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A comparison of molecular mass determination of hyaluronic acid using SEC/MALLS and sedimentation equilibrium

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Abstract Hyaluronic acid (HA) is a natural polysaccharide with importance in the pharmaceutical, medical and cosmetic industries. Determining factors in its final applications are its physicochemical properties, particularly molecular mass. A high molecular mass HA was degraded using five different hydroxyl free-radical starting concentrations chemically produced from ascorbic acid and hydrogen peroxide. The aims of the study were to investigate the effect of different hydroxyl free-radical concentrations on the chain length of HA and compare the molecular masses obtained from analytical ultracentrifugation using sedimentation equilibrium experiments and size exclusion chromatography/multi-angle laser light scattering (SEC/MALLS). The results indicated that their molecular masses varied, depending on the degree of hydroxyl free-radical starting concentration. Molecular mass values obtained from sedimentation equilibrium experiments for each sample showed the same trend as those obtained from the SEC/MALLS in the range of molecular masses studied. The molecular masses obtained from sedimentation equilibrium for high molecular mass samples from reciprocal plots of apparent weight average molecular mass against concentration gave values similar to those obtained by SEC/MALLS. In contrast, the molecular mass from conventional plots for high molecular mass samples were much lower than those from SEC/MALLS, even when high ionic strength buffers were used.

Keywords Hyaluronic acid · Multi-angle laser light scattering · Sedimentation equilibrium · Size exclusion chromatography

Introduction

Hyaluronic acid (HA) or hyaluronan, a naturally occurring linear polysaccharide chemically classified as a glycosaminoglycan, is composed of the repeating disaccharide units of [$\rightarrow 4$)- β -D-glucopyranosyluronic acid-(1 \rightarrow 3)-N-acetyl-2-amino-2-deoxy- β -D-glucopyranosyl-(1 \rightarrow]]. The polysaccharide contains between 500 and 50,000 monosaccharide residues per molecule; thus its molecular mass ranges from 10^4 to 10^7 Da (Fransson 1985; Robyt 1998).

HA is widely distributed in mammalian cells and tissue but is primarily found in synovial fluid, vitreous humour of the eye and loose connective tissue such as rooster comb, umbilical cord, dermis and arterial wall. It is also found in the capsular component of certain bacteria such as *Staphylococcus* and *Streptococci* (Tombs and Harding 1998). These have been biotechnologically developed and are now a main source of commercial HA for pharmaceutical, medical and cosmetic applications.

Pharmaceutically, HA has recently been studied for use in drug delivery applications (Lapcik et al. 1998). Medically, HA has been used as a surgical aid in ophthalmology and it also has therapeutic potential for the treatment of arthritis and wound healing (Goa and Benfield 1994).

HA has unique physicochemical properties; these properties are currently being studied and have drawn much attention in medical and pharmaceutical research for novel applications. Since these properties are governed greatly by molecular mass, a reliable method for its determination is of fundamental importance.

Studies on the molecular mass of HA, especially the weight average molecular mass (M_w), have been performed by several workers, mostly using gel permeation

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chromatography [GPC, also known as size exclusion chromatography (SEC)]. Intrinsic viscosity measurements can also be used to determine the molecular mass through the Mark-Houwink-Kuhn-Sakurada (MHKS) relationship and, recently, high-performance capillary electrophoresis has been introduced (Hayase et al. 1997). However, all three methods are relative and rely on calibration with appropriate standards.

In addition to relative methods, where the molecular mass values obtained are dependent on standards and the solvent systems used, absolute methods to characterize molecular mass such as light scattering, osmometry and sedimentation experiments in the analytical ultracentrifuge have been used (Lapcik et al. 1998).

