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Solution properties of high-molar-mass hyaluronans: the biopolymer degradation by ascorbate

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Abstract—An accurate molecular characterization, molar mass and size distributions, of 10 hyaluronan (HA) samples was performed by using a multi-angle light scattering detector connected on-line to a size exclusion chromatographic system. The dynamic viscosity η of the HA solutions was investigated using a rotational viscometer. On monitoring the sample dynamic viscosity for up to 5 h, a small however constant increase of the η value was observed, indicating rheopectic behavior of all 10 HA solutions. Addition of ascorbic acid to the HA solutions caused significant changes in the rheological properties of the samples investigated. The change of η values in the course of time was explained by the redox reactions (caused by the added ascorbate) that occur during the dynamic viscosity monitoring.

Keywords: Hyaluronan degradation; Rotational viscometry; Ascorbic acid

1. Introduction

Hyaluronan (HA) is a linear glycosaminoglycan built of the disaccharide repeating units comprising D-glucuronate linked through β-(1→3) glycosidic linkage to *N*-acetyl-D-glucosamine. These disaccharide structural units are linked via β-(1→4) glycosidic bonds (cf. Fig. 1).

HA is a polysaccharidic constituent of almost all tissues in the vertebrates: its physiological concentration, for example, in the synovial fluid (SF) is 2–3 mg/mL.[1] Due to the very high HA molar mass in the SF of healthy subjects—several million Daltons—a frictionless functioning of the joints is warranted. However, when, for example, during a joint inflammation the HA macromolecules are degraded by the action of free radicals, SF loses its lubricating properties, which leads to increased

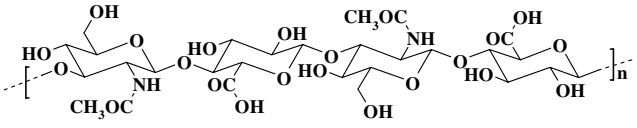

Figure 1. Hyaluronan—acid form.

wear of the joint and results in arthritic pain. The observed reduction of the hyaluronan molar mass in the SF of patients suffering from rheumatic diseases led to in vitro studies of HA degradation.[2] The earliest investigations go back to the 1940s.[3,4] Since then, numerous studies of this phenomenon have been reported.[5–10]

HA solution of high concentration possesses extraordinary rheological properties: it exhibits valuable viscoelastic properties, that is, its viscosity is strongly dependent on the applied shear-stress. Accordingly, rotational viscometry has been one of the simplest instruments of choice used to characterize the 'macroscopic' properties of HA solutions.[11] Dynamic viscosity—the

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output parameter of the measurements—depends, however, on numerous variables: primarily on the applied shear-stress, the HA molar mass, the concentration of the sample applied for the measurement, the temperature, and in the second place on, for example, impurities usually present in materials recovered from biological sources.

Although animal tissues, primarily rooster combs, were involved at the early stages of production of the commercially available hyaluronans, at the present time HA produced extracellularly by the microorganisms such as *Streptococcus zooepidemicus*, *Streptococcus equi*, etc., and isolated from the growth medium are marketed by many companies. Frequently the main drawback of both 'extractive' and 'fermentative' products is the lack of the Certificate of Analysis of the sample batch reflecting insufficient characterization of the HA molar mass distribution (MMD) and the impurities present in the biopolymer. Among the contaminating ingredients, the so-called 'link proteins' and traces of (transition) metal cations trapped by HA macromolecules should be mentioned.[12,13]

Size-exclusion chromatographic (SEC) device connected on-line to a multi-angle laser light scattering (MALS) detector belongs to the most appropriate equipments for the MMD analysis of the polymers. Continual dilution and a 'turbulent' flow character of the macromolecules in the SEC column(s) result in an efficient and rapid separation of the polymer species, whereas at the same time a sensitive MALS photometer directly renders molecular characteristics of the separated macromolecular species.

