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Degradation of high-molar-mass hyaluronan by an oxidative system comprising ascorbate, Cu(II), and hydrogen peroxide: Inhibitory action of antiinflammatory drugs—Naproxen and acetylsalicylic acid

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Abstract

Changes in dynamic viscosity of the solutions of a high-molar-mass hyaluronan (HA) were monitored using a rotational viscometer. The degradative conditions generated in the HA solutions by a system comprising ascorbate plus Cu(II) plus H_2O_2 were studied either in the presence or absence of a drug—naproxen or acetylsalicylic acid. Continual decrease of the dynamic viscosity of HA solution was indicative of the polymer degradation. Addition of the drug retarded/inhibited the HA degradation in a concentration-dependent manner. The characteristics of the fragmented polymers were investigated by FT-IR spectroscopy and by two different liquid chromatographic techniques, namely by size-exclusion chromatography equipped with a multi-angle light scattering photometric detector and by high-performance liquid chromatography connected on-line to a spectrofluorometer.

Keywords: Hyaluronan degradation; Rotational viscometry; FT-IR spectroscopy; Size-exclusion chromatography; HPLC; Non-steroidal antiinflammatory drugs

1. Introduction

Hyaluronan (HA; Fig. 1) is a glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. It is also one of the major components of the extracellular matrix. The term "hyaluronate" refers to the conjugate base of hyaluronic acid, and due to the fact that the HA macromolecule typically exists *in vivo* in its polyanionic form, it is most commonly referred to as hyaluronan. In synovial fluid (SF), high-molar-mass HA accounts for its viscosity; together with a glycoprotein lubricin, it is one of the SF's main lubricating components.

The physiological level of hyaluronan in SF of human beings is 2–3 mg/ml [1]. The mean molar mass of the HA in SF of healthy subjects is of several million Daltons. The degradation of high-molar-mass hyaluronan occurring under inflammation and/or oxidative stress is accompanied with the impairment and even loss of the viscoelastic properties of SF.

Non-steroidal antiinflammatory drugs (NSAIDs) exhibit analgesic, antipyretic, anti-oncogenic, and antiinflammatory actions. These effects are based on the inhibition of prostaglandin synthesis and their impact on the immune reactions. Acetylsalicylic acid (ASA; aspirin), a drug belonging to a group of salicylates, is, for example, often used as an analgesic (against minor pains and aches), antipyretic, and antiinflammatory medication. Aspirin was the first discovered member of the

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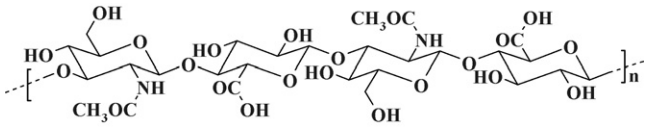
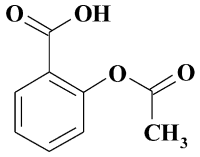


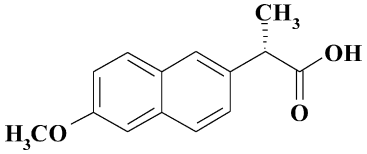
Fig. 1. Hyaluronan—the acid form.

NSAID class of drugs, not all of which are salicylates, though they all have similar effects and a similar action mechanism.



Acetylsalicylic acid (ASA)

Naproxen (NX) is a member of the 2-arylpropionic acid (profen) family of NSAIDs. Naproxen (and/or sodium naproxen) is commonly used for the reduction of mild to moderate pain, fever, inflammation, and stiffness caused by conditions such as osteoarthritis (OA), rheumatoid arthritis (RA), *etc*. Food and Drug Administration approved the use of sodium naproxen as an over-the-counter drug in 1991.



There are, however, several further potential actions of the NSAIDs including their capability to prevent the release of free radicals and degradative enzymes from synovial macrophages and polymorphonuclear leukocytes (PMNLs) [2,3]. These drugs (may) affect the mobility, chemotaxis, and aggregation of PMNLs and macrophages [2,4]. NSAIDs are also supposed to be direct scavengers of free radicals [5–9].

Hyaluronan solutions of sufficiently high concentrations form a (microheterogeneous) network resulting in a non-Newtonian flow behavior. To monitor this phenomenon, rotational viscometry might be one of the most efficient analytical tools. Rotational viscometry renders detailed dependencies of the dynamic viscosity (η) on time, therefore this methodology has often been applied to monitor the degradative action of low-molar-mass reactive oxygen species on the high-molar-mass hyaluronan [10–19].

Fourier-transformed infrared (FT-IR) spectroscopy using an arrangement to measure the IR irradiation reflectance from the surface of a solid sample belongs to the simplest analytical methodologies providing characterization of the material quality. In the case of hyaluronan samples, chemical changes in the structure are reflected in the altered values of the characteristic wavelengths of the functional groups of the component monosaccharides comprising the disaccharide repeating units of HA—D-glucuronate/D-glucuronic acid and *N*-acetyl-D-glucosamine (cf. Fig. 1).

