Combined low speed sedimentation equilibrium/gel permeation chromatography approach to molecular weight distribution analysis: application to a sodium alginate

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A new approach to molecular weight distribution analysis of commercial polysaccharide systems is described. The elution profile of a macromolecular solution from gel permeation chromatography is calibrated by extraction of a small number of narrow fractions from the eluate whose molecular weights are determined by short-column low speed sedimentation equilibrium in the analytical ultracentrifuge. The method represents a straightforward alternative to direct attempts at modelling distributions using analytical ultracentrifugation and is illustrated by application to a high mannuronate sodium alginate solution ($\bar{M}_r \sim 130\,000$), both native and heat treated forms. The form of the distribution agrees well with the whole solute weight average molecular weight determined separately by low speed sedimentation equilibrium. The method is not applicable to self-associative systems.

Keywords: Polysaccharides; sedimentation; gel permeation chromatography; alginate structure

Introduction

Although the determination of 'average' molecular weights or 'moments' of macromolecular systems is fairly well established (for example using sedimentation equilibrium, light scattering, osmotic pressure) the determination of molecular weight *distributions* is not normally as straightforward. This is in spite of the fact that knowledge of molecular weight distributions is important in a variety of commercial applications. For example, the thickening and gelling behaviour of polysaccharides used in the food and pharmaceutical industries will be related to such distributions[1]. It is, however, difficult to determine reliably molecular weight distributions (MWDs) for such commercial polysaccharides, which are generally highly polydisperse.

Methods based on techniques which have an inherent fractionation property with respect to molecular size have an obvious advantage, and both analytical ultracentrifugation and gel chromatography possess this property: the former based on differential separation under the influence of a centrifugal field, the latter based on size exclusion.

Whereas the determination of size or density distributions based on sedimentation *velocity* techniques depend on assumptions concerning particle shape, the technique of low speed sedimentation equilibrium (LSSE) provides an accurate measure of weight average molecular weight data independent of any assumptions concerning shape or solvation. The method is particularly well suited to the study of difficult heterogeneous macromolecular systems, unlike light scattering procedures which generally suffer from problems of sample clarification.

In the case of polysaccharides the presence of incompletely dispersed high molecular weight material can prove a major problem when light scattering methods are used (Zimm, low-angle or quasielastic measurements). For example Smidsrod and Haug[2] had to subject their alginate solutions to an ultracentrifugation clarification procedure to obtain satisfactory Zimm plots and much higher weight average molecular weights for galactomannans have been found using Zimm plots[3] compared with analytical ultracentrifuge techniques, both sedimentation-diffusion[4] and low speed sedimentation equilibrium[5]. Similar difficulties have been encountered in determining pectin molecular weights by light scattering[6].

An increasingly popular method is to use quasielastic light scattering (QLS) for both diffusion coefficient measurements and direct size-distribution analysis. However, besides being subject to the usual difficulties of sample clarification such distributions are only 'apparent' distributions (namely measured at a finite sample concentration). Further, for asymmetric scatterers because of the problems of finite contributions to the

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observed autocorrelation plots from rotational diffusional phenomena, extrapolation to zero angle is normally necessary*—precisely where the effects of contaminants are at their greatest. Such difficulties in the application of QLS have been illustrated for proteoglycans by Harper *et al.*[7]. A significant recent development is to use low angle light scattering on samples that have been passed through a gel chromatography column[8,9]. Although this removes large aggregates from the parent solution the technique is not without difficulty: the assumption that there is no reversible aggregation phenomenon has to be made and that scattering vessels are completely free of contaminant, since the low angle method is sensitive to even trace amounts of supramolecular material. Other problems have been cited[8,9], including that of thermodynamic non-ideality which is a difficult parameter to control with this procedure, largely because of the variable concentrations of material coming off the column.

Low speed sedimentation equilibrium (LSSE) is not normally subject to these difficulties, and in addition to providing basic molecular weight average information can also give molecular weight *distribution* information from both simple ratios of different averages or more complex modelling of the fringe and point average molecular weight data[10], although this generally requires automated data capture techniques and dedicated computer hardware.

