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An analytical ultracentrifugation based study on the conformation of lambda carrageenan in aqueous solution

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ABSTRACT

The conformation and heterogeneity of lambda-carrageenan, a sulphonated galactan from red seaweed, solubilised in aqueous solvent with the assistance of microwave irradiation, has been assessed by a combination of analytical ultracentrifugation, size-exclusion chromatography, light scattering and capillary viscometry. Preparations appeared generally unimodal on the basis of sedimentation coefficient distributions from sedimentation velocity although at the highest concentrations a shoulder appears with a sedimentation coefficient approximately 1.1 times greater than that of the main component. Even under conditions commensurate with charge suppression simple linear regression was insufficient to represent non-ideal concentration dependence and the extraction of the Grálen concentration dependence parameter k_s. A more general fitting algorithm was therefore employed. Mark–Houwink–Kuhn–Sakurada analysis of the change in intrinsic viscosity $[\eta]$ with molecular weight, together with the Wales–van Holde ratio (combination of k_s with $[\eta]$) point to an extended flexible conformation for lambda-carrageenan in the (weight average) molecular weight range M_w = 340,000–870,000 g/mol. The origin of the larger sedimentation coefficient component appearing at the higher concentrations is considered.

1. Introduction

The carrageenans are a family of linear polysaccharides with strictly alternating backbones of α(1–3) and β(1–4) galactose residues masked with sulphate half ester groups and 3.6 anhydro-bridges (Lawson & Rees, 1968; Imeson, 2009). Carrageenans are found in the "red" seaweeds (*Rhodophyceaea*) and the most important members of the family are kappa (κ), lambda (λ), and iota (ι). *Chondrus crispus* is the main source of kappa and lambda, and iota and kappa are extracted from *Eucheuma spinosum* and *cottonii* (see, for example, Campo, Kawano, Silva, & Carvalho, 2009). The characteristic differences between the carrageenans are due to the varying proportions and linkage positions of their 3,6-anhydrogalactose and ester sulphate substituents. These structural variations are responsible for the unique characteristics for each type of carrageenan, including gel strength, viscosity, temperature stability, synergism and solubility (Tombs and Harding, 1998).

Lambda-carrageenan – is the most soluble with three sulphate groups per disaccharide repeat – and as it contains no 3,6-anhydrogalactose residues it cannot form gels (Fig. 1). The high viscosities of its solutions – and its inability to form gels have made lambda-carrageenan a popular thickening agent in foods and biopharmaceutical products (Bonferoni et al., 1994; Therkelsen, 1995). Lambda-carrageenan has also shown potent immunogenic activity against HIV and some tumours (Nakashima et al., 1987; Zhou et al., 2004). Thus, critical to understanding the functional properties of this molecule is an understanding of its fundamental properties of homogeneity, molecular weight, conformation and flexibility, and oligomeric state.

Progress has been made in earlier studies on the properties of these molecules – for example Slootmaekers, van Dijk, Varkevisser, Bloys van Treslong, and Reynaers (1991a), Slootmaekers, Mandel,

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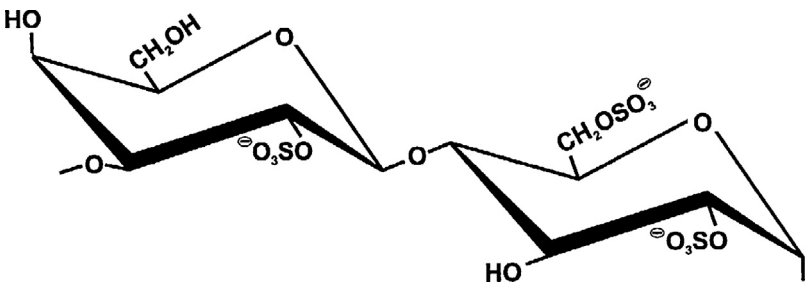

Fig. 1. Lambda-carrageenan repeat unit.

and Reynaers (1991b) have considered the sedimentation, viscosity and light scattering behaviour of a lambda-carrageenan of molar mass (molecular weight) $M \sim 610{,}000$ g/mol and Berth, Vukovic, and Lechner (2008) have considered the use of light scattering, viscosity and multi-angle laser light scattering coupled to size exclusion chromatography of a larger lambda-carrageenan ($M \sim 920{,}000$ g/mol), and how the way hydrodynamic parameters such as the intrinsic viscosity change with molecular weight. We take advantage of recent advances in analytical ultracentrifugation to investigate the properties of three preparations of lambda-carrageenan of different molecular weight, and in particular improvements in the extraction of sedimentation coefficients, dealing with the problem of the high concentration dependence through non-ideality.

