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Hydrodynamic characterisation of chemically degraded hyaluronic acid

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Abstract

Hyaluronic acid (HA) is a natural macromolecule with importance in the pharmaceutical, medical and cosmetic industries. The knowledge of its hydrodynamic properties is fundamentally important for further study on its applications. The aim of our study was to investigate and provide hydrodynamic parameters for six different molar mass samples chemically produced by depolymerisation of a high molar mass 'parent' HA using five different hydroxyl free radical concentrations. The main tools employed for these studies were size exclusion chromatography/multi-angle laser light scattering (SEC/MALLS), sedimentation velocity in the analytical ultracentrifuge and intrinsic viscosity. The results indicated that values for intrinsic viscosity, molar mass and sedimentation coefficient decreased with increasing hydroxyl free radical starting concentration. The six samples investigated here also represent a homologous series of a polysaccharide thus conformational information could be obtained, and the results indicate that HA adopts a 'stiffish' coil conformation in solution. Where appropriate our results were compared with previously published data.

Keywords: Hyaluronic acid; Hydrodynamic characterisation; Chemical degradation

1. Introduction

A great deal of research on hyaluronic acid (HA) has been conducted since it was discovered by Meyer and Palmer in 1934 in the vitreous humour of cattle eyes (Lapcik, Lapcik, De Smedt, Demeester, & Chabrecek, 1998). It is a chemically well characterised linear polysaccharide consisting of alternating $\beta(1 \rightarrow 4)$ linked *N*-acetyl-D-glucosamine and $\beta(1 \rightarrow 3)$ linked D-glucuronic acid (Jouon, Rinaudo, Milas, & Desbrieres, 1995; Lapcik et al., 1998).

The findings of its remarkable physicochemical properties, for example, its unique viscoelastic properties led to applications of HA especially in the pharmaceutical and medical fields. Ongoing pharmaceutical and medical research is now concentrating on its use in drug delivery systems in addition to its present therapeutic indications in ophthalmology, dermatology and osteoarthritis (Goa & Benfield, 1994; Lapcik et al., 1998).

Many applications of HA are based on its behaviour in aqueous solution, characterisation of its hydrodynamic properties is therefore an important prerequisite for further utilisation. But despite a considerable amount of research in this area some findings are still controversial (Lapcik et al., 1998).

Size exclusion chromatography (SEC) coupled to multi-angle laser light scattering is an absolute technique for the determination of molar mass (Harding, 1995b) and has been found particularly useful for HA as the dilution of the sample through the column system reduces some of the highly non-ideal behaviour of this polyelectrolyte. It has the added advantage that it can also give absolute molar mass distributions. The knowledge of molar mass alone is not sufficient to obtain information regarding the dilution solution conformation of macromolecules. To achieve this, additional measurements of intrinsic viscosity $[\eta]$ and sedimentation coefficient have to be made on a range of different molar mass samples to obtain the MHKS coefficients a and b, respectively.

The samples discussed in this article form a homologous series. A parent HA was degraded using ascorbic acid/hydrogen peroxide systems with different amounts of

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reactants resulting in HA samples with different molar masses depending on the amounts of reactants.

To our knowledge depolymerisation using ascorbic acid/hydrogen peroxide and subsequent characterisation by hydrodynamic methods has not been reported in the literature and it is hoped that this article will contribute to the understanding of the dilute solution conformation of HA.

2. Materials and methods

The following samples were supplied by Vitrolife UK Ltd HA 0.77 Fop/SH2, HA 0.5 Fop/SH, HA 0.37 Fop/SH, HA 0.18 Fop/SH, HA B5095 and HA ref. 1003B

The parent hyaluronic acid (HA ref. 1003B) was formulated as a 1% solution in phosphate buffered saline (PBS).

A 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 below:

B5095 0.07 ml of each reactant
0.18FOp/SH 0.18 ml of each reactant
0.37FOp/SH 0.37 ml of each reactant
0.5FOp/SH 0.50 ml of each reactant
0.77FOp/SH2 0.77 ml of each reactant

Each bottle was lidded and placed on a Tube Rotator (Bibby Stuart) to mix for two hours. 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 ambient conditions.

All samples were solubilised for approximately 24 h in PBS containing 0.145 M NaCl and 0.002 M Na phosphate, pH 7.3.

