On hydrodynamic methods for the analysis of the sizes and shapes of polysaccharides in dilute solution: a short review

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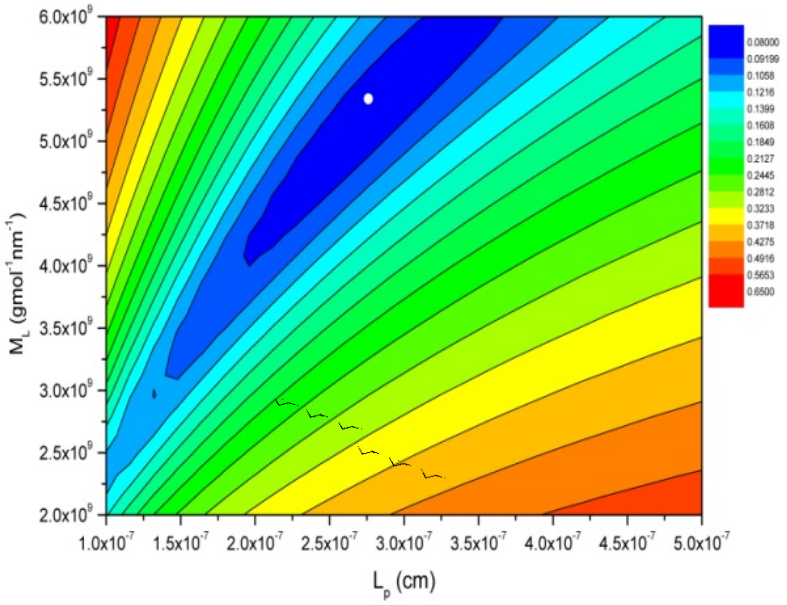
Abstract

Polysaccharides and their derivatives are increasingly being used by the food, cosmetic and pharmaceutical industries: physical properties like size and conformation are important contributors to their performance. Here the use of hydrodynamic tools such as sedimentation velocity, sedimentation equilibrium, size exclusion chromatography – multi-angle light scattering (SEC-MALS), and viscometry are considered highlighting some recent developments in methodology and the application of these to help better understand polysaccharide structure-function relationships.

Keywords: polysaccharides, size, conformation, structure - function relationships



Graphical Abstract: Solutions to the Bushin-Bohdanecky and Yamakawa-Fujii equations using equivalent radii approach for pullulan (inset). The x-axis and y-axis represent L_p (nm) and M_L ($g.mol^{-1}.nm^{-1}$) respectively. The calculated minimum is indicated (○). This result is consistent with random coil conformation, however excluded volume effects have *not* been taken into account.

Highlights

- Hydrodynamic methodologies for the characterisation of polysaccharides are reviewed
- Pullulan is used as a model “random coil” polysaccharide
- Simple estimates of conformation can be obtained from *e.g.* power-law coefficients
- Combining methods results in more sophisticated estimates *e.g.* persistence length

1. Introduction

The last two decades has seen considerable advances in hydrodynamic methodology for the analysis of the dilute solution properties of polysaccharides. Advances include improved ways in which we can ascertain the molecular weight (molar mass) or molecular weight distribution of polysaccharide systems using size exclusion chromatography coupled to multi angle light scattering (Wyatt, 1993) and sedimentation based techniques using the analytical ultracentrifuge (Harding, Abdelhameed and Morris, 2010; Schuck, Gillis, Besong, Almuntairi, Adams, Rowe and Harding, 2014). There have also been important advances in the way we can use these techniques in combination – and with other techniques like viscometry to characterize the shape and flexibility of polysaccharides in the environment in which many occur naturally – in solution. The focus of this article is to highlight some of the recent advances in hydrodynamic methodologies for estimating the size and conformation of polysaccharides.

2. Estimation of size

a. Sedimentation velocity (SV)

In a centrifugal field solute molecules will sediment towards the cell base, therefore the region near the meniscus will be depleted of solute and there will be a region nearer the cell base where the solute concentration is uniform and a transitional region (the "boundary region") where the solute concentration varies with distance from the axis of rotation is created. It is the rate of movement of the concentration distribution with time which allows the calculation of sedimentation coefficients and distribution of sedimentation coefficients (see *e.g.* van Holde, 1985; Ralston, 1993; Dam and Schuck, 2004). The progression of the concentration distribution with time is recorded by an optical system. Since polysaccharides are not usually absorbing in the visible or (near) ultraviolet, the refractometric or Rayleigh interference optical system is the most useful, using a laser light source. Double-sector cells are employed with solution and reference solvent (dialysate) in each channel and a series of parallel The Rayleigh interference fringes, captured on a CCD camera register the concentration distribution at regular time intervals throughout the experiment. The change in the distribution with time yields both the (weight average) sedimentation coefficient s (measured in seconds, s, or Svedberg units S, where $1\ S = 10^{-13}$ s) and the distribution of sedimentation distribution g(s).

(i) To facilitate comparisons, the s value – a measure of the size and shape of the polysaccharide - is usually corrected to standard conditions (density and viscosity of water at 20.0 ^{o}C), to give $s_{20,w}$, and this is usually easily done using a database algorithm known as SEDNTERP (Laue, Shah, Ridgeway and Pelletier, 1992).

(ii) to correct for non-ideality the s (or $s_{20,w}$) value is extrapolated to zero concentration to give $s^0_{20,w}$, using for example the Gralen relation:

$$\frac{1}{s_{20,w}} = \frac{1}{s^0_{20,w}}(1 + k_s c) \qquad (1)$$

where k_s (mL g^{-1}) is the concentration dependence regression coefficient. For more severely concentration dependent systems other relations such as the equation of Rowe (1992) can be used. Alternatively low loading concentrations can be employed (it is possible to make measurements below 0.1 mg mL^{-1}), when $s_{20,w} \sim s^0_{20,w}$ is a reasonable approximation.

(iii) besides non-ideality which needs to be accounted for as described above, the distribution g(s) vs. s will be affected by diffusion broadening (although polysaccharides are usually much slower diffusing compared to proteins). Dam and Schuck (2004) have described a procedure for making an approximate correction based on the assumption that all the species can be represented by an average frictional ratio. The diffusion corrected distribution is known as a c(s) vs. s plot.

(iv) g(s) and c(s) plots by themselves can provide a useful measure of heterogeneity (*e.g.* in mixed polysaccharide systems such as starch).

(v) g(s) vs. s (or c(s) vs s) plots can be converted into molecular weight distributions provided the conformation/ conformation type (sphere, rod, coil etc) of the polysaccharide is known or can be reasonably assumed. The procedure is known as the Extended Fujita method (Harding, Schuck, Abdelhameed, Adams, Kök, and Morris, 2011) and has recently been incorporated into

the highly popular SEDFIT platform of algorithms to estimate the molecular weight distribution (**Figures 1a and b**) of heterogeneous systems including polysaccharides and mucins (Harding, *et al.*, 2011; Gillis, *et al*, 2012).

Figure 1 here

One limitation is that this *Extended Fujita* method does need calibrating for each particular conformational system. The conformation coefficient *b* and constant $\square_s$ in the transformations:

$$M = (s/\kappa_s)^{1/b} \qquad (2)$$

and

$$f(M) = ds/dM.\ g(s) \qquad (3)$$

where

$$ds/dM = b.\square\kappa_s^{1/b}.s^{(b-1)/b} \qquad (4)$$

are needed; if the conformation is known then this will define b: random coils b ~ 0.4 - 0.5; spheres, b ~ 0.67, rod shaped molecules b ~ 0.2). Knowledge of the weight average sedimentation coefficient and corresponding weight average molecular weight from a sedimentation equilibrium experiment or SEC-MALS (Size Exclusion Chromatography coupled to Multi-Angle Light Scattering) can then be used to define κ_s, using Eq. 2.

If b is also unknown then a number of pairs of s-M values are required (see section 2.1 and **Figure 1b**).

b. Sedimentation equilibrium (SE)

In contrast to sedimentation velocity, sedimentation equilibrium requires lower angular velocities depending on the size of the macromolecule (van Holde, 1985). As the solute sediments towards the cell base the concentration therefore increases at base, this sets up a diffusion gradient, which opposes that of sedimentation. After a certain amount of time the two processes reach dynamic equilibrium leading to a steady state pattern of solute concentration increasing towards the cell base. As there is no net movement of solute at equilibrium the final pattern is not affected by frictional/conformation properties and is an absolute function of molecular weight and polydispersity. For thermodynamically non-ideal and polydisperse systems such as polysaccharides, solute distributions at sedimentation equilibrium can be analysed using the MSTAR algorithm (Cölfen and Harding, 1997), now recently incorporated into the SEDFIT platform of algorithms, as SEDFIT-MSTAR (Schuck, *et. al*., 2014). This yields an estimate for the apparent weight average molecular weight for the whole distribution, $M_{w,app}$ using both the M* function of Creeth and Harding (1982) and the hinge point method (the value of $M_{w,app}$ evaluated at the point in the distribution where the concentration = the initial loading concentration). An example of the output for pullulan P_{400} is given in **Figure 1c**.

