PART 1
POLYSACCHARIDES
Hyaluronic Acid: A Natural Biopolymer†

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Abstract
This chapter gives a brief overview on glycosaminoglycans, with a special focus on hyaluronic acid/hyaluronan – its structure, occurrence, and function, along with its broadly enlarging applications. Hyaluronan biosynthesis, catabolism, and degradation as well as its technological input in regenerative medicine where hyaluronan is applied at viscosurgery, viscoprotection, and viscosupplementation is presented as well. Also, special interest is focused on elucidating cellular mechanisms such as the effects of chemical pathways-driven oxidative stress.

Keywords: Glycosaminoglycans, hyaluronan biosynthesis, hyaluronan catabolism, hyaluronan oxidative degradation, inflammation, inflammatory mediators, regenerative medicine


†Dedicated to Dr. Grigorij Kogan in appreciation of his significant contribution to the studies of polysaccharides.

1.1 Glycosaminoglycans

Glycosaminoglycans (GAGs) are natural, very complex, unbranched, polydisperse polysaccharides composed of disaccharide units of D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) (keratan sulfate (KS) has galactose instead of uronic acid) linked to a D-glucosamine (GlcN) or D-galactosamine (GalN) residue (Figure 1.1). In general, GAGs are sulfated macromolecules [with the exception of hyaluronic acid (HA)] having different numbers of sulfate groups linked at different positions. They have very heterogeneous structures by considering relative molar mass, charge density and chemical properties generating various biological and pharmacological activities [1, 2]. Based on carbohydrate backbone structure, it is possible to distinguish four classes of GAGs: 1. HA, 2. KS, 3. chondroitin sulfate (CS)/dermatan sulfate (DS), and 4. heparan sulfate (HS)/heparin (Hep). HA is the only GAG containing an unmodified N-acetyl-D-glucosamine (GlcNAc)-GlcA repeating unit, while the other polysaccharides are generally modified through post-biosynthetic modifications, such as the addition of O-sulfo groups, C5-epimerization to form IdoA residues, and de-N-acetylation to produce GlcN-sulfo residues. These macro and micro modifications often play a key role in a wide variety of biological and pharmacological processes [1, 2].

GAG chains are covalently attached (with the exception of HA) at their reducing end to a core protein to produce macromolecules named proteoglycans (PGs) [3–5] localized at cellular and extracellular levels playing structural and regulatory roles due to their interaction with several proteins. In fact, PGs are not only structural components, but they participate in many cellular events and physiological processes, such as cell proliferation and differentiation, cell-cell and cell-matrix interactions [6–8] and are implicated in regulatory functions of development, angiogenesis, axonal growth, cancer progression, microbial pathogenesis, and anticoagulation [1, 2, 6–8] due to the specific interactions between structural GAGs and numerous proteins. As a consequence, these heteropolysaccharides are macromolecules of great importance in the fields of biochemistry, pathology and pharmacology.

KS was extracted and characterized for the first time from the bovine corneal stroma [9] and called KS-I having an alkali-stable bond between GlcNAc and asparagine. Skeletal KS with the alkali-labile bond between GalNAc and serine or threonine was designated as KS-II. This skeletal type has been further subclassified into articular, KS-IIA and KS-IIB [10], for the presence on the former of α(1-3)-fucose and α(2-6)-N-acetylneuraminic acid absent in the latter. The repeating disaccharide unit of KS [Gal (β1→4) GlcNAc (β1→3)], contains a galactose residue instead of uronic acid and the glycosidic bonds are reversed
in comparison with HA and CS/DS (Figure 1.1). Sulfate esters are present at the C-6 of one or both of the monosaccharides forming the disaccharide unit, but any other hydroxyl group may carry an esterified sulfate group.

CS and CS B, also known as DS, are constituted by the disaccharide unit \([\text{GlcA} \, (\beta 1 \rightarrow 3) \, \text{GalNAc} \, (\beta 1 \rightarrow 4)]_n\), variously sulfated in different positions of the hexosamine unit and/or uronic acid. Some, although relatively few, of these positions remain unsulfated. The regular disaccharide sequence of CS A, chondroitin-4-sulfate, is formed by the repeating unit sulfated in position 4 of the GalNAc unit, while CS C, chondroitin-6-sulfate, is composed of a disaccharide unit sulfated in position 6. Disaccharides with different numbers and
positions of sulfate groups can be located, in different percentages, inside the polysaccharide chains, such as the disulfated disaccharides in which two sulfate groups are O-linked in position 2 of GlcA and 6 of GalNAc (disaccharide D) or in position 4 and 6 of GalNAc (disaccharide E) [11].

In the case of DS, further enzymatic modifications complete the final structure, such as C-5 epimerization of GlcA to IdoA, and O-sulfation at C-2 of IdoA. As a consequence, polysaccharide chains of DS are formed of a prevailing disaccharide unit [IdoA (β1→3) GalNAc (β1→4)], with a minor concentration of disulfated disaccharides, in particular sulfated in position 4 of GalNAc and 2 of the IdoA unit [11] (Figure 1.1). These heterogeneous structures are responsible for the different and more specialized functions of these GAGs. Furthermore, IdoA imparts conformational flexibility to the DS chain altering the shape and spatial orientation of sulfate residues, endowing the chain with a higher negative charge density than the GlcA [11]. Although the principles of the biosynthetic process have not yet been fully elucidated, it is well known that this process results in the generation of highly modified oligosaccharide domains separated by regions of relatively low-degree structural modifications within the polymer chain. Thus, the DS chain has a hybrid co-polymeric structure consisting of low modified (CS) and highly modified (DS) domains [12]. The IdoA-containing units are often sulfated at C-4 of the GalNAc residue, while sulfation at C-6 is frequently associated with GlcA-containing disaccharides [13]. Twenty-three different CS/DS disaccharides have been identified so far [13].

Hep and HS possess a distinctly different repeating disaccharide structure compared with the previous GAGs [GlcA (β1→4) GlcNAc (α1→4)], (Fig. 1.1). Hep is sometimes considered to be synonymous with HS, but this is an oversimplification. They have been shown to differ in their degree of sulfation, with Hep being more negatively charged and displaying higher N- and O-sulfation than HS. They follow different biosynthetic paths in different cells and in different core proteins. The glycosidic linkage between uronic acid and GlcN is (β1→4) instead of (β1→3), and that between GlcN and uronic acid is (α1→4) instead of (β1→4) [6,7,14]. The growing GAG polymer chain is N-deacetylated and N-sulfated at the glucosamine residues, yielding regions in the chain particularly available for further structural changes, in particular C-5 epimerization of GlcA and O-sulfation mainly at C-2 of IdoA and C-6 of glucosamine [14]. Other more infrequent O-sulfations occur at C-2 of GlcA and C-3 of N-sulfated glucosamine. A few of the glucosamine amino groups may also remain unsubstituted. This process yields hybrid structures with hyper-variable, highly sulfated domains and poorly modified ones. As reported above, Hep has the highest charge density of any known biological macromolecule, while HS is generally less sulfated and possesses lower IdoA content. Both GAGs are highly polydisperse macromolecules, depending on tissue origin and status. Due to their properties, Hep and HS exhibit diverse biological functions and participate in a large number of interactions with other effective extracellular and cell membrane molecules, such as growth factors, virus proteins, enzymes, adhesion proteins, integrins, and thrombin/antithrombin [6, 7, 14].
1.2 Hyaluronic Acid/Hyaluronan – Structure, Occurrence

As stated above, hyaluronic acid (Figure 1.1), also called hyaluronan and sometimes presented as a hyaluronate (poly)anion from the chemical/structural viewpoint, is a non-sulfated GAG, while all other glycosaminoglycans are sulfated polysaccharides (cf. Table 1.1). Another fundamental and remarkable difference between HA and the other GAGs is the mean molar mass of the native biopolymeric chains. While the value of several MDa is the most common one for HA synthesized by hyaluronan sytheses (HAS1, HAS2, and HAS3), the molar mass of further glycosaminoglycans, on average, does not exceed the value of 50 kDa [8].

