Polysaccharide molecular weight determination: which technique?

Stephen E.Harding

Department of Applied Biochemistry & Food Science, University of Nottingham, Sutton Bonington LE12 5RD, UK

ABSTRACT

Average molecular weights and molecular weight distributions are important parameters affecting the performance of polysaccharides as thickening and gelling agents in foods and in other commercial applications. Difficulties encountered with presently widely used techniques for the determination of these parameters are highlighted. The potential of low speed sedimentation equilibrium in the analytical ultracentrifuge as an alternative is demonstrated, particularly in the light of some recent theoretical & experimental developments.

INTRODUCTION

Polysaccharides such as alginates, galactomannans, xanthan & carageenan gums are widely used as thickening and gelling agents in the food industry (1). Their potential in the pharmaceutical industry (drug delivery systems) and in oil mining (well bore technology) has also been identified (2,3). One of the most important factors governing the performance of such ´commercial´ polysaccharides is their molecular weights, and, since for a given preparation they are polydisperse, the distribution of molecular weights. For example, the performance of pectins in drinking yoghurts has been related to such distributions of M_r; some concern has also been expressed over the possible toxicity of low molecular weight species in carageenan. As a result, considerable attention has been paid to polysaccharide molecular weight determination. Unfortunately, the technique which has revolutionised protein biochemistry - SDS polyacrylamide gel electrophoresis (SDS PAGE) - cannot be applied to polysaccharides as a quantitative tool. Although certain charged polysaccharides satisfy similar charge per unit length criteria, gel electrophoresis of polysaccharides has thus far had only a fraction of the impact of its protein counterpart, through, for example, difficulties of calibration.

Other ´relative´ techniques (i.e. requiring calibration standards of known M_r and similar conformation, have however been applied widely, as have ´absolute´ (viz. not requiring standards) light scattering procedures. We now highlight some of the difficulties associated with the latter, which has contributed to considerable data variability (see, e.g., 4-6) and demonstrate the potential of some recent advances in the relatively under-used technique of low speed sedimentation equilibrium in the analytical ultracentrifuge.

LIGHT SCATTERING

For over four decades light scattering procedures have provided powerful tools for elucidating the size and conformation of biomolecular systems in solution. They are particularly well suited for the analysis of relatively large, fairly monodisperse systems, and indeed, we have extensively applied these techniques to systems of viruses and bacterial spores (see, e.g. ref. 7).

Two principle techniques have been applied to polysaccharide size determinations: (i) ´classical´ or ´differential´ light scattering, in which the intensity scattered by a dispersion is measured as a function of angle and (ii) ´quasi-elastic´ light scattering (QLS) in which the short-time (ns - µs) fluctuations in intensity at a given angle are measured. For molecular weight measurement in (i) a double extrapolation to zero angle and zero concentration (´Zimm´ plot) is normally employed, or measurements are made at very low angle and a single extrapolation to zero concentration is sufficient (´Low angle Light Scattering, LLS); in (ii) ´autocorrelation´ measurements of the intensity fluctuations can yield the (z-average) translational diffusion coefficient, D_z, after an extrapolation to zero concentration. This, when combined with the sedimentation coefficient, can also yield the molecular weight.

Despite their wide application, we believe these procedures have inherent difficulties when applied to heterogeneous systems such as polysaccharides, largely because of problems of dust and even trace amounts of large aggregates, particularly for measurements at low angles. QLS diffusion measurements are often performed at 90^o, to minimise such contamination effects. Although this does not lead to any appreciable error for rigid spherical particles, for polysaccharides extrapolation to zero-angle is normally necessary because of the finite contribution to the observed autocorrelation profiles from rotational diffusion - unfortunately at low angles, the effects of any dust/ aggregates are aggravated. Clarification procedures run the risk of not removing aggregates or removing part of the distribution of sizes that is actually being analysed. A good demonstration of the effects of aggregates has been given for glycoconjugates by Preston & coworkers (8). Further, apparent agreement between Zimm plots and the Svedberg equation (using D_z values measured by QLS) can be misleading in that the same effects producing high M_r values (and high Rg values) from the Zimm method would also produce lower D_z values (and hence higher M_r values) from QLS.

