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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