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Chapter 31

HYALURONIC ACID: A BIOPOLYMER WITH VERSATILE PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

G. Kogan[1], L. Šoltés[2], R. Stern[3] and R. Mendichi[4]

[1]Institute of Chemistry, Slovak Academy of Sciences, 84538 Bratislava, Slovakia
[2]Institute of Experimental Pharmacology, Slovak Academy of Sciences, 84104 Bratislava, Slovakia
[3]Department of Pathology, School of Medicine, University of California, San Francisco, CA 94143-0511, USA
[4]Istituto per lo Studio delle Macromolecole, Consiglio Nazionale delle Ricerche, 20133 Milan, Italy

ABSTRACT

Hyaluronic acid (hyaluronan, HA) is a linear polysaccharide formed from disaccharide units containing *N*-acetyl-D-glucosamine and glucuronic acid. Its molar mass can vary between 2×10^5 and 10×10^6 Da and its physiological properties are strongly influenced by its polymeric and polyelectrolyte character, as well by the viscous nature of its solutions. HA is abundantly present in almost all biological fluids and tissues and is also used as a diagnostic marker for many diseases including cancer, rheumatoid arthritis, and liver pathologies. In clinical practice it is widely used for supplementation of impaired synovial fluid in arthritic patients by means of intra-articular injections, as well as during certain ophthalmological and otological surgeries. In this paper, the functional roles of hyaluronan in living organisms, characterization of its polymeric properties, detection methods of HA degradation and of the resulting fragmentation products (e.g. viscometry, HPLC, mass spectrometry, NMR spectroscopy, light scattering, rheology, etc.), as well as the involvement of HA in various pathologies and inflammatory processes are reviewed. Finally, some important medical applications of HA will also be described.

Keywords: Hyaluronan, polysaccharide, viscosity, degradation, synovial fluid, arthritis, viscosupplementation.

INTRODUCTION

Hyaluronan (sodium hyaluronate, hyaluronic acid, HA) is a linear high-molar-mass, natural polysaccharide composed of alternating (1→4)-β linked D-glucuronic and (1→3)-β linked *N*-acetyl-D-glucosamine residues, Figure 1 [1]. The structure of HA is rather regular, the only deviation is a possible replacement of *N*-acetyl-D-glucosamine by deacetylated glucosamine residues.

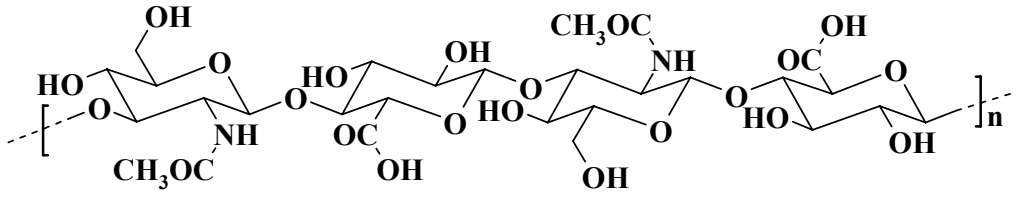

Figure 1. Structure of HA showing two repeating units.

HA is the simplest member of a group of substances known as glycosaminoglycans (GAGs). As the only GAG, it is not covalently associated with a core protein, not synthesized through the Golgi pathway, and the only non-sulfated one. The molar mass of HA can be as high as 10 MDa and such molar mass accounts for the important physiological roles of HA in living organism including maintenance of viscoelasicity of liquid connective tissues, such as synovial fluid (SF) in the joints or eye vitreous humor, control of tissue hydration, water transport, proteoglycan organization in the extracellular matrix (ECM), tissue repair, and various receptor-mediated functions in cell detachment, tumor development, and inflammation [2].

The HA macromolecule can adopt various shapes, sizes, and configurations, whereas under normal physiological conditions in the ECM it is believed to occur in a random coil form. It can circulate freely, or exist in a tissue-associated state. Due to its versatile biological properties and amazing viscoelastic properties, HA of both high and moderate or low molar mass has found numerous applications in medicine and in cosmetics preparations [3-5]. This chapter is focused on the occurrence and important biological functions of HA, methods of its detection and characterization, as well as some applications.

OCCURRENCE OF HA IN LIVING ORGANISMS AND ITS FUNCTIONS

Although HA occurs nearly ubiquitously (albeit in relatively small amounts) in the human body and in other vertebrates, the largest amounts of HA are found in the ECM of soft connective tissues [6]. Besides vertebrates, HA is also present in the capsules of some bacteria (e.g. strains of *Streptococci*), on the other hand, this polysaccharide is completely absent in fungi, plants, and insects. Although the history of the very first discovery of HA is

not established unambiguously, it was most probably the French chemist Portes who reported already in 1880 [7] that the mucin in the vitreous body which he named "hyalomucine" behaved differently from other mucoids occurring in cornea and cartilage. Later, a more detailed investigation was performed by Karl Meyer, who described isolation of high-molar-mass polysaccharide from the vitreous humor of cattle eyes and recognized a uronic acid and an aminosugar as its components [8]. He was also the first to propose the name "hyaluronic acid", from hyaloid (vitreous) and uronic acid.

The currently used term is "hyaluronan" and this name represents a combination of "hyaluronic acid" and "hyaluronate", in order to indicate the different charged states of this polysaccharide [9]. In the organism, HA occurs in many diverse forms, circulating freely, decorated with a variety of HA-binding proteins (hyaladherins), tissue-associated, inserted into the ECM by electrostatic or covalent binding to other matrix molecules. It constitutes a major portion of the glycocalyx that surrounds all cells. HA can be tethered to cell surfaces by any of a number of membrane-associated receptors. Recent evidence indicates that HA also exists within cells, though little is known of the form or function of such HA [10].

HA has also been assigned many functions. It may play a role of a space filler, an ion exchange filter, a medium for hydration, promoter of cell migration, a key to embryonic development, is involved in tissue repair and regeneration. HA molecules have size-specific functions such as mediation of inflammation, angiogenesis, promotion of wound healing, and induction of heat shock proteins. In this section, we review the occurrence and the variety of forms that HA molecules can adopt *in vivo*, as well as provide a brief overview of the many functions attributed to this simple, unadorned, however remarkable molecule. A comprehensive overview of the individual sources, from which HA can be isolated and the contribution of the potential impurities has recently been published [11]. A list of the most relevant sources of HA and its content is presented in Table 1.

Table 1. Occurrence of HA in different animal tissues and its content

Tissue or body fluid	*Concentration (μg/ml)*	*Remarks*
Rooster comb	*7500*	*The animal tissue with the by far highest HA content [12]*
Human umbilical cord	*4100*	*Contains primarily HA with a relatively high molar mass*
Human joint SF	*1400-3600*	*The volume of the SF increases under inflammatory conditions. This leads to a decreased HA concentration*
Bovine nasal cartilage	*1200*	*Used often as a cartilage model in experimental studies*
Human dermis	*200-500*	*Suggested as a rejuvenating agent in cosmetic dermatology [13]*
Human vitreous body	*140-340*	*HA concentration increases upon the maturation of this tissue*
Human epidermis	*100*	*HA concentration is much higher around the cells that synthesize HA*
Human thoracic lymph	*0.2-50*	*Low molar mass of this HA is explained by the preferential uptake of the larger molecules by the liver endothelial cells*
Human urine	*0.1-0.3*	*Urine is also an important source of hyaluronidases*
Human serum	*0.01-0.1*	*HA concentrations increase in serum from elderly people as well as in patients with rheumatoid arthritis and liver cirrhosis [14]*

LOCALIZATION OF HA IN THE ORGANISM

A major difference may exist between HA in vivo and HA chains that are extricated from the in situ situation. Very little is known about the properties of the HA within the ECM or in the restricted volume of the intercellular space. However, when HA is obtained by an aqueous extraction from major sources, such as from the rooster combs, from joint fluid, the umbilical cord (Wharton's jelly), or from bacterial capsules, the extraction fluid has very high viscoelasticity. Such HA is in a random coil conformation. However, HA is unlikely to be in such a conformation in vivo. It is probably much more structured, and probably has many additional functions that are unknown and lost when examined in vitro [15].

Hyaladherins

The HA-binding proteins are termed hyaladherins [16,17]. These constitute a wide range of proteins and glycoproteins, the majority of which are members of the superfamily of link proteins [18]. Various HA receptors, among which are CD44 and receptors for HA-mediated motility (RHAMM), are also hyaladherins. They secure the HA chain for its multitude of functions, and occur in a wide variety of isoforms. There is also a wide range of avidity of binding between HA and hyaladherins, from link proteins with a binding strength similar to that between avidin and biotin, to plasma proteins such as albumin [19-23] and fibrinogen [24], to which HA is loosely bound. HA also occurs covalently bound to proteins, as in inter-α-inhibitor [25].

Hyaladherins are also found intracellularly, where they may be involved in the intracellular and nuclear functions of HA. These include the cell-cycle control protein Cdc37 [26] and splicing factor SF2 [27]. In some cases, it may be the hyaladherins that confer HA-specific functions, rather than the strand of HA itself.

Extracellular Matrix

High-molar-mass HA serves as a structural support and scaffold, and is a central molecule in organizing the ECM. This was demonstrated in classic experiments by Knudson *et al.* (1993) [28]. COS cells, a line of African Green monkey kidney cells, produce virtually no ECM. If matrix components are added exogenously to cultures of CHO cells, nothing happens. However, if such cells are transfected with the cDNA for CD44, HA binds to the expressed CD44, and other matrix components then organize themselves around the tethered HA, resulting in the formation of a complex pericellular matrix.

Thus, many ECM polymers utilize strands of HA tethered to cell surfaces to organize complex structures. This is particularly prominent in the ECM of chondrocytes, where HA serves to bind aggrecan, other proteoglycans, and link protein [29]. Other proteoglycans that utilize HA as a scaffold are sometimes referred to as hyalectans [4].

Glycocalyx

There is an intimate glycocalyx that surrounds all cells, immediately adjacent to the surface plasma membrane, and quite separate from the general ECM. It has a vaguely filamentous ultrastructure and contains HA and heparan sulfate [30]. Multiple membrane tethers and receptors extend through the glycocalyx. Growth factors and extracellular molecules critical for signaling within cells are probably concentrated therein. This structure contains mechano-sensors, and is involved in macromolecular sieving, possibly functioning as an ion exchange resin. There is a highly developed glycocalyx on the apical surface of endothelial cells that extends into the vascular lumen [31,32]. The glycocalyx may be responsible for the blood-brain barrier, for the renal glomerular barrier, endowing it with selectivity and permeability, and may also control the rheology of the microcirculation. An argument can be made that the very nature of endothelial permeability is dependent on the HA and its associated molecules.