Size-exclusion chromatographic (SEC) device connected on-line to a molar-mass detector such as a multi-angle light scattering (MALS) photometer belongs to the most frequently used equipments for the analysis of the polymer molecular characteristics. While increasing retention time of the analyzed HA samples indicates reduction of the polymer molar mass, altered shape of the graph reflecting the dependence of gyration radius (R_g) on molar mass (M) of the eluted HA macromolecules [$R_g = f(M)$] implies that degradation of high-molar-mass HA involves not only its fragmentation but also certain changes in the primary chemical structure of the polymer.

Application of the high-performance liquid chromatography (HPLC) connected on-line to a specific detector such as e.g. spectrofluorometer (FL) that enables detection of the fluorescent naproxen and acetylsalicylic acid, allows a rapid and reliable monitoring/establishment of presence of these drugs in the fractions eluted from the chromatographic column.

The aim of the present paper is to report the results of the investigation of the inhibitory effect of the two compounds belonging to the class of NSAIDs, which are assumed to exert antioxidant effect against the HA degradation in a system, resembling the conditions occurring in an inflamed joint. Comparative study of the molar-mass values of the native HA sample and the polymer fragments was performed by means of SEC-MALS. FT-IR spectroscopy was used to detect changes in the chemical structure of the polymer fragments. At the same time, application of HPLC equipped with a spectrofluorometric detector enabled rapid and reliable information whether NX or ASA were embedded into hyaluronan macromolecule.

2. Material and methods

2.1. Chemicals

High-molar-mass HA sample LIFECORE P9710-2A [20] (cf. Table 1), used throughout the study was a generous gift of Dr. K. Thacker from LIFECORE Biomedical Inc., Chaska, MN, USA. The presence of 13 ppm of iron and 4 ppm of copper ions in this sample has been claimed ["Certificate of Analysis" (Lifecore Biomedical Inc.)].

Analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ used were from Slavus Ltd., Bratislava, Slovakia. Ascorbic acid was from Merck KGaA, Darmstadt, Germany. Aqueous solution of H_2O_2

Table 1
SEC-MALS results obtained with the native HA sample and the fragmented polymers

Sample	M_n (kDa)	M_w (kDa)	M_z (kDa)	M_w/M_n	M_z/M_w	R_g (nm)
I (LIFECORE P9710-2A)	495.1	808.7	1095	1.63	1.35	110.0
II	440.6	738.1	994.0	1.68	1.35	87.8
III	111.8	180.4	263.1	1.61	1.46	37.6

The averages of the molar masses—numeric: M_n, weight: M_w, and z: M_z, the dispersity indices (M_w/M_n and M_z/M_w), and the z-average of the R_g values of the macromolecules.

(≈30%) was purchased from Chemapol, Prague, Czech Republic. Sodium naproxen [(+)-*S*-enantiomer; the drug purity ≥98%] was a generous gift of Mr. M. Chityala from Matrix Labs., Ltd., Secunderabad, India. Acetylsalicylic acid was of pharmacopoeial quality. Water used for preparation of stock and working solutions in HA degradation studies was of bi-distilled deionized quality.

2.2. Preparation of stock and 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 factual concentration of the H_2O_2 solution was determined by a spectrophotometric method [21]. The stock solutions of sodium naproxen (100 mM) and acetylsalicylic acid (13.9 mM) were prepared in 0.15 M aqueous solution of NaCl. Aspirin dissolution was accelerated by stirring at moderate heating (<60 °C).

2.3. Study of uninhibited hyaluronan degradation

For the degradation studies, 20.0 mg of the HA sample was dissolved in 0.15 M aqueous NaCl overnight in the dark at room temperature in two steps: first, 4.0 ml solvent was added in the morning, next 3.85 ml portion of the solvent was added after 6 h. The following morning, 50 μl of 16.0 μM $CuCl_2$ solution was added to the gel-like HA solution 9 min before application of 50 μl of 16 mM ascorbic acid solution followed immediately by 50 μl of 8 mM H_2O_2 solution. Thus, the final concentrations of the components were: 0.1 μM $CuCl_2$, 100 μM ascorbic acid, and 50 μM H_2O_2.

2.4. Study of inhibited hyaluronan degradation

Four different arrangements were employed at the inhibitory studies of degradation of the high-molar-mass HA sample LIFECORE P9710-2A in the system comprising 0.1 μM $CuCl_2$ plus 100 μM ascorbic acid plus 50 μM H_2O_2: (a) containing NX, added to the system 1 h after the reaction onset to the final concentrations of 0.0, 0.143, 1.43, and 10 mM; (b) containing NX, added to the system before the reaction onset to the final concentrations as in system a; (c) containing ASA, added to the system 1 h after the reaction onset to the final concentrations of 0.0, 0.0143, and 1.43 mM; (d) containing ASA, added to the system before the reaction onset to the same final concentrations as in system c.

