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# VISCOMETRY, ANALYTICAL ULTRACENTRIFUGATION AND LIGHT SCATTERING PROBES FOR CARBOHYDRATE STABILITY

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## 1 INTRODUCTION

Hydrodynamic probes vary in their degree of sophistication and capability, although between them they provide a complementary set of tools for the assessment of polymer stability. This is particularly relevant for large carbohydrate polymer assemblies, whether it be resistance to chain scission or resistance against aggregative phenomena. All these probes involve motions of the polymer in solution – either in terms of the effect on the bulk flow properties in response to a shearing force (viscosity), or the motions of the polymer through a solution in response to a force field. The latter can be thermal/stochastic (light scattering) or gravitational (chromatographic flow and ultracentrifugation).

In this chapter we highlight some of the information obtainable from the modern implementation of these well-established methods, some limitations and the virtue of using these methods in combination. The types of information that can be reasonably expected are (i) heterogeneity information; (ii) molecular weight (molar mass) averages and distributions; (iii) the extent and reversibility of aggregation and degradative phenomena; (iv) conformation and conformational flexibility. In the following chapter by Morris *et al.* the application of some of these methods to the study of the stability of one important class of carbohydrate polymer – the pectins - is considered. We cover only an overview of the techniques here although some key references giving the necessary detail behind them are indicated.

## 2 VISCOMETRY

This is the simplest – and least expensive - of the hydrodynamic probes and can provide basic information about conformation, conformation change (e.g. in response to thermal treatment), and, for non-spheroidal particles molecular weight and the state of aggregation/ degradation. Some key viscosity parameters (see ref. 1 and references therein) are the absolute viscosity (or ratio of the shear stress to shear rate), $\eta$, the relative viscosity (ratio

of the solution viscosity at a concentration c (g/ml), to the solvent viscosity) $\eta_r = \eta_{solution}/\eta_{solvent}$ and the reduced viscosity $\eta_{red}$ (ml/g) = $(1 - \eta_r)/c$. To eliminate the effects of non-ideality the reduced viscosity – or alternatively the inherent viscosity = $\ln\eta_{rel}/c$ – is extrapolated to zero concentration (via the Huggins and Kramer plots respectively) to yield the intrinsic viscosity $[\eta]$ which is an intrinsic function of the properties of the macromolecule. If the viscometer is sensitive enough then a concentration extrapolation may not be necessary and $[\eta] \approx \eta_{red}$ at high dilution. A better approximation is given by the Solomon-Ciuta expression[2]

$$[\eta] \simeq (1/c).\ [2\{\eta_{sp} - \ln(\eta_{rel})]^{1/2} \qquad (1)$$

The relative viscosity itself can be measured using conventional capillary or "Ostwald" viscometers – controlled to a temperature of at least $\pm 0.01^{o}$C – provided there is no shear dependence or "non-Newtonian" behaviour of the measured $[\eta]$. If there is, then a rolling ball viscometer of the type for example manufactured by Anton-Paar Instruments (Graz, Austria) using a steel ball (coated with silane, teflon or other inert material) rolling through solution in a capillary at different set roll angles - with extrapolation to zero angle (where the shear rate$\rightarrow$0), may be useful[3]. Alternatively conventional "cone and plate" rheometers – where shear stresses and shear rates are measured directly - can be used to correct for any shear effects[4]. Another potential complication is one of coil overlap above a certain concentration, usually represented as c*. An approximate relation linking c* and $[\eta]$ has been given[5]:

$$c^* \approx \ 3.3/[\eta] \qquad (2)$$

The intrinsic viscosity depends on the conformation, flexibility and volume of the polymer. The strong hydrodynamic interactions between the segments of a polymer mean that effectively a polymer chain can normally be considered as a single hydrodynamic particle as considered for example by Tanford and others[6], with solvent between the segments of a polymer moving with the polymer. Departure from expected behaviour is sometimes explained in terms of partial draining of the solvent[7].

The dependence of the intrinsic viscosity on molar mass is strongly dependent on the conformation and flexibility, and the simplest representation of this is the Mark-Houwink-Kuhn-Sakurada (MHKS) equation (see for example refs 8 & 9 & references therein):

$$[\eta] = K_{visc}.M^{a} \qquad (3)$$

where $a$ is the MHKS or "power law" coefficient, and $K_{visc}$ is a coefficient which also depends on the conformation: both can be estimated from a plot of log$[\eta]$ vs logM. The most useful parameter is $a$: for a sphere $a$=0, for a (non-draining) random coil $a$~0.5-0.8 and for a stiff molecule $a$>1.0 with the limit $a$~1.8. It can be seen from these values that for spheroidal/globular molecules such as many proteins, although $[\eta]$ is very sensitive to change in conformation – as in the classical demonstration of the thermal denaturation of ribonuclease[10] – $[\eta]$ is not a useful measure of change in molar mass through degradation or aggregation. By contrast for most polysaccharides – whose conformations are generally between those of coils and rods – $[\eta]$ is a useful measure of change in M. An example of a straightforward stability evaluation using an Ostwald capillary viscometer (based on $\eta_{red}$ measurements at a concentration of 1 mg/ml) for the polycationic chitosan (poly N-acetyl glucosamine – chitin – deacetylated according to specification) is shown in Figure 1a. Here

the effects of different storage temperatures on the decay profiles for this material – being considered as a nasal mucoadhesive formulation[11] - were explored[12], showing that storage at 4°C rather than room temperature is desirable. Figure 1b shows an intrinsic viscosity determination using the Solomon-Ciuta equation for 3 concentrations of a lignin dissolved in dimethyl sulphoxide (DMSO)[13].

