

Study of Hyaluronan Degradation by Means of Rotational Viscometry: Contribution of the Material of Viscometer

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The molecular parameters of nine hyaluronan (HA) samples were determined by the method of size exclusion chromatography. The flow behaviour of the HA solutions was investigated by capillary and rotational viscometry. The dependences of the molecular mass on the radius of gyration or on the intrinsic viscosity number indicated that the HA samples investigated constitute a uniform set.

Rotational viscometry during the measurements showed, however, a continual increase of the apparent viscosity of the HA solution. Addition of hydrogen peroxide into the solution in the metal cup brought a rapid loss of sample viscosity. These observations indicate that the material of rotational viscometer presumably influences the measurements.

Hyaluronan (HA; Fig. 1) is a polysaccharidic constituent of numerous tissues in the organism. HA is one of the functionally most essential components of both cartilage and synovial fluid (SF). The physiological hyaluronan level in SF is 2–3 mg cm⁻³ [1]. The mean molecular mass of HA in SF of healthy subjects is of several million g mol⁻¹. The degradation of high-molecular-mass hyaluronan, occurring under inflammation and/or oxidative stress, is accompanied with the loss of the viscoelastic property of SF. Thus, viscoelasticity can be used as a marker of the extent of hyaluronan degradation.

As reported by *Deeble et al.* [2], HA degradation by oxygen-derived free radicals was first investigated by Pigman and Rizvi as early as in 1959. Since then a huge number of studies have been reported. The low resistance of HA itself against oxidative species determines this biopolymer to be exploited as an *in vitro* probe for investigating the damaging action of oxidants and/or to evaluate the effectivity of various substances to act as antioxidants. In principle, changes of viscoelasticity of the hyaluronan solution caused by oxidative action of various reactive species can be and have been monitored easily by viscometry and/or rheometry.

In 1999 *Jahn et al.* [3] published results of the

kinetics of decrease in viscosity of HA solution monitored by means of a rotational viscometer. At 37°C, within 14 h, the viscosity of pure phosphate-buffered HA solution (pH 7.4) was reduced by approx. 15 %. This observation coincides well with findings of *Miyazaki et al.* [4] on their viscosity measurements of HA solution by rotational viscometry [5] reporting hyaluronan degradation even when the sample solution was left in the (metallic) cup of the viscometer. Thus they anticipated that the degradation occurred due to the contact of the HA macromolecular chain with a solid-state metal. Their investigation performed with stainless steel, copper, or tungsten carbide beads demonstrated the loss of the HA solution viscosity also in contact with these metallic beads [4].

Findings of further groups of researchers [6–8] may lead to another plausible explanation of the above-mentioned phenomenon: The metal in the form of the cation (Fe³⁺, Fe²⁺, Cu²⁺, etc.) forms with the hyaluronate polyanion a salt – a macrochelate complex – demonstrating different solution properties from those of *e.g.* sodium hyaluronate.

All the above facts indicate the relevance of performing rotational viscometric measurements of pure HA samples, focusing attention on the action of oxidants the oxidative potential of which depends on the

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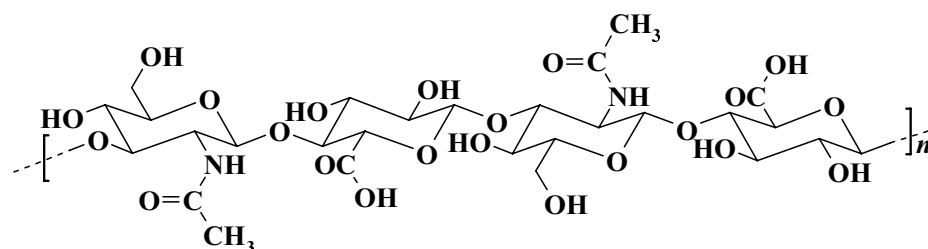


Fig. 1. Hyaluronan (acid form).