2.5. Rotational viscometry

The resulting reaction mixture (8.0 ml) was immediately transferred into the Teflon® cup reservoir of the rotational viscometer. Recording of the viscometer output parameters started 2 min after the experiment onset. The changes of η and torque values were monitored at 25 ± 0.1 °C by using digital rotational viscometer Brookfield LVDV-II+ PRO (Brookfield Engineering Labs., Inc., Middleboro, MA, USA) equipped with a cup-spindle pair built of Teflon® in our laboratory [15]. At the spindle rotational speed of 180 rpm, the shear rate was 237.6 s^{-1}. Degradation of LIFECORE P9710-2A sample was monitored in 3 min intervals for up to 3 h. Within the monitored interval of the η values, the torque varied within the range between 68 and 28%.

To assay the reaction products, the reservoir content was poured into 20 ml ethanol, which led to precipitation of the polymer. On the following day, the polymer precipitate was rinsed with 20 ml ethanol, centrifuged, and dried in a desiccator. The yields of the recovered polymers ranged between 15.7 and 17.7 mg, i.e. represented 78.5–88.5% of the initial HA amount. The pellets of the dried polymer were very firm.

2.6. FT-IR spectroscopy

The FT-IR spectra were acquired by using a NICOLET Magna 750 spectrometer equipped with DGTS detector and applying OMNIC 7.1 software (Thermo Electron Corp., Madison, WI, USA). ATR (Attenuated Total Reflectance) accessory with ZnSe crystal was used for running the spectral scans. Number of scans was 128 at a resolution of 4 cm^{-1}.

2.7. SEC-MALS characterization of the recovered polymers

The recovered dry polymer was dissolved overnight in aqueous NaCl (0.15 M). On the following morning, the sample solution was diluted to the required concentration by adding 0.15 M NaCl. Prior to analysis, each sample was clarified by filtration through a 0.45 μm Nylon filter (Millipore Corp., Bedford, MA, USA). The molar mass distribution (MMD) analysis of the samples was performed by using a modular multi-detector SEC system. The SEC system consisted of an Alliance 2690 separation module from Waters (Milford, MA, USA) equipped with two on-line detectors, namely with a MALS Dawn DSP-F photometer from Wyatt Technology (Santa Barbara, CA, USA) and a DRI 410 differential refractometer from Waters. The latter detector was used to determine the polymer concentration in the eluate. The setup of this multi-detector SEC system was serial in the following order: Alliance-MALS-DRI.

The experimental methodology for a reliable use of the MALS photometer has been already described in detail [22–24]. Briefly: the MALS photometer uses 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 $R(\theta) = 1.406 \times 10^{-5}$ 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, $R_g = 2.9$ nm), a globular protein that is assumed to act as an isotropic scatterer. The specific refractive index increment of the polymeric samples, d*n*/d*c*, with respect to the mobile phase at 35 °C and $\lambda = 632.8$ nm was set = 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, flow-rate = 0.4 ml/min; sample injection volume = 150 μl; the polymer concentration in the injected samples ranged from 0.1 to 2 mg/ml depending on the polymer molar mass.

The data acquisition and analysis softwares were EMPOWER PRO from Waters and ASTRA 4.73 from Wyatt Technology.

2.8. HPLC-UV/FL analyses of the samples

The pure sodium naproxen, the reference hyaluronans, as well as the dry recovered polymer fragments were dissolved overnight in the HPLC aqueous mobile phase, which contained 125 mM Na_2SO_4 and 2 mM NaH_2PO_4, and which pH was adjusted to 6.0 with 0.1 N NaOH. On the following morning, prior to the analysis, each sample was clarified by filtration through a 0.45 μm Nylon filter (Millipore Corp.). The analyses of the samples were performed by using a modular Jasco LC-1500 HPLC separation system (JASCO Corp., Tokyo, Japan; the pump—Model PU-1580), equipped with two detectors, namely with a UV–vis spectrophotometer (Model UV-1570) set for 214 nm, and/or with a spectrofluorometer (Model FP-1520) adjusted for 270/340 nm (excitation/emission wavelengths). The latter device was applied to identify sodium naproxen in the flow-through eluate.

The experimental conditions of the HPLC system were as follows: two stainless steel columns (7.5 mm × 30 cm and 7.8 mm × 30 cm) assembled in series; packings were Waters Protein Pak (types 300 and 125); separation temperature = ambient; the mobile phase flow-rate = 0.9 ml/min; sample injection volume = 10 μl; the polymer concentration in the injected samples was 10 mg/ml; sodium naproxen amount in the injected calibration standards ranged from 10 up to 1000 ng.

The data acquisition and analysis software was JASCO-BORWIN rel. 1.5.

