CHAPTER EIGHTEEN

Ultracentrifuge Methods for the Analysis of Polysaccharides, Glycoconjugates, and Lignins

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Abstract

Although like proteins, polysaccharides are synthesized by enzymes, unlike proteins there is no template. This means that they are polydisperse, do not generally have compact folded structures, and are often very large with greater nonideality behavior in solution. This chapter considers the relevant analytical ultracentrifuge methodology available for characterizing these and related carbohydrate-based systems and information this methodology supplies, in terms of sizes, shapes, and interactions using a comprehensive range of examples, including glycoconjugates and lignins. The relevance and potential of recent software developments such as SEDFIT-MSTAR, the Extended Fujita algorithm, and HYDFIT are considered.
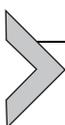
1. INTRODUCTION

Analytical ultracentrifugation (AUC) provides a powerful, matrix-free method for the characterization of the heterogeneity, molecular weight (molar mass) distribution, and interactions of "carbohydrate polymers," namely polysaccharides and glycoconjugates, and also related materials such as lignins (Harding, 2005a, 2005b, 2005c). In addition, when used in conjunction with viscometry and light scattering—particularly the powerful probe of size exclusion chromatography coupled to multiangle light scattering (Wyatt, 1992, 2013)—AUC provides a method for assessing the conformation and conformational flexibility (via the persistence length L_p and related parameters) of these macromolecules (García de la Torre & Harding, 2013; Harding, 1995a, 1995b, 1997a, 1997b, 2012a, 2012b, 2013a).

Compared with proteins, carbohydrate polymers provide a different challenge to characterization methodologies. Although they are synthesized by enzymes, there is no template to direct this synthesis (except for the protein or peptide components of glycoconjugates), and as a result they are polydisperse and do not usually have highly defined folded structures. Secondary structure may exist in the carbohydrate, e.g., as helices or double (or even triple) helices but very few are compact (glycogen and possibly gum Arabic are notable exceptions). Carbohydrate polymers are usually of higher molecular weight and are highly swollen in solution through solvation effects and in the case of polysaccharides can be very highly charged (Harding, 1994a, 1994b, 1994c). This means that besides being polydisperse and poorly defined, they can be very nonideal in the thermodynamic sense due to high exclusion volume effects and, if the concentration of supporting electrolyte is not sufficient, also have large polyelectrolyte behavior (Winzor, Carrington, Deszczynski, & Harding, 2004).

Like with protein research, for carbohydrate polymers, both sedimentation velocity and sedimentation equilibrium have strong complementary roles to play:

Sedimentation velocity

Simple matrix-free (no columns or membranes) and highly resolving assay for heterogeneity (sedimentation coefficient distributions), interactions (self-associations and ligand binding through co-sedimentation assays), and sedimentation coefficient determinations (Dam & Schuck, 2004; Harding, 1994b, 1994c, 2000; Stafford & Correia, 2015), in seconds, s or Svedberg units ($1S = 10^{-13}$ s).

Sedimentation equilibrium

Absolute molar mass or molecular weight (primarily the weight average) in g/mol or Da and molecular weight distribution determination (Harding, 1988, 1992, 1994a, 1994d).

Following the early pioneering work in the 1930s and 1940s on the AUC of carbohydrates by Svedberg and colleagues (see Jullander, 1987 for a review), work on carbohydrates became eclipsed by the great focus of biochemical science on proteins and nucleic acids. This led to carbohydrates, and polysaccharides in particular, acquiring a sort of "Cinderella" status (see Harding, 1993). This has dramatically changed in the last two decades, particularly with the increasing recognition of carbohydrates in molecular recognition phenomena (see Flint et al., 2004) and the increasing awareness of the importance of carbohydrate polymers or "glycopolymers" (Harding, 1994e) in food, pharmaceuticals, and biotechnology (Tombs & Harding, 1998). There has been a concomitant increase in awareness of the usefulness of AUC for the characterization of these materials—and its complementarity to other methodologies for characterizing the solution properties of polysaccharides such as light scattering and viscosity (Harding, 1994f; Harding, Vårum, Stokke, & Smidsrød, 1991, Jumel, Fiebig, & Harding, 1996, Jumel, Harding, & Mitchell, 1996; Jumel, Harding, Mitchell, To, et al., 1996; Morris & Harding, 2013). It has become particularly useful for the study of the stability of polysaccharide/glycoconjugate-based biopharmaceutical formulations (Harding, 2010a), particularly glycoconjugate vaccines (Harding, Abdelhameed, & Morris, 2010).

We consider the advances in analytical ultracentrifuge methodology for characterizing molecular weight, molecular weight distribution, conformation, and flexibility of carbohydrate (or carbohydrate derived or related) polymers, and for characterizing molecular interactions (self-associations and ligand interactions). First, we define the types of polymer we are considering (Fig. 1).
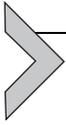
2. POLYSACCHARIDES

Polysaccharides (see Tombs & Harding, 1998) are polymers of hexose or pentose sugar residues, linked together by glycosidic bonds, resulting in expulsion of one water molecule per bond. A two-residue substance is known as a disaccharide (e.g., maltose, lactose, sucrose, cellobiose), 2–10 residues is an oligosaccharide (e.g., raffinose), and >10 residues a polysaccharide (Fig. 1A). Since the molecular weight of a residue is usually 160–250 g/mol, this means polysaccharides have (average) molecular weights usually in excess of ∼2000 g/mol. Heparin is a low molecular
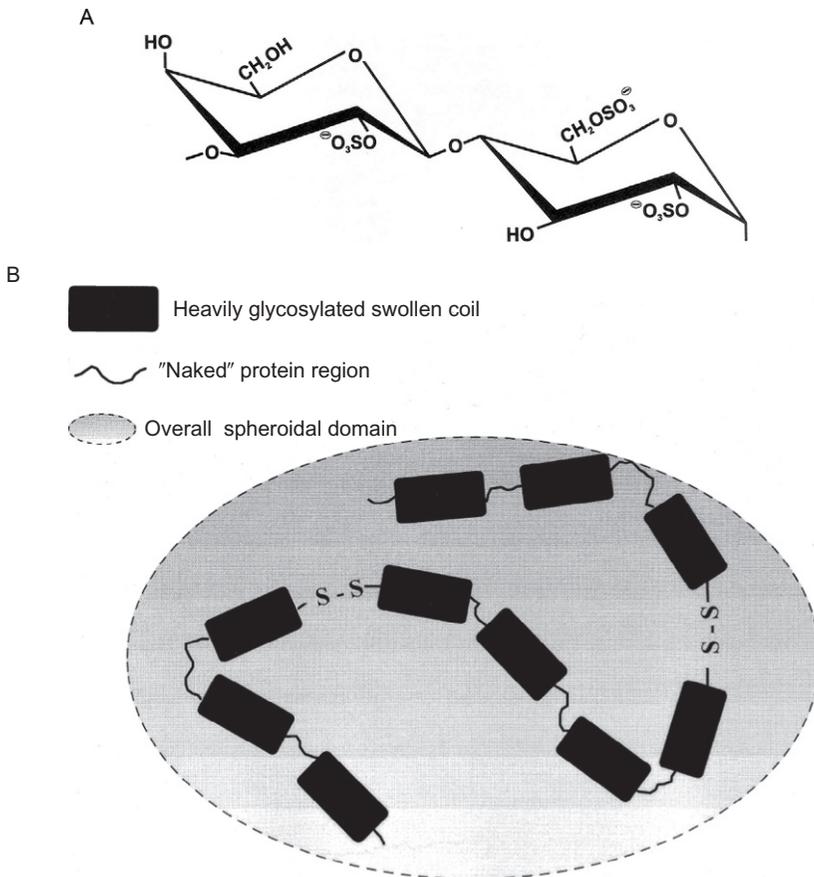
Figure 1 (A) A polysaccharide repeat structure: lambda-carrageenan. The degree of polymerization can be ∼3500 with considerable polydispersity. (B) Linear random coil model for pig colonic mucin. The shadowed area shows the "effective" overall spheroidal volume of influence of this glycoprotein.

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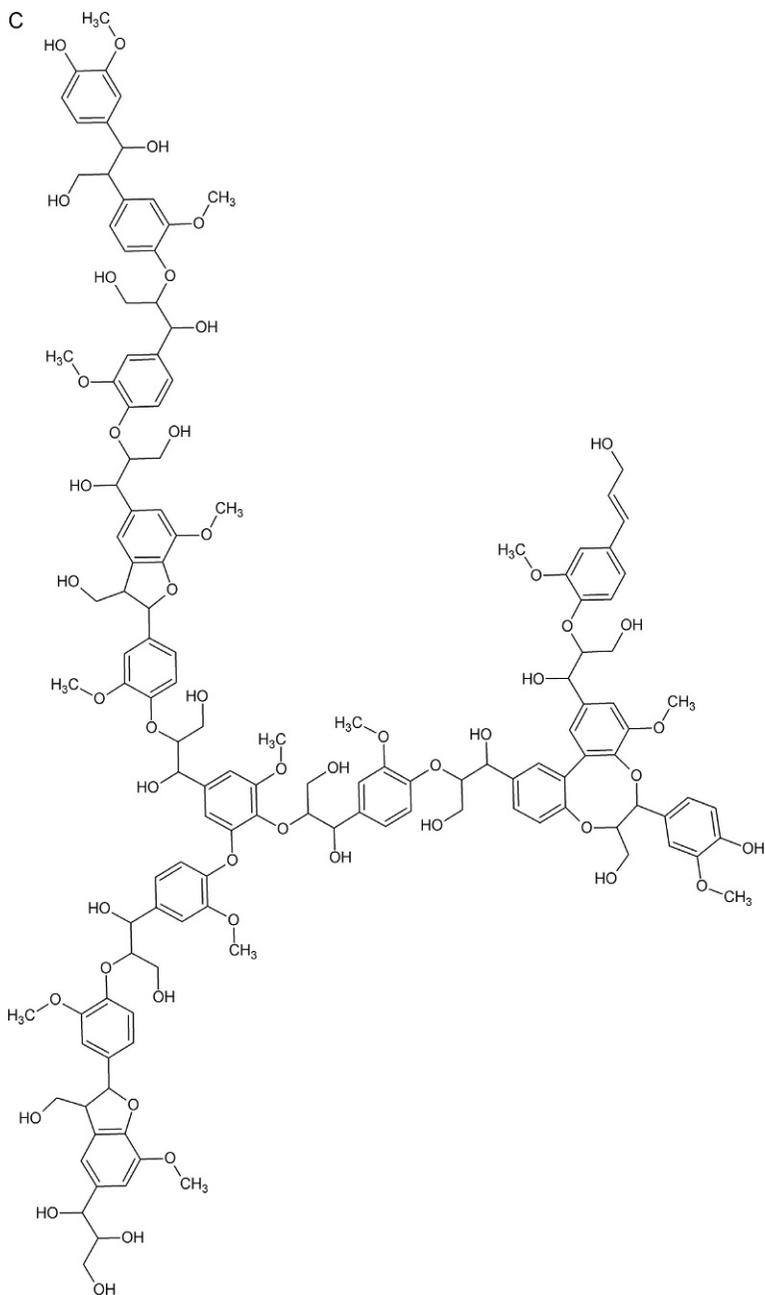
Figure 1—Cont'd (C) Complex aromatic structural model for part of a softwood lignin. *(A) Reprinted from Almutairi et al. (2013) with permission from Elsevier. (B) Reprinted with permission of Springer and Jumel et al. (1997). (C) Redrawn based on Brunow (2001). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.*

weight polysaccharide $\sim$8000 g/mol. The largest can be in excess of 50 million g/mol (e.g., amylopectin). They can be neutral (e.g., the galactomannans, pullulans, dextrans), polyanionic (alginate, pectin, xanthan), or polycationic (chitosans, aminocelluloses).