In aqueous solutions, at a physiological pH, HA is represented by negatively charged hyaluronate macromolecules ($pK_a = 3.21$) [15] with extended conformations. In a polyanionic form, hyaluronan functional groups make the biopolymer so hydrophilic that it binds 1000 times more water than is predicted from its molar mass. The heterogeneity and hydrophilicity of HA facilitate its interaction with a variety of tissue constituents inside and outside the cells. In the extracellular space, HA controls the retention of water, ionic and molecular diffusion and provides a 3D-structural meshwork [16].

HA is omnipresent in almost all biological fluids and tissues of the vertebrates, in which the highest amount is found in the extracellular matrix (ECM) of soft connective tissues [17]. In the skin, for example, there is slightly more than 50% of the total HA content present within the human body. The turnover

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*At the HA mean molar mass of about $1 \times 10^7$ Da, the polymer chain, if it is straightened, would exceed 15 μm
of hyaluronan in most tissues in the body is surprisingly rapid, with the exception of the vitreous body where its half-life is 20–70 days. Typical half-life in skin and pericardium is 2–5 days, whereas it is 0.5–1 day in joints and pleura, and 1–2 h in the anterior chamber of the eye. The synthesis of hyaluronan takes place locally in the tissues, while the breakdown is a process that partly takes place locally in the tissue, but also to a large extent in local lymph nodes and in the endothelial cells of the liver. The catabolism of hyaluronan takes place both by local degradation and drainage via the lymphatic system [18].

Generally, two fractions of hyaluronate macromolecules can be characterized in the living vertebrate organisms: (i) free, not associated with other tissue constituents such as proteins, and (ii) the protein associated fraction of HA [19].

1.3 Hyaluronan Synthases

Hyaluronan is the product of biosynthesis via articular chondrocytes and synovial cells on the inner surface of the plasma membrane-bound hyaluronan synthases (HASs) in eukaryotes. This fact has a great importance in physiology and pathology of joint inflammation and cancer progression [20, 21]. While the enzymes hyaluronidase-1 (HYAL1) and hyaluronidase-2 (HYAL2) have both predominant function in the HA catabolism and a high rate of this biopolymer turnover [17], HA itself is synthesized de novo in mammals by the three related integral membrane isoenzymes (HAS1, HAS2, HAS3) producing varying chain lengths [20, 22] in a dynamic continuous manner [21]. As atypical for glycosaminoglycans, HA is synthesized at the plasma membrane with the polymer being extended from the reducing end which results in its extrusion from the cell surface [23]. HASs utilize two sugar substrates, uridine diphosphate (UDP)-glucuronic acid and UDP-N-acetylglucosamine, to alternately add the corresponding UDP-sugar to the reducing end of the hyaluronan chain with release of the anchoring UDP [24]. Simultaneously with synthesis, the growing hyaluronan chain is translocated through plasma membrane into the extracellular space [25]. Being likely a part of a larger protein complex in a cell organelle regulating enzyme activity, the HASs, however, do not require primers when exerting their activity [17]. To perform specific biological purposes, HASs are responsible for the biosynthesis of various HA size distributions [26]. Depending on the catalytic rate and mode of regulation, HAS1 is the least active, and rules the HA synthesis from $2 \times 10^5$ to $2 \times 10^6$ Da; HAS2 is more active generating HA greater than $2 \times 10^6$ Da – this enzyme is probably responding to oxidative stress-induced increased HA synthesis. The enzyme is also involved in regenerative tissue processes and is also associated with cell migration, invasion, and proliferation. HAS3 is the most active HAS enzyme promoting the synthesis of HA chains from $0.2 \times 10^6$ to $2 \times 10^6$ Da. The pericellular glycocalyx might be provided by HAS3 products as well as the HA interacting with cell surface receptors [17]. The first HA synthase gene (42 kDa) from Group A Streptococcus bacteria was discovered in 1993 [27, 28]. The amount of HA synthesized in vivo is postulated that it should be strictly regulated by
a signal receptor CD44 which is a component of some feed-back mechanism [17]. The three HAS enzymes (Figure 1.2) are encoded by the three related tissue- and cell-specific HAS genes on three different chromosomes [29, 30]. Comparison of the deduced amino acid sequences exhibits a high degree of homology between the HAS enzymes, particularly HAS2 and HAS3. They all contain seven putative membrane-spanning domains, two of which are located at the N-terminal end of the molecules while the other five are located at the C-terminal end [31]. Regarding HA bioproduction, synovial cells preferentially utilize the HAS1 message, whereas chondrocytes and osteosarcoma cells the HAS2 message [20]. Cytokines such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin beta (IL-1β) may modulate HASs transcription activity and the variation of HA levels in normal and oxidative stress-driven fibroblasts. A synergistic effect was observed in normal fibroblasts when concomitantly exposed to the cytokines and oxidative stress; the increase

Figure 1.2 Regulation of hyaluronan amount and chain length by expression of a specific HAS protein. Biochemical characterizations of the vertebrate HAS enzymes expressed in mammalian cell culture have revealed similarities and differences between the respective mammalian hyaluronan synthase enzymes. The differences are depicted in this cartoon. HAS1 produces small amounts of high-molar-mass hyaluronan. HAS2 produces significantly more high-molar-mass hyaluronan. HAS3 is the most active of the hyaluronan synthases, yet produces low-molar-mass hyaluronan chains. The physiological significance of these differences in enzymatic activity is not yet known [33].
in HASs mRNA expression was detected. HASs stimulation-derived HA production in reactive oxygen species (ROS)-treated fibroblasts may represent a biological defending system against cell damage caused by ROS [32]. As to the HAS enzymes in synovial fibroblasts in osteoarthritic (OA) and rheumatoid arthritis (RA) patients, HAS1 mRNA is up-regulated by transforming growth factor (TGF-β), whereas HAS3 is up-regulated by TNF-α and IL-1β [17].