Polymer solutions of sufficiently high concentrations can, however, form a (microheterogeneous) network resulting in a non-Newtonian flow behavior. To monitor this phenomenon—that is, typical especially for HA solutions—the rotational viscometry might be one of the most efficient analytical tools to investigate the effects of the added 'contaminants' such as salts of transition metals, various oxidants, etc.

The aim of the present paper is to report the results of characterization of the 10 HA preparations using rotational viscometer and SEC–MALS methodology as well as to investigate changes of the dynamic viscosity of the different HA solutions in the course of prolonged measurement in the presence and in the absence of ascorbic acid—a natural inducer of redox reactions in vitro and under the physiological conditions, especially in the presence of transition metal ions.

2. Experimental

2.1. Biopolymers

Ten different native hyaluronans used throughout the study (cf. Table 1) were kindly donated by or purchased from the following HA manufacturers: Fidia Farmaceutici S.p.A., Abano Terme, Italy; Genzyme Corp., Cambridge, MA, USA; Lifecore Biomedical Inc., Chaska, MN, USA; Sigma–Aldrich Chemical Company, St. Louis, MO, USA; CPN Ltd (formerly Contipro), Ústí nad Orlicí, Czech Republic.

2.2. Chemicals

Analytical purity grade NaCl was from Slavus Ltd, Bratislava, Slovakia; ascorbic acid was from Merck KGaA, Darmstadt, Germany. Water used was of Milli-Q_{RG} quality (Millipore Corp., Bedford, MA, USA).

2.3. SEC–MALS characterization of native hyaluronans

The HA sample was dissolved overnight in aqueous NaCl (0.15 M). On the following morning, prior to analysis, the sample solution was clarified by filtration through a 0.45 μm Nylon filter (Millipore Corp.). The MMD analysis of 10 HA samples was performed by using a modular multi-detector SEC system. The SEC system consisted of an Alliance 2690 separation module

Table 1. Summary of characteristic parameters for the 10 HA samples

HA Sample code	Analytical method				
Parameter (unit)	SEC–MALS			Rotational viscometry	
	M_w (kDa)	M_w/M_n (–/–)	Rg (nm)	$\eta_{3'}$ (mPa s)	$\eta_{300'}$ (mPa s)
Altissimo	1553	1.87	152.0	11.75	12.33
F1750762	1378	1.61	148.7	11.13	11.93
B22157	1340	1.50	129.8	11.00	11.93
Oftalmico	1292	1.63	123.5	8.33	8.91
53H0439	1017	1.82	130.7	6.33	6.54
P9710-2A[a]	808.7	1.63	110.0	9.78	10.33
P9706-6	803.9	1.64	107.9	8.45	8.78
CPN	659.4	1.88	97.4	5.94	6.29
1-9100-1	426.2	1.84	77.2	4.20	4.72
Hylumed	90.2	1.60	24.8	1.50	1.78

[a] Aged HA sample.

from Waters (Milford, MA, USA) equipped with two on-line detectors, namely with a MALS Dawn DSP-F photometer from Wyatt (Wyatt Technology, Santa Barbara, CA, USA) and a DRI 410 differential refractometer from Waters. The later detector was used to determine the biopolymer concentration in the effluent. The setup of this multi-detector SEC system was serial in the following order: Alliance-MALS-DRI. It is well known that an on-line MALS detector coupled to a concentration detector allows to obtain the true molar mass value (M) and the size, that is, the root mean square radius ($\langle s^2 \rangle^{1/2}$), hereafter referred to as radius of gyration Rg, of each fraction of the eluted polymer.

