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Thermal degradation and stability of sodium hyaluronate in solid state

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ABSTRACT

The kinetics and mechanism of depolymerisation of solid sodium hyaluronate at elevated temperatures and various pH have been investigated. Depolymerisation was found to be governed by random chain scission. The activation energy at neutral pH was found to be 127 kJ/mol. The solid polymer was most stable at neutral pH. Results suggest the depolymerisation mechanism in solid- and solution state to be the same. Correlation of log intrinsic viscosity to log weight-average molecular weight was investigated to ensure high quality data for polymer size. Based on more than sixty hyaluronate samples spanning from 0.4 to 2.3 MDa, it was concluded that a second order polynomial regression gives a better fit than the linear regression offered by classical Mark–Houwink–Kuhn–Sakurada description. This finding was supported by literature data and could be expanded to other simple, well behaving linear polymers, such as polystyrene and polyethylene.

1. Introduction

Sodium hyaluronate (HA), often referred to as hyaluronic acid or hyaluronan, is a widespread glycosaminoglycan in vertebrates. HA is a linear polymer composed of repeating disaccharide units of $\beta(1,3)$ linked D-glucuronic acid and $\beta(1,4)$ linked *N*-acetyl-D-glucosamine (Lapcik, Lapcik, De Smedt, Demeester, & Chabrecek, 1998).

Historically, human umbilical cord has been used as a source for small scale purification of HA. Extraction from bovine vitreous humour and rooster comb have been used for large scale HA production (Kaye, 1950; Balazs, 1958; Laurent, Ryan, & Pietruszkiewicz, 1960). With increasing awareness of the risks related to extraction from animal sources (e.g., avian viruses), HA manufactured by fermentation of *Streptococcus* sp. was developed during the 1980s. In this process, organic solvents are used to extract HA (DeAngelis, 2012). Since *streptococci* are class II pathogens, a new recombinant source of HA was developed using the safe microorganism *Bacillus subtilis* in combination with an aqueous recovery process (Widner et al., 2005).

It has been found that HA is involved in a range of biological mechanisms, such as, cell migration (Laurent, Fraser, & Robert, 1992), cell adhesion (West, Hampson, Arnold, & Kuma, 1985), cell pH (Kathagen & Prhem, 2013) and angiogenesis (Koyama et al., 2008; Kobayashi et al., 2010). HA is suggested to play a potential role in cancer therapy (Misra et al., 2011; Sironen et al., 2011) and multidrug resistance (Cordo-Russo et al., 2010). The polymer has historically been used as a formulating agent to control viscosity, but has increasingly been used for more advanced applications, like drug stabilisation and delivery (Kogan, Soltes, Stern, & Gemeiner, 2007), e.g. by formation of HA-gels (Luo, Kirker, & Prestwich, 2000) or hydrophobic modifications (Tømmeraas, Mellergaard, Malle, & Skagerlind, 2011; Eenschooten et al., 2012). The size of the polymer has been shown to be of importance in order to produce an effect (Lokeshwar & Selzer, 2008; Stern, 2008).

The new application areas of HA have led to a demand for well-defined HA material in terms of purity, stability and size. Historically, intrinsic viscosity ($[\eta]$) has been used as the standard method for indirectly assessing the weight-average molecular weight (M_w) of HA by use of the Mark–Houwink–Kuhn–Sakurada (MHKS) equation. This states that a linear relationship shall exist for $\log[\eta]$ versus $\log M_w$ for an un-branched/linear polymer

Abbreviations: $[\eta]$, intrinsic viscosity; HA, hyaluronan; MALS, multi angle light scattering; MHKS, Mark–Houwink–Kuhn–Sakurada; M_w, weight-average molecular weight; RI, refractive index; SEC, size exclusion chromatography.

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(Flory, 1953; Tanford, 1961). Recently, size exclusion chromatography with dual detection of multi-angle light scattering and refractive index (SEC-MALS-RI) has become the preferred method of choice for determining M_w (Mendichi & Schieroni, 2000).

The current study presents an investigation of the kinetics and associated activation energy of depolymerisation of HA in solid state. Furthermore, release data from more than sixty HA batches has been used, and related to literature data, to develop an improved correlation between M_w and intrinsic viscosity for HA.