If QLS is being used for size distribution analysis, it is not normally possible to distinguish genuine polydispersity (viz. non-interacting species of different molecular size) from self-association phenomena; such analyses also generally fail to take into consideration the effects of thermodynamic non-ideality. Finally, an accurate measurement of concentration is normally necessary (for both the Zimm plot and diffusion coefficient extrapolations) and the measurement of refractive increments: concentrations can rarely be measured to better than 5%.

Because of these difficulties, light scattering techniques would not, where possible, be our method of choice: if light scattering has to be used, we feel that some form of confirmation of results using an independent procedure (such as low speed sedimentation equilibrium) would be desirable.

RELATIVE TECHNIQUES: CALIBRATED GEL CHROMATOGRAPHY (CGC) AND INTRINSIC VISCOSITY

Calibrated Gel Chromatography, like SDS PAGE, is another technique that has revolutionised protein biochemistry and polymer science as a whole. The major difficulty, once an appropriate gel material and detection system has been chosen for a given separation, is in the calibration, using standards of similar conformation and M_r to the sample being analysed. For globular proteins such standards are readily available. For polysaccharides this is not so easy, because of uncertainties concerning conformation: the popular use of dextrans as standards is not generally reliable. The only reliable way would appear to be to measure the molecular weight of isolated narrow fractions using an absolute technique such as light scattering, (given the limitations referred to above). The assumption has to be made that the distribution of molecular weights is due to genuine polydispersity (i.e. the presence of non-interacting components of different molecular weight) as opposed to a self-association.

The other widely used relative procedure is in the use of intrinsic viscosity measurements, calibrated by light scattering or some other ´absolute technique´ using the Mark-Houwink equation. This procedure again requires the use of standards of known M_r and similar conformation/solvation to the polysaccharide whose M_r is required.

LOW SPEED SEDIMENTATION EQUILIBRIUM

This is a technique that has been available in various forms for over four decades since its inception by Svedberg in the 1920´s (see, e.g., 9). Its routine use in biochemical laboratories has declined, primarily with the advent of SDS PAGE and CGC for protein work. For difficult heterogeneous thermodynamically non-ideal systems - the hallmark of polysaccharides - its usefulness is however retained, particularly in the light of recent advances. Nonetheless, unlike light scattering, there are very few centres of expertise left with this technique, both in Europe & in the USA. This is somewhat surprising considering alone its potential in the polysaccharide field. The length of time required to reach equilibrium (up to three days) and the shear size of the machinery required when compared with light scattering are no doubt contributory factors to its lack of popularity. In addition, the ´high speed´ or meniscus depletion method, which facilitates much easier data handling, is not generally suitable for polysaccharides because of the difficulty of depleting the meniscus of low molecular weight material, without losing optical registration at the cell base: a pitfall is to assume depletion conditions when this is not valid (10). I want to now describe some of the recent analytical developments involving the capture and analysis of the data that we have been involved with which I feel now make the technique particularly attractive. This includes an improved method for the extraction of the weight average molecular weight for the whole sample distribution and how we cope with thermodynamic non-ideality. Other developments include both an ´indirect´ and a ´direct´ approach to the characterisation of distributions of M_r and also a combined approach with gel chromatography. I will demonstrate how a new method for off-line fringe analysis has opened up the possibilty of using a series of most interesting methods derived over the last 20 years but previously very difficult to implement because of the severe requirements on the precision of the fringe data.

Determination of weight average molecular weights: the ´star average´.

One of the most fundamental pieces of information to be obtained from a sedimentation equilibrium experiment is the weight average molecular weight over the whole solute distribution, M_w^o. The distribution of solute at sedimentation equilibrium is most accurately recorded using Rayleigh Interference Optics: M_w^o can be obtained from the mean slope of a plot of Ln J (where J is the absolute fringe number displacement) versus the square of the radial displacement from the centre of the rotor, r^2. At equilibrium in a ´low speed´ experiment the concentration at the air/solution mensicus remains finite, but can be found without too much difficulty by mathematical manipulation of the fringe data (11).

