CHAPTER 7

Classical Light Scattering for the Determination of Absolute Molecular Weights and Gross Conformation of Biological Macromolecules

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1. Introduction

Classical light scattering, like sedimentation equilibrium in the analytical ultracentrifuge, can provide a powerful absolute method for the determination of molecular weights of macromolecules. By "classical" light scattering (as opposed to "dynamic" light scattering [cf. Chapter 8]) we mean the measurement of the total or time-integrated intensity scattered by a macromolecular solution compared with the incident intensity for a range of concentrations and/or angles; this information can be used to deduce the mol wt, M, gross conformation (from measurement of a parameter commonly referred to as the "radius of gyration," R_G), and thermodynamic nonideality parameters (particularly the thermodynamic second virial coefficient, B, which can also yield potentially useful information on molecular conformation). Classical light scattering is often referred to in the literature as "total intensity light scattering" ("TILS"), "static light scattering," "integrated light scattering," "differential light scattering," "traditional light scattering," or simply "light scattering."

Although a more rapid and, in principle, more convenient alternative to sedimentation equilibrium, the method has until relatively recently suffered greatly from the "dust problem"; viz. all solutions/scattering

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cells have to be scrupulously clear of dust and supramolecular particles, particularly for the analysis of solutes of low mol wt ($\lesssim$50,000). This has resulted in many cases in the requirement for unacceptably large amounts of purified material: experiments on incompletely purified solutions have been of little value.

Two developments have made the technique now worth serious consideration by biochemists or molecular biologists: (1) the use of laser light sources, providing high collimation, intensity, and monochromaticity. These, plus the additional property of high degree of coherence also form the basis of the "dynamic" light scattering technique (*see* Chapter 8). (2) The coupling of gel permeation high-pressure liquid chromatography ("GPC") systems on-line to a light scattering photometer via the incorporation of a flow cell facilitates considerably the analysis of polydisperse materials and, more significantly, provides a very effective on-line "clarification" system from dust and other supramolecular contaminants.

Other introductory chapters on classical light scattering are to be found in van Holde *(1)* and Tanford *(2)*. More detailed treatments are given in, for example, the highly used texts of Stacey *(3)*, van de Hulst *(4)*, and Kerker *(5)*. A useful introduction to the coupling of GPC to multiangle classical (laser) light scattering ("MALLS") has been given by Jackson et al. *(6)*.

2. Summary of Information Available

1. Molecular weight (if the solution is polydisperse this will be a weight average).
2. Gross conformation information (from measurement of the radius of gyration, R_G). Measurement of the thermodynamic second virial coefficient ("B" or "A_2") can also yield gross conformational information, provided that satisfactory account can be taken of contributions to B from polyelectrolyte behavior and also from possible self-association or aggregation behavior.
3. If a coupled GPC/multiangle light scattering system is employed for a polydisperse system (e.g., a mucus glycoprotein, proteoglycan, or a polysaccharide), mol-wt distribution, R_G distribution, and from double logarithmic plots of M vs R_G and other combinations of light scattering data, additional gross conformational information can be obtained.

Classical light scattering is generally most suitable for macromolecules within the mol-wt range 50,000–50 $\times$ 10^6 g/mol.

3. Summary of Limitations

1. Sample clarification from dust and supramolecular aggregates. This is particularly serious if solutions of macromolecules of $M \lesssim 50{,}000$ are being studied.
2. A separate, precise measurement of the refractive index increment, *dn/dc* is required, preferably at the same wavelength used in the light scattering photometer.
3. For very large (say ≈ 1 μm, $M \approx 10^{11} - 10^{12}$ g/mol) or optically dense macromolecular assemblies, the representation of the data in terms of molecular parameters can become exceedingly difficult since, because of the greater complexity of the theory involved, the so-called Rayleigh-Gans-Debye (RGD) approximation becomes inapplicable *(1)*.

4. Outline of Theory

For solutions of macromolecules or macromolecular assemblies, the basic equation for the angular dependence of light scattering is the Debye-Zimm relation:

$$Kc/R_\theta \cong \{1 + (16\pi^2 R_G^2/3\lambda^2) \sin^2[(\theta/2)]\} [(1/M) + 2Bc] \qquad (1)$$

where it is assumed that the second virial coefficient, B, is sufficient to represent nonideality (i.e., third and higher order terms are assumed negligible). 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_o (a cos θ correction term is needed if unpolarized light is used); K is an experimental constant dependent on the square of the solvent refractive index, the square of the refractive index increment (*dn/dc*, mL/g), and the inverse fourth power of the incident wavelength; R_G is extensively referred to as the "radius of gyration" of the macromolecule, c is the solute concentration (g/mL), and B is the thermodynamic second virial coefficient (mL · mol/g^2). If the solute is heterogeneous, M (g/mol) will be a weight average, M_w, and R_G a z-average. Equation (1) is generally a good representation for particles whose maximum dimension is $<\lambda$. For larger particles, much more complex representations are necessary.