3. GLYCOCONJUGATES

The most well-known glycoconjugates are deoxyribose nucleic acid (DNA) and ribonucleic acid (RNA) involving conjugates of sugar residues with nucleic acid bases, with the sugar backbone held together not by glycosidic bonds but by phosphodiester bonds. Historically, ultracentrifuge methods have been used, for example, to show that replication of DNA was semiconservative (Meselson & Stahl, 1958), and earlier to confirm the purity of calf-thymus DNA used by Creeth and colleagues in the first demonstration of hydrogen bonds in DNA using viscometry (Creeth, 1947; Creeth, Gulland, & Jordan, 1947; see also Harding, 2010b; Harding & Winzor, 2010; Watson, 2012). Modern application of ultracentrifuge methods to DNA has been covered by, for example, Clay, Carels, Douady, and Bernadi (2005) and for RNA by Mitra (2014) and will not be considered further in this chapter other than in terms of condensation interactions with polysaccharides.

Glycoproteins consist of a protein backbone with sugar residues attached. Ovalbumin and antibodies are examples of glycoproteins with low degrees of glycosylation: since their properties are dictated by the protein component, again they will not be considered in this review. Mucin glycoproteins (Harding, 1989; Fig. 1B) also have a protein backbone by which sugar chains are attached (through O-linkages via serine or threonine residues on the backbone). By contrast, these, however, are heavily glycosylated (generally >80%)—and their properties are close to those of polysaccharides—so they will be considered here.
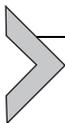
4. LIGNINS

Lignins are not carbohydrates but are closely associated with carbohydrate polymers in plant cell wall structures and are cross-linked racemic macromolecules (Fig. 1C). They are a class of natural, highly branched phenylpropanoid macromolecules, which have a random and amorphous 3D structure, in which the partly cross-linked chains are hydrophobic, heterogeneous, and polydisperse. The carbon content of the aromatic lignins is around 60–63%, i.e., higher than that of the accompanying polysaccharides

(see Alzahrani et al., 2015; Daly, Maluk, Zwirek, & Halpin, 2012). There is now a major push in the field of the enzymatic breakdown and manipulation of lignins and cellulosic type of materials to facilitate biofuel production from plant biomass (Daly, Maluk, Zwirek, & Halpin, 2012). There is also considerable interest now in the importance of these substances in wood and the mechanisms responsible for the decay, particularly in archaeological wood (Harding, 2012a). One outstanding example is the alum induced decay of cellulose in archaeological wood structures from the Oseberg ship find of 1904 in Norway—involving the search for alternative carbohydrate-based polymer consolidants to replace the cellulose and which will interact with the lignin structures and give long-term strength.
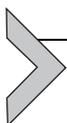
5. RELEVANT RECENT ADVANCES IN AUC AND RELATED PROCEDURES

5.1 Sedimentation Velocity: SEDFIT

The SEDFIT procedure (Dam & Schuck, 2004) for obtaining distributions of sedimentation coefficient is well known and has been widely applied mainly to protein-based systems. The procedure solves the Lamm equation describing the change of concentration distribution with radial position with time in terms of a distribution of sedimentation coefficients, $g(s)$ versus s, where s is the sedimentation coefficient (Fig. 2A). The (differential) distribution of sedimentation coefficients $g(s)$ can be defined as the population—weight fraction—of species with a sedimentation coefficient between $s + ds$ (Dam & Schuck, 2004). Corrections can be made for diffusion broadening of peaks of monodisperse and paucidisperse systems to yield a modified distribution known as a $c(s)$ versus s plot, and this can be further adjusted, based on assumptions involving the conformation/friction coefficient to a molar mass distribution plot $c(M)$ versus M. For polydisperse polymers—such as polysaccharides, which have a continuous rather than discrete distribution of sizes, the $c(s)$ versus s method is not so suitable (except for discrete components of narrow distribution); however, since many polysaccharide/glycoconjugate systems are quite large ($M > 100,000$ g/mol), diffusive effects are much slower and the $g(s)$ versus s is more representative of the true distribution of sizes. In addition, there is a method for transforming $g(s)$ versus s to a molecular weight distribution $f(M)$ versus M for polysaccharides and other polymers with a quasicontinuous polydispersity—the *Extended Fujita Method*—which we consider below.

To eliminate the effects of nonideality (co-exclusion and any residual polyelectrolyte effects), distributions should be obtained at the lowest possible
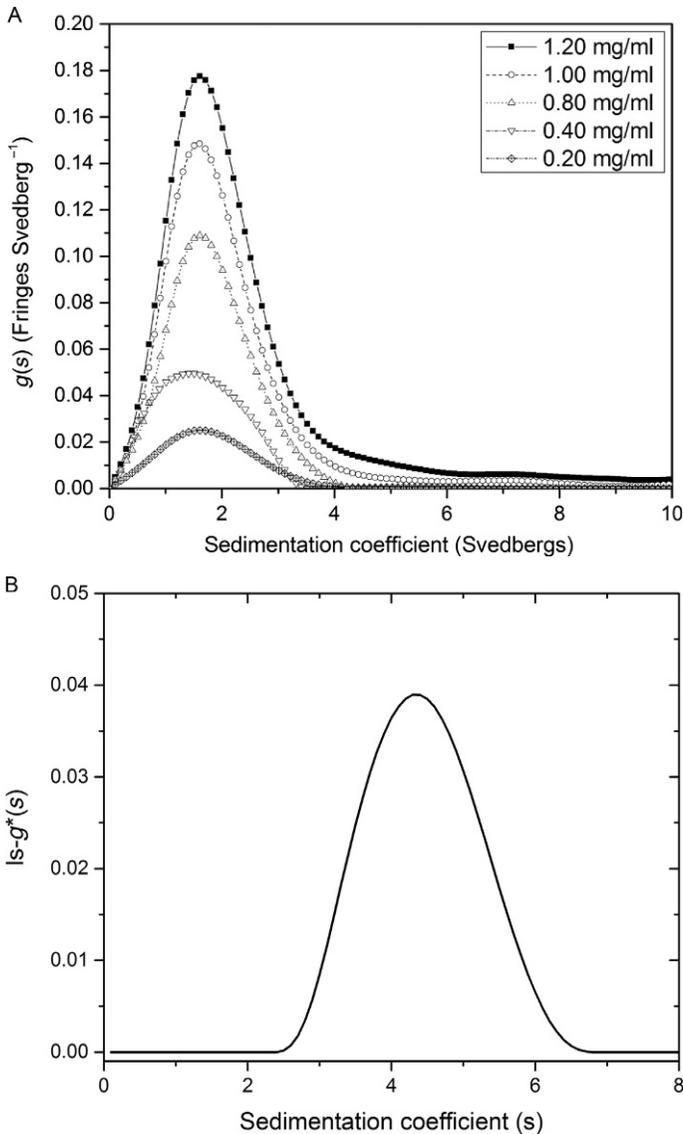
Figure 2 Sedimentation coefficient $g(s)$ versus s distribution profiles (A) chitosan in 0.2 M acetate buffer, pH 4.3, for a range of concentrations; (B) for an alginate in 0.3 M NaCl at a loading concentration of 0.03 mg/ml.

concentration (concentrations as low as 0.03 mg/ml are possible—Fig. 2). The weight average sedimentation coefficient s (corrected to standard conditions of the density and viscosity of water at 20.0 °C, to give $s_{20,w}$) can then be used to obtain other information (such as conformation or flexibility). Alternatively,

an extrapolation can be made of $(1/s_{20,w})$ against concentration, c to infinite dilution $c=0$, to yield $s°_{20,w}$ (Fig. 3A) using the (Gralen, 1944) relation

$$\left(\frac{1}{s_{20,w}}\right) = \left(\frac{1}{s°_{20,w}}\right)(1 + k_s c) \tag{1}$$

where k_s is the "Gralen" coefficient (ml/g), or equations with higher order in c in cases of more severe nonideality, such as for alginate (Fig. 3B).
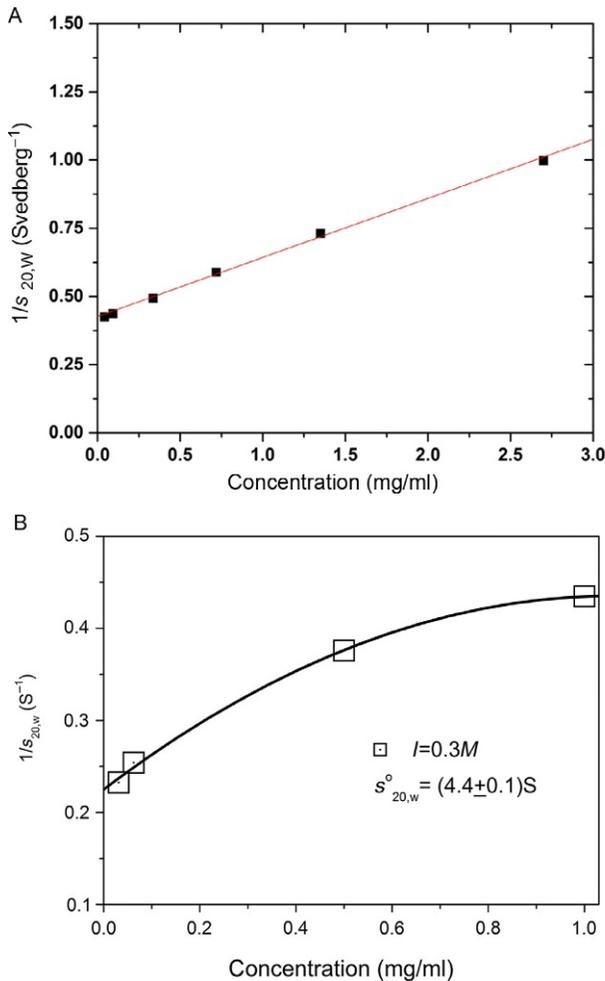

