Degradative Action of Reactive Oxygen Species on Hyaluronan

L. Šoltés,*,† R. Mendichi,‡ G. Kogan,§ J. Schiller,‖ M. Stankovská,† and J. Arnhold‖

Institute of Experimental Pharmacology, Slovak Academy of Sciences, SK-84104 Bratislava, Slovak Republic, Istituto per lo Studio delle Macromolecole, Consiglio Nazionale delle Ricerche, I-20133 Milan, Italy, Institute of Chemistry, Slovak Academy of Sciences, SK-84538 Bratislava, Slovak Republic, and Institute of Medical Physics and Biophysics, Faculty of Medicine, University of Leipzig, D-04107 Leipzig, Germany

Received November 14, 2005; Revised Manuscript Received December 21, 2005

Many human diseases are associated with harmful action of reactive oxygen species (ROS). These species are involved in the degradation of essential tissue or related components. One of such components is synovial fluid that contains a high-molecular-weight polymer—hyaluronan (HA). Uninhibited and/or inhibited hyaluronan degradation by the action of various ROS has been studied in many in vitro models. In these studies, the change of the molecular weight of HA or a related parameter, such as HA solution viscosity, has been used as a marker of inflicted damage. The aim of the presented review is to briefly summarize the available data. Their correct interpretation could contribute to the implementation of modern methods of evaluation of the antioxidative capacity of natural and synthetic substances and prospective drugs—potential inflammatory disease modifying agents. Another focus of this review is to evaluate briefly the impact of different available analytical techniques currently used to investigate the structure of native high-molecular-weight hyaluronan and/or of its fragments.

1. Introductory Remarks

Reduction of the molecule of oxygen is one of the main reactions, by which animal cells, including human ones, produce metabolic energy

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \quad (1)$$

The substrate (O_2) is, by a cascade of enzymatically driven reactions, reduced within subcellular organelles, mitochondria, to a completely harmless substance, the waste product water.

Along with this four-electron reaction 1, several specialized cells—or more precisely, their specific (sub)cellular structures—reduce O_2 molecules, producing the superoxide anion radical ($O_2^{\bullet-}$)

$$O_2 + 1e^- \rightarrow O_2^{\bullet-}$$

which in aqueous (acidic) milieu can form perhydroxyl radical ($^{\bullet}O_2H$)

$$O_2^{\bullet-} + H^+ \leftrightarrow {}^{\bullet}O_2H \quad (2)$$

Since the reverse direction (←) of reaction 2 represents dissociation of a weak acid of the perhydroxyl radical, its pK_a value, equaling 4.8,[1] and the pH value of the aqueous milieu govern the actual molar ratio between the two forms, i.e., between $O_2^{\bullet-}$ and $^{\bullet}O_2H$. Under slight acidosis accompanying inflammation processes, e.g., at pH 6.8, the ratio of [$O_2^{\bullet-}$]:[$^{\bullet}O_2H$] equals 99:1.

Nitrogen monoxide ($^{\bullet}$NO), a (bioactive) free radical, is produced in various cells/tissues by the enzyme NO synthase. The level of $^{\bullet}$NO increases markedly during inflammation, a process accompanied with abundant production of the superoxide anion radical.[2−4]

The two radical intermediates, $O_2^{\bullet-}$ and $^{\bullet}$NO, serve as precursors of various reactive oxygen species (ROS), including hydrogen peroxide, peroxynitrite, hypochlorous acid, and so forth (see section 5.1). On respiring air, human beings by utilizing 1 mol of O_2 ingest 6.023×10^{23} molecules of oxygen, of which approximately 1−3% are assigned to the generation of ROS that defends the organism against viral/bacterial invaders.[12] In some cases, however, the intermediate and/or the "final" reactive oxidative species may also damage cells/tissues of the human host. Imbalance between the extent of damage and self-repair of the functionally essential structures may result in a broader host tissue injury, eventually leading to a specific disease.

There are numerous diseases, which pathology involves reactive oxidative/oxygen-derived species at the onset and/or at later stages of the disease.[13] One of the classes of such diseases includes arthritic conditions—inflammatory diseases of joints. A substantial amount of evidence exists for an increased generation of oxidants in patients suffering from acute and chronic inflammatory joint diseases.[14−17]

A joint is formed by the ends of two (or more) bones connected by connective tissues. The fundamental function of joints in the human organism is to ensure mutual motion of the adjacent bones in the plane (bending $x \leftrightarrow y$) as well as in space (rotation $x \leftrightarrow y \leftrightarrow z$).

One of the firmest tissues in the human body, along with the teeth, are bones. Vertebral bones are made of "nanocomposites" with hard mineral crystals embedded in a soft protein matrix. Thus, the biomaterial of bones can be classified as molecular composites of proteins and (bio)minerals. The bone ends that are linked in a joint are covered with a smooth layer called

* Correspondence: Tel. +421-(2)-5941-0670; Fax +421-(2)-5477-5928; E-mail ladislav.soltes@savba.sk.
† Institute of Experimental Pharmacology, Slovak Academy of Sciences.
‡ Consiglio Nazionale delle Ricerche.
§ Institute of Chemistry, Slovak Academy of Sciences.
‖ University of Leipzig.

10.1021/bm050867v CCC: $33.50
Published on Web 02/16/2006

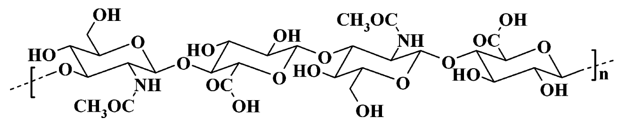


Figure 1. Hyaluronic acid. The term hyaluronic acid (HA) was coined by K. Meyer.[18] However, under physiological (pH) conditions, HA is a polyanion (with corresponding countercation(s) such as H^+, Na^+, K^+, etc.), and hence, the term hyaluronate appears to be more appropriate. Yet, irrespective of the degree of dissociation of its macrobiomolecules, the term hyaluronan, suggested by Balázs et al.,[19] has become predominantly used for the designation of this polysaccharide.[20]

cartilage. A specific property of these solid tissues—bones and cartilage—is their permanent biodegradation and reconstruction/regeneration, while maintaining the original shape and size. The cartilage layer of the bones constituting a joint, e.g., a knee joint, is one of the most mechanically stressed tissues in the human body. In fact, almost the total mass of the body rests on the two knee joints, and moreover, at walking or running the still body mass is multiplied by strokes with the frequency of approximately 0.5 Hz (walking), 2.5 Hz (jogging/running), and even more (sprinting).

Every joint is surrounded by a fibrous tissue envelope/capsule called synovium, which produces synovial fluid (SF) that reduces friction and wear and tear of the joint. The SF can be simplistically characterized as a (bio)lubricating solution consisting of an ultrafiltrate of blood plasma plus glycoproteins and hyaluronan (HA), a high-molecular-weight polysaccharide.

HA (Figure 1) is a non-branched non-sulfated glycosaminoglycan (cf. Table 1), which mean molecular weight reaches the megadalton values. In aqueous solutions, HA is represented by negatively charged macromolecules ($pK_a = 3.21$[23]) with extended conformations, which impart high viscosity/viscoelasticity, accompanied also by low compressibility, of the synovial fluid.[24]

A normal/healthy joint allows (practically) frictionless and pain-free movement. However, when damaged or affected by arthritis, joints become stiff and painful. Of the more than 100 arthritic diseases, osteoarthritis (OA) and rheumatoid arthritis (RA) are the most common chronic conditions affecting the elderly population. While OA is a degenerative disease of the cartilage and bone, resulting in pain and stiffness in the affected joint, RA is classified as a systemic inflammatory disease, in which pain of the joint(s) is often accompanied with degenerative changes in organs, such as lungs, heart, and blood vessels.

Although the etiology and pathogenesis of RA are as yet unknown, a progressive degradation of polymeric carbohydrates, including HA, in synovial fluid can be observed in the course of the disease. In acute phases, a high number of neutrophils are accumulated in the patient's synovial fluid. These cells alter the oxidative homeostasis, and their products, especially ROS, can contribute to the destruction of joint structures. Because of chronic inflammation of the joint (cf. Figure 2), the ROS alter/destroy the joint structure to such an extent that it is no longer functional. The altered tissues are recognized as "foreign", and subsequently, autoimmune reactions promote the disease and make rheumatoid arthritis a systemic ailment affecting the whole body.

2. In Vitro Studies of Uninhibited/Inhibited Hyaluronan Degradation by Reactive Oxygen Species

The observed reduction of hyaluronan molecular weight in the synovial fluid of patients suffering from rheumatic diseases led to in vitro studies of HA degradation by reactive oxygen species.[7] The first investigation was carried out by Pigman and Rizvi in 1959,[26] and since then, numerous studies have been reported (cf. Table 2).