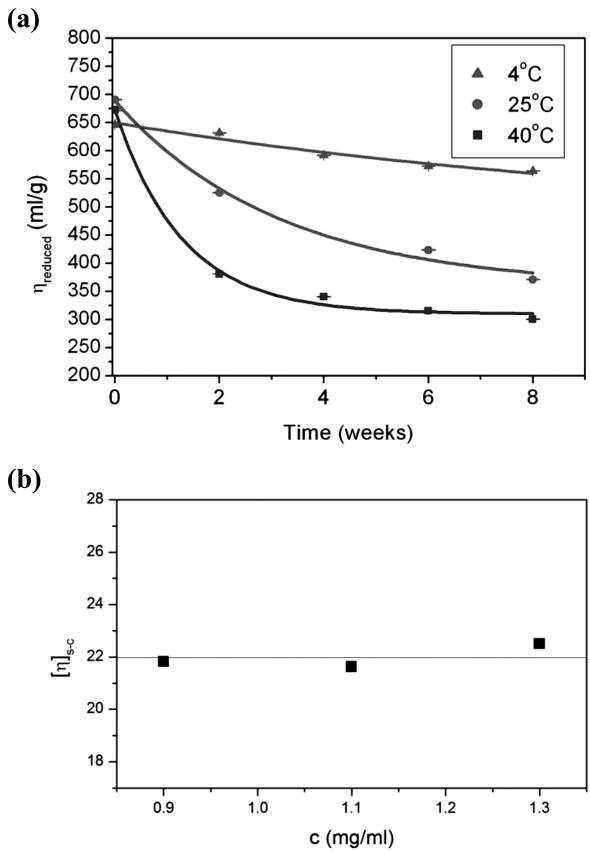


**Figure 1** *(a) Intrinsic viscosity [η] or reduced specific viscosity $\eta_{red}$ measurements can be used as a simple to use assay for stability of polysaccharide dispersions. Stability of chitosan (CL210) solutions (1 mg/ml in a pH 4.0, I=0.2M aqueous solvent) in response to long term storage at different temperatures[12]. Decay constants k = 0.087 week$^{-1}$ (4°C), 0.317 week$^{-1}$ (20°C) and 0.775 week$^{-1}$ (40°C). (b) Intrinsic viscosity measurements using the Solomon Ciuta equation for a lignin solublised in dimethyl sulphoxide[13]*

## 2.1 Viscometry pivotal to one of the greatest discoveries of the 20th Century

The most famous experiment using capillary viscometry on a carbohydrate polymer was that conducted by J.M. Creeth in 1947. Creeth – a PhD student at University College Nottingham supervised by D.O. Jordan and J.M. Gulland – examined the effects of extremes of pH on the relative viscosity of highly purified calf-thymus DNA, whose purity had been checked by the then fledging technique of analytical ultracentrifugation. At the

extremes there was a huge drop in relative viscosity corresponding to titration/ disruption of the hydrogen bonds between the bases and break-up of the DNA molecule. This experiment – proving conclusively the existence of H-bonds in DNA – was crucial to Watson and Crick's subsequent discovery of the double helix several years later. Creeth himself produced his own early model for DNA (Figure 2b) – two chains, sugar residues and phosphate links on the outside of the molecule – and paired bases on the inside, all the essential features apart from one important detail - the helix. Although Figure 2 appeared in his Doctoral thesis of 1947[14], only Figure 2a appeared in the publication in the *Journal of the Chemical Society*[14], his supervisors regarded Figure 2b as "too speculative". This work has generally remained un-recognised until very recently[16-18].

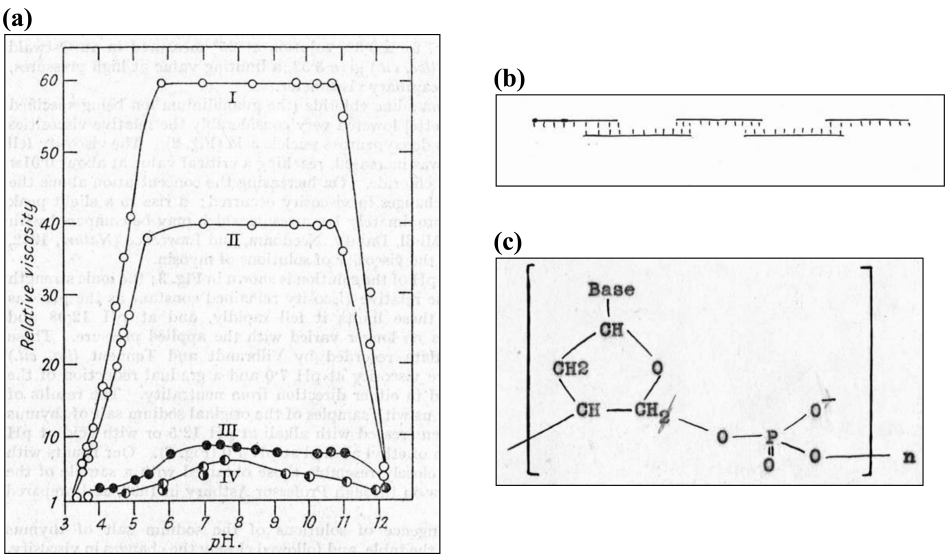


**Figure 2** *(a) Plot of relative viscosity versus pH of various preparations of calf thymus DNA showing the titration out of hydrogen (H) - bonds at low and high pH[15]. A similar diagram appears in Creeth's PhD thesis[14] (b) Sketch of a model for DNA from Creeth's PhD thesis of 1947 showing two broken chains held together by H-bonds, and (c) an expanded sketch of the sugar phosphate backbone with the phosphates on the outside of the chains*

### 2.2 Viscosity studies on stability against irradiation and thermal degradation

An earlier study on high molar mass carbohydrate stability was on the effects of irradiation of starches. Irradiation by γ-radiation is one of the methods used to preserve foods at low doses (<10 kGy) although as with other methods of preservation (particularly thermal processing) damage to the foodstuff itself is an inevitable risk. Greenwood and MacKenzie[19] measured the effects of γ-irradiation dose (up to 100kGy) on the intrinsic viscosity of potato amylose, an α(1-4) linked glucan. A dramatic decrease was observed with increase in dose, which these researchers interpreted in terms of decrease in degree of polymerization. To make this interpretation they first of all assumed values for $K_{visc}$ and *a* in the MHKS equation (Equation 1) to convert intrinsic viscosities to molar masses, and

then the degree of polymerisation *p* is simply M/162, where 162g/mol is the molar mass of a glucose residue (Table 1).