Over the past two decades, absolute methods for the molecular mass determination of biological macromolecules have greatly improved. For example, coupling of a multi-angle laser light scattering (MALLS) photometer to SEC (so-called SEC/MALLS), permitting on-line molecular mass determination by an absolute method, has been employed and is becoming increasingly popular in molecular mass determination of polyionic polysaccharides such as chitosan, where calibration with commercially available standards like dextran or pullulan is likely to give molecular masses greatly different from the true values (Churms 1996).

Analytical ultracentrifugation – more specifically the sedimentation equilibrium technique – has long been used for the molecular mass determination of proteins and is experiencing somewhat of a revival due to the development of modern instrumentation.

For polysaccharides in particular, the addition of the Rayleigh interference optical system has been an important step (Harding et al. 1991). Polysaccharides are almost always polydisperse and many such as HA are also polyelectrolytes. Both these factors affect molecular mass values and careful determination is required in the experimental design and data analysis.

The aim of our study was to determine and compare weight average molecular masses obtained by sedimentation equilibrium and SEC/MALLS for five different molecular mass HA samples chemically produced by different degrees of free radical depolymerization of a parent HA.

As HA is a polyelectrolyte, the effect of solvent ionic strength on the molecular mass values obtained was also investigated.

Materials and methods

The HA samples were supplied by Vitrolife UK and designated as HA1, HA2, HA3, HA4 and HA5.

The parent HA of $M_w \approx 3.25 \times 10^6$, previously determined using SEC/MALLS, was formulated as a 1% solution in phosphate buffered saline (PBS). 10 mL aliquots of 1% HA in PBS were measured into 25 mL plastic Universal bottles. Volumes of 0.1 M ascorbic acid and 0.1 M hydrogen peroxide (H_2O_2) were added to each bottle as indicated: HA5, 0.07 mL of each reactant; HA4, 0.18 mL of each reactant; HA3, 0.37 mL of each reactant; HA2, 0.50 mL of each reactant; HA1, 0.77 mL of each reactant.

Each bottle was lidded and placed on a Tube Rotator (Bibby Stuart) to mix for 2 h. The contents of each bottle were then transferred to 100 mL of isopropyl alcohol to stop the reaction and to precipitate the HA from the solution. Samples were centrifuged in a bench top centrifuge (MSE Centaur) at 3500 rpm for 10 min. The supernatant was decanted to waste and the HA recovered and dried at room temperature. The moisture content of these samples was found to be approximately 17% w/w and concentrations quoted in this article were calculated from dry mass.

All samples were solubilized for approximately 24 h in PBS (pH 7.3) in varying ionic strengths, i.e. 0.15, 0.3 and 0.5 M NaCl. Each contained 0.002 M sodium phosphate as buffer.

Determination of M_w by SEC/MALLS

SEC separates molecules according to their hydrodynamic volume, provided that there are no non-size exclusion mechanisms interfering with the separation. For a homologous series this results in a separation according to decreasing molecular mass. Dual detection with in-line refractive index and light scattering detectors allows the determination of absolute M_w (Jumel 1994; Wyatt 1992).

The chromatography system consisted of a HPLC pump (Model PU-1580, Jasco, Tokyo, Japan), a Rheodyne injection valve (Model 7125, Rheodyne, St Louise, Miss., USA) fitted with a 100 μ L loop and the following column system: Phenomenex guard column, TSK G6000PW and TSK G5000PW connected in series. Eluent identical to the solvent used to dissolve the samples was monitored using a Dawn DSP multi-angle laser light scattering photometer and an Optilab 903 interferometric refractometer (both instruments from Wyatt Technology, Santa Barbara, Calif., USA). A refractive index increment (dn/dc) of 0.167 mg/mL was experimentally determined using the same refractometer and solvent conditions as for the SEC/MALLS. Signals from the light scattering photometer and the refractometer were captured and analysed on a PC using the dedicated ASTRA software supplied by the manufacturer. Eluent was pumped at a flow rate of 0.8 mL/min. Sample concentrations of 5 mg/mL for HA1 to HA3, 2 mg/mL for HA4 and 1 mg/mL for HA5 (injection volume 100 μ L) were injected at room temperature.