Figure 3 Concentration dependence (reciprocal) of sedimentation coefficient plots for (A) chitosan in 0.2 M acetate buffer (Almutairi et al., 2015)—and a linear fit (B) alginate in 0.3 M NaCl. *Reprinted from Almutairi et al. (2015) with permission from Elsevier.*

5.1.1 Hypersharpening

Nonideality, besides lowering the apparent (weight average) sedimentation coefficient, can also lead to skewing of the distribution due to hyper-sharpening of the sedimenting boundary, namely faster moving components being slowed down by sedimenting through a solution of the slower moving components—see Harding (1989, 1992), Harding (1994a, 1994b, 1994c, 1994d, 1994e, 1994f), Dhami, Cölfen, and Harding (1995), Dhami, Harding, Jones, and Hughes (1995), and Harding et al. (1996). Figure 4A shows a classical hypersharpening of a sedimenting boundary from an experiment on a xanthan using the Schlieren optical system (Dhami, Cölfen, et al., 1995; Dhami, Harding, Elizabeth, et al., 1995; Dhami, Harding, Jones, et al., 1995). Figure 4B shows a modern equivalent—$g(s)$ versus s profile of a xanthan at a similar concentration (0.23 mg/ml). For comparison, Fig. 4B also shows how the hypersharpening disappears at very low concentration, 0.075 mg/ml (Erten, Adams, Foster, & Harding, 2014). Another excellent example of hypersharpening using the Schlieren system is for the polysaccharide xylinan in a study by Harding et al. (1996).

The situation is in some ways analogous to the existence of a steady state between the effects of diffusion broadening countered by nonideality, explored in detail by Creeth (1964) and more recently by Scott, Harding, and Winzor (2015). The observation of hypersharpening of boundaries reinforces the importance of either working at very low concentrations or performing an extrapolation to zero concentration to obtain a reliable sedimentation coefficient for further use in hydrodynamic modeling or molecular weight analysis.

5.2 Sedimentation Equilibrium: SEDFIT-MSTAR

The long-established MSTAR method (Cölfen & Harding, 1997; Harding, Horton, & Morgan, 1992), in which the M^* function is computed over the whole length of the solution column has recently been incorporated into the SEDFIT suite of algorithms (Schuck et al., 2014). The M^* function is defined (Creeth & Harding, 1982a, 1982b) as a function of radial position r from the center of rotation and concentration $c(r)$ by

$$M^*(r) = (c(r) - c_{\mathrm{m}}) \bigg/ \left\{ k c_{\mathrm{m}} (r^2 - r_{\mathrm{m}}^2) + 2k \int_{r_{\mathrm{m}}}^{r} (c(r) - c_{\mathrm{m}}) r \, \mathrm{d}r \right\} \quad (2)$$

where k is an experimental constant depending on the rotor speed, partial specific volume of the solute, and density of the solvent and the subscript
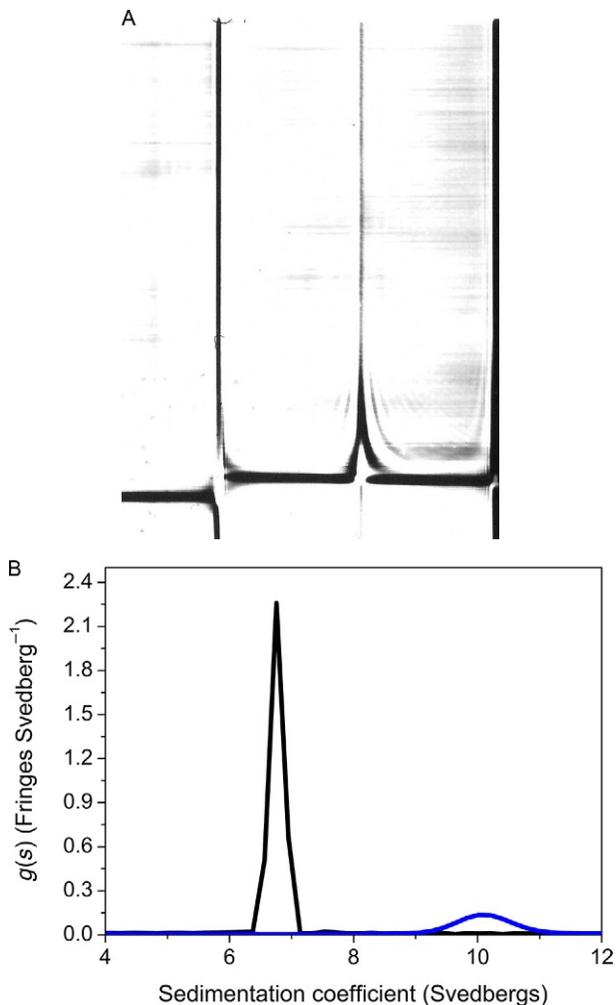
Figure 4 Hypersharpening of sedimentation profiles in polysaccharides (A) classical Schlieren pattern (Dhami, Harding, Jones, et al., 1995) for keltrol xanthan. Rotor speed 30,000 rev/min, temperature 20.0 °C; solvent $I=0.01$ *M*. The single hypersharp boundary is indicative of sample purity and also high thermodynamic nonideality, even at the low loading concentration used (0.2 mg/ml) (B) corresponding plots for TSF xanthan using $g(s)$ versus *s* (Erten et al., 2014). Note the strong hypersharpening again at low loading concentration of 0.23 mg/ml and high ionic strength $I=0.3$ *M* (black trace) and how it disappears at very low concentration (0.075 mg/ml) when non-ideality $\sim$ negligible (blue trace; dark gray in the print version). *(A) Reprinted from Dhami, Harding, Jones, et al. (1995), with permission from Elsevier. (B) Reprinted from Erten et al. (2014), with permission from Elsevier.*

"m" denotes the air-solution meniscus. The original Creeth and Harding (1982a, 1982b) paper lists several useful properties of $M^*(r)$, the most important of which is that extrapolation of $M^*(r)$ to the radial position at the cell base ($r = r_b$) yields an estimate of $M_{w,app}$ for the whole solution as loaded into the cell: a "whole cell weight average molecular weight." As described in Schuck et al. (2014), SEDFIT-MSTAR marks an advance on previous versions of MSTAR inasmuch as the (necessary) extrapolations of data values to the meniscus and cell base radial positions are now based upon the properties of the whole data set, rather than upon simple local polynomial "pieces," facilitating evaluation of M^* ($r = r_b$) $= M_{w,app}$ for the whole distribution.

The routine also evaluates $M_{w,app}(r)$ as a function of radial position in the cell, r, or the equivalent local concentration $c(r)$. The value of $M_{w,app}(r)$ at the "hinge point" of the cell, the radial position where $c(r) = c^o$, the original cell-loading concentration prior to redistribution, provides an alternative measure for the whole distribution $M_{w,app}$ (Schuck et al., 2014). SEDFIT-MSTAR employs a smart-smooth method for obtaining baselines using Savitsky–Golay filters, and also provides a (low–resolution) estimate of the molecular weight distribution $c(M)$ versus M. Figure 5 gives an example for lambda-carrageenan. Another recent example is the application to the absolute molecular weight characterization of wood and nonwood lignins (Alzahrani et al., 2015).

Molecular weights obtained from SEDFIT-MSTAR are apparent ones—that is not corrected for thermodynamic nonideality (through co-exclusion and polyelectrolyte behavior). Unfortunately, whereas for sedimentation velocity it is possible to run experiments at concentrations as low as 0.01 mg/ml, the minimum concentration for a sedimentation equilibrium experiment in conventional 12 mm optical path length cells (Beckman XL-I ultracentrifuge) is ~0.4–0.5 mg/ml. Thirty-millimeter path length cells were commercially available until the 1980s with the Beckman Model E analytical ultracentrifuge. Encouragingly, 20 mm path length cells have recently become available from Nanolytics Ltd. (Potsdam, Germany), allowing a minimum concentration of 0.2–0.3 mg/ml. These concentrations may still though be too high to be able to rule out the effects of nonideality for some materials. Harding (1992, 2005a) gives comparative tables assessing the underestimate of measured molecular weights for a range of polysaccharide materials, measured at these minimum concentrations without correction. For some polysaccharides $M_w \sim M_{w,app}$ measured at these
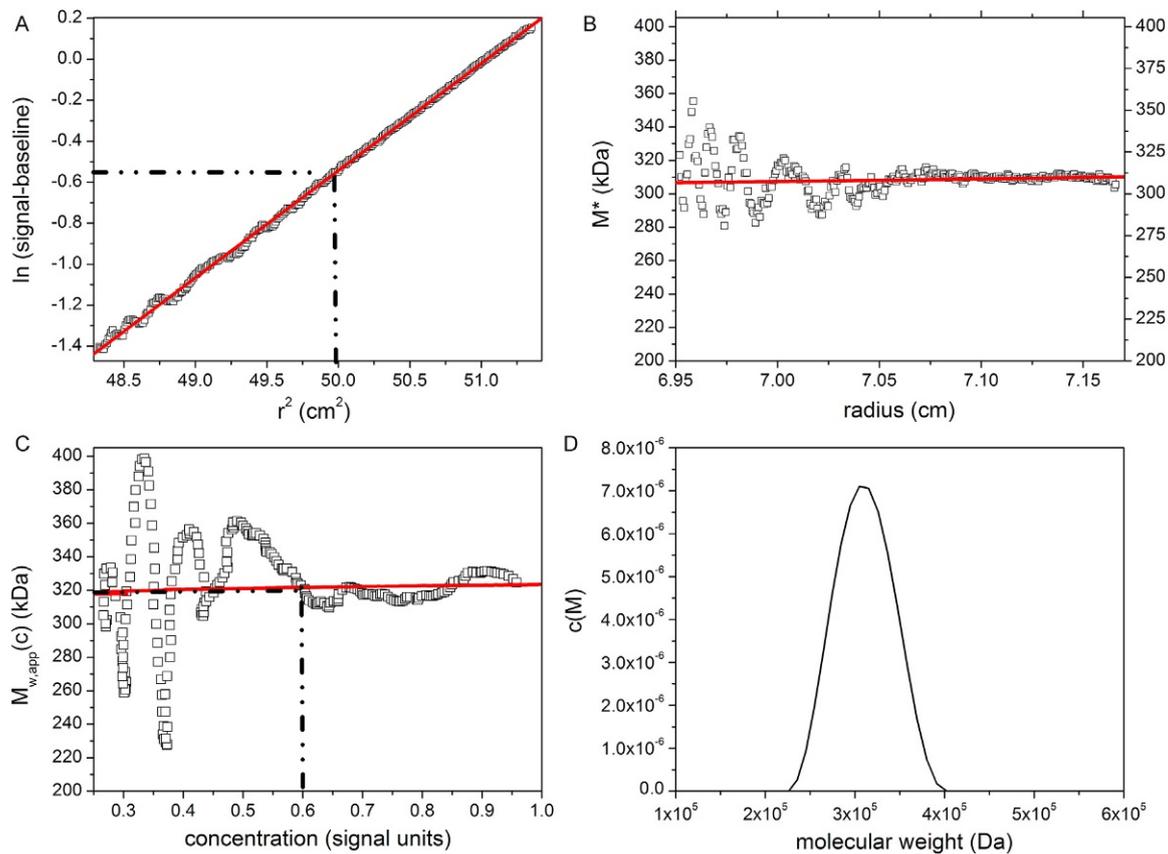
Figure 5 See legend on next page.