**Table 1** Viscometry study on the effect of γ-irradiation dose on amylose

| Dose (kGy) | Intrinsic viscosity [η] ml/g | Degree of polymerization *p* |
|---|---|---|
| 0 | 230 | 1700 |
| 0.5 | 220 | 1650 |
| 1 | 150 | 1100 |
| 2 | 110 | 800 |
| 5 | 95 | 700 |
| 10 | 80 | 600 |
| 20 | 50 | 350 |
| 50 | 40 | 300 |
| 100 | 35 | 250 |

Adapted from Greenwood and MacKenzie[19]

Bradley and Mitchell[20] also used the MHKS equation (Equation 3) to convert intrinsic viscosities to molecular weights in their comparative investigation of the kinetics of degradation of three polysaccharides, namely alginate, carboxymethylcellulose and κ-carrageenan – exposed to temperatures over 100°C. A specially designed high-temperature slit viscometer was used and zero shear viscosities were converted to intrinsic viscosities [η] using an approximate relation involving the coil overlap parameter c* (Equation 2). Then using the MHKS relation (Equation 3) and published values for $K_{visc}$ and the power law coefficient "*a*" they were able to obtain approximate estimates of molar masses and from the slope of the reciprocal molar mass 1/M versus time they were also able to obtain first order rate constants for the degradation of the polymers. Figure 3 shows the degradation of a 15 mg/ml solution of κ-carrageenan at high temperature (118°C) in terms of the molar mass calculated in this way. From the kinetic curves and Arrhenius type of analysis these researchers were able to estimate the activation energies of depolymerisation for the 3 polysaccharides showing that alginate was less stable than carboxymethylcellulose or κ-carrageenan.

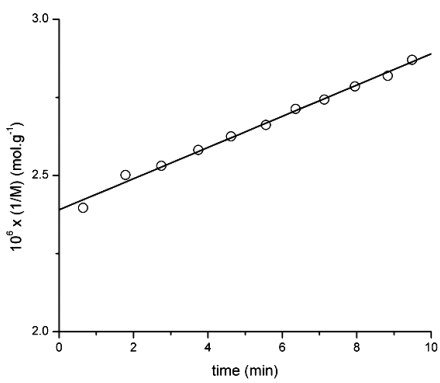


**Figure 3** *Thermal degradation of κ-carrageenan at 118°C, using a specially adapted slit viscometer. Apparent molecular weights M of 15 mg/ml solutions were estimated using approximate relations from zero shear viscosity measurements. Adapted from Bradley and Mitchell*[20]

Another very useful viscometer is the Differential Pressure viscometer (see ref 1 & references therein), which uses a sensitive pressure transducer to measure the difference in pressure between solution and solvent flow through metal capillaries to evaluate the relative viscosity $\eta_r$. The flow is driven by a pump at constant rate. The three advantages of this system for carbohydrate polymers are[1] (i) it can be coupled to a size-exclusion chromatography column so that polydisperse systems can in principle be separated (ii) a concentration detector can be coupled on-line so that relative viscosities can be converted to reduced viscosities, $\eta_{red}$ (ml/g), and (iii) because of the great sensitivity of the pressure transducers, low concentrations are usually only needed, often sufficient so that the approximation $[\eta] \approx \eta_{red}$ is valid. If it is not valid then the Solomon-Ciuta expression, Equation 1, can be utilised. The very low concentrations means also that the Newtonian approximation may also be more reasonable. A further advantage is that a multi – angle light scattering detector can also be coupled on-line to allow simultaneous measurement of $[\eta]$ and molar mass M, which can be very useful for carbohydrates as we shall see below. A disadvantage compared to the traditional glass capillary viscometers is that for aqueous systems in salt the metal surfaces can be more sensitive to corrosion over a period of time – appropriate coating is therefore advisable.

## 3 DYNAMIC LIGHT SCATTERING

Dynamic light scattering has become an increasingly popular tool for estimating the hydrodynamic radii of polymers and polymeric assemblies[21]: the instrumentation commercially available is relatively inexpensive (particularly for the single or dual angle instruments) and the method relatively rapid: this makes it particularly attractive for the investigation of changes in the molecular integrity of a system (through aggregation of degradation) with time.

From analysis of the fluctuations in intensity of scattered light caused by the (Brownian) motions of particles it is possible to estimate the translational diffusion coefficient $D_t$. For carbohydrate polymers and other polydisperse systems it is possible to specify both the *average* $D_t$ – which will be a z-average[22,23], but also from the routine CONTIN[24] and related types of analysis, a *distribution* of diffusion coefficients. $D_t$ values are popularly directly translated into hydrodynamic radii $r_h$ using the Stokes-Einstein relation

$$r_h = k_B T/\{6\pi\eta_o D_t\} \qquad (4)$$

with $k_B$ Boltzmann's constant, T the absolute temperature and $\eta_o$ the solvent viscosity. Great care has to be taken with ensuring the samples (and scattering vessels) are free of dust – choice of the appropriate filter (conventionally ~ 0.2 – 0.5um pore size) is important, although the modern versions of CONTIN are usually quite good at separating the signal of large particulates from the polymers in solution. With polysaccharides and other carbohydrate polymers though a word of caution is necessary particularly for the use of the single or dual angle photometers. The decay of the intensity autocorrelation function used to calculate the translational diffusion coefficient depends, for non-spherical particles on other factors most notably internal flexibilities and rotational diffusion contributions. Classically the way of eliminating these other contributions – as outlined clearly by Burchard[25] – is to extrapolate the first cumulant in the autocorrelation decay function (or equivalently the measured "apparent" translational diffusion coefficient, $D_{t,\theta}$)