2. Materials and methods

2.1. Materials

The carrageenan samples were kindly donated by CPKelco, Lille Skensved, Denmark. A stock solution of 3.0 mg ml^{-1} was made up in deionised distilled water then dialysed into phosphate chloride buffer solution supplemented with NaCl to an ionic strength 0.1 M and pH 7.0 (Green, 1933). The concentration was measured (after dialysis) using an Atago (Fairfax, Canada) DD-5 refractometer calibrated with glucose standards. This solution was then exposed to microwave heating (see, e.g., Wang, Wood, & Cui, 2002) in an 800 W microwave oven (Panasonic UK Ltd., Bracknell, UK) for 15 s and 30 s (Table 1). Heating for longer periods (30 s) led to discoloration of the samples.

2.2. Size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALS)

Molecular weight (weight averages) of the polysaccharide samples were determined by using SEC-MALS (Wyatt, 1992), a technique which has been successfully applied to polysaccharide characterisation since Horton, Harding, and Mitchell (1991). The present set-up comprised a series of size-exclusion chromatography (SEC) columns, namely TSK G6000PW and TSK G4000PW, protected by a similarly packed guard column (Tosoh Bioscience, Tokyo, Japan) coupled on-line to a "Helios" multi-angle light scattering (MALS) and refractive index (Optilab rEX) detectors (Wyatt Technology, Santa Barbara, USA). Solutions prepared at injected concentrations of 0.5 mg ml^{-1} and 1.0 mg ml^{-1} were filtered through a 0.45 μm syringe filter (Whatman, Maidstone, England) – to remove any insoluble material or dust prior to being injected into the columns. The buffer was pumped at a steady, pulse-free flow rate of 0.8 ml min^{-1} through the column system. ASTRA (Version 5.1.9.1) software (Wyatt Technology, Santa Barbara, USA) was used to analyse the data. A linear fit to the "Debye" plot of Kc/R_θ vs $\sin^2(\theta/2)$, where the terms have their usual meaning (see for example Wyatt, 1992) was used for calculating absolute weight average molecular weight (M_w) using a value for the refractive index increment $dn/dc = 0.116$ ml/g (Slootmaekers et al., 1991b). Because of the low concentrations after dilution on the columns no correction for non-ideality was assumed necessary.

2.3. Capillary viscometry

Relative viscosities η_{rel} were measured from the ratio of flow times of solution to solvent using a 2 ml Oswald viscometer. The U-tube viscometer was suspended in an accurate temperature regulated water bath. The temperature was kept constant at (20.0 ± 0.01) °C throughout by using a coolant system. Because of the low concentrations no correction for solution density was necessary (Harding, 1997). The reduced $\{\eta_{red} = (\eta_{rel} - 1)/c\}$ and inherent $(\eta_{inh} = \{\ln \eta_{rel})/c\}$ viscosities were extrapolated to infinite dilution to eliminate non-ideality effects using both the Huggins (1942) and Kraemer (1938) approaches:

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

$$\frac{\ln(\eta_{rel})}{c} = [\eta](1 - K_K[\eta]c) \quad (2)$$

where the intrinsic viscosity $[\eta]$ is taken as the mean of intercepts from Eqs. (1) and (2) and K_H and K_K are the Huggins and Kraemer constants, respectively. To avoid possible ambiguities through transition from the dilute to the semi-dilute region $[\eta]$ was also estimated at individual concentrations from the Solomon–Ciuta relation, essentially a combination of the Huggins and Kraemer linear relationships (see e.g., Harding, 1997):

$$[\eta] \approx \frac{1}{c}[2(\eta_{rel} - 1) - 2\ln(\eta_{rel})]^{1/2} \quad (3)$$

2.4. Sedimentation velocity in the analytical ultracentrifuge

Sedimentation velocity in the analytical ultracentrifuge was used to probe the heterogeneity of solutions as well as to

Table 1
Hydrodynamic properties of lambda carrageenans.