The experimental methodology for a reliable use of the MALS photometer has been already described in detail.[14–16] Briefly: The MALS photometer uses a vertically polarized He–Ne laser, $\lambda = 632.8$ nm, and simultaneously measures the intensity of the scattered light at 18 fixed angular locations ranging in aqueous solvent from 14.5° to 158.3° by means of an array of photodiodes. The MALS calibration constant was calculated using toluene as a standard assuming a Rayleigh factor $R(\theta) = 1.406 \times 10^{-5}\ \mathrm{cm}^{-1}$. Normalization of the photodiodes was performed by measuring the scattering intensity in the mobile phase of bovine serum albumin (BSA; $M \approx 67$ kDa, Rg = 2.9 nm), a globular protein that is assumed to act as an isotropic scatterer. The specific refractive index increment, dn/dc, of HA with respect to the mobile phase—at 35 °C and $\lambda =$ 632.8 nm—was obtained from the literature.[15] The dn/dc value for HA in 0.15 M NaCl is 0.15 mL/g.

The experimental conditions of the SEC–MALS system were as follows: Two stainless steel columns (both 7.8 mm × 30 cm) connected in series with a guard precolumn; packings were TSKgel of PW type (G6000 and G5000; 17 μm particle size; Tosoh Bioscience, Stuttgart, Germany); separation temperature = 35 °C; mobile phase = aqueous 0.15 M NaCl solution; sample injection volume = 150 μL; the biopolymer concentration in the injected samples was 0.1–2 mg/mL depending on the HA molar mass. Taking into account high molar mass of some HA samples, a relatively low flow rate, 0.4 mL/min, was used to avoid shear-degradation of the polymer in the SEC columns.

The data acquisition and analysis softwares were EMPOWER PRO from Waters and ASTRA 4.73 from Wyatt Technology. Table 1 contains data on the HA sample M_w average, the M_w/M_n polydispersity value (where the M_n represents the number average of the HA sample molar masses), and the sample radius of gyration (Rg) in aqueous 0.15 M NaCl solution at 35 °C.

2.4. Rotational viscometric analysis of native hyaluronans

For viscometric measurements, the HA sample was swollen and subsequently dissolved overnight in dark at laboratory temperature in 0.15 M aqueous NaCl solution to obtain the concentration of polymer 2.5 mg/mL. All measurements were carried out at 25 ± 0.1 °C by using a digital rotational viscometer Brookfield DV-II+ PRO (Brookfield Engineering Labs., Inc., Middleboro, MA, USA) equipped with a Teflon cup/spindle set of coaxial geometry constructed at our laboratory.[17] The sample viscosity was monitored in 3 min intervals for up to 5 h. At a rotational spindle speed of 180 rpm, the shear rate equaled 237.6 s^{-1}. Measurement of the instrument output parameters—the apparent dynamic viscosity values (η)—was performed within a torque interval 9–88%.

2.5. Rotational viscometric study of the effect of added ascorbate

The conditions of HA sample preparation were identical to those applied at rotational viscometric analysis of the native hyaluronans, however immediately before the measurement of the η value, ascorbic acid (100 μM) was admixed into the polymer solution. The viscosity-time dependence of the sample was monitored during 5 h.

3. Results

Table 1 summarizes the most important results obtained with the HA samples analyzed by using the SEC–MALS system and rotational viscometry. All hyaluronans have unimodal MMD (not shown) represented by low dispersity indexes (M_w/M_n) ranging approximately from 1.5 to 1.9. The M_w values determined cover a relatively broad range of molar masses (90.2–1553 kDa), while the values of Rg correspond to the size of the dissolved macromolecules in the range 24.8–152.0 nm. The estimated values Rg of the HA macromolecules correlate well with the M_w parameters of the sample (cf. also Fig. 2). The calculated intercept and slope of the conformational plot $\mathrm{Rg} = 2.25 \times 10^{-2}\ M_w^{0.621}$, where Rg is expressed in nanometers, is in a good agreement with the published relationship ($\mathrm{Rg} = 2.53 \times 10^{-2} M_w^{0.601}$) determined for 'monodisperse' HA fractions under similar experimental conditions.[18]