1.4 Enzymatic Catabolism of Hyaluronan

Hyaluronan degrading enzymes, the hyaluronidases (HYALs) are, in general, responsible for specific catabolic pathways involved in HA turnover. Karl Meyer was the pioneer scientist who described the in vivo occurrence as well as the biochemical functioning of HYALs [34]. The so-called Meyer's scheme consists of the three groups of HYALs due to their various endobiotic effects [35]. Bacterial HYALs, functioning as lyases, are β-endo-N-glucosaminidases, mammalian HYALs are of the same composition as the first ones, however, they function as hydrolases. The third group of HYALs, composed of hydrolitic β-endoglucuronidase, is related to the vertebrate enzymes. Isolation and characterization of HA degrading enzymes in eukaryotes was accomplished by Frost et al. [36]. Being present at subtle concentrations exhibiting high, however, unstable specific activities, HYAL enzymes activity is, during purification, usually kept by protease inhibitors and detergents [17].

Continuously ongoing HA turnover (5g daily from 15g of HA per 70 kg human individual) is a well-established phenomenon and is carried out by a rapid catabolic action of HYALs. HA catabolism is, in fact, realized via two simultaneously occurring mechanisms, the enzymatic and chemical (oxidative) scission whose correlative proportion is unknown.

HA catabolism/degradation results in the formation of different HA fragments. The parental biopolymer and the enzyme-mediated HA fragments, regardless of chain length, have both identical chemical structure whereas fragmented chains produced under oxidative stress contain e.g. aldehyde-, hydroperoxide-, and other chemical groups [17].

In humans, the HYALs constitute an enzyme protein family having a high degree of sequence homology. There are six genes tightly clustered at two chromosomal locations with HYAL-like sequences. The three genes, HYAL1, HYAL2 and HYAL3 coding for HYAL1, HYAL2 and HYAL3, are on chromosome 3p21.3 [37, 38]. They are organized in an extraordinarily complex and overlapping manner in an area densely packed with transcribed genes [39]. Regarding the cluster on chromosome 3p, HYAL1 (55 kDa glycoprotein monomeric unit) and HYAL2 (54 kDa glycoprotein monomeric unit) are probably the major eukaryotic hyaluronidases in connective tissues. HYAL1, being probably a lysosomal enzyme, cleaves HA predominantly to tetrasaccharides [37, 38, 40]. HYAL2 is attached to the plasma membrane via a glycosylphosphatidylinositol link. This enzyme cleaves high-molar-mass HA to approximately 20 kDa fragments [41, 42]. Widely expressed HYAL3 enzyme activity and function has not been
satisfactorily documented [43, 44]. The three genes, HYAL4, PHYAL1 and SPAM1 (Sperm Adhesion Molecule-1) are clustered in a similar fashion on chromosome 7q31.3, coding respectively for HYAL4, a pseudogene transcribed, but not translated in the human, and PH-20. The PH-20 enzyme facilitates penetration of sperm through the cumulus mass surrounding the ovum, and is also necessary for fertilization [45, 46]. It is a multifunctional enzyme protein which has a separate domain bound to zona pellucida. PH-20 can be detected in other positions in the male reproductive tract applying polymerase chain reaction analysis [47], the female genital tract [48], and moreover, in several malignancies [49, 50].

The β-endoglycosidase enzymes, HYAL1 and HYAL2, involved in HA catabolism in connective tissues, are the triggers of HA scission. High-molar-mass HA degradation to individual sugars (Scheme 1.1) is accomplished by the lysosomal exoglycosidases, β-glucuronidase, and β-N-acetyl-glucosaminidase. The HYAL enzymes and products of enzyme-like sequences have some other important functions – they also behave as receptors [51], or as adhesion/anti-adhesion molecules.

A pathway of intermediary metabolism is depicted in Figure 1.3 involving the HA catabolism. The cell surface hyaluronan receptor, CD44, two hyaluronidases, HYAL-1 and HYAL-2, and two lysosomal enzymes, β-glucuronidase and β-N-acetyl-glucosaminidase, are involved. This metabolic cascade begins in lipid raft invaginations at the cell membrane surface. Degradation of the high-molar-mass extracellular HA occurs in a series of discrete steps generating HA chains of decreasing sizes. The biological functions of the oligomers at each quantum step differ widely, from the space-filling, hydrating, anti-angiogenic, immunosuppressive 104-kDa extracellular biopolymer, to 20-kDa intermediate polymers that are highly angiogenic, immunostimulatory, and inflammatory. This is followed by degradation to small oligomers that can induce heat shock proteins and that are anti-apoptotic. The single sugar products, d-glucuronic acid and a glucosamine derivative are released from lysosomes to the cytoplasm, where they become available for other metabolic cycles [52].

1.5 Oxidative Degradation of Hyaluronan

There are basically two different reasons why the degradation of hyaluronan by free radicals is of interest: (a) many inflammatory diseases (for instance, RA or OA) are accompanied by the generation of free radicals that may lead to tissue damage [54] and (b) this is a frequently used method to generate smaller fragments of HA, i.e. with a defined number of polymer repeating units [55]. We especially focus here on the free radical-induced degradation of HA which is particularly useful if chemically modified HA is to be investigated because many modifications of HA (e.g. the introduction of sulfate groups) lead to partial or even complete inhibition of HA-depleting enzymes such as hyaluronidase(s) [56].
### Scheme 1.1 Hyaluronan catabolism.

'A proposed mechanism for HA catabolism: high-molar-mass extracellular biopolymer is gradually degraded into single sugars available to enter corresponding metabolic pathways.

Abbreviation: NHE1, Na⁺/H⁺ exchanger isoform 1.

Before some basic reactions are discussed in more detail, one short definition of the terms “free radicals” and “reactive oxygen species” is needed [57]: Basically, all “free radicals” are “ROS”, but not every “ROS” is a “free radical”. Free radicals are always paramagnetic compounds due to the presence of an
unpaired electron ("•"). Therefore, the compounds such as hypochlorous acid (HOCl) or hydrogen peroxide (H₂O₂) are ROS but do not represent free radicals because they lack the unpaired electron.

We focus here primarily on ROS that do also play a major role under in vivo, e.g. inflammatory conditions. Although there are many further species that can be generated by means of chemical reactions and are basically capable of degrading HA under in vitro conditions [58], these species are not discussed here in more detail. Additionally, superoxide anion radical (O₂•−), hydrogen peroxide (H₂O₂) and singlet oxygen (¹O₂) are not discussed. Although these are very important species, they possess only low reactivity and do not provide major oxidative damages to biomolecules.

The in vivo generation of all relevant species is discussed in the next paragraph, while some selected laboratory scale methods to study ROS generation are also discussed.

1.5.1 Reaction of HA with HO• Radicals

Hydroxyl radicals (HO•) represent one of the most reactive species and may be generated in vitro by the Fe²⁺ ion-induced decomposition of H₂O₂ [59], by the light-induced scission of H₂O₂ or by H₂O radiolysis [60]. Water hydrolysis is unequivocally the most specific method of HO• radical generation and the radical yield can be easily altered by the applied energy dose [61].