Although Table 2, column 2, implies that the given ROS source generates one single type of the oxidative species, this is not correct. For example, in the case of the generation of superoxide anion radicals due to their spontaneous dismutation, molecules of hydrogen peroxide are simultaneously present. The reaction between $O_2^{\bullet-}$ and the formed H_2O_2, catalyzed by ubiquitous transition-metal cations, yields $^{\bullet}OH$ radicals and molecular oxygen (cf. section 5.1). Another example, not listed in Table 2, is the $^{-}OCl/HOCl$ generating system comprising the enzyme myeloperoxidase plus H_2O_2 in the aqueous halide (Cl^-) milieu. Although the enzyme catalyzes the reaction (5; see section 5.1), the ubiquitous transition-metal cations may simultaneously—by decomposition of H_2O_2—generate further ROS (cf. section 5.1).

3. Analytical Methods of Investigation of Hyaluronan Degradation by Reactive Oxygen Species

Hyaluronan is a linear homopolymer built of the disaccharide repeating units of [D-glucuronic acid-β-(1→3)-*N*-acetyl-D-glucosamine] linked together with β-(1→4) glycosidic linkages (cf. Figure 1). Simple hydrolytic scission of the glycosidic bond (by action of the water molecule) in the HA macromolecule would yield hyaluronan molecules with lower molecular weights. Thus, by such a hypothetical hydrolytical reaction, the HA macromolecule is hydrolyzed/degraded; yet its primary, chemical, structure remains well-preserved. However, an attack on the backbone of the high-molecular-weight hyaluronan by an oxidative species can hardly be described as a simple hydrolytical reaction. For example, the radical, e.g., $^{\bullet}OH$, abstracts a hydrogen radical from the HA macromolecule, which results in the formation of a macroradical plus a molecule of water. The fate of the (intermediate) macroradicals formed is not univocal. The final reaction products usually include macromolecules of lower molecular weights than that of the native HA. The biopolymer fragments formed are often (chemically) modified; therefore, a simple description of the products as hyaluronan(s) is imprecise.

To explain the HA degradation by the action of any particular ROS is rather a challenging task. The simplest level of such a study is to measure changes of rheological properties of a hyaluronan solution, for example, the decrease of the solution viscosity is used as a "primitive" marker of the degradative processes inflicted by the action of the given ROS (cf. section 3.1). The molecular weight of the native hyaluronan as well as that of the products of HA degradation can be determined by using, e.g., a light-scattering (LS) device (cf. section 3.2). However, determination of the changes in the structure of a macromolecule usually requires a battery of analytical techniques (cf. sections 3.3 and 3.4). Modifications, which occur at higher structural levels than the primary one remain sometimes undetected because of limited performance of the analytical tools employed.

3.1. Rheological Parameters—Markers of Hyaluronan Degradation by Reactive Oxygen Species. All principal viscometric/rheometric methods fall into one of two classes: (1) involving a moving fluid or (2) involving a moving element. The first class is characterized by a liquid moving through a definite channel/capillary—the variable measured is the time, which relates to the kinematic (ν) viscosity of the fluid. Capillary viscometers, being the simplest and most widely used devices, are however not "true" rheological instruments. Capillary tube

Table 1. Glycosaminoglycans[a]

polysaccharide	structural components	location	mean molecular weight
hyaluronate	GlcNAc & GlcUA	synovial fluid; vitreous humor; ECM[b] of loose connective tissues	several MDa[21]
chondroitin-4-sulfate ≡ chondroitin sulfate A	GalNAc-4-sulfate and GlcUA	cartilage; bone; ECM; basement membranes of many tissues including intestines	
chondroitin-6-sulfate ≡ chondroitin sulfate C	GalNAc-6-sulfate and GlcUA	similarly to chondroitin-4-sulfate	
dermatan sulfate ≡ chondroitin sulfate B	GalNAc-4-sulfate and IdoA or GlcUA[c]	skin; blood vessels; heart valves; ECM	∼25 kDa[4,22]
keratan sulfate[d]	GlcNAc-6-sulfate and galactose or galactose-6-sulfate	bone; cartilage	∼5 kDa[16]
heparan sulfate and heparin[e]	*N*-sulfo-D-glucosamine-6-sulfate and D-glucuronate-2-sulfate[f]	ECM; basement membranes; components of cell surfaces; components of intracellular granules of mast cells lining arteries of lungs, liver, and skin	

[a] Glycosaminoglycans (GAGs) are the most ubiquitous non-branched heteropolysaccharides in the body of vertebrates, including human beings. The GAG macromolecules consist of repeating disaccharide units, in which *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) alternate with a uronic acid residue, i.e., D-glucuronate (GlcUA) or L-iduronate (IdoA). The majority of GAGs in the body are linked to core proteins, forming proteoglycans. [b] ECM = extracellular matrix. [c] Predominant disaccharide units: GalNAc-4-sulfate and IdoA. [d] Keratan sulfate is often aggregated with chondroitin sulfates. [e] One well-defined function of heparin is its role in preventing coagulation of blood. [f] Heparans contain fewer sulfate groups than heparins.

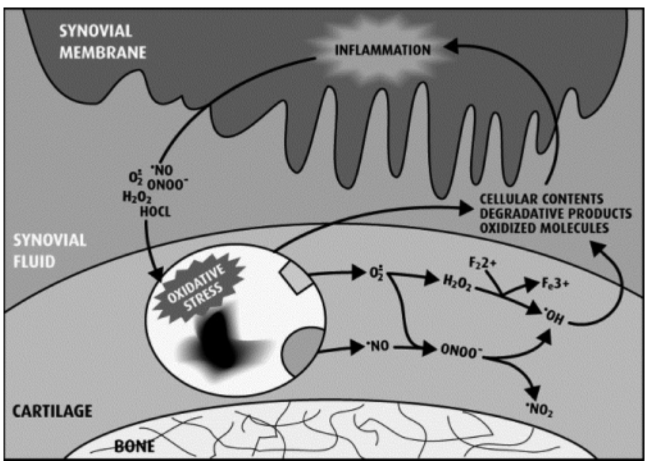


Figure 2. Involvement of reactive oxygen species in cartilage degradation and related synovium inflammatory reaction[25] (with permission).

viscometers, characterized by shear rates in the range of hundreds up to thousands of reciprocal seconds, are suitable only for use with Newtonian fluids.

The second class comprises either a linearly moving element, such as the falling ball, or a rotationally moving element. In the latter group of instruments, either the stress is controlled and the resulting rotational speed is measured, or the rotational speed is controlled and the stress is measured. Those instruments in which the rotational speed is controlled and stress is measured can certainly indicate dynamic (η) viscosity changes with time. Rotational rheometers, characterized by a very low shear rate, are addressed to characterize the rheological parameters of non-Newtonian fluids, including beyond controversy the hyaluronan solutions. Moreover, oscillatory (rotational) rheometers allow assessment of the storage (G') as well as loss (G'') moduli—the parameters, which provide information on polymer structure and might be related to molecular weight distribution, cross-linking, and so forth.[48]

Capillary viscometry has been applied to analyze the hyaluronan molecular weight (M_v average) itself and/or to follow its changes. This method was exploited in some studies of HA degradation caused, e.g., by •OH radicals.[43,44]

Although similar studies were performed on exploiting the rotational viscometric approach, the material of the sample reservoir and especially that of the rotating spindle—usually metallic—is a limiting factor for determination of exact data. Very recently, our group has attempted to apply the method of rotational viscometry for HA degradation studies.[49,50] In our studies, we used a device, which main parts were made from an inert material, Teflon. Figure 3 illustrates the kinetics of degradation of high-molecular-weight HA induced by the combined action of Cu^{2+} and ascorbic acid and of Cu^{2+}, ascorbic acid, and HOCl.

3.2. Methods to Determine the Hyaluronan Molecular Weight. The molecular weight is one of the most important characteristics of a given compound. In the case of polymers, the sample is exactly characterized by the distribution of molecular weights. Molecular weight averages—M_n = numerical average, M_v = viscosity average, M_w = weight average, etc. (where $M_n < M_v < M_w$), determinable, e.g., by osmometry (M_n), viscometry (M_v), and light scattering (M_w)—characterize the given polymer, however, incompletely.

