribution for colonic mucin glycoprotein (peak a) and its thiol-reduced (peak b) and papain digested (peak c) forms (from Jumel et al., 1997). The quantity g(M) is in arbitrary units.

ume can be constructed, thus enabling the elution volume versus concentration plot to be converted to a molecular weight distribution. Such molecular weight distributions thus found will be absolute in the sense that, unlike with conventional SEC, calibration standards of known *M* are not required. This is particularly useful not only for protein mixtures but for heavily glycosylated systems because of their polydispersity and the difficulty in obtaining standards of the appropriate (often uncertain) conformation.

For a more detailed account of the theory the reader is referred to Yau et al. (1979).

DYNAMIC LIGHT SCATTERING ANALYSIS OF PROTEIN SOLUTIONS

Basic Theory

Whereas static light scattering is concerned with the time-averaged scattering intensity properties, dynamic light scattering is concerned with time fluctuations in intensity caused by motions of macromolecules and macromolecular assemblies. Whereas lasers are highly desirable for static light scattering because of their high intensity, collimation, and monochromaticity—with dynamic light scattering they are mandatory because of the requirement for spatial and time ("temporal") coherence—light has to be emitted from the source as a continuous wave rather than as short bursts.

The physics underlying dynamic light scattering is rather complicated (Brown, 1993), although the basic principle and experimental setup is relatively simple (Fig. 7.8.1 and Fig. 7.8.6). Laser light is directed onto a thermostatted protein solution, and the intensity is recorded at either a single angle or multiple angles using a photomultiplier/photodetector. The moving biomolecules will "Doppler broaden" the otherwise monochromatic incident radiation. The scattered intensity recorded as the number of photons received by a detector (photomultiplier) will fluctuate because of "beating interference" of scattered waves of different but similar wavelength. The situation is analogous to the fluctuations in intensity of a radio channel caused by interference from another radio channel of very close wavelength. This "broadening" of the otherwise monochromatic incident radiation is why the technique is often referred to as "quasielastic light scattering" or QLS. The detector sends the intensity signal to a special computer called an autocorrelator, which compares or correlates the intensity at different times (hence the other name often used—photon correlation spectroscopy or PCS).

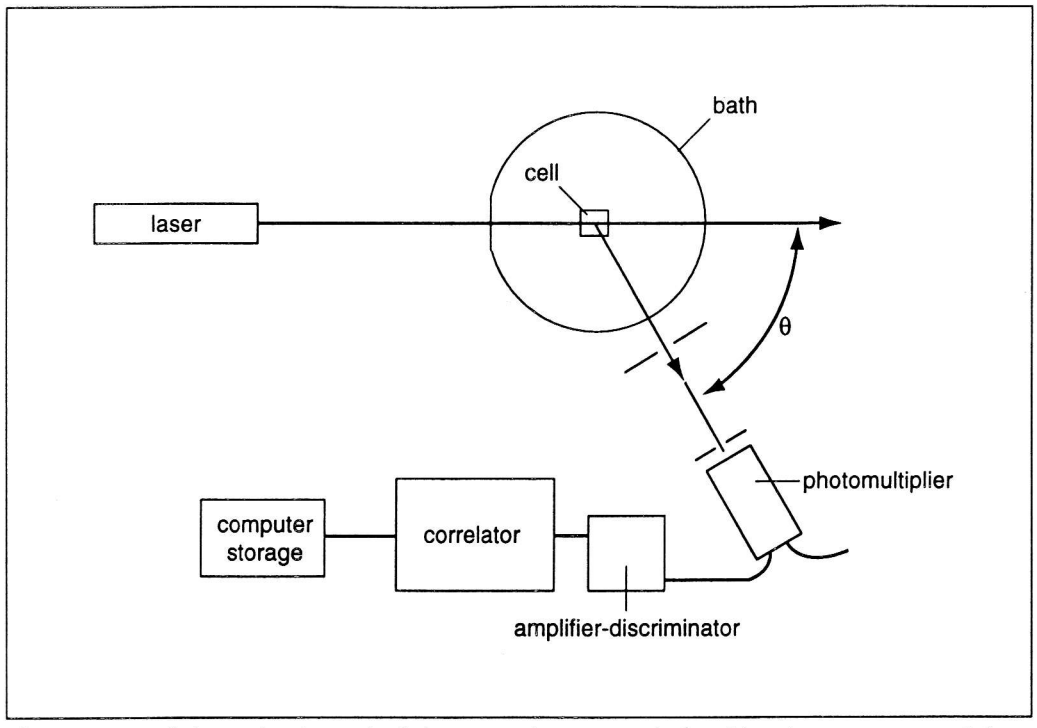

Figure 7.8.6 Schematic dynamic light scattering setup for multiangle measurement (see Johnson, 1993).