2. Materials and methods

2.1. Chemicals and reagents

Sodium chloride (NaCl), sodium dihydrogen phosphate ($NaH_2PO_4{\cdot}2H_2O$), disodium hydrogen phosphate ($Na_2HPO_4{\cdot}2H_2O$) and sodium azide (NaN_3) were of analytical grade and purchased from Sigma–Aldrich Co. Distilled water was purified via a Milli-Q system (Millipore) with a 0.2 μm filter. 0.1 μm filter (ExpressPLUS, Millipore) was used for filtration of the eluent. Hyaluronan was obtained as sodium hyaluronate from Novozymes Biopharma. The batches with different pH had their pH adjusted with dilute NaOH or HCl prior to the spray drying.

2.2. SEC-MALS-RI

Analysis was performed on a Waters Alliance 2695 HPLC system equipped with a cooled (5 °C) auto sampler and a column oven (30 °C). SEC was performed using three columns connected in series: TSKgel PWXL guard column (4 cm × 6.0 mm ID) followed by a TSKgel G5000PWXL (30 cm × 7.8 mm ID) and finally a TSKgel G6000PWXL (30 cm × 7.8 mm ID) column (Tosoh BioScience). The columns had been washed out for minimum one month prior to use to remove debris and particles from the columns. The mobile phase used was 150 mM NaCl, 50 mM NaH_2PO_4, 0.05% NaN_3, pH 7.0 that was 0.1 μm filtered prior to use. Analysis was done at a flow rate of 0.5 mL/min, which gave a run time of 60 min per analysis. Detection was performed by MALS (DAWN HELEOS-I, Wyatt) and subsequent RI (Optilab T-rEX, Wyatt). Data was collected and evaluated using Astra V software (Wyatt). For calculation of HA M_w the Berry model was used with fit degree 2. A dn/dc of 0.153 mL/g and a second virial coefficient of 2.3×10^{-3} were used based on internal method development (data not shown). Samples were diluted to target concentration (approx. 0.15 mg/mL) by dissolving approximately 17 mg of HA powder in 100 mL mobile phase. Samples were stirred for 16–24 h at 2–8 °C to ensure complete dissolving. The samples were 0.2 μm filtered prior to injection.

2.3. Intrinsic viscosity

Measurements were made using an adaptation of the procedure described in the European Pharmacopeia for HA (European Pharmacopeia, 2013) to fit the M_w range of the HA investigated. A suspended Ubbelohde viscometer (RPV-1, Rheotek) at 25.00 ± 0.03 °C was used for automated sampling and analysis. Radius of the R tube is 0.23 mm and volume of bulb C is 3 mL. A 150 mM NaCl, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.0 buffer solution was prepared. Samples were prepared by dissolving approximately 0.55 g HA powder in 50 mL buffer solution in a 100 mL flask. Dissolving was achieved by stirring for 24 h at 2–8 °C. From this stock solution 5.00 g was further diluted with 100 g buffers solution by shaking in a 250 mL flask for 20 min at 2–8 °C. This was denoted Test solution A. From the test solution A further test solutions were prepared by mixing 30.00 g of Test solution A with 10.00 g of buffer solution (Test solution B), 20.00 g Test solution A with 20.00 g buffer solution (Test solution C) and finally 10.00 g Test solution A with 30.00 g buffer solution (Test solution D). All the used amounts were weighed accurately and together with the water content in HA, HA assay and density of test solution (1.005 kg/m^3) used for the calculation of the HA concentration (c). Flow times were determined in triplicate for the four test solutions and the buffer solution. The uncorrected flow times together with the kinetic energy factor were used to calculate the relative viscosities (η_{rel}). The intrinsic viscosity was determined by linear regression of the four data points from plotting $\log(\eta_{rel} - 1)/c$ as a function of c at the intercept of the y-axis. To ensure reliable data, the flow times for one condition could not differ more than 0.35% from the mean value. Also the flow times of Test solution A must be between 1.6 and 1.8 times higher than that of the buffer solution, which had a flow time of approximately 190 s. Non-compliance with these requirements resulted in correction of the HA concentration. An estimate for correction can be given from that a change in sample weight of 0.1 g HA will change the ratio between the two flow times of Buffer solution and Test solution by approximately 0.1–0.15. Finally, it was included that the linear regression must give a $R^2 \geq 0.95$.

