Molecular Weight Distribution Evaluation of Polysaccharides and Glycoconjugates Using Analytical Ultracentrifugation

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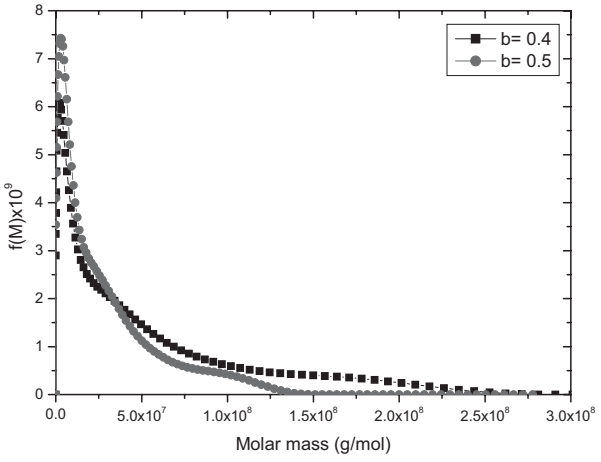
We review here the advances that have been made in methodology using the analytical ultracentrifuge for characterising polydisperse polymer systems with a quasi-continuous distribution of molecular weight. These advances include improved ways of defining the weight and z-average molecular weights of a distribution from sedimentation equilibrium experiments and hence the ratio M_z/M_w, off-line calibration of size exclusion chromatography by sedimentation equilibrium and conversion of a sedimentation coefficient distribution into a molecular weight distribution. Although these methods are applicable to other polymeric systems, we focus on polysaccharides, mucins and other glycoconjugates, systems which particularly benefit from the availability of recently available long optical path length cells for the minimisation of complications through thermodynamic non-ideality.



Introduction

Since the publication of the Svedberg 100th anniversary symposium held in 1984[1] there have been a number of significant advances in the methodology for characterising the molecular weight of polymeric materials which encompass quasi-continuous distributions of molecular weight using analytical ultracentrifugation. In biological systems, these include polysaccharides, mucins and other polydisperse glycopolymers using analytical ultracentrifugation. These advances have been complementary to advances in other techniques based on hydrodynamics, namely size-exclusion chromatography (SEC) coupled to multi-angle laser light scattering or so called ''SEC-MALS''.

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The advances include: 1) Improved ways of defining the weight and z-average ''whole distribution'' molecular weights and the ratio M_z/M_w, and for log-normal distributions M_w/M_n using sedmentation equilibrium in the analytical ultracentrifuge; 2) Off line calibration of size exclusion chromatography by sedimentation equilibrium; 3) Conversion of a sedimentation coefficient distribution into a molecular weight distribution using power law or ''scaling'' information (originally implemented for mucins). We consider here these advances and illustrate them with application to selected polysaccharides and glycoconjugates.

Weight and z-Average Molecular Weight Determination and the Herdan Relations

The principal average molecular weight obtainable from a sedimentation equilibrium experiment on a heterogeneous

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solution of polymers is the weight average molecular weight M_w.[2] Figure 1 illustrates the fundamental problem we have when attempting to analyse systems that have a greater than narrow distribution of molecular weights, namely the difficulty of choosing an appropriate speed giving an adequate concentration increment from meniscus to base without losing optical registration near the cell base. This is due to solute redistribution: neglect of the information near the base of the cell can lead to significant underestimates of the average molecular weight of what was in the original solution, whether it be a weight or some other average. Estimates for the absolute concentration $c(r)$ ($g \cdot ml^{-1}$) or in equivalent fringe displacement units $J(r)$ can also be difficult, particularly from Rayleigh interference optics which register only a record of concentration relative to other radial positions. For convenience, the reference radial position is normally taken as the meniscus, $r = a$. A procedure for estimating the meniscus concentration J_a is normally necessary, to define $J(r)$

$$J(r) = j(r) + J_a \tag{1}$$

where $j(r)$ is the fringe number concentration relative to the meniscus. The popular practice of floating this parameter along with M_w in the analyses of well defined proteins can be particularly unreliable for heterogeneous systems.[4] Furthermore, the high speed meniscus depletion method of Yphantis is clearly inapplicable,[5] although the long column variant of that method described by Chervenka may be useful for systems with a limited degree of polydispersity.[6] A whole suite of procedures have however been described by Creeth and Pain[2] and also Teller[7] who have given a comprehensive comparison of methods optical records, and more recently Hall et al.[4] We have found the ''intercept over slope'' method of Creeth