LS, elastic or total, evaluates the intensity of the light scattered by macromolecules in diluted solution state. This intensity is related to the molecular weight (M) of the sample by the following equation:

$$K \cdot c/\Delta R(\theta) = 1/[M \cdot P(\theta)] + 2A_2 \cdot c + ...$$

where $\Delta R(\theta)$ is the scattering excess (Rayleigh factor) of the solution with regard to the pure solvent; θ means the angle between the incident light and the detector; c, the concentration; A_2, the second virial coefficient; $P(\theta)$, the form factor; $K = [4\pi^2 \cdot n_0^2 \cdot (\mathrm{d}n/\mathrm{d}c)^2]/(N_A \cdot \lambda_0^4)$, the optical constant, where n_0 is the refractive index of the solvent, dn/dc is the refractive index increment of the polymer solution, N_A is Avogadro's number, and λ_0 is the wavelength of the light in vacuum. The reciprocal of the form factor $P(\theta)$ can be written as $1/P(\theta) = 1 + {}^1/_3 \cdot \mu^2 \cdot \langle s^2 \rangle$, where $\mu = 4\pi/\lambda \cdot \sin(\theta/2)$, $\lambda = \lambda_0/n_0$ is the wavelength of

Table 2. Systems of High-Molecular-Weight Hyaluronan *Plus* Reactive Oxygen Species (ROS)

ROS	sources of ROS	action of inhibitors investigated	refs
$O_2^{\bullet -}$	cellular		
	for example, PMN leukocytes[a]		
	enzymatic		
	for example, xanthinoxidase + xanthine[a]		
	chemical		
	KO_2[b]	not	27
•NO	cellular		
	endothelial cells[c]	not	3
	chemical		
	gaseous •NO (from a gas canister)[c]	not	3
$ONOO^-$	chemical		
	$NaNO_2$ + (H_2O_2 + HCl) + NaOH	yes	2
		not	7, 28
	NaN_3 + O_3	not	29
H_2O_2	chemical		
	aqueous H_2O_2[d]	not	30
^-OCl/HOCl	chemical		
	hypochlorite[e]	yes	32
		not	33−35
•OH	enzymatic		
	xanthinoxidase + xanthine + transitional metal cation[f]	yes	36
	physical and/or chemical		
	H_2O lysis by γ-rays	not	37−39
	H_2O_2 irradiation by the UV light	not	40
	H_2O_2 + transition metal cation[f]	yes	2, 41−44
		not	33, 34, 45, 46
$^1O_2$	physical and/or chemical		
	UV_{366} irradiated riboflavin[g]	yes	47

[a] Because of the spontaneous dismutation reaction of superoxide anion radicals, the action of $O_2^{\bullet -}$ may be infringed by the presence of hydrogen peroxide. [b] Potassium superoxide (KO_2) did not cause detectable fragmentation of hyaluronan.[27] [c] Endothelial-cell-derived •NO, as well as exogenous •NO gas, did not degrade hyaluronan.[3] [d] Because of the presence of transition-metal cations in the high-molecular-weight HA—although in trace amounts—it is plausible that along with hydrogen peroxide the studied system was "contaminated" with •OH. [e] Under the condition of an acute inflammation, the hypochlorite concentration (produced by stimulated PMN leukocytes) may increase up to 340 μM.[31] [f] Iron, copper, titanium, etc. [g] The system does not generate solely singlet oxygen.[47]

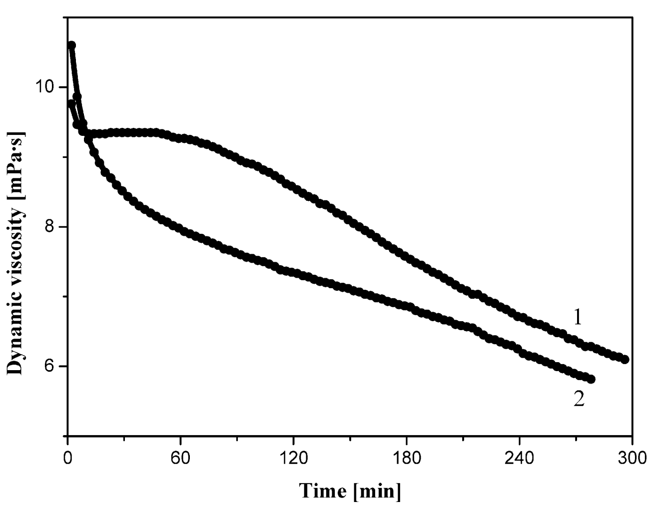


Figure 3. Kinetics of degradation of high-molecular-weight HA (2.5 mg/mL) induced by the combined action of 0.1 μM Cu^{2+} and 100 μM ascorbic acid (curve 1) and of 0.1 μM Cu^{2+}, 100 μM ascorbic acid, and 5.0 mM HOCl (curve 2).

the light in the solvent, and $\langle s^2\rangle^{1/2}$ is the radius of gyration of the macromolecules.

The experimental LS data are analyzed using the double extrapolation ($c \to 0$ and $\theta \to 0$) generally known as the Zimm plot. By applying this procedure to a polydisperse sample, three average values are obtained: the weight-average molecular weight M_w, the *z*-average root-mean-square radius of the macromolecules $\langle s^2\rangle_z^{1/2}$ (shortly referred to as radius of gyration R_g), and the second virial coefficient A_2.[51]

Lack of knowledge of one (or more) *M* average(s) may underlie the fact that polymers with different distributions of molecular weights are classified as identical substances owing to their equal *M* value(s). Analytical procedures, which can establish the distribution of molecular weights of the polymer are therefore more preferred.

SEC-MALS, a size exclusion chromatographic (SEC) fractionation device connected on-line to an MA (multiangle) LS detector, belongs to the top devices designed to characterize polymers—including high-molecular-weight hyaluronans.[52] Figure 4 illustrates the differential molecular weight distribution of two HA samples: Hylumed (M_w = 90.2 kDa, M_w/M_n = 1.8) and B22157 (M_w = 1.34 MDa, M_w/M_n = 1.5) determined by the SEC-MALS method.

SEC in combination with low-angle light scattering is another arrangement applicable for determining the distribution of HA molecular weights in a single run. When SEC is equipped with a detector monitoring only the HA concentration in the effluent, more than one run has to be performed. Application of a refractive index (RI) or UV absorbance detection to measure the HA concentration requires calibration of the apparatus. Although until recently only scarcely available, today the

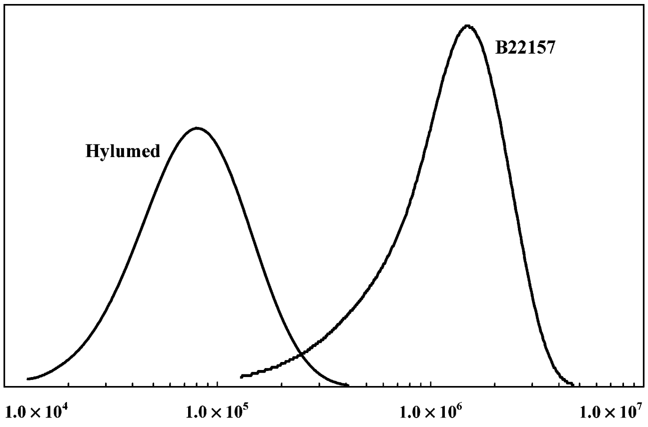
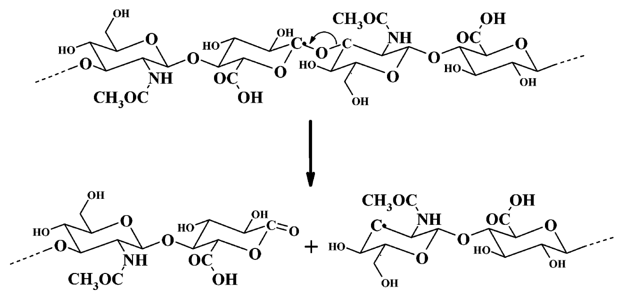


Figure 4. Differential molecular weight distributions of the HA samples Hylumed and B22157.

Scheme 1. HA Strand Cleavage May Be Due to β-Cleavage of the Radicals Formed at, e.g., C(1) on the GlcUA Ring



"monodisperse" HA standards/calibrants are commercially marketed (http://www.hyalose.com).

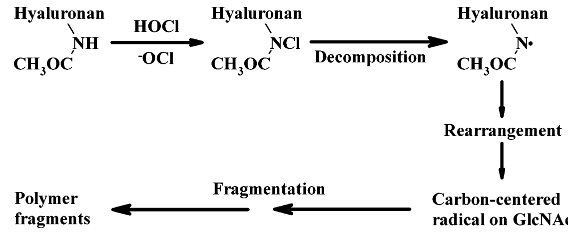
Since hyaluronan is a polyanion, the SEC separation principle can and has been altered by electrophoretic techniques, e.g., by agarose gel electrophoresis, capillary electrophoresis, and capillary gel electrophoresis,[53] the methods that are marked by the shortcomings mentioned at the description of the SEC-RI or SEC-UV equipments.