2.4. pH

Determination of the pH was done using a procedure that was compatible to that of the European Pharmacopeia (European Pharmacopeia, 2013). Water free of CO_2 was prepared by boiling water in an open flask for a few minutes. The flask was closed airtight and allowed to cool to room temperature before use. HA (0.5 g dry weight) was added to a 100 mL flask. Water free of CO_2 (100 mL) was added carefully in order to minimise reabsorption of CO_2. A magnet was added and the flask closed air tight. The HA was dissolved by stirring at room temperature for 16–24 h. The measurement of pH was done using a PHC2001 electrode connected to a PHM240 pH meter, both from Radiometer Analytical. The electrode was submerged in to the solution with very slow stirring.

2.5. Stability studies of HA powder

Powder samples of spray-dried HA were packed in 5 g pouches with an inner lining of polyethylene film. Pouches were welded to become airtight. Temperature incubation were done at 5 ±3 °C/ambient % relative humidity (RH), 25 ±3 °C/60 ±5% RH and 30 or 40 ±2 °C/75 ±5% RH in validated climate chambers from Binder. For the 105 °C incubation HA powder was placed directly in an oven (halogen moisture analyser HR83, Mettler Toledo) in aluminium trays and incubated instantly.

2.6. Theory

2.6.1. Polymer characterisation using SEC-MALS-RI and intrinsic viscosity measurements

One of the most important parameters for characterising a macromolecule, such as HA, is the molecular weight. Molecular weights are difficult to determine due to; a) polydispersity, b) conditions differing from θ conditions (i.e., ideal solution conditions) c) difficulty in defining the conformation and d) aggregation or self-association (Harding, 1992).

When a macromolecule is illuminated by a beam of light at wavelength λ, the polymer chains will scatter light in direct proportion to their M_w. The angular dependence of the scattered light at low angles can be related directly to z-average of the radius of gyration $\langle s \rangle_z$ (also referred to as $(R_g)_z$. MALS can be used to determine the M_w after extrapolating the scattering data to zero angle and subsequently using the standard expression $Kc/R_\theta = 1/M_w + 2A_2c$. The values of M_w obtained by SEC-MALS are direct determinations without use of standards. The M_w value obtained from the viscometry measurements on the other hand are indirect measurements

since the primary data are measurements of the intrinsic viscosity. In viscometry, the relationship between $[\eta]$ and M_w, for a linear polymer, is described by the MHKS equation; $[\eta]_w = KM_w^a$, which can be rearranged to $\log[\eta] = \log K + a \cdot \log M_w$.

The intrinsic viscosity can be used to determine the exponent a from the MHKS equation, which is a measure of the conformation of the polymer at the conditions measured (*i.e.*, ionic strength, temperature and solvent) as illustrated in Haug's triangle (Harding, 1992). and can take on values from 0 to 2. An a-value of 0.5–0.8 denotes a random coil and stiff rods have a values of 1.8. This parameter will to a large extent be dependent of the solvent system used for the measurement (i.e., the better solvent used, the higher a value as the polymer will expand with the quality of the solvent).

Another more indirect evaluation of the confirmation of the polymer can come from Huggins' constant. At very low shear rates, the specific viscosity, η_{sp}, of a well behaved linear polymer can be described by the concentration, c, and intrinsic viscosity of the polymer, see Huggins (1943) and references therein; $\eta_{sp} = c[\eta] + k'c^2[\eta]^2$. The k' is the Huggins constant which according to the Stokes–Einstein equation for calculating the viscosity of a suspension of spheres will be 0.4.

The uncertainty arising from the indirect determination of M_w by viscometry can be reduced by using a large number of data points where both $[\eta]$ and M_w have been determined to improve the constants in the MHKS equation. Further, such a fitting of $[\eta]$ versus M_w data gives a better understanding of how the conformation (i.e., the a-value) of HA changes in the chosen solution system as function of M_w.