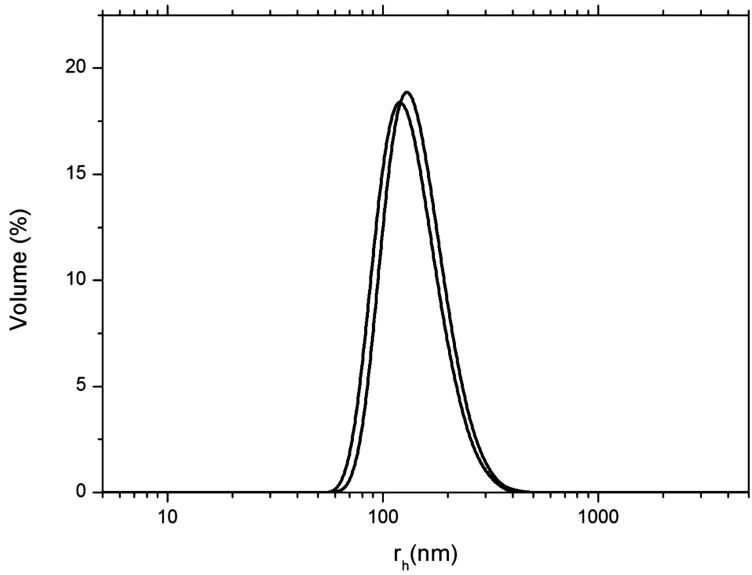


**Figure 4** *Distribution of hydrodynamic radius $r_h$ for an xanthan preparation in a phosphate chloride buffer (pH=6.8, I=0.3M) from a dynamic light scattering photometer (Malvern nano-S) at a scattering angle of 12$^o$ and a loading concentration of 0.4 mg/ml. Two measurements are shown with z-average $r_h$ values of 120nm and 130nm respectively. At the low angle and concentration used, non-translational diffusion and non-ideality effects are assumed respectively to be negligible. From ref. 26.*

to zero angle. This is possible with conventional multi-angle instruments, but not possible with the modern fixed angle instruments. If a fixed angle instrument is being used it is essential that the scattering angle of the detector is low enough: an example of a determination on a preparation xanthan at a scattering angle $\theta = 12^o$ is shown in Figure 4[26].

Care also has to be taken of concentration dependence (non-ideality) effects – although the effects are usually less severe compared to the case for intrinsic viscosity: the measured apparent translational diffusion coefficient at concentration c, $D_{t,c}$ is related to the true or "ideal" $D_t$ (=$D^o_t$) by the relation[27,28]

$$D_{t,c} = D^o_t (1 + k_D c) \quad (5)$$

where $k_D$ is the concentration dependence coefficient. Usually the approximation $D_{t,c} \approx D^o_t$ is made. In cases where both the angular approximation and also the concentration dependence approximation are inappropriate, Burchard[25] introduced the concept of a dual extrapolation plot known as a *Dynamic Zimm Plot*.

It is possible also to transform distributions of $D_t$ or $r_h$ into distributions of molar mass using a power law relation analogous to Equation (3)[8]:

$$D_t = K_{diff}.M^{-\varepsilon} \quad (6)$$

This is commonly employed for globular proteins[29] where $K_{diff}$ and ε are well defined, but not so useful for carbohydrate polymers where conformations are less well defined. Classical or "static" light scattering procedures where the time averaged intensity is measured as a function of angle are by contrast better suited for providing molar mass averages and distributions and without the need for calibration standards.

## 4 SEC-MALS: SIZE EXCLUSION CHROMATOGRAPHY COUPLED TO MULTI-ANGLE LIGHT SCATTERING

The idea proposed over two decades ago by P.J. Wyatt[30] of coupling a classical multi-angle laser light scattering or "MALLS" photometer to size exclusion chromatography (SEC) led to one of the most significant inventions for characterizing the molar mass and molar mass distributions of polymers (n.b. the acronym MALLS has now been changed to MALS since lasers are almost universally used as the light source for light scattering experiments). SEC provides separation of molecules for a polydisperse polymer system and the fractions elute on-line into a flow cell in the MALS designed so that an instantaneous snapshot of the angular scattered intensity envelope is measured for each elution volume (V) passing through. If the concentration c(V) is also known as a function of elution volume (by coupling a concentration detector – usually refractive index based – also on-line) then the (weight average) molar mass $M_w(V)$ as a function of V can be specified and the corresponding overall weight average $M_w$ (and also the number average $M_n$ and z-average $M_z$ molar masses) for the distribution - or between user specified limits of the distribution - specified. For carbohydrate polymers since the very first measurements on polysaccharides by Horton et al[31] and Rollings[32], and on mucins by Jumel et al[33] the method has become almost routine for these substances with large numbers of publications every year reporting its successful use. An early example of application to the study of glycoconjugate stability was the effect of treating mucin glycoproteins to degrading agents[34].

As with viscosity and DLS non-ideality is a consideration – measured molecular weights at a finite concentration, c, will be apparent ones, and normally an extrapolation to c=0 is necessary. However the high sensitivity of the photodiode detectors is such that single low concentrations can be injected, which are then diluted by the columns, rendering non-ideality effects small.

### 4.1 Effect of irradiation on guar

Jumel and colleagues[35] also used intrinsic viscosity measurements reinforced by SEC-MALS measurements of the weight average molar mass $M_w$ and molar mass distribution, reinforced by sedimentation coefficient measurements using the analytical ultracentrifuge to evaluate the effects of gamma irradiation on the macromolecular integrity of guar, a galactomannan with a β(1-4) linked mannan backbone with α(1-4) linked galactose side chains. $M_w$ values for the guar in response to level of irradiation (up to 9kGy) showed a 5 fold drop (Table 2) and the distributions of molar mass became correspondingly broader (Figure 5). On the basis of the molecular weight data these researchers defined an index for quantifying the incremental degree of disruption called the scission index $G_{scission}$:

$$G_{scission} = \{S_{1000} \times 100\} / \{dose(eV/g) \times g(1000\ bonds)^{-1}\} \quad (7)$$

**Table 2** *Effect of γ-irradiation dose on guar (adapted from ref. 35)*

| Dose (kGy) | $10^{-6}$ x $M_w$ g.mol$^{-1}$ | Intrinsic viscosity [η] ml.g$^{-1}$ | Scission index $G_{scission}$ |
|---|---|---|---|
| 0 | 2.70 | 1576 | - |
| 0.11 | 2.03 | 1467 | 10.34 |
| 0.20 | 2.32 | 1360 | 2.96 |
| 0.37 | 1.78 | 1360 | 5.00 |
| 0.50 | 1.87 | 957 | 3.14 |
| 0.65 | 1.66 | 1092 | 3.37 |
| 0.86 | 1.49 | 894 | 3.40 |
| 1.70 | 1.24 | 964 | 2.48 |
| 5.07 | 0.866 | 736 | 1.49 |
| 9.07 | 0.565 | 471 | 1.48 |

where 1 Gray (Gy) = 6.24 x $10^{15}$ eV.g$^{-1}$. $S_{1000}$ is the number of scissions per 1000 glycosidic bonds, defined by $S_{1000} \sim 1000\{p_o^{-1} - p^{-1}\}$ where $p_o$ is the degree of polymerization of non-irradiated guar. Table 2 shows the incremental G values as the dose is increased. It can be seen that as the chains become shorter and shorter, the chains become more difficult to break.