3.3. Methods to Disclose Changes in the Structure of Degraded Hyaluronans. It is very plausible that during the degradation of the native high-molecular-weight HA, along with the decreasing sample molecular weight the ROS and/or the inhibitor/drug tested will bring about certain changes in the structure of polymer fragments.[54,55] Modification of the chemical structure of the component monosaccharides of HA, due to, e.g., extraction of hydrogen atoms, oxidation of functional groups, scission of certain chemical bonds, and so forth, should be detectable by some analytical methods.[56−58]

EPR Spectroscopy. The •OH radical attack on hyaluronan and on its two structural components—GlcUA and GlcNAc—was studied by direct (rapid flow) electron paramagnetic resonance (EPR) spectroscopy and spin trapping EPR spectroscopy.[1,40,46,59] Evidence has been obtained for random hydrogen atom abstraction at all the ring C−H bonds within glucuronic acid, as well as at all sites except the *N*-acetyl group and C(2) within the *N*-acetylglucosamine unit. Results of EPR spectroscopic studies support the hypothesis that the HA strand cleavage may be due to β-cleavage of the radicals formed either at C(1) of the monosaccharide ring (cf. Scheme 1), at C(3) of the *N*-acetylglucosamine, or at C(4) of the glucuronic acid ring.

Reaction of HOCl with hyaluronan and with other glycosaminoglycans (GAGs) yields chloramides derived from the *N*-acetyl function of the *N*-acetylglucosamine rings (cf. Scheme 2[60]). The

Scheme 2. Outline of Events that May Occur on Reaction of HOCl/$^{-}$OCl with the *N*-Acetylglucosamine (GlcNAc) Moiety of Hyaluronan[a]



[a] The generation of chloramide species and their subsequent decomposition yields nitrogen-centered radicals, which on rearrangement lead to the production of carbon-centered radicals. These are believed to result in fragmentation of the HA macromolecule. Adapted from Hawkins and Davies.[60]

results of spin trapping EPR spectroscopic studies are consistent with the formation of amidyl radicals from these chloramides via both metal-ion-dependent and ion-independent processes. In the case of glycosaminoglycan-derived amidyl radicals, evidence has been obtained that these radicals undergo rapid intramolecular abstraction reactions yielding carbon-centered radicals at C(2) on the *N*-acetylglucosamine rings (via a 1,2-hydrogen atom shift) and at C(4) of the neighboring uronic acid residues (via 1,5-hydrogen atom shifts). The C(4) carbon-centered radicals undergo pH-independent β-scission reactions that result in glycosidic bond cleavage.[35]

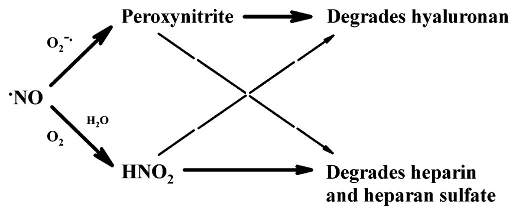
NMR Spectroscopy. Classical NMR spectroscopy has not been proven successful in establishing minor chemical changes introduced into HA at oxidative degradation. The most serious problem facing an investigator attempting to assess the structure of high-molecular-weight hyaluronan by means of ^{1}H NMR spectroscopy is that because of a very restricted motion and fast relaxation of the polymer macromolecule no signals can be observed in the spectrum.

Schiller et al.[16] investigated the action of hypochlorous acid on polymeric components of cartilage and observed that hypochlorite affected mainly *N*-acetyl groups in chondroitin sulfate and its action led to the appearance of two new signals in the spectra with a simultaneous decrease of the resonance intensity at about 2.0 ppm of the *N*-acetyl methyl groups. The appearance of a broad signal at 2.35 ppm was assigned to a chlorinated product of *N*-acetyl groups, while the final formation of free acetate was characterized by a signal at 1.90 ppm. The resonances, clearly visible in the case of chondroitin sulfate, were observable in the HA solutions only upon prolonged incubation with hypochlorous acid. The conclusion of the primary action of HOCl on the *N*-acetyl groups was confirmed also by a study using ^{13}C NMR spectroscopy, which showed that application of a large excess of hypochlorous acid led to the degradation of carbohydrate rings with formate as the final product.[61]

As further methods appropriate for studies of changes occurring during the degradation of hyaluronans by ROS, the following physicochemical and analytical techniques come into consideration: infrared (IR) spectroscopy in both solution and solid states as a non-destructive methodology[62] and matrix-assisted laser desorption ionization−time-of-flight (MALDI−TOF) mass spectrometry as a destructive methodology. Although the latter technique provides a precise and fast tool for the determination and characterization of carbohydrates, on analyzing the HA degradation products, so far only the structures of small oligomeric fragments have been established.[63,64]

In summary, it seems legitimate to say that chemical changes introduced into the structure of HA at degradation reactions

Scheme 3. In the Presence of Superoxide Anion Radical ($O_2^{\bullet -}$), $^{\bullet}NO$ Is Converted into Peroxynitrite, and in the Presence of O_2 in Aqueous Solutions, $^{\bullet}NO$ is Converted into HNO_2[a]



[a] Each of these products has different glycosaminoglycan specificity: peroxynitrite degrades hyaluronan (solid arrow) but does not cleave heparin or heparan sulfate (dashed arrow); HNO_2 cleaves heparin and heparan sulfate (solid arrow) but does not degrade hyaluronan (dashed arrow). Adapted from Vilar et al.[3]

usually occur in a low ratio with respect to the overall high-molecular-weight polymer, and because of this, their detection by the above-mentioned analytical procedures is rather complicated and does not yield unambiguous results.

3.4. Complementary Analytical Methods for Deeper Insight into Oxidative Reactions Damaging High-Molecular-Weight Hyaluronans. The hyaluronan degradation can be stopped by pouring the reaction vessel content into an appropriate volume of ethanol. By such a step, the HA macromolecules and/or their derivative(s) precipitate and can be subsequently recovered, e.g., by centrifugation. The two fractions—the sediment containing precipitated macromolecules and the water-ethanol supernatant—can be further analyzed by complementary analytical methods.

The macromolecular component(s) in solid form or in solution can be further investigated by techniques such as thermogravimetric analysis, differential scanning calorimetry, UV-vis spectroscopy, circular dichroism, and so forth. The easiest way to test changes in the recovered biopolymer structure is to perform its degradation by specific hyaluronidase enzyme(s)[65] and compare the results with those obtained with an original hyaluronan sample having a similar molecular weight. The greater the structural changes are, the slower the modified polymer degradation by the enzyme.[66,67]

The water-ethanol phase can be analyzed by different techniques, such as high-performance liquid chromatography with a mass spectrometric detector (HPLC-MS), gas chromatography with a mass spectrometric detector (GC-MS), and so forth. On applying the latter technique, oxidation of HA with NaOCl proved to yield *meso*-tartaric acid; in addition, arabinaric acid and glucaric acid were obtained by oxidation with the H_2O_2/Fe^{2+} system. Arabinuronic acid, arabinaric acid, *meso*-tartaric acid, and glucaric acid were identified by GC-MS as oxidation products of glucuronic acid. When GlcNAc was oxidized, erythronic acid, arabinonic acid, 2-acetamido-2-deoxy-gluconic acid, glyceric acid, erythrose, and arabinose were formed.[33]

4. Concluding Remarks

Reactive oxygen species were originally thought to be released only by phagocytic cells during their involvement in host defense mechanisms. In many ways, these species are, however, ideally suited to be signaling molecules, since

- they are small;
- they can usually migrate within short distances;
- there are several rapid and controllable mechanisms for their production;
- there are numerous mechanisms for their rapid removal.