Determination of M_w using sedimentation equilibrium

Sedimentation equilibrium experiments were performed using the Optima XL-I analytical ultracentrifuge (Beckman, Palo Alto, Calif., USA) fitted with Rayleigh interference optics to record the concentration distribution of the solute at equilibrium. 80 μ L of each, solution and solvent, were filled into the respective channels of multi-channel 12 mm optical path length cells and run at speeds of 17,000 rpm for HA1 and HA2, 10,000 rpm for HA3 and HA4 and 8,000 rpm for HA5 until equilibrium was achieved. Data was subsequently analysed using the model-independent software MSTARI (Cölfen and Harding 1997). For the calculation of molecular mass from sedimentation equilibrium, a value for the partial specific volume is required. Partial specific volumes ranging from 0.60 to 0.64 mL/g for the HA investigated here were used, which were individually determined using the mechanical oscillator technique according to the procedure by Kratky et al. (1973).

Results and discussion

Molecular mass (M_w) obtained from SEC/MALLS

At $I=0.15$, the lowest ionic strength used in this study, the elution profiles for all samples appeared as a single peak (see Fig. 1a and b). The small peaks appearing in the refractive index (RI) chromatogram at higher elution

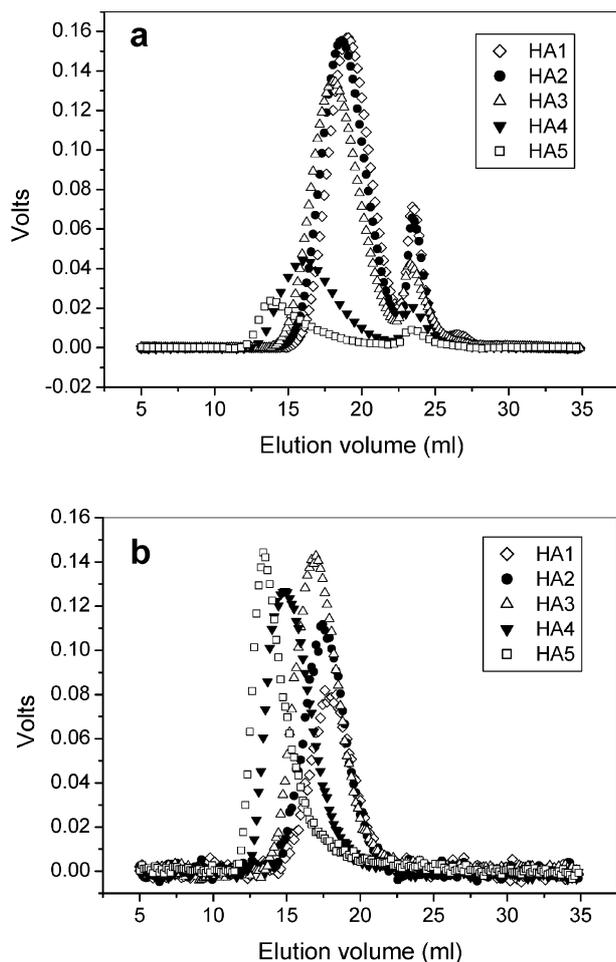


Fig. 1 Chromatograms from SEC/MALLS for all samples: (a) from refractive index (RI) detector; (b) from 90° light scattering detector

volume indicate the position of the total permeation volume and are not taken into further consideration. The peak elution volumes from the RI traces decrease from HA1 to HA5 (Fig. 1a), indicating a decrease in hydrodynamic volume and molecular mass.

The molecular mass values obtained from SEC/MALLS for each sample are given in Table 1, which agree with the qualitative assumption from the RI traces and confirm that by increasing the amounts of reactants added to the parent HA, depolymerization also increases.