A double logarithmic Mark-Houwink-Kuhn-Sakurada plot of [η] with $M_w$ yielded an MHKS coefficient $a$ ~0.73, consistent with other estimates for guar indicating a very flexible conformation[36-38]. The conclusion was that for guar, although irradiation produces significant chain scission, it seems to have little effect on chain conformation, at least for doses below 10kGy.

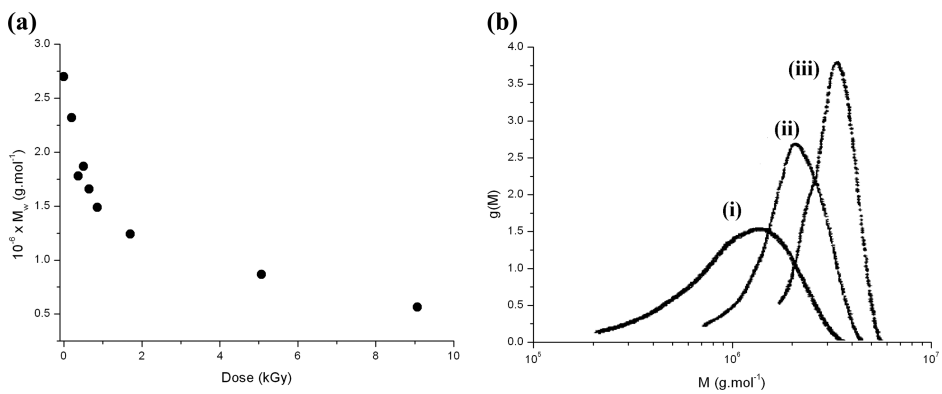


**Figure 5** *SEC-MALS evaluation of the effect of γ-irradiation degradation of guar. (a) Weight average molar masses fall dramatically, and (b) the distributions also become correspondingly broader (i) 1.70kGy irradiated guar (ii) 0.20kGy irradiated (iii) non-irradiated guar. Adapted from Jumel et al*[35]

### 4.2 Coupling an on-line differential pressure viscometer

It is possible to couple on-line a differential pressure viscometer as described in section 2 above. This permits simultaneous determination of [η](V) and $M_w$(V) to be made allowing (i) a distribution of [η] to be specified and hence weight and other average values for [η] to be estimated (ii) a plot of [η](V) versus $M_w$(V) can be used to estimate the MHKS "*a*" parameter and an example is shown in Figure 6.

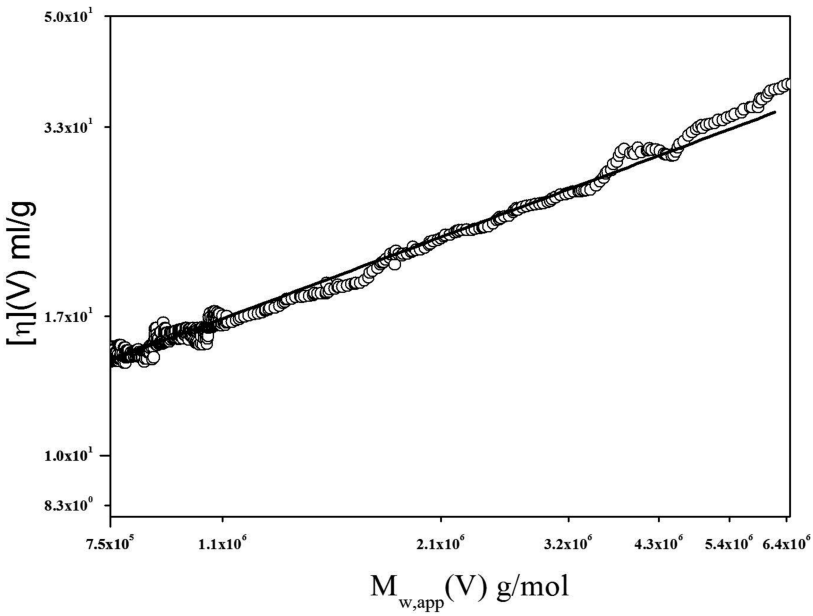


**Figure 6** *Plot of [η](V) versus $M_w$(V) for Kikar gum polysaccharide in phosphate chloride buffer, pH=6.8, I=0.1M. The slope of this plot = (0.431±0.002). From ref. 39*

### 4.3 Radius of gyration

For polymers of size larger than 1/20$^{th}$ of the wavelength λ of the laser light used (this is normally between 500-600nm, so particles with maximum dimension larger than ~ 25nm) it is possible to also obtain from the MALS – either in batch mode[25] or coupled with SEC[30] - a useful conformation parameter from the angular dependence of the scattered light – the radius of gyration $R_g$ - both as an average over the distribution and, with SEC-MALS, also as a function of elution volume, $R_g$(V) vs V. This too can be related to molar mass via a power-law relation[8,9] analagous to equations (3) and (6)

$$R_g = K_{LS}.M^c \qquad (8)$$

for a sphere *c*~0.33, for a random coil *c*~0.5-0.6 and for a stiff rod *c*~1.

### 4.4 Larger molar masses

A limitation of the SEC-MALS approach is the separation range of the SEC columns which tend to have an upper limit of molar mass of ~ 3 x $10^6$ g/mol. For carbohydrate assemblies above this size alternative separation media can be employed, and the use of Field Flow Fractionation with appropriate membranes has been a popular choice, successfully used to characterize solubilized starches up to a molar mass[40] of ~ 5x$10^8$ g/mol. For some types of carbohydrate polymer – particularly cationic ones like amino-celluloses – it can be difficult finding columns (or membranes) that are sufficiently inert, and column/membrane based methods are difficult to apply to the study of reversibly associating or dissociating systems because of the variability in concentration. For these systems analytical ultracentrifugation provides a complementary approach, giving separation without the need for a separation medium.