Gel permeation chromatography on the other hand can provide a *direct* way of visualizing the distribution of molecular sizes, provided that adequate standards are available to calibrate the gel columns being used. These standards need to be of both similar (and known) molecular weight and conformation to the species whose molecular weight or MWD is being sought[11]. These conditions are generally satisfied for globular proteins, but problems arise with other classes of macromolecule, polysaccharides in particular. For polysaccharides, attempts have been made in the past to use dextrans (and in some instances pullulans) as general polysaccharide standards, but this procedure has led to results that are widely in error—largely because of the variable conformation of polysaccharides—and is now widely accepted as inaccurate. Considerable attention has therefore been paid to determining the key parameter affecting separation. Although this has been an area of considerable debate, the strongest evidence appears to have been given (see e.g. Ref. 12) in favour of using the 'hydrodynamic volume' (taken as $\sim[\eta]\cdot M_r$, where $[\eta]$ is the intrinsic viscosity and M_r is the molecular weight). A 'universal calibration curve' of $\log_{10}([\eta])\cdot M_r)$ *versus* elution volume, V_e, has been produced[12], presumed valid for a range of macromolecular systems.

We now described a *combined* approach to molecular weight distribution determination which avoids such assumptions and difficulties, using low speed sedimentation equilibrium (LSSE) and gel permeation chromatography (g.p.c.). The method does not require the use of dextrans nor the need for resort to a light scattering method. The procedure is related to two other procedures given before involving analytical ultracentrifugation and g.p.c. (i) for acid mucopolysaccharides[13], and (ii) for chondroitin sulphate[14]. In (i), molecular weights of individual fractions were determined from the Svedberg equation by combining measurements of sedimentation coefficient and diffusion coefficient, both after extrapolation to zero concentration. In (ii), both the Svedberg equation method and 'conventional' sedimentation equilibrium at concentrations $\sim$1–3 mg/ml[15] extrapolated to zero concentration were used. The present method uses low speed sedimentation equilibrium at very low concentrations ($\sim$0.2 mg/ml) where an extrapolation from multiple measurements is not normally necessary.

Method

The basic principle of the method is quite straightforward, and is to 'self-calibrate' the g.p.c. column using the macromolecule itself as the calibration standard. We do this in the following way:

(1) Obtain the elution profile of the macromolecule in solution from the gel column (packed with a polymer of desired exclusion properties) in the standard way (see e.g. Ref. 16). These elution profiles can be recorded in terms of optical density if the macromolecule has a chromophore or in terms of optical density after a chemical assay: for example, in the case of polysaccharides, we employ the phenol/sulphuric acid assay for total sugar content[17]. Of increasing popularity for materials non-absorbing in the visible or (usable u.v.) is the use of refractive index detectors, appropriately thermostatically controlled[18].

(2) Isolate a number of fractions of narrow bandwith from the eluate containing the macromolecule. After concentrating the solute where necessary the weight average molecular weights of these fractions are determined using short column sedimentation equilibrium at low solute loading concentration c^0 ($\sim$0.1–0.2 mg/ml). Multiple determinations can be made from single equilibrium runs using cells with wedge windows or multichannel cells of the Yphantis type[19].

(3) Determine the 'Andrews plot'[16] ($\log_{10} M_r$ *versus* V_e) for the system and use this information to convert the elution profile into a molecular weight distribution.

We now illustrate the method by its application to the determination of the molecular weight distribution of an industrially important polysaccharide (the alginate Manucol DM) and a heat treated sample of the same.

Experimental

Materials

A commercially available sodium alginate (Manucol DM/Kelco A.I.L.) from *Ascophyllum nodosum* sp. (a gift of Kelco A.I.L.) was used. The solvent used for both gel chromatography and low speed sedimentation equilibrium was a standard phosphate chloride buffer, pH 6.5 containing Na_2HPO_4 and KH_2PO_4 made up to an ionic strength of 0.30 by adding the relevant proportion of NaCl in accordance with Green[20]. For the experiment on heat treated Manucol DM, the sample had been heated for 30 min at 140°C.