concentrations are a reasonable approximation; for others, like xanthan and alginate, it is not, even at 0.2 mg/ml. In these cases, an extrapolation of $1/M_{w,app}$ versus c, the loading concentration to $c=0$ is necessary, with the hinge point estimates for $M_{w,app}$ giving the optimal extrapolation, as explained in Schuck et al. (2014). This extrapolation is normally linear:

$$\left(\frac{1}{M_{w,\,app}}\right) = \left(\frac{1}{M_w}\right)(1 + 2BM_w c) \qquad (3)$$

where B is the second thermodynamic virial coefficient. Although useful for many systems, Eq. (3) does not universally apply, particularly for strongly polyanionic or polycationic polysaccharides. For example, even in high ionic strength (0.3 M or higher) solvents, an extra virial term in c^2 has been shown necessary for alginates (Harding, 1992; Horton, Harding, & Mitchell, 1991).

   This requirement for an extrapolation is a disadvantage for sedimentation equilibrium compared with SEC–MALS as with the latter, due to dilution on the SEC–columns, nonideality effects can be very small and such extrapolations are unnecessary.

5.3 Higher Resolution Molecular Weight Distribution Evaluation—the *Extended Fujita* Approach

For a higher resolution of the molecular weight distribution, two other approaches are possible. First—and assuming thermodynamic non-ideality is negligible—the best fit to the concentration distribution to a mul-

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Figure 5—Cont'd SEDFIT-MSTAR output for analysis of lambda-carrageenan (at a loading concentration of 0.3 mg/ml) by sedimentation equilibrium (A) log concentration ln $c(r)$ versus r^2 plot, where r is the radial distance from the center of rotation; (B) M^* versus r plot (open squares) and fit: the value of M^* extrapolated to the cell base $=M_{w,app}$ the apparent weight average molecular weight for the whole distribution. (C) Point or local apparent weight average molecular weight at radial position r (open square) plotted against the local concentration $c(r)$ for different radial position (D) molecular weight distribution $c(M)$ versus M plot. The dash-dot line gives the estimate for $M_{w,app}$ at the hinge point. The two estimates for $M_{w,app}$ are in good agreement, (310,000 $\pm$ 5000) g/mol from the M^* extrapolation and (320,000 $\pm$ 20,000) g/mol from the hinge point. *Reproduced from Schuck et al. (2014) with permission from the Royal Society of Chemistry.*

ticomponent system can be applied to the data in the routine MULTISIG, and a quasicontinuous distribution can be generated. A description of this method is given in Gillis, Adams, Heinze, et al. (2013).

Second, the *Extended Fujita* method (Harding, Schuck, et al., 2011) is recommended. This involves transformation of the sedimentation coefficient $g(s)$ versus s profile into a molecular weight distribution $f(M)$ versus M. The method was originally provided by Fujita for randomly coiled types of polymer and then recently extended by Harding, Schuck, et al. (2011) to cover any polymer conformational type. To transform the $g(s)$ versus s profile requires three extra pieces of information. First, the weighted average molecular weight M_w from either sedimentation equilibrium (SEDFIT-MSTAR) or SEC-MALS, and second, the corresponding weighted average sedimentation coefficient from sedimentation velocity. Third, some knowledge of the approximate conformation of the polymer is required (sphere, rod, coil, or something in between). The method, like SEDFIT-MSTAR, has been built into the SEDFIT routine (Harding, Schuck, et al., 2011).

The transformation is as follows:

$$f(M) = \left(\frac{ds}{dM}\right) g(s) \tag{4}$$

with

$$M = \left(\frac{s}{\kappa_s}\right)^{1/b} \tag{5}$$

and

$$\frac{ds}{dM} = b\kappa_s^{1/b} s^{(b-1)/b} \tag{6}$$

b is the conformation/scaling parameter, with limits of ~ 0.15 for a rod, 0.4–0.5 for a random coil, and 0.67 for a compact sphere (see Harding, Vårum, et al., 1991) and κ_s can be found from Eq. (5) provided that at least one value of M (e.g., M_w from sedimentation equilibrium or SEC-MALS) is known for one value of s (e.g., the weight average s value).

Figure 6A shows an example of a determination for sodium alginate in $I = 0.3\,M$ solvent and at very low loading concentration (0.03 mg/ml) to
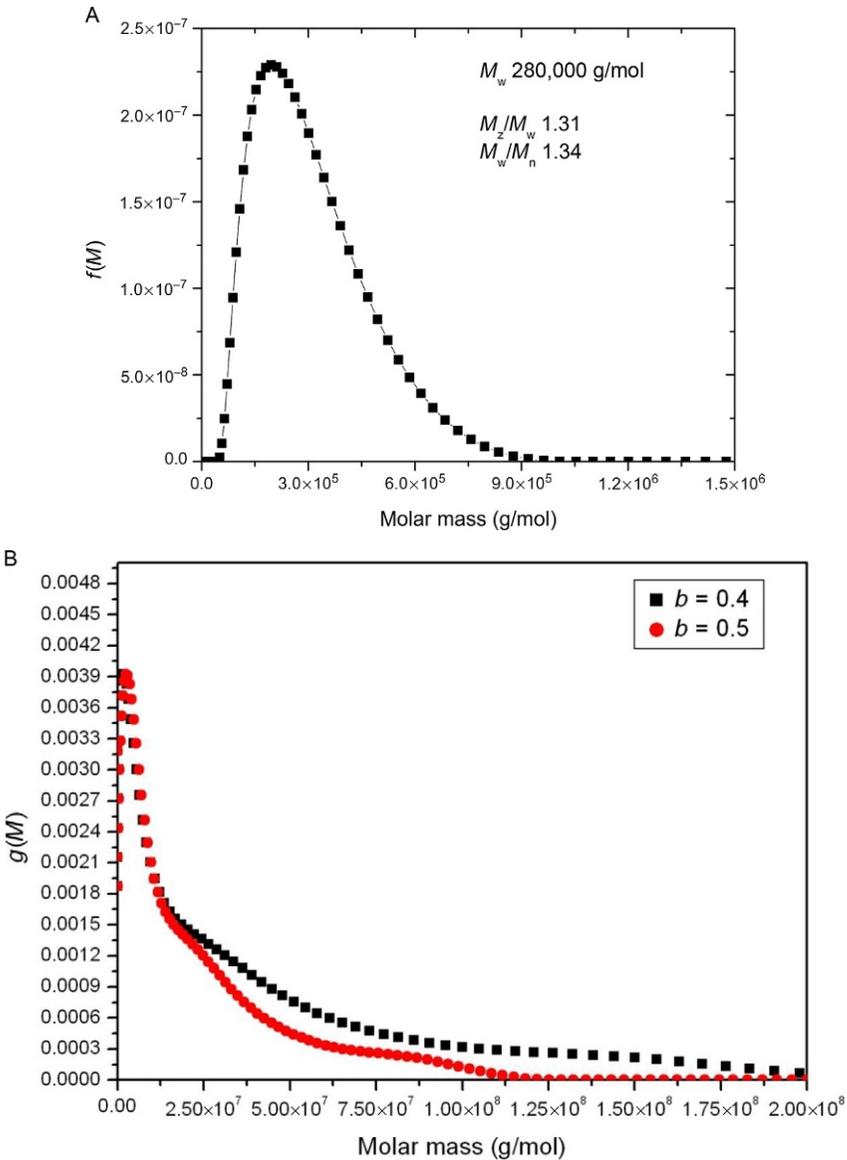
**Figure 6** Normalized molecular weight (molar mass) distribution for (A) alginate in 0.3 M NaCl obtained from transformation of the $g(s)$ versus s distribution at concentration (0.03 mg/ml), and (B) a large glycoconjugate (for 2 different values of the power law coefficient b), using the Extended Fujita method of Harding, Schuck, et al. (2011). B is reprinted from Harding, Schuck, et al. (2011) with permission from Elsevier.

render nonideality effects negligible. Nonideality, besides lowering the apparent weight average molecular weight of a distribution, can also lead to skewing of the distribution due to hypersharpening of the sedimenting boundary, namely faster moving components slowed down by sedimenting through a solution of the slower moving components—as considered in Section 5.1.1—another reason for working at as low a concentration as is possible (see Section 5.2).

Taking a value for b for alginate ∼0.33 (Harding, Schuck, et al., 2011; Harding, Smith, et al., 2011), and taking $M_w = 280,000$ (from SEC-MALS) and $s = s_{20,w}$ (at 0.03 mg/ml) = 4.3S, this yields a value for $\kappa_s = 0.0685$. The distribution $f(M)$ versus M obtained in Fig. 6A corresponds to the $g(s)$ versus s distribution of Fig. 2B, and estimates for M_z/M_w and M_w/M_n are also given. The method has a large dynamic range, covering molecular weights $>10^8$ g/mol: Fig. 6B gives the distribution for a large glycoconjugate of *Streptococcus pneumoniae* capsular polysaccharide with tetanus toxoid protein. If there is uncertainty in b, then this can be adjusted to monitor effects on the distribution, as shown in Fig. 6B. If b is adjusted, it is important that κ_s is adjusted too in Eq. (5) so that the known weighted average values for M and s used to calibrate the plot still correspond.

5.4 Conformation and Flexibility Analysis

There are a number of options open for the analysis of the overall conformation of a carbohydrate-based macromolecule (in terms of general models such as sphere, rod, and coil) and in terms of the flexibility of the macromolecule (Harding, Vårum, et al., 1991). The inherent polydispersity of these substances appears to make measurements more difficult, although it can actually be beneficial as measurements of the sedimentation coefficient (and other parameters from other hydrodynamic measurements, such as the intrinsic viscosity [η] or radius of gyration R_g) as a function of molecular weight within a homologous series can provide conformation and flexibility information. Complications can exist, such as through solvent potentially draining through a macromolecule, although as Tanford (1961) and others have long ago pointed out, the strong hydrodynamic interactions between elements within the particle and interstitial solvent generally dominate over these effects. Conformation and flexibility analysis is however always of low resolution.