Table 1. Characteristics of High-Molecular-Mass HA Materials Used

Method	HA material code								
Parameter	ALTISSIMO	F1750762	GENZYME B22157	OFTALMICO	LIFECORE P9710-2	SIGMA 35H0439	CPN	LIFECORE 1-9100-1	GENZYME
Size exclusion chromatography									
$M_m/(\text{kg mol}^{-1})$	1553	1378	1340	1292	1215	1017	659.4	426.2	90.2
M_m/M_N	1.87	1.61	1.50	1.63	1.79	1.82	1.88	1.84	1.60
R_g/nm	152.0	148.7	129.8	123.5	120.1	130.7	97.4	77.2	24.8
Capillary viscometry ^a									
$[\eta]/(100 \text{ cm}^3 \text{ g}^{-1})$	16.0	13.3	14.3	14.1	14.0	9.32	10.3	7.31	1.86
Rotational viscometry									
$\eta/(\text{mPa s})$	22.60 ^b	17.62 ^b	20.80 ^b	22.40 ^b	17.45 ^b	9.88 ^c	8.32 ^c	5.35 ^c	1.77 ^c
Producer data									
Iron/ppm	NA*	NA	NA	NA	13	NA	NA	NA	NA
Copper/ppm	NA	NA	NA	NA	4	NA	NA	NA	NA

a) Shear-rate estimated = 2050 s^{-1} .

b) Shear-rate = 138.6 s^{-1} ; value read at 12th min.

c) Shear-rate = 264.0 s^{-1} ; value read at 12th min.

* NA = not available.

presence of metal in the form of solid state or in that of dissolved cations.

EXPERIMENTAL

Hyaluronan samples, covering by their mass average of the molecular mass (M_m) the range from 90.2 to 1553 kg mol^{-1} (Table 1), were kindly gifted by or purchased from HA material producers: Fidia Farmaceutici S.p.A., Abano Terme, Padova, Italy; Genzyme Corp., Cambridge, MA, U.S.A.; Lifecore Biomedical Inc., Chaska, MN, U.S.A.; Sigma Chem. Co., St. Louis, MO, U.S.A.; CPN Ltd., formerly Contipro, Ústí nad Orlicí, Czech Republic.

Anal. grade NaCl was from Slavus Ltd., Bratislava, Slovak Republic. Aqueous H_2O_2 solution (30 %), anal. grade, was purchased from Chemapol, Prague, Czech Republic. Water used was of Milli-Q_{RG} quality (Millipore Corp., Bedford, MA, U.S.A.).

Hyaluronan sample for capillary viscometry was swollen and further dissolved overnight in dark at laboratory temperature in 0.2 mol dm^{-3} aqueous NaCl solution. Namely in the case of ALTISSIMO, F1750762, GENZYME B22157, OFTALMICO, LIFE-

CORE P9710-2 the stock sample concentration was 0.5 mg cm^{-3} and that of HA samples, having lower molecular mass, was 1.0 mg cm^{-3} . The diluent used was a 0.2 mol dm^{-3} aqueous NaCl solution.

HA sample for rotational viscometry was swollen and further dissolved overnight in dark at laboratory temperature in 0.15 mol dm^{-3} aqueous NaCl solution to a working concentration of 3.0 mg cm^{-3} .

Molecular parameters of the HA samples were determined by size exclusion chromatography using an Alliance 2690 separation module (Waters, Milford, MA, U.S.A.) equipped with two on-line detectors, namely with a UV VIS spectrophotometer (Model 996 PDA; Waters) and a MALS photometer (DAWN DSP-F; Wyatt Technology, Santa Barbara, CA, U.S.A.). The setting of variables was as follows: Two stainless-steel columns (both $7.8 \text{ mm} \times 30 \text{ cm}$) connected in series with a guard precolumn; packings were TSK-gel of PW type (G6000 and G5000; $17 \mu\text{m}$ particles; Toso Haas, Montgomery-ville, PA, U.S.A.); temperature 35.0°C ; mobile phase 0.15 mol dm^{-3} aqueous NaCl solution; flow-rate $0.4 \text{ cm}^3 \text{ min}^{-1}$; injected sample volume 0.2 cm^3 ; concentration of the injected sample 0.1 mg cm^{-3} . Each HA sample, prior to its anal-

ysis, was clarified by filtration through a 0.45 μm filter (Millipore Corp.). Detector settings: Absorbance at $\lambda = 206\text{ nm}$; MALS photometer operated at $\lambda = 632.8\text{ nm}$. The angular distribution of the intensities of the scattered light was monitored simultaneously by means of an array of photodiodes.

The specific refractive index increment of the HA sample solution was determined at 35.0°C , at $\lambda = 632.8\text{ nm}$, by a KMX-16 differential refractometer (LDC Milton Roy, Rochester, NY, U.S.A.). The data acquisition and analysis softwares used were MILLENNIUM 2.15 (Waters) and ASTRA 4.50 (Wyatt Technology). Table 1 shows the mass average of the HA sample molecular mass, the sample polydispersity (M_m/M_N , where M_N is the number average of the sample molecular mass), and the gyration radius (R_g) of the HA samples.