Gel permeation chromatography

The polysaccharides were made up to a volume of 5 ml and applied to a column of Sephacryl S-400 (1.6 × 75 cm). The loading concentrations were approximately 0.5–0.8 mg ml^{-1} (not corrected for moisture content) and were run in the phosphate-chloride solvent described

* For larger (Mie) particles the situation is more complex (see e.g. Ref. 38)

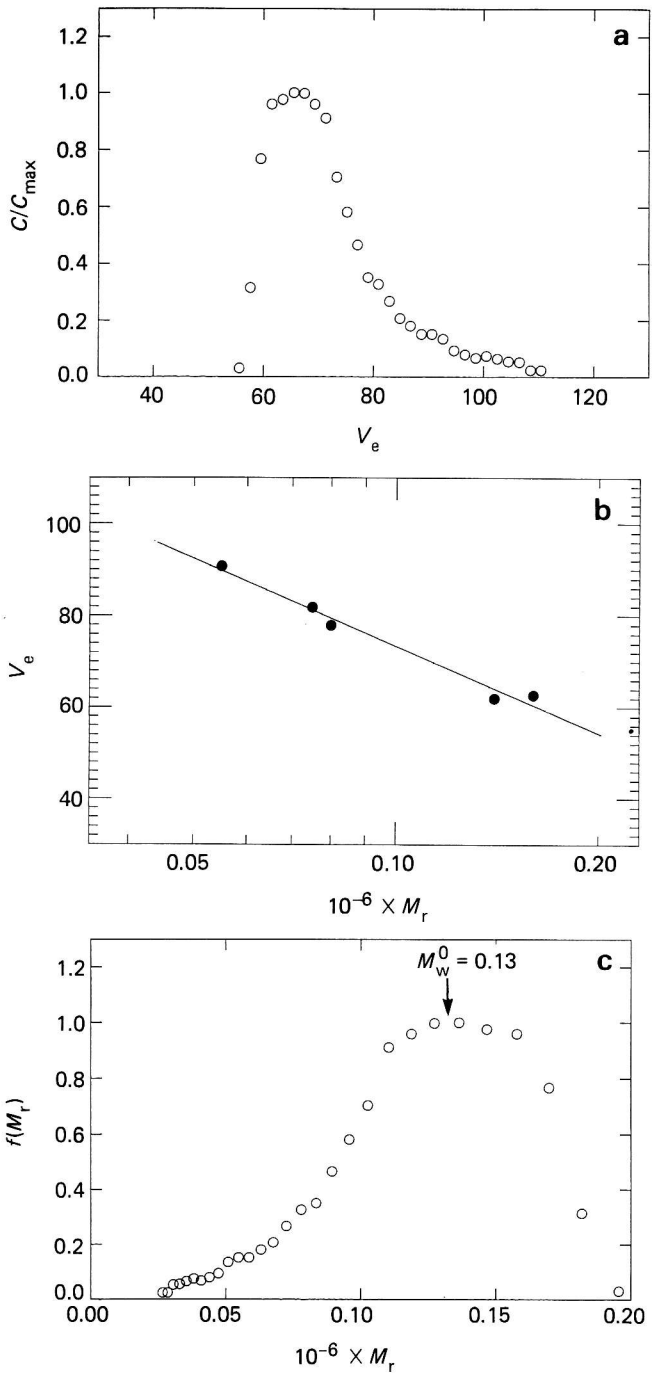

Figure 1 Calibrated gel chromatography for the alginate Manucol DM. (a) Elution profile from a Sephacryl S-400 column ($V_0 = 60$ ml; $V_t = 142$ ml). Loading concentration: ~ 0.8 mg·ml^{-1}, phosphate buffer, $I = 0.3$, pH 6.5, flow rate $\sim$ 10 ml·h^{-1}. (a) Calibration plot, using low speed sedimentation equilibrium on isolated fractions of narrow ($\Delta V_e \sim 2$ ml) bandwith of Manucol DM. Points fitted to the line described by $V_e = a - K_c \log_{10} M_r$ with $a = 389$ ml and $K_c = 63.3$ ml. (c) Corresponding molecular weight distribution. The value indicated for M_w^0 corresponds to the weight average molecular weight of the whole solute distribution determined from a sedimentation equilibrium experiment on unfractionated material