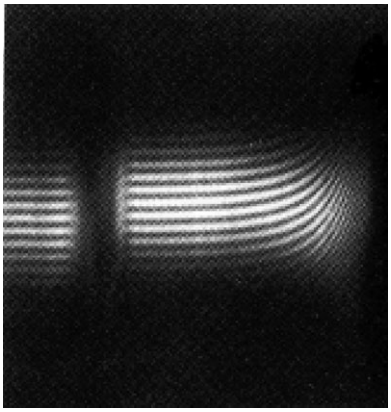

Figure 1. Steeply rising Rayleigh interference fringes at the cell base, but finite slope at the meniscus, as shown here for a low speed sedimentation equilibrium experiment on a bronchial mucus glycoprotein (BM-GRE, $M_w \sim 6.0 \times 10^6$) from a patient suffering from chronic bronchitis.[3]

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and Harding[8] to be useful in this regard; this yields estimates of J_a to $\sim +/- 20\%$ which does not translate to significant uncertainty in the estimates for M_w providing that there is a fringe increment of at least ~ 3 between the meniscus and base. For lower values it is recommended that this method is supplemented by the synthetic boundary method, as described by Hall et al.[4]

Conventional estimates of the weight average molecular weight over the whole distribution of solute in the original solution prior to ultracentrifugation also require an estimate of the concentration at the cell base, for example the widely used equation:

$$M^0_{w,app} = \frac{1}{k}\left\{\frac{c_b - c_a}{c_0(b^2 - a^2)}\right\} \tag{2}$$

where $k = (1 - \overline{v}\rho_0)\omega^2/2RT$ (these symbols having their usual meaning), c_a and c_b the concentrations at the cell meniscus and base respectively, or the related equation (in terms of the corresponding fringe number concentrations J_o, J_a and J_b respectively)

$$M^0_{w,app} = \frac{1}{k}\left\{\frac{J_b - J_a}{J_0(b^2 - a^2)}\right\} \equiv \frac{1}{k}\left\{\frac{j_b}{J_0(b^2 - a^2)}\right\} \tag{3}$$

where j_b is the fringe concentration (relative to the meniscus) at the cell base. These evaluations require an

estimate for the initial cell loading concentration, J_o (or c_o), although for short columns, <1 mm, this is approximately equal to $c(r)$ or $J(r)$ at the "hinge point" of the distribution, i.e., at a radial position $r_h \sim [(a^2+b^2)/2]^{1/2}$ for short columns. A much more serious problem is estimating j_b (or c_b) because of the form of the extrapolation of $j(r)$ to the cell base for situations, as in Figure 1.∼

For polydisperse systems, we found an operational point average molecular weight, the "star average" M^* particularly useful. This was introduced by J. M. Creeth in 1980[9] and its properties examined in detail by Creeth and Harding in 1982.[8] It has also been considered in some detail by Harding and coworkers[10,11] and Cölfen and Harding.[12]

The M^* function at a radial position (r) is defined by:

$$M^*(r) = \frac{j(r)}{\left\{kJ(a)(r^2-a^2) + 2k\int_a^r r.j(r)dr\right\}} \quad (4)$$

and one of its interesting properties is that the limiting value of $M^*(r)$ at the cell base $= M_{w,app}$, the apparent weight average molecular weight over the distribution of solute in the cell. Generally it provides a much more reliable extrapolation to the cell base than the corresponding extrapolation of $j(r)$ to the cell base to yield j_b (Equation (2)), and Creeth and Harding[8] gave a comprehensive comparison of the two forms of the extrapolation. Furthermore, an independent estimate of the initial loading concentration J_o is not required. The method was incorporated into the MSTAR program, originally in FORTRAN for use on mainframe computers,[8] and later upgraded for PCs by Cölfen and Harding,[12] and more recently a version for Microcal ORIGIN has been made available by K. Schilling of Nanolytics Ltd.[13] The algorithm incorporates the possibility of assessing J_a via the intercept/slope procedure above, and also the option of using the conservation of mass principle as a guide if J_o is known (from, for example, synthetic boundary measurements).

The M^* function has two other useful properties, namely: i) $M^*(a) = M_{w,app}(a)$ the apparent point weight average at the meniscus; ii) $M^*(J=0) = M_{n,app}(a)$, the apparent number average at the meniscus.