As can be seen in Fig. 1a, the higher molecular mass samples have a broader elution range despite the lower injection concentrations. This is most likely due to viscous drag, a non-size exclusion effect found for viscous samples. Three different ionic strength solvents were used and although their elution patterns are very similar, a slight increase in elution volume with increasing solvent ionic strength can be observed (see Fig. 2). These changes indicate a decrease in hydrodynamic volume which would be expected for polyelectrolytes like HA: the repulsion between the carboxyl groups in HA

Table 1 Summary of M_w values for all samples obtained from SEC/MALLS and sedimentation equilibrium determined at different ionic strengths

	Hyaluronic acid samples				
	HA1	HA2	HA3	HA4	HA5
$10^{-4} \times M_w(\text{g/mol})^a$	4.8	6.5	12.3	42.2	145
$10^{-4} \times M_w(\text{g/mol})^b$	5.0	7.0	11.0	43.9	139
$10^{-4} \times M_w(\text{g/mol})^c$	4.9	7.1	11.7	43.6	135
$10^{-4} \times M_w(\text{g/mol})^d$	5.3	6.8	12.6	27.9	44.2
$10^{-4} \times M_w(\text{g/mol})^e$	5.4	7.6	14.2	49.9	165
$10^{-4} \times M_w(\text{g/mol})^f$	4.9	7.2	11.9	24.3	46.6
$10^{-4} \times M_w(\text{g/mol})^g$	4.9	7.6	12.5	41.8	123
$10^{-4} \times M_w(\text{g/mol})^h$	5.2	6.7	12.9	25.5	58.1
$10^{-4} \times M_w(\text{g/mol})^i$	5.2	6.8	14.1	44.5	123

^aSEC/MALLS at $I=0.15$

^bSEC/MALLS at $I=0.30$

^cSEC/MALLS at $I=0.50$

^dSedimentation equilibrium at $I=0.15$ from conventional plot

^eSedimentation equilibrium at $I=0.15$ from reciprocal plot

^fSedimentation equilibrium at $I=0.30$ from conventional plot

^gSedimentation equilibrium at $I=0.30$ from reciprocal plot

^hSedimentation equilibrium at $I=0.50$ from conventional plot

ⁱSedimentation equilibrium at $I=0.50$ from reciprocal plot

present as carboxylate anions in aqueous solution is diminished when the ionic strength of the solvent is increased.

However, no significant differences in the M_w values determined at different ionic strengths were observed (see Table 1), which is in keeping with expectations. Contributions due to shape are eliminated by extrapolations to zero angle and non-ideality effects become negligible due to the very low sample concentration after fractionation and dilution on the columns.

M_w obtained from sedimentation equilibrium

When the molecular mass of a macromolecule is determined by sedimentation equilibrium, the effects of thermodynamic solution non-ideality have to be taken into account. Solution non-ideality arises because of charge effects and excluded volume effects and both of these are relevant to the behaviour of HA in solution because it is highly charged and exhibits strong solvent-polymer interactions which increase with increasing concentration. As physical measurements on macromolecules have to be made at finite concentrations, these effects will contribute to the final result. When molecules are separated through a column system (as is the case with SEC/MALLS) they are greatly diluted and the concentrations at which measurements are taken are usually in the order of 10^{-5} g/mL. Non-ideality effects can therefore be considered negligible for this technique. However, in sedimentation equilibrium, non-ideality (or concentration dependence) can no longer be ignored and measurements at several concentrations and subsequent extrapolation to zero concentration are necessary.