5.4.1 Scaling or Power Law

Simple scaling law analysis uses the power law equation (cf. Eq. 5)

$$s = K_s M^b \tag{7}$$

with the limiting values of b for different conformation types given above. The variation in the exponent for different conformation types is however not as great compared with the variation of $[\eta]$ with M or R_g with M. Equation (7) can however be used in conjunction with other hydrodynamic scaling relations with M, for example, with $[\eta]$ and M or R_g and M (see Harding, 1995b; Harding, Vårum, et al., 1991). The relatively low dependence of the sedimentation coefficient on conformation is on the other hand advantageous in terms of the *Extended Fujita* method for molecular weight distribution analysis from sedimentation velocity (Section 5.3).

5.4.2 Wales–van Holde Relation

Another useful conformation parameter is the Wales–van Holde (Wales & van Holde, 1954) ratio R of the limiting (at $c=0$) concentration dependence coefficient k_s to the intrinsic viscosity $[\eta]$

$$R = \frac{k_s}{[\eta]} \tag{8}$$

This has approximate limits $R \sim 0.2$ for a rod and ~ 1.6 for a random coil/ compact sphere (Creeth & Knight, 1965; Rowe, 1977), although for charged polysaccharides, the ionic strength of the supporting electrolyte needs to be sufficient such that polyelectrolyte effects are fully suppressed. An excellent recent example has been the application to chitosans of differing degrees of acetylation by Schütz, Käuper and Wandrey (2012), in agreement with earlier estimations on different chitosans by Morris, Castile, Smith, Adams, and Harding (2009a, 2009b).

5.4.3 Persistence Length Estimation

A more quantitative representation of particle flexibility comes from measurement of the *persistence length* L_p which has theoretical limits of 0 for a random coil and ∞ for a stiff rod (see García de la Torre & Harding, 2013). Practically, the limits are $\sim 1–2$ nm for a random coil and $\sim 200–300$ nm for a very stiff rod-shaped macromolecule. Persistence lengths, L_p, can in principle be estimated several different ways using sedimentation coefficient, intrinsic viscosity, or radius of gyration-based approaches. For example, the Yamakawa–Fuji relation (Yamakawa & Fujii, 1973):

$$s^0 = \frac{M_L(1 - \bar{v}\rho_0)}{3\pi\eta_0 N_A}\left[1.843\left(\frac{M_w}{2M_L L_p}\right)^{1/2} + A_2 + A_3\left(\frac{M_w}{2M_L L_p}\right)^{-1/2} + \cdots\right]$$

(9)

where M_L is the mass per unit length, $\bar{v}$ the partial specific volume, ρ_o the solvent density, η_o the solvent viscosity, N_A Avogadro's number, and A_2 and A_3 are coefficients. The corresponding equation for viscosity is the Bushin–Bohdanecky equation (Bohdanecky, 1983; Bushin, Tsvetkov, Lysenko, & Emel'yanov, 1981):

$$\left(\frac{M_w^2}{[\eta]}\right)^{1/3} = A_0 M_L \Phi^{-1/3} + B_0 \Phi^{-1/3}\left(\frac{2L_p}{M_L}\right)^{-1/2} M_w^{1/2}$$

(10)

where Φ is the Flory–Fox coefficient (2.86×10^{23}/mol) and A_0 and B_0 are tabulated coefficients.

Yamakawa and Fujii (1973) showed that A_2 in Eq. (9) can be considered as $-\ln(d/2L_p)$ and $A_3 = 0.1382$ if the persistence length L_p is much higher than the chain diameter, d. Difficulties arise if the mass per unit length is not known, although both relations (Eqs. 9 and 10) have been built into an algorithm Multi-HYDFIT (Ortega & García de la Torre, 2007) which estimates the best range of values of L_p and M_L based on minimization of a target function Δ. An estimate for the chain diameter d is also required but extensive simulations have shown that the results returned for L_p are relatively insensitive to the value chosen for d (see García de la Torre & Harding, 2013 & references therein). An example for a *Streptococcal* capsular polysaccharide is given in Fig. 7 yielding a value for L_p of (6.2 ± 0.6) nm—a semiflexible structure.

5.4.4 Sedimentation Conformation Zoning

A check for consistency of the above results can be obtained from a *sedimentation conformation zone* plot of $k_s M_L$ versus $[s]/M_L$ introduced by Pavlov, Rowe, and Harding (Pavlov, Harding, & Rowe, 1999; Pavlov, Rowe, & Harding, 1997) where

$$[s] = \frac{s^0\eta_o}{(1 - \bar{v}\rho_o)}$$

(11)

and k_s is the concentration dependence or Gralen coefficient. The "zones" A: rigid rod, B: rod with limited flexibility, C: semiflexible coil, D: random coil, E: compact/globular are based on a series of polysaccharides of known
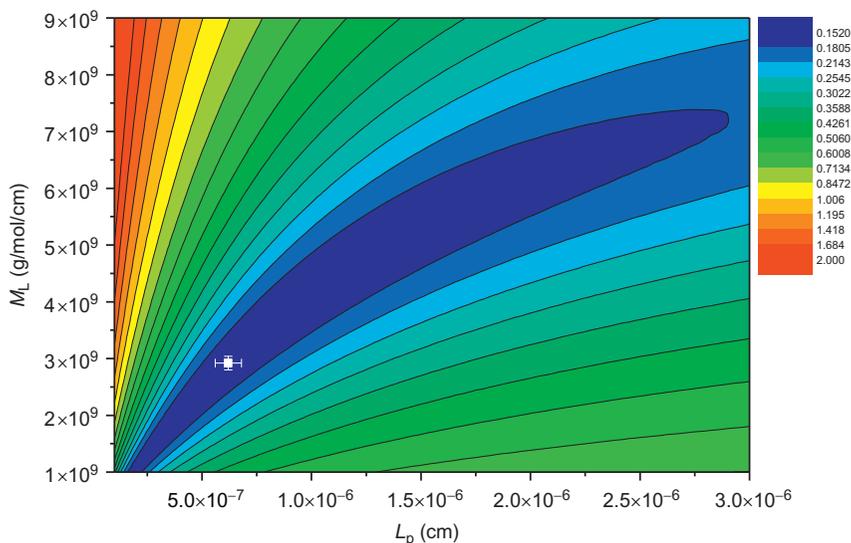
Figure 7 Global or "HYDFIT" estimation of the persistence length L_p versus mass per unit length M_L for a capsular *Streptococcus pneumoniae* polysaccharide SP(4). The white cross represents the minimum of a target function based on fitting the Yamakawa–Fujii and Bushin–Bohdanecky equations. The plot yields $L_p \sim$6.2 (nm) and $M_L \sim$2.92 $\times 10^9$ (g/mol/cm) at the minimum target (error) function value of 0.15. *Reprinted from Harding et al. (2012), with permission from Elsevier.*

[s], k_s and M_L, and known conformation type. Figure 8 shows the results for capsular polysaccharides from *S. pneumoniae*—all those studied are clearly semiflexible chains. Similar results have been found for example for chitosans (Morris et al., 2009a, 2009b).

5.4.5 Quasi-Rigid Particle Modeling
The intrinsic viscosity and sedimentation coefficient can also give valuable shape information on the protein components of glycoconjugates, for example, those used in the production of glycoconjugate vaccines. As recently reviewed by García de la Torre and Harding (2013), a whole rafter of modeling strategies are available ranging from simple whole body or ellipsoid representations of the structures to more complex bead modeling (the latter usually requiring additional information such as from solution X-ray scattering). The software for this simple modeling—the "ELLIPS" suite of programs (Harding & Cölfen, 1995; Harding, Horton, & Cölfen, 1997) is now very simple to use (Harding, 2013b; Harding, Cölfen, & Aziz, 2005), and Fig. 9 shows such a representation for the tetanus toxoid protein in terms of a prolate ellipsoid of revolution (an ellipsoid with one major and
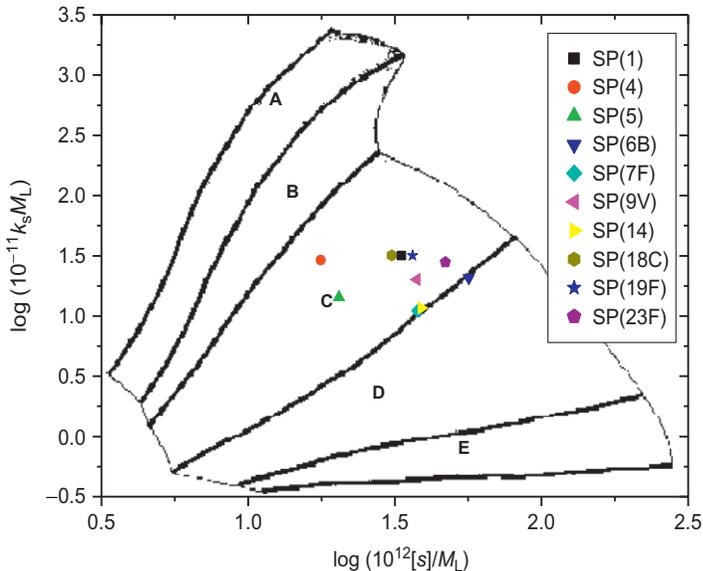
Figure 8 Conformation zoning plot. k_s is the concentration dependence sedimentation coefficient (ml/g), M_L is the mass per unit length and $[s]$ is the intrinsic sedimentation coefficient. All 10 *Streptococcus pneumoniae* capsular polysaccharides have conformations in Zone C (semiflexible coil) or near the boundary with Zone D (highly flexible). *Reprinted from Harding et al. (2012), with permission from Elsevier.*

two equal minor axes) of axial ratio ∼3:1. Coincidentally, the representation looked remarkably like the "guessed" cartoon representation given earlier by Astronomo and Burton (2010)!

