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Hyaluronan degradation by copper(II) chloride and ascorbate: rotational viscometric, EPR spin-trapping, and MALDI–TOF mass spectrometric investigations

Ladislav Šoltés,[a,*] Monika Stankovská,[a] Vlasta Brezová,[b] Juergen Schiller,[c] Juergen Arnhold,[c] Grigorij Kogan[d] and Peter Gemeiner[d]

[a] *Institute of Experimental Pharmacology, Slovak Academy of Sciences, SK-84104 Bratislava, Slovakia*
[b] *Institute of Physical Chemistry and Chemical Physics, Faculty of Chemical and Food Technology, Slovak University of Technology, SK-81237 Bratislava, Slovakia*
[c] *University of Leipzig, Medical Faculty, Institute of Medical Physics and Biophysics, D-04107 Leipzig, Germany*
[d] *Institute of Chemistry, Slovak Academy of Sciences, SK-84538 Bratislava, Slovakia*

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Abstract—The degradation of high-molar-mass hyaluronan (HA) by copper(II) chloride and ascorbate was studied by means of rotational viscometry. It was found that even small amounts of $CuCl_2$ present in the oxidative system led to the pronounced degradation of HA, reflected in a rapid decrease of the dynamic viscosity of the biopolymer solution. Such degradation was induced by free radicals generated in elevated amounts in the presence of copper ions. Electron paramagnetic resonance investigations performed on a model oxidative system containing Cu(II) and ascorbic acid proved the formation of relatively stable ascorbate anion radicals resulting from the reaction of ascorbic acid with hydroxyl radicals. In this way, by scavenging the hydroxyl radicals, ascorbic acid protected HA from their degradative action. Matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometry was applied to analyze the degraded HA. The results showed that only regular fragmentation of hyaluronan occurred using the mentioned oxidative system that led to the formation of HA oligomers with unaffected primary chemical structure.

Keywords: Hyaluronan degradation; Rotational viscometry; EPR spectroscopy; Spin-trapping technique; MALDI–TOF mass spectrometry; Ascorbate; $CuCl_2$; Hydroxyl radicals

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1. Introduction

Biomacromolecules, such as nucleic acids and proteins, have often been employed to study the in vitro degradative actions of various oxidants. In these studies, both the biopolymers and the applied oxidative conditions are adjusted to mimic 'pathological' events, such as, arteriosclerosis and rheumatic diseases. The key task is to detect chemical and/or physical changes occurring in the target biomacromolecule.[1] In the case of an enzyme, reduction of its activity has been employed as a simple marker of the extent of functional impairment.[2]

Under arthritic inflammatory conditions of the affected joint, synovial fluid (SF) undergoes a significant reduction of its viscoelastic properties. Thus, changes of the kinematic and/or dynamic viscosity have been subjected to extensive investigations to disclose the agent(s) primarily responsible for the observed decrease of the SF viscoelasticity. In order to simplify the system studied, that is, to exclude effects of further molecules, investigators concentrated mainly on high-molar-mass

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[*] Corresponding author. Fax: +421 2 5477 5928; e-mail: ladislav.soltes@savba.sk

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hyaluronan (HA) since the degradation of this biopolymer is accompanied by a significant loss of viscoelastic properties of its solutions.[3]

The applied oxidative systems usually involved redox reactions of transition metal ions, for example, iron, copper, and titanium, although application of the latter does not have any physiological relevance. A serious drawback in employing iron(II) salts is, however, the spontaneous oxidation of Fe^{2+} cations to the Fe^{3+} under aerobic conditions. For example, iron(II) chloride is very unstable when dissolved in aqueous solutions. It is oxidized by the action of atmospheric oxygen, and the solution gradually darkens, yielding a brownish-colored liquid. Therefore, common generators of free hydroxyl radicals ($^{\bullet}OH$) based on the use of Fe^{2+} salts usually avoid the Fe^{3+} precipitation [e.g., in the form of FeO(OH)] by complexing Fe^{2+} ions with ethylenediamine tetraacetate (EDTA) and, simultaneously, recycle the oxidized Fe^{3+} ions by adding ascorbate (vitamin C). A typical example of such an $^{\bullet}OH$ generating system is Udenfriend's reagent consisting of 142 μM ascorbic acid, 80 μM EDTA, and 15 μM $FeSO_4$ in 0.1 M phosphate buffer, pH 5.5.[4] As shown by Deguine et al.,[5] a modified Udenfriend's reagent containing 48 mM ascorbic acid, 28 mM EDTA, and 5 mM $FeCl_2$ even at high dilutions (up to 12,000-fold) efficiently degraded high-molar-mass sodium hyaluronate.

Copper is another transition metal of high pathophysiological relevance, which exists in two distinct redox states, with Cu^{2+} being much more stable than Cu^{+}. Recently, we have demonstrated that even traces of $CuCl_2$ in the presence of hydrogen peroxide are effective generators of $^{\bullet}OH$ radicals:[6] In the systems comprising $CuCl_2$ and H_2O_2, at the initial stage Cu^{2+} ions are reduced by hydrogen peroxide, and subsequently the produced Cu^{+} ions react with the excess H_2O_2 yielding $^{\bullet}OH$ radicals.