above at a flow rate of ~ 10 ml·h^{-1}. Fractions of ~ 2 ml were assayed for total sugar content using a phenol–sulphuric acid procedure similar to that described by Dubois *et al.*[17]. Elution volumes were determined by weight (calculated from the loading of the last of the sample), the void volume V_0 was determined using blue dextran 2000 and the total volume, V_t using sucrose. Column recoveries were normally between 90 and 100%.

Low speed sedimentation equilibrium

A Beckman Model E analytical ultracentrifuge was used employing Rayleigh interference optics and an RTIC temperature measurement system. The low or 'intermediate' speed method was employed[21]; in this method the speed is sufficiently low to allow adequate resolution of the fringes near the cell base. We consider the 'meniscus depletion' or high speed method unsuitable for these materials, because of (1) the normal impossibility of depleting the meniscus without losing optical registration of the fringes at the cell base, and (2) the possible problem of 'speed dependence' effects on the apparent molecular weight at higher speeds, reported for polydisperse polymeric systems[22,23]. Instead the 'low' speed method was used where, at equilibrium, the concentration at the air/solution meniscus remains finite; this was obtained by mathematical manipulation of the fringe data[21]. Determinations were made in 12 or 30 mm optical path length cells at the lowest possible loading concentration (~ 0.1–0.5 mg·ml^{-1}) to minimize possible effects of thermodynamic non-ideality and/or associative phenomena. Molecular weight determinations on the fractions were normally performed in triplicate using an appropriate combination of 12 mm pathlength cells with wedge windows. Unfractionated material was analysed separately using 30 mm optical pathlength cells. All samples prior to sedimentation equilibrium had been made up in the solvent as described above and dialysed against this solvent (in accordance with Cassassa and Eisenberg[24]). The value used for the partial specific volume was 0.44 ml·g^{-1} (Ref. 25; see also Ref. 26).

Whole cell weight average molecular weights, M_w^0 were extracted by using the limiting value at the cell base of a particularly directly determinable point average (the 'star' average, M^* [21]); an independent estimate for the initial solute concentration was not required.

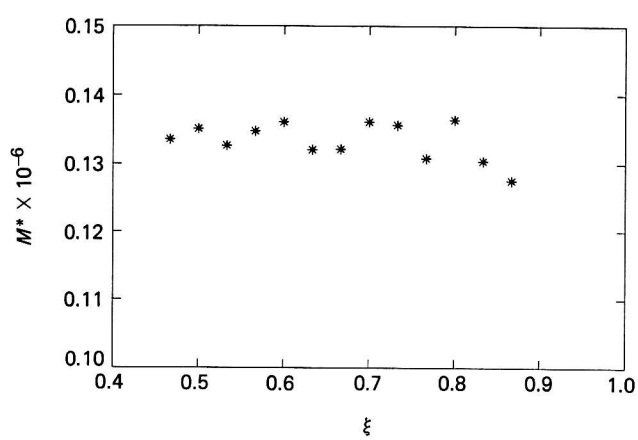

Figure 2 Plot of the star average molecular weight, M^*, *versus* the normalized radial displacement squared parameter ξ for unfractionated Manucol DM. $\xi = (r^2 - a^2)/(b^2 - a^2)$, r being the radial displacement of a given point in the cell from the centre of the rotor and a, b, corresponding radial positions of meniscus and base respectively. Rotor speed = 9339 rev/min^{-1}, Temp = 25.0°C, Initial loading concentration, $c^0 \sim 0.5$ mg·ml^{-1}; solvent conditions as *Figure 1*