Sample	$10^{-3} \times$ Molar mass M_w (g/mol)	Intrinsic viscosity $[\eta]$ (ml/g)	$s^o_{20,w}$ (S)	k_s (ml/g)	$k_s/[\eta]$
λ870	870 ± 40	1080 ± 40	4.9 ± 0.1	1060 ± 20	1.0 ± 0.1
λ730	730 ± 40	960 ± 10	5.3 ± 0.1	1200 ± 40	1.2 ± 0.1
λ340	340 ± 20	640 ± 30	3.9 ± 0.1	510 ± 30	0.8 ± 0.1

measure the sedimentation coefficient (see Harding, 2005a,b; Harding, Rowe, & Horton, 1992). Polysaccharide samples 400 μl of the polysaccharide samples (after dialysis against the phosphate chloride buffer at pH 7.0 and ionic strength 0.1 M, and then serial dilution with the dialysate) and (400 μl) of the phosphate buffer dialysate were injected into sample and reference channels respectively, of double sector 12 mm optical path length cells. The Rayleigh interference optical system was used for recording concentration profiles and the movement of the sedimentation boundary in the analytical ultracentrifuge cell (see, e.g., Harding, 2005b). An initial low rotor speed of 3000 rpm was accelerated to a rotor speed of 40,000 rpm. Scans were taken at 2 min intervals for a run time of ~24 h. The data was analysed using the SEDFIT procedure (Dam & Schuck, 2003) which solves the Lamm equation describing the change of concentration distribution with radial position with time in terms of a distribution of sedimentation coefficients, $g(s)$ vs s, where s is the sedimentation coefficient. The (differential) distribution of sedimentation coefficients $g(s)$ can be defined as the population – weight fraction – of species with a sedimentation coefficient between $s + ds$ (Dam & Schuck, 2003; Harding, 2005a).

Sedimentation coefficients (in Svedberg units, S) were then corrected to standard conditions of the density and viscosity of water at 20.0 °C using the SEDNTERP procedure of Laue, Shah, Ridgeway, and Pelletier (1992) to yield $s_{20,W}$. Finally data has to be corrected for hydrodynamic non-ideality. Because of the severity of the concentration dependence of the sedimentation coefficient, standard extrapolations of the reciprocal $1/s_{20,W}$ to $c = 0$ (Schachman, 1959) were inapplicable. Instead we have used a non-linear fitting procedure (Robust fitting in the curve-fitting package proFit™, QuantumSoft, Zürich) to achieve stable estimates for k_s (the Gralén (1944) concentration regression parameter) even with only 5 data points. The equation fitted is to a general equation for concentration dependence (Rowe, 1992):

$$s(c) = s^o \left(1 - \frac{k_s c - (c^2 v_s^2 (2\phi_p - 1)/\phi_p^2)}{(k_s c - 2cv_s + 1)}\right) \quad (4)$$

where $s(c)$ and s^o are the sedimentation coefficient at $c = c$ and $c = 0$ respectively, ϕ_p is the critical packing fraction and v_s (ml/g) the swollen specific volume (the volume of a macromolecule swollen through solvation per unit anhydrous mass). Eq. (4) applies to solutes where polyelectrolyte (charge) effects are 'swamped' by the presence of neutral salt, as is the case in this study. For spherical particles, the equation gives results near-identical to those provided by the numerical solution of Brady and Durlovsky (1988). Statistical errors in resultant k_s values have been determined by bootstrapping (200 fits, confidence limits 68.3%), and found to be negligible