2.6.2. Determination of kinetic rate constants

The kinetic rate constants for the degradation process of HA were determined as described in Tømmeraas and Melander (2008). For polymers degraded by random scission, the rate at which bonds are broken is proportional to the total number of intact bonds (Tanford, 1961): $-d(N_0p)/dt = kN_0p$, where N_0p is the number of bonds at time t, and k is the rate constant. The solution of this equation for a single strand linear polymer with random degradation gives the following equations: $DP_{n,t} \propto kt$ and $1/M_{w,t} \propto k't$. From the last equation, we get the expression $1/M_{w,t}(t) = k_h t + 1/M_{w,t=0}$, where k_h is the kinetic rate constant of the degradation process and $M_{w,t=0}$ is the molecular weight at the beginning of the degradation process. To obtain information about the kinetics of the degradation processes the change in the inverse value of the weight-average molecular weight $(1/M_w - 1/M_{w,0})$ as function of time. The rate constant (k_h) is found by determining the slope of the curve. A non-linear curve indicates a non-random degradation process.

3. Results and discussion

3.1. Kinetics of HA degradation in solid state

The degradation rate of HA powder (i.e., M_w loss) as a function of time was monitored for a range of batches at different conditions. At low temperatures, the HA degradation was limited compared to the accuracy of the M_w determinations from SEC-MALS. Therefore, no significant trend can be seen at 5 °C the first two years. Data from elevated temperatures gave linear relationships for $(M_{w(t)}^{-1} - M_{w(0)}^{-1})$ as a function of time (data not shown). This is characteristic for a random degradation process (Tanford, 1961). Random degradation of HA has previously been shown for HA at various pH values and temperatures both in solution (Tømmeraas & Melander, 2008) and as slurry in EtOH/water/HCl (Melander & Tømmeraas, 2010).

The depolymerisation rate of HA powder was studied at temperatures ranging from 5 to 105 °C. An Arrhenius plot of the degradation rates of HA in solid state is shown in Fig. 1. The activation energy for the HA in pH neutral powder was determined to be 127 kJ/mol. This is close to the activation energy determined for acid hydrolysis of HA in solution, 137 kJ/mol (Tømmeraas & Melander, 2008), which indicated that the degradation process in solid state is following a mechanism similar to that in hydrolysis.

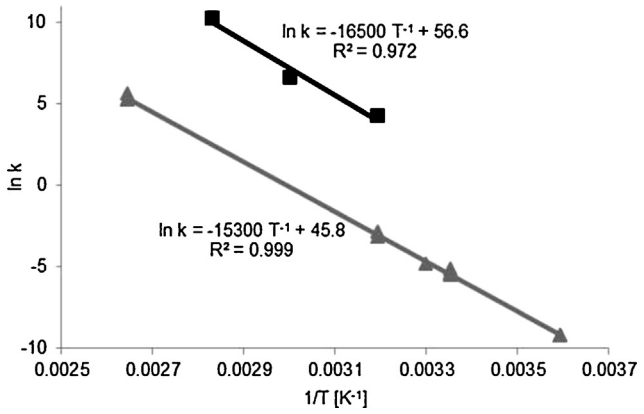

Fig. 1. Arrhenius plot of degradation of hyaluronan with neutral pH (grey triangles). For comparison, data of HA degradation in 0.1 M HCl solution are included (black squares) (Tømmeraas & Melander, 2008).

The powder stability of HA at 40 °C as function of pH is shown in Fig. 2. Data for the retained molecular weight in % after one and three months is given in Fig. 2. The relationship between pH and stability is similar to what has previously been observed for alginate, another uronic acid containing polysaccharide, in solution (Haug, Larsen, & Smidsrød, 1963) and solid state (Holme, Limdmo, Kristiansen, & Smidsrød, 2003). A parabolic stability behaviour as a function of pH was observed in Fig. 2. The choice of parable was not based on mechanism but merely a visual description of data. The noise in the data is an effect of the uncertainty in determining pH of un-buffered HA solutions.

The parabolic relationships indicate an optimal stability of pH around 7.2–7.3 for one and three months storage at 40 °C, respectively. A stability optimum at neutral pH is in agreement with a hydrolysis mechanism being catalysed by increasing concentration of oxonium (H_3O^+) ions at low pH (Bemiller, 1967) and hydroxide (OH^-) ions, through β-elimination, at high pH (Haug et al., 1963).