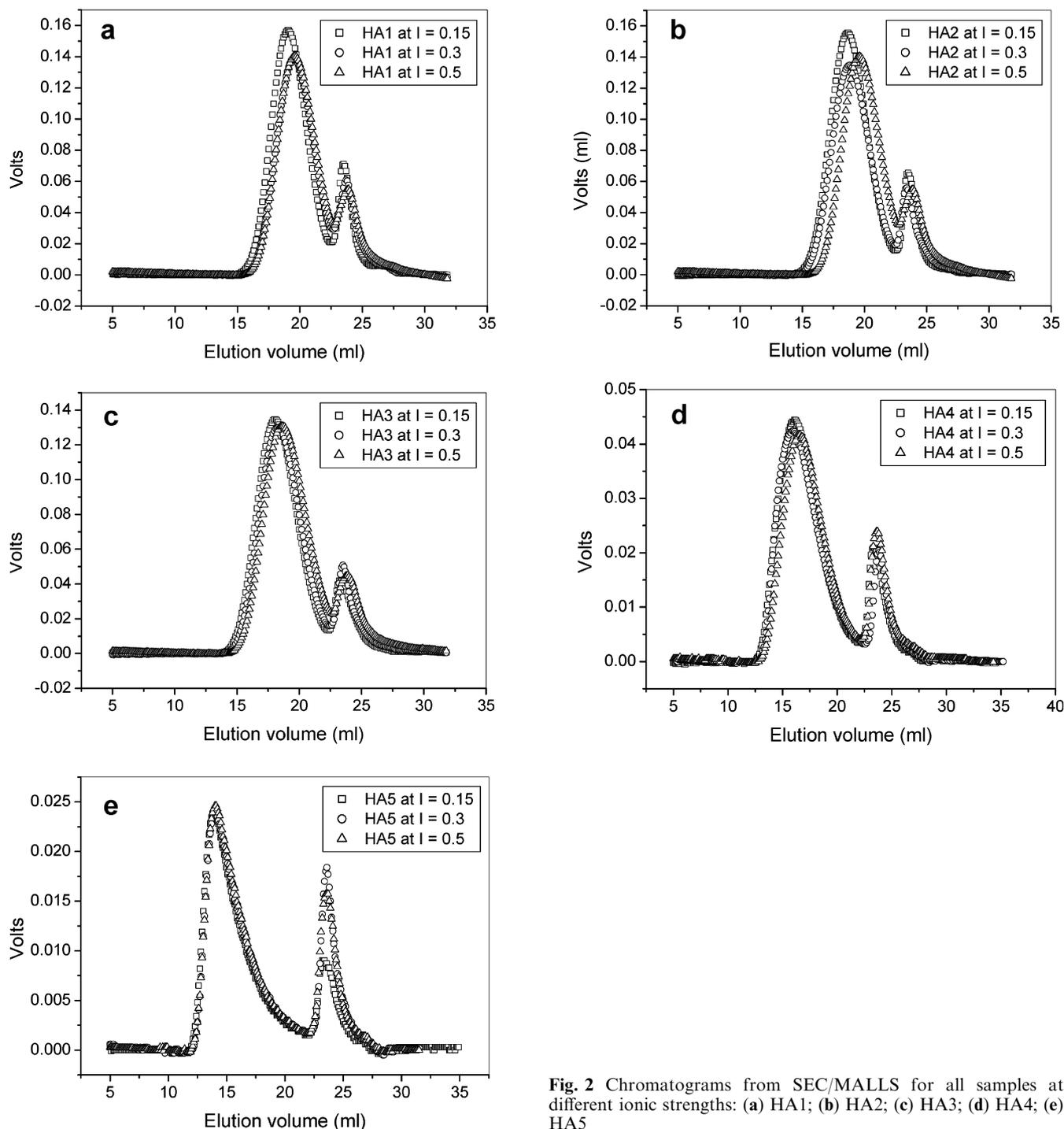


Fig. 2 Chromatograms from SEC/MALLS for all samples at different ionic strengths: (a) HA1; (b) HA2; (c) HA3; (d) HA4; (e) HA5

The $\ln J(r)$ versus normalized radius (ξ) plots generated by the model-independent software MSTARI give an indication as to the non-ideality and/or polydispersity of a macromolecular system. Figure 3 shows an overlay of such a plot for all samples at a concentration of 1 mg/mL, indicating non-ideal behaviour (see Harding and Creeth 1982; Harding 1985).

