CHAPTER 8

Determination of Diffusion Coefficients of Biological Macromolecules by Dynamic Light Scattering

Stephen E. Harding

1. Introduction

The importance of transport phenomena in biological processes is indisputable, whether they be concentration gradient driven, "active," or Brownian diffusion processes. Light scattering can provide a rapid probe into these processes, particularly if the technique of *dynamic* light scattering is used.

The technique of dynamic light scattering is possible because of the high *coherence* of laser light—that is to say, the light is emitted from the source as a continuous wave train. The wavelength of the otherwise highly monochromatic incident radiation can be Doppler broadened by the motion of the scattering particles: This broadening can be measured by a wave analyzer, and from the wavelength spread, diffusion coefficients can be measured. More commonly now, however, instead of a wavelength analyzer, the short-time fluctuations in *intensity*—caused by the movements of the macromolecules—are measured. These changes in intensity (or numbers of photons received by a detector) are recorded using a "Correlator," and from suitable analysis of the change of the "autocorrelation function" with time, translational and, in some cases, rotational diffusional information about the macromolecule can be obtained. Because of these features, "dynamic light scattering," like

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classical light scattering (*see* Chapter 7), also comes with a plethora of alternative names: "intensity fluctuation spectroscopy" (IFS), "light beating spectroscopy," "photon correlation spectroscopy" (PCS), or "quasielastic light scattering" (QLS).

Other introductions to the technique can be found, for example, in van Holde *(1)* or from a short article by Johnson *(2)*. A detailed review of the methodology has been given by Bloomfield and Lim *(3)*. Applications to biochemistry can be found in, for example, refs. *4–6*. More complete mathematical texts are by Berne and Pecora *(7)* and Chu *(8)*. For a good description of how classical light scattering equipment can be modified for dynamic work, the reader is referred to Godfrey et al. *(9)*.

2. Summary of Information Obtainable

1. Translational diffusion coefficient, D (this will be a "z-average" if the system is heterogeneous).
2. The effective hydrodynamic or "Stokes" radius, r_H.
3. Gross conformational information in terms of bead modeling from diffusion coefficient data (in much the same way as the sedimentation coefficient is used—*see* Chapter 5).
4. An estimate for the polydispersity of a macromolecular solution (from, e.g., the "polydispersity factor," PF [normalized z-average variance of the diffusion coefficients]), or from various types of multiexponential inversion procedures.
5. Molecular weight (weight average), from the combination of D (z-average) with the (weight average) sedimentation coefficient.
6. An estimate for the rotational diffusion coefficient, D_R.

D and the corresponding r_H can be obtained relatively rapidly (a measurement can take <1 min in some cases), although D_R for asymmetric particles is much more difficult to obtain. D can be obtained to a precision of up to ~±0.2%; D_R to only ~±5% at best. The technique is particularly useful for looking at the time-course changes in size (and where appropriate, polydispersity) of assembling/disassembling system: for example, the kinetics of head-tail associations of T-type bacteriophages *(10)* or the effect of removal of Ca^{2+} ions on the swelling of southern bean mosaic virus *(11)*; another good example is the self-association of tubulin *(12)*.

3. Summary of Limitations

3.1. Sample Clarification: The "Dust" Problem

The smaller the macromolecule, the greater the problem: The technique is best suited for macromolecular assemblies ($M \gtrsim 100{,}000$).

3.2. Asymmetry

Measurement at a single angle (conventionally 90°) gives insufficient information to obtain D for asymmetric scatterers—extrapolation to zero angle is necessary, which can cause problems, since at low angles, the dust problem is at its greatest.

3.3. Sedimentation

For very large particles (e.g., microbes), sedimentation under gravity can also contribute to the observed autocorrelation function.

4. Outline of Theory

Consider a scattering element in the fluid. Over short time intervals (~ns – μs), the positions and phase contributions of the particles within that element will fluctuate and, hence, the intensity of light scattered from that volume element will also fluctuate (Fig. 1). An "autocorrelator"—a purpose built computer—correlates intensities, $I(t)$, or equivalently numbers of photons $n(t)$, at time t with subsequent times $t + b\tau_s$, where b is the "channel number" (taking on all integral values between 1 and 64, or up to 128 or 256, depending on how expensive the correlator) and τ_s is a user-set sample time (typically 100 ns for a rapidly diffusing low-mol-wt [M ~ 20,000] enzyme, and increasing up to ~ ms for microbes). The product $b\tau_s$ is referred to as the "delay time" τ.