Ellipsoidal representations also appear appropriate in describing the conformations of lignins from wood and nonwood sources. Favis and Goring (1984) had suggested that they may have plate-like structures and if so, Alzahrani et al. (2015) have recently found that a flat oblate model (two major axes and one minor axis) of axial ratio of ∼30:1 seems appropriate, again on the basis of viscometry and sedimentation measurements.
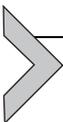
6. EXAMPLES OF APPLICATIONS TO SPECIFIC CARBOHYDRATE SYSTEMS

Table 1 gives a comprehensive (but not exhaustive) summary of applications of AUC to carbohydrate systems from the work of Svedberg and Gralen on the molecular weights of cellulose in cuprammonium sulfate to modern applications to the molecular weight distribution and conformation of glycoconjugate vaccines. It should be stressed that AUC applies

a/b= 3.00   b/c= 1.00
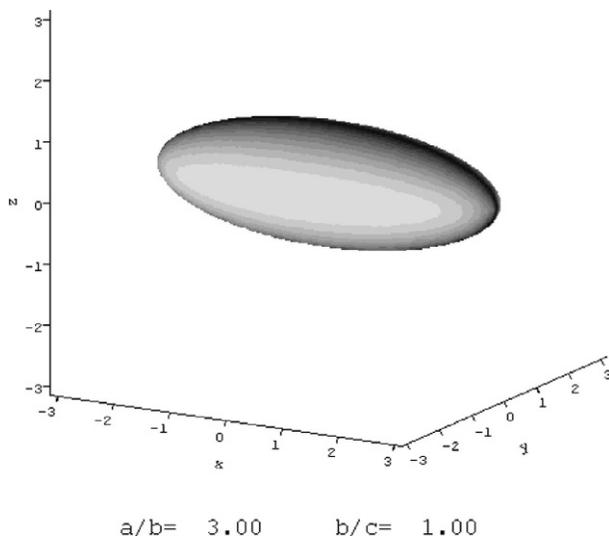
Figure 9 Low-resolution structure of tetanus toxoid conjugation protein using the simple ellipsoid modeling routine ELLIPS1 showing an extended form of (prolate) axial ratio ~3:1. *Reprinted from Abdelhameed et al. (2012), with permission from Elsevier.*

principally to systems of macromolecules or macromolecular assemblies *in solution*, although particles of sedimentation coefficient >3000S (such as mucin–chitosan complexes) can be registered (Deacon et al., 1998). However for such very large particles, "disc centrifugation" procedures (essentially preparative sedimentation in a sucrose or other density gradient, with a turbidity detector to register the sedimentation rate) are more appropriate, as, for example, used in the study of the stability of chitosan–tripolyphosphate particulate systems considered for use in drug delivery (Morris et al., 2011).

Besides characterization of molecular weight, conformation, and flexibility, the most important application of AUC in carbohydrate-based systems has been in the study of macromolecular interaction phenomena, and for this final section of this chapter we will now consider some aspects of this.

7. ANALYTICAL ULTRACENTRIFUGE ANALYSIS OF INTERACTIONS INVOLVING CARBOHYDRATE POLYMERS

7.1 Carbohydrate Self-Association

Certain classes of carbohydrate polymer are known to form double or triple helices (for example, xanthan and schizophyllan, respectively) but these tend

Table 1 Analytical Ultracentrifuge Studies on Carbohydrate Polymers

Macromolecular System	Type of Study	References
Acanthus ebracteatus polysaccharides	Molecular weight, conformation	Hokputsa,Harding, Inngjerdingen, et al. (2004)
Alginate	Novel combination of sedimentation equilibrium with size exclusion chromatography for molecular weight distribution	Ball, Harding, and Mitchell (1988)
	Thermodynamic nonideality study	Horton, Harding, Mitchell, & Morton-Holmes (1991)
	Novel use of XL-A ultracentrifuge for Schlieren study	Dhami, Cölfen, et al. (1995)
Aminocelluloses	Protein-like self-association	Nikolajski et al. (2014)
	Completely reversible tetramerization of AEA-1	Heinze et al. (2011a, 2011b)
Amylovoran	Molecular weight and shape	Jumel, Geider, and Harding (1997)
Arabinoxylan	Effect of uv-tagging on properties	Morris, Ebringerova, Harding, and Hromadkova (1999)
	AUC study of a weak self–association	Patel et al. (2007)
Blue dextran	Use of absorption optics on the XL-A analytical ultracentrifuge	Errington, Harding, and Rowe (1992)
Capsular polysaccharides from *Streptococcus pneumoniae*	AUC/hydrodynamic analysis of mol wt., heterogeneity, and conformation of a series of serotypes	Harding et al. (2012)
Carbohydrate-coated dendrimers	Solution conformation	Pavlov, Korneeva, Jumel, et al. (1999)

Continued

Table 1 Analytical Ultracentrifuge Studies on Carbohydrate Polymers—cont'd

Macromolecular System	Type of Study	References
Carbohydrate–protein recognition	Ligand-mediated dimerization	Flint et al. (2004)
Carboxymethyl chitin	Molecular weight and conformation	Korneeva, Vichoreva, Harding, and Pavlov (1996)
	Solution conformation at high ionic strength	Pavlov, Korneeva, Harding, and Vichoreva (1998); Pavlov, Korneeva, Vikhoreva, and Harding (1998)
Carboxymethyl cellulose	Molecular flexibility study on dendronized derivatives	Pohl, Morris, Harding, and Heinze (2009)
Charged polysaccharides	Extent of charge screening	Winzor et al. (2004)
Cellulose	Sedimentation velocity and diffusion measurements	Gralen (1944)
	Nitrocellulose	Jullander (1945, 1987)
Chitosan	Interactions with DNA and xanthan	Almutairi et al. (2015)
	Effect of fluorophore 9-anthraldehyde on hydrodynamic properties	Cölfen, Harding, and Vårum (1996)
	Interactions with lysozyme	Cölfen, Harding, Vårum, and Winzor (1996)
	Interactions with mucin—sedimentation and atomic force microscopy study	Deacon et al. (2000)
	Specific interactions with mucins from different regions of stomach	Deacon et al. (1999)
	Correlation of AUC with SEC-MALS data	Fee et al. (2003)
	Interactions with mucin	Fiebrig, Harding, Stokke, et al. (1994); Fiebrig, Harding, and Davis (1994); Fiebrig, Davis, and Harding (1995)

Table 1 Analytical Ultracentrifuge Studies on Carbohydrate Polymers—cont'd

Macromolecular System	Type of Study	References
	Depolymerization with temperature	Morris et al. (2009a)
	Global hydrodynamic analysis of flexibility	Morris et al. (2009b)
	Sedimentation behavior in relation to other properties/ nanogels	Schütz, Käuper, and Wandrey (2012)
	Analysis by sedimentation velocity of mucin complexation	Harding (1997a)
	Comparison of different degrees of acetylation	Errington, Harding, Vårum, and Illum (1993)
	Storage stability study of chitosan-tripolyphosphate nanoparticles	Morris, Castile, Adams, and Harding (2011)
Chondroitin sulfate	Structural integrity after laser irradiation	Jumel et al. (2002); Sobol et al. (2000)
Citrus pectins	AUC and light scattering evidence for extended conformation	Harding, Berth, Ball, and Mitchell (1990)
	Molecular weight distribution and conformation	Harding, Berth, Ball, Mitchell, and Garcia de la Torre (1991)
	Global hydrodynamic analysis of flexibility	Morris et al. (2008)
Cyanobacterial exopolysaccharides	Solution properties in relation to use	Li, Harding, and Liu (2001)
	Comparison of hydrodynamic properties of exopolysaccharide from *Aphanothece halophtica* GR02 with those of xanthan	Morris et al. (2001)
Cystic fibrosis mucin	Self-association, polydispersity, and nonideality study	Harding and Creeth (1982)

Continued

Table 1 Analytical Ultracentrifuge Studies on Carbohydrate Polymers—cont'd

Macromolecular System	Type of Study	References
Desert algae polysaccharides	Molecular weight, conformation	Hokputsa, Hu, Paulsen, and Harding (2003)
Dextran	Protein diffusion through incompatible PEG/dextran phase systems	Harding and Tombs (1989, 2002)
Dextran T-500	Novel combination of sedimentation equilibrium with size exclusion chromatography for molecular weight distribution	Ball, Harding, and Simpkin (1990)
Diethylaminoethyl-dextran	Interactions with mucin	Anderson, Harding, and Davis (1989)
Di–iodotyrosine dextran	Characterization using the XL-A analytical ultracentrifuge	Errington, Harding, Illum, and Schact (1992)
Dietary fiber polysaccharides—various	AUC for probing possible interactions with gliadin	Kök, Harding, Adams, Tatham, and Morris (2010); Kök et al. (2012); Adams et al. (2012)
Durian rinds polysaccharides	Molecular weight, conformation	Hokputsa, Hu, Paulsen, et al. (2004)
Food polysaccharides	Analysis by AUC	Harding (1994a, 1995c)
Galactomannans	Global hydrodynamic analysis of flexibility	Morris et al. (2008)
	Effect of pressure assisted solubilization on AUC	Patel, Picout, Harding, and Ross-Murphy (2006)
	Interactions with gliadin studied by sedimentation velocity	Seifert, Heinevetter, Cölfen, and Harding (1995)
Glycoconjugate vaccine: tetanus toxoid protein	Dimerization and conformation study	Abdelhameed et al. (2012)
Glycogen	Molecular weight and conformation	Morris et al. (2008)

Table 1 Analytical Ultracentrifuge Studies on Carbohydrate Polymers—cont'd

Macromolecular System	Type of Study	References
Guar	Thermal degradation effects on sedimentation coefficient and molecular weight	Bradley, Ball, Harding, and Mitchell (1989)
	Effect of gamma irradiation on macromolecular integrity	Jumel, Harding, Mitchell (1996)
Heteroxylan	Molecular weight, conformation	Dhami, Harding, Elizabeth, et al. (1995)
Hyaluronic acid	Comparison of AUC with SEC-MALS	Hokputsa, Jumel, Alexander, and Harding (2003a)
	Molecular weight, conformation of chemically degraded material	Hokputsa, Jumel, Alexander, and Harding (2003b)
Hydroxypropylmethyl cellulose	Molecular weight and shape	Jumel, Harding, Mitchell, et al. (1996)
Inulin	Molecular weight and shape in DMSO	Azis, Chin, Deacon, Harding, and Pavlov (1999)
Kappa-carrageenan	Size, shape, and hydration	Harding, Day, Dhami, and Lowe (1997)
Konjac glucomannan	Molecular weight and flexibility using global analysis	Kök, Abdelhameed, Ang, Morris, and Harding (2009)
Lambda-carrageenan	Conformation in solution	Almutairi et al. (2013)
Lignin	AUC and viscometry study of molecular weight distribution and conformation of soda, Alcell, kraft, and soda lignins	Alzahrani et al. (2015)
Locust bean gum	Comparison of hot and cold water-soluble fractions	Gaisford, Harding, Mitchell, and Bradley (1986)
Methyl galactan (synthetic)	Molecular weight	Koschella et al. (2008)

Table 1 Analytical Ultracentrifuge Studies on Carbohydrate Polymers—cont'd
Macromolecular