Each HA sample was run at a range of concentrations: 0.3–1.0 mg/mL for HA5 and 0.4–1.0 mg/mL for all other samples, and the apparent weight average

molecular mass values ($M_{w,app}$) obtained were extrapolated to zero concentration, giving M_w^0 . The conventional plot of $M_{w,app}$ versus concentration gave a linear extrapolation for samples HA1, HA2 and HA3 at all ionic strengths investigated (i.e., $I=0.15, 0.3, 0.5$) and M_w^0 values were very similar to the M_w values obtained by SEC/MALLS. However, M_w^0 values obtained for HA4 and HA5 by this extrapolation were considerably lower than those from SEC/MALLS and were no longer linear (Fig. 4a, c, e). In general, an increase in ionic

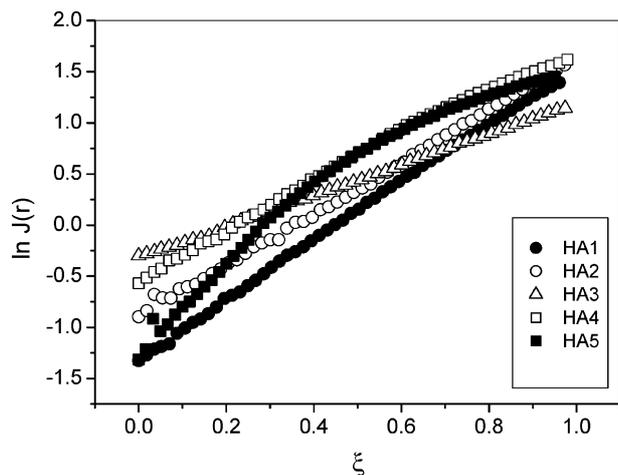


Fig. 3 Plot of $\ln J(r)$ versus ξ [$\xi = (r^2 - r_m^2)/(r_b^2 - r_m^2)$] from MSTARI for all samples at a concentration of 1 mg/mL, $I=0.15$

strength led to an increase in $M_{w,app}$ at each concentration for these samples, but the M_w^0 values were still considerably lower than those from SEC/MALLS because a linear relationship was not obtained.

The reciprocal plot of $M_{w,app}$ versus concentration is also frequently used to eliminate the concentration dependence of molecular mass in sedimentation equilibrium and it also allows the extraction of the second virial coefficient, B . Figures 4b, d, and f show these reciprocal plots for all samples and demonstrate the increase in linearity, even at the lowest ionic strength, for samples HA4 and HA5. The M_w^0 values obtained from these plots are shown in Table 1 and it is apparent that they are very similar to the M_w values obtained by SEC/MALLS.

Previous work by Preston and Wik (1992) investigated the effect of ionic strength on the second virial coefficient of low molecular weight HA by sedimentation equilibrium. A high dependence was shown when increasing the ionic strength from 0.001 to 0.2 M NaCl with values of B at $I=0.05$ and 0.2 being much smaller than those determined at lower ionic strength. In our investigation, only three different ionic strengths were used and, as Table 2 shows, our values for the second virial coefficient are in good agreement with that previously reported by Preston and Wik at $I=0.2$. There is little variation in the values obtained over the range of ionic strengths, which seems to indicate that $I=0.15$ is sufficient to suppress the effects of non-ideality. This is supported by the fact that M_w^0 values are no longer dependent on ionic strength, provided a linear extrapolation to zero concentration can be obtained. It must be noted that the higher concentration solutions of samples HA4 and HA5 are at the limit (or slightly above in the case of HA5) of the Debye criterion of $c[\eta] < 1$. This means that intermolecular interactions contributing to non-ideality should be taken into account through the third virial coefficient. Second-order polynomial fits were applied to the reciprocal $M_{w,app}$ versus