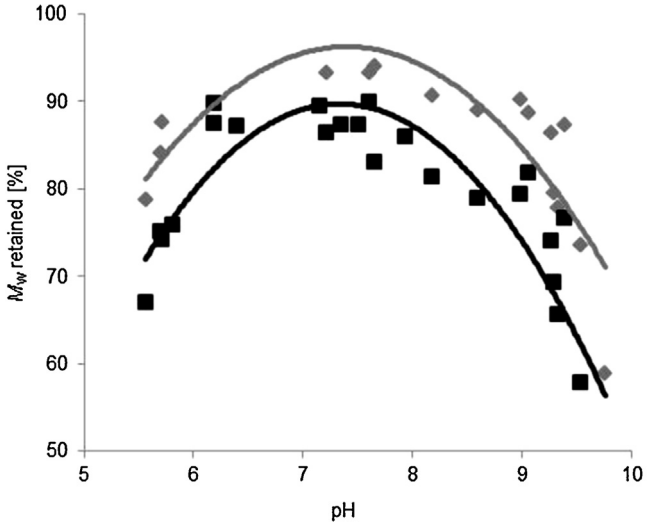

Fig. 2. Remaining % of weight average molecular weight as a function of pH. Grey diamonds and black squares denote one month and three months incubation at 40 °C, respectively.

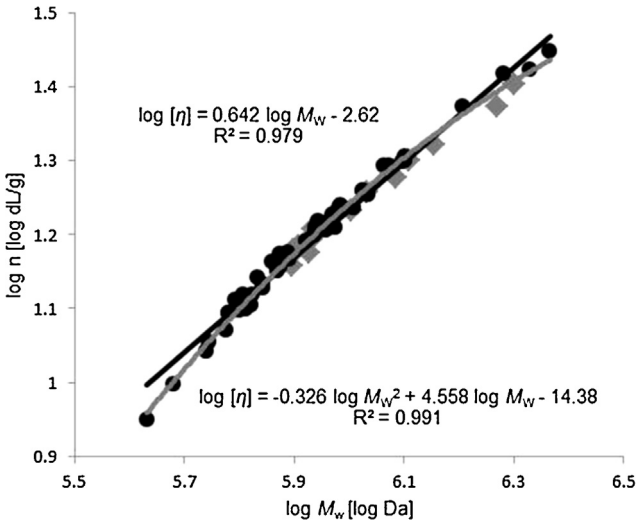

Fig. 3. Mark–Houwink–Kuhn–Sakurada relationship for HA in 10 mM Phosphate 150 mM NaCl pH 7.0 at 25 °C. Black circles are from Laboratory 1 and grey diamonds from Laboratory 2. Fitting of a linear curve (black) or second order polynomial (grey) was done for the combined data set.

3.2. Molecular weight determination of HA

At two different laboratories more than sixty batches of HA, that spanned from 400 kDa to 2.3 MDa, were analysed for intrinsic viscosity and M_w by SEC-MALS-RI. The shear rate was calculated for representative samples across the entire mass range giving values from 1160 to 1210 s^{-1}. From the intrinsic viscosity data, Huggins' constant was found to be between 0.34 and 0.38, which is in accordance with values for HA from the literature (Shimada & Matsumura, 1975; Fouissac, Milas, & Rinaudo, 1993). The resulting values for $[\eta]$ versus M_w are given in Fig. 3 in a log–log plot to yield the MHKS relationship. Based on a good inter laboratory accuracy for both methods no distinction, with regards to source of data, was made. Accordingly, the data set was treated as a whole in the remainder of the study. An acceptable linear fit with a R^2 of 0.98 yielded the MHKS values of $a = 0.642$ and $K = 0.0024$.

In Table 1 a list of MHKS values is presented to allow for comparison. According to the MHKS theorem, given the same experimental conditions, MHKS values should be the same. It is notable that even for those experiments where the same conditions have been used there are significant variations in the reported MHKS values. This variation in a and K values make the use of the MHKS equation for the assignment of M_w from $[\eta]$ precarious. This is illustrated in Table 1 by calculating the resulting M_w from an intrinsic viscosity of 15 dL/g.