For particles $\lesssim\lambda/20$ ($\equiv M \lesssim 50{,}000$) the angular term in Eq. (1) is small: No angular dependence measurements are necessary to obtain M (although R_G cannot be obtained). When this is not the case, 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" (Fig. 1) or measurement at a single angle assumed low enough so that $\sin^2(\theta/2) \approx 0$ may be adequate. Alternative methods of representing the data have in the past been in terms of "disymmetry," $z(\theta)$ (the ratio of the scattering intensity at an angle θ [typically 45°] to that at 180–θ), vs angle plots. Both $z(\theta)$ and R_G provide useful guides to the gross conformation of a macromolecule (between the extremes of sphere, rod, or random coil). Two further useful representations of the scattering data (*see*, e.g., ref. *7*) are the "Cassassa-Holtzer plot," used for example for the estimation of mass per unit lengths of rod-shape macromolecules *(8)*, and the "Kratky plot."

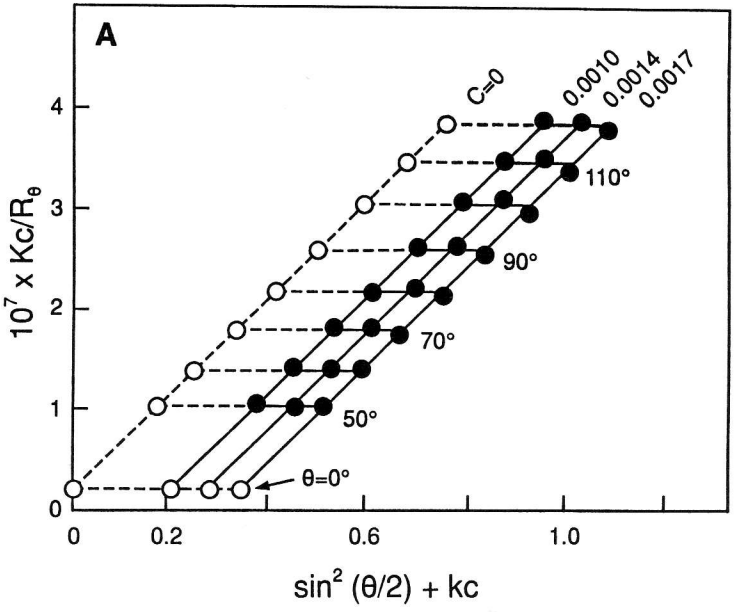
For fairly rigid macromolecules, R_G can be used directly to model gross conformation: (1) as an additional parameter to the sedimentation coefficient (*see* Chapter 16) and other hydrodynamic parameters for representing the structure of complex macromolecules in terms of "bead modeling" *(9)*, and (2) as a parameter, after combination with the second virial coefficient, B, and intrinsic viscosity parameters for representing structures in terms of general triaxial ellipsoids *(10)*.