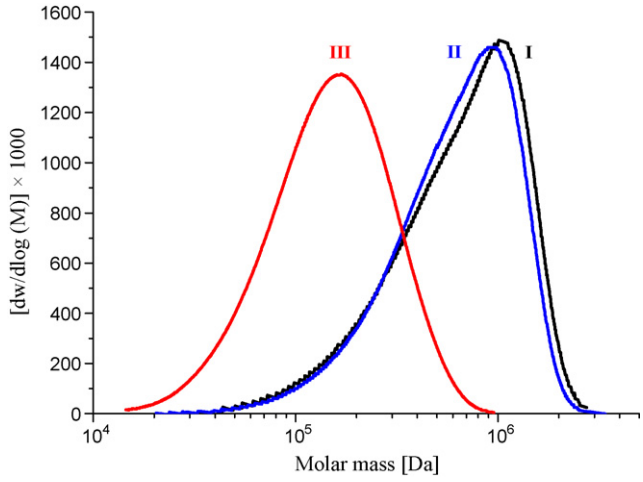


Fig. 3. Comparison of the differential MMDs of the native LIFECORE P9710-2A sample (I); the sample degraded by the system containing 0.1 μM $CuCl_2$ plus 100 μM ascorbic acid plus 100 μM H_2O_2 in absence of sodium naproxen (III), and the sample degraded by the system containing 0.1 μM $CuCl_2$ plus 100 μM ascorbic acid plus 50 μM H_2O_2 in the presence of 10 mM sodium naproxen (II); sodium naproxen was added to the system prior to the start of the HA sample degradation (cf. also curve 3, panel B in Fig. 2).

3. Results

3.1. Uninhibited/inhibited HA degradation

Fig. 2 (panels A and B, curves coded 0) illustrates the dynamic viscosity *versus* time dependence of the native LIFECORE P9710-2A sample monitored during a 3 h degradative action of the system containing 0.1 μM $CuCl_2$ plus 100 μM ascorbic acid plus 50 μM H_2O_2. At the degradation onset (2 min), the nominal η value of the solution was equal to 12.0 mPa s. However, as can be seen, decrease of the solution dynamic viscosity was steady and monotonous during the whole monitored period of 3 h. As demonstrated by the SEC-MALS analysis of the recovered polymer (coded III), 3 h action of a similar degradative system resulted in a reduction of the molar mass (compare Fig. 3, curves I and III); the M_w value of the native LIFECORE P9710-2A sample being initially equal to 808.7 kDa dropped to 180.4 kDa (cf. Table 1).

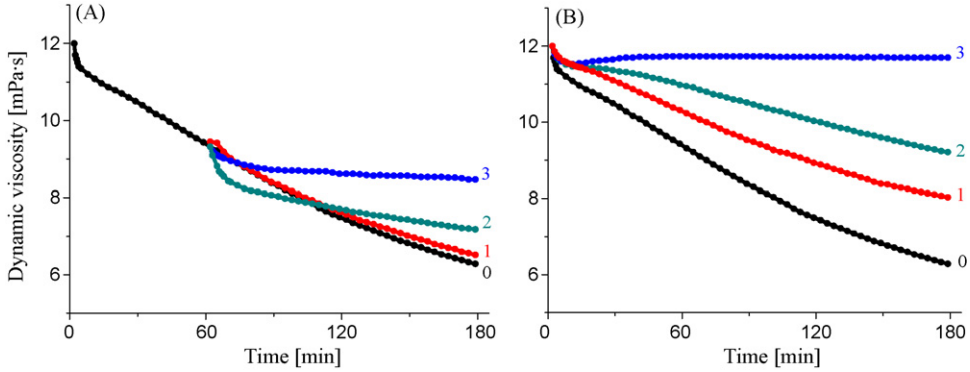


Fig. 2. Effect of sodium naproxen on HA degradation induced by the system containing 0.1 μM $CuCl_2$ plus 100 μM ascorbic acid plus 50 μM H_2O_2. Concentrations of NX added into the system at 1 h (A) or before the start of degradation (B), in mM: 0.0 (0); 0.143 (1); 1.43 (2); and 10 (3).

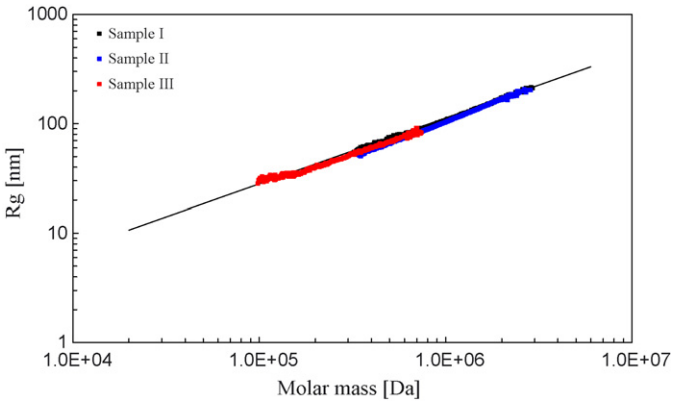


Fig. 4. Comparison of the experimental $R_g = f(M)$ plot of the native HA sample LIFECORE P9710-2A (I) and the two recovered polymers (II and III).