The correlator calculates the normalized intensity correlation function $g^{(2)}(\tau)$ as a function of the delay time τ:

$$g^{(2)}(\tau) = [\langle I(t) \cdot I(t+\tau)\rangle / \langle I\rangle^2] \quad (1)$$

The angular brackets indicate that the products are averaged over long times (user set as the "experiment duration time," which can be of the order of 1 min or higher depending on the size of the scattering particles and the power of the laser) compared with τ. As $\tau \rightarrow 0$, $g^{(2)}(\tau)$ can theoretically be as high as 2 *(13)* and decays with increasing τ to a lower limit of 1.

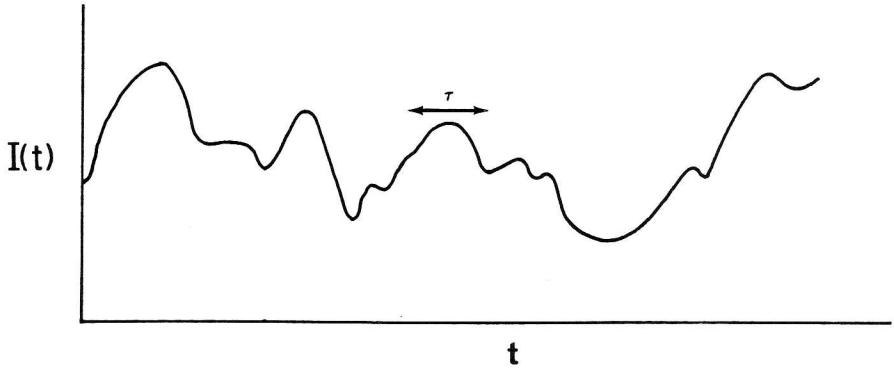

Fig. 1. Fluctuation in scattered intensity, I(t) with time, t. The variable τ is defined as the "delay time." At short delay times, there is good correlation in the scattered intensity; at long delay times, poor correlation.

For dilute Brownian systems (i.e., macromolecules and macromolecular assemblies with $M \leq 100 \times 10^6$), which are also quasispherical, the normalized autocorrelation function, $g^{(2)}(\tau)$ is related to the translational diffusional coefficient, D_2 by:

$$[g^{(2)}(\tau) - 1] = e^{-Dk^2\tau} \tag{2}$$

where k (or sometimes symbol "q" in the literature) is the Bragg wave vector whose magnitude is defined by:

$$k = \{4\pi n/\lambda\} \sin(\theta/2) \tag{3}$$

(n being the refractive index of the medium and λ the wavelength of the incident laser light). Thus, D can be found from a plot of $\mathrm{Ln}[g^{(2)}(\tau) - 1]$ vs τ, and an example is given for the motility protein dynein in Fig. 2.

The translational diffusion coefficient so obtained will be a function of solvent conditions, so as with the sedimentation coefficient (Chapter 5), it is usual to correct to standard conditions (water at 20.0°C), to give $D_{20,w}$ (*see*, e.g., ref. *1*). $D_{20,w}$ at a finite concentration will be an apparent value, and hence measurement at several concentrations and extrapolation to zero concentration to give an "infinite dilution" value, $D^\circ_{20,w}$, is normally necessary. However, the concentration dependence of $D_{20,w}$ is usually much smaller compared to other hydrodynamic parameters, such as $s_{20,w}$, and measurement at a single, dilute concentration may suffice.

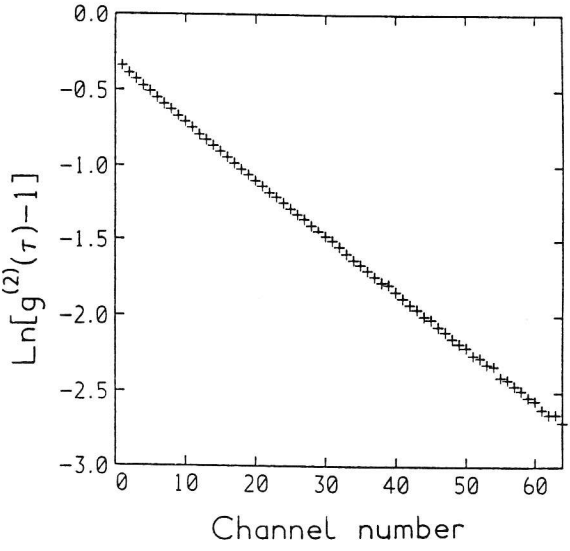

Fig. 2. Plot of Ln $[g^{(2)}(\tau) - 1]$ vs "channel number" for the protein assembly dynein. τ is the delay time; $g^2(\tau)$ is the normalized autocorrelation function; "Channel number" = delay time/sample time.