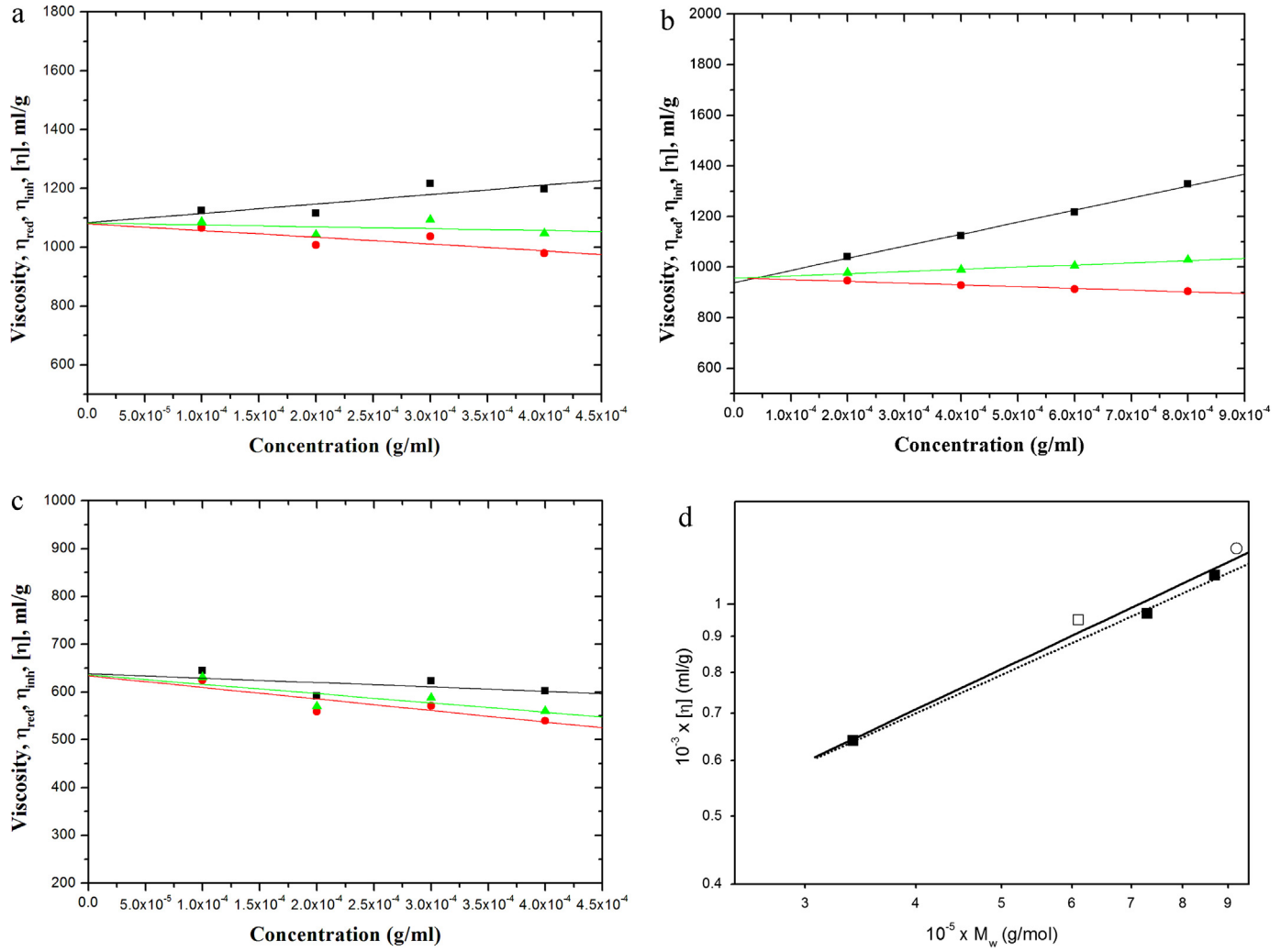

Fig. 2. Huggins (black line), Kraemer (red line) and Solomon–Ciuta (green line) extrapolations for (a) λ870, (b) λ730, (c) λ340 and (d) Mark–Houwink–Kuhn–Sakurada intrinsic viscosity plot. Filled circles – λ870, λ730, λ340; open circle – data of Berth et al. (2008); open square: data of Slootmaekers et al. (1991a). Solid line fitted to all 5 points yields a slope ($a = 0.6 \pm 0.1$) which within error is the same for a 3 point fit (dotted line) to λ870, λ730 and λ340. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(<±1%) – uncertainties in absolute sedimenting concentration estimates will be larger than that.

3. Results

3.1. Estimation of molecular weight (M_w)

For each lambda-carrageenan sample we present the molecular weight results obtained from SEC-MALS experiments in (Table 1). Intriguingly the value for the 15 s microwave heated sample (λ870) yielded a slightly higher molecular weight than for the unheated sample (λ730). This may reflect the final dispersion of higher molecular weight material. Heating for a short time (15 s) exhibited good dispersibility without degradation. Heating for an additional 15 s led to clear chain scission with a clear reduction in molecular weight (sample λ340). Further application of heat led to discoloration of the sample, and such samples were not used for further characterisation.