System	Type of Study	References
Methylcelluloses	Global hydrodynamic analysis of flexibility	Patel, Morris, Garcia de la Torre, et al. (2008)
Microbial polysaccharides	AUC in relation to structure and other properties	Morris and Harding (2009)
Mucins	New approach to molecular weight distribution analysis	Gillis, Adams, Wolf, et al. (2013)
	Interactions with mussel glue protein	Deacon, Davis, White, Waite, and Harding (1997), Deacon, Davis, Waite, and Harding (1998).
	Mucoadhesive delivery systems	Fiebrig et al. (1995); Fiebrig, Harding, and Davis (1994)
	Solution structure of pig colonic mucins	Fogg, Allen, Harding, and Pearson (1994); Fogg et al. (1996); Jumel, Fogg, et al. (1997)
	Use of AUC in mucoadhesion analysis	Harding (2003, 2006)
	Linear swollen coil model from AUC and electron microscopy	Harding, Creeth, and Rowe (1983); Harding, Rowe, and Creeth (1983)
	Sedimentation equilibrium analysis	Harding (1994a)
	Diffusion coefficients from AUC and correlation with gel hydration and dissolution	Dodd, Place, Hall, and Harding (1998)
	Study of polyelectrolyte behavior	Harding and Creeth (1983)
	Heterogeneity analysis	Harding (1984)
	Macrostructure from AUC and other methods	Harding (1989)
	Analysis by AUC of mucoadhesion	Harding (1998); Harding, Davis, Deacon, et al. (1999)
	Analysis by AUC of mucins and polysaccharides	Harding (1994b)

Table 1 Analytical Ultracentrifuge Studies on Carbohydrate Polymers—cont'd

Macromolecular System	Type of Study	References
Pectic polysaccharides from *Biophytum petersianum* Klotzsch	Solution properties in relation to immunology	Inngjerdingen et al. (2008)
Pectic polysaccharides from *Glinus oppositifolius*	Solution properties in relation to immunology	Inngjerdingen et al. (2005, 2007)
Pectin	Elevated temperature AUC and depolymerization	Morris, Butler, Foster, Jumel, and Harding (1999); Morris, Foster, and Harding (2002)
	Effect of degree of esterification on AUC properties	Morris, Foster, and Harding (2000)
	Effect of different storage temperatures on solutions and comparison with gels	Morris, Castile et al. (2010)
	Effect of uv-tagging on properties	Morris, Hromadkova, et al. (2002)
	Solution properties in relation to immunology of pectin and pectic arabinogalactan from *Vernonia kotschyana*	Negard et al. (2005)
	Stability	Morris, Adams, Harding, Castile, and Smith (2012)
Polysaccharide encapsulants	Review of properties including AUC	Wandrey, Bartkowiak, and Harding (2009)
Polysaccharide stability against bioprocessing	Use of AUC with other hydrodynamic tools	Harding (2010a, 2012a)
Polysaccharide–protein complexes	AUC and other analyses	Harding et al. (1993)
	Solution studies of interaction products after dry-heating of mixtures	Jumel, Harding, Mitchell, and Dickinson (1993); Kelly, Gudo, Mitchell, and Harding (1994)

Continued

Table 1 Analytical Ultracentrifuge Studies on Carbohydrate Polymers—cont'd

Macromolecular System	Type of Study	References
Pullulans	Characterization of standard samples	Kawahara, Ohta, Miyamoto, and Nakamura (1984)
	Solution properties of pullulans recovered from agricultural waste	Israilides, Scanlon, Smith, Harding, and Jumel (1994)
Schizophyllan	Hydrodynamic study in water	Yanaki, Norisuye, and Fujita (1980)
Starch	Heterogeneity of starches from somatic embryo- and seed-derived plants	Azhakanandam et al. (2000)
	Preparative/analytical ultracentrifuge study on density gradient fractionation	Majzoobi, Rowe, Connock, Hill, and Harding (2003)
	Molecular weights of amyloses and amylopectins	Ong, Jumel, Tokarczuk, Blanshard, and Harding (1994)
	Sedimentation study on the effect of damage on starch solutions	Tester, Patel, and Harding (2006)
	Heterogeneity of starch from modified waxy maize	Desse et al. (2009)
	Sedimentation velocity characterization of heterogeneity—comparison with SEC-MALS for rice starch	Tongdang, Bligh, Jumel, and Harding (1999)
	Sedimentation velocity characterization of starch-modified magnetite nanoparticles	Soshnikova et al. (2013)
Stewartan	Molecular weight and shape	Jumel, Geider, Harding (1997)
Sugar beet pectins	Solution characterization	Morris, Ralet, Bonnin, Thibault, and Harding (2010)

Table 1 Analytical Ultracentrifuge Studies on Carbohydrate Polymers—cont'd

Macromolecular System	Type of Study	References
Ternary mixtures of konjac glucomannan, xanthan, and alginate	AUC study of ionic strength-dependent interactions	Harding, Smith, et al. (2011)
	Interaction—and effect of ionic strength	Abdelhameed et al. (2010)
Tomato pectin	Effect of genetic modification on molecular weights	Seymour and Harding (1987); Seymour, Harding, Taylor, Hobson, and Tucker (1987); Smith et al. (1990)
Xanthan	Molecular weight, heterogeneity	Dhami, Harding, Jones, et al. (1995)
	Comparative heterogeneity with different pyruvate and acetate content	Erten et al. (2014)
Xylinan (acetan)	AUC and DLS study	Harding et al. (1996)
Xyloglucans (irradiated)	Global hydrodynamic analysis of flexibility of irradiated material	Patel, Morris, Ebringerova, et al. (2008)
	Solution properties of pressure solubilized material from tamarind seed and detarium gum	Picout, Ross–Murphy, Errington, and Harding (2003)

to be nonreversible: i.e., lowering the concentration does not cause a reversible dissociation. Two systems have however been shown to give reversible behavior—one a very weak dimerization (heteroxylan) and the other a stronger oligomerization (aminocelluloses).

A very weak reversible dimerization was observed for heteroxylan—these are $\beta(1-4)$-linked xylose polymers with arabinose (sometimes esterified with phenolic acids) side chains (Patel et al., 2007). Evidence for dimerization comes not from the appearance of an extra component in the sedimentation coefficient distribution plots but from the concentration dependence of the sedimentation coefficient after allowing for nonideality. For example, one heteroxylan (PO2) gave a dissociation constant value $K_d \sim (340 \pm 50)\,\mu M$ at

20 °C. Intriguingly decreasing the temperature to 5 °C greatly suppressed the interaction ($K_d > 3000 \, \mu M$), whereas raising the temperature to 30 °C increased the dimerization strength ($K_d \sim 140 \, \mu M$): this decrease in K_d (increase in dimerization) with increase in temperature is systematic of a reversible hydrophobic interaction.

Even more unusual *protein-like* association has been observed in solutions of the aqueous soluble carbohydrates known as the 6–deoxy–6–(ω–aminoalkyl) aminocelluloses which had previously been reported to produce controllable self-assembling films for enzyme immobilization and other applications. $c(s)$ versus s distributions show multiple components (up to pentamers) all linked according to $s \sim M^{2/3}$ (Fig. 10). Not only had such oligomerization—of the sort seen, for example, in antibody aggregation processes—never been seen in polysaccharides before, but also the power law coefficient of 2/3 is more appropriate for globular proteins rather than carbohydrates (Heinze et al., 2011a, 2011b). The oligomerization was at least partially reversible (the proportion of the lower molecular weight components decreasing as the total concentration is increased). One particular aminocellulose exhibited a completely reversible tetramerization step in the associative process (Nikolajski et al., 2014) as deduced on the basis of (i) point average molecular weights (local estimates of molecular weight at individual radial positions, r), $M_n(r)$, $M_w(r)$, and $M_z(r)$ all converging to the same (monomer value) $M_1 = 3250 \, g/mol$ at zero concentration (and converging to the tetramer value
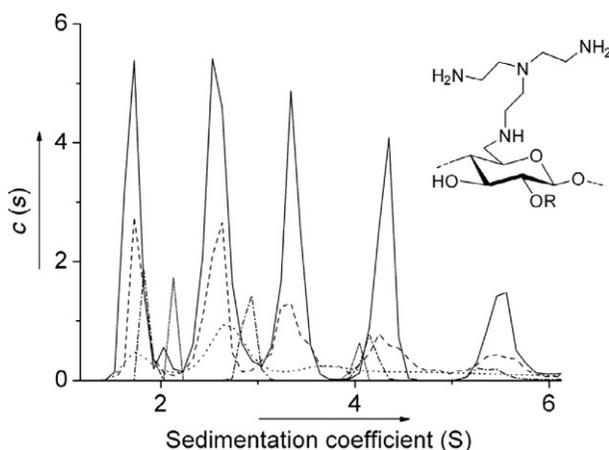

Figure 10 Sedimentation coefficient distributions of a 6-deoxy-6-amino cellulose (BAEA cellulose) at various concentrations: solid (—) 2.0 mg/ml; dash (− −) 1.0 mg/ml; dot (······) 0.5 mg/ml; dash dot (− · − ·) 0.25 mg/ml; short dot (······) 0.125 mg/ml. *Reproduced from Heinze et al. (2011a) with permission from Wiley.*

at higher concentration) and (ii) overlap of plots of $M_z(r)$ versus $c(r)$ for different loading concentrations. This completely reversible tetramerization (and subsequent assembly into larger structures) resembled more like the behavior of a protein self-assembly process—such as sickle cell deoxyhemoglobin formation— than the behavior of any carbohydrate, possibly revising traditional views of what is "protein-like" and what is "carbohydrate-like" behavior (Nikolajski et al., 2014).

7.2 Carbohydrate-Protein Interactions: Co-Sedimentation in the Analytical Ultracentrifuge

The principle of co-sedimentation for the evaluation of the extent of "binding" or association of macromolecules to other macromolecules or smaller ligands—using different optical systems or the UV-absorption optical system tuned at different wavelengths to pick up different chromophores— has been described by Harding and Winzor (2001) and applied to the quantification of protein–cofactor interactions (Marsh & Harding, 1993; Marsh, Harding, & Leadley, 1989) for the elucidation of protein–cofactor interactions. In addition, this has also been applied to encapsulation processes (Morgan, Harding, & Petrak, 1990a, 1990b; Wandrey et al., 2009), mucoadhesive systems (Harding, 2003, 2006), and the study of protein–polysaccharide interactions.

Recent work on carbohydrate–protein interactions has been focusing on the possible use of fiber polysaccharides as a macromolecular block trying to stop gliadins reaching the mucosal epithelia and causing an immune response in people with gluten intolerance or coeliac disease. The work is currently ongoing, but an assay procedure based on sedimentation velocity in the analytical ultracentrifuge monitoring for co-sedimentation has been developed (Kök et al., 2012). The assay procedure takes advantage of the fact that gliadins absorb ultraviolet light at a wavelength ∼280 nm, whereas polysaccharides generally do not. Figure 11 shows an example for an interaction between iota-carrageenan and gliadin. A comprehensive survey of fiber polysaccharides as potential macromolecular barriers to gliadin will be published in the near future.