In order to account for thermodynamic non-ideality, calculated apparent molecular weights should be extrapolated to zero concentration to yield the value corrected for non-ideality, M_w.

$$\frac{1}{M_{w,app}} = \frac{1}{M_w} + 2Bc \qquad (5)$$

where B is the 2nd thermodynamic (osmotic pressure) virial coefficient. At very low loading concentrations (the minimum is ~ 0.2 - 0.3 mg mL^{-1} using 20 mm path length cells), for some systems the approximation $M_w \sim M_{w,app}$ can be made. Conversely at higher concentrations and/ or highly non-ideal solutions such as alginate or xanthan higher order terms may be necessary.

c. Capillary viscometry

Viscosity can be measured in many different ways, the simplest being using an Ostwald viscometer. The rate of flow of a solvent through a capillary when driven by pressure will follow

Poiseuille's law. From this the ratio of viscosities can be given and is known as the relative viscosity,

$$\eta_{rel} = \left(\frac{t}{t_0}\right)\left(\frac{\rho}{\rho_0}\right) \tag{6}$$

where t is the flow time for the macromolecular solution, t_o is the flow time for the solvent. Due to the low concentration used (ρ/ρ_o) can often be taken as unity (see *e.g.* Harding, 1997). The specific (η_{sp}), viscosity is defined as follows:

$$\eta_{sp} = \eta_{rel} - 1 \tag{7}$$

and this, divided by concentration, c (g mL^{-1}) is known as the reduced specific viscosity, η_{sp}/c (mL g^{-1}). To eliminate non-ideality effects, measurements are made at different concentrations are extrapolated to infinite dilution using for example the Huggins (1942) or Kraemer (1938) approaches, or both:

$$\frac{\eta_{sp}}{c} = [\eta](1 + K_H[\eta]c) \tag{8a}$$

$$\frac{\ln(\eta_{rel})}{c} = [\eta](1 - K_K[\eta]c) \tag{8b}$$

where the intrinsic viscosity [η] is taken as the is the mean of the intercepts from equations (7a) and (7b) and K_H and K_K are the Huggins and Kraemer constants respectively.

A useful method for measuring intrinsic viscosities is to calculate the relative and specific viscosities at one concentration and utilise the Solomon-Ciutâ approximation (Solomon and Ciutâ, 1962). The intrinsic viscosity can then be accurately estimated (error generally ~1 %) by a single measurement at low concentration (see for example Morris, 2001).

$$[\eta] \approx \frac{\left(2\eta_{sp} - 2\ln(\eta_{rel})\right)^{1/2}}{c} \quad (8c)$$

d. Size exclusion chromatography (SEC)

Size exclusion chromatography (or "Gel Permeation Chromatography", GPC) is based on the simple principle of the separation of molecules due to size (hydrodynamic volume). The chromatographic column consists of a matrix of porous polymer beads and solute molecules will penetrate in and out of these pores, thus setting up equilibrium between the concentration (of solute) inside and outside the polymer beads. The volume of mobile phase inside and outside the pores is collectively known as V_M, and the internal pore volume V_i is essentially the stationary phase. The remaining mobile phase the interstitial liquid between the packing particles is the void volume, V_0.

The partition of solvent between phases can be described K_D ($0 \leq K_D \leq 1$), which is the ratio of average solute concentration inside and outside the pores and is independent of flow rates or column length. Therefore the total accessible volume for the solute is the retention volume V_R. If $K_D = 0$, then $V_R = V_0$ and the molecule is therefore too large to diffuse into the column matrix, this is known as the total exclusion volume, and when $K_D = 1$ the polymer can penetrate the entire bead matrix and $V_R = V_M$, which is called to total permeation volume. Retention in an SEC system is governed by changes in entropy between phases. However, the major disadvantage of a standalone SEC system is that one can only assign *relative* molecular weights (or relative hydrodynamic radii) by comparison with known standards, this relies on both the standards and sample of interest behaving at least similarly in the SEC columns and that non-size exclusion processes due to molecular charge etc are kept to a minimum. However *absolute* estimates of hydrodynamic properties can be calculated with the appropriate detection system:

i. Multi-Angle Light Scattering (MALS)

Light scattering is one of the few absolute, thermodynamically rigorously founded methods for the determination of molar masses and is therefore one of the most fundamental methods in polymer science. More detailed explanations of the principles of light scattering can be found in Wyatt (1993). However in brief most polysaccharides (with a molecular weight greater than ~

150 000 g mol^{-1}) have a radius of gyration $R_g > \lambda/20$. Larger molecular dimensions mean that a single molecule can have many scattering points and the light from these different scattering points will reach the detectors in different phases, due to intramolecular interference. Therefore as the Rayleigh factor, R_θ is a function of θ, the scattering intensity is reduced due to interference at all angles except zero. However, this internal interference depends on the size and shape of the macromolecule. Therefore the angular dependency in itself can yield important information on size and conformation. In practice R_0 is difficult to measure and is usually calculated by extrapolation of $R_\square$ to zero angle (Debye, 1946; Zimm, 1948). This has the added advantage of calculating R_g without any prior assumptions of shape (Tanford, 1961). With the addition of an on-line differential refractive index detector (or UV detector) one can calculate absolute concentrations and therefore M_w furthermore due to the high column dilution the extrapolation in infinite dilution is not required. Simultaneous determination of $M_w(V_e)$ and $R_g(V_e)$ for each value of the elution volume V_e in the chromatogram can be used to determine the power-law coefficients (see section 2a).

ii. Differential Pressure Viscometer (DPV)

This based on the theory of the 4-capillary bridge design (Haney, 1985a,b) and the differential pressure transducers measure both the inlet pressure (P_i) and the differential pressure across the midpoint of the bridge (ΔP). The application of Poiseuille's Law for the flow of fluids to these values for pressure can be used to calculate the specific viscosity.

$$\eta_{sp} = \frac{4\Delta P}{P_i - 2\Delta P} \qquad (9)$$

There the intrinsic viscosity can be estimated for as a function of elution volume V_e using the Solomon-Ciutâ approximation (eqn. 4c). Simultaneous determination of $M_w(V_e)$ and $[\eta](V_e)$ at each slice in the chromatogram can be used to determine the Mark-Houwink-Kuhn-Sakurada coefficients (see section 2a). Furthermore the weight-average viscosity called across the entire peak corresponds to bulk intrinsic viscosity measured using a traditional Ostwald capillary viscometer.

e. Dynamic light scattering (DLS)

Dynamic Light Scattering is the technique used to calculate translational diffusion coefficients, D_t. The hydrodynamic radius can also be calculated from the Stokes-Einstein equation.

$$r_H = \frac{k_B T}{6\pi\eta D_t} \qquad (10)$$

where k_B is the Boltzmann constant (1.381 x 10^{-16} erg K^{-1}); T is the absolute temperature (293 K) and η is viscosity of the solvent.

DLS measures the diffusion of a macromolecule within a solution due to Brownian motion and measures the intensity fluctuations of scattered light as a function of time (see, e.g. Harding, 1999). The rapidity of this fluctuation over time, is represented by the normalised intensity autocorrelation function, $g^{(2)}(\tau$ where the superscript (2) is indicative of intensity fluctuation. the decay in $g^{(2)}(\tau$ with "delay time" τ can be repeated many times and averaged and used to calculate the translation diffusion coefficient, D_t (cm^2s^{-1})

As with sedimentation coefficients, diffusion coefficients are concentration dependent and extrapolation to zero concentration may be necessary.

$$D_{20,w} = D^0_{20,w}(1 + k_D c) \qquad (11)$$

where $D^o_{20,w}$ is the translation diffusion coefficient at infinite dilution, $D_{20,w}$ is the value at concentration, c (g mL^{-1}) and k_D (mL g^{-1}) is the concentration dependency (Harding and Johnson, 1985).

There is a complication from the contribution of rotational diffusion effects and other anisotropic contributions. These effects extrapolate to zero at zero scattering angle, and Burchard (1992) has suggested a double extrapolation "Dynamic Zimm plot" to zero angle and zero concentration, illustrated with application to glycogen. Many modern instruments have only one or two fixed

angles, not permitting such an extrapolation, although measurement at a low angle (< 15^{o}) may provide a value close to the true value.