The capillary viscometric measurements were performed at $(25.0 \pm 0.05)^\circ\text{C}$ using an Ubbelohde dilution viscometer (Schott Glas, Mainz, Germany). The diameter of the viscometer capillary was 0.53 mm and the flow-through time of the diluent (η_0) was 85.1 s. The flow-through times of the diluent and of the investigated HA solutions (η_i) were measured with a precision of 0.1 s for the run. The HA solution was diluted directly in the viscometer reservoir so as to fulfil the condition $1.1 \leq \eta_i/\eta_0 \leq 2.0$. The viscometry data were evaluated according to the equations introduced by Kraemer and by Huggins [9]. The determined intrinsic viscosity values ($[\eta]$) are listed in Table 1.

The rotational viscometric measurements were carried out at $(25 \pm 0.1)^\circ\text{C}$ by using a digital rotational viscometer Brookfield DV-II+ PRO (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). Into the metal cup reservoir 8 cm^3 of the HA solution (3.0 mg cm^{-3}) was loaded. The instrument output parameters – the apparent viscosity, shear stress, and torque – were monitored during the time period of up to 5 h. The determined apparent viscosity values (η) of the investigated HA samples are listed in Table 1.

UV absorption spectra of the samples investigated were scanned with a UV VIS spectrophotometer Specord 40 (Analytik Jena AG, Jena, Germany).

The amount of 30.0 mg of the HA sample (LIFECORE P9710-2) was swollen and further dissolved overnight in dark at laboratory temperature in 9.0 cm^3 of 0.15 mol dm^{-3} aqueous NaCl solution. The batch hydrogen peroxide solution was prepared by dissolving NaCl in the concentrated H_2O_2 to a salt concentration equaling 0.15 mol dm^{-3} . The batch H_2O_2 solution (1 cm^3) was added to the above HA solution, stirred for 20 s, and 8 cm^3 of the solution was transferred into the cup of the rotational viscometer. The monitoring of the viscometer output parameters started 2 min after the solution stirring. The monitoring was stopped 1 h after the experiment onset. A similar sample mixture was kept during the whole time period of 1 h in a glass vessel and then the solu-

tion was evaluated by taking 8 cm^3 for the rotational viscometry measurement.

RESULTS

Table 1 lists the molecular characteristics of nine hyaluronan samples investigated. As evident, the values of M_m ranged between 90.2 and 1553 kg mol^{-1} . The values of polydispersity of the samples, M_m/M_N , fall within an interval from 1.50 up to 1.88. The R_g values (Table 1) as well as the resulting dependence of the radius of gyration on the HA sample molecular mass, written in the form of a power law function

$$\{R_g\} = 2.45 \times 10^{-2} \{M_m\}^{0.613} \quad (r = 0.9883) \quad (1)$$

well correspond to $\{R_g\} = 2.75 \times 10^{-2} \{M_m\}^{0.596}$ determined for another set of high-molecular-mass HA samples [10].

The relationship between the limiting viscosity number $[\eta]$ of a polymeric sample solution and the viscosity average of the polymer molecular mass (M_v) is represented by a well-known Kuhn—Mark—Houwink—Sakurada equation $[\eta] = K \times \{M_v\}^a$, where K and a are the parameters related to the system polymer—solvent—temperature. Similarly, the $[\eta]$ vs. M_m relationship is often described by using a power law functional equation. However, since high-molecular-mass HA biopolymer solutions behave like non-Newtonian liquids, the use of the power law function calculated ($[\eta]$ being in $100\text{ cm}^3\text{ g}^{-1}$) from the data given in Table 1

$$\{[\eta]\} = 5.489 \times 10^{-4} \{M_m\}^{0.721} \quad (r = 0.9817) \quad (2)$$

should be applied with a care [11].