The data presented in Fig. 3 exhibits a clear curvature. This brings about a continuous change in a and K values across the investigated M_w. Over the years, several authors have noticed this apparent none-linear behaviour of the $\log[\eta]$ versus $\log M_w$ data and the concomitant variation in a and K values. Many authors have resorted to dividing the MHKS plot into ranges with a decreasing with increasing M_w (Cowman & Matsuoka, 2005; Hokputsa, Jumel, Catherine, & Harding, 2003; Gatta, De Rosa, Marzaioli, Busico, & Schiraldi, 2010; Mendichi, Soltes, & Schieroni, 2003; Bothner, Waaler, & Wik, 1988). The continual change in a and K values across single HA samples has also been reported (La Gatta et al., 2010; Terbojevich, Cosani, Palumbo, & Pregnolato, 1986). It is therefore proposed that a second order polynomial; $\log[\eta] = a'(\log M_w)2 + b'(\log M_w) + c'$ will give a better fit of the data, thereby providing more accurate M_w determinations of HA from viscometry measurements. No physical meaning for the experimental constants a', b' and c' is offered at this point. A second order polynomial fitting of the data is shown in Fig. 3.

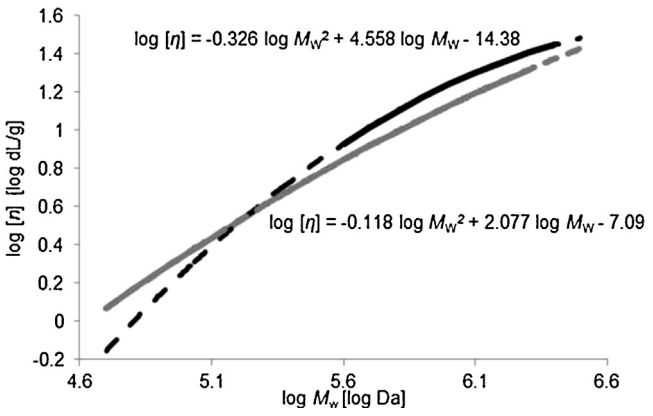

Fig. 4. Comparison of polynomial equations for $\log[\eta]$ versus $\log M_w$ relationship for HA polymer. Full curve denotes the part of the curve where raw data are available and the broken part extrapolation from the raw data. Black curve is from this work and grey is Equation 4 from Podzimek et al. (2010).

The use of a polynomial fit for $\log[\eta]$ versus $\log M_w$ has previously been suggested (Podzimek, Hermannova, Bilerova, & Bezakova, 2010). Using a range of different methods and solvents, the authors found polynomial relationships for the correlation of $\log[\eta]$ versus $\log M_w$ by measuring across individual HA samples. In Fig. 4, the polynomial fit from this work is compared to Equation 4 in Podzimek et al. (2010). Equation 4 has been chosen since it covers M_w by SEC-MALS and $[\eta]$ determined in 100 mM phosphate buffer pH 7.0 at 25 °C, which is the condition most similar to the conditions of this work. The c' has been corrected by −2 to account for the change in units from mL/g to dL/g for intrinsic viscosity used in this work.

As can be seen, the two curves in Fig. 4 differ both in terms of curvature and absolute values. Predicting M_w using the polynomial equation for an intrinsic viscosity of 15 dL/g (as used in Table 1) with these equations gives 1181 kDa (Podzimek et al., 2010) and 803 kDa (this work). This underlines the need for alignment of analysis methods and equipment across laboratories as the variation in M_w determined from intrinsic viscosity is not lessened by simply introducing more parameters.