For polysaccharides a plot of LnJ versus r^2 is not generally linear, because of polydispersity (presence of non-interacting components of different molecular weight) and, sometimes, self-associative phemomena, both which produce upward curvature; and also thermodynamic non-ideality (through exclusion volume & charge effects) which produces downward curvature. [Occasionally these effects can cancel each other out and linear ´pseudo-ideal´ plots are obtained which can be misleading]. The principle difficulty in obtaining a value for the mean slope lies in establishing an accurate value for J at the cell base. This can be avoided by using a new type of point average molecular weight, the star average, M* (11). The M* function has many interesting properties, one of which is that its value extrapolated to the cell base = M_w^o, and provides a more accurate way of determining this latter parameter (to within ± 5% if a conventional light source and manual data capture procedures are used): we have applied this function for determining for example, the molecular weights of pectins (12) and galactomannans (13). The M* function also facilitates the determination of the point number average at the meniscus (11), where the precision in the data justifies this. Point weight average molecular weights, M_w can be readily obtained (without the use of M*) using sliding strip fits to the Ln J versus r^2 plots. Another potential pitfall if sedimentation equilibrium is used is a failure to allow properly for the effects of thermodynamic non-ideality, which tends to diminish measured molecular weights and mask heterogeneity. The effects on M_w^o are normally minimized by using the lowest possible loading concentration (∼0.2 mg/ml in a 30mm path length cell), and this normally suffices. Alternatively, point weight averages can be extrapolated to zero concentration (J=0) to yield a value essentially independent of thermodynamic non-ideality or associative phenomena. The ´ideal´ value obtained in such a way may however be biased towards the lower end of the molecular weight distribution, but this bias can be minimised by using short columns and (in extreme cases) by extrapolating the value so obtained to zero gravitational field.

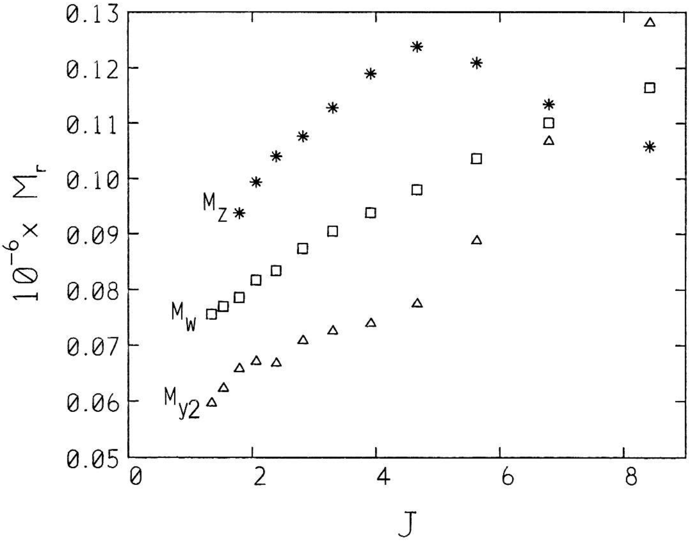

Figure 1. Point average molecular weights plotted versus fringe concentration for a sedimentation equilibrium experiment on a tomato fruit polyuronide (17,12). Rayleigh fringe data had been captured on an LKB Ultroscan & fringe increments determined using the PASCAL routine ´ANALYSER´.

Another way of dealing with non-ideality is to combine weight average values with number , z- and higher order averages (14,15) if the precision in the data justifies this: it is possible to obtain number and z whole cell average molecular weights (M_n^o and M_z^o respectively) and also point M_n, M_z and ′compound′ point average molecular weights:

$$M_{y1} = 1/(2/M_n - 1/M_w), \text{ and}$$

$$M_{y2} = M_w^2 / M_z.$$

M_{y1} and M_{y2} point averages are free of first order non-ideality effects: experimental values are generally reliable, however, only if a laser light source can be employed to generate the interference fringes, or if accurate on- or off-line data capture procedures are available. We have recently adapted a commercially available laser gel scanner (LKB ′Ultroscan′) for data capture and written a Fourier series algorithm ′ANALYSER′ (UCSD PASCAL) for data analysis (16,17): the improvement in precision over manual microcomparators is remarkable, and enables the realistic determination of M_z and M_{y2} values. Fig. 1 illustrates an example of this for tomato fruit polyuronides.