How rapidly the intensity fluctuates over short time periods or "delay times," τ (in nsec to msec depending on how mobile the scattering biomolecules/assemblies are), is represented by how a parameter known as the normalized intensity autocorrelation function—$g^{(2)}(\tau)$—decays as a function of τ. The superscript "(2)" is used to indicate that it is an intensity as opposed to an electric field or "(1)" autocorrelation function, and many data sets of $g^{(2)}(\tau)$ as a function of τ are accumulated and averaged. The amount of averaging necessary depends on the incident laser intensity and the size and concentration of the scattering biomolecule (at a given concentration, larger molecules scatter more). For globular proteins, sufficient data can usually be acquired over the time scale of one to several minutes. The calculation of the correlation function from the intensity fluctuations is performed after the signal has passed through an amplifier-discriminator via the autocorrelator. Calculation of the diffusion coefficient from the decay of $g^{(2)}(\tau)$ with τ is performed on a computer. Some of the most modern instrumentation (particularly fixed-angle photometers) have all of the units depicted in Figure 7.8.6 built into one instrument.

Analysis of how the normalized intensity autocorrelation function $g^{(2)}(\tau)$ decays as a function of τ can be used to evaluate the translational diffusion coefficient, D. For dilute systems of spherical or near-spherical (i.e., globular) biomolecules and assemblies, the variation of $g^{(2)}(\tau)$ with τ can be represented by the simple logarithmic equation

$$\ln\left[g^{(2)}(\tau-1)\right] = -2Dg^{(2)}\tau$$

Equation 7.8.6

where q is known as the Bragg wave vector whose magnitude is defined by $q = \{4\pi n/\lambda\}\sin(\theta/2)$—$n$ being the refractive index of the medium, θ being the scattering angle, and λ being the wavelength of the incident light. Thus D can be found from a plot of $\ln[g^{(2)}(\tau) - 1]$ versus τ, and Figure 7.8.7 shows an example for the motility protein dynein.

D can then be converted to standard conditions (i.e., the viscosity and temperature of water at 20.0°C) to give $D_{20,w}$ as noted above, and then extrapolated to zero concentration to give $D^0_{20,w}$. An additional extrapolation is necessary if the biomolecule is not globular: this is because at finite angles θ there will be an extra term on the right-hand side of Equation 7.8.6 deriving from rotational diffusional phenomena. This term approaches 0 as θ approaches 0; therefore true D can be measured according to Equation 7.8.6 so long as measurements of the apparent D are made at a

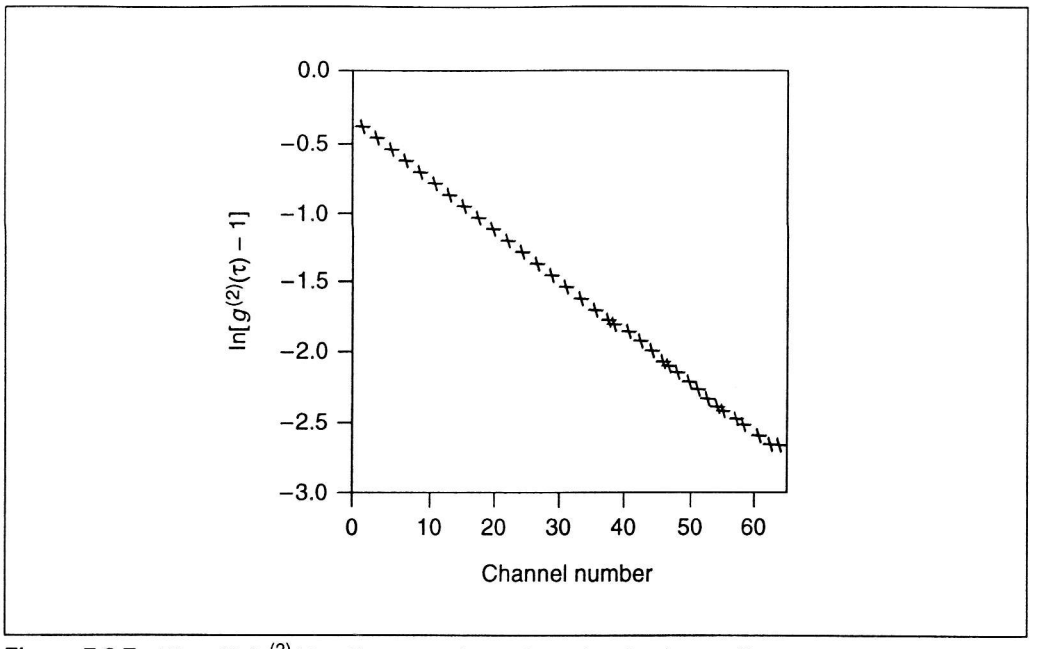

Figure 7.8.7 Plot of $\ln[g^{(2)}(\tau) - 1]$ versus channel number for the motility protein dynein. Channel number = delay time/sample time (Wells et al., 1990).