The ECMs of endothelial cells, embryonic cells, fibrous connective tissue and mesenchymal cells, mesothelial cells [33], peritumor stromal cells, the peri-oocyte cumulus cells, and stem cells have a high concentration of HA, suggesting that these may be variants of a glycocalyx, rather than a true ECM structure. The glycocalyx has many adhesion molecules, and the loss of adhesion as cells enter mitosis may be attributed to a change in their HA structure and content [34,35].

Intracellular HA

Intracellular HA is now well documented, though the functions of this material are unknown [10,36-38]. It occurs not only in cytoplasm, but also within nuclei and nucleoli. Intracellular HA may function in regulating the cell cycle, modulating the trafficking of specific kinases [39,40] thereby regulating cell behavior.

Another curious form of HA are the stress cables that appear to emanate from the peri-Golgi apparatus, with interweaving strands from multiple strands that are exuded, and to which inflammatory cells bind. These are documented in the inflammatory reactions associated with inflammatory bowel disease, including Crohn's disease and ulcerative colitis [41].

Biosynthesis and re-uptake of HA are rather puzzling. It is not clear whether the intracellular HA is a product of synthesis by that cell, or whether the HA has been taken up secondarily.

TISSUE-SPECIFIC LOCATIONS AND FUNCTIONS

Hematogenous HA

Circulating HA in the human organism occurs at very low levels, approximately 15 μg/liter, with a range of 4-40 μg/l [42]. Elevated levels of plasma HA have been observed in a remarkable variety of diseases, including malignancies, arthritis, scleroderma, psoriasis,

septicaemia, shock, in burn patients, and particularly in liver or kidney failure (for review see [43]).

In transplantation, levels of HA are often used in the early diagnosis of organ rejection. Liver and kidney are the two major organs involved in the final steps of total-body HA catabolism. When arteries to kidneys or liver are ligated, the circulating levels of HA increase immediately [44]. The HA concentration in the circulation of cancer patients is also often elevated [45].

Fetal Tissues

HA is also prominent in embryogenesis. It helps to maintain the undifferentiated state, and its removal by hyaluronidases is essential for the onset of differentiation [14]. There are very high levels of HA in the fetal circulation [46], as well as in amniotic fluid [47] and in fetal tissues. This may account, in part, for the immunosuppression of the fetus.

HA in Skin

Half the HA of the human body occurs in skin [48], with 0.5 mg/g wet tissue in the dermis and 0.1 g/g wet tissue in the epidermis, and has a rapid rate of turnover, with a half-life of 1.5 days [49]. Most of the HA in skin resides in the intracellular space, where it must have a concentration approaching 2.5 g/l. The major function of HA in skin is to maintain hydration and proper homeostasis of moisture. Interestingly, while the dermis consists primarily of extracellular matrix with a sparse population of cells, the situation in epidermis is reverse; the keratinocytes fill all but a few percent of the tissue. The actual concentration of HA in the matrix around the cells in the epidermis is about one order of magnitude higher than in the dermis: thus, the matrix around the keratinocytes may contain HA concentration as high as that in umbilical cord ($\approx$ 4 mg/ml) considered to be the mammalian tissue with one of the highest HA contents. HA is also present in many glands e.g. sweat glands [50] and their products.

Skin is a large complex tissue with a wide range of functions. Most importantly, it represents an interface with a hostile environment. A major function of skin is to protect underlying tissues from the harmful actions of the environment. These include ultraviolet (UV) light, pollutants, infectious organisms, and oxidative stress. The UV rays from sunlight are generators of harmful oxygen-derived species including a range of free radicals. It is apparent that HA in skin is an absorber, scavenger, or "sink" for such free radicals, as is discussed in another part of the chapter.

HA in Cartilage

The collagenous network of the articular cartilage matrix is filled with hyaluronan-dependent aggregates of proteoglycans. The large cartilage proteoglycan (often termed "aggrecan") binds HA with a specific site close to its *N*-terminus [51]. The attachment is stabilized by a link protein that binds both aggrecan and hyaluronan [52]. A link protein-

aggrecan complex occupies 24-30 monosaccharide residues on the HA chain [53]. Though HA is present in reasonable quantities in cartilage (about 0.2-2 mg/g wet weight), there have been technical difficulties in its analysis due to the prevailing amounts of other GAGs.

While representing only a minor portion of the tissue, HA serves as an essential structural element in the matrix. However, aggrecan is present at a much higher concentration (25-50 mg/g wet weight). Therefore, "free" HA does not play a major role in cartilage.

HA in Joint Fluid

SF in the joint capsule has a very high HA content, where it serves as both a lubricant and as a shock absorber. The level and molecular size of HA decreases with inflammatory and degenerative forms of arthritis. A major commercial use of HA, in a modified form, is to replace such degraded joint HA, as discussed in another part of this review.

HA in Brain

The ECM of the adult brain tissue has a unique composition. The striking feature of this matrix is the prominence of lecticans (a family of chondroitin sulfate proteoglycans, comprising aggrecan, versican, neurocan, and brevican) that contain a lectin domain and a hyaluronic acid-binding domain. In addition, HA is also abundant. Matrix proteins common in other tissues are nearly absent in adult brain. The brain ECM appears to have trophic effects on neuronal cells and affects neurite outgrowth.

HA in Cancer

The levels of HA surrounding tumor cells correlate with tumor aggressiveness [54]. The HA does not necessarily come from the tumor cells, but is often provided by the surrounding peri-tumor stroma. The stromal HA also correlates with tumor aggressiveness and poor prognosis [55]. HA is an effective space-filler and tissue expander, opening up spaces for cancer cell movement and metastasis, as well as stimulating anchorage-independent growth [56]. Interaction of HA with cancer cell surface receptors enhances tumor cell survival and invasiveness [57,58]. It is clear that the interaction of HA with the malignant process occurs at multiple levels.

MULTIPLE FUNCTIONS OF HA

The large volume occupied by high molecular size HA chains, including the aqueous solvent volume underlies the ability to distend and maintain extracellular spaces and tissue hydration. In fact, the moisture of skin is attributed to the rich HA content of the dermal compartment. For example, HA is present in significant amounts in hyaline cartilages, enough

to fill the tissue volume in the absence of other constituents. Therefore, HA has been assigned various roles in the homeostasis of the extracellular space [17].

Steric Interactions with other Molecules

At physiological concentrations, the HA molecules are entangled and form random networks. It can be easily understood that such networks interact sterically with other macromolecular components present in the corresponding tissue. Accordingly, HA excludes other molecules, especially the larger ones, from the network space. The high concentration of HA and the resulting considerable viscosity of its solutions also retard the diffusion of other molecules, for instance, inside and outside cartilage [59].

The determination of the diffusion behavior of larger molecules in a given tissue prior to and after the digestion with hyaluronidase is an established method to investigate the limitations of diffusion in a given tissue [60].

The fetal circulation contains high levels of HA, as do fetal tissues, and amniotic fluid [47]. Some of the immune suppression of the fetus is provided by HA, which, as a space filler, sequesters immune cell receptors and prevents interactions with ligands.

At the cellular level, bursts of HA synthesis correlate with the onset of mitosis [34,35,61]. This disengages the cell from the ECM and tissue organization, and prepares the cell for the semi-autonomous situation required for cell division. At the completion of mitosis, or at the beginning of G_0 phase of the cell cycle, a burst of hyaluronidase expression may occur, removing the shell of pericellular HA, preparing the cell for re-association with the ECM and the social contract. This hypothesis, however, has, to date, not been tested in a synchronized cell culture system. Below some of the most important physiological roles of HA in living organism are presented in brief.

Maintaining Matrix Structure

HA plays an extremely important structural role in cartilage and other connective tissues. For instance, the most important cartilage proteoglycan (PG), aggrecan, is bound specifically to HA chains with the help of special link proteins. The formed aggregates have masses of about 100 MDa and are deposited within the collagen framework in a highly ordered manner [62].

It is commonly accepted that without the PG/HA interaction, the PGs would not be retained within cartilage. Since the principal task of the negatively charged polymers of the proteoglycans is the binding of water, absence of HA would result in a loss of water and subsequently in a loss of the mechanical properties of cartilage [63]. The interaction with collagen is, however, primarily mediated by the keratan and chondroitin sulfates.

HA Fragments of Varying Lengths Have Size-Specific Functions

Despite their exceedingly simple primary structure, HA polymers have extraordinarily wide-ranging and often opposing biological functions. Some of these functions are size-

specific. HA ranges from large matrix polymers of 10^4 to 10^7 Da size that are space-filling, anti-angiogenic [64], and immunosuppressive [65,66], to intermediate-sized, comprising 25-50 disaccharide fragments, that are inflammatory [67], immunostimulatory, and highly angiogenic [68], to decasaccharides that compete with larger fragments for receptors [52], and to even smaller tetrasaccharides that are anti-apoptotic and inducers of heat shock proteins [69]. These low-molar-mass oligosaccharides appear to function as endogenous danger signals. Some of the variably sized fragments trigger different signal transduction pathways. Thus, the unadorned HA polymers have size-specific functions that constitute a very information-rich system. A recent paper by Stern et al. [70] renders a review of the size-dependent activities and functions of HA oligomers and polymers as well as the difficulties associated with the study of the short HA fragments.

Lubrication

Concentrated solutions of HA possess extraordinary rheological properties: HA solutions exhibit viscoelastic properties, i.e. their viscosities are strongly dependent on the applied shear-stress. Accordingly, rotational viscometers and oscillatory rheometers are the instruments of choice to characterize the macroscopic properties of HA solutions [71]. The shock absorbing properties of SF are based primarily on the viscoelasticity of its main constituent, HA.

Signal Transduction and Cell-Cell Interaction

The interactions of HA with its CD44 receptor is one of the most studied interactions in the field. A vast variety of signal transduction pathways are initiated by that interaction [72]. CD44 also interacts with the cytoskeleton, and as with RHAMM, can confer motility upon cells. Thus, in cell movement HA is involved at two levels: opening up tissue spaces, through which cells may move, particularly embryonic and tumor cells, as well as enabling the cells to move. RHAMM is also involved in a network of signal transduction pathways, and can exist not only upon the cell surface, but also in multiple intracellular forms [73]. Of recent interest is the observation that RHAMM may substitute for CD44 when the latter has undergone genetic deletion [74]. It has been also recently shown that HA as well as its degrading enzymes play important roles in the fertilizing process of the oocyte with sperm [75].