$D^{o}_{20,w}$ can then be combined with the sedimentation coefficient $s^{o}_{20,w}$ to provide an estimate for M_w via the Svedberg equation (Svedberg and Pedersen, 1940).

$$M_w = \frac{s^0 RT}{D^0(1-\bar{v}\rho)} \qquad (12)$$

where R is the universal gas constant (8.314 x 10^7 erg K^{-1} mol^{-1}); ρ is density of the solvent and $\bar{v}$ is the partial specific volume of the polysaccharide. Dynamic light scattering detectors can also be integrated into on-line with size exclusion chromatography system, although caution should be expressed with regards the angular extrapolation (or lack of). It is of upmost importance to keep solutions and cuvettes free from dust and/ or supramolecular material, although modern software can to some extent deconvolute this contribution from the overall scattering.

f. Asymmetric Flow Field Flow Fractionation (AF4)

Asymmetric flow field-flow fractionation (AF4) which is one of the sub-techniques in the field-flow fractionation (FFF) family is an analytical technique used for separating a wide range of macromolecules and colloidal particles at high resolution (Wahlund and Giddings, 1987). This method of separation is based on differences in the diffusion coefficient, which in turn reflects their size and shape (Nilsson, Birnbaum, and Wahlund, 1996). This technique is coupled to one or more detectors such as light scattering and refractive index. Unlike liquid chromatography, AF4 has no stationary phase and the separation is achieved solely by a flow in an empty channel, where a perpendicular flow force is applied. The channel consists of an upper solid wall which is impermeable to solvent and a lower (accumulation) wall permeable to solvents (Wahlund and Giddings, 1987; Pauck and Cölfen, 1998). Because the channel height is low, the flow through the channel is laminar. The laminar flow of the mobile phase creates a parabolic flow profile

within the channel; that is, the stream moves slower close to the channel walls than it does in the channel centre. Since separation is based on diffusion coefficient, the smaller molecules tends to elute faster than the larger molecules because they form less compressed dense zones than larger ones and will therefore occupy faster velocity vectors than larger molecules (Runyon, Ulmius and Nilsson, 2013).

Since the separation is governed by the translational diffusion coefficient D_t, it is therefore possible to calculate the diffusion coefficient from the retention time using the equation below:

$$D_t = \frac{w^2 V_c t_0}{6 V_0 t_r} \qquad (13)$$

where *w* is the channel thickness, V_0 the channel volume, t_r the retention time, t_0 the void time, V_c the applied cross flow. The above relationship is valid within 10 % if $t_r/t_0 \geq 2.4$. As with dynamic light scattering the effect of non-ideality on D_t needs to be considered carefully.

Table 1 details some estimates on the size of some important commercial polysaccharides.

Table 1 here

3. Estimation of solution conformation

Although in the previous section the main hydrodynamic techniques have in general been discussed individually it is of course possible to combine two or more different types of measurement to give a more detailed picture of hydrodynamic structure (Harding 1995, Amorós, Ortega and García de la Torre, 2011).

For instance one can compare the M_w values from the two independent and absolute techniques of SEC-MALS and low speed sedimentation equilibrium. Molecular weights can also be related to $[\eta]$, $s^0_{20,w}$, r_g (r_H) and $D^0_{20,w}$ through a series of Mark-Houwink-Kuhn-Sakurada (MHKS) or "power law relations" (equations 10a – d). Although strictly speaking MHKS only applies to the

viscosity relation the relations are now popularly called MHKS power law relations (Harding, Vårum, Stokke, and Smidsrød, 1991).

g. Mark-Houwink-Kuhn-Sakurada (MHKS) or power law relations

For a homologous series of polysaccharides of different molecular weights the conformation can be estimated from the molecular weight dependency of a number of hydrodynamic parameters *e.g.* intrinsic viscosity ([η]), sedimentation coefficient ($s^0_{20,w}$), root-mean-square radius (R_g), translational diffusion coefficient ($D^0_{20,w}$) (Mark, 1938; Kuhn and Kuhn, 1945) (**Figure 2a-d**).

$$[\eta] = \kappa_\eta M^a \qquad (14a)$$

where κ_η and a are obtained from the intercept and slope of the double log plot of [η] vs. M_w (**Figure 2a**). The value of a can be used as an estimation of gross macromolecular conformation and hence a values of ~0 correspond to spheres, 0.5 - 0.8 to random coils, and up to 1.8 to rigid rods (see, *e.g.*, Smidsrød and Andresen, 1988).

$$s^0_{20,w} = \kappa_s M^b \qquad (14b)$$

where κ_s and b are obtained from the intercept and slope of the double log plot of $s^0_{20,w}$ vs. M_w (**Figure 2b**). The value of b can be used as an estimation of gross macromolecular conformation and hence b values of ~0.67 correspond to spheres, 0.4 - 0.5 to random coils, and ~0.15 to rigid rods (see, *e.g.*, Smidsrød and Andresen, 1988).

$$r = \kappa_r M^c \qquad (14c)$$

where κ_r and c are obtained from the intercept and slope of the double log plot of r vs. M_w (**Figure 2c**). The value of c can be used as an estimation of gross macromolecular conformation and hence ε values of ~0.333 correspond to spheres, 0.5 - 0.6 to random coils, and 0.85 to rigid rods (see, *e.g.*, Smidsrød and Andresen, 1988).

$$D^0_{20,w} = \kappa_D M^{-\varepsilon} \qquad (14d)$$

where κ_D and ε are obtained from the intercept and slope of the double log plot of $D^0_{20,w}$ vs. M_w respectively (**Figure 2d**). The value of ε can be used as an estimation of gross macromolecular conformation and hence ε values of ~0.333 correspond to spheres, 0.5 - 0.6 to random coils, and 0.85 to rigid rods (see, *e.g.*, Smidsrød and Andresen, 1988).

Figure 2 here

The inter-validity of the MHKS parameters can be further explored by the calculation of their corresponding Tsvetkov, Eskin and Frenkel (TEF) relations (Tsvetkov, Eskin and Frenkel, 1970).

$$a = 2 - 3b \qquad (14e)$$

$$b = 1 - c \qquad (14f)$$

$$c = \frac{a+1}{3} \qquad (14g)$$

As can be seen from **Figure 2** there is a high degree of consistency in the MHKS exponents for pullulan.

h. Conformation zoning (Normalised scaling relations)

Conformation zoning (or normalised scaling relations) can be used to represent semi-empirically the conformation of a polymer based on a series of hydrodynamic measurements. For example, in *Sedimentation Conformation Zoning* (Pavlov, Rowe and Harding., 1997, Pavlov, Harding and Rowe, 1999) a plot of $k_s M_L$ versus $[s]/M_L$ is used to facilitate an estimate of the "overall" solution conformation of a macromolecule in solution ranging from Zone A (extra rigid rod) to Zone E (globular or branched) - see **Figure 3a**. Pavlov, *et. al*. (1999) have described a further

procedure for representing the conformation of polymers in solution based on the relationship between their molar mass, intrinsic viscosity and mass per unit length, M_L (**Figure 3b**).

Figure 3 here

i. The ρ parameter

A further estimate of molecular conformation can be obtained the ρ parameter which has theoretical limits of 0.78, 1.7 and 2 for hard spheres, random coils (θ-conditions) and rigid rods, respectively (Burchard, 1988).

$$\rho = \frac{r_g}{r_H} \tag{15}$$

From **Table 1** the published values for pullulan are consistent with other data and typical of a random coil.

j. Translational frictional ratio and Perrin function

The translational frictional ratio, f/f_O is a parameter which depends on conformation *and* molecular expansion through hydration effects (Tanford, 1961). It can be measured experimentally from the sedimentation coefficient, hydrodynamic radius or translational diffusion coefficient and molecular weight:

$$\frac{f}{f_0} = \frac{M_w(1-\bar{v}\rho_{20,w})}{(N_A 6\pi\eta_{20,w}s^0{}_{20,w})}\left(\frac{4\pi N_A}{3\bar{v}M_w}\right)^{1/3} \tag{16a}$$

$$\frac{f}{f_0} = r_H\left(\frac{4\pi N_A}{3\bar{v}M_w}\right)^{1/3} \tag{16b}$$

$$\frac{f}{f_0} = \frac{k_B T}{(6\pi\eta_{20,w} D^0{}_{20,w})} \left(\frac{4\pi N_A}{3\bar{v} M_w}\right)^{1/3} \quad (16c)$$

where N_A is Avogadro's number and k_B is the Boltzmann constant. f is the friction coefficient of the molecule and f_0 the corresponding value for a spherical particle of the same mass and (anhydrous) volume (Tanford, 1961).