Fig. 4 shows the comparison of the experimentally established $R_g = f(M)$ scaling law – generally known as conformation plot – for the native LIFECORE P9710-2A sample and the two recovered polymers. It is evident that all three conformation plots could be described by a single scaling law. As shown, the scaling law published already for a set of nine hyaluronan samples (cf. black line in Fig. 4) [25] support the statement that the recovered polymer samples II and III behave in solution identically to native HA macromolecules.

Addition of a degradation inhibitor, i.e. naproxen, to the solution, in which degradation of HA macromolecules has been already taking place for 1 h, resulted in slowing down (curves 1 and 2, panel A in Fig. 2) or even in almost complete cessation of a process of decline of dynamic viscosity of the solution (curve 3). Analogically, addition of NX to the solution prior to the start of HA degradation (cf. Fig. 2, panel B) revealed a dose-dependent retardation/inhibition, whereas the highest applied dose of NX completely suppressed the decrease of η value within the whole monitored time interval of 3 h. As also demonstrated in Fig. 3 and by the data presented in Table 1, the molecular parameters of the recovered polymer coded II in fact very closely resembled those of the sample I, i.e. of the native LIFECORE P9710-2A hyaluronan. Here, it should be however noticed that while addition of 0.143 mM NX (cf. also curves 1, panels A and B in Fig. 2) corresponds to a common drug concentration in the synovial fluid of the treated patients [26], higher additions of NX used in this *in vitro* study – up to 10 mM – almost 70-fold exceeds the therapeutic drug level.

An analogous investigation of the ASA application as a retarder/inhibitor of the degradation of LIFECORE P9710-2A sample revealed changes in the shapes of the curves (cf. Fig. 5, panels A and B, curves 1 and 2), however certain facts should be mentioned: (i) addition of ASA to the solution after 1 h was less efficient concerning the degradation inhibition than the corresponding addition of NX; (ii) if ASA was added to the solution prior to degradation start, its "therapeutic" drug level [27] (cf. curve 1, panel B in Fig. 5) did not reveal any significant retarding/inhibitory effect; (iii) similarly to NX, ASA exerted dose-dependent degradation retardation/inhibitory effect (cf. Fig. 5, panel B), however due to the limited drug solubility it was not feasible to achieve complete inhibition of the degradation of LIFECORE P9710-2A sample in contrast to the study involving NX. Yet, in the case of ASA, the highest used dose, i.e. 1.43 mM, exceeded 100-fold the therapeutic drug level.

3.2. FT-IR analyses

The recovered pellets of the polymers obtained upon a 3 h degradative treatment in absence (sample coded III) or in the presence of varying amount of the drugs (NX and ASA) were analyzed in solid state by means of FT-IR (ATR) spectroscopy. Comparison of the measured spectra of the sample LIFECORE P9710-2A, its fragments, and those of the pure drugs (data not shown) indicated that amount of the free drugs or NX and ASA molecules bound onto HA in the recovered polymers, if any, was below the detection limit of this fast and simple screening techniques.

3.3. HPLC-UV/FL analyses

Exclusion size of the macromolecules, which can be separated by the used HPLC packings, namely by Waters Protein Pack (types 300 and 125), in the case of the native globular proteins is up to ≅400 kDa. However, at the separation of other polymers that similarly to HA adopt random coil conformation in the solution, the above mentioned chromatographic column

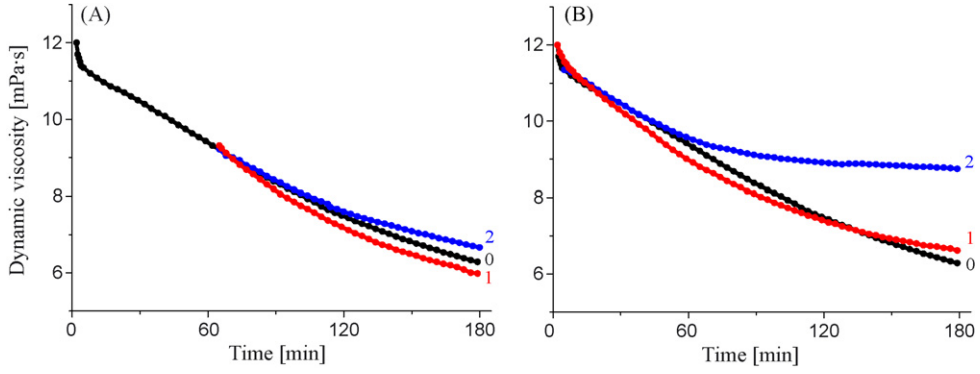


Fig. 5. Effect of acetylsalicylic acid on HA degradation induced by the system containing 0.1 μM $CuCl_2$ plus 100 μM ascorbic acid plus 50 μM H_2O_2. Concentration of ASA added into the system at 1 h (A) or before the start of degradation (B), in mM: 0.0 (0); 0.0143 (1); and 1.43 (2).