3.2. Intrinsic viscosity [η]

The intrinsic viscosity values for lambda-carrageenan samples, reported in Table 1, showed a good linear extrapolation in all cases from the Huggins, Kraemer and Solomon–Ciuta plots (Fig. 2a–c). The values for the intrinsic viscosity, particularly for the λ870 sample, are similar to other non-gelling viscosifying agents used in industry, such as the galactomannans and glucomannans.

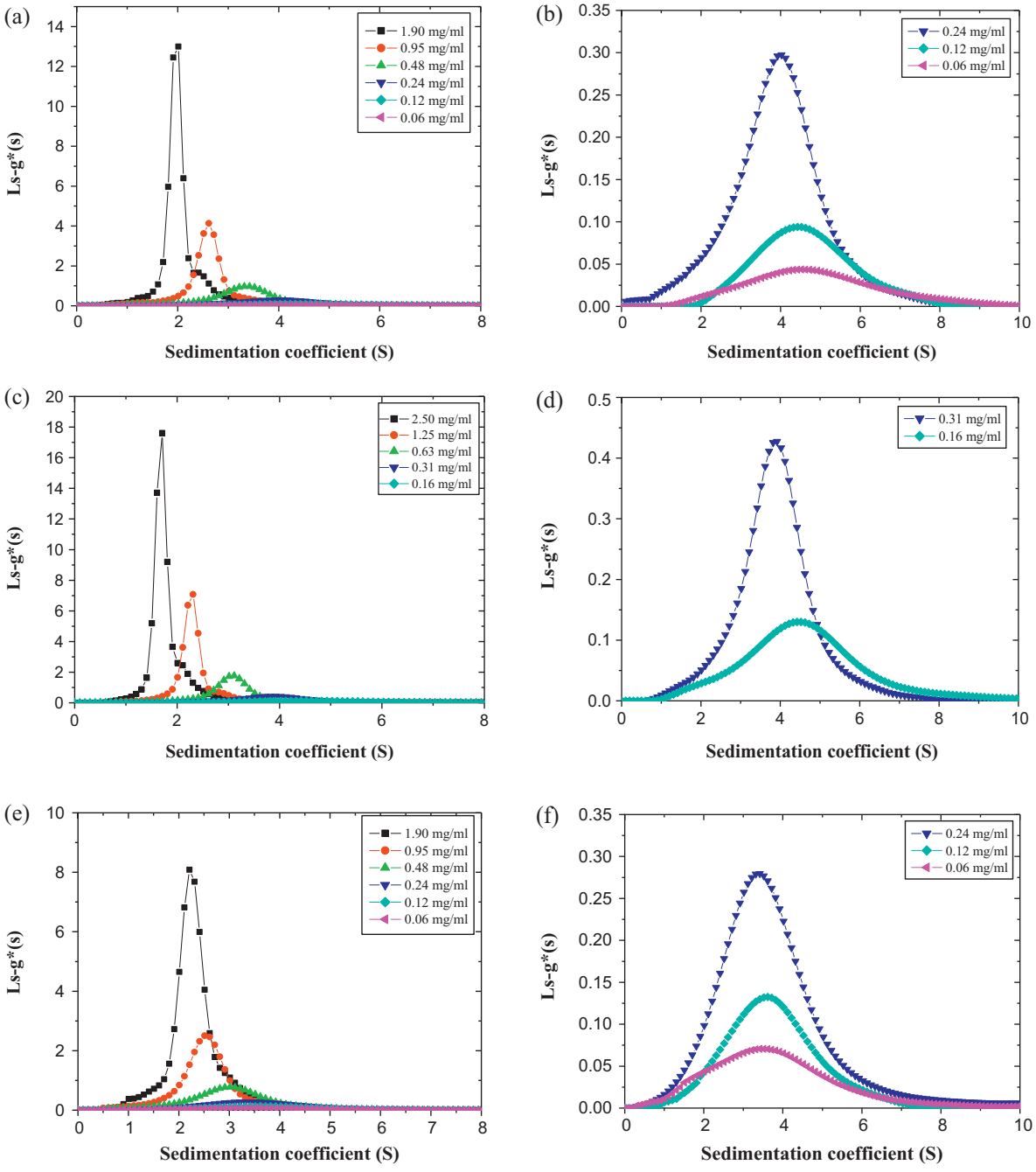

Fig. 3. Sedimentation coefficient distributions for (a) and (b) λ870 (c) and (d) λ730 (e) and (f) λ340. Distributions were obtained from the least squares $g(s)$ procedure of SEDFIT (Dam & Schuck, 2003).