It is now clear that ROS have a cell signaling role in many biological systems, both in animals and in plants. These species induce programmed cell death or necrosis, induce or suppress the expression of many genes, and activate cell signaling cascades.[5]

Contrary to the above-mentioned roles of ROS, excessive amounts of $O_2^{\bullet -}$ and $^{\bullet}NO$ are formed during inflammatory processes. These two species may recombine, producing peroxynitrite, which degrades the high-molecular-weight hyaluronan, but not heparin/heparan sulfate (cf. Scheme 3). Degradation of hyaluronan of joint synovial fluid has been linked to rheumatoid arthritis. Thus, the balance between the formed $O_2^{\bullet -}$ and $^{\bullet}NO$ "precursors", the "intermediates" such as H_2O_2, and the degradative species, e.g., $^{\bullet}OH$, determines which glycosaminoglycan component of the extracellular matrix is destroyed and may be important in regulating disease processes.[3,4] On the other hand, the chemical modification of GAGs mediated by ROS flux, e.g., during periodontal disease state, is not only of importance in considering connective tissue destruction but may have also severe consequences upon extracellular matrix synthesis, organization, and repair.[57,58]

The two main antioxidatively acting enzymes—superoxide dismutase and catalase—are barely detectable in rheumatoid synovial fluid.[7,68] Their levels in synovial fluid do not exceed 1 μg/mL and 50 ng/mL, respectively.[69] Further proteins present in SF, such as albumin, transferrin, and ceruloplasmin, can be classified as high-molecular-weight non-enzymatic "antioxi-

Table 3. Antioxidatively Acting Low-Molecular-Weight Xenobiotics[a]

xenobiotic	properties/action(s)
Endogenic/Exogenic Substances	
carotenoids	free radical scavenger and singlet oxygen quencher
coenzyme Q_{10} (ubiquinone)[b]	transports electron in mitochondria
flavonoids	scavenge superoxide anion radical and hydroxyl radicals
glucose	scavenger of $^{\bullet}OH$ radicals
glutathione	scavenges peroxyl radicals, peroxynitrite, and H_2O_2; it conjugates with $^{\bullet}NO$
melatonin	scavenges $^{\bullet}OH$ and peroxyl radicals, $^{\bullet}NO$, $ONOO^-$, as well as $^1O_2$[44]
uric acid[c]	HOCl and peroxyl radical scavenger, singlet oxygen quencher, transition metal ion chelator
vitamin C[d]—essential nutrient	free radical and HOCl scavenger; it regenerates vitamin E
vitamin E—essential nutrient	peroxyl free radical scavenger, chain-breaking antioxidant
Drugs	
NSAIDs[e]	
steroidal anti-inflammatory drugs[e]	

[a] Compiled from refs 12, 13 and http://www.alexis-corp.com/. [b] The reduced form, ubiquinol, is an effective antioxidant in cellular membranes. [c] Reaction of uric acid with oxidants, such as $^{\bullet}OH$, may generate secondary radicals, which may damage biomolecules, including HA. [d] Ascorbic acid is a significant antioxidant in the absence of transition-metal ions, while in their presence, its prooxidative effect is markedly pronounced.[71] [e] Anti-inflammatory drugs have often been proposed to decrease free radical production, although they might also enhance it.[72]

dants". Their protective function is related to their high affinity to bind cations of transition metals (Fe, Cu, etc.).[70]

From the point of view of their antioxidative properties, several low-molecular-weight xenobiotics present in SF may function protectively against the degradative action of ROS on hyaluronan (cf. Table 3). Drug action for optimized therapeutic purposes can be manipulated by understanding the role of physicochemical parameters, such as redox properties and features, which control drug distribution.

It can therefore be concluded that intervention in a process of generation and/or distribution of ROS is a sensitive task. Precise knowledge of the mechanisms controlling formation and, in the case of longer-living species, distribution is a primary prerogative for proper action. Identification of the factors governing the distribution and fate of xenobiotics/drugs in microenvironments, as, e.g., synovial fluid, are further prerequisites of rational pharmacological intervention.

Acknowledgment. The grants 2/5002/5 and 2/4143/04 from the Grant Agency of the Ministry of Education and the Slovak Academy of Sciences (VEGA), Bratislava, Slovak Republic, the grant D/04/25701 from the German Academic Exchange Service, and financial supports from the Interdisziplinäre Zentrum für Klinische Forschung—IZKF Leipzig—at the Faculty of Medicine of the University of Leipzig (Project A 17 and Project A19) are gratefully acknowledged.

Abbreviations

Nomenclature

cNOS − constitutive •NO synthase
DNA − desoxyribonucleic acid
ECM − extracellular matrix
EPR − electron paramagnetic resonance (spectroscopy)
GAG − glycosaminoglycan
GalNAc − *N*-acetylgalactosamine
GC-MS − gas chromatography with a mass spectrometric detector
GlcUA − D-glucuronate
GlcNAc − *N*-acetylglucosamine
GSSG•− − oxidized glutathione anion radical
HA − hyaluronan
HPLC-MS − high-performance liquid chromatography with a mass spectrometric detector
IdoA − L-iduronate
iNOS − inducible •NO synthase
IR − infrared (spectroscopy)
LS − light scattering
MALDI−TOF − matrix-assisted laser desorption ionization−time-of-flight (mass spectrometry)
M_n − numerical average of the molecular weight
M_v − viscosity average of the molecular weight
M_w − weight average of the molecular weight
NADPH − reduced nicotinamide adenine dinucleotide phosphate (oxidase)
NMR − nuclear magnetic resonance (spectroscopy)
NOS − •NO synthase
NSAIDs − non-steroidal anti-inflammatory drugs
OA − osteoarthritis
PMN − polymorphonuclear (leukocytes)
RA − rheumatoid arthritis
RI − refractive index
ROS − reactive oxygen species
SEC − size exclusion chromatography
SEC-MALS − size-exclusion chromatography with a multiangle light-scattering detector
SEC-RI − size-exclusion chromatography with a refractive index detector
SEC-UV − size-exclusion chromatography with a UV light detector
SF − synovial fluid
SOD − superoxide dismutase
UV − ultraviolet
vis − visible

5. Appendix

5.1. Low-Molecular-Weight Reactive Oxygen Species. *Superoxide Anion Radical*, $O_2^{\bullet-}$, is formed in neutrophils, monocytes, macrophages, and eosinophils because of the action of NADPH oxidase—the enzyme is also called respiratory burst oxidase. NADPH oxidase, a highly regulated enzyme complex composed of a number of proteins, reduces oxygen to superoxide anion radical at the expense of NADPH

$$2O_2 + NADPH \rightarrow 2O_2^{\bullet-} + NADP^+ + H^+$$

Another source of superoxide anion radical is xanthine oxidoreductase, also called xanthinoxidase. This molybdenum- and iron-containing flavoprotein catalyzes oxidation of hypoxanthine to xanthine and then to uric acid. Molecular oxygen is the substrate, and the products include the superoxide anion radical.[5] Other cellular sources for $O_2^{\bullet-}$ are mitochondria in stressed cells, the formation of met-hemoglobin, and the reduction of oxygen by quinone radical or by oxidized glutathione anion radical (GSSG•−).

Superoxide anion radical is both a one-electron oxidant and a one-electron reductant. This reactive oxygen species does not have direct toxic effects on living targets. Its toxicity is exerted by penetration to important sites where it is converted to hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and possibly to hydroxyl radical (•OH). Superoxide anion radical also plays a decisive role by converting nitrogen monoxide (•NO) to the powerful oxidant peroxynitrite anion ($ONOO^-$).

Nitrogen Monoxide, •NO, a short-living radical, can play a dual role in physiology. By interacting with the iron-containing prosthetic group of guanylate cyclase, it has a regulatory function as an endothelium-derived relaxation factor. It can also be converted to other nitrogen oxides and thus become a toxic or inflammatory agent.

Enhanced •NO synthesis was reported to occur in inflammatory responses initiated by microbial products or autoimmune reactions and also in the systemic inflammatory response, referred to as sepsis. •NO probably participates in the inflammatory reaction and subsequent damage of joint tissues in certain types of arthritis. For instance, synovial fluid from patients with arthritis exhibits elevated nitrate concentrations (nitrate is the end-product of the L-arginine-NO synthase pathway).

NO synthase enzymes (NOS) are P_{450}-related hemoproteins that oxidize L-arginine to L-citrulline and nitrogen monoxide.[6] Three distinct isoforms of NOS representing three distinct gene products have been isolated and purified. Two of the enzymes are permanently present and termed constitutive NOS (cNOS). The third one is an inducible NOS (iNOS). Stimuli typically include cytokines and/or lipopolysaccharide, and once expressed, the enzyme generates large amounts of •NO.

Peroxynitrite anion, also called *oxoperoxonitrate*, $ONOO^-$, is formed at sites of inflammation by the rapid reaction of superoxide anion radical with nitrogen monoxide

$$^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$$

$ONOO^-$ is a highly reactive oxidizing species capable of damaging cellular lipids, carbohydrates, proteins, and DNA. The reaction of $ONOO^-$ with tyrosine residues in proteins results in the formation of 3-nitrotyrosine, a suggested biomarker of $ONOO^-$ production in vivo. Indeed, increased levels of 3-nitrotyrosine have been detected in numerous human diseases, such as rheumatoid arthritis, Parkinson's disease, Alzheimer's disease, and asthma.