The Role of Pericellular HA

The existence of HA layer covering certain cells (e.g. chondrocytes and epithelial cells) is known since a long time [76] and has attracted considerable research interest because this coat protects the cells against lymphocytes and viruses. This is maybe the reason why *Streptococci* strains represent important sources of HA: These cells are protected from macrophages by an envelope of HA. Interestingly, it could also be shown that the formation of HA coats can be induced in cells with HA receptors on their surface by the simple addition of exogenous HA [77].

It has been also suggested that the HA cover of chondrocytes protects these cells against the attack of reactive oxygen (ROS) as well as reactive nitrogen species (RNS) [78] that play an important role in tissue destruction during inflammation.

BIOLOGICAL SOURCES OF THE EXPERIMENTALLY USED HA

As has been described above, HA is an essential functional component of almost all tissues in the vertebrate organism. Thus, various animal tissues – e.g. rooster combs, shark skin, bovine eyeballs – have been used as sources of isolation and production of high-molar-mass HAs. Since in the biological materials HA is usually present in a complex linked to other biopolymers, several separation procedures have to be applied in order to obtain a pure compound, such as protease digestion, HA ion-pair precipitation (with e.g. cetylpyridinium chloride), membrane ultrafiltration, HA non-solvent precipitation and/or lyophilization [79,80]. The mean molar mass of the commercially available "extractive" HA preparations obtained from animal tissues is mostly in the range from several hundred thousands Da up to approximately 2.5 MDa. To date, the demand for HA materials approved for applications in human medicine has been satisfied by high-molar-mass HAs prepared from the rooster combs. For example, Healon® (Pharmacia and Upjohn, Inc., Peapack, NJ, USA) – used in viscosurgery at eye implant insertion – has a mean HA molar mass of about 2.5 MDa.

Although animal tissues, primarily rooster combs, were involved at the early stages of production of the clinically utilizable materials approved by the Food and Drug Administration (FDA), e.g. in eye surgery (Healon®), HA secreted by microorganisms such as *Streptococcus zooepidemicus, S. equi*, *etc*. is currently offered by many companies at the market in the amount of several tons per year, as well. Some of these "fermentative" HA preparations meet the demand on molar mass in the range of several MDa [81,82]. However, the risk of mutation of the bacterial strains, possible coproduction of various toxins, immunogens, *etc.* hamper the broader application of fermentative HA in clinical practice. This is also the reason why HA samples originating from rooster combs are still currently more preferred for human treatment in cases when the HA material is to be introduced into the organism, e.g. in the eye, knee joint, *etc*. Microbial HA has been approved for treatment of superficial wounds, as well as for the use in cosmetic industry.

The mean molar mass of the commercially available HAs covers a wide range, yet this parameter is often insufficiently specified for the marketed HA biopolymers. Moreover, a frequently neglected fact is that both fermentative and extractive HAs may contain certain contaminating ingredients. A trace amount of proteins, e.g. in extractive HA samples originates usually from the link proteins. The presence of embedded water along with traces of transition metal cations in HA samples could pose a potential risk of the reduction of the high molar mass of hyaluronans (even of those stored in the solid form) most probably due to their degradation by atmospheric oxygen [83] and subsequent change of their properties. Not only the ubiquitous bacteria or molds, but also the accompanying contaminating substances (proteins, metal cations, *etc*.) must be critically assessed for their potential to degrade the HA polysaccharidic chain.

Under homeostatic conditions, HA exists as a high-molar-mass polymer that plays important roles in maintaining structural integrity of the tissue. Under stress conditions, such

as tissue injury, HA becomes degraded and a significant amount of lower molar mass fractions appears. The biological properties of these HA fragments are different from those of the larger precursor molecules. While the high-molar-mass HA possesses anti-inflammatory, anti-angiogenic, and immunosuppressive properties, intermediate-sized fragments act predominantly in an opposite way, i.e. they are highly angiogenic, inflammatory, and immunostimulatory. Therefore, recently Camenisch and McDonald [84] have pointed out the necessity to control the biological activity of commercial "intact" extractive and fermentative HAs of different molar masses, as well as that of the HA fragments prepared by either physico-chemical methods or by partial digestion with hyaluronidases. They also proposed to validate the identity/differences of HA samples by a set of certain bioanalytical procedures.

BIOLOGICAL ACTIVITY OF HA AND ITS USE IN MEDICINE

HA has been used in diverse fields in medicine. HA as well as its breakdown products have a range of properties that lend it specifically to medical applications. It is non-antigenic and is manufactured in a wide range of forms, ranging from gels, tubes, sheets of solid material, to lightly woven meshes. HA engraftment is superior to most other biotechnologies. HA and its derivatives have enormous promise in a wide range of medical applications, in tissue engineering, for drug delivery, in the ability to enhance angiogenesis and improve the quality of healing wounds, and as a medical grade structural support and material for augmentation and viscosupplementation. It is biodegradable, biocompatible, and has both supportive and hydrating effects that are ideal for human use.

The uses of HA and its derivatives can be summarized in the following manner [15]:

1) viscosurgery - to protect delicate tissues and provide space during surgical manipulations, as in ophthalmological surgeries,
2) viscoaugmentation - to fill and augment tissue spaces, as in skin, sphincter muscles, vocal and phayngeal tissues,
3) viscoseparation - to separate connective tissue surfaces traumatized by surgical procedures or injury, in order to prevent adhesions and excessive scar formation,
4) viscosupplementation - to replace or supplement tissue fluids, such as replacement of synovial fluid in painful arthritis, and to relieve pain,
5) viscoprotection - to protect healthy, wounded, or injured tissue surfaces from dryness or noxious environmental agents, and to promote the healing of such surfaces.

Chemical Modifications of HA

Because of its unique physicochemical properties and distinctive biological qualities, this polyanionic polymer is enormously versatile. The naturally occurring material is unfortunately, too soluble and is too rapidly degradable, hence certain modifications are necessary for its practical use. There are several functional groups on HA that are exploited for chemical modifications for use in the preparation of medical materials. Such

modifications are too extensive to review here, but are accessible in excellent overviews (e.g. [85]).

Applications of HA in Clinical Practice

The overwhelming majority of medical applications of HA is based on the viscoelasticity of the concentrated HA solutions. Viscoelasticity means that the fluid can absorb mechanical impact by elasticity or dissipate it by viscous flow. Viscoelasticity is characteristic for body fluids with higher HA concentrations of a considerable molar mass, especially synovial fluid and the vitreous liquid of the eye.

The individual HA molecules are present in solutions in random coil conformation and occupy large hydrated volume. This depends on the molar mass of HA as well as on its concentration: When the concentration increases, the motion of the molecular segments becomes more restricted [86]. As an important consequence, such crowded molecular system has significant viscous and elastic properties. The biological role of the HA in vitreous body and in the joint was interpreted according to these rheological properties [87].

Due to a limited space of this review, only a few applications will be discussed here. The interested reader is referred to the more extended discussions in [15] or [88], where medical applications of HA with low or high molar mass are comprehensively described.

Dermatology and Plastic Surgery

HA occurs in high concentrations within skin, where it has been extensively studied. Approximately 50% of body HA occurs in the skin, with most occurring in the dermis [48]. The moisture of skin is attributed in part to its HA content. With increasing age, HA does not decrease. Instead, the proportion of tissue bound HA increases, as the freely soluble HA decreases [89].

Preparations of slightly cross-linked HA are currently commonly used for augmentation, to fill facial wrinkles and depressed scars. Such HA gels are more effective in maintaining cosmetic corrections than collagen-based products [90]. Restylane®, produced by the Medicis Corp. (Scottsdale, AZ, USA) is prominent among such HA-based injectable materials [91]. Unlike collagen-based fillers, HA is extremely elastic, providing the elasticity required by spaces in which it is injected, such as facial wrinkles and depressed scars, at vocal cord augmentation, laryngeal and glottal reconstruction, or sphincter muscle support. The HA preparations are also longer lasting.

Obstetrics and Gynecology

Wharton's jelly found in the umbilical cord is one of the major sources of vertebrate HA. The HA occurs in the perivascular space surrounding the three umbilical vessels. The abundance of HA in that structure and its associated enormous volume of water serves perhaps as a protective mechanism against ties and strictures of the cord. The HA may have

evolved as a selective mechanism, as a survival technique protecting against strangulation of the fetus.

HA cross-linked with ferrous ions (Lifecore. Biomedical Inc. Chaska, MN, USA) forms a viscous solution that is used intraperitoneally after gynecological surgery to protect against post-surgical adhesions.

Ophthalmology

HA is a major component of the vitreous body, and a key macromolecule in ophthalmology. Because of its viscoelastic properties, HA is used in a number of key ophthalmologic surgeries. Preparations of HA protect delicate eye tissues and provide space during surgical manipulations. Its major use, however, is as a substitute or replacement for the vitreous fluid lost during procedures such as cataract surgery or lens placement. For many years, Healon®, derived from rooster combs, manufactured initially by Biotrics, Inc. (Arlington, MA, USA) and later by Pharmacia, Sweden, was utilized for this purpose. This preparation was also used as a viscoelastic protector of the corneal endothelium during corneal transplantation. These cells do not regenerate after being damaged. Currently, a number of preparations, of varying molecular size HA chains, are available, including an HA and chondroitin sulfate combination, termed Viscoat® (Alcon Labs, Inc., Fort Worth, TX, USA).

HA is found as a component of the normal ocular surface, in tears, lacrimal tissues, conjunctiva, and cornea, and is frequently used in the treatment of ocular surface disorders. The HA is used either alone, or in association with other molecules. In addition, hylans, a family of cross-linked high-molar-mass HA derivatives, have been used in artificial tear preparations for the treatment of dry eye disease, *Keratoconjunctivitis sicca.* HA has been also used in assisting excimer laser surgeries for the correction of irregular astigmatism resulting from previous corneal refractive surgery, being able to cover depressions and irregularities remaining after the performed surgery.

HA is also useful as a vehicle for the delivery of topical treatments for the ocular surface. It improves the effect of pilocarpine on miosis, and facilitates delivery of gentamicin sulfate for the treatment of severe ocular infections.