The aim of this communication is to present further results on the efficiency of $^{\bullet}OH$ radical generating systems by employing only traces of $CuCl_2$ as the source of transition metal ions. Further components investigated as $^{\bullet}OH$ radical generators were ascorbic acid, hydrogen peroxide, as well as a combination of these compounds. As a marker of the degradative action of the generated $^{\bullet}OH$ radicals, the changes in the dynamic viscosity of a high-molar-mass hyaluronan solution were monitored using a rotational viscometer. In order to obtain more information on the reaction pathways, the $^{\bullet}OH$ radical generating system(s) were also investigated by electron paramagnetic resonance (EPR) spectroscopy. The chemical changes that occurred in the hyaluronan macromolecule were assessed by applying enzymatic degradation of the biopolymer, followed by matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometric analysis of the HA oligosaccharides that were formed.

2. Experimental

2.1. Biopolymers

The two samples of intact high-molar-mass hyaluronan used throughout the study were provided by Dr. K. Thacker from Lifecore Biomedical Inc., Chaska, MN, USA (sample coded LIFECORE P9710-2; $M_w = 1215$ kDa, $M_w/M_n = 1.79$) and by the company CPN Ltd, Ústí nad Orlicí, Czech Republic (sample coded CPN; $M_w = 659.4$ kDa; $M_w/M_n = 1.88$).[7] In the LIFECORE P9710-2 sample, the presence of 13 ppm of iron and 4 ppm of copper ions has been claimed ['Certificate of Analysis' (Lifecore Biomedical Inc.]. Bovine testicular hyaluronate lyase (product No. 53718 with an activity of about 0.25 U/mg) purchased as lyophylizate from Fluka Chemie AG (Buchs, Switzerland) was used without further purification.

2.2. Chemicals

Analytical purity grade NaCl and $CuCl_2{\cdot}2H_2O$ were from Slavus Ltd, Bratislava, Slovakia. Methanol and ascorbic acid were from E. Merck KGaA, Darmstadt, Germany. Ethanol (~96%, v/v) and an aqueous solution of H_2O_2 (~30%) were purchased from Chemapol, Prague, Czech Republic. Spin-trapping agents, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; Sigma Chemical Co., St. Louis, MO, USA) and 5-(diisopropoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DIPPMPO; Radical Vision, Marseille, France), were stored under argon at −18 °C. DMPO was distilled before application. The MALDI matrix (2,5-dihydroxybenzoic acid, DHB), obtained from Fluka, was used as supplied. The water used for all solutions was of redistilled deionized quality grade.

2.3. Preparation of the working solutions

The stock hydrogen peroxide solution (8.82 M) was prepared by dissolving NaCl in commercial H_2O_2 to a salt concentration of 0.15 M. The stock $CuCl_2$ solution (16.0 μM) and that of ascorbic acid (16.0 mM) were prepared in 0.15 M NaCl. These solutions, freshly prepared each day, were appropriately diluted with 0.15 M NaCl. The actual concentrations of aqueous H_2O_2 solutions were determined by a spectrophotometric method.[8]

2.4. Degradation studies by rotational viscometry

For the degradation studies, 20.0 mg of the high-molar-mass HA sample (LIFECORE P9710-2) was dissolved in 0.15 M aq NaCl overnight in the dark at room temperature in two steps: First, 4.0 mL solvent was added in the morning, the next 4.0 mL portion of the solvent was added after 6 h. [A similar procedure was applied to dissolve the HA samples (LIFECORE P9710-2 and, CPN) at the investigation of the rheological properties of the solution.]

Four different reaction systems were employed in the studies of the degradation of the sample LIFECORE P9710-2: (a) containing ascorbic acid with the initial ascorbate concentrations of 1, 10, or 100 μM; (b) containing 882 mM hydrogen peroxide; (c) containing 100 μM ascorbate plus 0.1 μM $CuCl_2$ added 9 min before application of ascorbic acid; and (d) containing 0.1 μM $CuCl_2$ and 100 μM ascorbic acid, followed by addition of hydrogen peroxide to yield initial H_2O_2 concentrations of 55 μM, 0.55 mM, or 55 mM. The resulting solution (8.0 mL) was immediately transferred into the Teflon® cup reservoir of the rotational viscometer. The record of the viscometer output parameters started 2 min after the onset of the experiment. The changes of dynamic viscosity (η) and torque were monitored 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 cup–spindle pair built of Teflon® at our laboratory. At the spindle rotational speed of 180 rpm, the shear rate was 237.6 s^{-1}. The LIFECORE P9710-2 sample degradation was monitored in 3-min intervals for up to 5 h or until the nominal η value 5.8 mPa s was reached. Within the monitored interval of the η values, the torque ranged between 72% and 36%.

To assay the reaction products, the reservoir content was poured into 20 mL of EtOH, which led to precipitation of the polymer. On the following day, the polymer precipitate was washed out with 20 mL of EtOH, centrifuged, and dried in a desiccator.