Figure 3 represents the plot of M_w values of the HA samples versus their dynamic viscosities recorded at the 3rd min ($\eta_{3'}$). As evident, the data of the $\eta_{3'} = f(M_w)$ scaling law are more scattered. This fact is not surprising because it is well known that good correlation between η and M_w parameters exists only if the Newtonian dynamic viscosity η_0 value (i.e., the first plateau of the flow curve) is used. The rheopectic behavior of the HA solutions thus represents a real limitation. As evident from Figure 4, left panel, the continuous increase of the monitored η value for any of the investigated HA

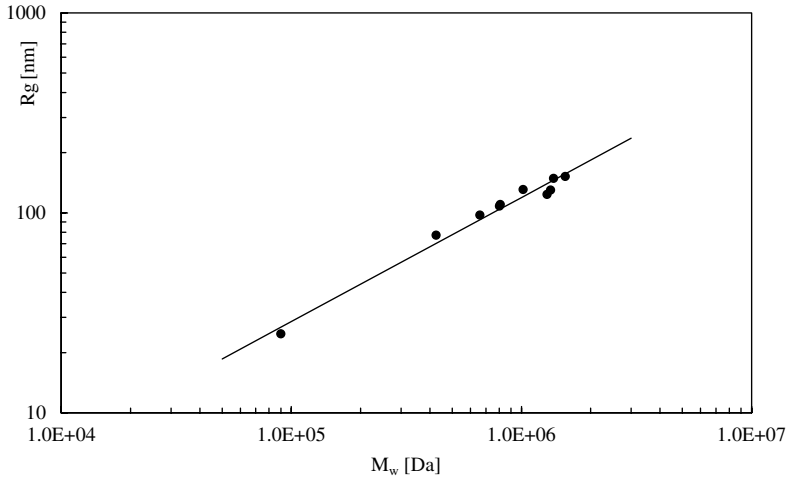


Figure 2. The conformational plot Rg = $f(M_w)$ constructed from the experimental data obtained with the 10 HA samples. Experimental data (●) and linear regression (—) throughout the whole molar mass range.

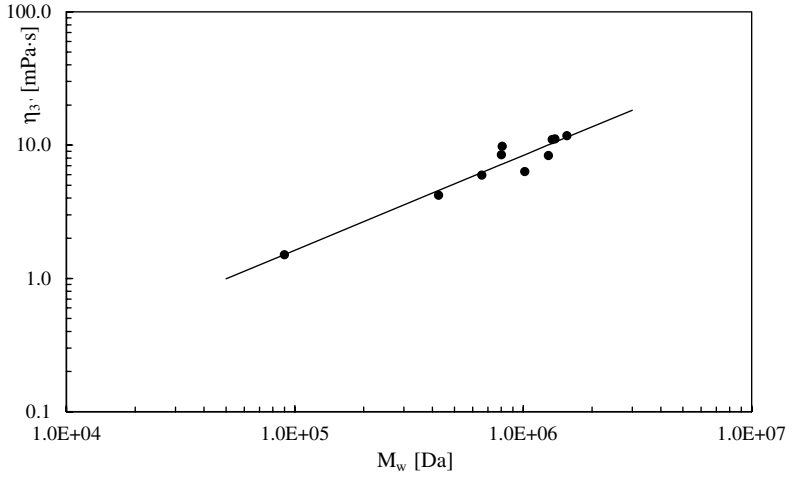


Figure 3. The scaling law relationship $\eta_{3'} = f(M_w)$ constructed from the experimental data of the 10 HA samples. Experimental data (●) and linear regression (—) throughout the whole molar mass range.

samples reduces the applicability of the generic dynamic viscosity value as a physico-chemical parameter. Therefore, the data listed in Table 1, columns 5 and 6 have only tentative values. In any case, however, the scaling law relationship between $\eta_{3'}$ and M_w calculated as $\eta_{3\prime} = 4.47 \times 10^{-4} M_w^{0.712}$, where $\eta_{3'}$ is expressed in mPa s, indicates a high regression ($r = 0.9627$).

