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Molecular weight distribution analysis by ultracentrifugation: Adaptation of a new approach for mucins

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ABSTRACT

Mucins are the key macromolecular component of mucus, nature's natural lubricant, and one of the most important physical properties is their molecular weight distribution. A new approach for polydisperse polymers was recently published based on sedimentation velocity in the analytical ultracentrifuge and converts a distribution of sedimentation coefficient $g(s)$ vs. s plot into a distribution of molecular weight utilising the power-law or scaling relationship between the sedimentation coefficient and molecular weight, $s = \kappa_s M_w^b$ where s is the sedimentation coefficient, M_w is the weight average molecular weight and κ_s and b are characteristic coefficients related to conformation. We investigate the possibility of using a large database of previously published values of s an M to define κ_s and b for both aqueous solution and aqueous solution supplemented by 6 M guanidine hydrochloride (a solvent which helps to minimise sample degradation). These values are then applied to a study of the molecular weight distributions of preparations of human gastric mucin in the different solvents and at different stages of purification.

1. Introduction

Mucin glycoproteins are the major macromolecular component of mucus – nature's natural lubricant – protecting the underlying epithelia from physical damage, as well as providing a chemical barrier. Although their backbone is a polypeptide – coded by MUC genes (in humans there are over 20) – generally over 80% of the macromolecule is carbohydrate. The carbohydrate or glycan component consists of side chains of up to 30 residues O-linked to serine or threonine. The carbohydrate residues are N-acetyl galactosamine (galNAc), N-acetyl glucosamine (glcNAc), N-acetyl Neuraminic acid (NANA) – a sialic acid, galactose and fucose (Creeth, 1978). The carbohydrate component confers on the mucins polysaccharide like properties of high viscosity and water immobilisation – critical to the performance of these substances. Also critical is the average molecular weight and distribution of molecular weight: despite their importance, measurement of both these is not easy, primarily because of the large size – which can go as high as 20×10^6 g/mol, the large polydispersity and high degree of thermodynamic non-ideality of these substances (Harding, 1989). A breakthrough came in the late 1980s with the development of SEC–MALs (size exclusion chromatography coupled to multi-angle light scattering) (see Wyatt, 1992, 2012), providing separation and absolute molecular weight analysis. The first application to polysaccharides was in 1991 (Harding, Vårum, Stokke, & Smidsrød, 1991; Horton, Harding, & Mitchell, 1991), followed not long after by the first application to mucin glycoproteins in 1996 (Jumel, Fiebrig, & Harding, 1996; Jumel et al., 1997), a technique which has now become the method of choice for many polymeric systems.

However there are two limitations to the types of substance SEC–MALs can be successfully applied to – one is the separation range of the columns (usually up to a maximum of $2–3 \times 10^6$ g/mol) and the other problems of inertness of the columns used. An alternative is to use the different separation method of field flow

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fractionation, and FFF–MALs has successfully been applied to for example starches, but again there may be problems due to non-inertness of the membranes used.

Sedimentation equilibrium in the analytical ultracentrifuge has provided an important alternative (Creeth & Harding, 1982; Harding, 1989) that does not require any separation columns or membranes or separation medium such as sucrose or caesium salts. However this method has thus far only been able to yield average molecular weights (usually the apparent weight average molecular weight $M_{w,app}$ if conventional Rayleigh optics are used, although z-average molecular weights $M_{z,app}$ can be routinely obtained). The values are apparent values because of the effects of non-ideality (unless very low concentrations can be used) – and an extrapolation to zero concentration is often necessary to yield M_w and M_z, which can take a considerable amount of time and effort. The ratio of M_z/M_w can be used to define the polydispersity of a distribution and the (weight average) standard deviation of the distribution σ_w (whatever form this may take) can be evaluated from the classical Herdan relations (Herdan, 1949).

A new alternative – based on an original method of Fujita (1962) given for one particular class of macromolecules, but recently extended to cover all classes of macromolecule (Harding et al., 2011) – is now possible which lends itself particularly to mucins. It is also an analytical ultracentrifuge technique but is based on sedimentation velocity rather than sedimentation equilibrium.