Table 2 Second virial coefficients for all samples obtained from $1/M_{w,app}$ versus concentration plots at different ionic strengths

Samples	$10^3 \times B$ (mol mL/g ²) ^a		
	$I=0.15$	$I=0.3$	$I=0.5$
HA1	2.2	0.3	0.6
HA2	4.9	3.3	1.9
HA3	3.3	1.7	2.7
HA4	3.7	2.3	3.3
HA5	3.6	3.0	2.3

^aValue of $10^3 \times B$ (mol mL/g²) = 4.1 for HA ($M_w = 72,000$) at $I=0.2$ reported by Preston and Wick (1992)

concentration plots; however, these fitting procedures were unsuccessful and values for the third virial coefficients are therefore not quoted here.

A previous study has shown that molecular masses of the polydisperse polyelectrolyte pectin determined by sedimentation equilibrium and SEC/MALLS were in good agreement and linear extrapolations to zero concentration from the plots of $M_{w,app}$ against concentration obtained from sedimentation equilibrium were adequate (Morris et al. 2000). However, in this study, only a narrow range of molecular masses with values of $(16.5-21.1) \times 10^4$ was investigated. Our results from the HA samples in the low molecular mass range agree very well with those findings. However, for the higher molecular mass HA samples, such a simple approach to the sedimentation equilibrium data analysis is no longer valid.

Our observations show that the molecular masses of HA samples obtained from sedimentation equilibrium were in keeping with the results from SEC/MALLS over the range of molecular mass studied; however, further investigation will be necessary, especially for high molecular mass HA samples when using sedimentation equilibrium, because of their very non-ideal and viscous solution properties and the problems with higher order extrapolations of the reciprocal molecular mass to zero concentration.

Concluding remarks

Our work on the effect of different free-radical hydroxyl concentrations on the depolymerization of HA has shown that an increase in the free-radical concentration will decrease the molecular mass of HA. Comparison of molecular mass values obtained from SEC/MALLS and sedimentation equilibrium demonstrates that the results of these techniques are in good agreement over the range of M_w studied, provided that the extra problems due to non-ideality are dealt with in the sedimentation equilibrium results. Determination of the molecular mass of HA by sedimentation equilibrium relies heavily on eliminating the effects of solution non-ideality. This can be achieved by running samples over a range of concentrations and extrapolating to zero concentration,