Point Weight Average Molecular Weights $M_w(r)$: Non-interacting Polydispersity Versus Self-association

These still provide a useful guide, for example to assess whether a heterogeneous system is due to a non-interacting mixture of materials or the presence of reversible association phenomena (using the criterion of overlap of datasets of $M_w(r)$ vs. $c(r)$ for different loading concentrations). This was used, for example, to prove that preparations of mucin glycoproteins were not, as previously suspected, involved in isodesmic or indefinite self-association reactions under the conditions studied.[14,15]

The differential form of the fundamental equation for sedimentation equilibrium is used:[2]

$$\frac{d\ln J(r)}{dr^2} = kM_{w,app}(r) \quad (5)$$

where $M_{w,app}(r)$ is the (apparent) point weight average molecular weight. The Microcal ORIGIN MSTAR algorithm[13] also has this facility using sliding strip procedures. It is not suitable for short columns (<1 mm) since if the strip width is too small the data becomes too noisy.

Measurement of z-Average Molecular Weights

Unfortunately, the lack of availability of the Schlieren optical system in modern instrumentation compared to the Model E and MOM centrifuges is a great pity: the direct record of concentration gradient the Schlieren system provides allows for the determination of M_z, the z-average, as the principal parameter.[16] Nonetheless, both point and whole cell z-average molecular weights can be found from Rayleigh interference data, although to a lower precision. The point z-average molecular weights can be obtained from

$$M_{z,app}(r) = M_{w,app}(r) + \frac{1}{k}\left\{\frac{d\ln M_{w,app}(r)}{d(r^2)}\right\} \quad (6a)$$

$$\equiv \frac{1}{k}\frac{d}{d(r^2)}\left(\ln\left\{\frac{1}{r}\frac{dj(r)}{dr}\right\}\right) \quad (6b)$$

(see Equations 2.12 and 2.42 in ref. [2]) and is available as an option in the MSTAR programme. The equivalent of a double differentiation of the data is involved, so the data is less precise than for weight average molecular weights; despite this, a value for the meniscus concentration J_a is not required. Regression analysis across the whole distribution (from $r=a$ to $r=b$) provides an estimate of $M_{z,app}$ (the equivalent of Equation 2.50 in ref. [2]) and has been incorporated into a new algorithm MFIT.[17]

To obtain reasonable estimates for either point number average molecular weight or the whole cell number average molecular weight is very difficult, as an estimate of the number average molecular weight at the meniscus $M_{n,app}(a)$ is necessary, although the identity $M^*(J\rightarrow 0) = M_{n,app}(a)$ may prove useful.

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Non-ideality, and an Important Limitation of the XL-I Now Overcome

The apparent weight average molecular weight $M_{w,app}$ is related to the ideal molecular weight by

$$\frac{1}{M_{w,app}} = \frac{1}{M_w}(1 + 2BM_w c) \quad (7)$$

and for z-average molecular weights

$$\frac{1}{M_{z,app}} = \frac{1}{M_z}(1 + 4BM_z c) \quad (8)$$

assuming there are no speed dependence effects on B at the slow speeds employed and a 2nd virial coefficient is adequate to describe the non-ideality. If higher speeds are employed, a speed corrected effective B should be used in these equations (see for example, ref. [11] or ref. [18]).

For polysaccharides and glycoconjugates, theromodynamic non-ideality is a much more serious problem than for proteins, and until recently this has been magnified because of a significant limitation with the modern XL-I ultracentrifuge compared to the classical Model E, namely the longest path length cell available was only 12 mm, 2.5 times smaller than for the 30 mm cell available for the Model E. This has meant that, until recently, the lowest possible concentration for the XL-I for equilibrium experiments was only 0.5 mg · ml^{-1}, compared with 0.2 mg · ml^{-1} for the Model E. The situation has been partially resolved with the launch of 20 mm path length cells by Nanolytics Limited, (Potsdam, FRG) allowing us to work at a minimum of ~0.3 − 0.4 mg · ml^{-1}. The reason why this is important is in Table 1, which compares the magnitude of the expression $1 + 2BMc$ (which represents the factor by which the apparent weight average molecular weight underestimates the true molecular weight) for a range of polysaccharides and a mucin. For example for the bronchial mucin CFPHI, at 0.3 mg · ml^{-1}, the underestimate is ~18%, whereas at 0.5 mg · ml^{-1} it is 30%. For the sodium alginate at 0.3 mg · ml^{-1} it is 61% and at 0.5 mg · ml^{-1} it is as high as 100%. For some preparations of the latter, it has been found that a 3rd virial coefficient was required to account for the concentration dependence behaviour.[19]