3.3. Sedimentation coefficient and sedimentation coefficient distribution

The sedimentation velocity experiments mostly yielded unimodal distribution plots, $g(s)$ vs s (Fig. 3a–c): a shoulder at the highest concentration peaks were seen for all three preparations, suggestive of the presence of higher molecular weight material. The weighted average $s_{20,W}$ values however all increased with decrease in concentration (Fig. 4a–c), showing no evidence of reversible associative behaviour. Even across the small range of concentrations used there was considerable concentration dependence of the sedimentation coefficient through the effects of non-ideality, and conventional linear fitting of plots of $1/s_{20,W}$ vs c proved inadequate as found earlier by Slootmaekers et al. (1991a). Instead we used Robust fitting in the curve-fitting package proFit^{TM}, (QuantumSoft, Zürich) to apply the generalised form of the concentration dependence Eq. (4), to extract $s_{20,W}$ and k_s, and in general satisfactory fits were found, although the lowest concentration value for the λ870 sample (marked as an × in Fig. 4a) had to be excluded from the fitting procedure. Statistical errors in the extraction of the resultant k_s values have been determined by bootstrapping (200 fits, confidence limits 68.3%), and found to be negligible (<±1%) although systematic errors arising from uncertainty in absolute sedimenting concentration estimates will be larger than that. An example of the evaluation procedure is provided in Fig. 4d. The values of $s^{o}_{20,w}$ and k_s obtained from Eq. (4) are given in Table 1.

3.4. Conformational analysis

One of the simplest methods of assessing the conformation of a polymer chain is the "Mark–Houwink–Kuhn–Sakurada" MHKS power law relation

$$[\eta] = K'M^a \tag{5}$$

with the limits for $a \sim 0$ for a sphere, 0.5–0.8 for a flexible coil and 1.8 for a rigid rod. Solvent draining effects – where the solvent within the domain of a coiled molecule is free to travel at its own velocity – can also affect these parameters but these are usually small compared to the strong hydrodynamic forces between the macromolecular segments and following Tanford (1961) we assume these are negligible. In Fig. 2d we plot the values for λ870, λ730, λ340 and obtain a value for a of (0.6 ± 0.1) consistent with a flexible coil conformation. A very similar value is obtained if additional values of $[\eta]$ and M from Slootmaekers et al. (1991b) and Berth et al. (2008) are included.

The Wales–van Holde ratio $R = k_s/[\eta]$ provides another useful indicator of conformation. The limits are ~1.6 for a compact sphere or a (non-draining) random coil (Creeth & Knight, 1965), and ~0.15 for rods (see, for example, Tombs & Harding, 1998). All the values are ~1 (Table 1) consistent with a flexible structure – possibly more extended than a random coil, and consistent with a value previously estimated for kappa-carrageenan (Harding, Day, Dhami, & Lowe, 1997).