2.5. EPR spin-trapping technique

The solutions for EPR spin-trapping experiments containing various concentrations of Cu^{2+} ions, constant initial concentrations of spin-trapping agents (0.025 mM), and ascorbic acid (100 μM) were prepared using 0.15 M NaCl under air immediately before measurements, and placed into a 50-μL boron capillary. Its reproducible position in the EPR cavity was maintained by a defined tight fitting with silicon rings in a broad EPR tube. The EPR measurements at the X-band were performed with a Bruker EMX EPR spectrometer equipped with a standard TE_{102} (ER 4102 ST) rectangular cavity. The recording of EPR spectra started 60 s after addition of ascorbic acid into the reaction mixture. The EPR spectra were recorded at 22 °C. The EPR spectrometer settings were as follows: microwave frequency, 9.44 GHz; microwave power, 10.034 mW; center field, 335.2 mT; sweep width, 8–20 mT; gain, 2×10^6; modulation amplitude, 0.1 mT; modulation frequency, 100 kHz; scan, 21 s; time constant, 10.24 ms, number of scans, 5–10. The experimental EPR spectra acquisition and simulation were carried out using WIN EPR and SimFonia standard programs (Bruker).

2.6. MALDI–TOF mass spectrometry

The HA samples investigated were dissolved in distilled water to a final concentration of 5 mg/mL and subsequently digested by hyaluronate lyase (1 mg/mL final concentration) at 37 °C for 24 h. Afterwards, all digests were diluted 1:1 (v/v) with the matrix (0.5 M DHB in MeOH) and spotted onto a mate steel target for MALDI–TOF analysis. DHB is the matrix of choice for the analysis of comparably small molecules because this matrix provides only relatively weak signals.[9] Subsequently, the solvent was quickly evaporated by a heat gun to achieve homogeneous crystallization between the matrix and the analyte.

All MALDI–TOF MS measurements were carried out using an Autoflex MALDI–TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany). This instrument uses a pulsed N_2 laser operating with an excitation wavelength of 337 nm. To improve the resolution, all measurements were carried out in the reflector mode under delayed extraction conditions. More details of the measurements have been provided in a recent publication.[10]

3. Results

3.1. Rotational viscometry

Figure 1, panel A, curves a and b, demonstrates the dynamic viscosity measurements of the tested high-molar-mass HA samples CPN and LIFECORE P9710-2. As can be seen, at the beginning of the monitoring the dynamic viscosity values corresponded to the molar mass of the sample. However, it is plausible that during the spindle rotation the macromolecular chains of HA tend to attain a distinct orientation—a process, which results in a continuous increase of η—that is, rheopexy. As has been demonstrated in the previous studies, the relative changes of η at the sample stress are more evident when an HA sample with higher molar mass is used.[7,11]

In the case of the LIFECORE P9710-2 sample, taking into account the biopolymer concentration (2.5 mg/mL) used and the transition metal content declared by the manufacturer, it is reasonable to consider the iron ion concentration (0.58 μM) comparable to those applied by Deguine et al.[5] at the highest dilutions of the Udenfriend's reagent, namely 0.63 μM at 8000-fold dilution or 0.42 μM at 12,000-fold dilution. Addition of ascorbic acid at both 1 and 10 μM concentrations to the HA sample LIFECORE P9710-2 resulted in an increase of the η value, and as can be seen from Figure 1, panel A, curves c and d, the magnitude of dynamic viscosity gradually increased, most probably due to the effect of rheopexy.

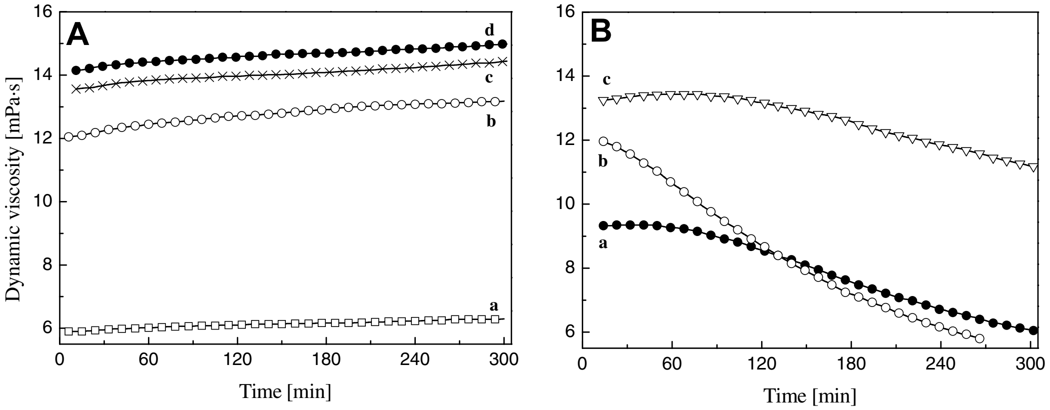

Figure 1. (A) The dynamic viscosity versus time profiles of the HA samples CPN (a); LIFECORE P9710-2 (b); LIFECORE P9710-2 incubated with 1 μM (c) or 10 μM ascorbic acid (d). (B) HA degradation of sample LIFECORE P9710-2 in the system 100 μM ascorbic acid plus 0.1 μM Cu^{2+} (a); 55 mM H_2O_2 plus 0.1 μM Cu^{2+} (b); HA plus 100 μM ascorbic acid (c).