Orthopedic Surgery and Rheumatology

HA is the basis of the lubricant and "shock absorber" properties of synovial fluid. Osteoarthritis (OA) is the most common disease of joints, and correlates with a deterioration of synovial HA. Intra-articular administration of HA is a widely used therapy for OA, providing relief of pain, and other symptoms. The first arthroscopic viscosurgical application of HA was in 1989 [92]. There are several preparations of partially cross-linked HA that are now used in this context. However, only one preparation will be discussed here. Synvisc®, also known as hylan G-F 20, is a viscoelastic fluid containing modified HA produced from rooster combs. Hylans are cross-linked derivatives of HA. Synvisc® contains hylan A (average molecular size 6×10^6 Da) and hylan B, a hydrated gel in a buffered salt solution. Recently a new approach has been suggested, in which two HA derivatives bearing host and

guest complexing substituents are injected in the joint, where they form an aggregate with increased molar mass due to formation of multiple inclusion complexes (clathrates) by the appended substituent groups [93,94].

Otolaryngology

HA preparations with various viscoelastic properties promote healing of perforated tympanic membranes [95,96]. Films of HA esters, such as HYAFF® provided by Fidia Farmaceutici, S.p.A (Abano Terme, Italy) are used in ear and sinus surgery. These preparations promote wound healing of the tympanic membrane, facilitate re-epithliazation, as well as prevent adhesion between layers of mucous tissues.

Viscoaugmentation of the vocal cord, the repair of injured or scarred vocal cords, and treatment of glottal insufficiency are additional uses of HA derivatives. Hyal B slurries injected into vocal cords produce no inflammatory reactions, and the material continues to be present even after one year [97].

Pharmacology and Drug Delivery

The carboxylate group of HA's glucuronic acid is the most commonly modified functional group. It can be used to create a cross-linked hydrogel for DNA entrapment and also for drug delivery. HA has been conjugated directly to drugs using the carboxylate as a drug carrier [98]. Low-molar-mass HA oligomers have not been used as commonly as the high molecular size polymer, however, precedents do exist. By providing homing molecules, by attaching specific HA receptors, the potential exists for tropic delivery of drugs to specific sites. The CD44 molecule, the predominant HA receptor, exists in a myriad of isoforms, and is susceptible to a host of post-translational modifications that impose varying affinities for tissues. Such homing devices have much potential for drug targeting [99].

Surgery and Wound Healing

High molecular size HA preparations, applied topically, promote healing of fresh skin wounds [100]. They also promote the healing of venous leg ulcers [101] and are useful in the management of chronic wounds [102].

A new product, a combination of HA with iodine, Hyiodine®, is effective in the healing of severe chronic wounds, as found in the extremities of diabetic patients [103].

Tissue Engineering

Tissue engineering is the field, in which the greatest advances have occurred in the medical use of HA and its derivatives, and the field with the greatest potential. HA-based sheets serve as a matrix for soft tissue, cartilage, bone, and skin growth, and as a substrate for

tissue regeneration and remodeling. Three-dimensional scaffolds of HA-based materials can facilitate restructuring of tissues and assist in regaining function. These materials are ideal for tissue reconstruction, as there is no host immune response, and are particularly useful for burn and trauma patients.

Stem cells require an HA-rich environment for maintaining the undifferentiated state [104]. Only one in 10^5 to 10^6 bone marrow cells are stem cells. These can be isolated by flow cytometry, using appropriate stem cell surface markers, and expanded on HA-enriched scaffolds. These can be triggered to become osteogenic, chondrogenic, or mesenchymal cells by seeding onto specialized membranes and by utilizing various HA-binding mechanisms. Vascular endothelial cells can be selected, as well as aortic smooth muscle cells for the construction of heart valves, by seeding onto HA sheets and membranes.

An in situ crosslinkable HA hydrogel for tissue engineering has also been devised. This injectable cell-containing hydrogel supports cell proliferation and growth and can be used in vivo [105].

Urology

Hylan B is used in a bulking procedure for urinary sphincter muscles and as a treatment modality for certain kinds of urinary incontinence [106]. It is also used in combination with dextran sulfate for the treatment of children with vesico-ureteral reflux [107].

DEGRADATION OF HYALURONIC ACID BY ROS IN INFLAMMATORY DISEASES

Many diseases are accompanied by inflammatory processes and in some of them the degradation of high-molar-mass HA is also involved. Such processes include, for instance, osteoarthritis (cartilage), wound healing (skin), asthma (airways), and urological malignancy. Usually the affected patient will complain about the so-called "cardinal" symptoms of inflammation "color, dolor, rubor, and tumor", indicating that the affected part of the body is painful, swollen, slightly reddish, and feels warm [108].

Besides the activation of proteolytic enzymes, reactive oxygen species (ROS) primarily contribute to these symptoms, ROS are 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 [109].

Neutrophils [110] are accumulated in the synovial fluid of the inflamed joints in large amounts [111], although the prime reasons for the accumulation and activation of neutrophils to generate ROS are not yet completely clarified [112]. The increased oxygen consumption by neutrophilic granulocytes upon stimulation under inflammatory conditions is commonly termed "respiratory burst" [113].

Although a large variety of ROS is known to be generated under *in vivo* conditions, the initial events are quite similar. The first step is the enzymatic reduction of "normal" oxygen

into superoxide anion radicals ($O_2^{\bullet-}$) catalyzed by the enzyme NADPH oxidase (also termed "respiratory burst oxidase"), a highly complex enzyme of different protein subunits [114]:

$$2O_2 + NADPH \rightarrow 2O_2^{\bullet-} + NADP^+ + H^+ \quad (1)$$

Although this is the most relevant pathway, other cellular sources for $O_2^{\bullet-}$ are the mitochondria in stressed cells, the generation of met-hemoglobin, and the reduction of oxygen by quinone radicals or by oxidized glutathione radicals ($GSSG^{\bullet-}$). The generation of $O_2^{\bullet-}$ is usually accompanied by hydrogen peroxide (H_2O_2) generation, the concentration of which is controlled by the enzyme catalase.

Superoxide anion radicals dismutate either spontaneously or especially in the presence of the enzyme superoxide dismutase to produce hydrogen peroxide:

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

Since $O_2^{\bullet-}$ as well as H_2O_2 are simultaneously present, they are often assumed to react with each other:

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

This reaction is the famous "Haber-Weiss" reaction that does, however, play only a minor role in physiology, because the products of this reaction have a strong inhibitory effect on their generation.

Both, superoxide as well as hydrogen peroxide are rather slow-reacting species that are as such not capable of damaging carbohydrates such as HA [62]. These compounds are, however, deleterious in the presence of traces of transition metals, especially Fe^{2+} [115]. Accordingly, hydrogen peroxide is the starting material for other ROS, including singlet oxygen (4), hypochlorous acid (5) and hydroxyl radicals (6) [116]:

$$H_2O_2 + HOCl \rightarrow {}^1O_2 + H_2O + HCl \quad (4)$$

$$H_2O_2 + Cl^- \rightarrow HO^- + HOCl \quad (5)$$

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + HO^- + Fe^{3+} \quad (6)$$

The latter reaction (6) is the so-called Fenton's reaction that is well known for over 100 years but still poses many mysteries [117]. Briefly, its biological significance is limited because under physiological conditions "free" iron does not exist, while all the iron is rather firmly bound into protein complexes: In blood, iron is associated with the protein transferrin and in cells with the protein ferritin [62].

The situation becomes even more complex, when species as nitric oxide ($NO^{\bullet}$) are additionally considered. $NO^{\bullet}$ and some nitrogen-derived species are analogously termed "reactive nitrogen species" [118]. The reader interested in details on these ROS and RNS is referred to a recent review by Šoltés *et al.* [119].

A highly simplified schema of the generation of individual ROS and RNS is shown in Figure 2:

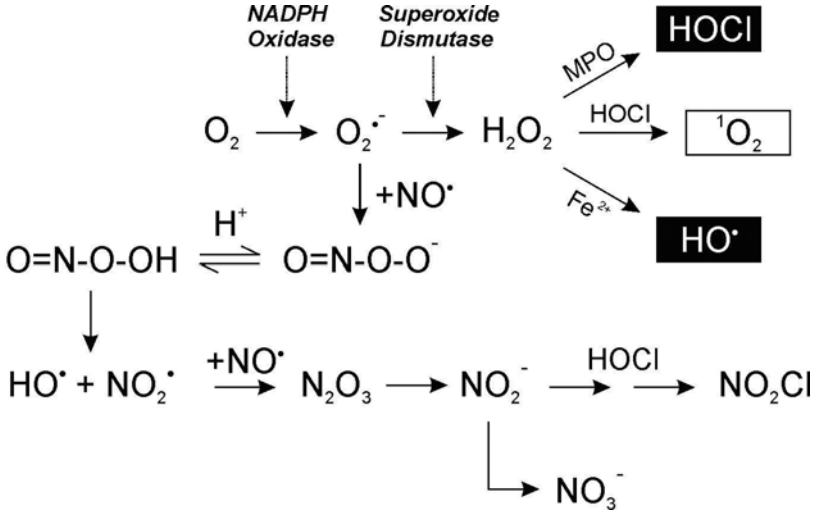

Figure 2. Scheme of ROS and RNS generation under *in vivo* conditions. Please note that this is a very simplified summary of potential chemical reactions that does not take into consideration the different locations of enzymes and their substrates.

Since its discovery, $NO^{\bullet}$ has become one of the most frequently investigated biomolecules, because it functions as a second messenger molecule [118]. $NO^{\bullet}$ possesses regulatory functions as endothelium-derived relaxation factor by interacting with the iron-containing prosthetic group of guanylate cyclase [120]. $NO^{\bullet}$ is generated under *in vivo* conditions by the enzyme $NO^{\bullet}$ synthase (NOS), of which several forms are known to exist [121]. NOS are P_{450}-related hemoproteins that oxidize L-arginine to L-citrulline and $NO^{\bullet}$.

Although $NO^{\bullet}$ is characterized by a considerable half-life [122] (a survey of the half-life of many ROS and RNS is provided in [123]), it is known that $NO^{\bullet}$ reacts with other ROS under the generation of more reactive RNS: For instance, when $NO^{\bullet}$ reacts with the simultaneously present $O_2^{\bullet -}$ ($k = 3.7 \times 10^5$ l mol^{-1} s^{-1}) [124], peroxynitrite is formed.