number of angles and at an additional extrapolation to zero angle (or Bragg vector q) is performed. The extrapolations to zero concentration and zero angle (or q) can be performed simultaneously on a biaxial extrapolation plot known as a dynamic Zimm plot (Burchard, 1992). If an angular extrapolation is not necessary, a scattering angle of 90° is usually chosen, and fixed-angle instruments are usually set at this angle (see Claes et al., 1992). At lower angles the problems due to supramolecular contamination are accentuated.

If a system is heterogeneous it is possible, at least in principle, to obtain a distribution of diffusion coefficients after various assumptions and mathematical manipulations of the autocorrelation data. The various methods of manipulation have been reviewed by Johnsen and Brown (1992) and several commercially available computer routines are available for performing these. A more simple way of representing heterogeneity is the polydispersity factor (PF) which is obtained by comparing linear with quadratic or quadratic with cubic-order fits of the normalized autocorrelation function decay data (Pusey, 1974).

Translational Diffusion Coefficient

The translational diffusion coefficient, $D_{20,w}$ or $D^0_{20,w}$, obtained by dynamic light scattering or boundary spreading in the ultracentrifuge, can be used to provide a number of useful characteristics about a biomolecular system.

Equivalent hydrodynamic radius (r_H)

The simplest deduction one can make from $D^0_{20,w}$ is the size of the biomolecule as represented by the equivalent hydrodynamic radius (also known as the Stokes radius)—r_H.What this means is, although the biomolecular system may not be a sphere at all, its diffusive behavior can be represented by an equivalent spherical particle of radius r_H. The value of r_H can be easily obtained from $D^0_{20,w}$ via the Stokes-Einstein relation:

$$r_H = \frac{k_B T}{6\pi\eta_{20,w} D^0_{20,w}}$$

Equation 7.8.7

where k_B, the Boltzmann constant, is equal to 1.379×10^{-16} erg/°K, T = 293.15°K and $\eta_{20,w}$ (the viscosity of water at 20.0°C) is equal to 0.01 poise. If D values are not corrected to standard conditions then the appropriate values for T and η have to be used.

Frictional coefficient (f)

The frictional coefficient provides a handle on molecular weight and shape as discussed below. Like r_H it can also be calculated simply from $D^0_{20,w}$ according to Equation 7.8.8.

$$f = \frac{RT}{N_A D^0_{20,w}}$$

Equation 7.8.8

where T = 293.15°K and R = 8.314 × 10^{-7} erg/mol °K and N_A is Avogadro's number (6.022137 × 10^{23} mol).

Molecular weight

A more absolute description of biomolecular size than r_H is the molecular weight, *M*. Calculation of *M* from dynamic light scattering is less direct than from static measurements. However, combination of the $D^0_{20,w}$ value with the analogous parameter from sedimentation velocity experiments (*UNIT 7.5*) in the analytical ultracentrifuge—$s^0_{20,w}$ (in sec)—provides a popular route for obtaining the molecular weight of a biomolecule. The analogous equation to Equation 7.8.8 for the sedimentation coefficient is Equation 7.8.9.

$$f=\frac{M\left(1-\bar{v}\rho_{20,w}\right)}{N_A s^0_{20,w}}$$

Equation 7.8.9

Elimination of *f* between Equation 7.8.8 and Equation 7.8.9 yields the Svedberg (1927) equation (Equation 7.8.10):

$$M=\left(\frac{s^0_{20,w}}{D^0_{20,w}}\right)\left(\frac{RT}{1-\bar{v}\rho_{20,w}}\right)$$

Equation 7.8.10

where again T = 293.15°K. The variable $\bar{v}$ is the partial specific volume of the biomolecule (which can be measured from densimetry or from the composition of the biomolecule) and $\rho_{20,w}$ is the density of water at 20.0°C (0.9982 g/ml). It is possible in principle to measure both $D^0_{20,w}$ and $s^0_{20,w}$ simultaneously from analysis of the boundary shape in sedimentation velocity analytical ultracentrifugation, although in practical terms this needs data of very high quality and the absence of any effects of sample heterogeneity. Equation 7.8.10 also of course provides a route for measurement of $D^0_{20,w}$ if $s^0_{20,w}$ and *M* are known.