M_z/M_w and the Herdan Relations

Assuming that non-ideality effects have been adequately catered for, then the measured M_z and M_w can be used to define the polydispersity of a distribution. Furthermore, if the distribution of molecular weight is assumed to be of a log-normal type then, as shown by Fujita (page 297 of ref. [20]) $M_z/M_w = M_w/M_n$. It is therefore possible to estimate also M_n for a log-normal distribution if M_z and M_w are known. Furthermore, these ratios can be related to the width of a distribution σ_w or σ_n (whatever form this may

Table 1. Comparative non-ideality of polysaccharides for loading concentrations of 0.3 and 0.5 mg · ml^{-1} (adapted from ref. [11] and references therein).

Polysaccharide	$10^{-6} \times M$	$10^4 \times B$	BM	$1 + 2BMc$[a)]	$1 + 2BMc$[b)]
	g·mol^{-1}	ml·mol·g^{-2}	ml·g^{-1}		
Pullulan P5	0.0053	10.3	5.5	1.003	1.005
Pullulan P50	0.047	5.5	25.9	1.015	1.025
Xanthan (fraction)	0.36	2.4	86	1.053	1.088
β-glucan	0.17	6.1	104	1.063	1.105
Dextran T500	0.42	3.4	143	1.086	1.142
Pullulan P800	0.76	2.3	175	1.105	1.175
Chitosan (Protan 203)	0.44	5.1	224	1.135	1.225
Pullulan P1200	1.24	2.2	273	1.164	1.273
Mucin glyco-protein (CFPHI[c)])	2.0	1.5	300	1.180	1.300
Pectin (citrus fraction)	0.045	50.0	450	1.270	1.450
Scleroglucan	5.7	0.50	570	1.342	1.570
Alginate	0.35	29.0	1015	1.609	2.015

[a)]Based on the lowest possible concentration in a 20 mm centrepiece (~0.3 mg·ml^{-1}); [b)]Based on the lowest possible concentration in a 12 mm centrepiece (~0.5 mg·ml^{-1}); [c)]From the bronchial secretion of a patient with Cystic Fibrosis.

take) using the Herdan relations:[21,2]

$$\frac{\sigma_w}{M_w} = \left[\left(\frac{M_z}{M_w}\right) - 1\right]^{1/2}; \quad \frac{\sigma_n}{M_n} = \left[\left(\frac{M_w}{M_n}\right) - 1\right]^{1/2} \quad (9)$$

Off-line Calibration of Size Exclusion Chromatography by Sedimentation Equilibrium

Perhaps the simplest procedure for defining a molecular weight distribution (although this loses the special feature of analytical ultracentrifugation as a method free of columns or membranes) is to use sedimentation equilibrium in conjunction with preparative size exclusion chromatography (SEC). Fractions of relatively narrow (elution volume) band width are isolated from the eluate and their M_w values evaluated by low speed sedimentation equilibrium in the usual way. The SEC columns can thereby be "self-calibrated" and elution volume values converted into corresponding molecular weights. A distribution can therefore be defined in a way which avoids the problem of using inappropriate standards (see Figure 2). This method was described in 1988 and applied to alginate,[23] and subsequently dextrans and pectins;[24,25] with the latter, excellent agreement with a comparative off-line SEC-light scattering procedure was observed. The ability to run several 20 mm path length cells simultaneously now renders this method as an attractive alternative to procedures using SEC-MALS.

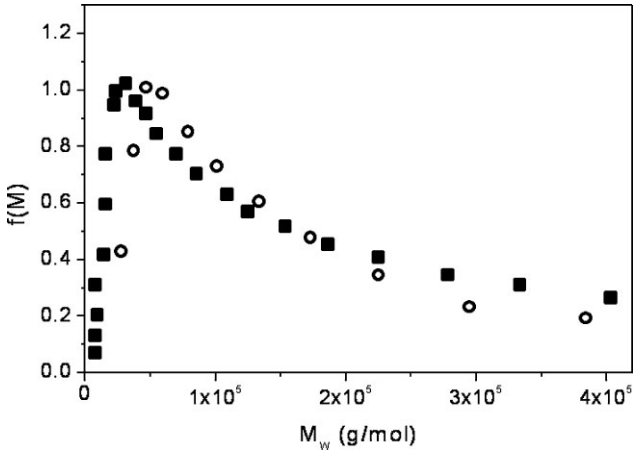

Figure 2. Distribution of molecular weight for a citrus pectin using size exclusion chromatography calibrated off-line by low speed sedimentation equilibrium (open circles) and light scattering (filled squares). Reproduced, with permission, from ref. [24].