4.1. Manipulation of $D^\circ_{20,w}$

1. The $D^\circ_{20,w}$ so obtained can be related to the equivalent hydrodynamic radius r_H of the macromolecule by Stokes equation:

$$r_H = (k_B T)/(6\pi\eta_{20,w} D^\circ_{20,w}) \quad (4)$$

   where $\eta_{20,w}$ is the viscosity of water at 20.0°C.
2. Like $s^\circ_{20,w}$, it can be used to obtain the frictional coefficient of the macromolecule and, from this, sophisticated "bead models" for macromolecular conformation (ref. *14*; *see also* Chapter 5).
3. $D^\circ_{20,w}$ can be combined with $s^\circ_{20,w}$ to yield an absolute value for the mol wt via the Svedberg equation *(15)*.

5. Experimental

A schematic dynamic light scattering setup is shown in Fig. 3. Collimated laser light from typically a helium-neon or argon ion laser light source is focused onto the center of a square (typically 1 cm^2) or circular cuvet, placed at the center of a goniometer so that the scattering angle can be varied from typically 3° to 120°. Scattered light is

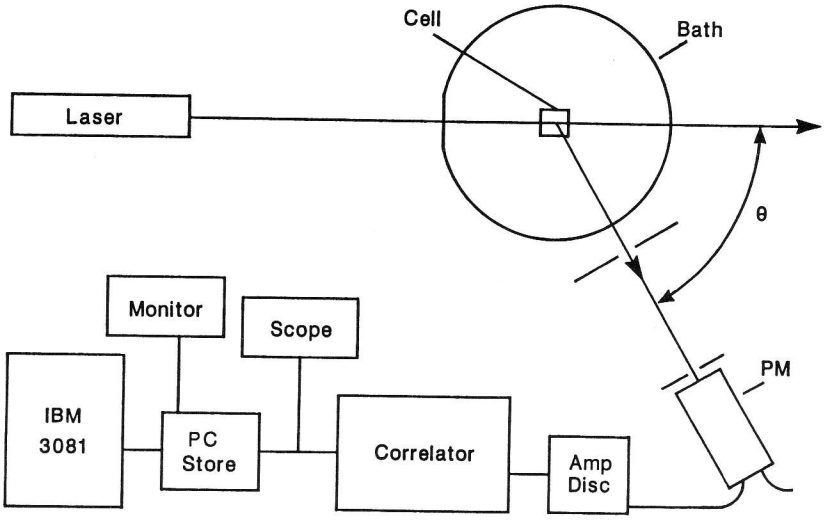

Fig. 3. Schematic dynamic light scattering apparatus (redrawn from ref. *16*). θ: scattering angle; Amp Disc: amplifier-discriminator; PM: photomultipliers; PC: IBM PC-compatible computer; IBM-3081: mainframe computer (only necessary for more sophisticated analyses).

collected by well collimated slits to reach the photomultipliers. Output from this is then processed via an amplifier-discriminator into a form suitable for processing in a digital correlator. Digital output from the correlator can be displayed directly onto an oscilloscope or via a microcomputer. In our laboratory, we store the basic correlation data on floppy disk via an IBM-PC compatible computer. The data can then be analyzed directly on the microcomputer or, for more detailed analysis, transferred to a mainframe computer. It is mandatory that the cell be kept in a thermostatted bath during measurement, since the diffusion coefficient is dependent on solvent viscosity, which itself is a strong function of temperature.

6. Commercial Availability of Instrumentation

Several dynamic light scattering photometers are available, and many of these are considered in ref. *5*. The principal manufacturers include Malvern Instruments and Biotage (UK), Coulter Electronics and Brookhaven Instruments (USA), Peters-ALV (Germany), and

Otsuka Electronics Ltd. (Japan). Most of the dynamic light scattering instrumentation also facilitates total intensity (i.e., "classical") light scattering measurements.