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^- \quad (7)$$

This compound is in equilibrium with its corresponding acid, the pK_a value of which is 6.8 [125]:

$$ONOO^- + H^+ \leftrightarrow ONOOH \quad (8)$$

Peroxynitrous acid, however, is not stable and decomposes to yield $HO^{\bullet}$ and $NO_2^{\bullet}$ [124]. When $NO_2^{\bullet}$ reacts with $NO^{\bullet}$, N_2O_3 is generated. A comprehensive discussion of these RNS is outside the scope of this paper. However, it should be noted that all these RNS are highly reactive, short-lived species. Therefore, their quantitative assay is a challenging task particularly under *in vivo* conditions: Nitrite (NO_2^-) that results e.g. from the decomposition

of N_2O_3, is often used as a marker of the $NO^{\bullet}$ production under *in vivo* conditions and it is known that under inflammatory conditions the concentration of nitrite is strongly elevated. For instance it was shown that nitrite concentrations up to 4 mmol/l can be detected in SF of the patients suffering from rheumatoid arthritis (RA) [126].

In the next several paragraphs, the reaction between HA and different ROS and RNS will be discussed. The special emphasis will be made on HOCl and $HO^{\bullet}$ radicals that represent extremely reactive species.

Reaction of HA with $O_2^{\bullet-}$

In many papers the term "superoxide" ($HOO^{\bullet}$) is used simultaneously with "superoxide anion radicals" ($O_2^{\bullet-}$). However, this is under the physiological conditions incorrect: The pK_a value of this acid-base equilibrium is 4.8 [127] and, therefore, there is only a very small contribution of $HOO^{\bullet}$ at physiological pH (7.4). Therefore, the term "superoxide anion radical" should be exclusively used. The superoxide anion radical is both, a one-electron oxidant and a one-electron reductant. The reactions of $O_2^{\bullet-}$ with many different biological substrates were studied in detail by the radiation chemists and a summary of the obtained second order rate constants is provided in [128]. However, not a single carbohydrate is mentioned in this comprehensive survey since no reaction could have been observed [129].

Although often suggested, it is nowadays clear that neither $O_2^{\bullet-}$ nor $HOO^{\bullet}$ are able to react with carbohydrates as such. However, there is one recent study showing that $O_2^{\bullet-}$ plays an important role in the decomposition of the initial products generated by the reaction of other ROS, e.g. HOCl, with glycosaminoglycans [130].

The "toxicity" of $O_2^{\bullet-}$ is exerted primarily by its penetration to important sites, where it is converted into further, more reactive oxygen species. When a reaction between $O_2^{\bullet-}$ and carbohydrates was observed, this was nearly exclusively the case when complex biomaterials, for instance cartilage [131] or body fluids, for instance SF [132], were used for the experiments. Such complex materials do always contain at least traces of transition metals. This was convincingly demonstrated by Carlin and Djursater [133]. These authors have shown that there is no reduction of the viscosity of a given HA solution when solely the enzyme xanthine oxidase is used as source of $O_2^{\bullet-}$. In contrast, a marked depolymerization of HA occurs in the presence of ferritin-bound iron. The authors concluded that ferritin could catalyze the Haber-Weiss reaction (3), leading to the formation of highly damaging hydroxyl radicals. This means that $HO^{\bullet}$ but not $O_2^{\bullet-}$ is responsible for the observed deleterious effects.

Additionally, the recombination of two $O_2^{\bullet-}$ molecules (9) occurs much faster ($k = 8.3 \times 10^5$ l mol^{-1} s^{-1}) than other reactions.

$$HO_2^{\bullet} + O_2^{\bullet-} + H_2O \rightarrow H_2O_2 + O_2 + HO^- \quad (9)$$

Reaction of HA with H_2O_2

Pure hydrogen peroxide, similarly to $O_2^{\bullet-}$ is not capable of reacting with carbohydrates. Even if there are indications of the opposite [134], these results have been usually obtained in the presence of very small amounts of ferrous and other low-valent transition metals, which catalyze decomposition of H_2O_2 under the generation of $HO^{\bullet}$ radicals.

The reader should note that avoiding Fenton chemistry is a very important task and a number of papers dealing with elimination of the impurities of transition metal ions have already been published [135-137].

Reaction of HA with $HO^{\bullet}$ Radicals

These ROS represent the most reactive species. $HO^{\bullet}$ radicals are generated under *in vivo* conditions by the Fenton's reaction (6), i.e. *via* the Fe^{2+} ion-induced decomposition of hydrogen peroxide [138]. Because details of the Fenton's reaction still remain unknown [117], reactions of $HO^{\bullet}$ radicals are primarily investigated using radicals generated under *in vitro* conditions by water radiolysis [139]. Under these conditions, the kind and the yield of the generated radicals can be carefully controlled [140]. $HO^{\bullet}$ radicals react in a diffusion-controlled way ($k \approx 6 \times 10^9$ l mol^{-1} s^{-1}) with virtually all compounds containing C-H groups under the abstraction of one hydrogen ($H^{\bullet}$) radical [141] leading to the generation of the corresponding alkyl radical.

The reaction between $HO^{\bullet}$ and even simple carbohydrates is very complex [141]. Therefore, some basic assumptions will be made on the example of glucose as a simple model compound: The first step of the reaction is the abstraction of one $H^{\bullet}$. Nearly all positions are affected to the same extent, while the positions C-1, C-2, and C-6 are slightly preferred [141]. Please note that under these conditions, twelve different radicals are generated because in aqueous solution, glucose exists as α- and β-anomers. Molecular oxygen is subsequently added to the alkyl radical, whereby peroxyl radicals are generated. The addition of O_2 is also diffusion-controlled. The decomposition of these initially generated radicals yields a considerable variety of reaction products, which are listed in the book of von Sonntag [141].

The second order rate constant of the initial reaction between the $HO^{\bullet}$ radicals and HA is about one order of magnitude ($k \approx 7 \times 10^8$ l mol^{-1} s^{-1}) smaller in comparison to that of the reaction with glucose [142]. This is most probably caused by the considerable viscosity of solutions of high-molar-mass HA.

Since HA is a copolymer of the alternating residues of glucuronic acid and *N*-acetylglucosamine, glycosidic linkage between the two sugar pyranosyl rings is a preferred reaction site for $HO^{\bullet}$ radicals leading to depolymerization of HA under retention of the general structure. It has been demonstrated that the glycosidic linkage is especially preferred as the reaction site when HA is irradiated in the solid state [143], as well as in aqueous solution. The individual reaction steps summarized in Figure 3 are quite similar to those occurring with the participation of glucose:

1) Abstraction of one H radical from a C-H group under formation of the corresponding alkyl radical,

2) Addition of molecular oxygen to the alkyl radical under generation of the corresponding peroxyl radical,
3) Elimination of $O_2^{\bullet-}$,
4) Cleavage of the intermediate radicals under generation of the corresponding carbonyl compound.

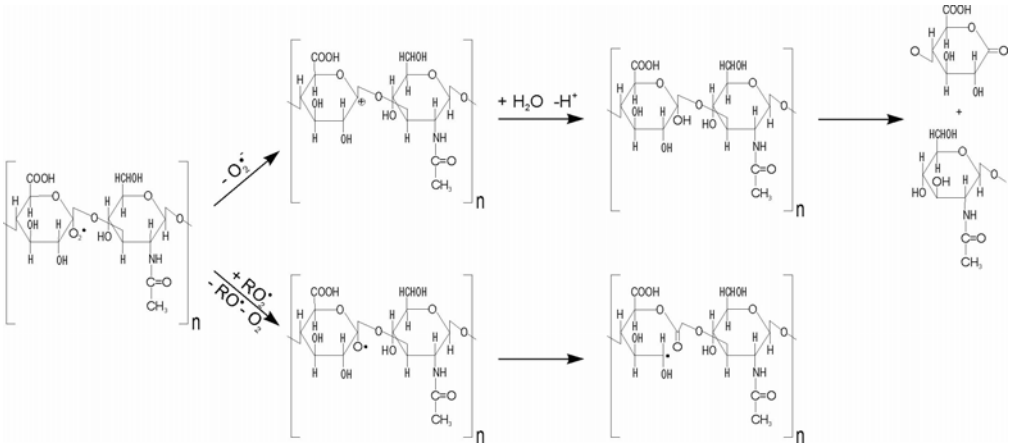

Figure 3. Fragmentation patterns of HA under the influence of hydroxyl radicals (upon γ-irradiation).

The individual events are summarized in Figure 3. Since the decrease of the molar mass of HA is the principal effect of the action of $HO^{\bullet}$ radicals, while functional groups are retained, methods enabling determination of the molar mass were primarily applied in order to study the $HO^{\bullet}$ radical-induced depolymerization of HA. For instance, Šoltés et al. used viscometry in order to study the effects of H_2O_2 and Cu^{2+} on HA solutions [144] as well as to study the inhibitory ("scavenging") effect of the ibuprofen isomers (ibuprofen is an anti-inflammatory drug).

Viscometry is a very sensitive method that enables detection of even very small changes of the molar mass. For instance, viscometry was also successfully used to study the (much weaker) effects of ozone and sunlight on HA solutions [145].

Changes of the molar mass of HA could also be monitored by a number of further methods. For instance, size exclusion chromatography/multi-angle light scattering (SEC-MALS) [146] and high performance liquid chromatography (HPLC) [115] were also used to study the radical-induced depolymerization of HA.

Nuclear magnetic resonance (NMR) spectroscopy is also a suitable tool of analyzing polymer degradation, although in contrast to the above mentioned methods, the detection of the native polymer is not possible due to its extremely high molar mass and viscosity. Since NMR represents a kind of a "mobility filter", less mobile, rigid molecules such as HA with molar masses in the MDa range are not detectable at all [147]. The lower the molar mass of the compounds under investigation is, the more efficiently they are detected by using the NMR technique, when HA samples were subjected to increasing doses of γ-irradiation and their NMR spectra were recorded [147]. The *N*-acetyl side chain of HA is a very good NMR marker of this polysaccharide because this side group exhibits a relatively high mobility being not entrapped in the relatively rigid carbohydrate ring system. The *N*-acetyl residue is represented by the resonance at about 2.04 ppm [147]. It is evident that the intensity of this

resonance increases under the influence of γ-irradiation due to the scissions induced by the $HO^{\bullet}$ radicals along the carbohydrate backbone. The generated degradation products possess higher flexibility and are, therefore, more sensitively detectable by NMR.

It is also obvious that the intensity of the resonance at about 2.04 ppm decreases when very high doses of irradiation are applied. In contrast to smaller doses, the signal of formate at 8.44 ppm is detectable only under very harsh conditions. Formate is a well-known product of the radiolysis of aqueous carbohydrate solutions [141]. Therefore, it is evident that at high irradiation doses - in addition to the reduction of the molar mass - fragmentation of the pyranose ring systems also occurs [147].