2.1. Determination of absolute molar mass by size exclusion chromatography/multi-angle laser light scattering (SEC/MALLS)

SEC separates molecules according to decreasing occupied 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 molar mass. Dual detection with in-line mass and light scattering detectors allows determination of apparent absolute weight average molar mass ($M_{w,app}$) (Jumel, 1994; Wyatt, 1992). Because of the low concentration after dilution on the column it is reasonable to assume non-ideality effects are small, and $M_{w,app} \approx M_w$.

The chromatography system consisted of a HPLC pump (Model PU-1580, Jasco Corp., Tokyo, Japan), a Rheodyne injection valve (Model 7125, Rheodyne, St. Louis, MS) fitted with a 100 μl loop, and the following column system: Phenomenex guard column, TSK G6000PW and TSK G5000PW connected in series. Eluent (phosphate buffered saline) was monitored using a Dawn DSP multi- angle laser light scattering photometer (MALLS) and an Optilab 903 interferometric refractometer (both instruments from Wyatt Technology, Santa Barbara, CA). Signals from the light scattering photometer and the refractometer were captured and analysed on a PC using the dedicated ASTRA™ software. Eluent was pumped at a flow rate of 0.8 ml/min. Injections of samples (injection volume 100 μl) were performed at room temperature.

The determination of the molar mass by SEC/MALLS requires the refractive index increment (dn/dC) of the HA solution. Therefore, the refractive indices of a series of five dilutions of HA in the solvent with concentrations between 0.1–0.5 mg/ml were determined at 633 nm using the Optilab 903 interferometric refractometer (see above) and the experimental dn/dC of HA was calculated using the DNDC for windows 5.00 software.

2.2. Intrinsic viscosity

Measurements on all samples ranging from 0.1 to 5 mg/ml were performed using an Automated Viscosity Measuring Unit (AVS 310, Schott Geräte, Hofheim, Germany) at a temperature of 20.00 ± 0.01 °C.

The relative, η_{rel}, and reduced viscosities, η_{red} were calculated from equation below (see, e.g. Harding, 1997):

$$\eta_{rel} = \left(\frac{t}{t_0}\right)\left(\frac{\rho}{\rho_0}\right) \approx \frac{t}{t_0} \quad (1)$$

where t, and ρ refer to the flow time and density for HA, respectively, and t_0 and ρ_0 to solvent flow time and density. At the low concentrations used, the density correction is not required and η_{rel} can be calculated from t/t_0. The reduced specific viscosities were found from

$$\eta_{red} = (\eta_{rel} - 1)/c \quad (2)$$

where c is the concentration of HA (g/ml). The value of η_{red} when extrapolated to zero concentration is defined as intrinsic viscosity $[\eta]$ (ml/g).

2.3. Partial specific volumes

Partial specific volumes of HA solutions were obtained using the mechanical oscillator technique according to the procedure by Kratky, Leopold, and Stabinger (1973) and calculated using the equation below:

$$\bar{v} = \frac{1 - d\rho/dC}{\rho_0} \quad (3)$$

Where $\bar{v}$ is the partial specific volume (ml/g), $d\rho/dC$ is the density increment which can be obtained from the limiting slope of the plot of solution density versus concentration and ρ_0 is the density of the solvent (g/ml).

2.4. Sedimentation velocity

Sedimentation velocity experiments were performed using the Optima XL-I analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA). Solutions and solvent (400 μl each) were filled into their respective channels into 12 mm optical path length double sector cells and run at 20 °C and at rotor speeds of 50,000 rpm (sample HA 0.77 Fop/SH2), 40,000 rpm (sample HA 0.5 Fop/SH), 32,000 rpm (sample HA 0.37 Fop/SH) and 30,000 rpm (HA 0.18 Fop/SH, HA B5095 and HA ref. 1003B). Apparent sedimentation coefficient distributions $g(s^*)$ were obtained using the DCDT + software (Philo, 2000). Apparent sedimentation coefficients (s^*) were converted to standard conditions according to the standard equation:

$$s_{20,w} = s^* \frac{(1 - \bar{v}\rho_{20,w})\eta_{T,b}}{(1 - \bar{v}\rho_{T,b})\eta_{20,w}} \quad (4)$$