Since the rheopectic behavior of the HA solutions could be influenced by trace amounts of non-metal admixtures and/or metal cations, the reducing ability of ascorbic acid was exploited to estimate the presence or absence of transition metal cations in the investigated HA samples. As can be seen (cf. Fig. 4, left and right panels), the corresponding initial η values vary in most cases. Moreover, the dynamic viscosity of the HA samples with higher M_w values (>659.4 kDa) indicates presence of two distinct regions: (i) rheopectic and (ii) the region, which should be assigned to the degradation of HA macromolecules due to well known oxidative–reductive properties of the system comprising ascorbate and transition metal cation(s).

4. Discussion

The 10 HA samples used in the present work had M_w values in the range that currently attracts increasing interest of many researchers. This is due to the recent findings that while the high-molar-mass HA molecules have anti-angiogenic, anti-inflammatory, and immunosuppressive properties, intermediate-sized fragments act predominantly in an opposite way, that is, they are highly angiogenic, pro-inflammatory, and immunostimulating.[12,13]

As can be concluded from the data provided in Table 1, all 10 HA samples represent polymers with relatively

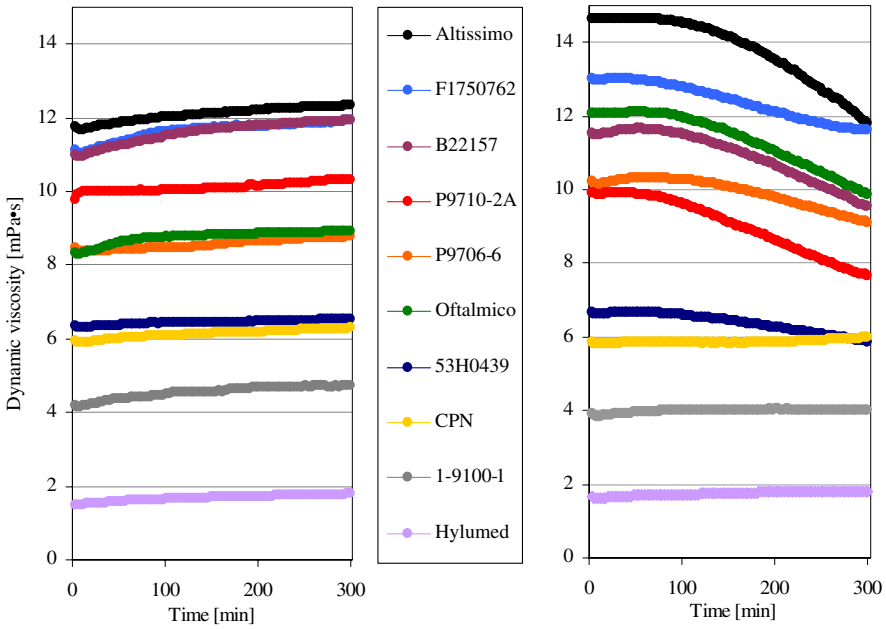


Figure 4. Time dependences of dynamic viscosity of the HA solutions. Left panel: Solutions of native hyaluronan samples. Right panel: Solutions of hyaluronan samples with addition of 100 μM ascorbic acid.

narrow MMD. The value of M_w/M_n ratio lies in the range 1.5–1.9, and the chromatograms of all samples reveal single-peak symmetric distribution. Moreover, the dependence $Rg = f(M_w)$ calculated from the data presented in Table 1 that is valid for the investigated series of 10 HA samples resembles very closely the one determined by means of the SEC–MALS analyses of 'monodisperse' HA fractions under similar experimental conditions.[18]