Knowledge of the hydration, δ (g or solvent per g of macromolecule) allows the estimation of the Perrin (frictional ratio due to shape) parameter, P.

$$P = \left(\frac{f}{f_0}\right)\left[\frac{\bar{v}}{(\bar{v} + \delta)}\right]^{1/3} \quad (17)$$

For quasi-rigid molecules the axial ratio (a/b) can be calculated from the Perrin parameter using for example the ELLIPS1 routine (Harding and Cölfen, 1995), and this type of modelling has been successfully applied to a globular/ heavily branched structure like glycogen (Ang, Kogulanathan, Morris, Kök, Shewry, Tatham, Adams, Rowe and Harding, 2010).

k. Wales – van Holde ratio

The Wales-van Holde ratio, R is a hydration independent estimation of conformation which related the concentration dependence of sedimentation with the intrinsic viscosity (Wales and van Holde, 1954).

$$R = \frac{k_s}{[\eta]} \quad (18)$$

As with the Perrin function molecules the axial ratio (a/b) can be calculated from the Wales – van Holde ratio using for example the ELLIPS1 routine (Harding and Cölfen, 1995).

l. Smidsrød-Haug stiffness parameter

This is another very simple conformational parameter based on the intrinsic viscosity; however it is *only* applicable for polyelectrolytes. In brief the stiffness of polyelectrolytes can be estimated by measuring the intrinsic viscosity at a number of different ionic strengths and then extrapolation to infinite ionic strength (Pals and Hermans, 1952).

$$[\eta] = [\eta]_{\infty} + (SI^{1/2}) \qquad (19)$$

where $[\eta]_{\infty}$ is the intrinsic viscosity at infinite ionic strength and S is a so-called *Stiffness Parameter* which can be used to estimate the conformation of different polyelectrolyte polymers, but with the constraint that they are of the same molar mass and in identical solvent conditions. Smidsrød and Haug (1971) suggested a new parameter (B) which removed these restrictions by comparing the intrinsic viscosity at a fixed ionic strength (typically 0.1 M). The Smidsrød-Haug stiffness parameter, *B* – not to be confused with the2nd thermodynamic virial coefficient (equation 5) is defined as (Smidsrød and Haug, 1971):

$$S = B([\eta]_{0.1M})^{\nu} \qquad (20)$$

where *v* has been shown experimentally to be approximately 1.3 ± 0.1. Therefore *B* can be estimated from a plot of [η] versus $I^{-1/2}$.

m. Estimation of persistence length

The linear flexibility of polymer chains can also be represented quantitatively in terms of the persistence length, L_p of equivalent *worm-like chains* (Kratky and Porod, 1949) where the persistence length is defined as the average projection length along the initial direction of the polymer chain. In the case of a theoretical perfect random coil $L_p = 0$ and for the equivalent perfect rod (Harding, 1997) $L_p = \infty$, although in practice limits of ~ 1 nm for random coils (*e.g.* pullulan) and 200 nm for a rod (*e.g.* schizophyllan) are more appropriate.

Figure 4 here

i. Burchard – Stockmayer – Fixman (BSF)

This is perhaps the simplest way of estimating the persistence length. It involves plotting $[\eta]/M_w^{1/2}$ versus $M_w^{1/2}$ and the persistence length is calculated from the intercept (**Figure 4a**), K_θ (Stockmayer and Fixman, 1963), although knowledge of the mass per unit length M_L is required.

$$K_\theta = \Phi\left(\frac{2L_p}{M_L}\right)^{3/2} \tag{21}$$

where ϕ is the Flory constant ~ 2.86 x 10^{26} mol^{-1}.

ii. Bushin, Tsvetkov, Lysenko and Emel'yanov (1981) – Bohdanecky (1983)

This is a popular method for estimating chain persistence lengths particularly for semi-flexible polymers, and has been applied to range of polysaccharides. In its simplest form, the Bushin-Bohdanecky method involves plotting $\left(M_w^2/[\eta]\right)^{1/3}$ versus $M_w^{1/2}$ and from the slope L_p can be calculated using the following relation and tabulated values **(**Bohdanecky, 1983**)** of the coefficient B_0 (**Figure 4b** and **Figure 4c**).

$$\left(\frac{M_w^2}{[\eta]}\right)^{1/3} = A_0 M_L \Phi^{-1/3} + B_0 \Phi^{-1/3}\left(\frac{2L_p}{M_L}\right)^{-1/2} M_w^{1/2} \tag{22}$$

iii. Yamakawa – Fujii (1973)

Hearst and Stockmayer (1962) first reported the sedimentation coefficient in relation to wormlike chain parameters, later refined by Yamakawa and Fujii (1973).

$$s^0 = \frac{M_L\left(1-\bar{v}\rho_0\right)}{3\pi\eta_0 N_A} \times \left[1.843\left(\frac{M_w}{2M_L L_p}\right)^{1/2} + A_2 + A_3\left(\frac{M_w}{2M_L L_p}\right)^{-1/2} + \ldots.\right] \tag{23}$$

Yamakawa and Fujii (1973) showed that $A_2 = \ln(d/2L_p)$ and $A_3 = 0.1382$ if the L_p is much higher than the chain diameter, d. The persistence length is then calculated from the slope of $s^0_{20,w}$ versus $M_w^{1/2}$ (**Figure 4d**).

iv. Combined (Global) approach

The way these approaches are implemented can lead to significant variability in the results, *i.e*. contrary to expectation, *L*p is model dependent (Bohdanecky and Petrus, 1991; Ortega and García de la Torre, 2007). This is ably demonstrated by the different persistence lengths calculated by the Burchard–Stockmayer–Fixman, Hearst, Bushin-Bohdanecky and Yamakawa-Fujii approaches (Kök, et al., 2009): realiance on a single measurement is unwise. The persistence length and mass per unit length can be estimated using, Multi-HYDFIT program (Ortega and García de la Torre, 2007) which considers data sets of hydrodynamic parameters for different molecular weights. It then performs a minimisation procedure finding the best values of M_L and L_p and chain diameter d satisfying the Bushin-Bohdanecky (Bushin, et al., 1981; Bohdanecky, 1983) and Yamakawa-Fujii (1973) equations (equations 18 and 19) (**Figure 4e** and **Figure 4f**).

There is also a semi-quantitative relationship between L_p/M_L ($nm^2 mol\ g^{-1}$) and the conformation as estimated by conformation zoning (Morris and Ralet, 2012) in that the transition from rigid rod to semi-flexible coil seems to occur at ~ 0.01 $nm^2 mol\ g^{-1}$.

Table 2 here

4. Limitations

Thermodynamic (sedimentation equilibrium and light scattering) and hydrodynamic (sedimentation velocity) has to be dealt with for either conformation or molecular weight work (Schuck, et. al., 2014). Structures are of necessity only of low resolution. Complications through molecular slip and draining effects can also obscure interpretations in terms of shape and flexibility and should be considered in certain cases (see, for example, Berth, et, al, 1998) although these effects are generally small compared with the strength of the hydrodynamic interactions within a polysaccharide (see Tanford 1961).

5. Conclusions

The size and shape of polysaccharides in solution can be estimated in a variety of ways, as illustrated in **Table 2**. Molecular weights and heterogeneities can be estimated to a good precision by Sedimentation velocity, Sedimentation equilibrium and SEC-MALS. An approximate idea of conformation and flexibility can be obtained from power-law coefficients and the Wales van Holde parameter. More sophisticated estimates can be obtained by combining methods together to yield the persistence length.

6. References

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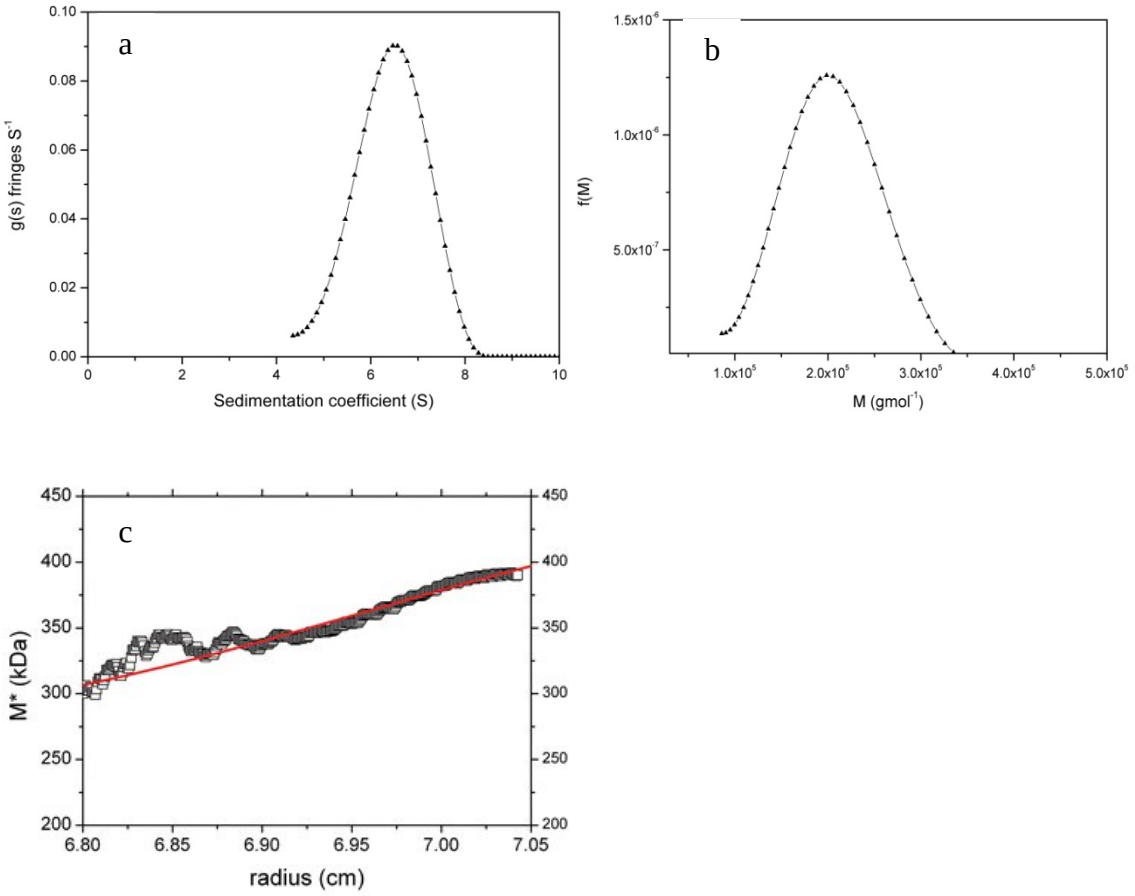