Peroxynitrite anion in aqueous milieu exists in an acid-base equilibrium with peroxynitrous acid

$$ONOO^- + H^+ \leftrightarrow ONOOH$$

It can be relevant to point out that the hydrogenated form, ONOOH, is a very weak acid with a pK_a value of 6.8.[7] Under slight acidosis accompanying inflammation processes, i.e., at, e.g., pH 6.8, the ratio of $[ONOO^-]$:[ONOOH] is 50:50.

The chemistry of peroxynitrous acid may be of high importance, taking into account that the molecule of ONOOH

- can "decompose" by homolytic fission to $ONO^\bullet + {}^\bullet OH$;
- can "decompose" by heterolytic fission to $ONO^+ + HO^-$;
- can isomerize, yielding $H^+ + NO_3^-$, i.e., HNO_3;
- can react with the one-electron reductant $O_2^{\bullet -}$

$$ONOOH + O_2^{\bullet -} \rightarrow ONO^\bullet + HO^- + O_2$$

- can react with a transition-metal ion, e.g., ferrous ion

$$ONOOH + Fe^{2+} \rightarrow ONO^\bullet + HO^- + Fe^{3+}$$

- can react with CO_2 to yield the nitrating species $ONOOCO_2^-$.

Undoubtedly, the radicals formed ($ONO^\bullet$, ${}^\bullet OH$), the cation (ONO^+), the anions (NO_3^-, $ONOOCO_2^-$), and also the in situ generated molecule of oxygen may play an important role in physiological/pathophysiological processes.

Hydrogen Peroxide, H_2O_2, is simply produced from two superoxide anion radical species, which undergo spontaneous dismutation, producing the molecule of hydrogen peroxide—a non-charged non-radical oxidative species—and a molecule of oxygen

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

Therefore, once superoxide anion radicals are formed in situ, the presence of hydrogen peroxide becomes almost inevitable. Reaction 3 occurs spontaneously, especially at low pH values; however, in vivo, this reaction is catalyzed by a family of enzymes known as superoxide dismutase (SOD). The cytosolic SOD form contains Cu and Zn (CuZn-SOD), while a mitochondrial form contains Mn (Mn-SOD). In addition to SOD, another heme-containing enzyme, catalase, converts the hydrogen peroxide to oxygen and water.

Since both superoxide anion radical and hydrogen peroxide are simultaneously present within the same microenvironment, they may undergo the so-called Haber-Weiss reaction

$$H_2O_2 + O_2^{\bullet -} \rightarrow HO^\bullet + HO^- + O_2 \quad (4)$$

The Haber-Weiss reaction, although very often cited, is actually of low importance, since the product of reaction 3—the molecule of oxygen—and the products of reaction 4—$O_2 + HO^-$—inhibit both processes represented by reactions 3 and 4.

Hypochlorous Acid, HOCl, and/or *Hypochlorite*, ^-OCl. The molecule of hydrogen peroxide itself is a weak ("inert") oxidative species. The heme enzyme myeloperoxidase released from stimulated neutrophils is able to oxidize (pseudo)halides in the presence of hydrogen peroxide under formation of (pseudo)hypohalous acids.[8] In the case of Cl^-, hypochlorous acid will be formed

$$H_2O_2 + Cl^- \rightarrow HOCl + HO^- \leftrightarrow {}^-OCl + H_2O \quad (5)$$

Hypochlorous acid in aqueous solution is in equilibrium with chlorine and hypochlorite. Under neutral conditions, a mixture of HOCl and ^-OCl dominates. This "bleach" (HOCl/^-OCl) readily kills any invading microorganism.

It can be relevant to take into consideration that the HOCl molecule is a weak acid with pK_a = 7.53.[9] Under slightly acidic conditions, i.e., at, e.g., pH = 6.53, which may accompany inflammation, the [HOCl] to [^-OCl] ratio is very high, indicating the prevailing existence of the non-dissociated hypochlorous acid molecule (90%). One could thus hypothesize that in particular cases the HOCl molecules could serve as precursors for the formation of further oxidative species

$$HOCl + O_2^{\bullet -} \rightarrow {}^\bullet OH + Cl^- + O_2 \quad \text{(cf. refs 10, 11)}$$

$$HOCl + H_2O_2 \rightarrow {}^1O_2 + H_2O + H^+ + Cl^- \quad \text{(cf. ref 10)} \quad (6)$$

Hypochlorous acid is a powerful oxidizing and chlorinating species, formed at sites of chronic inflammation, capable of oxidizing proteins, DNA, lipids, and so forth, and/or of chlorinating DNA, cholesterol, lipids, and so forth. That is why HOCl-induced cell death occurs very rapidly in comparison to that mediated by other ROS. (In addition, once activated, myeloperoxidase is also able to oxidize a large variety of small molecules including amino acids, phenols, indoles, sulfhydryls, nitrite, xenobiotics, and other substances generating different reactive radicals and contributing thus to a progressive damage of biomolecules at inflammatory sites.)

Hydroxyl Radical, ${}^\bullet OH$, could in principle be the product of homolytic fission of the H_2O_2 molecule; however, in vivo, the direct route (→) of the reaction

$$HO{-}OH \leftarrow HO^\bullet + {}^\bullet OH$$

is not plausible. Yet, the probability of "heterolytic" fission of the H_2O_2 molecule with participation of a transition-metal cation (e.g., Fe^{2+}) should be taken into the consideration

$$HO{-}OH + Fe^{2+} \rightarrow {}^\bullet OH + Fe^{3+} + HO^- \quad (7)$$

Reaction 7, most frequently suggested as a source of free ${}^\bullet OH$ radicals in biological systems, is termed the Fenton reaction. Under physiological (healthy) conditions, the iron ions are however always firmly bound: In blood, they circulate associated with the protein transferrin, and in cells, they are stored linked to the protein ferritin. Yet under stress conditions, an increase of the so-called "labile iron pool" is observed in cells.

The ${}^\bullet OH$ radical can be classified as an "ultimate" reagent oxidizing almost all low- and high-molecular-weight substances. However, because of its extremely short half-life ($\sim 10^{-9}$ seconds), the action should be site-specific. The toxicity of ${}^\bullet OH$ radicals results from their ability to abstract electrons from a large variety of compounds

$$R + {}^\bullet OH \rightarrow R^\bullet + HO^-$$

with the formation of a new radical—$R^\bullet$—which consecutively can oxidize other substances.

Singlet Oxygen, $^1O_2$, is an oxygen form, which electrons are excited at a higher energy level compared to the normal triplet oxygen. When returning to the ground state, the molecules of singlet oxygen emit energy, which may have antimicrobial and cytotoxic effects.

There are several reactions in which the generation of singlet oxygen molecules occurs (reaction 6) or could be anticipated.

References and Notes

(1) Deeble, D. J.; Bothe, E.; Schuchmann, H. P.; Parsons, B. J.; Phillips, G. O.; von Sonntag, C. The kinetics of hydroxyl-radical-induced strand breakage of hyaluronic acid. A pulse radiolysis study using conductometry and laser-light-scattering. *Z. Naturforsch.* **1990**, *45c*, 1031.

(2) Li, M.; Rosenfeld, L.; Vilar, R. E.; Cowman, M. K. Degradation of hyaluronan by peroxynitrite. *Arch. Biochem. Biophys.* **1997**, *341*, 245.

(3) Vilar, R. E.; Ghael, D.; Li, M.; Bhagat, D. D.; Arrigo, L. M.; Cowman, M. K.; Dweck, H. S.; Rosenfeld, L. Nitric oxide degradation of heparin and heparan sulphate. *Biochem. J*. **1997**, *324*, 473.

(4) Hassan, M. S.; Mileva, M. M.; Dweck, H. S.; Rosenfeld, L. Nitric oxide products degrade chondroitin sulfates. *Nitric Oxide* **1998**, *2*, 360.

(5) Hancock, J. T.; Desikan, R.; Neill, S. J. Role of reactive oxygen species in cell signaling pathways. *Biochem. Soc. Trans.* **2001**, *29*, 345.

(6) Reutov, V. P.; Sorokina, E. G. Review: NO-synthase and nitrite-reductase components of nitric oxide cycle. *Biochemistry (Moscow, Russ. Fed.)* **1998**, *63*, 874.