HO• radicals react in a diffusion-controlled manner (k ≈ 6×10⁹ l mol⁻¹ s⁻¹) with virtually all compounds containing C-H groups under the abstraction of one hydrogen (H•) radical [62] leading to the generation of the corresponding alkyl
radical. The reaction between HO\textsuperscript{*} and even simple carbohydrates such as glucose is very complex and not yet completely understood because there are many possible rearrangements of the initial products. A summary of these reactions is provided in [62]. The second order rate constant of the initial reaction between HA and HO\textsuperscript{*} radicals is about one order of magnitude (k = 7 \times 10^{8} \text{ mol}^{-1} \text{ s}^{-1}) smaller in comparison to glucose and other simple monosaccharides [63]. This is due to the high viscosity of high-molar-mass HA solutions that reduce the diffusivity of HO\textsuperscript{*} radicals according to the Stokes-Einstein equation.

The glycosidic linkage between D-glucuronic acid and N-acetyl-D-glucosamine repeating unit of HA is one preferred reaction site of HO\textsuperscript{*} radicals leading to HA degradation under retention of the structure of the monosaccharides. It was shown that the preference of the glycosidic linkage particularly holds if the \gamma-irradiation is performed in the solid state [64]. The reaction mechanism between HA and HO\textsuperscript{*} radicals can be explained as follows:

1. Abstraction of one hydrogen radical from a (widely unspecific) C-H group under formation of the corresponding alkyl radical. The H\textsuperscript{*} abstraction occurs nearly randomly and there is only a very slight preference for selected positions.
2. Addition of O\textsubscript{2} to the alkyl radical under generation of the corresponding peroxyl radical. The second order rate constant of the O\textsubscript{2} addition is very similar to the H\textsuperscript{*} abstraction, i.e. close to diffusion control.
3. Elimination of O\textsubscript{2}\textsuperscript{•−} from the initially generated peroxyl radicals.
4. Cleavage of the oxyl intermediate under generation of the corresponding carbonyl compound. This is one reason why the increase of carbonyl groups is often considered as a marker of "oxidative damage" [54].

The individual steps of the reaction are summarized in Scheme 1.2.

Methods allowing the precise determination of the molar mass were primarily applied to study the HO\textsuperscript{*} radical-induced degradation of HA. For instance, Šoltés and co-workers used rotational viscometry to study the effects of H\textsubscript{2}O\textsubscript{2} and Cu\textsuperscript{2+} on HA solutions [65] as well as the inhibitory ("scavenging") effect of selected ibuprofen isomers (ibuprofen is a commonly used analgesic and anti-inflammatory drug). Rotational viscometry is a very sensitive method and enables the detection of even very small changes of the polymer molar mass. In a similar way, the effect of \textalpha;-penicillamine on HA degradation was also studied [66]. It is shown that the application of \textalpha;-penicillamine results in two very different effects: there is an initial anti-oxidative action, but this effect is followed by the induction of pro-oxidative effects mediated by an enhanced generation of free radicals. It is assumed that this is a beneficial effect of \textalpha;-penicillamine because HO\textsuperscript{*} radicals are also strong inhibitors of proteases, which are believed to be responsible for the destruction of joint cartilage under chronic conditions of disease [67] for example.
Scheme 1: HO•-radical (generated by γ-irradiation) induced fragmentation of HA. Note that the glycosidic linkage is the preferred reaction site, whereas the remaining C-H groups react rather unspecifically.
SEC (size exclusion chromatography)/MALLS (multi-angle laser light scattering) [68] and HPLC (high performance liquid chromatography) [69], respectively, were also successfully used to study the radical-induced degradation of HA. Finally, NMR (nuclear magnetic resonance) spectroscopy is also a useful tool for analyzing HA degradation, although – in contrast to the above mentioned methods – the detection of the native HA is not possible due to the extreme line-widths high mass compounds are providing. In Figure 1.4, the influence of different γ-radiation doses on the 1H NMR spectra of high-molar-mass HA solution is shown.

Since the NMR method represents a form/concept of a “mobility filter”, less mobile, rigid molecules such as high-molar-mass HA are not detectable at all [70]. In contrast, NMR detects compounds more sensitively, the lower their molar mass is. The N-acetyl side chain of HA (at about 2.04 ppm) is a very good marker because this side group exhibits a relatively high mobility as it is not entrapped in the rigid carbohydrate ring system. It is evident that the intensity of this resonance increases upon γ-irradiation due to the induced HA degradation. However, it is also doubtless that the N-acetyl intensity decreases if very high γ-doses are applied and formate at 8.44 ppm is increasingly detectable. The formate is a well-known final product of the radiolysis of carbohydrates [62]. Therefore, it is evident that at high doses – in addition to the reduction of the molar mass – fragmentations of the pyranose ring systems also occur [70].

The NMR method is not only applicable for the characterization of the solutions of isolated HA, but also for the analysis of human body fluids, e.g. the synovial fluids from patients suffering from RA [71]: subsequently to γ-irradiation, increased N-acetyl intensities could be monitored [72]. Concomitantly, the intensity of formate at 8.44 ppm increased. It is one considerable advantage of NMR that both, high- and low-molar-mass compounds can be simultaneously detected. Of course, NMR also offers another additional advantage that even completely unexpected metabolites can be monitored [73]. The contribution of

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**Figure 1.4** 1H NMR spectra of aqueous solutions of HA after exposition to irradiation from a 60Co source. (a) no irradiation, (b) 5.15 kGy, (c) 10.3 kGy and (d) 20.6 kGy. Abbreviations: TSP, Trimethylsilyl-propionate (standard). Reprinted with (slight modification) permission from [70].
HO\textsuperscript{*} radicals to inflammatory processes in synovial fluids from patients with RA has been recently proven by ESR (electron spin resonance) as well as using spin traps such as 5,5-dimethyl-1-pyrroline-N-oxide to convert the highly reactive, short-lived HO\textsuperscript{*} radicals into a more stable, more simple detectable compound [74].

Some authors use a completely different approach: HA or its fragments were not directly detected, but the competition between HA and another (artificially added) compound for potential deactivating of HO\textsuperscript{*} radicals was used as the measure of reactivity. Among other methods, luminol-amplified chemiluminescence was applied [75]: luminol (5-amino-2,3-dihydro-phthalazine-1,4-dione) reacts with HO\textsuperscript{*} radicals under the emission of light. When luminol is present in excess over the generated radicals, the intensity of this light emission depends directly on the number of in situ generated radicals. When HA is added to the system, HO\textsuperscript{*} radicals are partially consumed by the reaction with HA. If the second order rate constant for the reaction between HO\textsuperscript{*} and luminol is known, the reactivity of the HA can be calculated by using a Stern-Vollmer plot [76]. The reactivities of different ROS with HA as well as with several other GAGs have been recently compared [77].

1.5.2 Reaction of HA with HOCl

HOCl is a molecular agent with strongly different reactivities. It exists as free acid as well as a salt and the ratio between HOCl and NaOCl is strongly pH-dependent [78]. Thus, the HOCl/NaOCl ratio can be easily altered by changing the pH value. It has been shown that there is a very gradual reactivity order if simple amino acids are considered [54]: cysteine or methionine are the most reactive amino acids due to their -SH or -S-S- groups and the second order rate constants of the reactions with HOCl are of the order of \(10^8\) M\(^{-1}\) s\(^{-1}\). An amino group exhibits much lower reactivity (\(k\approx10^4\) M\(^{-1}\) s\(^{-1}\)), whereas other functional groups are nearly inert against HOCl. Accordingly, in the repeating unit of HA, the glucosamine moiety represents the most reactive site [73].