Molecular Weight Distributions

There are four approaches using sedimentation equilibrium I want to highlight

1. Molecular weight ratios

The whole cell number and z averages (M_n^o & M_z^o) have been hitherto obtainable with considerably less precision than M_w^o, but again, with the availabilty of improved off-line processing referred to above, this should no longer be a limiting factor. The M_z^o/M_w^o or M_w^o/M_n^o ratios can be related to standard deviations of distributions (whatever form they may take) via the ′Herdan relations′ (see, e.g. 18)

2. Modelling the concentration distributions for a non-ideal polydisperse system.

Predicting the concentration distribution for a non-ideal polydisperse system at sedimentation equilibrium has until recently not been possible because of the nature of the non-linear equations characterising such distributions. An interdependent minimization procedure has now however been developed (19) and successfully applied to a particular glycoconjugate system (a chronic bronchitic glycoprotein) for a discrete distribution of molecular weights. The procedure at the present time takes a considerable toll on computer resources (19), and as a result has not yet been applied to quasi-continuous distributions of M_r which are characteristic of polysaccharides, although we are exploring its possibilies.

3. Modelling the concentration distribution: ′effective′ association constants.

This makes use of the fact that it is normally impossible to distinguish in one experiment the effects of a polydisperse

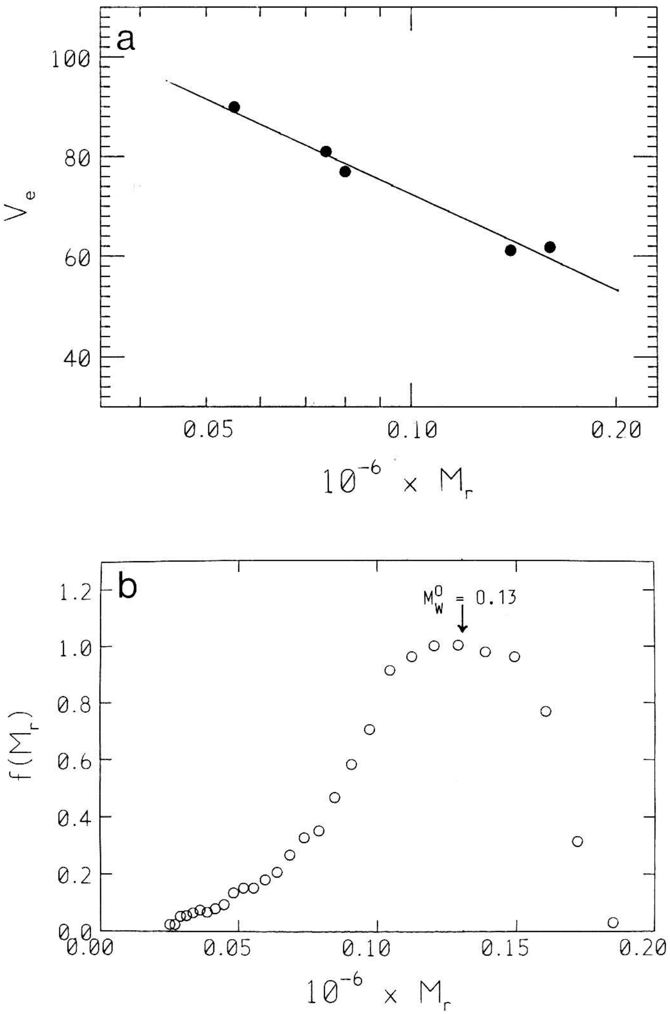

Figure 2. Molecular weight distribution of manucol DM in standard phosphate chloride buffer, pH 6.8, I=0.3

a: Calibration plot for a Sephacryl S-400 column using low speed sedimentation equilibrium on isolated fractions of manucol DM; b: Molecular weight distribution for the whole elution profile. Alginate assayed using the Phenol/sulphuric acid method. M_w^o for the whole distribution (from sedimentation equilibrium) as shown.

system (viz. non-interacting components of different M_r and/or density) and a self-associating system which contains the same distribution of molecular weight. It is therefore possible to apply the equations of e.g., a (first order) non-ideal isodesmic association to calculate a constant (an ´effective´ association constant) which, when applied to a static system, will define a distribution of molecular weight for the polydisperse case. Again, this method has been succesfully applied to glycoconjugates (pig gastric & chronic bronchitic mucins) (20) and we are currently exploring its potential application to polysaccharides.