We now outline the specific adaptation of the method to mucins and illustrate its application to a range of human gastric mucins of differing degrees of purity, and in both aqueous solvents and aqueous solvents supplemented with 6 M guanidine hydrochloride, a solvent often used to minimise the enzymatic degradation of mucins by denaturing the enzymes through the disruption of hydrogen and electrostatic bonds (Harding, 1989).

2. The Extended Fujita approach of Harding, Schuck and coworkers

Fujita (1962) provided the basis of transforming a (differential) distribution $g(s)$ of sedimentation coefficient s into a (differential) distribution $f(M)$ of molecular weight M for linear polymers based on the assumption that the polymers had a random coil conformation. $g(s)$ is defined as the population (weight fraction) of species with a sedimentation coefficient between s and $s+ds$ and $f(M)$ is defined as the population (weight fraction) of species with a molecular weight between M and dM. The $g(s)$ profiles could be obtained experimentally using procedures based on conversion of concentration gradient (Schlieren) optical records (Bridgman, 1942; Rinde, 1928). The transformation from $g(s)$ versus s is obtained as follows:

$$g(s)ds = f(M)dM \tag{1}$$

and so

$$f(M) = g(s)\cdot\left(\frac{ds}{dM}\right) \tag{2}$$

For the case of random coils

$$s = \kappa_s M^{0.5} \tag{3}$$

where the pre-exponential factor κ_s is taken as a constant for that particular polymer under a defined set of conditions and the exponent 0.5 corresponds to a randomly coiled polymer under theta solvent or "pseudo-ideal" conditions (i.e. conditions where exclusion volume effects are matched by associative effects – see e.g. Tanford, 1961). The differential is then

$$\frac{ds}{dM} = \frac{\kappa_s^2}{2s} \tag{4}$$

The method was applied to pig gastric mucin by Harding (1989) based on data of Pain (1980). Using a sedimentation coefficient distribution for pig gastric mucin and the assumption of a random coil conformation under ideal conditions ($b = 0.5$), and a known pair of values for s and M, namely an s value of 33×10^{-13} s (33 S) is approximately equivalent to a molecular weight of 2.5 million Da, it was possible to perform the transformation to obtain the equivalent molecular weight distribution. Gratifyingly the form of the distribution was shown also to be similar to that of the distribution of contour lengths estimated from electron microscopy studies on this mucin.

A straightforward extension of the method was recently provided catering for general conformation types (Harding et al., 2011). The extension is based on use of the general scaling relation (see, for example Harding et al., 1991):

$$s = \kappa_s M^b \tag{5}$$

where $b = 0.4$–0.5 for a coil, ~0.15–0.2 for a rod and ~0.67 for a sphere, and hence

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

For random coils $b = 0.5$ and so Eq. (6) reduces to Fujita's formula (Eq. (4)). So to do the transformation the conformation type or b needs to be known under the particular solvent conditions and at least one pair of s–M values is needed to define the κ_s from Eq. (6). Care needs to be expressed concerning thermodynamic non-ideality but these effects can be avoided by working at low concentrations, taking advantage of the fact that sedimentation velocity experiments can be performed at concentrations as low as 0.1 mg/mL, where such effects are usually negligible. The method has now been built into the popular sedimentation velocity analysis platform known as SEDFIT (Brown & Schuck, 2006; Schuck, 2000, 2010; Schuck, Perugini, Gonzales, Howlett, & Schubert, 2002; Schuck & Rossmanith, 2000).