This was proven by measuring the HOCl/NaOCl consumption by different isolated monosaccharides [79,80]. As time-dependent data are obtained, the relative second order rate constants can be easily calculated. As resulted from one earlier study, viscometry in combination with gel chromatography was used to evaluate (a) the effects of the reagent HOCl and (b) effects of the complete myeloperoxidase (MPO)/H\(_2\)O\(_2\)/Cl\textsuperscript{-} system on HA solutions [81]. It was shown that even very small HOCl concentrations (in the \(\mu\)M range) led to a considerable reduction of the HA viscosity, whereas much higher concentrations of HOCl were required to induce the polymer fragmentation. This discrepancy was explained by structural changes in the HA polymer matrix that do already occur in the presence of very small amounts of HOCl [81].

Using \(^1\)H NMR spectroscopy [82], and, a few years later \(^1^3\)C [79] NMR as well, it is evident that N-chloroamides are the prime products of the reaction between HA and HOCl. However, N-chloroamide is a transient product that decomposes under the generation of acetate, i.e. a cleavage of the N-acetyl side
chain occurs. This is an interesting result because the generation of acetate and formate enables the differentiation between the effects induced by HOCl and HO\(^*\) radicals, respectively. It was also shown that the acetate content is a potent marker of the MPO activity in the synovial fluid from patients with RA [83] and a close correlation between the corresponding NMR peak intensities and the MPO activity could be established.

The generation of the HA-derived N-chloroamide was later also confirmed by ESR [89]: it was shown that this initial product is converted into an N-centered radical that isomerizes into a C-centered radical located in the pyranose ring. This is the initial event for the reduction of molar mass of the HA polymer. A few years later, these results were extended by the same authors [84]: it was shown that the initially generated N-centered radicals undergo rapid intramolecular abstraction reactions to give C-centered radicals at the C-2 position on the N-acetylglucosamine rings (via 1,2-hydrogen atom shift), and at the C-4 position on the neighboring uronic acid residues (via 1,5-hydrogen atom shifts). The C-4-centered radicals, and analogous species derived from model glycosides, undergo pH-independent \(\beta\)-scission reactions that result in a glycosidic bond cleavage [84].

The stability of the initially generated HA-derived N-chloroamide has been, so far, controversially discussed. On the one hand, N-chloroamides are assumed to be "transient products" [82], and on the other hand, they were described to represent "long-lived species" [85]. This obvious discrepancy might result from the different experimental methods that were used: Schiller \textit{et al.} [82] used the NMR spectroscopy while Rees \textit{et al.} applied the TNB (5-thio-2-nitrobenzoic acid) assay [86] to monitor the stability of N-chloroamides. It is not yet clear, whether this assay is capable of differentiating N-chloroamides and N-chloroamines which are generated by the cleavage of the initially generated N-chloroamides. Secondly, the presence of small amounts of transition metals (present as impurities in the used buffers) also affects the stability of N-chloroamides.

Another study investigated final products of the HA degradation [87]: it was shown that the NaOCl oxidation of HA yields primarily \textit{meso}-tartaric acid. In contrast, arabinonic acid and glucaric acid are obtained by the oxidation of HA with the Fenton reagent. It was suggested that \textit{meso}-tartaric acid represents a useful biomarker of HA oxidation since it is produced by both HOCl and Fenton chemistry.

### 1.5.3 Reaction of HA with Peroxynitrite

Although by far less frequently investigated than HO\(^*\) radicals or HOCl, peroxynitrite is also capable of degrading HA. However, it is so far unknown if peroxynitrite or one of the products derived thereof are primarily responsible for the observed effects. An overview of the reactive species derived from peroxynitrite is shown in Scheme 1.3. Peroxynitrite-induced effects on HA are similar to the effects by HO\(^*\) radicals. Thus, it is assumed that peroxynitrite decomposes, under applied experimental conditions, yielding, among other
things, HO* radicals [88]. A more detailed investigation using spectroscopic methods as well as MS (mass spectrometry) was recently published [89]: surprisingly, neither NMR nor MS provided any evidence of a peroxynitrite-mediated modification of HA. On the other hand, simultaneously performed ESR experiments gave evidence of C-centered radicals that are most probably generated by the way of HO* radical-like reactivity of peroxynitrite [90]. The reasons for this unequivocal discrepancy are so far unknown.

Although the structures of HA and further GAGs are similar, it must be emphasized that NO* and particularly its derivatives are capable of cleaving heparan, heparan sulfate [91] and chondroitin sulfate [92]. In contrast, however, these ROS are not able to induce fragmentations of HA.

1.6 Hyaluronan Degradation under Inflammatory Conditions

The word "arthritis" (the disease that is the most important one regarding HA) is composed of the Greek words "arth" which refers to joints and "itis" which means inflammation. "Arthritis" is a general term for a group of different diseases that are characterized by the inflammation of one or more joints [54]. What does inflammation mean? From the viewpoint of a physician, the affected patient complains about the so-called "cardinal" indications of inflammation "color, dolor, rubor, and calor", indicating that the affected part of the body is painful, swollen, slightly reddish and feels warm [93].

Besides the activation of proteolytic enzymes, ROS primarily contribute to these symptoms, and they are also generated in many different cell types under stress conditions. For instance, in the inflamed joint, fibroblasts, chondrocytes, macrophages, and especially neutrophilic granulocytes are discussed as the most important sources of ROS [94]. Neutrophils are accumulated in the synovial fluid of the inflamed joints in huge amounts [95], although the prime
reasons for the accumulation and the mechanisms of activation of neutrophils to generate ROS and to release proteolytic enzymes are not yet completely clarified [96]. The increased oxygen consumption by neutrophilic granulocytes upon stimulation under inflammatory conditions is commonly termed "respiratory burst" [97]. A very coarse scheme of the events in the inflamed joint under inflammatory conditions is shown in Figure 1.5.

It is obvious that many various products are generated under these conditions that have different interactions. For instance, ROS generated under these conditions react with functional groups of enzymes that may significantly influence their enzymatic activities. Clearly, as the majority of ROS is generated by the enzymes, this fact also influences the yield of ROS. Thus, evaluation of the detailed role of ROS under physiological conditions is assuredly a rather difficult task.