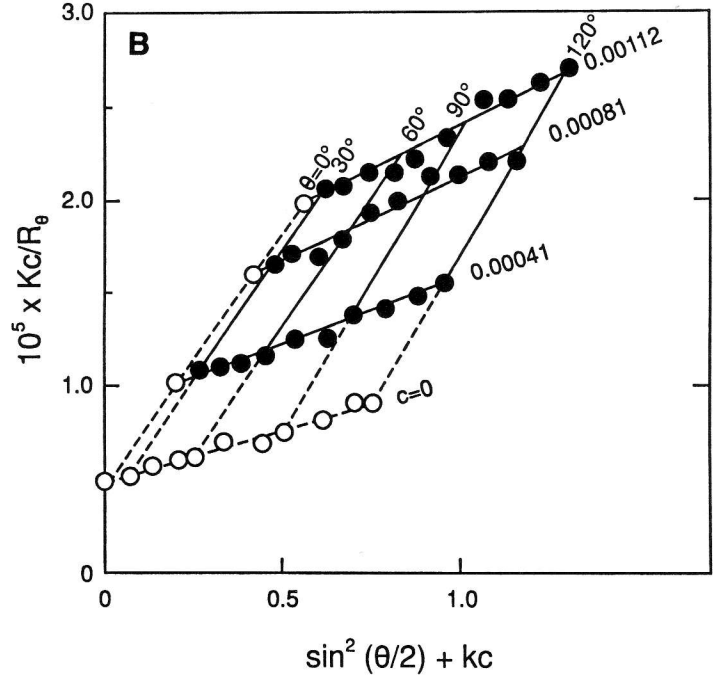
5. Principal Types of Measurement

5.1. Turbidimetry

This involves the measurement of the total loss of intensity by a solution through scattering, summed over the entire angular intensity envelope, compared with the intensity of the incident radiation. This type of measurement can be performed on a good-quality spectrophotometer (whose detector does not accept appreciable amounts of scattered light). Measurements are made at wavelengths away from absorption maxima. Turbidimetry is generally suitable for macromo-

Fig. 1 (*opposite page*). Zimm Plots. The second virial coefficient, B, can be evaluated from the slope of the $\theta = 0$ line; R_G from the limiting slope of the $c = 0$ line. The common intercept is I/M. R_θ is the scattered intensity function (the "Rayleigh excess ratio"), and K a constant depending on the square of the refractive index increment, the refractive index of the solvent, and the wavelength of the incident radiation. k is an arbitrary constant (positive or negative) chosen to "space out" the data. Note either the $c = 0$ line (usual) or the $\theta = 0$ line can have the maximum slope, depending on the relative magnitudes of R_G and B. (A) Zimm plot for a (diptheria) antigen-antibody aggregate. k = 200 mL/g. M ~78×10^6. (Data replotted from ref. *20*). (B) Zimm plot for a polysaccharide (*L. hyperborea* sodium alginate). k = 500 mL/g. M = (217,000 ± 10,000). B ~7.0×10^{-3} mL · mol / g^2. R_G ~59 nm. (Data replotted from ref. *12*.)


A
C=0
0.0010
0.0014
0.0017
110°
90°
70°
50°
θ=0°
10^7 x Kc/R_θ
$\sin^2(\theta/2) + kc$


B
120°
0.00112
0.00081
0.00041
θ=0°
30°
60°
90°
c=0
10^5 x Kc/R_θ
$\sin^2(\theta/2) + kc$

lecular assemblies of $M \geq 10^5$, and has been used for the measurement of molecular weights of viruses and estimating the number concentrations of bacteria and bacterial spores (*see*, e.g., ref. *11*).

5.2. Low-Angle Light Scattering

Scattering measurements are performed at only one fixed small angle ($\leq 8°$). The angle is assumed low enough such that no angular correction of the scattering data is required, although extrapolation to zero concentration of (Kc/R_θ) may still be necessary. The method can provide values for M and B of a system, but not R_G, since no record is made of the angular dependence of Kc/R_θ. Although at low angles scattering intensities are higher and hence solute concentrations can be correspondingly lower, the dust problem is correspondingly far more severe. The on-line coupling of a low-angle (laser) light scattering, "LALLS," photometer to GPC has largely circumvented this problem and facilitates the measurement of mol-wt distributions for heterogeneous materials *(12,13)*.

5.3. Multiangle Light Scattering

This can involve a goniometer arrangement or fixed detectors at multiple angles (*see* Fig. 2A). 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. 1). The method can yield M, B, and R_G. Plots of (Kc/R_θ) are only linear over a wide range of angles for randomly coiled macromolecules. For globular macromolecules, "Guinier plots" of $\text{Ln}(Kc/R_\theta)$ vs $\sin^2(\theta/2)$ can facilitate the angular extrapolation, or for highly branched macromolecules (for example, for some polysaccharides), "Berry" plots" $[(Kc/R_\theta)^{1/2}$ vs $\sin^2(\theta/2)]$ can be used.