3. Evaluating universal scaling parameters for mucins

We can now seek to establish the procedure for mucins by looking to see if the κ_s and b parameters can be defined across a wide range of mucins and in different solvents. This would save researchers the time needed to (i) estimate b and (ii) to obtain κ_s from a pair of M–s values, with M determined from a separate experiment using sedimentation equilibrium. Tables 1a and 1b we have collected together 26 pairs of s–M values from the literature. 8 of these data pairs correspond to sedimentation coefficient data measured in aqueous solvent, corrected to standard conditions of the density and viscosity of water at a temperature of 20 °C and extrapolated to zero concentration to eliminate any non-ideality effects ($s^0_{20,w}$). Four of the values correspond to measurements in 6 M guanidine hydrochloride (GuHCl). We performed a linear regression on the complete data set of 26 values (Fig. 1a) and the two subsets of $s^0_{20,w}$ data only (Fig. 1b) and 6 M GuHCl data only (Fig. 1c). The "all samples" and "$s^0_{20,w}$ data" subset give (Table 1b), after allowance for error identical values for b (0.519 ± 0.037 and 0.520 ± 0.059 respectively) and close values for κ_s (0.0088 ± 0.0038) and (0.0100 ± 0.0058) respectively – although the experimental uncertainties are considerably larger – whereas the values in 6 M GuHCl are significantly different ($\kappa_s = 0.0366 \pm 0.0032$ and $b = 0.433 \pm 0.006$). All values of b are within the range for random coils (0.4–0.5).

4. Application to human gastric mucins

To illustrate application of the method we look at two preparations of a human gastric mucin (HGM) in aqueous

Table 1a
Sedimentation coefficient-molecular weight data for mucins.

Mucin	M_w (kDa)	$s_{20,w}$ (S)	Reference
Human cervical mucin (HCM)	9700	50.1[a]	Carlstedt, Lindgren, Sheehan, Ulmsten, and Wingerup (1983)
Pig gastric mucin (PGM)	9000	60[a]	Deacon et al. (1998)
Rat oscites	650	14.9[a]	Sherblom et al. (1980)
HCM whole mucin	10,800	40.4[b]	
HCM subunits	200	19.2[b]	Sheehan and Carlstedt (1984)
HCM T-domains	30	8.7[b]	
Human airway mucin	19,000	48.9[a]	Davies et al. (1996)
	29,000	54.8[a]	
	21,000	55.8[a]	
	20,000	48.2[a]	
Human bronchial mucin	5100	16.1[a,c]	Creeth et al. (1977)
	7000	20.8[a,c]	
	5800	17.6[a,c]	
	3300	17.8[a,c]	
	5100	15.6[a,c]	
Armadillo submandibular glycoproteins	78	1.5[d]	Wu and Pigman (1977)
	31	1.8[d]	
Pig gastric mucin	1850	16.7[c]	Snary, Allen, and Pain (1970)
	110	4.4[c]	
Bovine cervical mucin	1640	65.8	Meyer (1983)
Guinea pig trachial epithelial mucin	4700	28.5	Dodd, Place, Hall, and Harding (1998)
	3300	28.5	
	4500	35.5	
Human cystic fibrosis mucin	14,700	47.1[b]	Thornton, Sheehan, Lindgren, and Carlstedt (1991)
Squid mucin	2600	16.9	Kimura, Gohda, and Sakurai (2003)
	2200	14.3	

[a] Extrapolation to s^0 not specified.
[b] 6 M GuHCl in solvent.
[c] Originally given as s_{25} values, corrected with SEDNTERP (Laue, Shah, Ridgeway, & Pelletier, 1992).
[d] s not corrected to standard solvent conditions.

solvent–phosphate buffered saline supplemented with inhibitors to enzyme degradation where prepared for analysis, and a further preparation where one of the samples had been dialysed against 6 M GuHCl.

4.1. Experimental

4.1.1. Purification

Gastric mucus aspirates were purified using ultra-filtration, dialysis and ultracentrifuge density gradients, as outlined by Creeth et al. (1977). The density gradients were analysed with blot dot tests with an anti-MUC5AC-HRP conjugated monoclonal antibody. Two regions of high MUC5AC affinity (characteristic for human gastric mucin), were identified: these were pooled separately (HGMYanH, HGMYanL) and dialysed twice into phosphate buffered saline (ionic strength I = 0.1 M pH 7.0) with an inhibitor cocktail of 1 mM sodium azide and 1 mM EDTA to minimise degradation. A further aliquot of HGMYanH was dialysed against 6 M GuHCl.