## 5 ANALYTICAL ULTRACENTRIFUGATION

Like viscosity and light scattering this method is not new – T. Svedberg received the Nobel Prize for its invention in 1926 – although its modern implementation is proving a very useful tool for probing molecular integrity. At lower rotor speeds, the sedimentation force due to the centrifugal field and the backforce due to diffusion are comparable. After a considerable period of time (usually >24h) the two forces come to equilibrium: a *sedimentation equilibrium* experiment can provide absolute molar mass - primarily weight (mass) and z-averages - and molar mass distribution, analogous to what MALS can provide. The method is column (or membrane) free, but takes much longer than a MALS experiment. At much higher rotor speeds (up to 50,000 rev/min) the centrifugal force dominates, and a *sedimentation velocity* experiment can provide us with information on the physical homogeneity of a, sample, conformation and flexibility information – and if the conformation type of the polymer is known (sphere/rod/coil) an estimate of the molar mass distribution. It can also provide us with interaction information if, for example we assay for what is called “co-sedimentation” phenomena, namely different species sedimenting at the same rate[41,42].

### 5.1 Sedimentation equilibrium

Rotor speeds of between 2000 and 20000 rev/min are usually chosen depending on the size range of the polymer/polymer assembly, and equilibrium concentration solute distributions c(r) as a function of radial displacement r from the axis of rotation are, for carbohydrate polymer systems usually recorded using Rayleigh interference optics (Figure 7a). For mucins and other glycoproteins with sufficient protein/ uv absorbing amino acids like tyrosine, tryptophan and phenylalanine, or lignins which absorb in the uv/visible, absorbtion optics can also be used. Concentration c(r) vs radial position, r, distributions can be transformed into whole distribution apparent weight average molar masses $M_{w,app}$ using a transformation parameter known as the M* function[44] (Figure 7b), popularly incorporated into a routine known as MSTAR[43]. In addition weight average molar masses $M_{w,app}(r)$ and z-average molar masses $M_{z,app}(r)$ plots can be calculated for individual radial positions r and hence $M_{w,app}(r)$ and $M_{z,app}(r)$ as a function of concentration c(r) can be obtained, particularly important for the evaluation of reversibly aggregating systems. Such representations have recently been used to help demonstrate a reversible association in aminopolysaccharides (Figure 7c)[45].

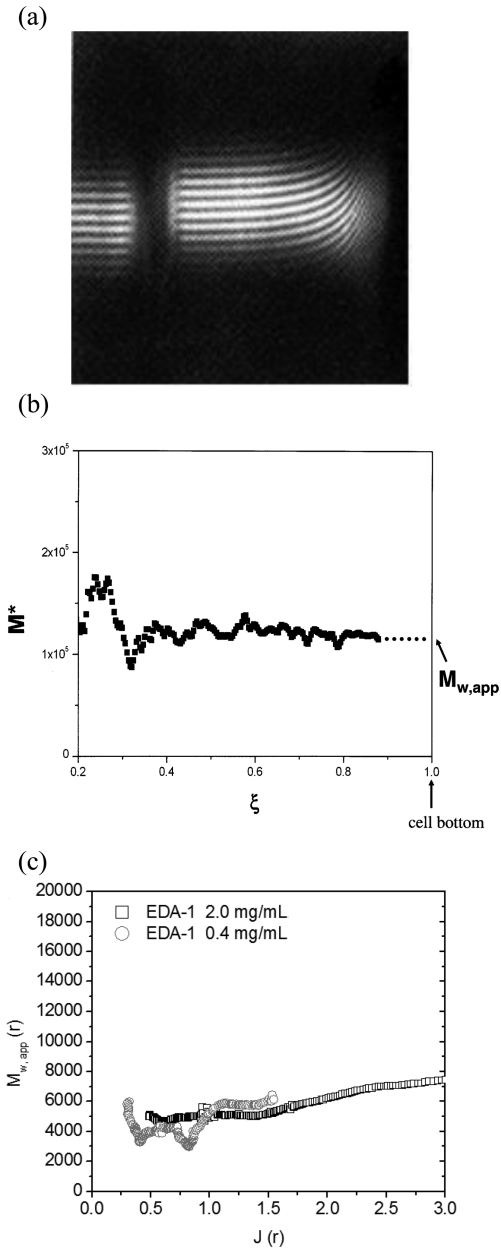


**Figure 7** *Sedimentation equilibrium of carbohydrate polymers.*

*(a) Rayleigh interference fringes for a bronchial mucin ($M_w$ ~ 6.0 x $10^6$ g/mol), at a loading concentration of ~0.2 mg/ml and rotor speed 1967 rev/min. Each profile represents a plot of solute concentration relative to the meniscus*[43].

*(b) MSTAR analysis for evaluation of $M_{w,app}$ for a chitosan (loading concentration 1.0mg/ml in 0.2M acetate buffer). Fringe displacements in the vertical direction can be converted to an operational molar mass parameter $M^*(\xi)$*[44] *where $\xi$, is a normalised radial displacement (r) squared parameter: $\xi = (r^2 - r_a^2)/(r_b^2 - r_a^2)$ and where $r_a$ and $r_b$ the radial positions at the solution meniscus and cell base respectively. $M_{w,app}$ – the whole distribution (apparent) weight average - is obtained from the identity $M_{w,app} = M^* (\xi \rightarrow 1) = 110{,}000$ g/mol.*

*(c) It is possible also to measure the weight average molecular weights at different radial positions and hence different concentrations in the centrifuge cell: plot of "point" average molecular weight versus concentration (in fringe displacement units J(r) for an amino-cellulose at 2 different loading concentrations. The overlap of the two data-sets is symptomatic of a reversible self-association*[45]

As with viscometry and light scattering, corrections for non-ideality are required, and so to evaluate $M_w$, $M_z$ values from $M_{w,app}$, $M_{z,app}$, an extrapolation to c=0 is performed. At dilute solution for example:

$$(1/M_{w,app}) = (1/M_w)\,(1 + 2BM_wc) \tag{9}$$

where B is the osmotic pressure 2nd virial coefficient. At very high dilution (~0.2mg/ml) the approximation $M_w \approx M_{w,app}$ can often be made (and $M_z \approx M_{z,app}$). Conversely at higher concentrations additional virial terms may be required as shown for κ-carrageenan (Figure 7c)[46].