(7) Parsons, B. J.; Al-Assaf, S.; Navaratnam, S.; Phillips, G. O. Comparison of the reactivity of different oxidative species (ROS) towards hyaluronan. In *Hyaluronan: Chemical, Biochemical and Biological Aspects*; Kennedy, J. F., Phillips, G. O., Williams, P. A., Eds.; Hascall, V. C., Guest Ed.; Woodhead Publishing Ltd: Cambridge, 2002; Vol. 1, pp 141−150.

(8) Arnhold, J. Properties, functions, and secretion of human myeloperoxidase. *Biochemistry (Moscow, Russ. Fed.)* **2004**, *69*, 4.

(9) Ullrich, O.; Reinheckel, T.; Sitte, N.; Grune, T. Degradation of hypochlorite-damaged glucose-6-phosphate dehydrogenase by the 20S proteasome. *Free Radical Biol. Med*. **1999**, *27*, 487.

(10) Halliwell, B. Production of superoxide, hydrogen peroxide and hydroxyl radicals by phagocytic cells: a cause of chronic inflammatory disease? *Cell Biol. Intern. Rep*. **1982**, *6*, 529.

(11) Lindvall, S.; Rydell, G. Influence of various compounds on the degradation of hyaluronic acid by a myeloperoxidase system. *Chem. Biol. Interact*. **1994**, *90*, 1.

(12) Fang, Y.-Z.; Yang, S.; Wu, G. Free radicals, antioxidants, and nutrition. *Nutrition* **2002**, *18*, 872.

(13) Martínez-Cayuela, M. Oxygen free radicals and human disease. *Biochimie* **1995**, *77*, 147.

(14) Halliwell, B.; Hoult, J. R.; Blake, D. R. Oxidants, inflammation, and antiinflammatory drugs. *FASEB J.* **1988**, *2*, 2867.

(15) Kvam, B. J.; Fragonas, E.; Degrassi, A.; Kvam, C.; Matulová, M.; Pollesello, P.; Zanetti, F.; Vittur, F. Oxygen-derived free radical (ODFR) action on hyaluronan (HA), on two HA ester derivatives, and on the metabolism of articular chondrocytes. *Exp. Cell Res.* **1995**, *218*, 79.

(16) Schiller, J.; Fuchs, B.; Arnhold, J.; Arnold, K. Contribution of reactive oxygen species to cartilage degradation in rheumatic diseases: Molecular pathways, diagnosis and potential therapeutic strategies. *Curr. Med. Chem.* **2003**, *10*, 2123.

(17) Lepperdinger, G.; Fehrer, C.; Reitinger, S. Biodegradation of hyaluronan. In *Chemistry and Biology of Hyaluronan*; Garg, H. G., Hales, C. A., Eds.; Elsevier Press: Amsterdam, 2004; pp 71−82.

(18) Meyer, K. Chemical structure of hyaluronic acid. *Fed. Proc*. **1958**, *17*, 1075.

(19) Balázs, E. A.; Laurent, T. C.; Jeanloz, R. W. Nomenclature of hyaluronic acid. *Biochem. J.* **1986**, *235*, 903.

(20) Šoltés, L.; Mislovičová, D.; Sébille, B. Insight into the distribution of molecular weights and higher-order structure of hyaluronans and some β-(1→3)-glucans by size exclusion chromatography. *Biomed. Chromatogr.* **1996**, *10*, 53.

(21) Armstrong, S. E.; Bell, D. R. Measurement of high-molecular-weight hyaluronan in solid tissue using agarose gel electrophoresis. *Anal. Biochem.* **2002**, *308*, 255.

(22) Ofman, D.; Slim, G. C.; Watt, D. K.; Yorke, S. C. Free radical induced oxidative depolymerisation of chondroitin sulphate and dermatan sulphate. *Carbohydr. Polym.* **1997**, *33*, 47.

(23) Ryabina, V. R.; Vasyukov, S. E.; Panov, V. P.; Starodubtsev, S. G. Obtaining, properties and application of hyaluronic-acid. *Khim.-Farm. Zh.* **1987**, *21*, 142.

(24) Hardingham, T. Solution properties of hyaluronan. In *Chemistry and Biology of Hyaluronan*; Garg, H. G., Hales, C. A., Eds.; Elsevier Press: Amsterdam, 2004; pp 1−19.

(25) Henrotin, Y. E.; Bruckner, P.; Pujol, J. P. L. The role of reactive oxygen species in homeostasis and degradation of cartilage. *Osteoarthritis Cartilage* **2003**, *11*, 747.

(26) Deeble, D. J.; Parsons, B. J.; Phillips, G. O.; Myint, P.; Beaumont, P. C.; Blake, S. M. Influence of copper ions on hyaluronic acid free radical chemistry. In *Free Radical Metal Ions and Biopolymers*; Beaumont, P. C., Deeble, D. J., Parsons, B. J., Rice-Evans, C., Eds.; Richelieu Press: London, 1989; pp 159−182.

(27) Rees, M. D.; Hawkins, C. L.; Davies, M. J. Hypochlorite and superoxide radicals can act synergistically to induce fragmentation of hyaluronan and chondroitin sulphates. *Biochem. J.* **2004**, *381*, 175.

(28) Corsaro, M. M.; Pietraforte, D.; Di Lorenzo, A. S.; Minetti, M.; Marino, G. Reaction of peroxynitrite with hyaluronan and related saccharides. *Free Radical Res.* **2004**, *38*, 343.

(29) Al-Assaf, S.; Navaratnam, S.; Parsons, B. J.; Phillips, G. O. Chain scission of hyaluronan by peroxynitrite. *Arch. Biochem. Biophys.* **2003**, *411*, 73.

(30) Stankovská, M.; Šoltés, L.; Lath, D.; Vikartovská, A.; Gemeiner, P.; Kogan, G.; Bakoš, D. Degradation of high-molecular-weight hyaluronan: a rotational viscometry study. *Biologia* **2005**, *60* (Suppl. Issue no. 17), 149.

(31) Katrantzis, M.; Baker, M. S.; Handley, C. J.; Lowther, D. A. The oxidant hypochlorite (OCl^-), a product of the myeloperoxidase system, degrades articular cartilage proteoglycan aggregate. *Free Radical Biol. Med*. **1991**, *10*, 101.

(32) Baker, M. S.; Green, S. P.; Lowther, D. A. Changes in the viscosity of hyaluronic acid after exposure to a myeloperoxidase-derived oxidant. *Arthritis Rheum*. **1989**, *32*, 461.

(33) Jahn, M.; Baynes, J. W.; Spiteller, G. The reaction of hyaluronic acid and its monomers, glucuronic acid and *N*-acetylglucosamine, with reactive oxygen species. *Carbohydr. Res*. **1999**, *321*, 228.

(34) Rees, M. D.; Hawkins, C. L.; Davies, M. J. Polysaccharide fragmentation induced by hydroxyl radicals and hypochlorite. In *Hyaluronan: Chemical, Biochemical and Biological Aspects*; Kennedy, J. F., Phillips, G. O., Williams, P. A., Hascall, V. C., Eds.; Woodhead Publishing, Ltd.: Cambridge, 2002; Vol. 1, pp 151−160.

(35) Rees, M. D.; Hawkins, C. L.; Davies, M. J. Hypochlorite-mediated fragmentation of hyaluronan, chondroitin sulfates, and related *N*-acetyl glycosamines: evidence for chloramide intermediates, free radical transfer reactions, and site-specific fragmentation. *J. Am. Chem. Soc.* **2003**, *125*, 13719.

(36) Mendichi, R.; Audisio, G.; Maffei-Facino, R.; Carini, M.; Giacometti-Schieroni, A.; Saibene, L. Use of size exclusion chromatography to study the protective effect of radical scavengers on oxygen free-radical-induced degradation of hyaluronic acid. *Int. J. Polym. Anal. Charact.* **1995**, *1*, 365.

(37) Myint, P.; Deeble, D. J.; Beaumont, P. C.; Blake, S. M.; Phillips, G. O. The reactivity of various free radicals with hyaluronic acid: steady-state and pulse radiolysis studies. *Biochim. Biophys. Acta* **1987**, *925*, 194.

(38) Al-Assaf, S.; Phillips, G. O.; Deeble, D. J.; Parsons, B.; Starnes, H.; Von Sonntag C. The enhanced stability of the cross-linked hylan structure to hydroxyl (OH) radicals compared with the uncross-linked hyaluronan. *Radiat. Phys. Chem.* **1995**, *46*, 207.