A good example of the experimental arrangement involving a laser light source, how the instrument is calibrated, and application to an associative/dissociative system (hemoglobin) has been given by Johnson and McKenzie *(14)*. As with LALLS, multiangle (laser) light scattering (MALLS) photometers have been coupled on-line to GPC, facilitating the analysis of heterogeneous solutions and also largely circumventing the dust problem *(6,15)*. The simultaneous measurement at multiple angles permits the detection of possible remaining problems at low angle (through, for example, the shedding of debris from the columns). Distributions of R_G—provided the maximum dimen-

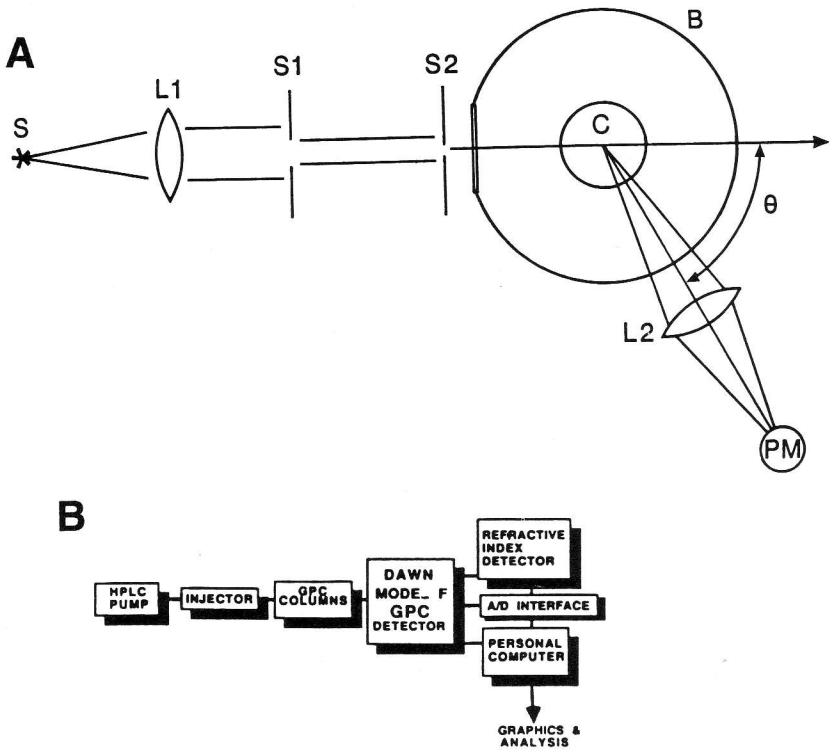


Fig. 2. Schematic light scattering systems. (A) Light scattering photometer. S light source; S1, S2 collimating apertures; L1, L2 lenses, B thermostatted bath (designed to minimize stray reflections); C cuvet (square or cylindrical); PM photomultiplier. If S is a well collimated laser, the collimating lens, L1, is not required. (Data replotted from ref. *20*.) (B) Configuration of an on-line GPC/multiangle laser light scattering photometer system. (From ref. *15*.)

sion of the macromolecule is $> \lambda/20$—as well as mol wt can be obtained, and, using the instrument in so-called batch (i.e., not coupled to GPC) mode, B can be estimated. Nonideality effects are not usually as severe for GPC-MALLS, since concentrations of volume "slices" passing through the flow cell are much smaller than the initial loading concentration: In many cases an extrapolation to zero concentration or knowledge of the second virial coefficient is not necessary to obtain a satisfactory estimate for M. One final advantageous feature for heterogeneous systems is that, since for a given M the corresponding R_G is estimated, for a homologous or quasi-homologous polymer distribu-

tion (e.g., DNA or a polysaccharide), the form of a plot of Log [M] vs Log [R_G] can provide an estimate for the gross conformation between the extremes of sphere, rigid rod, and random coil *(16)* as we have already indicated above. Examples of the use of GPC-MALLS for the determination of the mol-wt distribution of a well characterized polysaccharide are given in Fig. 3.

6. Availability of Instrumentation

This is less of a problem compared with analytical ultracentrifugation (*see* Chapters 5 and 6). A survey of recent instrumentation can be found in ref. *17*. Most of the instruments available permit dynamic as well as classical light scattering, or incorporate a flow cell and can be coupled on-line to a GPC system.

7. Materials

7.1. Choice of Solvent Media

As with sedimentation equilibrium, solutions should be dialyzed against an appropriate buffer of defined pH and ionic strength, I. For polyelectrolytes, ionic strengths of at least 0.3 are recommended.

7.2. Concentrations/Volume Requirements

This depends on:

1. The mol wt of the macromolecule, since the scattering is approximately proportional to concentration × mol wt;
2. The output of the laser; and
3. The clarity of the solutions.