Figure 1a g(s) distribution for pullulan P_{200}; **b** the corresponding f(M) molecular weight distribution f(M) versus M after implementation of the extended Fujita approach. Loading concentration $c_o \sim 1 \times 10^{-4}$ g mL^{-1}. [illegible] 0.025 and b = 0.44. Sample was centrifuged at 45000 rpm at a temperature of 20.0 °C in 0.1 M, pH 6.8, phosphate buffer. M_w = 197 000 g mol^{-1} (adapted from Harding, Schuck, Abdelhameed, Adams, Kök and Morris, 2011) and **c** analysis of pullulan P_{400} at a loading concentration of 2 mg mL^{-1}. True M_w = 400000 g mol^{-1}. Retrieved $M_{w,app}$ (from extrapolation of M* to the cell base = 400000 g mol^{-1} (adapted from Schuck, Gillis, Besong, Almuntairi, Adams, Rowe and Harding, 2014).

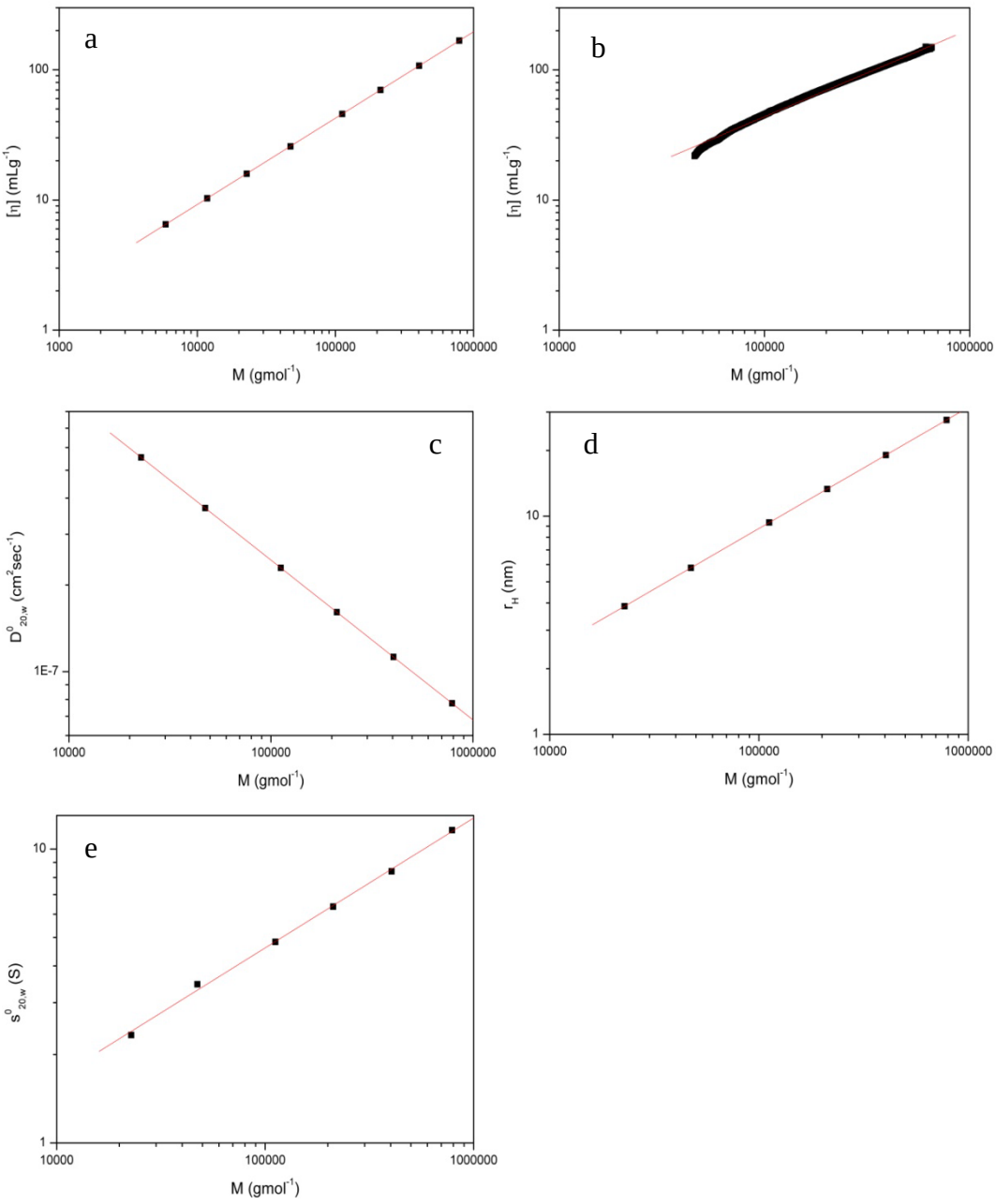


Figure 2 The Mark-Houwink-Kuhn-Sakurada (MHKS) plots for pullulan (adapted from Kato, Tsunehisa and Takahashi, 1984; Kawahara, Ohta, Miyamoto and Nakamura, 1984; Nishinari, Kohyama, Williams, Phillips, Burchard and Ogino; Pavlov, et al., 1997; Kasaai, 2006b). The slopes of all four plots are consistent with a semi-flexible coil conformation (Zone C).

a: the MHKS viscosity plot (a = 0.66)**; b**: the online MHKS viscosity plot (a = 0.67)

c: the MHKS diffusion plot (**; d**: the MHKS r_H plot (c = 0.55)**;**

e: the MHKS sedimentation plot (b = 0.44)

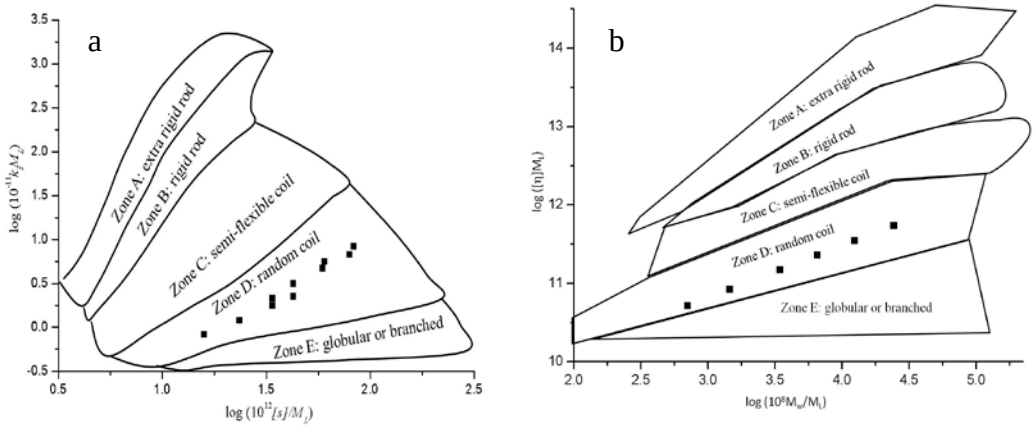


Figure 3 Idealised conformation zoning plots (adapted from Pavlov et al., 1997; Pavlov et al., 1999). Zone A: extra-rigid rod; Zone B: rigid rod; Zone C: semi-flexible; Zone D: random coil and Zone E: globular or branched: **a** – sedimentation conformation zoning and **b** – viscometric conformation zoning. Data shown for pullulan (adapted from Kato, Tsunehisa and Takahashi, 1984; Kawahara, Ohta, Miyamoto and Nakamura, 1984; Nishinari, Kohyama, Williams, Phillips, Burchard and Ogino; Pavlov, et al., 1997; Pavlov et al., 1999).