It is necessary to mention that these results are slightly different from those presented by Deguine et al.,[5] who have reported significant decrease of η value within the 0–60 min interval with 8000-fold and 12,000-fold dilutions of the Udenfriend's reagent, that is, at 6 or 4 μM concentrations of ascorbic acid. On the other hand, differences in the composition and in the experimental conditions should be considered as well, since all solutions in our investigations were prepared in NaCl solution (0.15 M), as the chloride anions are capable to react nearly diffusion-controlled with hydroxyl radicals with the second-order rate constant of $3.0 \times 10^9\ M^{-1}\ s^{-1}$.[12]

As can be seen in Figure 1, panel B, curve c, increased addition of ascorbic acid to the HA sample up to 100 μM concentration led in the monitored time period to a decline of the η value from 13.2 mPa s observed at 14 min to 11.2 mPa s at 302 min. The induction period occurring in the time interval up to approximately 80 min (cf., Fig. 1, panel B, curve c) involves chemical reactions, which account for the generation of a sufficient amount of HA-degrading species—most probably $^{\bullet}OH$ radicals—for example, by a sequence of the following reactions:

$$\text{Metal}^{(n+1)+} + \text{ascorbic acid} \rightarrow \text{Metal}^{n+} + \text{ascorbate anion radical} \quad (1)$$

$$\text{Metal}^{n+} + O_2 \rightarrow \text{Metal}^{(n+1)+} + O_2^{\bullet -} \quad (2)$$

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$\text{Metal}^{n+} + H_2O_2 \rightarrow \text{Metal}^{(n+1)+} + {}^{\bullet}OH + HO^- \quad (4)$$

$$^{\bullet}OH + \text{ascorbic acid} \rightarrow H_2O + \text{ascorbate anion radical} \quad (5)$$

$$2\ \text{ascorbate anion radical} \rightarrow \text{ascorbate} + \text{dehydroascorbate} \quad (6)$$

where $\text{Metal}^{(n+1)+}$ represents Cu^{2+} and/or Fe^{3+} ions.

The addition of just a very small amount of $CuCl_2$ (0.1 μM) to the studied oxidative system resulted in a significant reduction of the sample η value during the whole subsequent time interval (cf., Fig. 1, panel B, curve a). Moreover, in accordance with the law of mass action, the higher metal ion content generated a larger immediate amount of the reactive oxygen species, particularly superoxide anion radicals (Reactions 1 and 2), which finally resulted in an increased $^{\bullet}OH$ radical flux and in a reduced lag time (compare curves a and c in Fig. 1, panel B). However, it should be pointed out that the oxidative system consisting of 0.1 μM $CuCl_2$ plus 100 μM ascorbic acid under aerobic conditions is practically equally efficient as that in which ascorbic acid is replaced by a much higher concentration of H_2O_2, that is, 55 mM (curves a and b in Fig. 1, panel B). Obviously, comparable viscosity values $\eta = 6.1$ or 5.8 mPa s have been attained within 302 min on applying an oxidative system containing a relatively low ascorbate level or within 267 min when employing a 500-times higher hydrogen peroxide level.

As can be seen in Figure 2, curve a, the treatment of the HA sample with a tri-component oxidative system

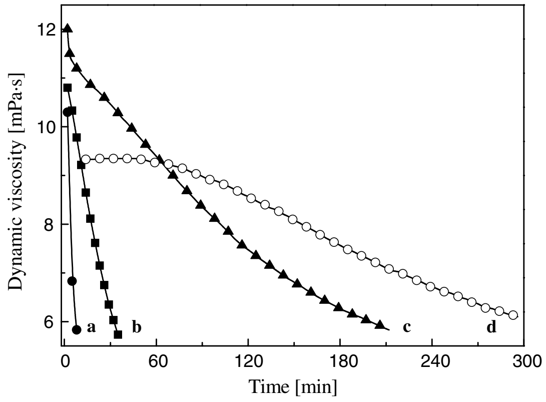

Figure 2. HA degradation of sample LIFECORE P9710-2 in the system 0.1 μM Cu^{2+} plus 100 μM ascorbic acid with addition of 55 mM H_2O_2 (a), 0.55 mM H_2O_2 (b), 55 μM H_2O_2 (c), 0 mM H_2O_2 (d).

comprising 0.1 μM $CuCl_2$ plus 100 μM ascorbic acid and 55 mM H_2O_2 leads to an instant rapid decrease of the dynamic viscosity value, which reaches 5.8 mPa s in 8 min. Even a 100-fold reduction of H_2O_2 concentration did not result in a significant decline of the sample degradation, and the viscosity value 5.8 mPa s was achieved in ca. 34 min (Fig. 2, curve b). The subsequent 10-fold dilution of H_2O_2 (i.e., to 55 μM) led to a marked retardation of HA degradation. However, as can be seen from the comparison of curves c and d in Figure 2, apparently the major effect of the H_2O_2 addition to $CuCl_2$ plus ascorbic acid is a rapid and continuous diminution of the η value without a significant lag phase. The differences observed in the courses of these two curves corroborate, albeit indirectly, the relevance of Reactions 1–4.

3.2. EPR spin-trapping measurements

The EPR experiments employing the model solutions containing a constant initial concentration of ascorbic acid (100 μM) and various concentrations of Cu^{2+} ions (0.1–5 μM) were carried out with the aim to monitor generation of the paramagnetic species that are potentially able to initiate HA degradation. Formation of the relatively stable ascorbate anion radical ($ASC^{\bullet-}$), characterized by spin Hamiltonian parameters ($a_H = 0.182$ mT; $g = 2.0053$; inset in Fig. 4), was observed in all systems, although the time profiles of the $ASC^{\bullet-}$ signal relative intensity were significantly dependent on the initial concentration of Cu^{2+} ions, as can be seen in Figure 3a–e.