where $s_{20,w}$ is the sedimentation coefficient expressed in terms of the standard solvent water at 20 °C; s^* is the measured sedimentation coefficient at experimental conditions (i.e., T, b); $\eta_{T,b}$, $\eta_{20,w}$ are, the viscosities of solvent (b) at temperature T and water at 20.0 °C, respectively, and $\rho_{T,b}$, $\rho_{20,w}$ are the corresponding densities. The $s_{20,w}$ values were evaluated at various concentrations ranging from 0.3 to 1.2 mg/ml. In order to remove the effects of non-ideality, plots of $s_{20,w}$ versus concentration were extrapolated to zero concentration to give $s^0_{20,w}$ and the concentration-dependence coefficient (Gralen parameter), k_s, was obtained using Eqs.5.1 and 5.2 (see e.g. Ralston, 1993).

$$s_{20,w} = s^0_{20,w}(1 - k_s C) \quad (5.1)$$

$$1/s_{20,w} = 1/s^0_{20,w}(1 + k_s C) \quad (5.2)$$

3. Results and discussion

3.1. Weight average molar mass (M_w)

The experimental dn/dC of HA solutions investigated here was 0.167 ml/g which is close to the value of 0.164 determined under similar conditions (0.20 M NaCl) by Preston and Wik (1992). However, the dn/dC values as determined by other workers ranged between 0.155 and 0.176 (Theisen, Johann, Deacon, & Harding, 2000). These variation in dn/dC values are most likely due to differences in solvents and wavelengths used.

The weight average molar mass (M_w) values obtained from SEC/MALLS for each sample are given in Table 1. The results show that the degree of depolymerisation depends on the amount of ascorbic acid/H_2O_2 added. The polydispersity values (M_w/M_n) for all samples were in the range of 1.3–1.8.

3.2. Intrinsic viscosity

Intrinsic viscosity $[\eta]$ is a measure of the occupied hydrodynamic volume of a macromolecule in solution and therefore a reflection of its size. The value for $[\eta]$ can be obtained from η_{red} when extrapolated to zero concentration as is demonstrated by the Huggins plots for all samples in Fig. 1. Table 1 demonstrates that the intrinsic viscosity varies greatly depending on the M_w. A comparison with data obtained by other workers (Gura, Huckel, & Mullet, 1998; Preston & Wik, 1992) shows good agreement for samples of similar molar mass in solvents of similar ionic strength (see Table 2).

The intrinsic viscosity can, in theory, be used to determine the molar mass of macromolecules through the Mark–Houwink–Kuhn–Sakurada (MHKS) relationship. However, the validity of the molar mass values obtained in this way depends greatly on the K and a parameters which

Table 1
Hydrodynamic data for HA samples

Hyaluronic acid samples						
	HA ref. 1003B	HA B5095	HA 0.18 Fop/SH	HA 0.37 Fop/SH	HA 0.5 Fop/SH	HA 0.77 Fop/SH2
$[\eta]$ (ml/g)	2850 ± 100	1470 ± 10	770 ± 10	220 ± 3	190 ± 3	120 ± 2
$\bar{v}$ (ml/g)	0.58	0.60	0.61	0.63	0.60	0.64
$s^0_{20,w}$ (S) from plot $s_{20,w}$ vs. c	3.75 ± 0.20	4.09 ± 0.20	3.54 ± 0.10	2.22 ± 0.10	1.97 ± 0.04	1.90 ± 0.06
$s^0_{20,w}$ (S) from plot $1/s_{20,w}$ vs. c	4.76 ± 0.60	4.86 ± 0.30	3.97 ± 0.10	2.20 ± 0.09	1.99 ± 0.05	1.92 ± 0.09
k_s from plot $1/s_{20,w}$ vs. c	1050 ± 120	1140 ± 80	790 ± 30	230 ± 50	180 ± 30	210 ± 50
$k_s/[\eta]$	0.37 ± 0.04	0.78 ± 0.05	1.03 ± 0.04	1.05 ± 0.20	0.95 ± 0.20	1.75 ± 0.40
$10^{-4} \times M_w$ (g/mol)	325 ± 100	146 ± 40	49.2 ± 4.0	8.2 ± 0.6	6.2 ± 0.7	4.7 ± 0.3