### 5.2 Sedimentation velocity

The change in the radial concentration distribution profile (as with sedimentation equilibrium usually recorded using Rayleigh interference optics) with time under the influence of a strong centrifugal field (with rotor speeds usually much higher than for sedimentation equilibrium - up to 50,000 rev/min), can be transformed into a distribution of sedimentation coefficient s (in Svedberg units), or g(s) versus s plot, giving a direct measure of the heterogeneity of a sample[47]. An example is given in Figure 8 for starch[48] - the method has been used for example by Tester *et al.*[49] for the analysis of damage by ball milling processing to way, pea and maize starches. The width of a g(s) vs s profile will be affected by diffusion broadening. This can be corrected for (at least partially) to give what is known as a c(s) vs s profile, and this procedure has recently been used (reinforced by g(s) vs s measurements) to show protein-like oligomerisation of aminocelluloses[50]. Distribution software g(s) vs s or c(s) vs s is popularly incorporated into the routine SEDFIT by P. Schuck[51].

The sedimentation coefficient itself (measured at a concentration c) depends on the molar mass, shape and volume (including the effects of hydration) of the macromolecule. Like the other hydrodynamic paramers referred to above, it also needs correcting for non-ideality effects using for example this dilute solution equation to give the "ideal value"[42] $s^o$.

$$(1/s_c) = (1/s^o)(1+k_sc) \tag{10}$$

A combination of $s^o$ with M can yield the frictional coefficient ratio (ratio of the frictional coefficient of a macromolecule to a spherical particle of the same anhydrous mass) – measure of the conformation and hydration of the macromolecule. The ratio of $k_s/[\eta]$ – the "Wales van Holde ratio" is also a measure of conformation – these aspects are considered further in the following chapter in this volume by Morris *et al*. The sedimentation coefficient $s^o$ and translational diffusion coefficient $D^o_t$ can also be combined together (eliminating the frictional contribution) to yield an absolute measure of molar mass via the Svedberg equation[52]. For a polydisperse system Pusey has shown[53,54] that a combination of the (z-average) diffusion coefficient and (weight average) sedimentation coefficient yields the weight average molar mass $M_w$.

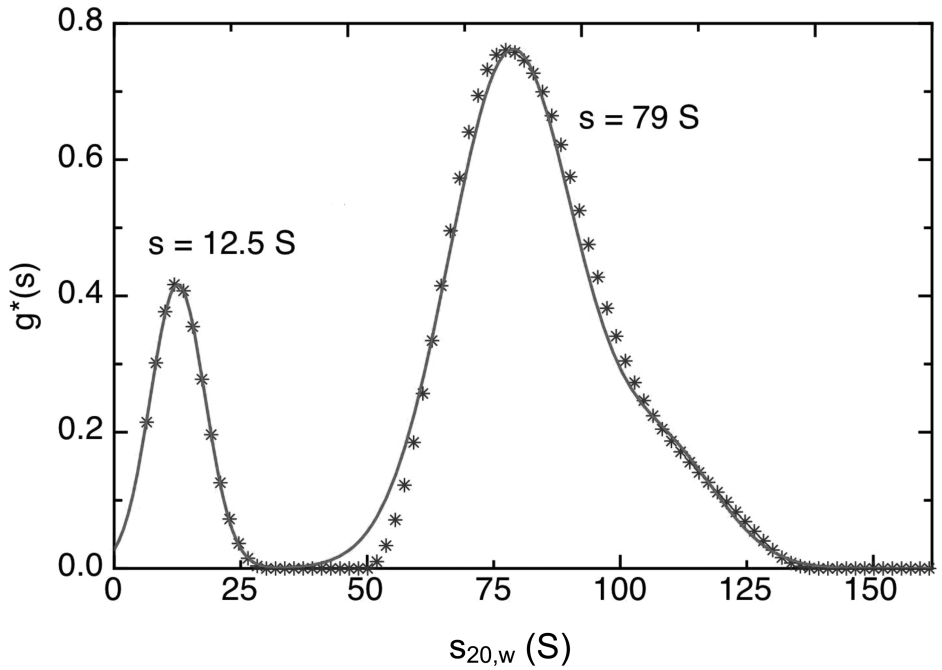


**Figure 8** *Sedimentation coefficient distribution: a g(s) versus s plot for wheat starch showing two components, namely amylose (left peak) and the faster moving amylopectin, (right peak). The total sample concentration 8 mg/ml dissolved in 90% dimethyl sulphoxide. Rotor speed was 35000 rpm at a temperature of 20 °C*[48].

### 5.3 Combination with SEC-MALS and viscometry

An example of how analytical ultracentrifugation, SEC-MALS and viscometry can be combined together to give useful information on polysaccharide stability was given in a recent study by Patel *et al.*[52] on the effects of γ-irradiation in the molecular integrity of xyloglucans. β(1-4) linked glucan with α(1-6) linked xylose single residue side chains sometimes capped with galactomannan. Molar mass, intrinsic viscosity and sedimentation coefficient all showed significant reductions with increase in dosage (Table 3). Unlike with guar (Table 2) there seemed to be no noticeable increase in the polydispersity ratio ($M_z/M_w$).