On the other hand, detection of the hydroxyl radicals using cw-(continuous wave)-EPR spectroscopy requires addition of spin-trapping agents into the experimental systems (Fig. 3b–d: DMPO; Fig. 3e: DIPPMPO). Application of DMPO results in the formation of a four-line EPR signal with parameters corresponding to a $^{\bullet}$DMPO–OH adduct ($a_N = a_H^{\beta} = 1.490$ mT; $g = 2.0058$; inset in Fig. 4), which is in a good agreement with the reference data.[13] The time-courses of EPR spectra monitored in the presence of DMPO and ascorbic acid (100 μM) using various Cu^{2+} concentrations (0.1–5 μM in Fig. 3b–d) indicate the subsequent generation of $^{\bullet}$DMPO–OH after disappearing of the $ASC^{\bullet-}$ EPR signal. The time period required for elimination of the ascorbate anion radical is in inverse proportion to the initial Cu^{2+} concentration. Dismutation of ascorbate anion radicals to yield ascorbate and dehydroascorbate (Reaction 6) is suggested to be the main reason for the disappearance of ascorbate anion radicals. It proceeds with a rate constant of 2.4×10^5 M^{-1} s^{-1}.[14] Higher initial amounts of Cu^{2+} accelerate both the formation of ascorbate anion radicals and its dismutation. Thus, only after a marked consumption of ascorbic acid, conditions are created that enable the detection of hydroxyl radicals by spin-trapping techniques. Thus, it can be suggested that competitive reactions between ascorbic acid and DMPO with hydroxyl radicals occur in the investigated system. As the concentration of hydroxyl radicals is higher when increased Cu^{2+} concentrations are used, the invariable concentration of ascorbic acid (100 μM) is depleted in a shorter time, and consequently, $^{\bullet}$DMPO–OH adducts are produced in higher concentrations as illustrated in Figure 3d. Figure 4 depicts the integral intensities of $ASC^{\bullet-}$ and $^{\bullet}$DMPO–OH signals extracted from the set of experimental EPR spectra shown in Figure 3d using double-integration procedure.

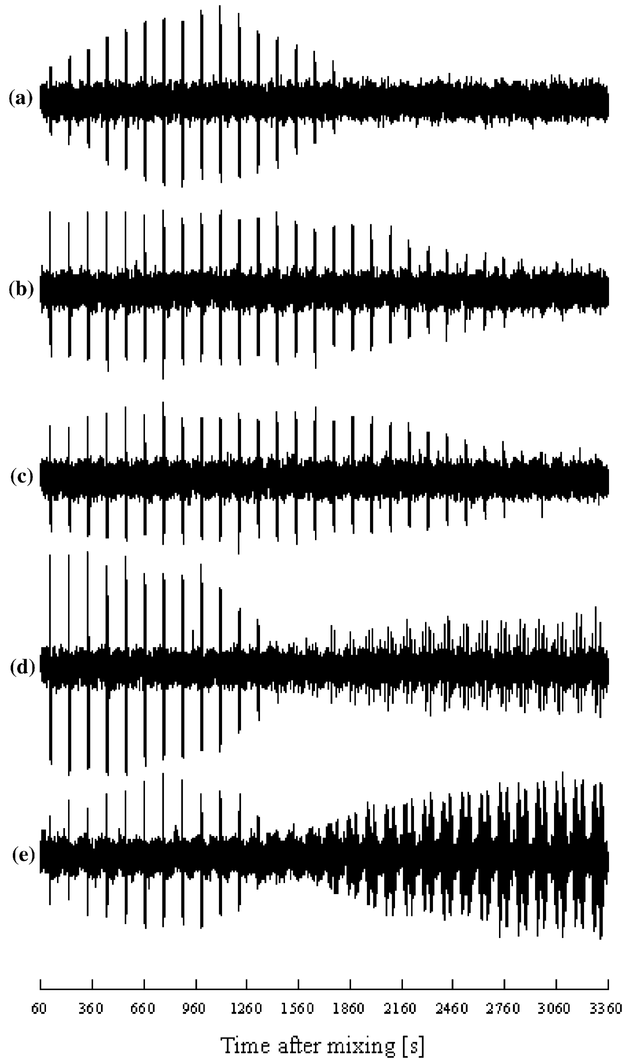

Figure 3. Time course of 30 EPR spectra (magnetic field width of 8 mT in a–d and 16 mT in e) monitored at 22 °C in 100 μM ascorbic acid aqueous solutions containing various initial concentrations of $CuCl_2$: (a) 5 μM; (b) 0.1 μM; (c) 1 μM; (d) 5 μM; (e) 5 μM. EPR spectra were measured (a) without spin-trapping agent; (b–d) c_0 (DMPO) = 0.025 M; (e) c_0 (DIPPMPO) = 0.025 M.