4.1.2. Analytical ultracentrifugation

Sedimentation coefficient distributions were evaluated using the Beckman Optima XL-I analytical ultracentrifuge (Beckman Instruments, Palo Alto, USA). A volume of 400 μL of serial dilutions (stock, half, quarter, etc.) of mucins were injected and matching amounts of buffer were injected into appropriate channels of 12 mm double sector aluminium epoxy cells with sapphire windows. Solutions were centrifuged at 40,000 rpm (45,000 rpm for the sample in 6 M GuHCl) at a temperature of (20.0 ± 0.1) °C. The weight average sedimentation coefficient 's' for a particular component was then corrected to standard solvent conditions (the density and viscosity of water at a temperature of 20.0 °C) to yield $s_{20,w}$ (S). A partial specific volume of 0.64 mL/g was used (Harding, 1989). Data were analysed using least squares $g(s)$ analysis for the stock solution and also four serial dilutions. To minimise the effects of non-ideality the data set for the lowest (measurable) concentration was used for the *Extended Fujita* transformation to give $f(M)$.

Table 1b
Collective κ_s and b parameters for mucins (from Fig. 1).

	Sample size, n	κ_s	b
All samples	26	0.0088 ± 0.0038	0.519 ± 0.037
$s^0_{20,w}$ data only	8	0.0100 ± 0.0058	0.520 ± 0.059
6 M GuHCl data only	4	0.0366 ± 0.0032	0.433 ± 0.006

4.1.3. SEC–MALs

For comparison purposes we also estimated the weight average molecular weight of HGMYanH by SEC–MALs. The SEC consisted of an X-Act 4 channel degassing unit (Jour Research, Onsala, Sweden), Jasco Intelligent HPLC Pump – PU-1580 (Jasco Corporation, Great Dunmow, UK), fitted with a Spark-Holland Marathon Basic autosampler (Spark Holland, Emmen, The Netherlands) combined with a guard column and TSK Gel G6000, 5000, and 4000 columns connected in series (Tosoh Biosciences, Tokyo, Japan), and a column temperature regulator (Anachem, Luton, UK). Light scattering intensity was detected using a DAWN® HELEOS™ II, light scattering photometer connected in series to an Optilab® rEX refractive index detector (Wyatt Technology Corporation, CA, USA). The stock solution of HGMYanH was filtered through a 0.45 μm syringe filter (Whatman, Maidstone, England) – to remove any insoluble material or dust prior to injection – and then injected into the autosampler. 100 μL of each solution was injected onto the columns at ambient temperature. The eluent employed was the PBS dialysate at a flow rate of 0.8 mL/min. ASTRA™ (Version 5.1.9.1) software (Wyatt Technology Corporation, Santa Barbara, USA) was used to estimate the weight average M_w and z-average M_z molecular weights with a 1st order Zimm extrapolation (Wyatt, 1992, 2012). Because of the low solute concentrations after dilution