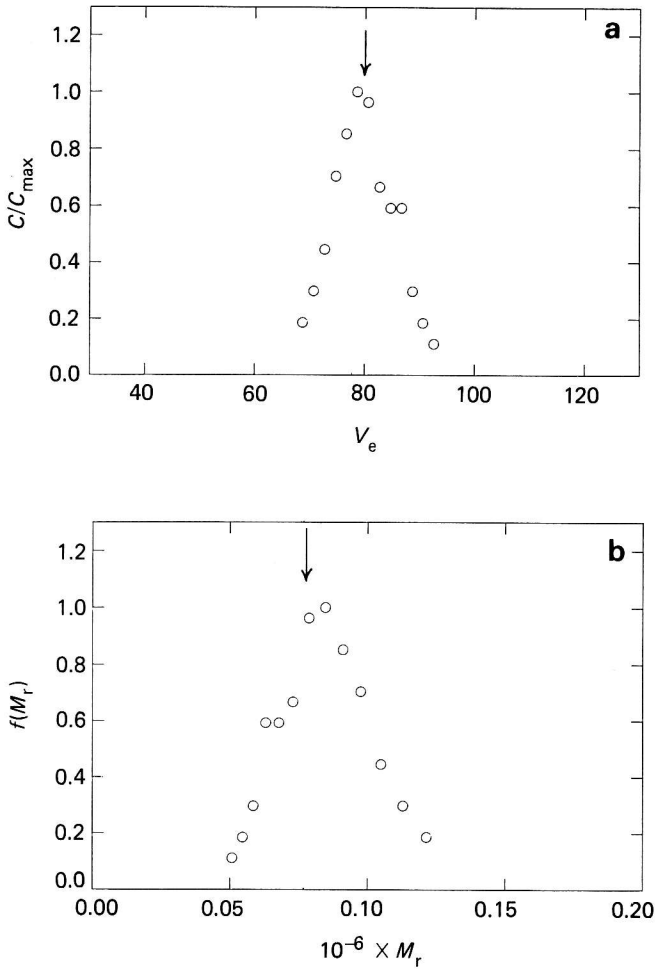

Figure 3 Rechromatographing of an isolated narrow fraction of Manucol DM. V_e, from original eluate 77–83 ml (fractions 40–42). (a) Elution profile. (b) Corresponding molecular weight distribution (not corrected for diffusional broadening due to rechromatographing) from *Figure 1b*. Values indicated correspond to the mean V_e and corresponding M_r value (80 ml and 76 000 respectively) prior to rechromatographing

Sedimentation velocity

Sedimentation velocity was performed using an MSE Centriscan 75 ultracentrifuge: the scanning schlieren optical system was used. Measurements were performed at 20.0°C, at a rotor speed of 49 000rev/min. The apparent sedimentation coefficient at a given concentration was evaluated using a computer digitizing tablet, and then corrected to standard conditions. For the extrapolation to zero concentration, concentrations were corrected for both moisture content and radial dilution effects.

Results

The plots of elution profile, calibration and molecular weight distribution for Manucol DM are given in *Figure 1* (*a*, *b*, *c* respectively). The polysaccharide was seen to elute clear of the void volume (*Figure 1a*). Five sets of fractions of narrow bandwith ($\Delta V_e \sim 2$ ml) were isolated from a series of runs, for a series of elution volumes. The set for each volume was combined and concentrated using Sartorius centrisart tubes to a concentration of 0.1–0.5 mg ml^{-1}. The weight average molecular weights for each of these five fractions were evaluated as described above, using short column (~ 2 mm) low speed sedimentation equilibrium.

A linear 'Andrews type'[16] plot of $\log_{10} M_r$ *versus* V_e was obtained, of form similar to that observed for globular proteins[16] within the range of molecular weights examined, although this of course does not extend over the full fractionation range of the gel.

Figure 1c gives the molecular weight distribution corresponding to this calibration. We have also determined the weight average molecular weight of the unfractionated sample by extrapolation of the M^* average to the cell base (*Figure 2*). The value obtained ($130\,000 \pm 5000$) is in good agreement with the expected mean whole solute average value from the MWD of *Figure 1c*. It should be emphasized that diffusion broadening should not normally be a problem with this procedure since the calibration is performed after the chromatography run.