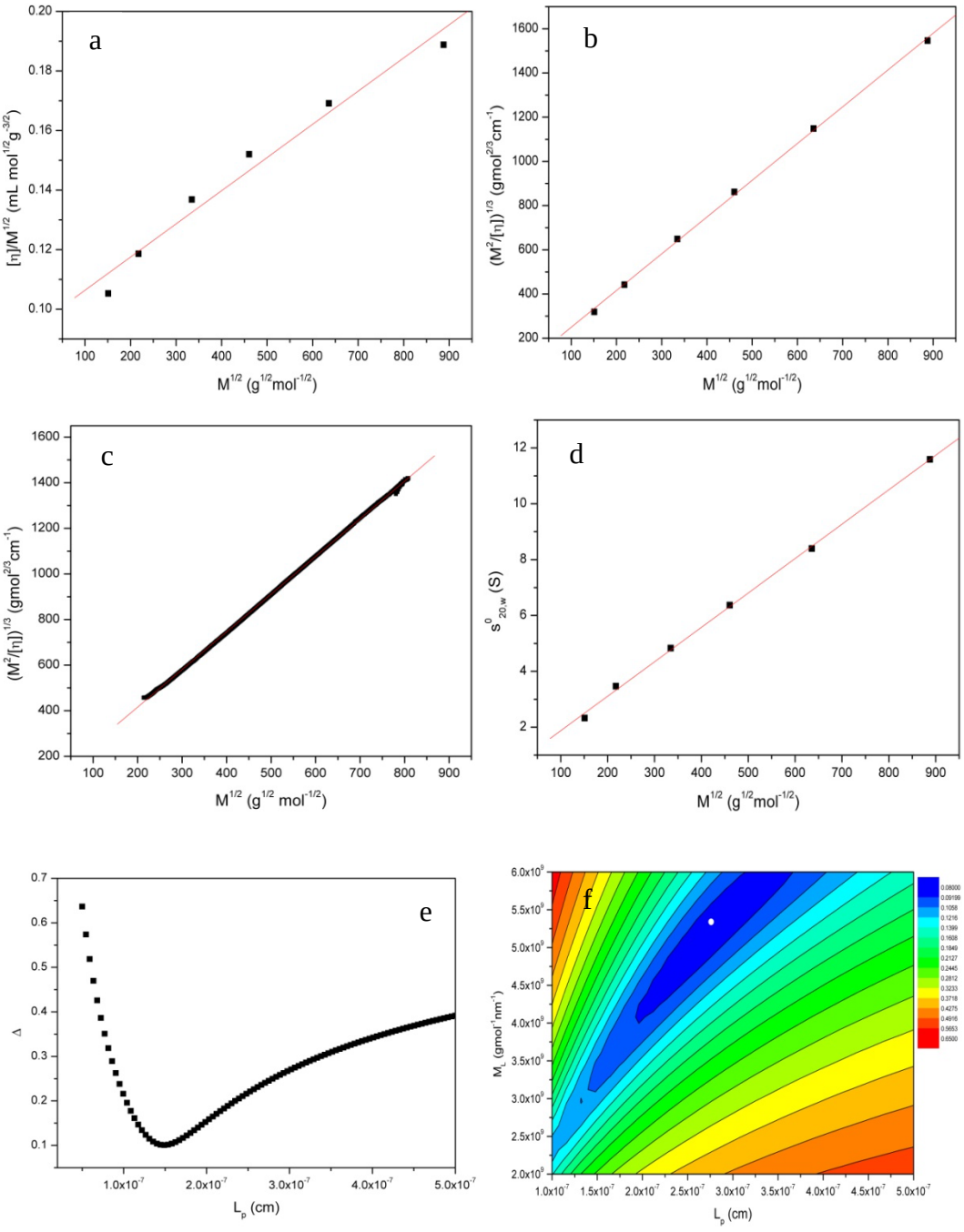

a
[η]/M^1/2 (mL mol^1/2 g^-3/2)
M^1/2 (g^1/2 mol^-1/2)
b
(M^2/[η])^1/3 (gmol^2/3 cm^-1)
M^1/2 (g^1/2 mol^-1/2)
c
(M^2/[η])^1/3 (gmol^2/3 cm^-1)
M^1/2 (g^1/2 mol^-1/2)
d
s^0 20,w (S)
M^1/2 (g^1/2 mol^-1/2)
e
Δ
L_p (cm)
f
M_L (gmol^-1 nm^-1)
L_p (cm)

Figure 4 The estimation of the persistence length, L_p, for pullulan (Zone C/D) using different approaches (adapted from Kato, Tsunehisa and Takahashi, 1984; Kawahara, Ohta, Miyamoto and Nakamura, 1984; Nishinari, Kohyama, Williams, Phillips, Burchard and Ogino; Pavlov, et al., 1997; Kasaai, 2006b)

a: BSF plot where L_p = 0.8 nm from the intercept.

b: Bushin-Bohdanecky plot where L_p = 1.6 nm from the slope.

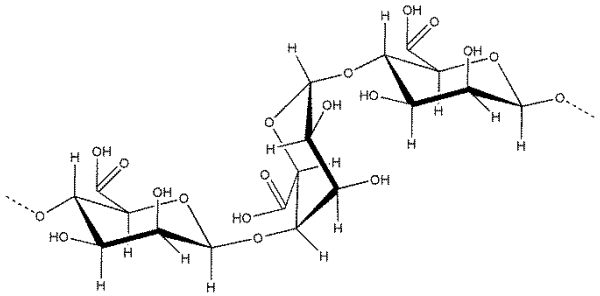
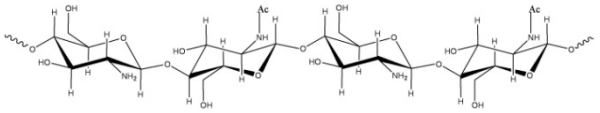
c: Bushin-Bohdanecky directly imported from multi-detection SEC where L_p = 1.6 nm from the slope.

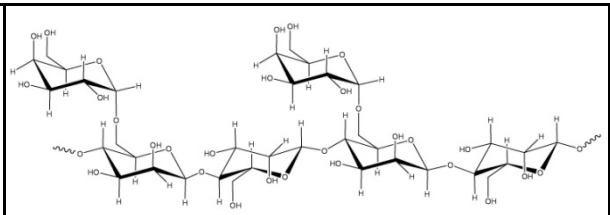
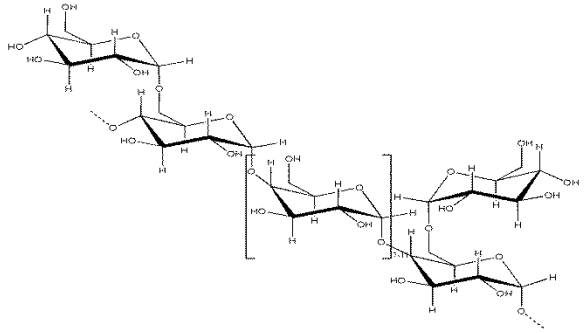
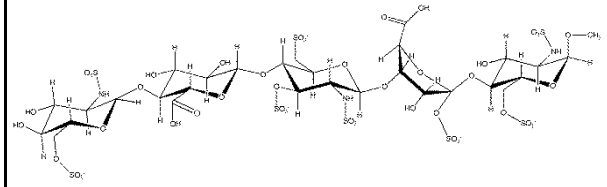
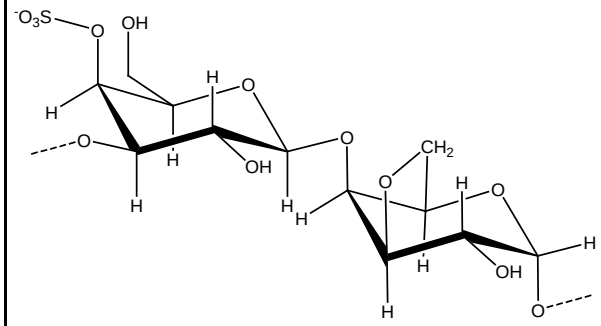
d: Yamakawa-Fujii plot where L_p = 1.8 nm from the slope.