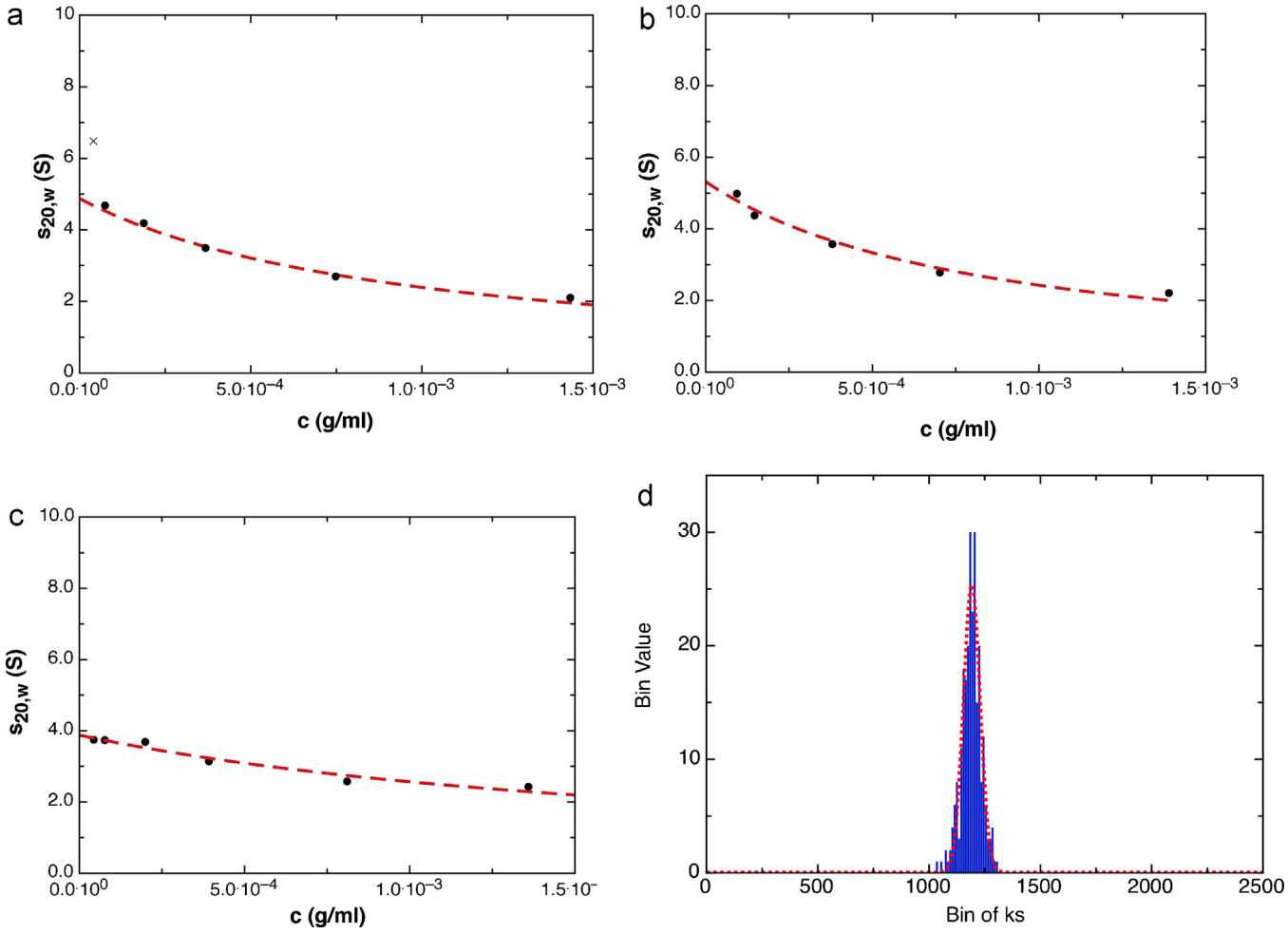

Fig. 4. Concentration dependence of the sedimentation coefficient (after correction to standard solvent conditions) for (a) λ870, (b) λ730 and (c) λ340, with lines fitted to Eq. (4). The lowest concentration point for λ870 (indicated by different symbol) was not used in the fit. (d) "Bin" for estimated values of k_s (ml/g) of λ870, from error analysis in the Levenberg–Marquardt (Marquardt, 1963) fitting procedure.