A simpler method for *M* measurement is possible if the conformation of the biomolecule is assumed and use is made of a power law relation, known as a Mark-Houwink relation (Equation 7.8.11):

$$D = KM^{-\varepsilon}$$

Equation 7.8.11

where ε = 0.333, 0.85, or 0.5 to 0.6 for sphere-, rod-, and coil-shaped molecules, respectively. From a calibration plot of log *D* versus log *M* for biomolecules of known *D* and *M* (Claes et al., 1992), *M* of an unknown can be found from its measured *D*. A conformation has of course to be assumed; the Equation 7.8.11 method for *M* evaluation is thus not as reliable as the method shown in Equation 7.8.10. Equation 7.8.11 can be taken even further in converting a distribution of diffusion coefficients into a molecular-weight distribution, although such distribution information is not as reliable (because of the approximations made) as that obtained with static light scattering methods coupled directly on-line to a gel-filtration column (Wyatt, 1992).

Conformation

The translational frictional coefficient (*f*) can be used directly to provide information on conformation. More convenient, however, is to use the corresponding dimensionless ratio called the frictional ratio (f/f_0), where f_0 is the frictional coefficient of a spherical particle of the same anhydrous mass and density as the biomolecule. The quantity f/f_0 can be calculated from $D^\circ_{20,w}$ by the relation in Equation 7.8.12.

$$f/f_0 = \left(\frac{k_B T}{6\pi\eta_{20,w}}\right)\left(\frac{4\pi N_A}{3\bar{v}M}\right)^{\frac{1}{3}}\left(\frac{1}{D^0_{20,w}}\right)$$

Equation 7.8.12

The frictional ratio depends intrinsically on the conformation, flexibility, and degree of solvent association (water plus other salt ions and any other solvent molecules) of the biomolecule. This degree of water association is termed the "hydration" of the biomolecule, δ, and is defined as the mass (in g) of associated solvent per g of anhydrous biomolecule. This associated solvent includes both chemically bound solvent and solvent physically entrained in the interstices of the molecule. The value of δ is typically between 0.2 and 0.5 g/g for proteins, although it is a notoriously difficult parameter to pin down with any accuracy.

The function defining the shape and flexibility of the biomolecule is the Perrin translational frictional function, *P*, illustrated by Equation 7.8.13:

$$P = (f/f_0)\left\{1+\frac{\delta}{\bar{v}\rho_0}\right\}^{-\frac{1}{3}}$$

Equation 7.8.13

where ρ_0 is the density (g/ml) of the bound solvent. For a molecule that is fairly rigid on a time-averaged basis, the gross conformation can be specified using P in terms of the axial ratio of the equivalent hydrodynamic ellipsoid or in terms of sophisticated arrangements of spheres called hydrodynamic bead models. Computer programs are available for both types of modeling strategy (Harding et al., 1997; Garcia de la Torre et al., 1997)—although particularly with the latter, the diffusion coefficient should be used in conjunction with other hydrodynamic measurements to minimize uniqueness problems of the determined solution conformation.

Limitations of Dynamic Light Scattering Methods

As with static light scattering, the main limitations here relate to sample clarification. The technique is most suited to larger proteins and protein assemblies (M >100,000 Da) where problems due to contamination are less, and, as with static light scattering, the problems become disproportionally higher at the lower scattering angles. If the protein systems are approximately globular, then to a good approximation these low angles can be avoided and measurements at a single higher angle (usually 90°) can suffice. For nonspheroidal systems, angular extrapolations are necessary to eliminate rotational diffusion contributions. The user should also be wary of interpreting data from fixed-angle photometers, which do not permit such angular measurements.

The other limitations concern direct molecular-weight evaluation via Equation 7.8.11 and interpretation of the autocorrelation data in terms of polydispersity or size distribution. The user should be aware that such distributions are obtained purely by mathematical manipulation of the autocorrelation data, instead of involving a physical separation of different sizes (as with SEC-LALLS or SEC-MALLS), and the user is advised to be extremely cautious when interpreting such information produced by computer packages.

Samples for Analysis in Dynamic Light Scattering

Aqueous solvents should be of sufficient ionic strength to suppress charge effects. The loading concentrations required—which, as with static light scattering, should be measured *after* clarification—will depend principally on the size of the scatterer and the output from the laser. For example, if a 25-mW He-Ne laser is used, a loading concentration of at least ~1 mg/ml (and a volume of 2 to 3 ml) is required for a large protein assembly whose molecular weight (M) is ~5 × 10^6 Da. For proteins of molecular weight down to a lower limit of ~10,000 Da, more powerful lasers (~100 mW) and/or higher concentrations and/or longer experimental duration times are generally necessary to obtain meaningful results.