NMR is not only applicable to the solutions of the isolated polysaccharide, but also can be used for the study of human body fluids, e.g., the synovial fluids from patients suffering from RA [148]: Subsequent to γ-irradiation, increased peak intensities of the *N*-acetyl groups of HA (at about 2.04 ppm) could be clearly monitored [149]. Concomitantly, the intensity of another peak at 8.44 ppm increased. This resonance is stemming from formate and represents another important (low molecular) degradation product of HA in the SF. It is one of the major advantages of NMR that both high- and low-molar-mass compounds can be simultaneously detected. Of course, NMR offers also the additional advantage that even completely unexpected metabolites can be monitored [150].

Due to the quite similar structure of HA and other GAGs, e.g. ChS, clear distinction between both species can hardly be established by ^{1}H NMR. This is, however, possible by using ^{13}C NMR that is characterized by higher resolution than ^{1}H NMR [151]. The considerable role of $HO^{\bullet}$ radicals in the synovial fluids from patients with RA was recently proven also by electron spin resonance spectroscopy (ESR) using the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide to convert the highly reactive $HO^{\bullet}$ radical into a more stable compound [152].

Some authors used a completely different approach: HA or the degradation products of HA were not directly detected, but the competition between HA and another (exogenously added) compound for the $HO^{\bullet}$ radicals was used as a measure of reactivity. In this context, the luminol-amplified chemiluminescence was often used [153]: Luminol (5-amino-2,3-dihydro-phthalazine-1,4-dione) reacts with $HO^{\bullet}$ radicals under the emission of light. When luminol is present in excess over the generated radicals, the intensity of light emission depends directly on the number of radicals. When HA is added to the system, some of the $HO^{\bullet}$ radicals will react with the HA and are thus consumed. Therefore, the light yield is reduced proportionally to the reactivity of HA with the hydroxyl radicals. This method may be even used to obtain second order rate constants [154].

Recently Rychlý et al. [155] used chemoluminiscence, differential scanning calorimetry, and thermogravimetry to characterize hyaluronan fragments obtained upon degradative action of several oxidative systems. It has been demonstrated that even hyaluronans with almost equal molecular masses and having similar rheological properties can be distinguished applying the mentioned methods provided different chemical changes took place in their primary structures during the oxidative degradation.

Reaction of HA with HOCL

Since myeloperoxidase (MPO), the enzyme that generates HOCl under *in vivo* conditions, is known to play a very important role in inflammation, the reaction between HOCl and HA has been studied very comprehensively. In the repeating unit of HA, the glucosamine moiety represents the most relevant site of reactivity while the glucuronic acid moiety is quite inert against the action of HOCl [150].

This was demonstrated using the individual component monosaccharides of HA by measuring their consumption of HOCl [156,157]. Since both HOCl and NaOCl provide a pH-dependent absorption in the UV range, spectrophotometry can be used to assess the HOCl concentration very conveniently [157]. The reaction between HOCl and glucosamine may even be used for the quantitative determination of amino sugars [158].

In one early study, viscometry in combination with gel chromatography was used to evaluate the effects of HOCl on the one hand, and the complete $MPO/H_2O_2/Cl^-$ system, on the other hand, on the solutions of HA [159]. It was shown that already very small concentrations (in the μM range) of HOCl lead to a considerable reduction of the viscosity of HA, whereas much higher concentrations of HOCl are required to observe fragmentation products of HA. This discrepancy is explained by structural changes of the HA polymer chain in the presence of very small amounts of HOCl [160].

Using ^1H NMR spectroscopy [161] and a few years later also ^{13}C NMR [162], Schiller *et al.* were able to show that *N*-chloroamides are the prime reaction products between HOCl and the *N*-acetyl-D-glucosamine component of the repeating units of HA. It could also be shown that these initial products were just transient products that decomposed under the generation of acetate, i.e. a cleavage of the *N*-acetyl side chain of the polymer occurred. This is an interesting result because generation of acetate, on the one hand, and formate, on the other hand, enables the differentiation between the effects induced by HOCl and $HO^\bullet$ radicals, respectively. It has been also shown that the acetate content is a potent marker of the MPO activity in the SF from patients with RA [162] and a close correlation between its peak intensity in the NMR spectra and the MPO activity could be established.

Generation of the *N*-chloroamide from HA was later confirmed also by means of ESR [157]: it was shown that this product degrades under the generation of an *N*-centered radical that subsequently isomerizes into a carbon-centered radical located in the pyranose ring. This represents the initial event for the reduction of the molar mass of the HA chain.

These results were extended a few years later by the same authors [163]: It was shown that the initially generated *N*-centered radicals underwent rapid intramolecular abstraction reactions to give carbon-centered radicals at *C*-2 on the *N*-acetyl-D-glucosamine rings (via a 1,2-hydrogen atom shift) and at *C*-4 on the neighboring uronic acid residues (via 1,5-hydrogen atom shifts). The *C*-4 carbon-centered radicals, and analogous species derived from the model glycosides, underwent pH-independent β-scission reactions that resulted in glycosidic bond cleavage [163].

Another study investigated the final products of the degradation of HA [164]: It was shown that the NaOCl oxidation of HA yields primarily *meso*-tartaric acid. In contrast, arabinaric acid and glucaric acid are obtained by the oxidation of HA with the Fenton reagent. It was suggested that *meso*-tartaric acid might be a useful biomarker of HA oxidation since it is produced by both NaOCl and Fenton chemistry.

Reaction of HA with Peroxynitrite

Although far less frequently investigated than $HO^{\bullet}$ radicals or HOCl, peroxynitrite is also capable of degrading high-molar-mass HA. However, it is not known whether peroxynitrite or one of its derivatives are primarily responsible for the observed effects. An overview of the reactive species derived from peroxynitrite is presented in Figure 4.

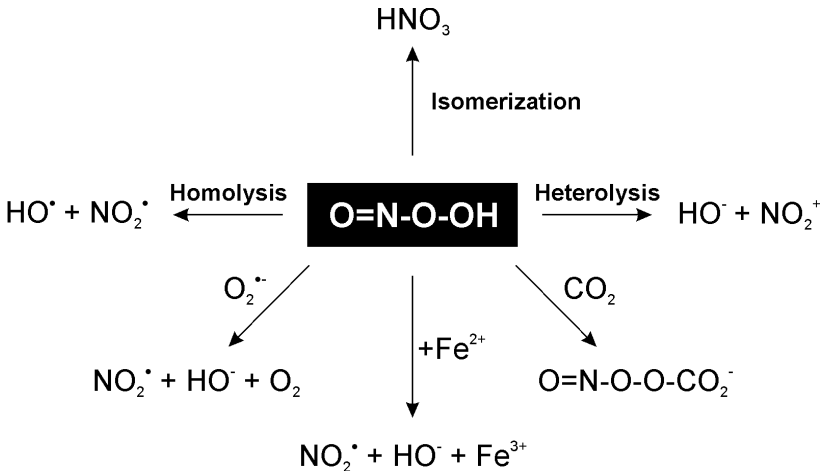

Figure 4. Scheme of the potential reactions of peroxynitrite under generation of further harmful species.

Most probably due to the capability of peroxynitrite to yield $HO^{\bullet}$ radicals, it was found that the induced effects are comparable to the effects elicited by hydroxyl radicals [165]. A more detailed investigation using spectroscopic methods as well as mass spectrometry (MS) has been published recently [166]: Neither NMR nor MS experiments provide any evidence of a peroxynitrite-mediated modification of HA. On the other hand, simultaneously performed ESR experiments give evidence for the generation of *C*-centered carbon radicals, most probably by the way of hydroxyl radical-like reactivity of peroxynitrite.

Although the structures of HA and other GAGs closely resemble each other, it should be noted that $NO^{\bullet}$ and especially its derivatives are capable of cleaving heparan and heparan sulphate [167] as well as ChS [168]. In contrast, however, these ROS are not able to induce fragmentation of HA. The reasons for this remarkable difference are not known.

Reaction of HA with Singlet Oxygen

Singlet oxygen ($^1O_2$) is an oxygen form, the electrons of which are excited to a higher energy level in comparison to "normal" triplet oxygen. When the molecule returns to the ground state, energy is emitted, which is responsible for the enhanced reactivity of $^1O_2$.

The effect of singlet oxygen on HA has been much less exhaustively studied in comparison to the action of other ROS. For instance, it was shown that the viscosity of HA is considerably reduced when HA solutions are irradiated in the presence of a suitable dye [169]. On the other hand, only small amounts of the fragmentation products could be

detected. It was suggested that singlet oxygen primarily changes the tertiary structure of HA, however this change is accompanied only by a minor depolymerization. Finally, the influence of singlet oxygen on HA is largely diminished by common scavenger molecules, e.g. radical quenchers or metal chelators [170].

METHODS OF CHARACTERIZATION OF PHYSICO-CHEMICAL PROPERTIES OF HYALURONAN

From a characterization point of view, considering its regular alternating structure, HA could be considered as a homopolymer, in which the repeating units are composed of disaccharides GlcNAc and GlcA. Moreover, the HA molecule in solution is not neutral but rather represents a polyelectrolyte (polyanion). Finally, HA macromolecular chains are not homogeneous in length, and consequently in molar mass, but reveal a certain extent of polydispersity. The physico-chemical characterization of HA must therefore take into account these peculiar macromolecular properties. HA characterization can be performed without or after fractionation. A characterization method used without fractionation (usually defined as off-line) furnishes only an average of the specific macromolecular properties such as number-average molar mass M_n (from osmometry), weight-average molar mass M_w (from light scattering), z-average molar mass M_z (from sedimentation), intrinsic viscosity $[\eta]$ and viscosity-average molar mass M_v (from viscometry), Newtonian dynamic viscosity η_0 and elastic or storage, or in-phase modulus G' and viscous or loss, or out-phase modulus G" (from rheometry). A characterization method used after an on-line fractionation (i.e. SEC, SEC-MALS, SEC-Visc) furnishes the whole molar mass distribution (MMD) and/or intrinsic viscosity distribution (IVD), and/or radius of gyration distribution (RGD) of the polymer. A great variety of methods, both off-line and/or on-line, are used at HA fractionation and characterization.