Although the software that comes with this instrumentation can considerably facilitate measurements, for the analysis of smaller macromolecular systems ($M \lesssim 100{,}000$), or for known asymmetric or self-associating macromolecules/assemblies, consultation with an expert user is recommended.

7. Preparation of Solutions

We would like to stress that the same attention to clarity of both sample and scattering cell that applies to the total intensity or "classical" light scattering method (*see* Chapter 7) is also necessary for dynamic light scattering. All solutions and cells need to be scrupulously free from even trace amounts of dust. We find that specially modified square-type fluorimeter cuvets (Fig. 4) are particularly useful for this purpose *(17)*. Repeated rinsing with ~ 0.2 μm filtered distilled water is necessary. If this procedure is not satisfactory, the use of acetone reflux apparatus may be necessary *(18)*.

Aqueous solvents should be of sufficient ionic strength to suppress charge effects. Loading concentrations required (which should be measured *after* clarification) will depend principally on (i) the size of the scatterer and (ii) the output of the laser. For example, if a 25-mW He-Ne laser is used, a loading concentration of at least ~1 mg/mL (and ~2–3 mL) is typically required, for a macromolecular assembly whose $M \sim 5 \times 10^6$. For macromolecules of mol wt down to ~10,000, more powerful lasers (~100 mW) and/or higher concentrations and/or longer experimental duration times are generally necessary to obtain meaningful results.

8. Notes

1. Choice of cuvet: Preferences vary, but we find that "square" cuvets are optically more reliable, since they do not suffer from total internal and stray reflection effects to the extent that can affect measurements using cylindrical cells. If measurement as a function of scattering angle is necessary, angles near the cell corners are obviously prohibited; if cylindrical cells have to be used, wide-diameter (i.e., ≥ 2 cm) cells are recommended.
2. Because of the enhanced effect of dust at low angles, a scattering angle of 90° is conventionally chosen for evaluation of D.

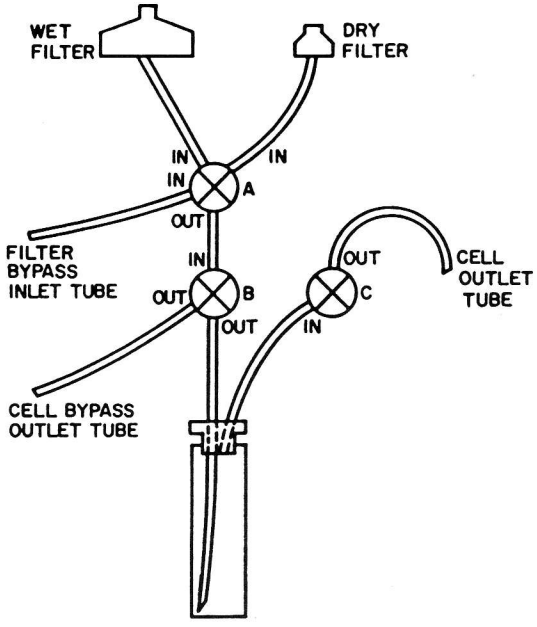

Fig. 4. Schematic cell filling apparatus for minimizing supramolecular contamination (reprinted with permission from ref. *17*).

3. Equation (2) is only exact for spherical particles. For nonspheroidal macromolecules scatterers, the contribution from rotational diffusional effects may not be negligible at higher angles, and the measured translational diffusion coefficient will be an apparent value with respect to angle. Therefore, in addition to measurement of $D_{20,w}$ as a function of concentration and extrapolation to zero concentration, a similar set of measurements as a function of angle and extrapolation to zero angle are necessary for asymmetric scatterers. These two extrapolations can be done on the same set of axes to give a "dynamic Zimm plot" (*19*). The form of the extrapolation to zero angle can, in principle, permit the determination of the rotational diffusion coefficient, D_R (*20*), although the precision with which D_R can be measured in this way is very limited. A good recent example of its careful measurement for a rod-shape virus is given in ref. *21*.
4. If the sample is polydisperse or self-associating, the logarithmic plot (Fig. 2) will tend to be curved, and the corresponding diffusion coefficient will be a z-average. The z-average $D^{\circ}_{20,w}$, when combined with