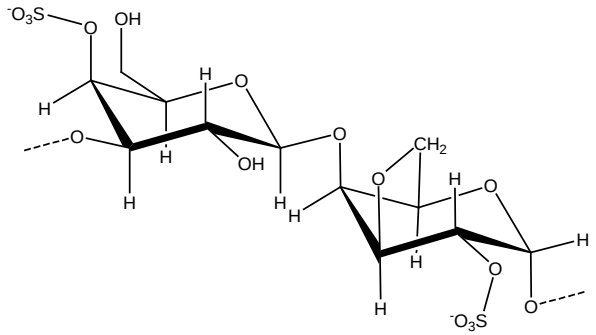
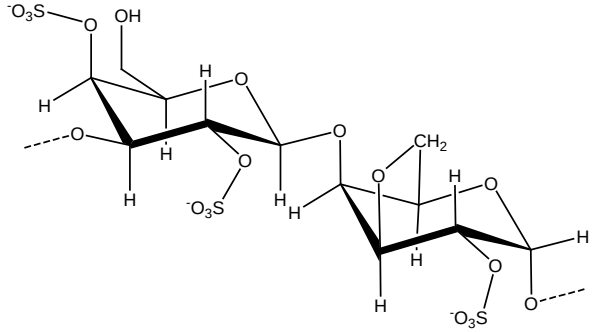
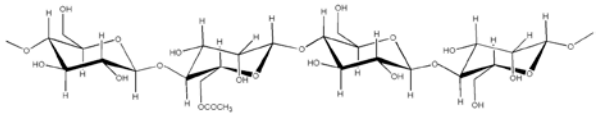
e: Solutions to the Bushin-Bohdanecky and Yamakawa-Fujii equations using equivalent radii approach. The target function, Δ is calculated over a range of values for L_p nm) and M_L (g mol^{-1} nm^{-1}) has been fixed at 320 g mol^{-1} nm^{-1}. The calculated minimum in L_p is found when nm.

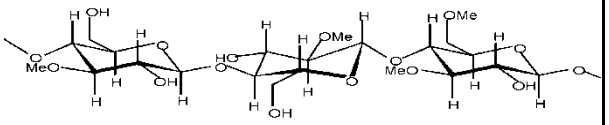
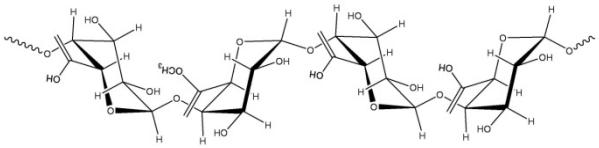
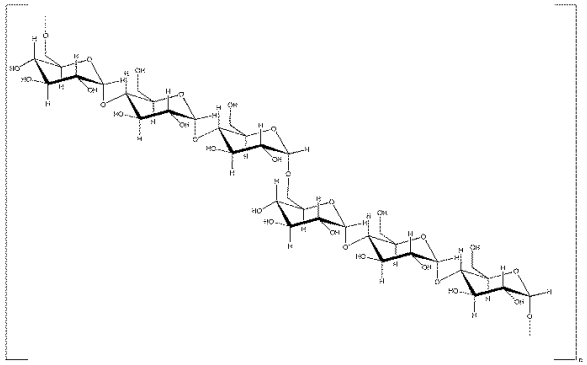
f: Solutions to the Bushin-Bohdanecky and Yamakawa-Fujii equations using equivalent radii approach. The x-axis and y-axis represent L_p (nm) and M_L (g mol^{-1} nm^{-1}) respectively. The target function, Δ is calculated over a range of values for M_L and L_p. In these representations, the values of Δ function are represented by the full colour spectrum, from the minimum in the target function in blue (L_p ≡ 2.08) nm and M_L = 525 g mol^{-1}nm^{-1}) is indicated (○).

Table 1 Commercial polysaccharides: Structures and applications

Polysaccharide	Structure	Charge	Properties	Applications	References
Alginate		Negative	Hydrocolloid - high viscosity; gelation; film formation	Hydrogels; wound dressing; drug delivery; tissue engineering; printing	Helgerud, Gåserød, Fjæreide, Andersen, and Larsen, 2010 (and references therein); Lee and Mooney, 2012 (and references therein)
Chitosan		Positive	Semi-crystalline; acid soluble; mucoadhesion	Drug delivery; hydrogels; fingerprint enhancement	Morris, Kök, Harding and Adams, 2010 (and references therein); Il Dueik Morris, 2013

Galactomannan		Neutral	Viscosity; synergistic interactions with other polysaccharides	Paper; textile; food; pharmaceutical; cosmetics	Srivastava and Kapoor, 2005 (and references therein)
Glycogen		Neutral	Compact	Glucose storage polysaccharide and animals	Ioan, Aberle and Burchard, 1999; Morris, Ang, Hill, Lewis, Shafer, Nobbmann and Harding, 2008a
Heparin		Negative	High negative charge density;	Anticoagulant	Pavlov, Finet, Tatarenko, Korneeva and Ebel, 2003
κ-Carrageenan		Negative	Gelation; interaction with □-casein; synergistic interactions	Food applications *e.g.* ice cream	Berth, Vukovic and Lechner, 2008; Blakemore and Harpell, 2010 (and references therein)

ι–Carrageenan		Negative	Gelation	Food applications *e.g.* dairy desserts	Berth, Lukovic and Lechner, 2008; Blakemore and Harpell, 2010 (and references therein)
λ-Carrageenan		Negative	Non-gelling	Thickening in dairy products	Almutairi, Adams, Kök, Lawson, Gahler, Wood, Foster, Rowe and Harding, 2013) Blakemore and Harpell, 2010 (and references therein)
Konjac glucomannan		Neutral	Gelling; synergistic interactions	Fat replacement; thickener; prebiotic fermentation	Parry, 2010 (and references therein)

Methyl cellulose		Neutral	Water soluble; GRAS (Generally Regarded As Safe); thermal gelation	Fat replacement; improve mouth feel in beverages	Cash and Caputo, 2010 (and references therein)
Pectin		Negative	Gelling; thickening; bioactivity	Jams; drug delivery; mucoadhesion	Morris, Kök, Harding and Adams, 2010 (and references therein);
Pullulan		Neutral	Non-toxic; odourless; tasteless	Starch replacement (not digested by mammalian amylases); denture adhesive	Israilides *et al*. (1999); Singh *et al*. (2008); Harding and Morris, 2013 (and references therein)

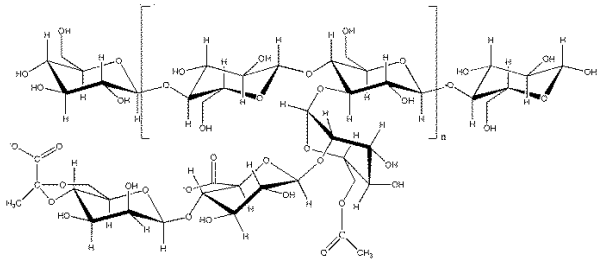
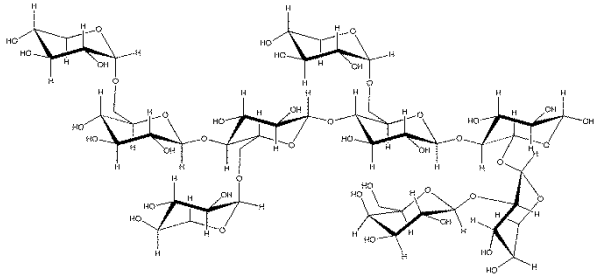
Xanthan		Negative	hydrocolloid - high viscosity yield at low shear rates even at low concentration; - stability over wide temperature, pH and salt concentration ranges	Foods; petroleum industry; pharmaceuticals; cosmetics and personal care products; agriculture	Dea *et al*. (1977); Morris *et al*. (1977); Dhami *et al*. (1995); Morris *et al*. (2001); Harding and Morris, 2013 (and references therein)
Xyloglucan		Neutral	Low viscosity; forms gels at high sugar concentration under acidic conditions	Drug-delivery; food technology; textiles industry	Mishra and Malhotra, 2009 (and references therein)

Table 2 Typical estimations of the size for selected polysaccharides

	M_w (kgmol^{-1})	$s^0_{20,w}$ (S)[a]	[□] (mLg^{-1})	r_H (nm)	r_g (nm)	$D^0_{20,w}$ (F)[b]	*References*
Alginate	15 - 2700	2.4	30 - 5500	□□□ □□□ □□	70 - 190	□□□□□ □□□□	Smidsrød, 1970; Harding, 1992; Ball, Harding and Mitchell, 1998; Vold, 2004; Bi, Mahmood, Arman, Taj and Iqbal, 2007; Storz, Muller, Ehrhart, Gomez, Shirley, Gessner, Zimmermann, Weyand, Sukhorukov, Forst, Weber, Zimmermann, Kulicke and Zimmermann, 2009; Villay, de Filippis, Picton, Le Cerf, Vial and Michaud, 2012
Chitosan	22 - 720	1.3 – 2.7	70 - 1770	11.2 – 24.5	20 - 70	0.9 – 1.5	Terbojevich, Cosani, Conio, Marsano and Bianchi, 1991; Errington, Harding, Vårum, and Illum, 1993; Ottøy, Vårum, Christensen,