The shape of the relationship between the apparent viscosity, η , and the shear rate applied at rotational viscometric measurements can be utilized to discriminate whether the given fluid behaves either like Newtonian or like non-Newtonian. In the case of an HA solution related to a high-molecular-mass sample the log-log scale plot of the power law function $\eta = A \times \{\text{shear rate}\}^B$, where A and B are the parameters, should be a straight line. However, as observed especially in the case of the HA sample ALTISSIMO, on measuring the η values by increasing the shear rate (spindle rotational speed from 12 up to 120 min^{-1}), the relationship $\{\eta\} = f\{\text{shear rate}\}$ exhibited a deviation from the linear dependence. Since the collection of the whole data set of η_i at various spindle rotational speeds takes some time, one should inspect whether the HA solution itself does not demonstrate changes related *e.g.* to structural modification of the HA chains. As expected, the time dependence of η , especially in the case of ALTISSIMO sample (Fig. 2, panel A), indicated such a potential. Thus, taking into account the observation with the sample ALTISSIMO,

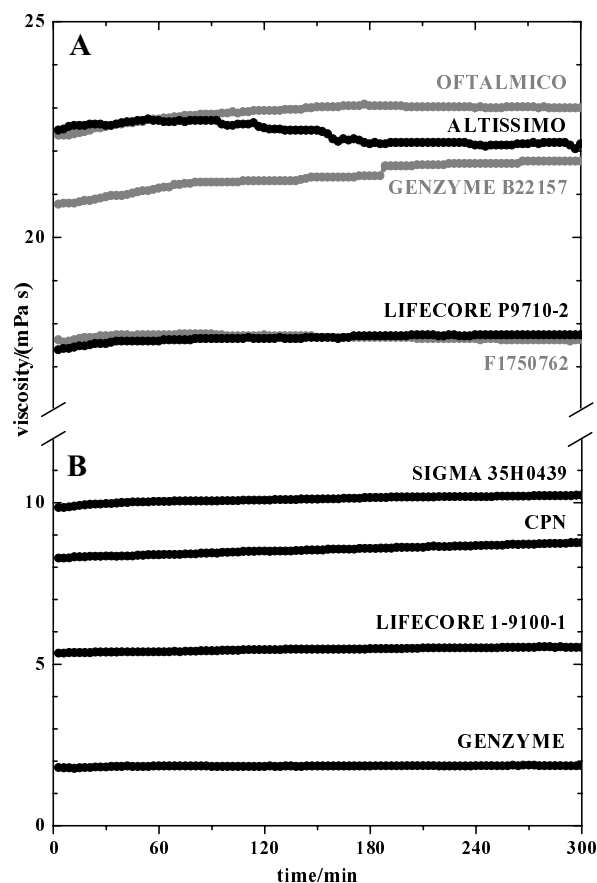


Fig. 2. Viscosity-time profiles of nine HA samples of the concentration 3.0 mg cm^{-3} during 5 h. Shear rates at 105 min^{-1} and 200 min^{-1} were respectively 138.6 s^{-1} (panel A) and 264.0 s^{-1} (panel B).

along with the findings of Miyazaki *et al.* [4, 5], the simplest preliminary conclusion would be that the shape of the η vs. time profile for ALTISSIMO disclosed degradation of the sample.

However, eight further HA materials inspected for the above phenomenon disclosed a different η vs. time profile (Fig. 2). As evident, each sample displayed a weak but continual increase of η within the time of measurement. One of the simplest and plausible explanations of the observed phenomenon is the hypothesis on continual dissolution of the metal from the viscometer cup/spindle into the HA sample solution. The polyvalent cations, *e.g.* Fe^{n+} might primarily act as a chelating cationic centre, which by intermolecular physicochemical interactions “crosslinks” the biopolymer.

To test the latter hypothesis, one way could be the determination of the metal content changes in the HA solution, however, the concentration variance would be of trace value [4]. To enhance the metal material leakage into the sample, heating, acidification/alkalization of the solution would be the “classical” mode. A more efficient agent, however, would be the hydrogen peroxide. In the case of dissolu-

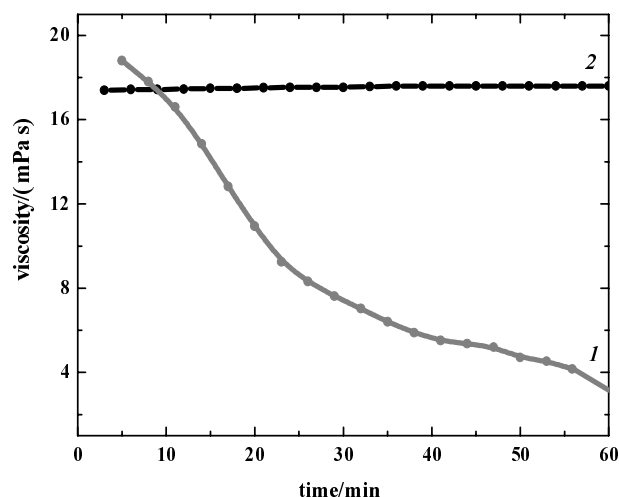


Fig. 3. Kinetics of the decrease in viscosity of a hyaluronan solution (3.0 mg cm^{-3}) incubated with 3 % H_2O_2 (curve 1). As control, the viscosity-time profile of the pure HA solution is shown (curve 2).