7.3 Carbohydrate—Mucin "Mucoadhesive" Interactions

Co-sedimentation has also been used extensively to the study of mucin–mucoadhesive interactions. Mucoadhesion has been considered as a route to making the delivery of drugs taken orally more efficient: by using a
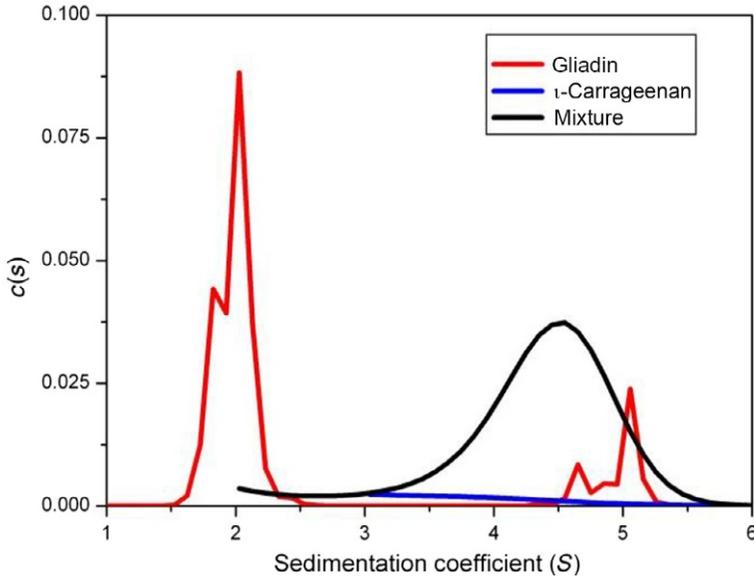
Figure 11 Sedimentation coefficient distribution diagrams for gliadins and iota-carrageenan in aqueous phosphate-chloride buffer. UV-absorption optics at 280 nm were used showing only the gliadins—and whatever they may have interacted with. Red line (light gray in the print version): gliadin only control at 5.0 mg/ml loading concentration showing material sedimenting at 2S and a small amount of aggregated material at ~5S. Blue line (dark gray in the print version): iota-carrageenan control at 1.0 mg/ml. The sedimenting material is almost transparent at 280 nm. Black line (same concentrations): mixture showing a substantial amount of material sedimenting at ~4.5S: this may indicate an interaction with gliadin. *Reproduced with permission from Kök et al. (2012).*

suitable mucoadhesive polymer carrier, the transit time through the maximum sites of absorption (usually the small intestine) can be increased through interactions of the carrier with the mucosal layer lining the gastrointestinal tract. Similar strategies are considered for application through the mucosal layer lining the nose. Table 1 includes a comprehensive list of the studies that have used co-sedimentation to probe the efficiency of candidate mucoadhesive polysaccharides—the most effective polysaccharide so far has been shown to be polycationic chitosan and the highly cationic mussel glue protein (see Harding, 2003, 2006 & references therein). The effects of changing the solvent environment (pH, ionic strength, bile salt concentration, etc.) can be easily probed, also the efficiency of binding of mucoadhesives with mucin glycoproteins from different regions of the alimentary tract (Deacon et al., 1999).

7.4 Carbohydrate—Nucleic Acid Interactions

There is increasing interest in the use of polycationic polysaccharides as histone analogues for condensing nucleic acids in DNA/RNA therapies against disease. Chitosans have been the focus of particular attention; these are soluble derivatives of chitin (poly N-acetyl glucosamine). Reducing the degree of acetylation (DA) yields the soluble polycationic form of chitin known as chitosan. In a recent study (Almutairi et al., 2015), the principle of co-sedimentation was used to explore the effectiveness of chitosans for binding to DNA, in a similar way to earlier studies applied to the study of chitosan "mucoadhesive" types of interaction with mucins. In the Almutairi et al. (2015) study, solutions of two chitosan samples of different degrees of acetylation, known as "CHIT5" (DA = 25%) and "CHIT6" (15%), and different weight average molecular weights M_w (95,000 and 170,000 g/mol, respectively) from sedimentation equilibrium were characterized and then studied in mixtures with the DNA of M_w, estimated from the *Extended Fujita* method of Harding, Schuck, et al. (2011) and Harding, Smith, et al. (2011) to be approximately 300,000 g/mol.

Sedimentation velocity of separate 1.0 mg/ml solutions of chitosans CHIT5, CHIT6, and DNA at a temperature of 20 °C all gave unimodal distributions with respective weight average sedimentation coefficients $s_{20,w}$ of 1.9S, 2.3S, and 6.8S, respectively. Each chitosan preparation was then mixed in a 1:1 w/w ratio with the DNA to a total concentration of 1.0 mg/ml. A clear shift to a high s value is observed in both cases, with nothing sedimenting at the rate of uncomplexed chitosan suggesting that the interaction had gone to completion. From the shoulder of the complex, some unreacted DNA appeared remaining. Multi-Gaussian analysis of the $g(s)$ versus s distribution for the complex suggests that ∼72% DNA had interacted with CHIT5 and ∼83% for CHIT6. The s distribution of the complex is very broad, even on a logarithmic scale, with material in excess of 100S, with the larger molecular weight and highest positively charged chitosan CHIT6 showing the greatest degree of complexation (Fig. 12).

7.5 Carbohydrate–Carbohydrate Interactions

7.5.1 Chitosan–Xanthan Interactions

The advantageous properties of mixtures of chitosan and xanthan as hydrogels have been considered for somewhile but only very recently has the biophysical basis behind this interaction been studied by AUC, again using co-sedimentation (Almutairi et al., 2015). Xanthan, like DNA, is a double
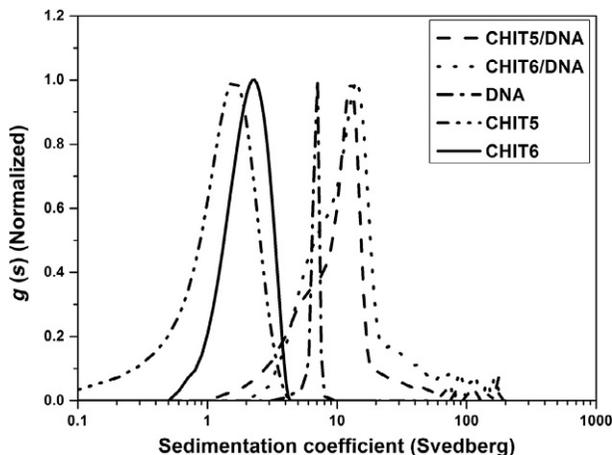
Figure 12 Normalized sedimentation coefficient distribution profiles obtained from sedimentation velocity experiments for unmixed controls and mixtures of chitosan with DNA. The shift of the complex to a higher sedimentation coefficient is seen. Multi-Gaussian analysis yields an estimate of the proportion of material complexed. *Reprinted from Almutairi et al. (2015), with permission from Elsevier.*

helical highly polyanionic polymer obtained from the microorganism *Xanthomonas campestris* and is widely used in the Food Industry as a thickener. The molecular weight of the particular xanthan used in the study of Almutairi et al. (2015)—known as "xanthan-STD"—had been found previously to be $\sim 3.2 \times 10^6$ g/mol using sedimentation equilibrium in the analytical ultracentrifuge (Erten et al., 2014).

As with the chitosan–DNA study, the sedimentation velocity profile of xanthan was first characterized at 1.0 mg/ml yielding a unimodal $g(s)$ versus s plot with weighted average $s_{20,w}$ of 4.8S. Then 1:1 by weight mixtures of chitosan with xanthan at a total concentration of 1.0 mg/ml were considered. As with chitosan–DNA, all the chitosan was complexed, but unlike chitosan–DNA, for xanthan–DNA there was no residual xanthan left behind for both CHIT5 and CHIT6 complexes (Almutairi et al., 2015). The weighted average $s_{20,w}$ for the complexes of 10.3S and 10.1S for CHIT5–xanthan and CHIT6–xanthan, respectively, was obtained.

7.5.2 PGX®

Sedimentation coefficient distribution profiling has also been used to good effect for detecting interactions in mixed polysaccharide systems used for health applications. An example is the proprietary commercial product

PGX® (PolyGlycopleX®) which is manufactured from konjac glucomannan, xanthan, and sodium alginate using a proprietary manufacturing process (EnviroSimplex®). This product has been successfully used as a dietary/satiety product for help toward obesity control. Detailed analysis by co-sedimentation (Abdelhameed et al., 2010; Harding, Smith, et al., 2011) showed clear evidence for a ternary interaction between the components at low ionic strength reinforcing experiments on the rheological properties of this system.
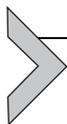
8. CONCLUSIONS

We have seen how important the analytical ultracentrifuge is for the characterization of large carbohydrate-based polymers. These materials still represent a considerable challenge, because of their polydispersity, great variety of conformation types, and large nonidealities, but there are nonetheless good strategies for dealing with these problems. One of the greatest recent impacts has been the development of software for the handling of both sedimentation velocity and sedimentation equilibrium data, greatly enhancing the resolving power of the instrument. In terms of future developments, one important area that is being explored is the reintroduction of Schlieren (refractive index gradient) optical system to complement the existing optical systems available. This optical system was once a feature of earlier commercial analytical ultracentrifuges such as the Beckman Model E, the MOM (Hungarian Optical Works) ultracentrifuge, and the MSE Centriscan ultracentrifuges (see Harding, Rowe, & Horton, 1992), instruments that are no longer available. For protein and nucleic acid work, the diversity of the optical systems present on current commercially available instruments—the Rayleigh interference, uv/visible absorption, and fluorescence optical systems—are particularly useful for protein-based systems. For polysaccharides, however, which lack appropriate chromophores or fluorophores (unless specifically labeled), only the Rayleigh system has been available, unless interactions with proteins or other materials with useable chromophores are being investigated—for example, in the interaction studies between polysaccharides with proteins, DNA, and mucins considered above. Incorporation of a Schlieren system—which like the Rayleigh system does not need chromophores—would greatly improve the power of the instrumentation for polysaccharide and synthetic polymer applications. Shortly after the introduction of the Beckman XL-A ultracentrifuge in 1990, it was shown that under certain situations, Schlieren profiles could be generated (Dhami,

Cölfen, et al., 1995; Dhami, Harding, Elizabeth, et al., 1995; Dhami, Harding, Jones, et al., 1995), and how this could now be extended into the production of a fully integrated system. Ultracentrifuge methods are also proving invaluable in increasing our understanding about the enzyme systems involved in both synthesis and degradation. These methods have already proven useful in early attempts at gene silencing of enzymes considered responsible for the softening of tomatoes on ripening (Seymour & Harding, 1987; Seymour et al., 1987; Smith et al., 1990) and now, taking advantage of the new developments in software it appears that they have helped with the identification of a specific pectate lyase (Uluisik et al., 2015), yet another example of the importance of the analytical ultracentrifuge.

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