Contrary to the above findings obtained on the basis of the measurements of the HA solutions using SEC–MALS method, that is, at high dilution, solutions of relatively high concentration (2.5 mg/mL) monitored by applying the method of rotational viscometry demonstrated a phenomenon called rheopexy (cf. Fig. 4, left panel). This phenomenon involves gradual orientation of the HA macromolecules in the space between two cylinders, the external of which is rotating while the internal one is at rest. The velocity gradient in this intercylinder space orientates the entangled macromolecules in the direction of the streamlines by 'unwinding' the individual molecules. This subsequently results in an increased probability of the occurrence of other intermolecular interactions of the HA macromolecules, which leads to a gradual raise of the monitored η value. For this reason, it seems to be lucid that any experimental dependencies of the type $\eta = A \times 10^B M_w^C$ are of little or no general applicability and can only serve as an auxiliary relationship used by the individual researchers at the conditions applied in their experiments.

Addition of 100 μM ascorbic acid to the solution of any HA sample resulted in an instant change of the η value. For example, with the sample Altissimo and Oftalmico the values $\eta = 11.75$ and 8.33 mPa s, respectively, were established in the 3rd min (cf. Table 1), while upon the addition of ascorbic acid an increase of the value η in the 3rd min was evidenced (14.66 mPa s for the sample Altissimo, that is, 24.8% increase, and 12.06 mPa s for the sample Oftalmico, that is, 44.8% increase). On the contrary, using samples CPN and 1-9100-1 a reduction of the $\eta_{3'}$ value from 5.94 and 4.20 mPa s to 5.85 and 3.92 mPa s—that is, 1.5% and 6.7% decrease, respectively—was observed.

Since ascorbate acts as a powerful reducing agent with a standard reduction potential of 0.282 V at pH 7 for the redox couple $Asc^{\bullet -}/AscH^-$,[19] one could assume that it reduces traces of transition metals present in the HA samples, particularly Fe(III) and partially also Cu(II). For example, as calculated from the Certificate of Analysis of the HA samples P9710-2 or P9706-6, for the HA concentration of 2.5 mg/mL, solutions of these samples contain along with a few other metal contaminants, the following concentrations of the two main transition metal ions—iron and copper: 0.582 and 0.157 μM (P9710-2) or 1.209 and 0.118 μM (P9706-6), respectively. In the aqueous solution at pH 7, the corresponding reduction potentials for the redox pairs Fe(III)/Fe(II) and Cu(II)/Cu(I) are 0.48 and 0.16 V, respectively.[20] Due to the chelating properties of the HA macromolecule, the values of the standard reduction potentials for these metal ions can, however, change.[21,22]

Lowering the oxidation state of all (transition) metal ions present in the investigated samples leads to changes in the HA solution viscosity due to modification of the

intra- and intermolecular physico-chemical cross-links caused by the metal ions in different oxidation states. In general, it is necessary to accept that transition metal cations can produce polyanionic salts with the hyaluronate—the so-called macrochelate complexes—which may form an 'ionotropic' hydrogel exhibiting solution properties different from those of, for example, a sodium hyaluronate solution.