1.6.1 Generation of ROS under In Vivo Conditions

The first step in the generation of all the ROS under in vivo conditions is the enzymatic reduction of "normal" oxygen forming \( \text{O}_2^- \) radicals catalyzed by NADPH oxidase (also termed "respiratory burst oxidase") [99]:

\[
2 \text{O}_2 + \text{NADPH} \rightarrow 2 \text{O}_2^- + \text{NADP}^+ + \text{H}^+ 
\]  \hspace{1cm} (1.1)

Although there are additional pathways of \( \text{O}_2^- \) generation, this is the most important mechanism under in vivo conditions. Please also note that

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**Figure 1.5** Proposed mechanism of cartilage degradation during rheumatic diseases: Neutrophils invade from the blood flow into the joint space. Upon stimulation they release different ROS and proteolytic enzymes. These damage-conferring products lead to the degradation of the high-mass components of articular cartilage under the formation of low-mass components. Reprinted with permission from [98].
Hyaluronic Acid: A Natural Biopolymer

$O_2^{•−}$ generation is always accompanied by $H_2O_2$ generation because $O_2^{•−}$ dismutate either spontaneously or in the presence of superoxide dismutase (SOD) yielding $H_2O_2$:

$$2O_2^{•−} + 2H^+ \rightarrow O_2 + H_2O_2$$ (1.2)

As $O_2^{•−}$ and $H_2O_2$ are simultaneously present, they are often assumed to react with each other (3):

$$H_2O_2 + O_2^{•−} \rightarrow HO^− + HO^+ + O_2$$ (1.3)

This reaction is the well-known Haber-Weiss reaction that is frequently mentioned in a physiological context and highly questioned [100]. Both $O_2^{•−}$ and $H_2O_2$ are rather slow-reacting species that are not capable of damaging carbohydrates such as HA as already outlined above [54]. These compounds are, however, deleterious in the presence of traces of transition metals, especially $Fe^{2+}$ [69]. Accordingly, $H_2O_2$ is the starting material for some further ROS formation, including singlet oxygen (4), hypochlorous acid (5) and hydroxyl radicals (6) [101]:

$$H_2O_2 + HOCl \rightarrow ^1O_2 + H_2O + HCl$$ (1.4)

$$H_2O_2 + Cl^- \rightarrow HO^− + HOCl$$ (1.5)

$$H_2O_2 + Fe^{2+} \rightarrow HO^+ + HO^− + Fe^{3+}$$ (1.6)

The last listed reaction (1.6) is the so-called Fenton reaction that is already known for more than 100 years but still possesses many mysteries [102]. In short, its biological significance is often indicated to be quite limited because under physiological conditions "free" iron does not exist, but all the iron is firmly bound to protein complexes: in the blood, iron is associated with the protein transferrin and in the cells with the protein ferritin [54].

The situation is even more complex, when species such as NO$^*$ are additionally considered. NO$^*$ and some derived species are analogously termed "reactive nitrogen species" (RNS) [103]. The reader interested in details of these ROS and RNS is referred to the timely review by Šoltés et al. [55].

A highly simplified scheme of the generation of physiologically relevant ROS and RNS is shown in Scheme 1.4 although we are focusing here on ROS.

1.6.2 Discussion of ROS Effects under In Vivo Conditions

It is extremely difficult to qualify a real role of ROS under in vivo conditions due to the following reasons:

1. There is normally a very complex environment comprising, in addition to HA, also a large variety of different proteins, low mass compounds such as amino acids or lipids as well as a variety of
Scheme 1.4 Generation of ROS and RNS under in vivo conditions. Note that this is a very simplified summary of potential chemical reactions that does not take into consideration the different locations of enzymes and their substrates. Reactions of HOCl and HO· with HA are emphasized in this chapter.

ions. Therefore, it cannot be expected that the results obtained under in vitro conditions with very simple systems can be transferred to the in vivo situation.

2. Under in vivo conditions, the majority of ROS is generated enzymatically in rather complex metabolic networks. This makes the assessment – which ROS is generated in what amount – very difficult. This particularly holds because there are also many effects of ROS on the related enzyme activities.

3. Finally, there are many antioxidants present in biological tissues that show important effects, not only antioxidative but also pro-oxidative effects.

1.6.3 Cell-derived Oxidants and Their Effects on HA

Neutrophilic granulocytes cannot be grown as a culture but must be freshly isolated from blood [96]. It is well-known that these cells generate ROS dependently on the conditions of stimulation. To treat HA solutions with the neutrophils and to see which fragments are generated, dependently on the conditions of cell stimulation, seems to be a reasonable approach. Such an experiment has already been performed in 1986 [104]: Using viscometry in combination with HPLC, the authors provided the evidence that there are comparable effects equally if a cell-free superoxide-generating system or a cellular system was used. However, as the results were obtained in the presence of biological buffers, it is questionable if the effects were really caused by superoxide and not by ROS derived thereof. It is, however, also remarkable that no major degradation of HA was observed if only the cell-conditioned media from PMA-(phorbol-myristoyl acetate)-stimulated neutrophils were applied.
Neutrophil-mediated HA degradation was shown to be increased in the presence of the MPO inhibitor azide [104] and is inhibited by dimethylsulfoxide [105] but not by metal chelators such as deferoxamine (DFO) or diethylentriamine pentaacetic acid (DTPA). Selected specific enzymes are able to prevent the degradation of HA by stimulated neutrophils [106]. Interestingly, the ability to degrade HA seems to be specific to PMA-stimulated PMNs and is not observed with neutrophils stimulated by other compounds such as formyl-methionyl-leucyl-phenylalanine, concanavalin-A or digitonin. This may be related to the increased concentrations of $H_2O_2$ generated in response to these stimuli, or a greater release of specific enzymes that consume the relevant reactive species [107].

In addition to neutrophils, endothelial cells were also used to degrade a variety of important GAGs. Although these cells were able to degrade heparin, that is very sensitive to a NO-dependent deaminative cleavage, HA is not susceptible to this pathway and thus, it can be concluded that endothelial cells are not capable of generating ROS that lead to the degradation of HA [91].

### 1.6.4 Synovial Fluids

Synovial fluids (SF) are highly viscous liquids that contain rather high amounts of high-molar-mass HA (normally about 2–3 mg/ml). Due to their profound biological significance, SF have been comprehensively studied. For instance, $\gamma$-irradiation of SF results in a dose-dependent degradation of HA [71, 72]. This effect can be effectively suppressed by common free radical quenchers such as $N$-acetyl-$L$-cysteine [108] and is, thus, mediated by ROS, not by enzymes.

Transition metal-dependent systems such as Fe$^{3+}$/O$_2$ or Cu$^+/O_2$ are also able to degrade the SF-present HA, and it has also been reported that even pure $H_2O_2$ is able to induce degradation of HA [109]. DFO and mannitol which were indicated to inhibit the degradation of purified HA do not prevent SF degradation, implicating site-specific degradation by metal ions that are not specific on HA. HA is also degraded by xanthine oxidase systems with the inhibitory effects of SOD and catalase, implicating effects of HO$^*$ radicals generated from O$_2$ in the presence of transition metal ions [110].