tion of a trace of the transitional metal, *e.g.* Fe^{2+} into the HA solution, the action of added H_2O_2 should speed up the changes in the η vs. time profile, due to the degradative action of OH radicals formed *in situ* ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{HO}^-$).

The result represented in Fig. 3 unambiguously shows that there is a massive change in the HA sample η values within a short time. For example, within the first 20 min after onset of H_2O_2 admixing the η value dropped by 42 %. This decrease of the solution viscosity continued at such a speed that 1 h after the onset the η value reached only 17 % from the initial viscosity value. Visual inspection of the solution treated in the reservoir of the viscometer disclosed a generation of bubbles and, by prolonging the sample treatment, a brown particulate material – rust – was found in the reservoir.

Parallel experiments, in which the HA solutions fortified with H_2O_2 were kept in a glass vessel, failed to indicate any of the above changes – no bubbles, no rust. The η value of the LIFECORE P9710-2 sample treated in the glass vessel with H_2O_2 , measured 1 h after the reaction onset, was 15.05 mPa s , *i.e.* 86 % of the initial viscosity value. Thus the most plausible conclusion of these series of experiments could be that due to the action of H_2O_2 the η value dropped and that this decrease was pronounced when the HA solution was in contact with a metal. It should be pointed out that the normalized UV spectra of the solutions drawn from the glass vessel were identical to the spectra of the samples drawn from the metal reservoir at any time interval inspected.

DISCUSSION

The size exclusion chromatographic (SEC) apparatus equipped with the MALS detector is classifiable as the most powerful tool to determine the molecular parameters of HA macromolecules [12]. The settings specified in the section Experimental are an appropriate compromise warranting proper SEC separation along with precise MALS molecular-mass analysis of the hyaluronan samples investigated. Thus, the molecular characteristics determined for all the nine high-molecular-mass HA samples listed in Table 1 can be classified as valid. Along with the M_m values, the estimation of the R_g parameter resulted in the power law $R_g = f\{M_m\}$ function represented by eqn (1). The slope of this function, calculated with a linear regression over the whole set of R_g vs. M_m data of the nine HA samples, was 0.613.

Viscosity belongs among the main characteristics of any fluid, and the method of capillary viscometry is ranked as the simplest and most precise technique of choice for determining viscosity characteristics of a liquid/solution. Capillary viscometry, falling among the moving fluid methods, measures variables in terms of time at a predetermined rate of the fluid flow. The capillary viscometer of the Ubbelohde type is, however, suitable for exact use only with Newtonian fluids.

The nonhomogeneous density of the entangled HA biopolymer chains, forming a microheterogenic polymer network, is the main phenomenon resulting in the non-Newtonian flow behaviour of the HA solutions. However, despite the relative meaning of the linear analysis of the $[\eta]$ vs. M_m data, the coefficients determined in this study (eqn (2)) are in agreement with published results [11, 13, 14].

It has been suggested that rheology is the most sensitive method for material characterization because the flow behaviour is responsive to properties such as molecular mass and molecular mass distribution [15]. Thus rotational viscometry might be the most efficient analytical method in investigating the degradation of high-molecular-mass hyaluronan caused by various oxidants. Changes of viscoelasticity of HA solutions during inhibited/uninhibited hyaluronan degradation studies could be of advantage in classifying the efficacy of various substances to act as antioxidants.

The Brookfield DV-II+ PRO digital rotational viscometer is one of those instruments in which rotational speed is controlled and stress measured. Moreover, on using this apparatus, a flow curve for the fluid can

be determined, which shows the viscous profile of the fluid over a wide range of shearing conditions [16]. Yet in the light of the series of experiments presented and taking into account the observations published [3–5], we strongly recommend to use rheometry by working with spindle as well as with the sample reservoir, where the material in contact with the HA solution is inert. The material of choice suggested is Teflon.

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