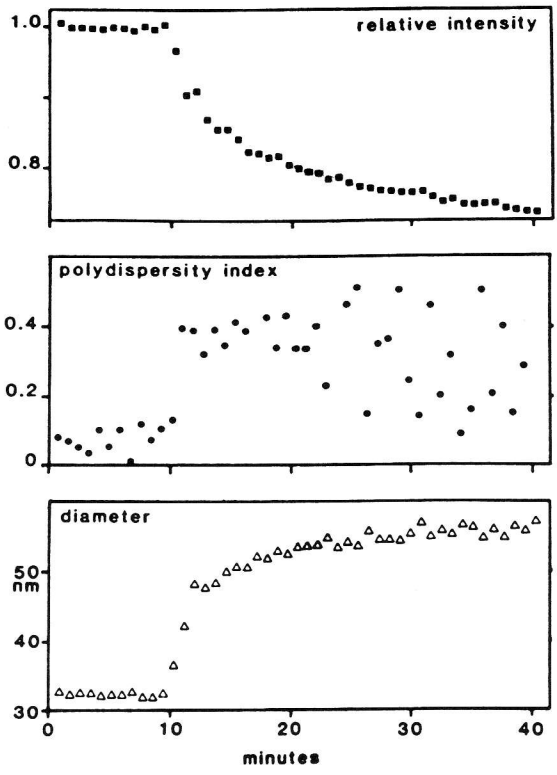

Fig. 5. Effect of removal of calcium ions (by adding EGTA at time $t = 10.5$ min) on southern bean mosaic virus. Top: total intensity (arbitrary units) scattered at 90°. Middle: polydispersity factor. Bottom: hydrodynamic diameter (from D). (redrawn from ref. *11*).

the (weight average) $s^{\circ}_{20,w}$ via the Svedberg equation, yields a *weight average* mol wt *(22)*, M_w.

5. Dynamic light scattering is particularly valuable for the investigation of *changes* in macromolecular systems. An example where it has been used to follow the swelling of a virus (southern bean mosaic virus) on removal of calcium ions is given in Fig. 5.
6. For a heterogeneous system, it is also possible as previously mentioned to obtain a parameter that indicates the spread of diffusion coefficients (the normalized z-average variance of the diffusion coefficients, referred to as the "polydispersity factor")—it is possible to relate this to the distribution of mol wt *(22)*. Commercial software, such as "CONTIN" *(23)*, is available for inverting the autocorrelation data directly to give

distributions of diffusion coefficient and equivalently particle size: These methods have recently been reviewed *(23,24)*. In addition, by analogy with LALLS/GPC and MALLS/GPC (Chapter 7), on-line coupling of dynamic light scattering to GPC has also been considered *(25)*.

7. For charged macromolecular systems, dynamic light scattering provides a useful tool for monitoring electrophoretic mobilities *(26)*. Commercial instrumentation is available for this purpose (*see*, e.g., ref. *5*).

Glossary of Symbols/Terms

QLS, Quasielastic light scattering; PCS, Photon correlation spectroscopy; IFS, Intensity fluctuation spectroscopy; D, Translational diffusion coefficient (cm^2/s) measured at a finite concentration; D_R, Rotational diffusion coefficient (s^{-1}) measured at a finite concentration; $D_{20,w}$, Translational diffusion coefficient at a finite concentration, c, and corrected to standard solvent conditions (i.e., water as solvent at a temperature of 20.0°C); $D°_{20,w}$, Infinite dilution translational diffusion coefficient; k_B, Boltzmann constant (1.38062×10^{-16} erg/K); T, Temperature (K); $s°_{20,w}$, Infinite dilution sedimentation coefficient (S or s); r_H, The effective hydrodynamic or "Stokes" radius of a particle (nm, μm, or cm); PF, Polydispersity factor (normalized z-average variance of the translational diffusion coefficients); M, Mol wt (g/mol); M_w, Weight average mol wt (g/mol); $n(t)$, Number of photons received by the photomultipliers at time t; b, Channel number; τ_s, Sample time (ns or μs); τ, Delay time (ns or μs) = $b\tau_s$; I, Intensity; $g^{(2)}(\tau)$, Normalized intensity autocorrelation function; k, Bragg wave vector (nm^{-1} or cm^{-1}); θ, Scattering angle; n, Refractive index of the medium; λ, Wavelength of the incident laser light (nm or cm).

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