A plot of the a and $\log K$ values associated with tangents of the polynomial fit in Fig. 4 is given in Fig. 5. The a and $\log K$ values of

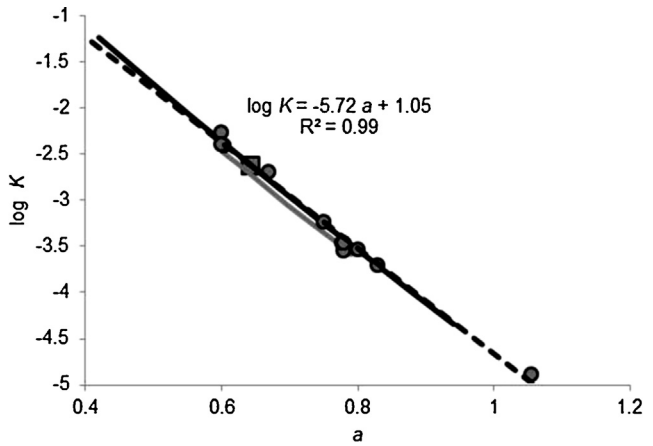

Fig. 5. Mark–Houwink–Kuhn–Sakurada constants from Table 1, literature values grey circles, this work grey square. The equation denotes the linear fit (broken black line) of all these constants. The two other lines are the MHKS constants as obtained from the tangents to the polynomial curves of the raw data in Fig. 4, grey line (Podzimek et al., 2010) and black line this work.

Table 1
Mark–Houwink–Kuhn–Sakurada values from literature.

a	K [dL/g]	Calculated M_w at 15 dL/g [kDa]	Condition for [η]	Ref.
0.642	0.0024	816	10 mM Phos, 150 mM NaCl pH 7.0 25 °C	This work
0.75	0.00057	783		Terbojevich et al. (1986)
0.67	0.0020	608	0.1 M $NaNO_3$ pH 7.0, 40 °C	La Gatta et al. (2010)
0.60	0.00537	549		
0.78	0.000278	1166	0.1 M $NaNO_3$, 25 °C	Soltes, Mendichi, Lath, Mach, and Bakos (2002)
0.829	0.000199	764	0.2 M NaCl, 25 °C	Yanaki and Yamaguchi (1994)
0.80	0.00029	780		Balazs (1965)
0.604	0.00395	844	0.15 M NaCl, 37 °C	Mendichi et al. (2003)
0.778	0.000339	937		
1.056	0.0000159	554		
0.779	0.000346	896	0.15 M NaCl, 25 °C	Bothner et al. (1988)
0.601	0.00397	896		

Table 1 have been added to the plot. It can be observed that the plot of log K versus a from the tangents of the polynomial fits produces almost linear traces and the literature values follow these. Thus, literature MHKS values across time, methods and even temperature and buffers can be said to support the second order polynomial fitting for the HA polymer. The reason for this polynomial correlation could be due to one of three things: analytical limitations, branching, as suggested in (Podzimek et al., 2010), or reflection of the actual behaviour of linear polymers when investigated across sufficiently large M_w ranges.

M_w has been determined by SEC-MALS for this work. M_w determined by SEC-MALS should not be significantly affected by the loss of separation of the SEC columns as opposed to the number-average molecular weight, M_n (Luan, Fang, Al-Assaf, Phillips, & Zhang, 2011). Also the measurement of intrinsic viscosity should not be affected by the M_w as further dilution or increasing concentration will ensure robust data. This was supported by that the Huggins' constant were derived from linear plots with $R^2 > 0.99$. Thus analytical limitations do not appear to be an immediate reason.

The sources and purification methods of the HA used in literature are wide and many. Branching as a reason would require that all of these HA materials should have similar limited, hitherto undetected, branching in order to yield a and log K values that follow the tangent of the polynomial from Fig. 4. Branching of HA cannot be ruled out based on this work, but does not appear an obvious cause to the authors.

Interestingly, the linear relationship for log K versus a in Fig. 5 can also be seen for MHKS values from the literature for some other linear polymers, such as polyethylene and polystyrene, Fig. 6, despite very different analytical conditions (Zeng, Du, Xue, & Frisch, 2007). However, no linear relationship is seen for other linear polymers such as chitosan and cellulose (data not shown). For chitosan this could be due to the variation in the distribution of the polysaccharide sub-units (i.e., blocks) from the chitosan production from chitin. For cellulose it could be due to its low solubility and tendency to form crystalline structures. Thus it can be postulated that a curved relationship between log[η] versus log M_w is the true relationship for linear polymers.