Furthermore, rechromatographing of individual narrow fractions (of slightly wider bandwidth, $\Delta V_e \sim 6$ ml, than used for the calibration plot) isolated from the eluate showed that a true separation had occurred (*Figure 3*) and that redistribution of solute upon fractionation was not significant.

Figure 4 shows an interesting application of the calibration to detect shifts in the MWD on heat treatment of the alginate; such observations help to identify changes

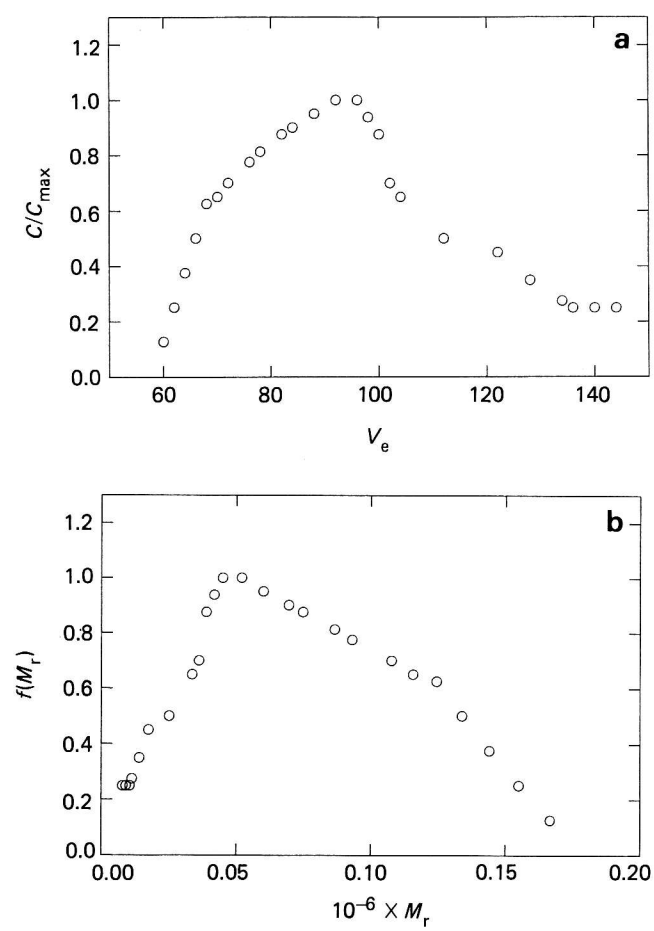

Figure 4 Elution profile (a) and corresponding molecular weight distribution (b) for heat treated Manucol DM. Other details as *Figure 1*

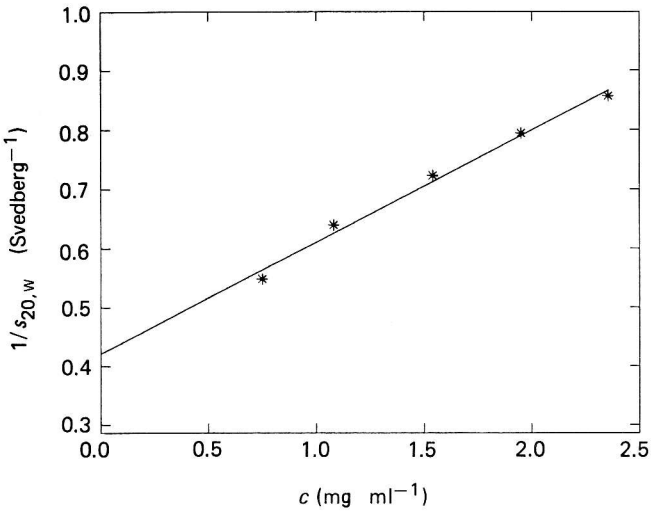

Figure 5 Plot of the reciprocal of the sedimentation coefficient (corrected to water at 20°C), s_c *versus* oncentration (corrected for radial dilution) for Manucol DM. (Anderson and Harding, unpublished). Data fitted to the equation $1/s_c = (1/s)\ (1 + k_s c)$, where s, the 'infinite dilution' value $= (2.37 \pm 0.10) \times 10^{-13}$ s and k_s (corrected to solution density (34)) $= (44.3 \pm 2.7)$ ml · g^{-1}

in the molecular size as a result of high temperature extrusion processes. A clear shift in the MWD is observed, although most of the molecular species are within the calibration range. More severe heat treatment shifts the MWD away from the calibration: this, and other aspects of the thermal degradation of alginates will be considered in a separate study (Oates *et al.* in preparation).