packings allow for distinguishing the molar masses only up to ≅150 kDa (Waters data sheets). These facts imply that at the HPLC analyses of the native high-molar-mass HA sample as well as of its fragments, the macromolecules will be eluted within a rather narrow range, i.e. in the vicinity of the void volume of the HPLC columns. This assumption was unambiguously proved at the HPLC analyses of the HA calibrants, which M_w values were in the range 90 kDa to 1.2 MDa. The retention times corresponding to the chromatographic peaks of these calibrants were in the interval 8–9 min. On the other hand, as demonstrated by the HPLC analysis of the low-molar-mass sample – naproxen – this analyte had significantly longer retention time of 31.6 min. Thus, a mixture of the high-molar-mass HA sample with the admixed naproxen has been efficiently separated by the used HPLC device (not shown).

At a chemical derivatization of the HA macromolecule with a fluorescent "label", such as for example with NX molecule, the size of the "HA-NX" derivative, most probably, will not change and the fluorescence due to the NX binding to HA will not be quenched completely. Thus, at the analysis of the polymer sample that may contain HA-NX derivative, detection of a fluorescent signal (Ex/Em = 270/340 nm) can be expected in the fraction having the retention time of 8–9 min. However, our HPLC-UV/FL analyses have shown that even at the highest sensitivity of the FL-detector, no peak corresponding to the fluorescent high-molar-mass polymer was detected. The analyzed samples of all native HAs (the HA calibrants and LIFECORE P9710-2A sample) were eluted as anticipated at the retention times of 8–9 min, as confirmed by the UV detection at 214 nm. At the analyses of samples fragmented by the systems containing naproxen as an inhibitor of the HA degradation, an insignificant amount (≤0.22%) of the fluorescent "contaminant", i.e. native or chemically modified naproxen, was however revealed by the FL detection at the retention time of 31.6 min. Similar analyses of the recovered polymer fragments produced by the systems, in which ASA was applied as the degradation inhibitor involving the FL detection could be also applied since ASA molecules are detectable due to their native fluorescence, too [Šoltés et al., unpublished].

4. Discussion

On studying the hyaluronan degradation, Al-Assaf et al. [28] formulated the following reaction of H_2O_2 with Cu(II):

$$2Cu(II) + H_2O_2 \rightarrow 2Cu(I) + O_2 + 2H^+$$

As can be seen, in this reaction hydrogen peroxide plays a role of a reductant. On the other hand, it is well established that ascorbate ($AscH^-$) acts as a powerful reducing agent with a standard reduction potential of +0.282 V at pH 7 for the redox couple $AscH^{\bullet -}/AscH^-$ [29], thus at applying ascorbate/ascorbic acid in the oxidative system used by us, a high excess of ascorbate (100 μM) results in an efficient reduction of the cupric ions (0.1 μM of $CuCl_2$) to cuprous ones, which subsequently react with H_2O_2 in a fast proceeding Fenton-type reaction: $Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + HO^-$ [30]. It should be, however, stated here that the physiological ascorbate concentrations in normal human adult serum and SF lie in the range 40–140 μM [31]. (On the other hand, the concentration of total ascorbate and the ascorbate to dehydroascorbate ratio are markedly subnormal in patients with advanced rheumatoid arthritis [32], whereas the total copper content is in these patients significantly higher than in healthy population [33,34].)

Thus, based on our observations it can be assumed that added ascorbate reduces transition metal ions—both added to a sample as $CuCl_2$ and those inherently present in the HA sample used. As can be calculated from the Certificate of Analysis of the sample LIFECORE P9710-2A, for the used HA concentration of 2.5 mg/ml, the solution contains the following iron and copper ion concentrations: ≈0.58 and ≈0.16 μM, respectively. Thus, it can be concluded that upon addition of 0.1 μM $CuCl_2$, the instantaneous concentrations of the two major transition metal ions equal 0.58 μM for iron and 0.26 μM for copper. Taking into consideration the known content of copper ions in the ultrafiltrate of SF of the RA patients, namely 0.125 ± 0.095 μM [35], concentration of copper ions in our solution containing a supplement of 0.1 μM $CuCl_2$ can be considered comparable to that present in SF of RA patients.

Although, 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 [36], due to the chelating properties of the HA macromolecule, the values of the standard reduction potentials for these two metal ions can, however, differ from the cited values [37,38].

4.1. Initiation

At the reaction initiation stage, there is more than 100-fold molar excess of ascorbate over the total concentration of the two metal ions. The reduced metal ions on reacting with the added H_2O_2 (50 μM) instantly initiate generation of $^{\bullet}OH$ radicals. The hydroxyl radical, due to its extremely high affinity for hydrogen, abstracts $H^{\bullet}$ radical from HA macromolecule resulting in formation of a radical, which we will further refer to simply as $A^{\bullet}$.