For scrupulously clean solutions, a 5-mW laser for a loading concentration of 3 mg/mL is sufficient for particle mol wt as low as ≈40,000. For smaller macromolecules, a proportionately higher concentration and/or higher laser power is required. If a flow cell arrangement is used on-line to GPC, depending on the extent of the fractionation and the extent of clarification the columns can provide, higher loading concentrations may also be required. If a flow cell arrangement is used, loading volumes can be as low as 100 µL; for standard fluorimeter cuvets, up to ≈ 3 mL; if cylindrical cuvets are used, small diameters (i.e., ≤ 2 cm) are to be avoided because of extraneous scattering/reflections from the glass walls, although large-diameter cuvets can be expensive in terms of quantity of solution required.

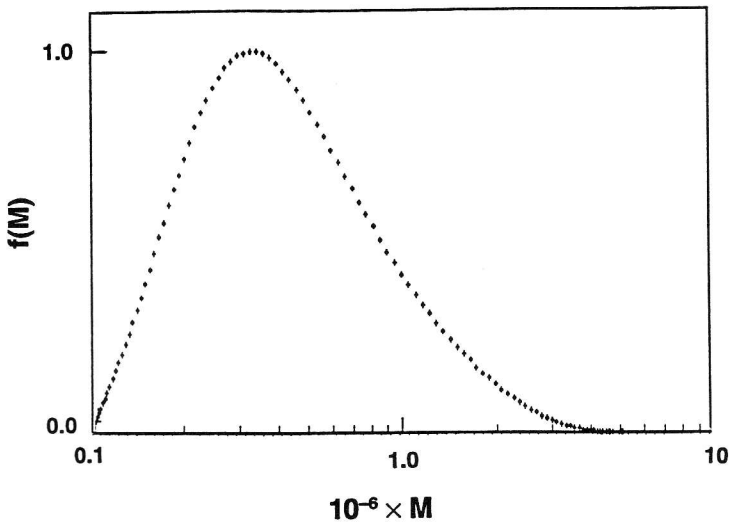


Fig. 3. Molecular-weight distribution of T-500 dextran obtained from GPC/MALLS. Weight-average mol wt for the whole distribution, M_w° ~480,000.

8. Methods

We describe briefly here the operation of a commercially available on-line GPC-MALLS system (Fig. 2B), since this in the author's opinion appears the most versatile (for more details, *see* ref. *15*).

1. Choose GPC columns/equipment as appropriate (*see* vol. 1, Chapter 2 in this series). A pulse-free high-pressure liquid chromatography pump is essential. A guard filter upstream is desirable, as is prefiltering solutions through an appropriate millipore filter (e.g., 0.22 μm). For the Dawn F system (Wyatt Technology, Santa Barbara, CA), a ≈ 100-μL microinjection loop is desirable. A column by-pass option can be installed if fractionation is not required (viz. if Zimm plot measurements are desired for a range of loading concentrations).
2. The light scattering photometer has to be "*calibrated*" usually with a strong Rayleigh (i.e., maximum dimension $<\lambda/20$) scatterer (e.g., toluene) whose scattering properties are known (*see*, e.g., refs. *14,18*). Calibration is necessary, because the ratio of the intensities of the scattered and incident beams is usually very small ($\approx 10^{-6}$).
3. For simultaneous multiangle detection, the detectors have to be "*normalized*" to allow for (i) the different scattering volumes as a function of angle and (ii) the differing responses of the detectors. This is normally

performed using a solution of macromolecules whose M is $\lesssim$ 50,000, or a solution of a larger macromolecule whose R_G is known (e.g., T-500 dextran).

4. A suitable concentration detector (UV absorbance for proteins/nucleic acids or a refractive index detector for polysaccharides) is incorporated downstream from the light scattering photometer. The volume delay between the light scattering photometer and the concentration detector needs to be accurately known.
5. The refractive index increment at the scattering wavelength used (and also, if appropriate, at the wavelength of the refractive index concentration detector), if not known, needs to be measured (*see* ref. *19*). Further, if the second virial coefficient, B, is not known, and if column loading concentrations are high, the Kc/R_θ ratio needs to be evaluated as a function of concentration as well as angle, and a double extrapolation to zero angle/zero concentration can be performed using a Zimm plot (*see* Fig. 1). As already mentioned, for the combined GPC/MALLS method, nonideality corrections are not usually significant, since after fractionation, the scattering concentrations are very small ($\approx$0.1 mg/mL or less).