The EPR spectra corresponding to the addition of $O_2^{\bullet-}$ or $^{\bullet}$OOH to DMPO were not observed. This is in agreement with literature data reporting very low stabil-

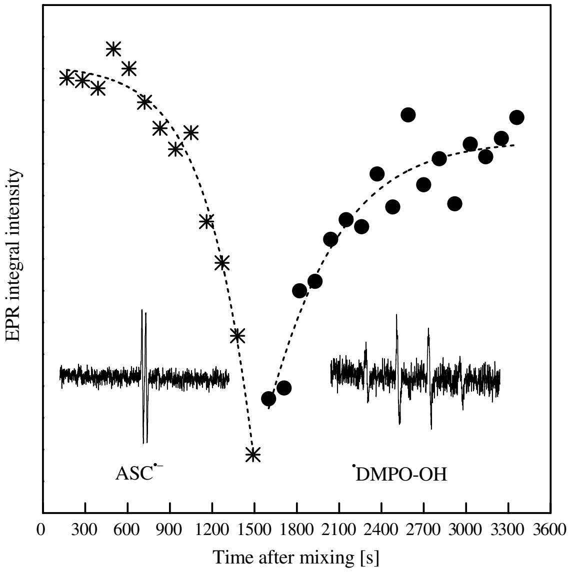

Figure 4. Time dependence of the EPR integral intensity of ascorbate anion radical (ASC$^{\bullet-}$) and of the $^{\bullet}$DMPO–OH adduct after mixing $CuCl_2$ solution with ascorbic acid in the presence of DMPO spin trap. The data were evaluated from the time course of EPR spectra depicted in Figure 3d. Insets show the experimental EPR spectra of ascorbate anion radical ($a_H = 0.182$ mT; $g = 2.0053$) and $^{\bullet}$DMPO–OH adduct ($a_N = a_H^{\beta} = 1.490$ mT; $g = 2.0058$) (magnetic field width 8 mT).

ity of $^{\bullet}$DMPO–O_2^{-}/OOH spin adducts in aqueous media and their conversion to $^{\bullet}$DMPO–OH.[15,16] For this reason, we carried out EPR experiments using DIPPMPO spin-trapping agent, which is suitable for the simultaneous detection of $^{\bullet}$OOH and $^{\bullet}$OH radicals.[17–20] The time-course of EPR spectra observed in the presence of DIPPMPO (0.025 M), $CuCl_2$ (5 μM), and ascorbic acid (100 μM) is shown in Figure 3e. Similarly, in this case, after mixing of solutions, only the EPR signal of ASC$^{\bullet-}$ was observed, whose relative intensity decreased to zero, and the time period corresponding to the depletion of the ascorbate anion radical was similar to that found in the presence of DMPO under the conditions involving identical $CuCl_2$ and ascorbic acid concentrations (Fig. 3d). Subsequently, the gradual generation of a symmetrical eight-line EPR signal was monitored, which was characterized with spin Hamiltonian parameters of $a_N = 1.407$ mT, $a_H^{\beta} = 1.320$ mT, $a_P = 4.665$ mT; $g = 2.0058$ (Fig. 5). These values are in accordance with the data previously published for $^{\bullet}$DIPPMPO–OH adduct.[17–20] The presence of the $^{\bullet}$DIPPMPO–O_2^{-}/OOH adduct has not been detected, most probably due to the slower reactions of $O_2^{\bullet-}$/$^{\bullet}$OOH radicals with the spin-trapping agent.

3.3. MALDI–TOF mass spectrometric investigation

In order to establish which final degradation products can be actually detected in HA samples treated with the above-mentioned agents, the individual HA samples were also investigated by MALDI–TOF MS. Since polysaccharides (especially the acidic ones) cannot be detected by this soft-ionization mass spectrometric method,[10] polymeric HA was preliminarily digested by testicular hyaluronate lyase prior to analysis. This enzyme is known to produce a mixture of di-, tetra-, hexa-, and octasaccharides.[21]

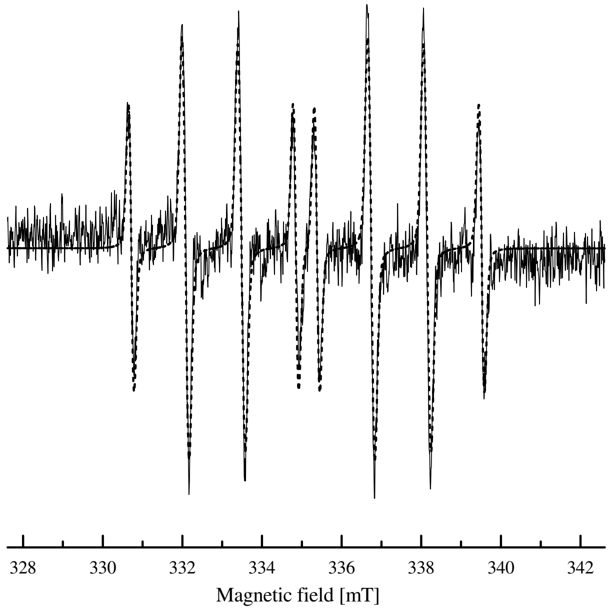

Figure 5. EPR spectrum of $^{\bullet}$DIPPMPO–OH adduct (solid line) monitored in solution of $CuCl_2$ (5 μM) with ascorbic acid (100 μM) in the presence of DIPPMPO spin trap (0.025 M) 1 h after mixing, along with the simulation (dashed line) calculated using the following spin Hamiltonian parameters: $a_N = 1.407$ mT, $a_H^{\beta} = 1.320$ mT, $a_P = 4.665$ mT; $g = 2.0058$ (magnetic field width 15 mT).