Discussion

The result we have obtained here for Manucol DM represents, in our opinion, the first reliable determination of the molecular weight distribution for an alginate type of polysaccharide. The value we obtain (130 000 ± 5000) for the weight average molecular weight for the unfractionated sample is somewhat lower than an earlier literature value of Wedlock *et al.*[26]. These workers obtained a value for the weight average molecular weight of 2.0×10^5 (no error estimate reported) using two different types of light scattering procedure: the Zimm plot method and the Svedberg equation, with the (z-average) diffusion coefficient determined by quasielastic light scattering. The difference from our result is in our opinion a result of classic difficulties associated with the application of light scattering to polydisperse materials (see, for example Refs 2, 6, 27), or alternatively could be due to sample variability.

We have checked for both (1) a speed dependence effect on our measured molecular weights, and (2) possible sample degradation during the time course of a sedimentation equilibrium run (2–3 days at 25°C). For the speed dependence (again in 30 mm cells, $c^0 \sim$ 0.5 mg/ml) we evaluated M_w^0 for speeds of 7923, 8745 and 10 583 rev/min. No change or trend in the measured values was observed with speed: the only observed effect was that at higher speeds the extrapolation of the M^* average to the cell base became more difficult. To check for degradation we determined the sedimentation coefficient at a single concentration and repeated the measurement 4 days later on the same material left at room temperature. No significant change was observed. The $s_{20,w}$ of the sample extrapolated to infinite dilution (*Figure 5*) was found to be $(2.37 \pm 0.18) \times 10^{-13}$ s. This corresponds to an $s_{25,w}$ of $2.67 \pm 0.20) \times 10^{-13}$ s, in reasonable agreement with Wedlock *et al.*[26].

The method described here provides a relatively easy to use supplement to other methods involving low speed sedimentation equilibrium procedures for representing polydisperse molecular weight distributions. These methods include for example (1) the use of the so-called 'Herdan' relations, involving ratios of the (whole solute) z-average to weight average, or weight average to number average molecular weights[28,29]; (2) direct modelling of the concentration *versus* radial displacement curves to equations describing a thermodynamically non-ideal polydisperse system[30], and (3) use of an 'effective' association constant, and use of the property of indistinguishability in a single sedimentation equilibrium experiment of the effects of polydispersity (non-interacting components of different molecular weight) and self-association even if thermodynamic non-ideality is present[31]. This latter procedure can of course be used to characterize a genuinely self-associating system. Johnson *et al.*[32] have recently considered in detail the problem of characterizing non-ideal self-associating systems, and Tindall and Aune[33] have considered the case of (thermodynamically ideal) polydisperse self-associating systems. Sedimentation velocity can also be used to give distribution information (see e.g. Refs 34, 35), but only after a number of assumptions, including those involving concentration dependence.

The presently defined approach provides a much simpler but nonetheless reliable alternative to the above procedures *provided* that self-association is absent. Obviously, if the observed heterogeneity in a macromolecular system is due to self-association, any attempts to obtain narrow fractions would be futile since the sample species would simply redistribute. Diagnostic procedures are available from sedimentation equilibrium however to assay for the presence of either self-association, polydispersity or both[36,37].