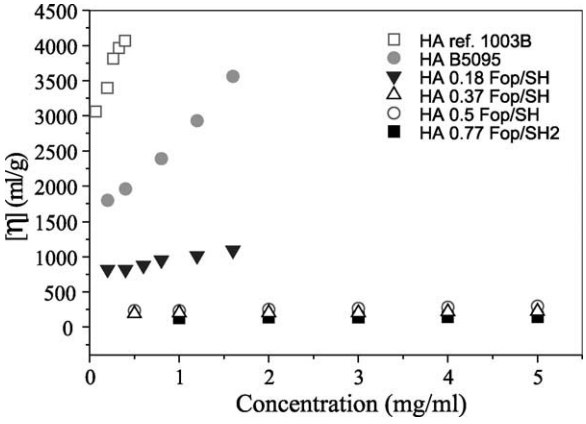


Fig. 1. Plot of reduced viscosity (η_{red}) against concentration for depolymerised samples and control, Solvent = Phosphate buffered saline, Temperature = 20 °C.

need to have been obtained using identical conditions (e.g. solvents, temperature) to those of the sample.

The a parameter which alone gives information regarding the conformation of a molecule has been reported by several workers and a comparison between our values and some published data are shown in Table 3.

Bothner, Waaler, and Wik (1988) suggested that the a parameter for HA was dependent on the molar mass of HA, we therefore present three areas for MHKS parameter a. For the first area the MHKS relationship between samples of molar mass $< 10^5$ g/mol was established and compared with values obtained by Cleland (1984) and Shimada and Matsumura (1975). The parameter a from our study in this area was found to be ~ 1 the same as reported by the above two workers. The second area covered samples of $M_w < 1 \times 10^6$ (all samples excluding HA B5095 and HA ref 1003B). The parameter determined in this region was 0.73 ± 0.01, which is in reasonable agreement with values determined by Bothner et al. (1988) and most published values. The third a parameter was calculated from the three highest M_w sample studied and found to be 0.69 ± 0.02 which is slightly lower than the other two mentioned above. This value again agrees with the finding of Bothner et al. (1988) who also determined a lower a parameter for higher molar mass HA samples. The a parameter is affected by many factors such as methods used for preparation of samples, determination of M_w, polydispersity of sample in addition to ionic strength of solvent and type of capillary viscometer used. This means that care has to be taken when estimating M_w from $[\eta]$ using published values of a in literature unless $[\eta]$ is determined under very similar conditions.

3.3. Partial specific volumes

The partial specific volume of a system is a characteristic property and can be calculated from density measurements. Values for partial specific volumes are required to correct the experimentally obtained sedimentation coefficients to standard conditions and for the calculation of molar mass from sedimentation equilibrium experiments. The values for the HA samples investigated were calculated according to Kratky et al. (1973) and are shown in Table 1. These values range from 0.58–0.64 for all samples and are therefore in the typical range of 0.59–0.65 for common polysaccharides (Ralston, 1993). These results show that the $\bar{v}$ values were independent of the molar mass of samples studied. As can be seen in Table 4, $\bar{v}$ values determined by various workers ranged between 0.51–0.66. Discrepancies in $\bar{v}$ were principally caused by the methods and solvents used. Gomez-Alejandre, de la Blanca, de Usera, Rey-Stolle, and Hernandez-Fuentes (2000) who investigated the solvent effect on $\bar{v}$ demonstrated a significant influence on the presence and type of cations and pH value. The $\bar{v}$ value of sample HA ref 1003B was very similar to that found by Gomez-Alejandre et al. (2000) and Preston and Wik (1992) who determined $\bar{v}$ using the same method and similar solvents.

3.4. Sedimentation velocity

From sedimentation velocity experiments, it appears that there is a general decrease in sedimentation coefficient ($s^0_{20,w}$) with molar mass (see Table 1). The actual $s^0_{20,w}$ values from plots of $s_{20,w}$ versus concentration and its reciprocal are quite similar for low M_w samples but vary considerably for the high M_w samples (see Table 1).