The MALDI–TOF mass spectra of the digestion products are shown in Figure 6. Since HA is an acidic (negatively charged) carbohydrate, besides the positive-ion spectra (right) the negative-ion spectra (left) were also recorded: It is known that oligomers of HA are more sensitively detected as negative ions.[22] Although there are many more peaks detected at higher mass values, only the mass region of the tetrasaccharide will be discussed. The molar mass of the (neutral) tetrasaccharide of HA (HA-4) is 776 Da. Accordingly, the signal at m/z 775.2 corresponds to HA-4 subsequent to the loss of one proton. The peak at m/z 797.2 corresponds to HA-4 following the exchange of one H^{+} by a Na^{+} ion.

The peaks at m/z 681, 641, 601, and 561 as well as at m/z 857 and 817 are stemming from ‘photoproducts’ of the applied matrix (DHB, with a molar mass of 154 Da) that undergoes oligomerization under conditions of laser irradiation accompanied by losses of NaOH (mass difference 40 Da).[23] Although all experiments were performed under identical conditions, it is obvious that the contribution of the matrix signals varied. This is particularly evident when the intensities of the matrix peaks

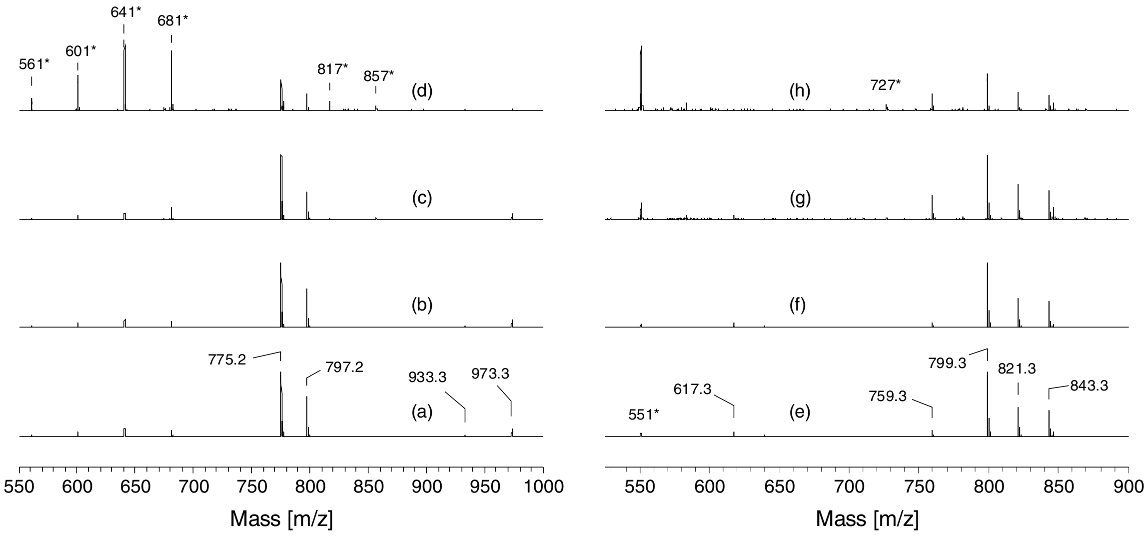

Figure 6. Negative-ion (left) and positive-ion (right) MALDI–TOF mass spectra of high-molar-mass HA treated with different agents. Panels (a) and (e) represent the native samples, (b) and (f) the samples treated with 882 mM H_2O_2, (c) and (g) treated with 0.1 μM $CuCl_2$ and 100 μM ascorbate, and (d) and (h) treated with 0.1 μM $CuCl_2$, 0.1 μM H_2O_2 and 100 μM ascorbate. Samples were digested prior to the analysis with hyaluronate lyase because the detection of native acidic polysaccharides by MALDI–TOF MS is impossible. Peaks are labeled according to their *m*/*z* ratios; peaks labeled with an asterisk are stemming from the applied DHB matrix.

(e.g., at *m*/*z* 681) are compared with the intensities of the peaks of the HA tetrasaccharide (*m*/*z* 775.2 and 797.2). In the positive-ion mode, the contribution of the matrix peaks increased in a similar manner as observed in the negative-ion spectrum.

This might indicate a complete breakdown of the HA molecule: From the previously performed NMR experiments, it is known that the action of $^{\bullet}OH$ radicals leads to the loss of the HA resonances under the formation of formate as one of the final products.[24] Unfortunately, products as formate are not detectable by MALDI–TOF MS due to their low molar masses and the resulting interference with the matrix peaks.

Another explanation might be an inhibitory effect of ascorbate on the applied hyaluronate lyase leading to a lower yield of the fragmentation products. Such an inhibitory effect of ascorbate has already been described.[25] In any case, the intensity of the matrix signals is much lower in traces 6a–c. Similar tendencies are evident from the corresponding positive-ion spectra.