HA Fractionation

The average molar mass of HA ranges from few kDa (oligomers, fragments) to about 10 MDa (ultra-high molar mass (UHMM) samples). Basing on the molar mass range, different on-line fractionation methods can be applied. Several methods have been used for the fractionation of low-molar-mass HA [171,172]: a) HPLC (i.e. SEC, ion-exchange chromatography (IEC), reverse-phase chromatography (RPC); b) electrophoresis (i.e. gel, capillary). Different methods could be used also for the fractionation of high-molar-mass HA samples [82,172-175]: a) SEC; b) flow-field flow fractionation (F-FFF); c) IEC; d) agarose gel electrophoresis. It is well known that SEC is the most important method for the fractionation of high-molar-mass HA samples. Mendichi and Schieroni [82] reported a successful SEC fractionation of medium, high, and also UHMM HA samples with the molar mass up to about 3 MDa (M_w). On the contrary, for the fractionation of UHMM HA samples (typically higher than 3 MDa) only the F-FFF method is effective [174]. Due to their importance and predominant application in practice, only SEC and briefly F-FFF and IEC fractionation methods will be described here.

SEC Fractionation of High-Molar-Mass HA

It is well known that SEC separates the macromolecules according to a decreasing hydrodynamic volume, i.e. according to a decreasing molar mass. However, the fractionation of high-molar-mass HA by SEC is rather very complex. Typical SEC experimental conditions applied to the fractionation of high-molar-mass HA samples present many drawbacks, such as shear degradation, concentration effects, anomalous elution, and in general poor resolution. As a rule, with high-molar-mass HA samples, each detail of the SEC experimental protocol has to be optimized methodically in order to obtain reliable results. An experimental protocol for a successful SEC fractionation of high and UHMM HA samples is described in detail by Mendichi and Schieroni [82]. Such SEC experimental protocols will not be described in detail here. Only the more important experimental conditions will be summarized. In shielding the anionic charge of HA chains, a 0.1 M - 0.2 M aqueous NaCl is an effective SEC mobile phase for HA. Flow rate and sample concentration should be as low as possible. Usually, one may use a flow rate ranging from 0.8 ml/min to 0.2 ml/min depending on the molar mass of the HA sample. Obviously, a flow rate value of 0.2 ml/min is used only with a UHMM HA sample. Moreover, concentration of the sample depends on its molar mass and in general, it ranges from 0.01 to 0.5 mg/ml. The critical point in SEC fractionation of HA is the column set, which is composed of one or two columns with larger particle size and larger pore size SEC packings suitable for HA fractionation.

F-FFF Fractionation of High-Molar-Mass HA

With UHMM HA sample, only F-FFF fractionation is effective. It has been demonstrated that if the M_w average of the HA sample is higher than ca. 2-3 MDa, the molar mass estimated by a SEC-MALS system is systematically underestimated due to shear degradation and anomalous elution in SEC columns [176]. The F-FFF fractionation technique encompasses a number of separation methods characterized by the transverse compression induced by an external field orthogonal to a laminar parabolic flow in a very thin flat channel. Because of the peculiar nature of the field, F-FFF is the most extensively employed sub-technique for the analysis of biological macromolecules.

On the contrary to SEC, F-FFF is a one-phase chromatography technique. High-resolution separation is achieved within a very thin laminar flow, against which a perpendicular force field has been applied. The flow is confined within a thin channel consisting of two plates and a thickness of about 80-100 μm. In an F-FFF system, the upper plate is impermeable, while the bottom plate is permeable and made of a porous frit and an ultra-filtration membrane (10 kDa). Within the FFF channel, a laminar parabolic flow profile is created and when a perpendicular flow is applied, the analytes are driven towards the so-called “accumulation wall” of the channel. Diffusion associated with Brownian motion, in turn, creates a counteracting motion, so that smaller particles, which have higher diffusion rates, tend to reach an equilibrium position farther away from the accumulation wall. The smaller particles move much more rapidly than the larger particles, due to their higher diffusion coefficients, which results in the smaller particles eluting before the larger ones. This is exactly the opposite to what happens at the SEC separation. With FFF separation there

is no porous media (packing) to interact with the samples and for UHMM HA this is an important advantage because shearing does not occur.

The F-FFF fractionation was successfully applied to high and UHMM HA samples [173]. Using this methodology, Cowman and Mendichi characterized molecular mass and the MMD of a HA sample (Hylan from Biomatrix, Ridgefield, NJ, USA) [172]. Hylan is a soluble slightly cross-linked HA with an exceptionally ultra-high molar mass (about 10 MDa or more) and broad MMD (D about 4.5 [174]). It is important to note that such a UHMM HA sample cannot be efficiently fractionated by the classical SEC columns.

IEC Fractionation of High-Molar-Mass HA

The IEC method can also be used for estimation of MMD of high and UHMM HA samples. Some HA samples, in the range of molar masses from 0.1 MDa to 5 MDa were successfully fractionated by Karlsson and Bergman by using an anion IEC chromatographic system [175]. A strongly anionic IEC column PL-SAX-4000 (Polymer Laboratories, Church Stretton, UK) was used and the fractionation was performed at 45 °C using a linear gradient of 10 mM sodium phosphate, 20 mM sodium sulfate, at pH 7.0 and 10 mM sodium phosphate, 225 mM sodium sulfate. Unfortunately, with such IEC chromatographic systems that use gradients, an on-line LS detector could not be used and the molar mass has to be evaluated by an indirect insufficiently accurate method.

CHARACTERIZATION OF MOLAR MASS AND SIZE OF HA

Molar mass and size distributions of a polymer are the most important parameters. In general, the complex biological functions of HA are closely related to the whole MMD. Taken alone, an average molar mass, hydrodynamic or size value (M_n, M_w, M_v, M_z, $[\eta]$, R_g, R_h) are not sufficient in describing the very complex biological functions of HA. After the on-line fractionation, one or more special detectors capable of measuring M and/or R_g, and/or $[\eta]$ of the HA fractions are required. Molar mass can be measured using the conventional methods, i.e. by means of a direct or universal calibration with narrow or broad MMD polymeric standards, or using "absolute" on-line detectors as for example LS and viscometer.

Determination of MMD of HA by Conventional SEC

Conventional SEC is the classical method for measuring the MMD of synthetic or natural polymers. Conventional SEC essentially means the use of an on-line single concentration detector – differential refractometer (DRI) or UV. In such SEC systems, the molar mass is calculated by means of a direct or universal calibration applying a set of appropriate narrow and/or broad MMD standards. Unfortunately, classical narrow MMD standards for aqueous SEC mobile phases such as pullulan, poly(ethylene oxide), poly(ethylene glycole), and dextran are not suitable for characterization of HA. The listed SEC standards are neutral molecules existing in solution as flexible random coils, and some of them are also branched

(dextrans). By contrast, HA in solution is a linear anionic polymer, having a semi-stiff conformation [82,177]. However, it is not difficult to find in the literature a description of the characterization of MMD of several HA samples with a direct calibration using narrow MMD pullulan standards. It is important to note that the M_w average of an HA sample estimated with this conventional SEC method is about five times higher than the true M_w average measured using an absolute LS detector [178]. In general, the MMD of HA cannot be estimated with a conventional SEC system but it is necessary to use an on-line LS detector. However, if an on-line LS detector is not available, for low- or medium-molar-mass HA samples it is possible to use a conventional SEC system and a direct calibration with well characterized broad and/or narrow molar mass HA standards [176]. Although until recently only scarcely available, today the "monodisperse" HA standards are commercially marketed (http://www.hyalose.com).

Determination of Molar Mass, Size, and Conformation of HA by Means of LS

An "absolute" direct method for measuring molar mass and size of a polymer is the use of a LS detector. LS methodology concerns the interaction of the electromagnetic field with the matter. LS theory is very complex but the technique is very useful for the characterization of macromolecules, because many important physical parameters can be measured. When a polymeric solution is irradiated with electromagnetic waves, the electric field component induces scattering. The light scattered from the polymeric solution can be analyzed using various detectors. Depending on the type of the analysis, the techniques are classified as elastic LS, quasi-elastic LS, etc. For the molecular characterization of macromolecules, only elastic LS and quasi-elastic LS are of interest. In an elastic LS experiment, the intensity of the scattering is measured. From the intensity of the scattering one can obtain the molar mass and from the angular variation of the intensity obtain the R_g value of the macromolecules can be estimated. In a quasi-elastic LS experiment, the fluctuations of the intensity of the scattering due to the Brownian movement of the macromolecules are measured. From these fluctuations, specifically from the correlation function, a translational diffusion coefficient D can be obtained, from which it is possible to calculate the value R_h using the Stokes-Einstein equation (I). There, k denotes the Boltzmann's constant, T the absolute temperature and η the solvent viscosity.

$$R_h = \frac{k \cdot T}{6\pi \cdot \eta \cdot D} \qquad \text{(I)}$$

The principal equations for elastic LS have been reviewed recently [172] and will be only summarized herein. In brief, following the Zimm theory [179], the intensity of the light scattering by a polymeric solution is in relation with the molar mass of the sample according to the following general equation:

$$\frac{K \cdot c}{\Delta R(\theta)} = \frac{1}{M \cdot P(\theta)} + 2A_2 \cdot c + ... \qquad \text{(II)}$$

where $\Delta R(\theta)$ denotes the scattering excess (Rayleigh factor) at angle θ of the solution with regard to the pure solvent, θ the angle between the primary incident light and the detector, c the concentration, A_2 the second virial coefficient, $P(\theta)$ an important parameter usually termed form factor, $K = (4\pi^2 \cdot n_0^2 \cdot (dn/dc)^2)/(N_a \cdot \lambda_0^4)$ - an optical constant, n_0 the refractive index of the solvent, dn/dc the refractive index increment of the polymer, λ_0 the wavelength of the light in vacuum, N_a the Avogadro's number. Furthermore, it is well known that the intensity of the scattering of large macromolecules brings about a destructive interference as a result of their unusual dimensions. In other words, the intensity of the scattering by large macromolecules (large with regard to the wavelength of the incident light) depends on the angle of measurement (angular variation). Taking into consideration such destructive interference, a form factor $P(\theta)$ has been introduced. $P(\theta)$ is defined as the ratio between $R(\theta)$ in the presence of interference, $\theta > 0°$, and $R(\theta)$ in absence of interference, $\theta = 0°$.

$$P(\theta) \equiv \frac{R(\theta)}{R(\theta = 0°)} \qquad \text{(III)}$$

$P(\theta)$ is very important because it allows to estimate the size of the macromolecules. Debye [180] found that the reciprocal of $P(\theta)$, in a certain range, could be approximated by the following equation:

$$P(\theta)^{-1} = 1 + \frac{1}{3} \cdot \mu^2 \cdot < s^2 > \qquad \text{(IV)}$$

where $\mu = 4\pi/\lambda \cdot \sin(\theta/2)$ and $\lambda = \lambda_0/n_0$ is the wavelength of the light in the solvent. Consequently, it is possible to measure the size of the macromolecules from $P(\theta)$. Indeed, combining the previous three equations, at infinite dilution ($c \rightarrow 0$), the general equation for the data analysis of an elastic LS experiment can be obtained.

$$\frac{K \cdot c}{\Delta R(\theta)} = \frac{1}{M} \cdot \left[1 + \frac{16\pi^2 \cdot \sin^2(\theta/2) \cdot < s^2 >}{3\lambda^2} \right] \qquad \text{(V)}$$

From the equation (V) it is evident that the intercept of the $K \cdot c/\Delta R(\theta)$ *vs*. $\sin^2(\theta/2)$ plot furnishes the reciprocal of the molar mass M and the initial slope furnishes the radius $<s^2>^{1/2}$ of the macromolecule generally known as radius of gyration (R_g). At estimating M from LS data, one needs to know $\Delta R(\theta)$ at zero angle. The intensity $\Delta R(\theta = 0°)$ cannot be estimated directly due to the interference with the primary incident light. Thus, at measuring $\Delta R(\theta = 0°)$, two different strategies may be used corresponding to two different LS instruments: low-angle LS (LALS) and multi-angle LS (MALS). A LALS photometer measures $R(\theta)$ at a scattering angle as low as possible at assumption that this value corresponds to $\Delta R(\theta = 0°)$.