f(*M*) vs. *M* Distribution from Sedimentation Velocity and a Power Law Conversion

In 1962, Fujita (pages 182–192 of ref. [20]) and earlier Cantow[26] had examined the possibility of transforming a distribution of sedimentation coefficient s into a distribution of molecular weight for linear polymers and concluded that the sedimentation velocity determination of $g(s)$ allows the evaluation of the molecular weight distribution of a given material if information about the relation between s and M is available from other sources. Essentially the transformation is as follows

$$g(s)ds = f(M)dM \quad (10)$$

and so

$$f(M) = g(s).(ds/dM) \quad (11)$$

In Fujita's work, the sedimentation coefficient s was given for the case of random coils as equal to $\kappa M^{0.5}$, where κ is taken as a constant for that particular polymer and the exponent 0.5 essentially corresponds to a randomly coiled polymer.

More generally $s = \kappa M^b$ where $b = 0.4$–0.5 for a coil, ~0.15–0.2 for a rod and ~0.67 for a sphere,[18,27] and

$$ds/dM = b \cdot \kappa^{1/b} \cdot s^{(b-1)/b} \quad (12)$$

For $b = 0.5$ (random coil) equation (12) reduces to Fujita's formula 3.139 in ref. [20].

To do the transformation, the conformation type or b needs to be known and at least one pair of s–M values is needed to define the κ. Furthermore, the method applies to the infinite dilution or non-ideality free sedimentation coefficient distribution, so is only valid for values of s (or a distribution of s values) extrapolated to zero concentration or s values measured at low enough concentrations where non-ideality effects are small. This is possible since sedimentation coefficients can be measured at much lower concentrations than those needed for a sedimentation equilibrium experiment. With 12 mm path length cells, it is possible to get reliable measurements at 0.1 mg · ml^{-1} and below. The new 20 mm path length cells allow us now to go to even lower concentrations, so non-ideality may not be an issue.

Table 2 illustrates some estimates of M_w, M_z and σ_w from sedimentation equilibrium for an alginate and some bacterial glycoconjugate constructs.

The method was first applied to glycopolymer systems in 1989.[28] Using a sedimentation coefficient distribution for pig gastric mucin (Figure 3(a))[29] and the assumption of a random coil conformation ($b = 0.5$), and a known pair of values for s and M, namely an s value of 33×10^{-13} s is

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Table 2. Estimates of M_w, M_z and σ_w from sedimentation equilibrium for an alginate and some bacterial glycoconjugate constructs.

Sample	M_w	M_z	M_z/M_w	σ_w
	kDa	kDa		kDa
Alginate (Manucol DM)[a)]	136	115	1.18	50
Glycoconjugate ASA_W	7 900	8 000	1.01	790
Glycoconjugate ASA_X	9 500	9 800	1.03	1 650
Glycoconjugate ASA_Y	9 800	10 300	1.05	2 190
Glycoconjugate ASA_Z	215	315	1.5	150
Glycoconjugate ASA_Z[b)]	240	375	1.6	190

[a)]From ref. [22]; [b)]from SEC-MALS for comparison.