(39) Al-Assaf, S.; Meadows, J.; Phillips, G. O.; Williams, P. A.; Parsons, B. J. The effect of hydroxyl radicals on the rheological performance of hylan and hyaluronan. *Int. J. Biol. Macromol.* **2000**, *27*, 337.

(40) Lapčík, L. Jr.; Chabreček, P.; Staško, A. Photodegradation of hyaluronic acid: EPR and size exclusion chromatography study. *Biopolymers* **1991**, *31*, 1429.

(41) Kataoka, M.; Tonooka, K.; Ando, T.; Imai, K.; Aimoto, T. Hydroxyl radical scavenging activity of nonsteroidal anti-inflammatory drugs. *Free Radical Res*. **1997**, *27*, 419. Erratum in *Free Radical Res*. **1998**, *28*, 108.

(42) Orviský, E.; Šoltés, L.; Stančíková, M. High-molecular-weight hyaluronan − a valuable tool in testing the antioxidative activity of amphiphilic drugs stobadine and vinpocetine. *J. Pharm. Biomed. Anal.* **1997**, *16*, 419.

CDV

(43) Šoltés, L.; Lath, D.; Mendichi, R.; Bystricky, P. Radical degradation of high molecular weight hyaluronan: Inhibition of the reaction by ibuprofen enantiomers. *Methods Find. Exp. Clin. Pharmacol*. **2001**, *23*, 65.
(44) Štetinová, V.; Smetanová, L.; Grossmann, V.; Anzenbacher, P. In vitro and in vivo assessment of the antioxidant activity of melatonin and related indole derivatives. *Gen. Physiol. Biophys*. **2002**, *21*, 153.
(45) Praest, B. M.; Greiling, H.; Kock, R. Effects of oxygen-derived free radicals on the molecular weight and the polydispersity of hyaluronan solutions. *Carbohydr. Res*. **1997**, *303*, 153.
(46) Al-Assaf, S.; Hawkins, C. L.; Parsons, B. J.; Davies, M. J.; Phillips, G. O. Identification of radicals from hyaluronan (hyaluronic acid) and cross-linked derivatives using electron paramagnetic resonance spectroscopy. *Carbohydr. Polym*. **1999**, *38*, 17.
(47) Frati, E.; Khatib, A. M.; Front, P.; Panasyuk, A.; Aprile, F.; Mitrovic, D. R. Degradation of hyaluronic acid by photosensitized riboflavin in vitro. Modulation of the effect by transition metals, radical quenchers, and metal chelators. *Free Radical Biol. Med.* **1997**, *22*, 1139.
(48) Milas, M.; Rinaudo, M.; Roure, I.; Al-Assaf, S.; Phillips, G. O.; Williams, P. A. Comparative rheological behavior of hyaluronan from bacterial and animal sources with cross-linked hyaluronan (hylan) in aqueous solution. *Biopolymers* **2001**, *59*, 191.
(49) Stankovská, M.; Šoltés, L.; Vikartovská, A.; Mendichi, R.; Lath, D.; Molnárová, M.; Gemeiner, P. Study of hyaluronan degradation by means of rotational viscometry: Contribution of the material of viscometer. *Chem. Zvesti (Chem. Pap.)* **2004**, *58*, 348.
(50) Šoltés, L.; Stankovská, M.; Kogan, G.; Gemeiner, P.; Stern, R. Contribution of oxidative-reductive reactions to high-molecular-weight hyaluronan catabolism. *Chem. Biodiversity* **2005**, *2*, 1242.
(51) Mendichi, R.; Giacometti-Schieroni, A.; Grassi, C.; Re, A. Characterization of ultra-high molar mass hyaluronan: 1. Off-line static methods. *Polymer* **1998**, *39*, 6611.
(52) Mendichi, R.; Giacometti-Schieroni, A. Fractionation and characterization of ultra-high molar mass hyaluronan: 2. On-line size exclusion chromatography methods. *Polymer* **2002**, *43*, 6115.
(53) Cowman, M. K.; Mendichi, R. Methods for determination of hyaluronan molecular weight. In *Chemistry and Biology of Hyaluronan*; Garg, H. G., Hales, C. A., Eds.; Elsevier Press: Amsterdam, 2004; pp 41−69.
(54) Chabreček, P.; Šoltés, L.; Kállay, Z.; Guttmann, M. A method of preparation of the hyaluronic acid conjugates with biologically active compounds. Slovak patent 2761, 1990.
(55) Orviský, E.; Šoltés, L.; Stančíková, M.; Vyletelová, Z.; Juránek, I. Assessment of antioxidative properties of hydrophilic xenobiotics on the basis of inhibition of the radical degradation of hyaluronan by reactive oxygen species. Slovak patent 2764, 1994.
(56) Uchiyama, H.; Dobashi, Y.; Ohkouchi, K.; Nagasawa, K. Chemical change involved in the oxidative reductive depolymerization of hyaluronic acid. *J. Biol. Chem*. **1990**, *265*, 7753.
(57) Moseley, R.; Waddington, R.; Evans, P.; Halliwell, B.; Embery, G. The chemical modification of glycosylaminoglycan structure by oxygen-derived species in vitro. *Biochim. Biophys. Acta* **1995**, *1244*, 245.
(58) Moseley, R.; Waddington, R. J.; Embery, G. Degradation of glycosylaminoglycans by reactive oxygen species derived from stimulated polymorphonuclear leukocytes. *Biochim. Biophys. Acta* **1997**, *1362*, 221.
(59) Hawkins, C. L.; Davies, M. J. Direct detection and identification of radicals generated during the hydroxyl radical-induced degradation of hyaluronic acid and related materials. *Free Radical Biol. Med*. **1996**, *21*, 275.
(60) Hawkins, C. L.; Davies, M. J. Degradation of hyaluronic acid, poly- and mono-saccharides, and model compounds by hypochlorite: Evidence for radical intermediates and fragmentation. *Free Radical Biol. Med.* **1998**, *24*, 1396.
(61) Schiller, J.; Arnhold, J.; Arnold, K. Action of hypochlorous acid on polymeric components of cartilage. Use of ^{13}C NMR spectroscopy. *Z. Naturforsch*. **1995**, *50c*, 721.
(62) Servaty, R.; Schiller, J.; Binder, H.; Kohlstrunk, B.; Arnold, K. IR and NMR studies on the action of hypochlorous acid on chondroitin sulphate and taurine. *Bioorg. Chem*. **1998**, *26*, 33.
(63) Schiller, J.; Arnhold, J.; Benard, S.; Reichl, S.; Arnold, K. Cartilage degradation by hyaluronate lyase and chondroitin ABC lyase: a MALDI-TOF mass spectrometric study. *Carbohydr. Res*. **1999**, *318*, 116.
(64) Capila, I.; Sasisekharan, R. Methods for analysis of hyaluronan and its fragments. In *Chemistry and Biology of Hyaluronan*; Garg, H. G., Hales, C. A., Eds.; Elsevier Press: Amsterdam, 2004; pp 21−40.
(65) Lepperdinger G.; Kreil G. Functional, structural and biological properties of hyaluronidases. In *Chemistry and Biology of Hyaluronan*; Garg, H. G., Hales, C. A., Eds.; Elsevier Press: Amsterdam, 2004; pp 585−598.
(66) Chabreček, P.; Šoltés, L.; Orviský, E. Comparative depolymerization of sodium hyaluronate by ultrasonic and enzymatic treatments. *J. Appl. Polym. Sci., Appl. Polym. Symp.* **1991**, *48*, 233.
(67) Šoltés, L.; Mendichi, R.; Machová, E.; Steiner, B.; Alföldi, J.; Sasinková, V.; Bystrický, S.; Balog, K. Cyclodextrin derivative of hyaluronan. *Carbohydr. Polym*. **1999**, *31*, 17.
(68) Wong, S. F.; Halliwell, B.; Richmond, R.; Skowroneck, W. R. The role of superoxide and hydroxyl radicals in the degradation of hyaluronic acid induced by metal ions and by ascorbic acid. *J. Inorg. Biochem*. **1981**, *14*, 127.
(69) McCord, J. M. Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. *Science* **1974**, *185*, 529.
(70) Conner, E. M.; Grisham, M. B. Inflammation, free radicals, and antioxidants. *Nutrition* **1996**, *12*, 274.
(71) Halliwell, B.; Gutteridge, J. M. C. The antioxidants of human extracellular fluids. *Arch. Biochem. Biophys.* **1990**, *280*, 1.
(72) Halliwell, B.; Evans, P. J.; Kaur, H.; Chirico, S. Drug derived radicals: mediators of the side effects of anti-inflammatory drugs? *Ann. Rheum. Dis*. **1992**, *51*, 1261.

BM050867V