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References

1 Launay, B., Doublier, J. L. and Cuvelier, G. in 'Functional Properties of Food Macromolecules', (Eds J. R. Mitchell and D. A. Ledward), Elsevier Applied Science, London, 1986, p. 1
2 Smidsrod, O. and Haug, A. *Acta. Chem. Scand.* 1968, **22**, 797
3 Robinson, G., Ross-Murphy, S. B. and Morris, E. R. *Carbohydr. Res.* 1982, **107**, 17
4 Sharman, W. R., Richards, E. L. and Malcolm, G. N. *Biopolymers* 1978, **17**, 2817
5 Gaisford, S. E., Harding, S. E., Mitchell, J. R. and Bradley, T. D. *Carbohydr. Polym.* 1986, **6**, 423
6 Hourdet, D. and Muller, G. *Carbohydr. Polym.* 1987, **7**, 301
7 Harper, G. S., Comper, W. D. and Preston, B. N. *Biopolymers* 1985, **24**, 2172
8 Martin, M. *Chromatographia* 1982, **15**, 426
9 Lecacheux, D., Mustiere, Y., Panaras, R. and Brigand, G. *Carbohydr. Polym.* 1986, **6**, 477

10 Harding, S. E. in 'Gums and Stabilisers for the Food Industry IV', (Eds G. O. Phillips, D. J. Wedlock and P. Williams), IRL Press, Oxford, 1988, p. 15
11 Nilsson, G. and Nilsson, K. *J. Chromatogr.* 1974, **101**, 137
12 Potschka, M. *Analyt. Biochem.* 1966, **28**, 350
13 Constantopoulos, G., Dekaban, A. S. and Carroll, W. R. *Analyt. Biochem.* 1969, **31**, 59
14 Wasteson, A. *Biochim. Biophys. Acta* 1969, **177**, 154
15 Wasteson, A. *Biochem. J.* 1971, **122**, 477
16 Andrews, P. *Biochem. J.* 1965, **96**, 595
17 Dubois, M., Giles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. *Anal. Chem.* 1956, **28**, 350
18 Waters Instruments manual no. 70240, 1986
19 Yphantis, D. A. *Biochemistry* 1964, **3**, 297
20 Green, A. A. *J. Am. Chem. Soc.* 1933, **55**, 2331
21 Creeth, J. M. and Harding, S. E. *J. Biochem. Biophys. Meth.* 1982, **7**, 25
22 Fujita, H. 'Foundations of Ultracentrifugal Analysis', John Wiley, New York, 1975, Ch. 5
23 Suzuki, H. in 'Physical Chemistry of Colloids and Macromolecules', (Ed. B. Ranby), Blackwell Scientific, Oxford, 1984, p. 101
24 Cassasa, E. F. and Eisenberg, H. *J. Phys. Chem.* 1961, **65**, 427
25 Cook, W. H. and Smith, D. B. *Can. J. Biochem. Physiol.* 1954, **32**, 227
26 Wedlock, D. J., Fasihuddin, B. A. and Phillips, G. O. *Int. J. Biol. Macromol.* 1986, **8**, 57
27 Berth, G. *Carbohydr. Polym.* 1988, **8**, 105
28 Herdan, G. *Nature* 1949, **163**, 139
29 Creeth, J. M. and Pain, R. H. *Prog. Biophys. Mol. Biol.* 1967, **17**, 217
30 Harding, S. E. *Biophys. J.* 1985, **47**, 247
31 Creeth, J. M. and Cooper, B. *Biochem. Soc. Trans.* 1984, **12**, 618
32 Johnson, M. L., Correia, J. J., Yphantis, D. A. and Halvorson, H. R. *Biophys. J.* 1981, **36**, 575
33 Tindall, S. H. and Aune, K. C. *Anal. Biochem.* 1982, **120**, 71
34 Sajdera, S. W. and Hascall, V. C. *J. Biol. Chem.* 1969, **244**, 77
35 Pain, R. H. *Symp. Soc. Exp. Biol.* 1980, **34**, 359
36 Roark, D. and Yphantis, D. A. *Ann. N.Y. Acad. Sci.* 1969, **164**, 245
37 Teller, D. C. *Methods Enzymol.* 1973, **27**, 346
38 Chen, S. H., Holz, M. and Tartaglia *Appl. Opt.* 1977, **18**, 187