4.2. Propagation

Under aerobic conditions, radicals $A^{\bullet}$ react with atmospheric oxygen yielding $AOO^{\bullet}$, which naturally can participate in reactions leading to further generation of $A^{\bullet}$ ($AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet}$). The progress of the latter propagation reaction along with the reaction of $A^{\bullet}$ with atmospheric oxygen lead to the steadily increasing fragmentation of the generated $AOO^{\bullet}$ radicals yielding the polymer fragments with reduced molar mass [16]. Hence, the propagation phase of the radical (degradation) reactions is accompanied with reduction of dynamic viscosity of the HA solution.

4.3. Transfer

Both reactions $A^{\bullet} + HA \rightarrow HA + A^{\bullet}$ and $AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet}$ can be classified as the reactions, by which the radical site is transferred to another macromolecule. This trans-

fer reaction, however, does not have as a rule a significant effect on possible retardation or cessation of the radical degradation/fragmentation of the HA macromolecules.

4.4. Termination

As demonstrated by the results of our studies (cf. Fig. 2, panel A, and in part panel A in Fig. 5), addition of the drug (NX or ASA) caused retardation/inhibition of the propagation phase of HA degradation. It is therefore reasonably to assume that molecules of both drugs most probably furnish $H^{\bullet}$ radical for the radicals $A^{\bullet}$ and $AOO^{\bullet}$ at the same time converting to the radicals, which can for example mutually recombine in the reaction mixture. The resulting decline in the number or a complete elimination of the radicals $A^{\bullet}$ and $AOO^{\bullet}$ leads to retardation or possibly cessation of polymer degradation.

As is evident from the results presented in the panels B of Figs. 2 and 5, addition of the sufficiently large amount of drug (NX, ASA) leads to a total inhibition of the whole reaction sequence – initiation, propagation, transfer, termination – starting most probably already in the primary phase "INITIATION". This hypothesis is corroborated mainly by the very high values of the rate constants k of the reaction between $^{\bullet}OH$ radicals and the molecules of naproxen and acetylsalicylic acid (for NX $k = 2.4 \times 10^{10}\ M^{-1}s^{-1}$ [39]; for ASA $k = 3.6 \times 10^{10}\ M^{-1}s^{-1}$ [40]). Thus, in regard to the definition: "So-called primary or preventive defenses diminish the initiation rate of radical reactions by decreasing free radical concentration. Secondary or chain-breaking defenses, on the other hand, trap propagator radicals, stopping their harmful effects in the early stages" [41], both drugs – ASA and NX – can be classified as the substances participating in both chain-breaking defenses and preventive defense mechanisms.

Summarizing the results obtained in the course of our extensive long-time experimental studies, it can be concluded that low resistance of HA against oxidative species predestines this polymer to be utilized as an *in vitro* probe for investigating the damaging action of different oxidants [19]. Changes of η of the HA solution during degradation studies could be of advantage in classifying the efficacy of various substances to act as antioxidants [42–44]. In principle, the changes of the dynamic viscosity of the polymer solution can be monitored easily by rotational viscometry, since the solution "flow" behavior is responsive to properties such as the polymer molar mass and molar mass distribution. The SEC-MALS apparatus can be considered the most powerful tool to determine the molecular parameters of both the native HA sample, as well as its fragments.