Moreover, in the negative-ion spectra the generation of matrix/analyte clusters is also apparent. The mass difference between *m*/*z* 973 and *m*/*z* 775 is 198. This corresponds exactly to the molar mass of DHB after the exchange of two H^+ with two Na^+ ions ($154 \rightarrow 176 \rightarrow 198$). This phenomenon has been already observed when phospholipids are analyzed; however, it has not been described so far for glycosaminoglycans. Summarizing, the MALDI–TOF MS results do not indicate the generation of characteristic products when HA is exposed to radical-generating systems. This is in good agreement with previously obtained data.[26]

4. Discussion

The prime difference between the chemical reactions taking place at using the Udenfriend's reagent and the Fenton-type reaction is the participation of ascorbic acid in the autoxidation of Fe^{2+} ions, whereas Fenton's reaction involves decomposition of hydrogen peroxide by Fe^{2+} cations. However, a common feature of the both mentioned reagents is most probably the generation of $^{\bullet}OH$ radicals, although excited iron complexes such as ferryl or perferryl compounds are also discussed to be the reactive species in these reactions.[27–29]

Although the value of the standard reduction potential E'° for the redox couple Fe^{3+}/Fe^{2+} at pH 7 equals +0.48 V,[30] in the case of complexation of iron ions with EDTA at pH 7, the E'° value is equal to +0.12 V,[31] that is very close to the standard reduction potential of the redox couple Cu^{2+}/Cu^{+} which is +0.16 V.[30] On the basis of the mentioned E'° values, it seems reasonable to assert that the pseudo-Udenfriend's reagent lacking EDTA, in which iron ions are replaced by Cu^{2+} should be similarly efficient as that suggested by Udenfriend more than half a century ago for the purpose of oxidation/hydroxylation of the aromatic compounds.[4]

As has been mentioned in Section 3, the findings of Deguine et al.[5] are only partially in agreement with our observations. The differences might result from the fact that at measuring the degradation kinetics of LIFECORE P9710-2 sample, a nonmetal sample reservoir and spindle of the rotational viscometer has been used, as well as from the different conditions of solutions preparation (0.15 M NaCl vs phosphate-buffered saline used by Deguine et al.[5] As pointed out by Miyazaki et al.,[32] the contact of HA solution with the metallic parts of the rotational viscometer might lead to HA degradation. Thus, the results presented by some researchers[5,33,34] should be considered with more caution, taking into consideration the variability in the oxidative systems composition, along with the experimental techniques and equipments applied.

Autoxidation of the transition metal ions, namely those of iron and copper, separately or in the same mixture, in the presence of ascorbic acid was investigated in detail in the pioneering work of Matsumura and Pigman.[35] However, it should be mentioned that at that time only insufficiently characterized hyaluronan samples (especially regarding the residual metal content) were available. More recent studies unambiguously confirmed that Cu^{2+} ions are sufficiently reactive with both H_2O_2 and ascorbic acid under aerobic conditions.[6,36,37] The LIFECORE P9710-2 sample used at the present work when applied at the physiological concentration of 2.5 mg/mL introduces about 0.16 μM copper ions into the system. This concentration is practically identical to that established by Naughton et al.[38] in the SF ultrafiltrate of patients suffering from rheumatoid arthritis (RA). As described by these authors, the copper ion content in the SF ultrafiltrate of the RA patients equals 0.125 ± 0.095 μM. Therefore, our experiments employing the addition of 0.1 μM $CuCl_2$ meet pathological conditions rather exactly.

The physiological level of ascorbic acid in SF ranges between 40 and 140 μM.[39] Hence, the 100 μM concentration of ascorbic acid along with a sub-micromolar level of the transition metal ions (0.1 μM $CuCl_2$) used in our experiments can adequately simulate the physiological conditions, under which the high-molar-mass HA is degraded in SF. It should also be pointed out that the synovial fluid is devoid of enzymes, which metabolize/catabolize HA. Thus, a fraction of oxygen supplied into the joint space is consumed at oxidation of the reduced metal ions (Reaction 2). The generated $O_2^{\bullet -}$ is converted by dismutation to hydrogen peroxide (Reaction 3), which is subsequently decomposed, whereby the formed $^{\bullet}OH$ radicals (Reaction 4) degrade the high-molar-mass hyaluronan. Changes in the joint homeostasis along with the diffusion of lower sized HA fragments outside the joint environment can serve as markers (indicators) of physiological/healthy functioning of the joint.

Under rheumatoid arthritis conditions, both the SF and plasma concentrations of total ascorbate and the ascorbate to dehydroascorbate ratio are markedly subnormal.[40] The number of polymorphonuclear leukocytes (PMNLs) infiltrated into the joint space rises considerably during the acute phases of rheumatoid arthritis,[41–44] and therefore the concentration of generated $O_2^{\bullet -}$ is also increased. The higher content of superoxide anion radicals can act in two ways (i) compensating the ascorbate deficit at reducing the oxidized transition metal cations, that is, $\mathrm{Metal}^{(n+1)+} + O_2^{\bullet -} \rightarrow \mathrm{Metal}^{n+} + O_2$ and (ii) by its dismutation: a higher H_2O_2 flux could be monitored in the immediate vicinity of the PMNLs. Due to degradation of the high-molar-mass HA by the action of H_2O_2, the viscosity of SF is significantly reduced, which alleviates the transport of leukocytes from the synovial membrane towards the cartilage. Although it might appear that the PMNLs would subsequently cause cartilage damage, leukocytes and their products—oxidative species, namely HOCl—might also act to deactivate/damage the proteases, increased levels of which have been reported under RA conditions.[1,10]

Although this hypothesis might seem rather speculative, functioning of the PMNLs as cartilage protectors from the degrading action of proteases cannot be excluded.[1,10] Control of the levels of transition metal and ascorbate, modulation of the activity of PMNLs, and regulation of the conversion of H_2O_2 to HOCl are the factors, which better understanding and management should contribute to the development of novel efficient therapeutic agents for the treatment of inflammatory joint diseases. However, at present, we are still a long way from reaching this aim.

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