Temperature Control

D is very sensitive to temperature, mainly because of the dependence of diffusion on the viscosity of the solvent. Temperature needs to be controlled or, at the very least, monitored accurately during the measurement, and a water bath is highly desirable.

Clarification of Solutions and Scattering Cells

As with static light scattering, the scattering signal in dynamic light scattering is very sensitive to the presence of trace amounts of dust or supramolecular aggregation products; solutions and scattering vessels (called scattering cells or cuvettes) need to be scrupulously clean and free of particulates. Appropriate filtration, centrifugation of solutions, and washing of vessels with solvent is necessary. Special in-house cell-filling devices can be used along the lines described by Sanders and Cannell (1980); these can also be used for static light scattering.

Choice of Scattering Cuvette/Cell

The same criteria apply as with static light scattering (see Samples for Analysis in Static Light Scattering). The cell design depends on whether the photometer is a fixed-angle or multi-angle design.

Procedure for Making Scattering Measurements Using a Fixed-Angle Instrument with a Flow Cell

The following steps describe the injection procedure for a fixed (90°) angle photometer with a 20-mW infrared (780 nm) semiconductor laser (Claes et al., 1992).

1. The user should be satisfied that the protein/protein assembly is approximately globular. If not, then a multiangle instrument should be used (see Procedure for Making Scattering Measurements Using a Multiangle Instrument with a Conventional Cell).

2. Switch on the instrument and allow the laser to warm up 5 to 10 min.

3. Inject pure ultrafiltered water or buffered solvent via a 0.1-μm filter to get the clean water count rate.

4. If the count rate is below the manufacturer's threshold, check the instrument alignment or increase the protein concentration.

5. Inject the protein solution in the same way as the water or buffer, using the appropriate filter, and check that the count rate meets the criteria specified by the manufacturer.

6. The choice of sample time and experimental duration time is normally done automatically.

7. Use the instrument's software to obtain the diffusion coefficient, and, where appropriate, the in-built calibration to directly obtain the approximate molecular weight.

8. Rinse and dry the flow cell of the photometer.

Procedure for Making Scattering Measurements Using a Multiangle Instrument with a Conventional Cell

1. Choose the appropriate cuvette or cell, using the same criteria (square versus cylindrical) as discussed above for static light scattering (see Samples for Analysis in Static Light Scattering).

2. Ensure that the cell is clear and free from dust and perform a check with pure ultrafiltered water, which should give only a negligibly small number of photon counts. Visually check for any "sparkling" in the laser beam passing through the water.

3. Remove the water from the cuvette, dry with ultrafiltered air, and inject the sample solution using the appropriate filter.

4. Set the goniometer for a scattering angle of 90°.

5. The choice of sample time and experimental duration time is normally done automatically.

6. Use the instrument's software to obtain the apparent diffusion coefficient, D_{app}. Inspect the autocorrelation decay plots (see Fig. 7.8.7). Nonlinearity can be due to sample polydispersity, particle asymmetry (effect of rotational diffusion phenomena), or particle settling (usually only observed for cellular systems, not for macromolecules). If it is not reasonable to assume an approximately globular system, then measure D_{app} at a series of angles and extrapolate to zero angle, but be aware of enhanced supramolecular contamination problems at the lower angles.

7. Remove the solution, rinse the cell with ultrafiltered pure water (after, if necessary, prior rinsing with detergent and mild acetic acid), dry with ultrafiltered air, and dry the cuvette.

Electrophoretic Light Scattering

Electrophoretic light scattering (ELS) uses dynamic light scattering to measure the velocity of migration, V, of a protein or other macromolecular system under the influence of an electric field, E (Langley, 1992; McNeil-Watson and Parker, 1992) The velocity V is related to the Doppler broadening, $\Delta\nu$, of the frequency of the incident laser radiation due to the velocity of the macromolecule. $\Delta\nu$ is related to V by Equation 7.8.14.

$$\Delta\nu = \left(\frac{2nV}{\lambda_0}\right)\sin\left(\frac{\theta}{2}\right)$$

Equation 7.8.14

The mobility, $U = V/E$, can thus be defined and hence the zeta potential, ζ:

$$U = \varepsilon\zeta/\eta$$

Equation 7.8.15

This procedure is however used more for the investigation of the stability of colloids rather than protein structure and so will not be discussed further here.

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