### 1.6.5 Extracellular Matrix

Oxidative damage to the extracellular matrix (particularly to cartilage), and its role in human pathologies has been recently comprehensively reviewed [111]. Although there are many important problems that must still be overcome, the extracellular compartments of most biological tissues are, in fact, significantly less well-protected against oxidative damage than intracellular sites. There is considerable evidence for such compartments being subject to a greater oxidative stress and an altered redox balance. However, with some notable exceptions (e.g. plasma) oxidative damage within these compartments has been neglected and is only poorly understood so far.
In particular, information on the nature and the consequences of the oxidative damage to ECM is lacking despite growing changes in the matrix structure. This can play a key role in the regulation of cellular adhesion, proliferation, migration, and cell signaling. Furthermore, ECM is widely recognized as being a key site of cytokine and growth factor binding. Modification of the matrix structure might be expected to alter such behavior. However, due to the limited available space, no major discussion can be given here and the interested reader is referred to one of the available, excellent reviews [111].

1.7 Interaction of Hyaluronan with Proteins and Inflammatory Mediators

There is increasing evidence that HA, as well as some further GAGs, play an active role in the development of a variety of diseases by their interaction with different cells and proteins [112]. Although the following list should not be expected to provide a complete survey, the aspects outlined below seem to be particularly important:

1. HA is able to interact in an autocrine manner with its cell surface receptors that are located on the same cell, i.e. the cell is influenced by a product produced by this cell itself. For instance, it has been recently shown [113] that low-molar-mass HA induces the proteolytic cleavage of CD44 from the surface of tumor cells and promotes tumor cell migration significantly in this manner.

2. HA interacts in a paracrine manner with a variety of ECM molecules on neighboring cells, and, due to the size of the HA polysaccharide, one HA molecule is capable of interacting directly with different cells, whereby one HA molecule is able to bind hundreds of ECM proteins. This interaction seems to be very important for the structure and the assembly of many different tissues, particularly for the development of cancer [114].

3. De novo synthesized HA may be secreted from the cell and subsequently interact with different cell surface receptors, such as CD44, the receptor for HA-mediated motility (RHAMM), the HA receptor for endocytosis (HARE) and many others. It has become evident that these processes mediate at least three different, very important physiological processes: signal transduction, formation of pericellular coats, and the receptor-mediated internalization.

A very short overview on the HA-binding proteins is given here, but due to the limited available space this should be regarded as only a very crude survey. The reader particularly interested in these aspects is referred to one of the excellent reviews that have recently appeared [115, 116]. Please note that due to the importance of HA oligosaccharides, HA-degrading enzymes, i.e. hyaluronidases, are now the focus of intense research.
1.7.1 HA Binding Proteins and Receptors

Hyaladherins are a relatively heterogeneous group of proteins with the common property that they are able to bind HA. These hyaladherins can be differentiated according to their localization, i.e. if they occur extra- (e.g. Versican, Aggrecan) or intracellularly (e.g. the CD44 family or RHAMM) or by the sequence of the HA binding site: The majority of the so far known HA binding proteins and receptors share a common 100 amino acid globular binding domain that is commonly called the “link module” and was first described in the context of the proteoglycans from articular cartilage that also contains a “link protein” mediating the interaction between a HA strand and the GAG. The link module region comprises two different regions: (a) an immunoglobulin domain and (b) two adjacent link modules. Nowadays it is widely accepted that (a) primarily mediates the link protein-proteoglycan interaction, whereas (b) mediate the binding to HA [117].

1.7.2 HA Receptors – Cellular Hyaladherins

Although other HA receptors are also known, we focus here exclusively on the most important ones, namely CD44 and RHAMM. CD44 is a very central molecule and seems to be involved in the development of many various diseases, mainly but not exclusively, different types of cancer. Although there are different isoforms that show different affinities to HA [118], CD44 is basically a transmembrane glycoprotein. Only a small part of the distal extracellular domain seems to be responsible for the ability to bind HA. Mutations of this region significantly decrease HA binding. The binding between CD44 and HA has been elucidated among other methods by means of crystallography and NMR spectroscopy [119]. The crystallography revealed that the CD44 interaction with HA is dominated by shape as well as hydrogen-bonding and identified two conformational forms of the receptor that differ from each other regarding the orientation of a crucial hyaluronan-binding residue (Arg45, equivalent to Arg41 in human CD44). Investigations by multi-dimensional NMR indicated that a conformational transition is induced by the HA binding. This seems a very important mechanism of CD44 regulation.

It must be explicitly stated that the HA binding is not specific at all and the CD44 interacts with many other molecules (often molecules that are, per se, capable of interacting with carbohydrates) such as osteopontin [120] or different matrix metalloproteinases [114]. CD44 is up-regulated by pro-inflammatory cytokines such as IL-1 and growth factors such as TGF-β or bone morphogenetic protein (BMP-7). HA-CD44 interactions participate in a large variety of cellular functions and these interactions have been summarized by Girish and Kemparaju [112].

Due to this significant importance, there were many attempts to influence HA-protein interactions: firstly, the overexpression of soluble hyaladherins that may displace HA completely from its endogeneous cell surface receptors [121]. Secondly, the administration of defined HA oligosaccharides that
compete with high-molar-mass HA for the binding site resulting in a replacement of the high affinity, polyvalent interaction by a low affinity, low-valency interaction. This strategy leads, for instance, to the inhibition of the \textit{in vivo} growth, local invasion and metastasis of melanoma cells [122]. Although the presence of smaller HA oligomers under \textit{in vivo} conditions is still rather speculative, it has been recently shown that HA fragments can be detected in a sterile UV-B induced inflammation under \textit{in vivo} conditions [123]. Thirdly, the treatment with antibodies that block HA-CD44 interactions, leads to the inhibition of tumor growth and invasion [124].

RHAMM is a HA receptor that can be present either on the cell surface, in the cytoplasm or even in the nucleus. Interactions between RHAMM and HA may trigger a lot of cellular signaling pathways, including those that involve protein kinase C, MAP kinases, phosphatidylinositol and tyrosine kinases [125]. Several recent studies have clearly demonstrated the involvement of RHAMM in the locomotion of TGF-β-stimulated fibroblasts, smooth muscle cells, macrophages as well as Ras-transformed fibroblasts [126], i.e. the processes that are most likely important in the development of cancer.

1.7.3 Extracellular Hyaladherins

These comprise a group of HA-binding proteoglycans that include, for instance, aggrecan and brevican. PGs are the components of ECM and show a characteristic distribution with versican present in different soft tissues, aggrecan predominately in the cartilage, while neurocan and brevican are primarily located in central nervous nerve tissues. We focus here exclusively on the study of cartilage tissue. HA stabilizes the ECM structure through its interaction with several matrix hyaladherins. The most important cartilage PG component, aggrecan, interacts strongly with HA through the HA-binding domain (link module). The binding of PGs to HA is largely mediated by the link protein. The attempts to understand such interactions are being currently intensively investigated [127]. However, it seems clear that such processes are also involved in the pathogenesis of cartilage-affecting diseases.

Finally, it is of interest to note that in addition to HA, other GAGs seem to also be involved in signal transduction events in the extracellular matrix. Shortly, a "GAG code" is coming into consideration [128] that is highly important in the tissue organization as well as in the development of diseases. Thus, many further related investigations may be expected in the future.