Glossary of Symbols/Terms

TILS, Total intensity light scattering; GPC, Gel permeation chromatography; LALLS, Low-angle laser light scattering; MALLS, Multiangle laser light scattering; RGD, Rayleigh-Gans-Debye; M, Mol wt (g/mol); M_w, Weight average mol wt (g/mol); B or A_2, Second thermodynamic (or "osmotic pressure") virial coefficient (mL · mol/g^2); R_G, Root mean square radius about the center of mass ("Radius of gyration") (nm or cm); θ, Scattering angle; $z(\theta)$, Disymmetry ratio; n, Refractive index; c, Solute concentration (g/mL); dn/dc, Refractive index increment (mL/g); λ, Wavelength of the incident light (nm or cm); R_θ, Rayleigh excess ratio; K, Experimental constant (mL · mol/g^2); I_o, Intensity of incident light; i_θ, Excess scattered light intensity from a solution (compared to pure solvent) at an angle θ.

References

1. Van Holde, K. E. (1985) *Physical Biochemistry*. Prentice-Hall, Englewood Cliffs, NJ.
2. Tanford, C. (1961) *Physical Chemistry of Macromolecules*. Wiley, New York.
3. Stacey, K. A. (1956) *Light Scattering in Physical Chemistry*. Academic, New York.

4. Van de Hulst, H. C. (1957) *Light Scattering by Small Particles.* Wiley, New York.
5. Kerker, M. (1969) *The Scattering of Light and other Electro-magnetic Radiation.* Academic, New York.
6. Jackson, C., Nilsson, L. M., and Wyatt, P. J. (1989) Characterization of biopolymers using a multi-angle light scattering detector with size-exclusion chromatography. *J. Appl. Polym. Sci., Appl. Polym. Symp.* **43,** 99–114.
7. Burchard, W. (1992) Static and dynamic light scattering approaches to structure determination of biopolymers, in *Laser Light Scattering in Biochemistry* (Harding, S. E., Sattelle, D. B., and Bloomfield, V. A., eds.), Royal Society of Chemistry, Cambridge, UK.
8. Chapman, H. D., Chilvers, G. R., and Morris, V. J. (1987) Light scattering studies of solutions of the bacterial polysaccharide (XM6) elaborated by *Enterobacter* (NCIB 11870). *Carbohydr. Polym.* **7,** 449–460.
9. Garcia de la Torre, J. G. (1989) Hydrodynamic properties of macromolecular assemblies, in *Dynamic Properties of Biomolecular Assemblies* (Harding, S. E. and Rowe, A. J., eds.), Royal Society of Chemistry, Cambridge, UK.
10. Harding, S. E. (1987) A general method for modeling macromolecular shape in solution. *Biophys. J.* **51,** 673–680.
11. Harding, S. E. (1986) Applications of light scattering in microbiology. *Biotech. Appl. Biochem.* **8,** 489–509
12. Martinsen, A., Skjåk-Braek, G., Smidsrød, O., Zanetti, F., and Paoletti, S. (1991) Comparison of different methods for determination of molecular weight and molecular weight distribution of alginates. *Carbohydr. Polym.* **15,** 171–193.
13. Corona, A. and Rollings, J. E. (1988) Polysaccharide characterization by aqueous size exclusion chromatography and low angle light scattering. *Separation Size Technol.* **23,** 855–874.
14. Johnson, P. and McKenzie, G. H. (1977) A laser light scattering study of haemoglobin systems. *Proc. R. Soc.* **199B,** 263–278.
15. Wyatt, P. J. (1992) Combined differential light scattering with various liquid chromatography separation techniques, in *Laser Light Scattering in Biochemistry* (Harding, S. E., Sattelle D. B., and Bloomfield, V. A., eds.), Royal Society of Chemistry, Cambridge, UK, pp. 35–58.
16. Smidsrød, O. and Andresen, I.-L. (1979) *Biopolymerkjemi.* Tapir Press, Trondheim, p. 136. *See also* p. 309 of ref. *2.*
17. Harding, S. E., Sattelle, D. B., and Bloomfield, V. A. (eds.) (1991) *Laser Light Scattering in Biochemistry.* Royal Society of Chemistry, Cambridge, UK.
18. Stacey, K. A. (1956) *Light Scattering in Physical Chemistry.* Academic, New York, pp. 86,87.
19. Stacey, K. A. (1956) *Light Scattering in Physical Chemistry.* Academic, New York, pp. 98–100.
20. Johnson, P. (1985) Light scattering–traditional and modern–in the study of colloidal systems. *J. Surf. Sci. Technol.* **1,** 73–85; *see also* Johnson P. and Ottewill, R. H. (1954) *Disc. Faraday. Soc.* **18,** 327–337.