4. A combined approach with CGC.

This is a much simpler method to implement, but is nonetheless of considerable use in visualising a distribution. Gel chromatographic methods provide a very easy to use way for giving an estimate of the extent of size variation in a sample, once a suitable column pore size has been found. We have used it uncalibrated to compare the change in size distribution of tomato fruit polyuronides on ripening (12): the change agrees well with the change in M_r measured from sedimentation equilibrium, used here independently.

If calibrated gel chromatography is to be used, the particular column being used requires calibration using standards of similar conformation to the unknown M_r sample. We have used an approach (Ball, Harding & Mitchell, unpublished) using fractions of the same polysaccharide as standards: we isolate 5-6 narrow fractions from the eluant, measure the M_r of each fraction using short column sedimentation equilibrium, work out the calibration constants for the gel from a V_e versus log M_r plot (within the range of the gel) , and thus able to convert the elution profile into a molecular weight distribution. An example of such a distribution obtained in this way is given in Fig 2 for a high mannuronate sodium alginate (manucol DM): the distribution agrees well with the M_w^o for the whole distribution, measured using sedimentation equilibrium alone.

ACKNOWLEDGEMENTS

I would like to thank Dr. John Mitchell for many learned discussions and Miss A. Ball for expert assistance with the experimental work.

REFERENCES

1. Glicksman, M. (ed.) (1982) Food Hydrocolloids, Vol. I, CRC Press, Florida.
2. Cartlidge, S.A., Duncan, R., Lloyd, J.B., Kopeckova-Rejmanova, P. & Kopecek, J. (1987) J. Controlled Rel. 4, 253
3. Gabriel, A. (1979) in Microbial polysaccharides and polysaccharases (Berkeley, R.C.W., Gooday, G.W. and

Ellwood D.C. eds.), Chap. 8, Academic Press, London
4. Launay, B., Cuvelier, G. & Martinez-Reyes, S. (1984) in Gums & stabilisers in the food industry II, (Phillips, G.O. ed.), p79, Pergamon Press, Oxford.
5. Lecacheux, D., Mustiere, Y., Panaras, R. (1986) Carbohyd. Polym. 6, 477
6. Robinson, G., Ross-Murphy, S.B. & Morris, E.R. (1982) Carbohyd. Res. 107, 17
7. Harding, S.E. (1986) Biotechnology & Applied Biochemistry 8, 489
8. Harper, G.S., Comper, W.D. & Preston, B.N. (1985) Biopolymers 24, 2172
9. Ranby, B. (ed.) (1987) Physical Chemistry of Colloids & Macromolecules: the Svedberg symposium, IUPAC, Oxford
10. Hinnie, J. & Serafini-Fracassini, A. (1986) Biopolymers 25, 1095
11. Creeth, J.M. & Harding, S.E. (1982) J. Biochem. Biophys. Meth. 8, 25-34
12. Seymour, G.B. & Harding, S.E. (1987) Biochem. J. 245, 463
13. Gaisford, S.E., Harding, S.E., Mitchell, J.R. & Bradley, T.D. (1986) Carbohyd. Polym. 6, 423
14. Roark, D. & Yphantis, D.A. (1969) Ann. N.Y. Acd. Sci. 164, 245
15. Teller, D.C. (1973) Meth. Enz. 27, 346
16. Harding, S.E. & Rowe, A.J. (1987) Optics & Lasers in Eng. (in press)
17. Harding, S.E. & Rowe, A.J. (1987) Biochem. Soc. Trans. 15
18. Creeth, J.M. & Pain, R.H. (1967) Prog. Biophys. Mol. Biol. 17, 217
19. Harding, S.E. (1985) Biophys. J., 47, 247
20. Creeth, J.M. & Cooper, B. (1984) Biochem. Soc. Trans. 12, 618