1.8 Hyaluronan and Its Derivatives in Use

The most distinctive property of HA is its viscoelasticity in the hydrated state which is able to vary with the rate of shear or oscillatory movement. In fact, the viscosity of a 1% HA solution of \((3-4) \times 10^6\) Da, is about 500000 times more than that of water at low shear rate, but can drop 1000-fold when forced through a fine needle. As a consequence, rapid movement reduces HA viscosity, and also
increases the elasticity, which stores energy and permits recovery from deformation [8]. The anomalous viscosity of HA solutions suggests that it should be an ideal biological lubricant, at least by reducing the work load during rapid movements. Indeed, the lubricant role of HA in the soft tissue lining of joints is well established [129], but its contribution to the stiff mass-bearing cartilaginous surfaces is less clear. Finally, both viscosity and elasticity properties are positively related, in a complex way, to molar mass and the concentration, a point that must be considered in the HA surgical and medical uses.

1.8.1 Viscosurgery

The main requirements for a solution to be used intraocularly at surgical interventions can be classified as follows: a) high viscosity at low shear rate to maintain space and manipulate tissues; b) moderate viscosity at medium shear rates to allow easy manipulation of surgical instruments and intraocular lenses within the polymer solution; c) very low viscosity at high shear rates to minimize the pressure needed to expel the solution through a thin cannula; d) high degree of elasticity to protect intraocular tissues, especially the endothelial cells of the cornea, from contact with surgical instruments; e) the pH and osmolality within the ranges 6.5–8.5 and 200–400 mOsm.

At the present, several companies are marketing various HA solutions, which compete for the attention of eye surgeons, and today the extraction and/or replacement of a damaged lens can be classified as a routine ophthalmologic intervention. Damage to ocular tissues might be hereditary-based or even occur during different pathophysiological events. For example, cataracts account for approximately 42% of all blindnesses and, due to the alarming prevalence of Diabetes mellitus worldwide, the diabetic cataract attracts ever-increasing attention [130, 131]. Today HA [Healon® (sodium hyaluronate), Pharmacia, Uppsala, Sweden] is used as a soft surgical instrument for cataract extraction, intraocular lens implantation, keratoplasts, glaucoma surgery, trauma and posterior segment surgery throughout the world.

1.8.2 Viscoprotection

The highly viscoelastic HA available for therapeutic purposes, has also proved to be very efficient in alleviating discomfort in “dry eye syndrome”. Although HA is not present in tears, in many aspects sodium hyaluronate is similar to mucin, a major component of tears. Mucin with a mean-molar-mass of about 2 MDa exhibits, similarly to HA, typical viscoelastic and shear-thinning behavior. This glycoprotein plays an important role in the lubricating, cleansing, and water-retaining properties of tears. The usefulness of a HA solution as a tear substitute resides in its water-entrainment capacity (hydration) and its function as a viscoelastic barrier between the corneal and conjunctival epithelia and noxious environmental factors (dust, smoke, etc.) [132]. During eye blinking, the HA eye drops are elastically deformed but not removed from the surface of the eye due to blinking movements.
1.8.3 Viscosupplementation

Osteoarthritis is one of the most common forms of arthritic diseases. It affects more than 10% of the world population. OA occurrence is age-progressive and, if not cured, it may result in severe disabilities. It has been claimed that 50% of people over 65 have evidence of OA of the knee, and nearly 100% of the population at the age of more than 75 have diagnosed changes in at least one joint [17].

The idea of intra-articular application of HA has been supported by the fact that the synovial fluid in OA joints lacks sufficient shock absorption and lubrication properties mostly due to the presence of HA of low mean-molar-mass, as a consequence of an absence of viscous high-molar-mass HA [133]. Thus, the so called “viscosupplementation”, a series of HA injections, has been designed to change the character of the joint fluid. Although the mechanism of the affection of HA injection(s) applied into the OA knee joints is not yet fully established [17], it is claimed that the viscosupplemented high-molar-mass HA increases the joint fluid viscoelasticity, stimulates the production of endogenous HA, inhibits the effects of inflammatory mediators, decreases cartilage degradation, and promotes cartilage matrix synthesis. Currently, HA injections (sodium salts) are approved for the treatment of OA in those patients who have persistent pains or are unable to tolerate conservative treatment or joint replacement. Viscosupplementation for other joints (e.g. shoulder) is currently being investigated.

1.8.4 Vehicle for the Localized Delivery of Drugs to the Skin

HA has also been investigated as a drug delivery agent for various routes of administration, including ophthalmic, nasal, pulmonary, parenteral, and topical. In fact, regulatory approval in the USA, Canada, and Europe has been granted recently for 3% diclofenac in 2.5% HA gel, Solaraze®, for the topical treatment of actinic keratoses, which is the third most common skin complaint in the USA [134]. The gel is well tolerated, safe and efficacious and provides an attractive, cost-effective alternative to cryoablation, curettage or dermabrasion, or treatment with 5-fluorouracil.

1.8.5 Electrospinning for Regenerative Medicine

Electrospinning techniques enable the production of continuous fibers with dimensions on the scale of nanometers from a wide range of natural and synthetic polymers [135]. The number of recent studies regarding electrospun polysaccharides and their derivatives, which are potentially useful for regenerative medicine, is dramatically increasing.

As a major component of the natural extracellular matrix, HA has also attracted considerable attention in electrospinning. However, it is very difficult to electrospin an aqueous HA solution because its unusual high viscosity and surface tension both hinder the electrospinning process. In addition, the
strong water retention ability of HA leads to the fusion of electrospun nanofibers on the collector due to the insufficient evaporation of the solvent during electrospinning. The fabrication of HA into nanofibrous membranes from aqueous solution was successfully carried out only after the development of blowing-assisted electrospinning (electro-blowing system) [136]. HA nanofibers were fabricated using a dimethylformamide (DMF)/water mixture, and the use of DMF significantly decreased the surface tension without changing the viscosity of the HA solution. HA/gelatin nanofibrous matrices can also be produced by this method.

HA-based nanofibrous membranes have been extremely attractive as biomimetic tissue engineering scaffolds and wound healing materials. In order to mimic the architecture of the natural ECM using electrospinning, a thiolated-HA derivative was synthesized and electrospun to form nanofibrous matrices [137].

1.9 Concluding Remarks

It could be hard to find such a unique biopolymer that exhibits the many excellent properties of hyaluronan/hyaluronic acid. Hyaluronan is, from a commercial viewpoint, a remarkably valuable biopolymer whose production is still growing through microbial fermentation. The research development has been focused on improving the key quality parameters – purity and molar mass. Having made exceptional progress over the last decade, metabolic engineering tools for hyaluronan production represent novel engineering challenges. Reflecting a variety of natural functions, hyaluronan has found a number of applications in, for example, medicine, cosmetics, and biotechnology. The investigation into the important role which hyaluronan plays in biological systems has recently led to numerous publication activities meeting all aspects of physiological and pathological areas of interest.

An enormous challenge still remains to reduce disease-derived human suffering if a biological specificity against deleterious actions of oxidants in living systems is better understood. The management of the pathogenesis of various diseases may eventually lead to the discovery of novel therapeutic and clinical strategies.

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