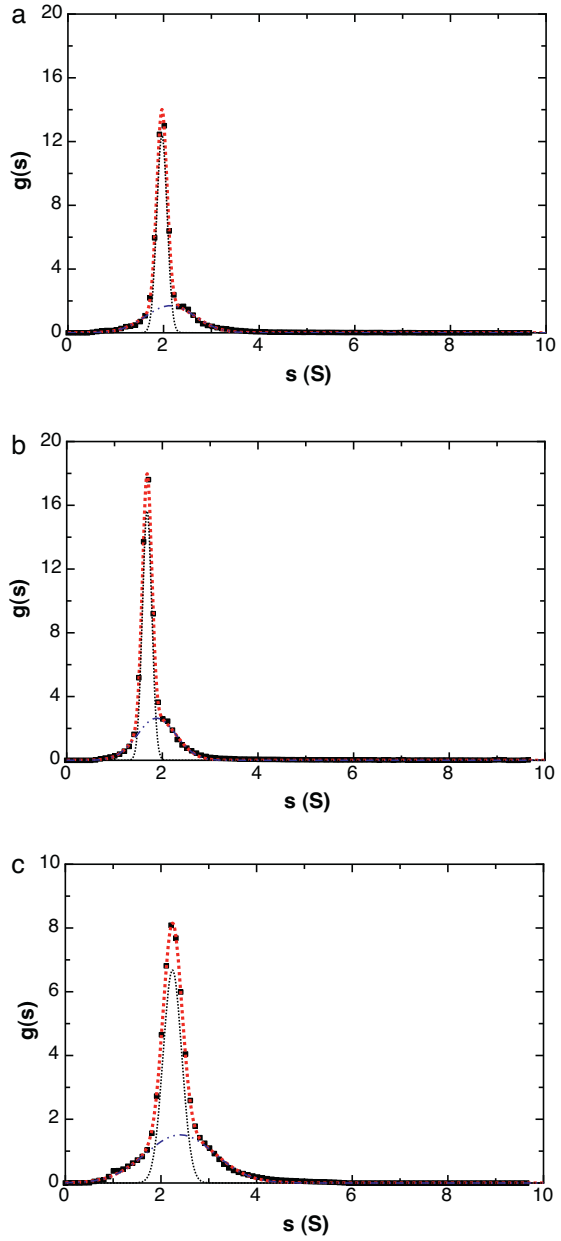

Fig. 5. Multi-Gaussian fit (dotted blue lines) of the highest concentration $g(s)$ vs s profiles for (a) λ870, (b) λ730 and (c) λ340. In each case two components are resolved, with the sedimentation coefficient of the faster component being approximately 1.1× greater than that of the slower component. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

Lambda-carrageenans, even under conditions which normally suppress polyelectrolyte effects, show very strong non-ideality behaviour, presumably through their large exclusion covolumes. Simple linear regression was insufficient to represent the non-ideal concentration dependence of the sedimentation coefficient for lambda-carrageenan, at least within the size range we have considered, but a more general form of the dependence equation (Rowe, 1992) yielded good fits and stable estimates for both $s^{o}_{20,w}$ and k_s.

Evidently this molecule has considerable flexibility, as judged from both the viscometric and analytical ultracentrifuge behaviour. This conclusion seems to reinforce earlier observations by Slootmaekers et al. (1991b) and Berth et al. (2008). The concentration dependence of the (weight average) sedimentation coefficient (with clear decrease with increase in concentration) in itself shows no clear evidence of any significant reversible association or dissociation in solution, at least under the conditions studied: the behaviour seen here for lambda carrageenan is different from the associative behaviour seen using analytical ultracentrifugation in for example arabinoxylans (Patel et al., 2007) – which shows a tendency for weak reversible self-association – and also more recently in amino-celluloses, the latter which exhibits step-wise, protein like association into dimers, trimers, tetramers, etc. (Heinze et al., 2011). An earlier study using analytical ultracentrifugation on kappa-carraageenan also showed no evidence for reversible associative phenomena (based on both sedimentation velocity and also sedimentation equilibrium analysis). It is possible that this may have been obscured by the strong co-exclusion (molecular obstruction) non-ideality in solution, requiring more than one non-ideality virial term for the representation of solute distributions at sedimentation equilibrium (Harding et al., 1997).

In this regard it is interesting to consider what the source of the shoulder observed at the highest concentration observed in this study for lambda carrageenan might be. Multi-Gaussian analysis of the $g(s)$ vs s profiles (Fig. 5) shows for all three samples the 2nd component has a sedimentation coefficient approximately 1.1× that of the main component, and the relative proportion of the 2nd component relative to the main component is approximately 40%. This component may be an impurity although its appearance only at the highest concentration indicates it might be an association product.

In perspective, it would be interesting to compare the properties of the different classes of carrageenans using modern analytical ultracentrifugation, with its now greater resolving power over a wide range of solvent and preparation conditions to see if this method provides any evidence of dimerization/double helix formation as originally suggested by Rees, Morris, Thom, and Madden (1992) for all three main classes (iota, lambda and kappa).

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