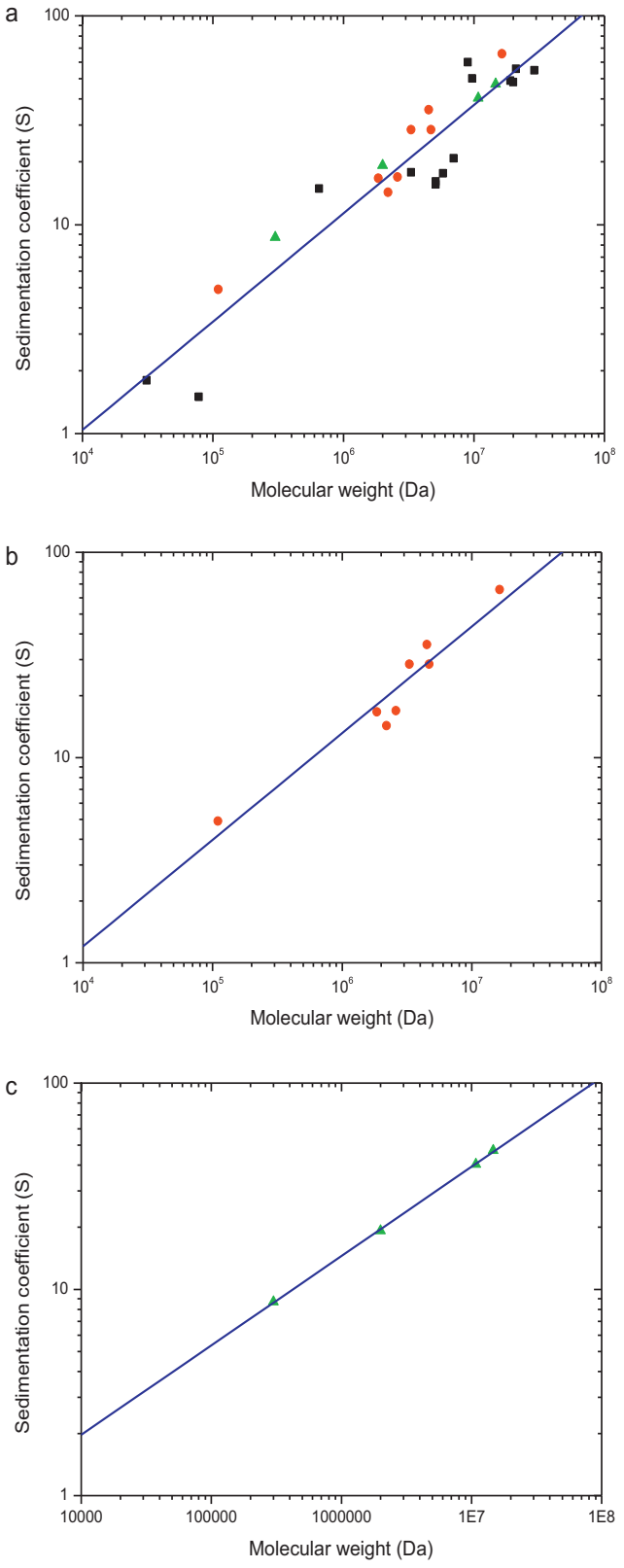

Fig. 1. Mark Houwink Kuhn Sakurada (MHKS) type of power law plots corresponding to the data of Tables 1a and 1b. (a) All data, (b) data based on $s^0_{20,w}$ values and (c) data based on samples measured in 6 M GuHCl.

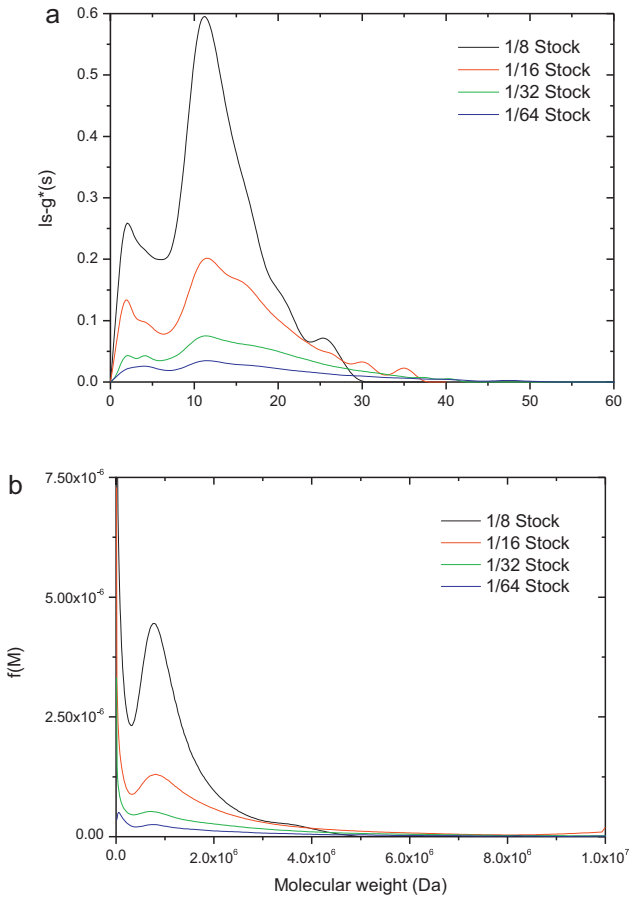

Fig. 2. Sedimentation coefficient (a) and molecular weight distribution plots (from the Extended Fujita procedure) for unpurified HGMYan in phosphate–chloride buffer, I = 0.1 M, pH 7.0.