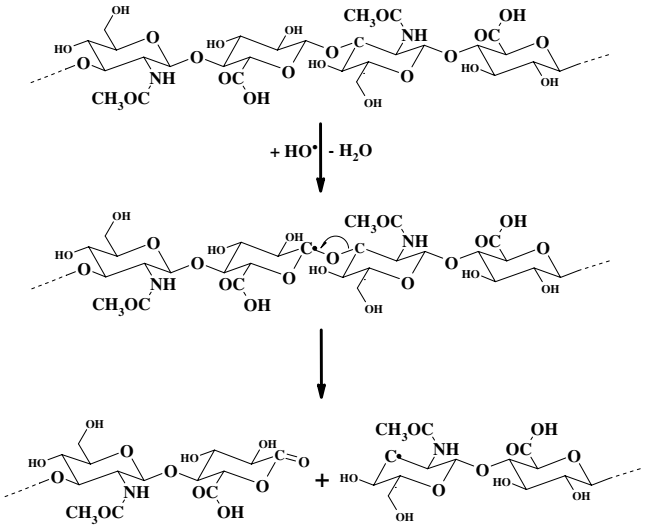
The most intriguing observation is, however, detection of the phase of a gradual decline of the η value of the high-molar-mass HA samples in a longer time interval. It is practically impossible for a character of the flow to change spontaneously from rheopectic to an opposite one. The most plausible explanation of this phenomenon is the effect of dioxygen, which is present in the prepared HA solutions as well as of that absorbed from the air in the process of measurement. Generally speaking, ions of transition metals in lower oxidation state, that is, $Metal^{(n-1)+}$, are able to reduce dioxygen. Considering the standard reduction potentials of the redox pairs involved, the one-electron reduction of dioxygen to superoxide anion radical is thermodynamically unlikely. At pH 7, the redox pair $O_2(aq)/O_2^{\cdot-}$ has a standard value of -0.16 V.[23] However, at pH 7 the two-electron reduction of gaseous dioxygen to hydrogen peroxide is characterized by the reduction potential of 0.28 V.[23] Taking into account the solubility of dioxygen in water of 1.17 mM[24] at pH 7, 25 °C, and physiological ion strength, a standard value of the redox potential of the pair $O_2(aq)/H_2O_2$ equals 0.37 V. Thus, taking into account that the reduction potential of the pair Cu(II)/Cu(I) = 0.16 V,[20] Cu(I) should be able to reduce O_2 molecules to yield directly H_2O_2.[25–29] Similar conclusion should apparently be valid for Fe(II) considering the above mentioned dependence of the redox properties of the chelated couple Fe(III)/Fe(II). Alternatively, complexes between Fe(II) ions and dioxygen are also assumed to yield reactive species of unknown nature, which are subsequently able to oxidize biological material.[30,31] This alternative pathway, however, is also based on the reduction of Fe(III) ions by ascorbate.

Because the excess of ascorbic acid over the total concentration of the metal ions is very high, it can be concluded that the transition metal ions in lower oxidation state react with H_2O_2 rendering $^{\cdot}OH$ radicals according to the following equation:

$$Metal^{(n-1)+} + H_2O_2 \rightarrow Metal^{n+} + {}^{-}OH + {}^{\cdot}OH$$

Excited iron complexes such as ferryl or perferryl compounds are also assumed as reactive products of this reaction.[32,33]

In aqueous milieu, hydroxyl radicals represent the most reactive species, which react with virtually all compounds containing C–H groups under the abstraction of one hydrogen radical ($H^{\cdot}$) leading to the generation of the corresponding alkyl radical; in the case of hyaluronan a C-centered radical is primarily formed. The continuation of the reaction—for example, fragmentation of the C-centered radical—can be described as shown in Scheme 1; however, concurrent reactions between the C-centered radical and (atmospheric) oxygen followed by the (transition metal ion catalyzed) decomposition of the formed intermediate peroxy radical yield HA fragments, carbonyl compounds, etc. Hydroxyl radicals, naturally, can attack the glucuronic acid or *N*-acetylglucosamine moieties on HA leading to the 'opening' of the pyranose ring(s) without breaking the polymer chain.[34,35] However, the subsequent radical reactions or rearrangement of generated C-centered radicals produce biopolymer fragments of lower molar mass, which naturally possess a decreased dynamic viscosity, which values can be very effectively monitored by using the Brookfield rotational viscometer (cf. Fig. 4, right panel).



Scheme 1. Scission of the HA chains may be due to β-cleavage of the C-centered radical formed at, for example, C(1) of the glucuronic acid residue.

In accord with the recently published findings,[36,37] it can be stated—in agreement with the title of the article by Buettner and Jurkiewicz[38]—'Catalytic metals, ascorbate and free radicals: Combinations to avoid'. On the other hand, addition of ascorbic acid, the known biogenic reducing agent, could be utilized as an especially suitable means of preparation of a series of HA samples with a specifically decreased molar mass.

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