Table 2
Comparison of intrinsic viscosity values of HA samples with similar M_w from previous and this study

Study	$10^{-4} \times M_w$ (g/mol)	$[\eta]$ (ml/g)	Solvent
Preston and Wik (1992)	8.2	225	0.25 M NaCl
This study	8.2[a]	250	0.145 M NaCl + 0.002 M Na phosphate
Gura et al. (1998)	47.9[b]	724	0.2 M NaCl
This study	49.2	767	0.145 M NaCl + 0.002 M Na phosphate
Gura et al. (1998)	142	1627	0.2 M NaCl
This study	146[c]	1470	0.145 M NaCl + 0.002 M Na phosphate

[a] HA 0.37 Fop/SH.
[b] HA 0.18Fop/SH.
[c] HA B5095.

Table 3
MHKS parameters *a* and *b* of HA

Study	MHKS*a*	MHKS*b*	Solvent
Gamini et al. (1992)	0.81	–	0.15 M NaCl
Gura et al. (1998)	0.716 (Ubbelohde viscometer)	–	0.2 M NaCl
	0.770 (Zimm-Crothers viscometer)		
Balazs, Briller, and Denlinger (1981)	0.8	–	0.2 M NaCl
Cleland and Wang (1970)	0.816	–	0.2 M NaCl
Laurent, Ryan, and Pietruszkiewicz (1960)	0.78	–	0.2 M NaCl
Shimada and Matsumura (1975)	0.76	–	0.2 M Na phosphate
Bothner et al. (1988)	0.601 ($M_w \geq 1 \times 10^6$)	–	0.15 M NaCl
	0.779 ($M_w < 1 \times 10^6$)	–	0.15 M NaCl
This study	0.69[a]	0.11[a]	PBS
	0.73 ($M_w < 1 \times 10^6$)	0.32	PBS
	1.10 ($M_w < 1 \times 10^5$)	0.24	PBS

[a] Values obtained from the three highest samples.

Fig. 2a and b demonstrate the concentration dependence of the sedimentation coefficient, with the Gralen parameter, k_s, from the reciprocal plot decreasing with molar mass (see Table 1). $s^0_{20,w}$ and k_s values from reciprocal plots are considered to be more reliable for asymmetric molecules in non-ideal systems (Harding, 1995a) and this is why those values are used for further conformational studies (see below). The reason for the very high concentration dependence of high M_w HA lies probably in its high viscosity which is not only due to solute-solvent interactions but also to solute-solute interactions.

There is a slight anomaly in that $s^0_{20,w}$ and k_s values for HA B5095 are larger than those of the parent sample which had a higher molar mass. This may again be due to viscosity effects, i.e., the higher molar mass exhibits much higher viscosity (see Table 1) and if these effects are intramolecular they will not be eliminated by extrapolation to zero concentration.

3.5. Gross overall solution conformation of hyaluronic acid

The HA samples investigated here form a homologous series, i.e., they are obtained from one 'parent' sample which has been depolymerised to varying degrees. The gross conformation of HA can therefore be probed using the following Mark–Houwink–Kuhn–Sakurada (MHKS) relationship (see, e.g. Tombs & Harding, 1998).

$$[\eta] = k' M^a \quad (6)$$

$$s^0_{20,w} = k'' M^b \quad (7)$$

The MHKS parameters *a* and *b* are obtained from the slopes of double logarithmic plots of intrinsic viscosity, $[\eta]$, and sedimentation coefficient, $s^0_{20,w}$, respectively versus molar mass, M_w (see Figs. 3 and 4).

The MHKS parameter *a* cannot only be used to calculate M_w as mentioned in the intrinsic viscosity section, but it can also be used to probe the gross conformation of macromolecules in solution—the same is true for parameter *b*.

The Haug Triangle representation of macromolecular conformation (see, e.g. Harding, 1992) is an equilateral triangle, the corners of which represent the three standard models of macromolecular gross solution conformation, i.e. compact sphere, random coil and rigid rod as shown in Fig. 5. The *a* and *b* parameters are then used to locate the actual conformation of the system under investigation along the sides of the triangle.