							Anthonsen and Smidsrød, 1996; Berth, Dautzenberg and Peter, 1998; Berth and Dautzenberg, 2001; Cölfen, Berth and Dautzenberg, 2001; Brugnerotto, Desbrières, Roberts and Rinaudo, 2001; Fee, Errington, Jumel, Illum, Smith and Harding, 2003; Schatz, Viton, Delair, Pichot, and Domard, 2003; Mazeau and Rinaudo, 2004; Vold, 2004; Lamarque, Lucas, Viton and Domard, 2005; Rinaudo, 2006; Kasaai, 2006a; Velásquez, Albornoz and Barrios, 2008; Morris, Castile, Smith, Adams and Harding, 2009

Galactomannan	80 – 2700	3.3 – 8.3	110 - 2000	22 - 47	7 - 200	0.4 – 1.0	Jumel, Harding and Mitchell, 1996; Beer, Wood and Weisz, 1999; Picout, Ross-Murphy, Errington and Harding, 2001; Morris, 2001; Picout, Ross-Murphy, Jumel and Harding, 2002, Risica, Dentini and Crescenzi, 2005; Patel, Picout, Ross-Murphy and Harding, 2006 ; Pitkänen, 2011 ; Villay, et al., 2012
Glycogen	450 - 36000	15 – 123	6.5 – 8.5	7 - 65	10 - 54	0.3 – 3.0	Bridgman, 1942; Ioan, Aberle and Burchard, 1999; Morris, Ang, Hill, Lewis, Shafer, Nobbmann and Harding, 2008a; Fernandez, Rojas and Nilsson, 2011
Heparin	3.9 – 37	1.3 – 3.2	7.9 – 40.3	1 - 5		3.9 – 15	Pavlov, et al., 2003

κ-Carrageenan	265 – 950	3.6 - 4.2	420 - 630		75 - 105		Vreeman, Snoeren and Payens, 1980; Harding, Day, Dhami and Lowe, 1997; Morris, 2001; Berth, Vukovic and Lechner, 2008
ι-Carrageenan	130 – 300	6.9	1270		90 - 110		Morris, 2001; Berth, Vukovic and Lechner, 2008
λ-Carrageenan	340 – 870	3.9 – 5.3	640 - 1080				Almuntairi, Adams, Kök, Lawson, Gahler, Wood, Foster, Rowe and Harding, 2013.
Konjac glucomannan	50 - 1200	1.7 - 3.3	200 - 3000		25 - 120		Prawitwong, Takigami and Phillips, 2007; Kök, Abdelhameed, Ang, Morris and Harding, 2009

Methyl cellulose	19 – 1200	0.9 – 3.6	67 - 2500	5 - 30	80 - 95	0.7 – 4.4	Pavlov, Michailova, Tarabukina and Korneeva, 1995; Pavlov, et al, 1997; Patel, Morris, Garcia de la Torre, Ortega, Mischnick and Harding, 2008b
Pectin	13 – 560	1.4 – 2.3	80 - 1600	12 - 55	13 - 45	0.4 - 1.8	Anger and Berth, 1985; Axelos, Lefebvre and Thibault, 1987; Axelos and Thibault, 1991, Berth, Anger and Linow, 1977; Harding, Vårum, Stokke and Smidsrød, 1991b; Garnier, Axelos and Thibault, 1993; Malovikova, Rinaudo Milas, 1993; Morris, Foster and Harding, 2000, 2002; Morris, García de la Torre, Ortega, Castile, Smith and Harding , 2008c; Fishman,

							Chau, Kolpak and Brady, 2001; Fishman, Chau, Hoagland and Hotchkiss, 2006; Morris, Ralet, Bonnin, Thibault, and Harding, 2010; Fishman, Chau, Qi, Hotchkiss and Yadav, 2013
Pullulan	6 – 1600	2.3 – 11.6	6 - 170	4 - 28	8 - 58	0.8 – 5.5	Kato, Tsunehisa and Takahashi, 1984; Kawahara, Ohta, Miyamoto and Nakamura, 1984; Nishinari, Kohyama, Williams, Phillips, Burchard and Ogino, 1991; Pavlov, et al., 1997; Kasaai, 2006b
Xanthan	2000 - 50000	10 - 13	1300 - 11400		30 – 200		Sato, Norisuye and Fujita, 1984; Dhami, Harding, Jones, Hughes, Mitchell and To, 1995; Milas, Reed and Prinz, 1996; Morris, Puaud, Li, Lui, Mitchell and

							Harding, 2001
Xyloglucan	45 – 2200	2.6 – 7.2	75 - 2600		33 - 136		Picout, Ross-Murphy, Errington and Harding, 2003; Ren, Picout, Ellis and Ross-Murphy, 2004; Freitas, Martin, Santos, Valenga, Buckeridge, Reicher, Sierakowski, 2005; Patel, Morris, Ebringerová, Vodenicarová, Velebny, Ortega, Garcia de la Torre and Harding, 2008a

[a] 1 S = 1 x 10^{-13} s

[b] 1 F = 1 x 10^{-7} cm^2s^{-1}

Table 3 Estimations of the dilute solution conformation of selected polysaccharides

	a	***b***	***c***	***□***	***k_s/[□***	***□***	***f/f_0***	***L_p (nm)***	***Zone***	***References***
Alginate	0.73 - 1.31	-	0.52 - 0.54	□	0.6	□	9	12 - 15	B/C	Smidsrød, 1970; Harding, 1992; Ball, et al., 1998; Vold, 2004; Bi, et al., 2007; Storz, et al., 2009
Chitosan	0.77 – 1.10	0.24 – 0.25	0.55 – 0.56	-	0.16 – 0.73	-	11 - 16	4 - 35	B/C	Terbojevich, et al., 1991; Errington, et al., 1993; Ottøy, et al., 1996; Berth, et al., 1998; Berth and Dautzenberg, 2001 ; Cölfen, et al, 2001; Brugnerotto, et al., 2001; Fee, et al., 2003; Schatz, et al., 2003; Mazeau and Rinaudo, 2004; Vold, 2004; Lamarque, et al., 2005; Rinaudo, 2006; Kasaai, 2006a; Velásquez, et al., 2008; Morris, et al., 2009

Galactomannan	0.70 – 0.77	0.12 – 0.65	0.54 – 0.57	-	0.15 – 0.41	-	8 - 17	2 - 12	C	Jumel, et al., 1996; Beer, et al., 1999; Picout, et al., 2001, 2002; Risica, et al., 2005; Patel, et al., 2006; Morris, et al., 2008b
Glycogen	-0.07 - 0	0.71	0.31- 0.33	0.38 - 0.40	-	0.7 – 1.0	1.7 – 2.8	-	E	Bridgman, 1942; Reiner, 1981; Ioan, et al., 1999; Morris ,et al., 2008a
Heparin	0.90	0.38	0.38	0.62	1.04 – 2.98	1.34 - 1.52	1 - 3	4 - 6	C	Pavlov, et al., 2003
κ-Carrageenan	0.67 – 0.90	-	0.68	-	0.39 - 0.9	-	7 - 9	2 - 3	B/C	Vreeman, et. al., 1980; Harding, et al., 1997; Morris, 2001; Berth et al., 2008
ι-Carrageenan	0.77	-	0.68	-	0.16	-	5	4	B/C	Berth, et al., 2008
λ-Carrageenan	0.6									Almuntairi, et al., 2013.

Konjac glucomannan	0.74 – 0.78	0.32	-	-	0.4	-	9 - 14	1 - 34	C	Prawitwong, et al., 2007; Kök, et al., 2009;
Methyl cellulose	0.83	0.39	-	-	0.30 – 0.75	-	10 - 12	10 - 17	C	Pavlov, et al, 1995; Pavlov, et al, 1997; Patel, et al., 2008b
Pectin	0.62 – 0.94	0.17	0.57	-	0.10 – 0.85	0.6 – 1.0	7 – 10	10 - 15	A/B/C	Berth et al., 1977; Anger and Berth, 1985; Axelos, et al., 1987; Axelos and Thibault, 1991, Harding, et al., 1991b; Garnier, et al., 1993; Malovikova, et al., 1993; Morris, et al., 2000, 2002, 2008c; Fishman, et al., 2001, 2006
Pullulan	0.66 – 0.67	0.44	0.55 - 0.58	0.51 – 0.55	1.27 – 1.49	1.40 – 1.66	2 - 5	1 - 3	C/D	Kato, et al., 1984; Kawahara, et al., 1984; Nishinari, et al., 1991; Pavlov, et al., 1997; Kasaai, 2006b

Xanthan	1.23	0.26	1.00	-	0.28	-	14 - 19	100 - 150	B	Sato, et al, 1984; Dhami, et al., 1995; Pavlov, et al., 1997; Morris, et al, 2001
Xyloglucan	0.55 – 0.67	0.42	0.51	-	0.12 - 1.44	-	2 - 6	4 - 15	C/D	Picout, et al, 2003; Ren, et al., 2004; Freitas, et al., 2005; Patel, 2007; 2008a

NB – some of results in the literature have been re-evaluated to calculate parameters not originally quoted in the paper.