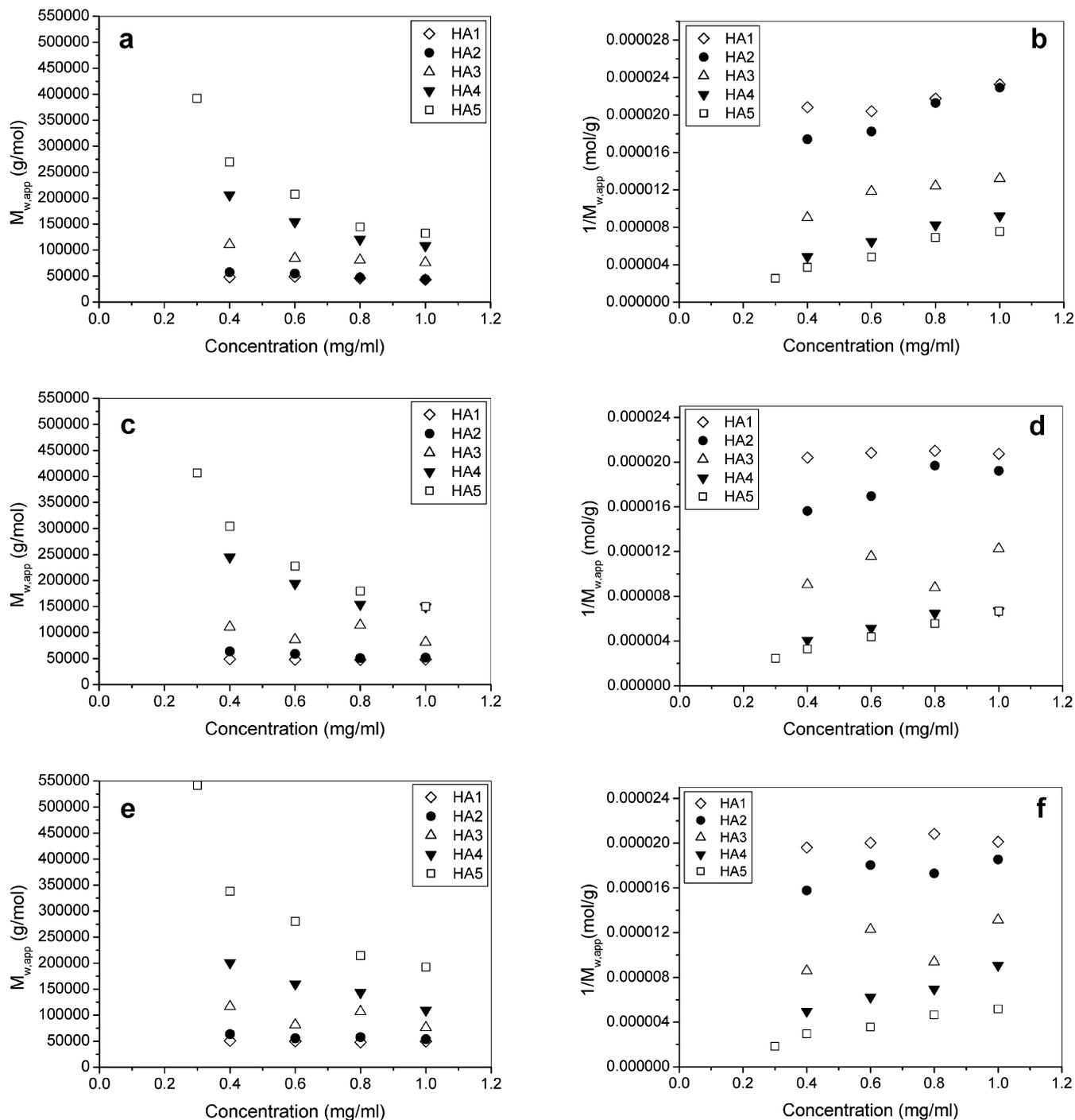


Fig. 4 Plots of $M_{w,app}$ against concentration for all samples: (a) conventional plot at $I=0.15$; (b) reciprocal plot at $I=0.15$; (c) conventional plot at $I=0.3$; (d) reciprocal plot at $I=0.3$; (e) conventional plot at $I=0.5$; (f) reciprocal plot at $I=0.5$

and/or increasing the ionic strength of the solvent. It was found that the extrapolation routine to zero concentration affected the results obtained for the higher molecular mass samples. Whilst for the (relatively) low molecular mass samples an extrapolation of $M_{w,app}$ versus concentration was sufficient, the reciprocal plot of

$1/M_{w,app}$ versus concentration was required for the two highest molecular mass samples. Investigations on the effect of varying ionic strengths from $I=0.15$ to $I=0.5$ have shown that this has little effect on the M_w values obtained from either technique, and second virial coefficients obtained from sedimentation equilibrium are in good agreement with previously published data. However, the authors are aware that in order to eliminate the effects of intermolecular interactions of the higher molecular mass samples, the third virial coefficient should also be taken into account. This work demon-

strates the validity of either technique for the determination of the molecular mass of HA; however, it may be preferable to use the much more rapid technique of SEC/MALLS for routine determinations. Further investigations regarding the molecular mass determination of high molecular mass HA by sedimentation equilibrium will be of interest owing to the unique properties of this biomacromolecule.

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