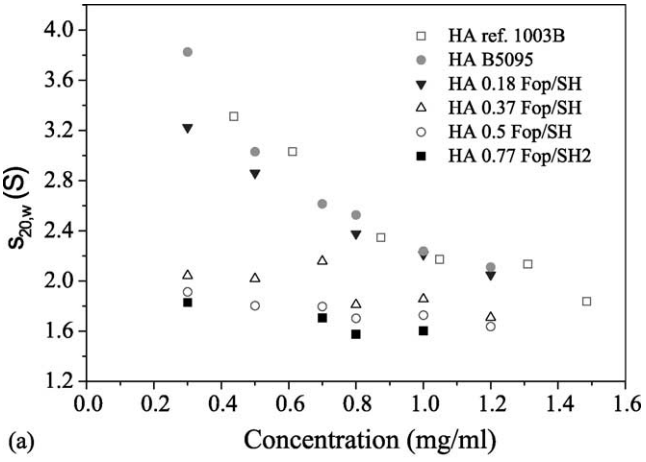
As can be seen in Table 3 all the values of parameter *a* from various authors and our own observations from different M_w ranges vary between 0.6–0.8, thus suggesting that the gross conformation of HA in aqueous solution is a random coil.

Double logarithmic plots of $s^0_{20,w}$ (values from plots of $1/s_{20,w}$ versus concentration) versus M_w were constructed

Table 4
Partial specific volumes of HA

Study	$\bar{v}$ (ml/g)	Method	Solvent
Varga (1955)	0.66	Pycnometry	Phosphate buffer[a]
Silpananta, Dunstone and Ogston (1968)	0.653	Density gradient centrifugation	–
Gomez-Alejandre et al. (2000)	0.512	Densimetry	Water
Gomez-Alejandre et al. (2000)	0.548	Densimetry	0.142 M NaCl
Gomez-Alejandre et al. (2000)	0.579	Densimetry	0.284 M NaCl
Preston and Wik (1992)	0.57	Densimetry	0.2 M NaCl
This study	0.58–0.64	Densimetry	0.145 M NaCl + 0.002 M Na phosphate

[a] 0.006 M phosphate buffer adjusted with NaCl to $I = 0.12$ M.



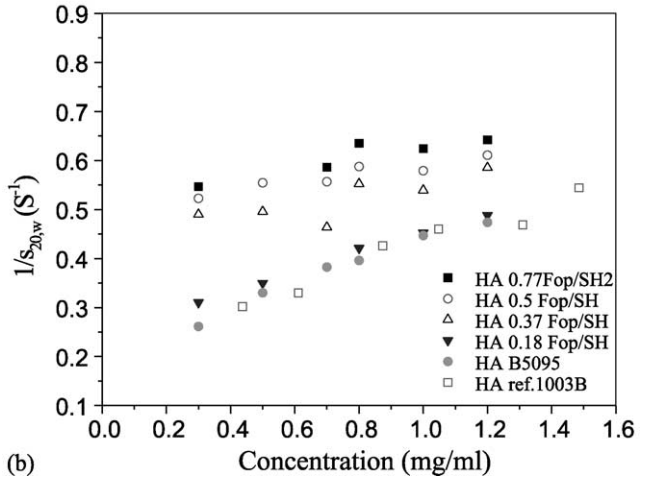


Fig. 2. (a) Concentration dependence of sedimentation coefficient conventional plot for depolymerised HA samples and control. (b) Concentration dependence of sedimentation coefficient reciprocal plot for depolymerised HA samples and control.

and the b parameters obtained using M_w areas as described in the intrinsic viscosity section. Values of (0.24 ± 0.09), (0.32 ± 0.01) and (0.11 ± 0.03) were obtained for the $M_w < 10^5$, 10^6 and the highest three molar mass samples

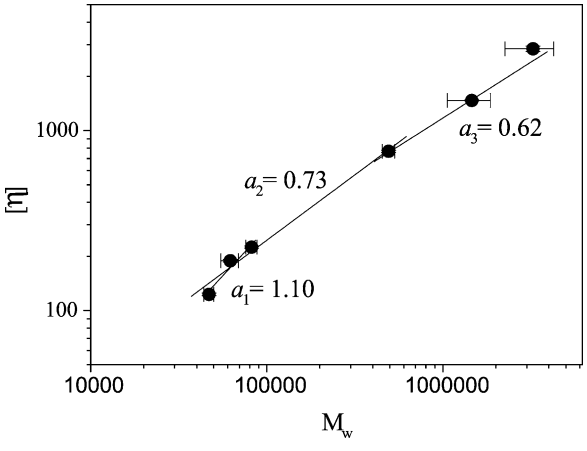


Fig. 3. Double logarithmic plot of intrinsic viscosity versus weight average molar mass.