on the columns non-ideality effects were assumed as negligible. A refractive increment (dn/dc) ~0.165 mL/g was used (Jumel et al., 1996).

4.2. Results

4.2.1. Unpurified HGMYan

After dialysis and ultrafiltration, a sedimentation velocity experiment was performed to assess the macromolecular integrity of the partially purified mucin solution prior to the final density gradient purification stage. The $g(s)$ shows that there were protein impurities at ~2 S (Fig. 2a). The transformation using the κ_s and b values from Fig. 1b to the $f(M)$ plot (Fig. 2b) shows a peak around 50 kDa, however this is not very pronounced. The $g(S)$ also shows a broad peak between 25 and 40 S which represents either a nucleic acid impurity or a high molecular weight mucin. The $f(M)$ shows a shoulder present for the 1/8 dilution (Fig. 2b), which may represent the nucleic acid impurity, but is obscured in the further dilutions. The κ_s and b values for the mucin used in the transformation may not however be applicable to the non-mucin components.

4.2.2. HGMYanH

Two peaks were identified in the $g(s)$ plots (Fig. 3a). On the stock concentration plot, peak 1 stretches from 5 to 25 S, and the second 25 to 30 S. The κ_s and b values from Fig. 1b were used in SEDFIT to convert the sedimentation data into a molecular weight distribution (Fig. 3b).

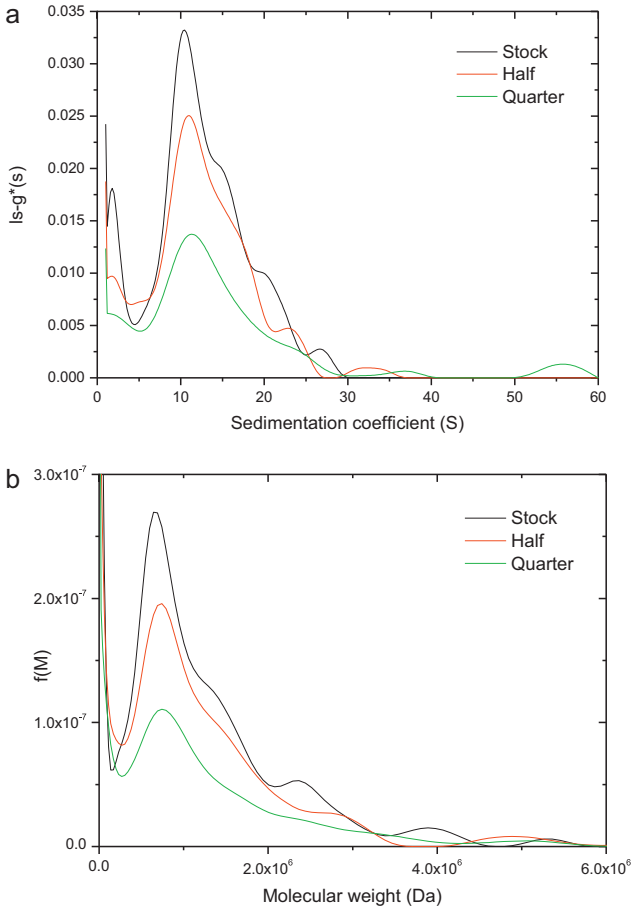

Fig. 3. Sedimentation coefficient (a) and molecular weight distribution plots (from the Extended Fujita procedure) for purified HGMYanH in phosphate–chloride buffer, $I = 0.1$ M, pH 7.0.