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References

[1] E.A. Balazs, D. Watson, I.F. Duff, S. Roseman, Arthritis Rheum. 10 (1967) 357–376.
[2] H.B. Kaplan, H.S. Edelson, H.M. Korchak, W.P. Given, S. Ambramson, G. Weissmann, Biochem. Pharmacol. 33 (1984) 371–378.
[3] B.M. Twomey, M. Dale, Biochem. Pharmacol. 43 (1992) 413–418.
[4] S.B. Abramson, G. Weissmann, Arthritis Rheum. 32 (1989) 1–9.
[5] A.K. Parkash, J.K. Sugden, Z. Yucan, W. Mingxin, Int. J. Pharm. 92 (1993) 151–155.
[6] N. Parij, J. Néve, Eur. J. Pharmacol. 311 (1996) 259–264.
[7] Y. Ikeda, K. Matsumoto, K. Dohi, H. Jimbo, K. Sasaki, K. Satoh, Headache 41 (2001) 138–141.
[8] E. Fernandes, D. Costa, S.A. Toste, J.L.F.C. Lima, S. Reis, Free Radic. Biol. Med. 37 (2004) 1895–1905.
[9] D. Costa, A. Gomes, S. Reis, L.F.C. José, E. Fernandes, Life Sci. 79 (2005) 2841–2848.
[10] S.F. Wong, B. Halliwell, R. Richmond, W.R. Skowreneck, J. Inorg. Biochem. 14 (1981) 127–134.
[11] E. Gura, M. Hückel, P. Müller, Polym. Degrad. Stab. 59 (1998) 249–302.
[12] M. Jahn, J.W. Baynes, G. Spiteller, Carbohydr. Res. 321 (1999) 228–234.
[13] K. Yamazaki, K. Fukuda, M. Matsukawa, F. Hara, K. Yoshida, M. Akagi, C. Hamanishi, Pathophysiology 9 (2003) 215–220.
[14] M. Stankovská, L. Šoltés, A. Vikartovská, R. Mendichi, D. Lath, M. Molnárová, P. Gemeiner, Chem. Pap. 58 (2004) 348–352.
[15] M. Stankovská, L. Šoltés, A. Vikartovská, P. Gemeiner, G. Kogan, D. Bakoš, Biologia 60 (2005) 149–152.
[16] J. Rychlý, L. Šoltés, M. Stankovská, I. Janigová, K. Csomorová, V. Sasinková, G. Kogan, P. Gemeiner, Polym. Degrad. Stab. 91 (2006) 3174–3184.
[17] L. Šoltés, V. Brezová, M. Stankovská, G. Kogan, P. Gemeiner, Carbohydr. Res. 341 (2006) 639–644.
[18] L. Šoltés, M. Stankovská, V. Brezová, J. Schiller, J. Arnhold, G. Kogan, P. Gemeiner, Carbohydr. Res. 341 (2006) 2826–2834.
[19] L. Šoltés, R. Mendichi, G. Kogan, J. Schiller, M. Stankovská, J. Arnhold, Biomacromolecules 7 (2006) 659–668.
[20] L. Šoltés, K. Valachová, R. Mendichi, G. Kogan, J. Arnhold, P. Gemeiner, Carbohydr. Res. 342 (2007) 1071–1077.
[21] D.P. Nelson, L.A. Kiesow, Anal. Biochem. 49 (1972) 474–478.
[22] P.J. Wyatt, Anal. Chim. Acta 272 (1993) 1–40.
[23] R. Mendichi, A. Giacometti-Schieroni, C. Grassi, A. Re, Polymer 39 (1998) 6120–6611.
[24] R. Mendichi, A. Giacometti-Schieroni, Polymer 43 (2002) 6115–6121.
[25] R. Mendichi, L. Šoltés, A. Giacometti-Schieroni, Biomacromolecules 4 (2003) 1805–1810.
[26] R. Bruno, A. Iliadis, I. Jullien, M. Guego, H. Pinhas, S. Cunci, J.P. Cano, Br. J. Clin. Pharmacol. 26 (1988) 41–44.
[27] A. Soren, Eur. J. Clin. Pharmacol. 16 (1979) 279–285.
[28] S. Al-Assaf, G.O. Phillips, D.J. Deeble, B. Parsons, H. Starnes, C. Von Sonntag, Radiat. Phys. Chem. 46 (1995) 207–217.
[29] N.H. Williams, J.K. Yandell, Aust. J. Chem. 35 (1982) 1133–1144.
[30] J.M. Gutteridge, S. Wilkins, FEBS Lett. 137 (1982) 327–330.
[31] B. Halliwell, M. Wasil, M. Grootveld, FEBS Lett. 213 (1987) 15–17.
[32] B. Halliwell, P.J. Evans, H. Kaur, S. Chirico, Ann. Rheum. Dis. 51 (1992) 1261–1263.
[33] W. Niedermeier, J.H. Griggs, J. Chron. Dis. 23 (1971) 527–536.
[34] S. Hippeli, E.F. Elstner, FEBS Lett. 443 (1999) 1–7.
[35] D.P. Naughton, J. Knappitt, K. Fairburn, K. Gaffney, D.R. Blake, M. Grootveld, FEBS Lett. 361 (1995) 167–172.
[36] W.H. Koppenol, in: C.A. Rice-Evans, R.H. Burdon (Eds.), Free Radical Damage and its Control, Elsevier Science B.V., 1994, pp. 3–24.
[37] W.H. Koppenol, Bioelectrochem. Bioenerg. 18 (1987) 3–11.
[38] G.R. Buettner, Arch. Biochem. Biophys. 300 (1993) 535–543.

[39] M.A. Oturan, J. Pinson, J. Bizot, D. Deprez, B. Terlain, J. Electroanal. Chem. Interfacial Electrochem. 334 (1992) 103–109.
[40] X. Shi, M. Ding, Z. Dong, F. Chen, J. Ye, S. Wang, S.S. Leonard, V. Castranova, V. Vallyathan, Mol. Cell. Biochem. 199 (1999) 93–102.
[41] M. Martínez-Cayuela, Biochimie 77 (1995) 147–161.
[42] E. Orviský, L. Šoltés, M. Stančíková, J. Pharm. Biomed. Anal. 16 (1997) 419–424.
[43] L. Šoltés, D. Lath, R. Mendichi, P. Bystrický, Methods Find. Exp. Clin. Pharmacol. 23 (2001) 65–71.
[44] M. Stankovská, J. Arnhold, J. Rychlý, H. Spalteholz, P. Gemeiner, L. Šoltés, Polym. Degrad. Stab. 92 (2007) 644–652.