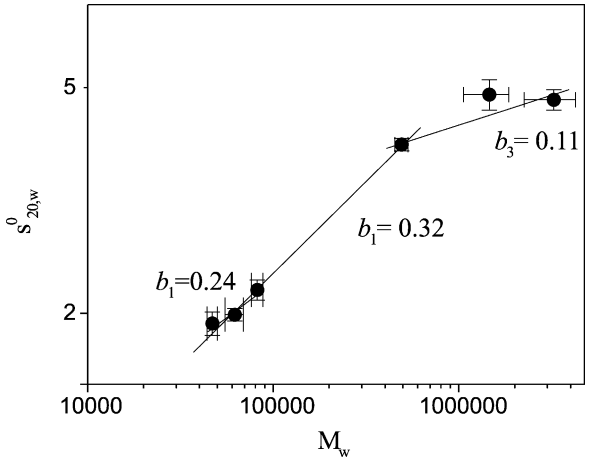


Fig. 4. Double logarithmic plot of sedimentation coefficient versus weight average molar mass.

respectively. The b parameter from the highest three samples is lower than those of the other two due to the unreliable $s^0_{20,w}$ value of HA ref 1003B as mentioned above. For the other M_w areas the b parameters are quite similar suggesting that HA adopts a somewhat stiff random coil conformation.

The values of a and b shown in Table 3 suggest that the overall solution conformation of the HA is between random coil and rigid rod or it can be said that HA is considered to behave as a stiffish coil in aqueous solution.

Additionally, the experimental Wales–van Holde ratio, $k_s/[\eta]$, is a useful guide to the gross conformation of macromolecules in solution. Its value is approximately 1.6 for random coils and compact spheres (i.e. symmetrical conformations) and ~0.2–0.5 for more extended or rod conformations (Rowe, 1977). The values from samples $M_w > 10^6$ range from 0.37–0.78 and $M_w < 10^6$ range from 0.95–1.75. This suggests that the depolymerised samples adopt a rigid rod/ random coil conformation and the high M_w samples adopt a much more extended conformation

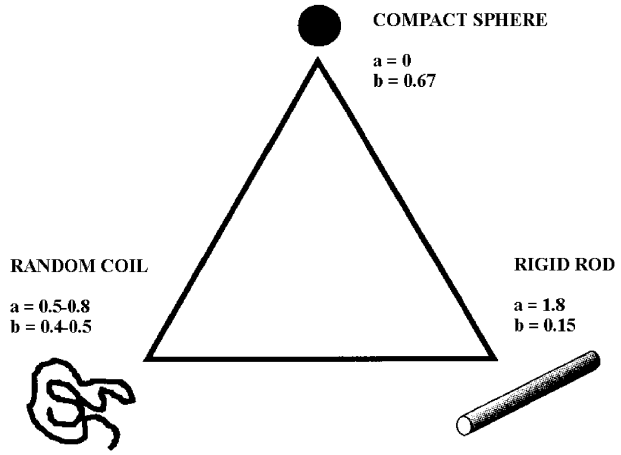


Fig. 5. Haug triangle representation of the gross conformation of macromolecules, a and b are the exponents in the MHKS viscosity and sedimentation equation respectively (from Harding, 1992).

which is typical for highly polyanionic polysaccharides as suggested by Tombs and Harding (1998). Evaluation of the Wales–van Holde ratios has therefore confirmed the findings from the MHKS coefficients *a* and *b*, i.e. that HA adopts a stiffish coil conformation in aqueous solution.

4. Concluding remarks

Our work using a number of hydrodynamic techniques has shown that hyaluronic acid can be degraded by an ascorbic acid/hydrogen peroxide system. The extent of depolymerisation depends on the amount of this system added to HA. The samples obtained in this way form a homologous series of molar mass samples, which can be used to probe dilute solution conformation through the MHKS relationship. Our values are in good agreement with those of other workers and consolidate previous findings suggesting that HA adopts a stiffish coil conformation in solution.

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