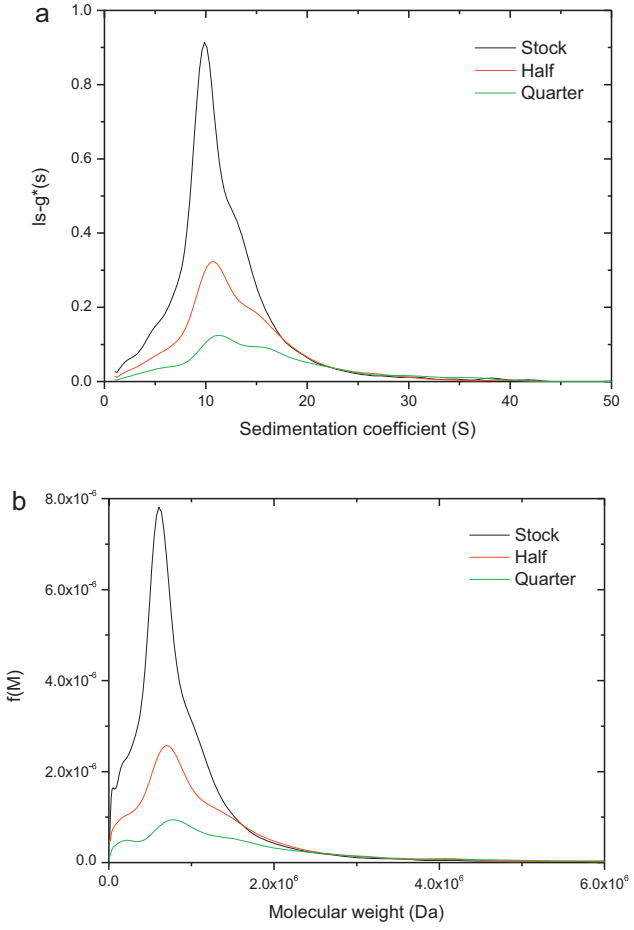

Fig. 4. Sedimentation coefficient (a) and molecular weight distribution plots (from the Extended Fujita procedure) for purified HGMYanL in phosphate–chloride buffer, $I = 0.1$ M, pH 7.0.

4.2.3. HGMYanL

The same procedure used for HGMYanH was used to analyse HGMYanL (Fig. 4a). The $g(s)$ distributions are similar to that of HGMYanH (Fig. 3a), showing a peak at ~12 S, and there is a shoulder at ~14 S, with no sign of material between 25 and 30 S at the stock concentration used, although a second peak could be found at lower concentrations. Again using the κ_s, b data of Fig. 1b the $g(s)$ profile for 3 dilutions of mucin was transformed into the $f(M)$ molecular weight distribution plot (Fig. 4b). Compared to Fig. 3b, there is a significant amount of material below 1 MDa.

4.2.4. HGMYanH in 6 M GuHCl

The $g(s)$ vs. s profiles are shown in Fig. 5a. To transform to $f(M)$ in this case we used the κ_s and b values appropriate for this solvent (Table 1b and Fig. 1c). There is a definite shift in the molecular weight distribution to higher molecular weights, with the weight average for the whole distribution M_w increasing from $\sim 1.9 \times 10^3$ kDa to $\sim 2.8 \times 10^3$ kDa, a difference consistent with the suggested use of GuHCl in the past as a storage medium for mucin glycoproteins – in this environment potentially degradative enzymes are fully inactivated (see Harding, 1989).

4.2.5. HGMYanH analysed by SEC–MALs

HGMYanH in PBS was also analysed using SEC–MALs to check for consistency with the $f(M)$ method. Excellent agreement was obtained for the whole distribution weight average molecular weights ($M_w \sim 1.9 \times 10^3$ kDa and $\sim 1.9 \times 10^3$ kDa for the $f(M)$ procedure and SEC–MALs respectively) with good agreement also for the polydispersity ratio ($M_z/M_w \sim 1.3$ and 1.5 respectively).