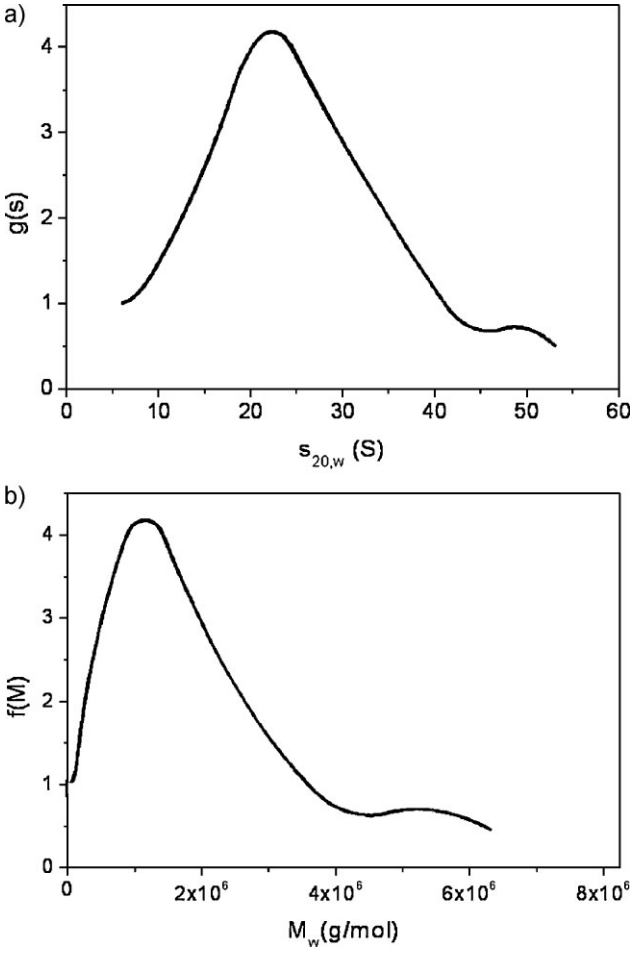

Figure 3. Sedimentation coefficient distribution for pig gastric mucin (a) and corresponding molecular weight distribution (b) based on $s \sim M^b$, with $b = 0.5$, obtained from a $g(s)$ versus distribution (above) and a fixed value of $s = 33S$ for $M = 2.5 \times 10^6\,g \cdot mol^{-1}$. Reproduced from ref. [28].

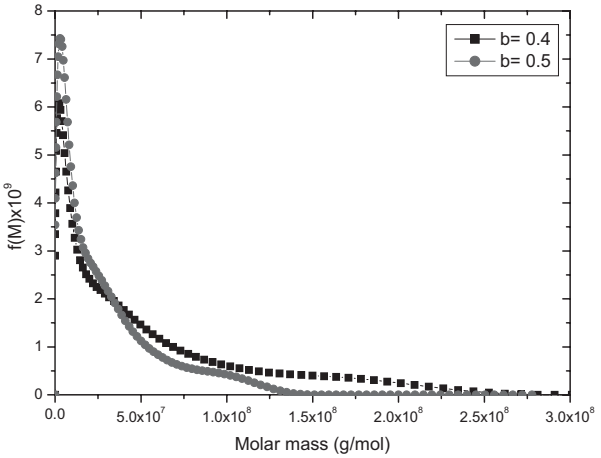

Figure 4. Molecular weight distribution for a large glyconjugate construct of a protein and bacterial polysaccharide, obtained from a $g(s)$ versus distribution using the SEDFIT procedure. Loading concentration $c_o \sim 3 \times 10^{-4}\,g \cdot ml^{-1}$. The distributions for two different selections of the power law coefficient b are shown.

approximately equivalent to a molecular weight of 2.5 million, so it was possible to perform the transformation to obtain the equivalent molecular weight distribution (Figure 3(b)). The form of the distribution is similar to that of the distribution of contour lengths estimated from electron microscopy studies on this polymer.[28,30]

The method can now be relatively easily applied with the advent of reliable procedures for obtaining sedimentation coefficient distributions, such as the SEDFIT procedure, for both natural and synthetic polymeric materials. To illustrate the power of the method we have taken as an example a large glycoconjugate construct of a protein and a bacterial polysaccharide, too large to be properly characterised by SEC-MALS or sedimentation equilibrium. Figure 4 shows the transformation of the sedimentation coefficient distribution measured at low loading concentration ($c_o \sim 3 \times 10^{-4}\,g \cdot ml^{-1}$) to a molecular weight distribution for two differing values of the power law coefficient b. In that regard, if there is uncertainty in the conformation type then it is worth attempting the distribution for differing values of b. Work is in progress with P. Schuck to have a special option inbuilt into the SEDFIT algorithm[31] to make this procedure generally available, and into extending the procedure to cases where non-ideality is significant.

Conclusion

It can be seen that the insights of Svedberg and many other distinguished early operators of the analytical ultracentrifuge, such as J. M. Creeth and H. Fujita, are still proving to be of great value in the modern "on-line" era of the

technique applied to heterogeneous biological systems such as polysaccharides, mucins and other glycoconjugates. Besides the interesting challenge of dealing with distributions of molecular weight rather than single molecular weight materials, the added complexity of the greater non-ideality of these substances is now being adequately dealt with, particularly with recent further advances in equipment and cell design. The absence of the requirement of columns and or membranes, and the ability to analyse very large molecular weights makes the method an attractive alternative or complementary procedure to SEC-MALS.

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