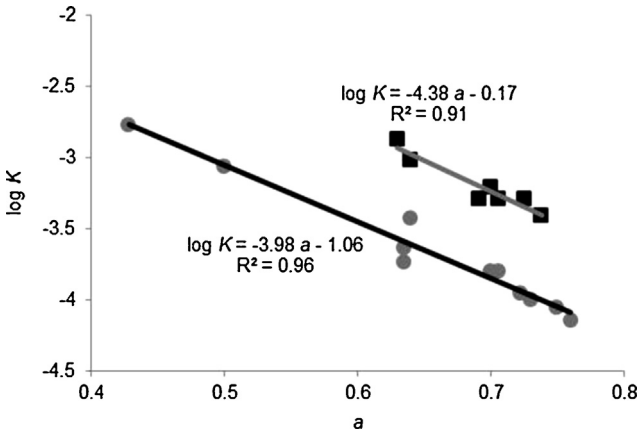

Fig. 6. Mark–Houwink–Kuhn–Sakurada constants for polystyrene (grey circles) and polyethylene (black squares) from Zeng et al. (2007) and references therein.

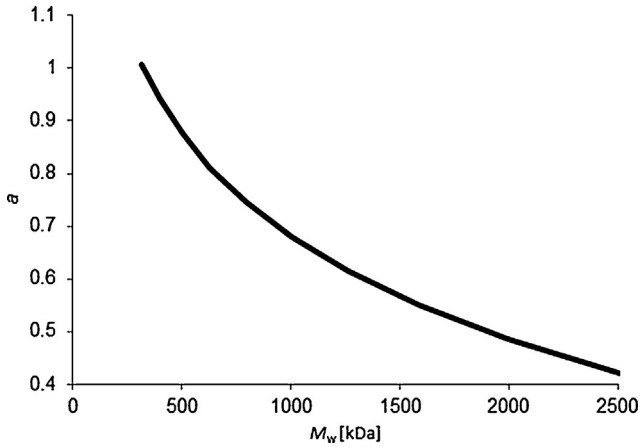

Fig. 7. The a Mark–Houwink–Kuhn–Sakurada exponent as found from the tangent to the second order polynomial fit in Fig. 4 (black line) plotted as a function of M_w.

This perspective fits well with the earlier findings (Hayashi, Tsutsumi, Nakajima, Norisuye, & Teramoto, 1995) that showed that the M_w dependence of intrinsic viscosity, at high ionic strength, can be described almost quantitatively by the combination of the Yamakawa–Fujii–Yoshizaki theory for intrinsic viscosity of an unperturbed wormlike chain and the Yamakawa–Stockmayer–Shimada theory for excluded-volume effects. This means that the longer the HA chain is, the more prominent the intramolecular excluded volume-effects will be. This would also mean that one should expect a decrease of a with increasing M_w, as is what is seen in this work, Fig. 7, and by other authors (Cowman & Matsuoka, 2005; Hokputsa et al., 2003; La Gatta et al., 2010; Mendichi et al., 2003; Bothner et al., 1988).

4. Conclusion

This work has shown that the depolymerisation of sodium hyaluronate in solid proceeds by a random degradation process with an activation energy at neutral pH of 127 kJ/mol. This indicates that it is following a hydrolysis type mechanism similar to

that seen for HA in solution. Furthermore, the pH stability optimum of solid HA is at neutral pH with accelerated degradation at alkaline and acidic pH.

The Mark–Houwink–Kuhn–Sakurada relationship for HA in 10 mM phosphate 150 mM NaCl pH 7.0 at 25 °C solution has been investigated for more than sixty HA batches with M_w ranging from 0.4 to 2.3 MDa. It has been shown that there is a second order polynomial relationship for $\log[\eta]$ versus $\log M_w$ for HA. This confirms the previous finding of a non-linear relationship due to increased chain flexibility with increasing M_w for HA at high ionic strength (Hayashi et al., 1995). The second order polynomial will potentially allow for a more accurate determination of M_w over the entire span of HA sizes compared to using the linear MHKS model. But it is important to realise that the introduction of more parameters does not alleviate the need for alignment of analytical methods across laboratories.

Support for the use of a polynomial equation for the description of the $\log[\eta]$ versus $\log M_w$ is found from that the MHKS data obtained from the tangents to the second order polynomial curve fit MHKS data from the literature. Furthermore, this linear behaviour of $\log K$ versus a was seen for other well-behaving linear polymers

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