5. Discussion

The method is seen as complementary to SEC (or FFF)–MALs, which has been established as the method of choice for polymeric molecular weight distribution analysis. However the latter method is not useful when non-inertness of the columns (SEC) or membranes (FFF) are suspected, or the separation range has been exceeded. In the normal operation of the *Extended Fujita* procedure (Harding et al., 2011) the κ_s is evaluated from a separate sedimentation equilibrium or SEC–MALs experiment to define a pair of M–s values and hence κ_s. A problem with the alternative "universal" scaling approach suggested here for mucins is the amplification of the experimental uncertainty in κ_s converting from the double logarithmic plot (by contrast uncertainties in b are not amplified). Despite this, in the case of the 'universal' plot for mucins in aqueous solvents supplemented by 6 M GuHCl the uncertainty is still relatively small. Although the uncertainty is much larger with the universal plot in the absence of GuHCl it is nonetheless re-assuring that the M–s pair approach applied to HGMYanH in PBS, with an experimentally measured M_w from SEC–MALs of $(1.9 \pm 0.4) \times 10^3$ kDa used with an $s^0_{20,w}$ from sedimentation velocity of (13.5 ± 0.6) S yields a comparable value of κ_s but with a much lower degree of uncertainty, $\kappa_s = (0.0074 \pm 0.0010)$ compared with (0.00100 ± 0.0058).

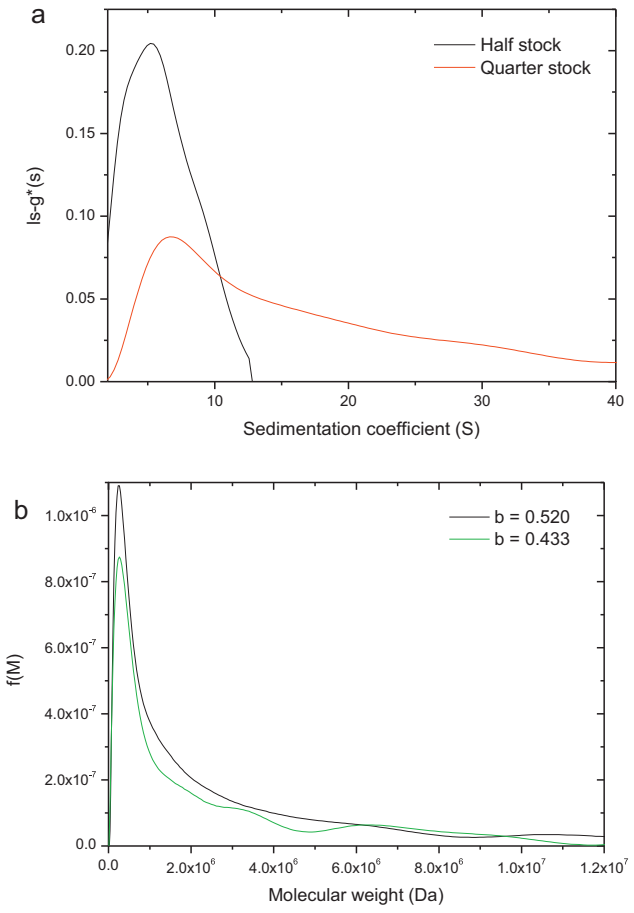

Fig. 5. Sedimentation coefficient (a) and molecular weight distribution plots (from the Extended Fujita procedure) for purified HGMYanH in phosphate–chloride buffer, I = 0.1 M, pH 7.0 and 6 M GuHCl.

Our recommendation for future application to these substances would be to utilise the scaling coefficient b from this study (appropriate for aqueous solvents or aqueous solvents supplemented with 6 M GuHCl) and then to evaluate κ_s for a particular mucin using Eq. (6) prior to performing the transformation of $g(s)$ vs. s to $f(M)$ vs. M. If operating in other solvents, b would need to be measured too. Care has also to be expressed in terms of the appropriate choice of parameters depending on the solvent, and also that concentrations are low enough so that complications through thermodynamic non-ideality can be reasonably ignored. If these issues are taken into consideration it would seem that the new Extended Fujita approach can be useful for characterising the molecular weight distributions of preparations of mucins.

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