As with guar, it is possible to use the data-sets for intrinsic viscosity and molecular weight together to assess the conformation using the MHKS type of relation, and in so doing Patel *et al.*[55] obtained a value of 0.55±0.03, within the expected range of a random coil (0.5-0.8). Furthermore, the sedimentation coefficient – molecular weight dataset allowed a further power-law type of analysis

$$s = K''M^{b}. \qquad (11)$$

**Table 3** *Effect of γ-irradiation dose on the physical properties of xyloglucans*

| Dose (kGy) | $10^{-3}$ x $M_w$ g.mol$^{-1}$ | Polydispersity $M_w/M_n$ | Intrinsic viscosity [η] ml.g$^{-1}$ | Sedimentation coefficient $s^o_{20,w}$ (S)[a] | Persistence length $L_p$ (nm) |
|---|---|---|---|---|---|
| 0 | 700±5 | 1.1±0.1 | 405±35 | 7.21±0.03 | 6±1 |
| 10 | 270±10 | 1.3±0.1 | 210±10 | 4.66±0.03 | 6±1 |
| 20 | 158±3 | 1.4±0.1 | 170±10 | 3.10±0.04 | 9±1 |
| 30 | 127±10 | 1.3±0.1 | 140±10 | 3.30±0.01 | 6±1 |
| 40 | 97±10 | 1.3±0.1 | 135±5 | 2.82±0.04 | 8±1 |
| 50 | 60±4 | 1.3±0.1 | 100±5 | 2.80±0.08 | 6±1 |
| 70 | 45±3 | 1.1±0.1 | 75±5 | 2.61±0.02 | 6±2 |

Adapted from Patel *et al.*[55] [a]The superscript "o" means extrapolated to zero concentration to eliminate the effects of non-ideality. The subscript 20,w means the sedimentation coefficient has been corrected or normalized to standard conditions, namely the density and viscosity of water at 20°C. S is the Svedberg unit = $10^{-13}$ sec

yielding an estimate for *b* of 0.42±0.01, again between the expected limits for a random coil (0.4 – 0.5). All three datasets for $M_w$, [η] and $s^o_{20,w}$ could be combined into a recently developed global or HYDFIT plot procedure[56] – from combinations of the Bushin-Bohdanecky relations linking [η] with $M_w$ and the Yamakawa-Fujii relations linking $s^o_{20,w}$ with $M_w$ - and global minimization of a target function to yield estimates of a flexibility parameter known as the chain persistence length $L_p$ (the practical limits are ~2nm for the highly flexible pullulan and ~ 200nm for a stiff rod like conformation like the triple helical polysaccharide schizophyllan): values obtained for this procedure for xyloglucan samples as a function of dose damage are given in (Table 3) and it appears again, as with guar (Table 2), despite chain scission there is little change in the conformational flexibility of the chain, with individual values deviating little across the whole range of molecular weight. A further example for pectins is described in the following chapter by Morris *et al*.

### 5.5 Molecular weight distribution from sedimentation velocity.

In the original implementation of the SEDFIT algorithm, P. Schuck[51] provided the possibility of transforming the sedimentation coefficient distribution into a molar mass distribution for paucidisperse systems such as mixtures of proteins. This has been recently extended by us to the case of quasi-continuous polydisperse systems – the hallmark of carbohydrate polymers and mucins and glycoconjugates. This method, known as the "*extended Fujita approach*" is based on an earlier method given by Fujita[57] for random coils (applied to mucins[58]) but extended to cover general conformation types[59]. An example is given for a glycoconjugate vaccine characterization in Figure 10, a system too large for characterization by SEC-MALS. Further recent examples of its use have included mucins[60].

The new "Extended Fujita" approach has now been incorporated into the SEDFIT programme. A further version of the SEDFIT algorithm will have the MSTAR procedure (section 5.1) also incorporated into it[61].

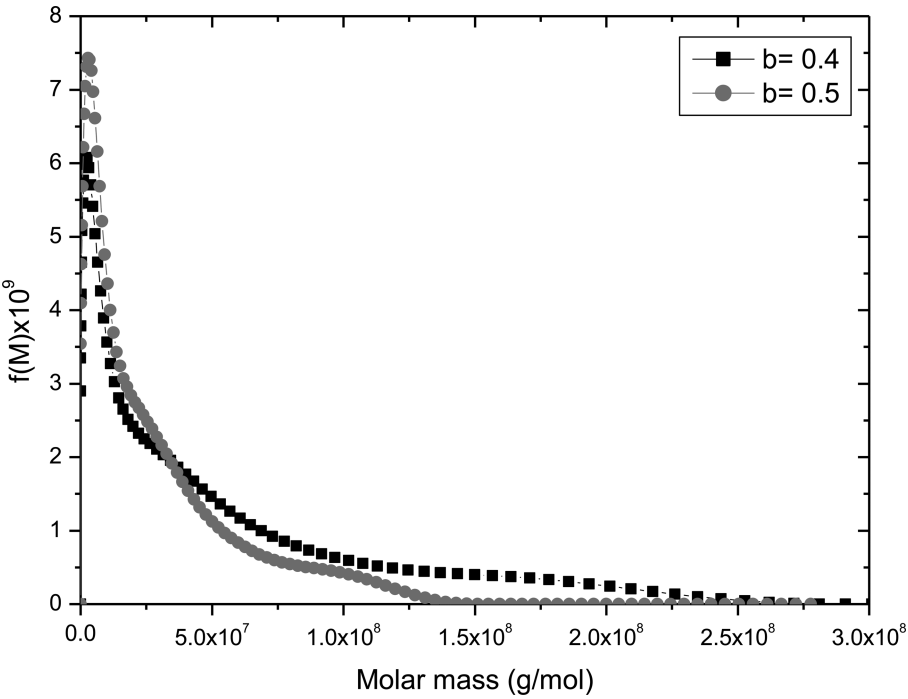


**Figure 9** *Molecular weight distribution for a large glycoconjugate vaccine construct of a protein and bacterial polysaccharide obtained from sedimentation velocity data and the method described in Harding et al.*[59]*. Loading concentration c ~ 0.3 mg/ml. The method requires an approximate idea of the overall conformation: distributions for two reasonable selections of the power law coefficient b (Equation 11) are shown*

## 6 PERSPECTIVES

This short overview has reviewed the principal hydrodynamic based methods for characterizing the molecular integrity (molar mass, state of aggregation/ degradation, conformation, flexibility and volume) of carbohydrate polymers and assemblies thereof. There are many aspects we haven't covered – mucoadhesive assemblies for example but hopefully the impression has been given that the methods considered are complementary and are particular powerful when combined together in a study, with each method providing different information on the one hand, but internal checks on the other. The following chapter by Morris *et al*. illustrates application of some of the methods described here to the study of pectins assemblies in the context of their use for efficient delivery of drugs.

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