On the contrary, a MALS photometer measures R(θ) in a wide range of angles by means of an array of photodiodes, and ΔR(θ = 0°) is calculated by an extrapolation. Consequently, at measuring the angular variation of the scattering, in order to obtain the size R_g, a MALS photometer is required. On the contrary, by using a LALS photometer only the molar mass of the polymer could be measured. An LS photometer can be used both off-line and on-line to perform SEC, HPLC, or F-FFF. Below, only the MALS photometer will be considered.

Off-Line Elastic LS (Batch Mode)

By using a MALS photometer in off-line mode (batch), only certain average values of the macromolecular properties are obtained, namely the weight-average molar mass M_w, the z-average root mean square radius $\langle s^2 \rangle_z^{1/2}$, and the second virial coefficient A_2. In a batch mode, three to five different concentrations are prepared and the intensity of the scattering is measured [172]. The experimental MALS data are processed using the classical double extrapolation (infinite dilution $c \rightarrow 0$ and zero-angle $\theta \rightarrow 0$), generally known as Zimm plot.

Elastic LS on-Line to a SEC System

If all distributions of an HA sample (MMD, RGD, IVD) are required, use of absolute on-line detectors as MALS and Visc is needed. A multidetector SEC-LS-Visc system is composed of an LS detector (LALS or MALS), a viscometer (differential viscometer DV or SCV), and a concentration detector (DRI or UV). As a result, from the record of the on-line MALS detector, values of M and Rg can be obtained, while from the on-line Visc detector one can calculate [η]. Figure 5 shows the experimental relationships $M = f(V)$, $R_g = f(V)$, and $[\eta] = f(V)$ obtained using a SEC-MALS-SCV system for an HA sample (M_w = 1314 kDa, D = 1.6). The use of absolute on-line detectors, as MALS and Visc, for the characterization of HA samples was described previously [172,181].

The experimental dependence $M = f(V)$, i.e. the classical SEC calibration curve usually obtained by using narrow standards, in such a case can be obtained directly without calibration from the on-line LS detector. By combining the experimental function $M = f(V)$ and the concentration profile (from DRI), one can construct the complete MMD of the HA sample. The differential and cumulative MMD of a high-molar-mass HA sample (M_w = 652 kDa, D = 2.1) are shown in Figure 6. Starting from the initial MMD, the molar mass averages and dispersity index (M_n, M_w, M_z, and D) could be easily calculated using the appropriate definitions. Similarly to the molar mass, using other experimental functions ($Rg = f(V)$ determined from MALS and $[\eta] = f(V)$ from Visc), the respective distributions (RGD and IVD) and averages (Rg_n, Rg_w, Rg_z, and $[\eta]_n$, $[\eta]_w$, $[\eta]_z$) can be obtained [181].

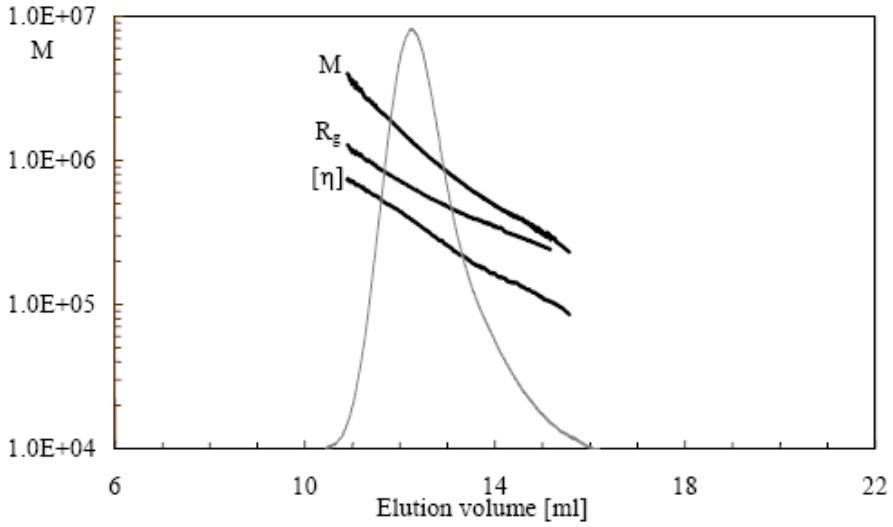

Figure 5. M = f(V), R_g = f(V) and [η] = f(V) experimental dependencies obtained from a SEC-MALS-SCV system for a high-molar-mass HA sample (M_w = 1314 kDa, D = 1.6).

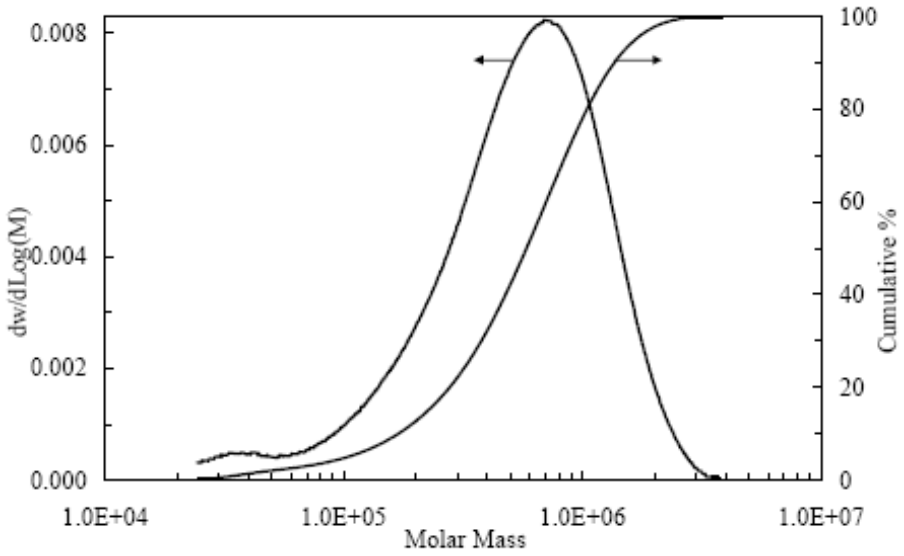

Figure 6. Differential and cumulative MMD for a high-molar-mass HA sample (M_w = 652 kDa, D = 2.1) estimated using a SEC-MALS system. Arrows indicate assignment of the curves to y axes.

Furthermore, by using absolute on-line detectors (MALS and Visc), another important piece of information on the HA conformation can be obtained. Because the on-line MALS detector measures both M_i and Rg_i values for each fraction of a sample, it is possible to obtain the Rg = f(M) scaling dependence, generally known as conformation plot. The conformation plot is a very important function for the proper understanding of the stiffness (conformational rigidity) of the polymer. The conformation plot for HA is shown in Figure 7. This plot was obtained by the superimposition of data of the SEC-MALS system applied to four HA

samples having M_w = 0.43, 0.66, 1.06, and 1.44 MDa. The slope of the conformation plot is about 0.6, which is a typical value for semi-stiff polymers as HA [177].

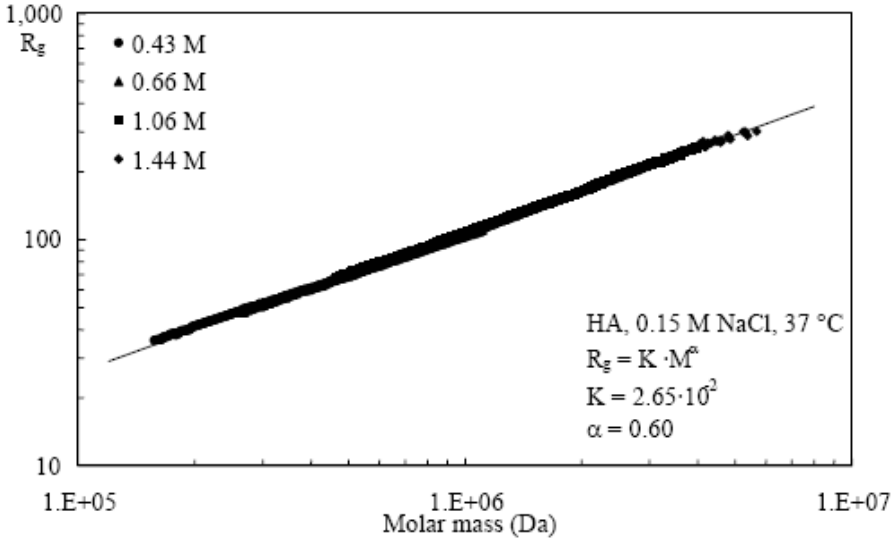

Figure 7. Conformation plot, $R_g = f(M)$, from a SEC-MALS system, obtained from the superimposition of the data of four high-molar-mass HA samples.

Intrinsic Viscosity and Mark-Houwink-Sakurada Plot from Viscometry

Measurement of viscosity [η] is more complicated than estimation of the molar mass, due to the non-Newtonian behaviour of HA solutions during the flow. It is well known that the HA viscosity strongly depends on the shear rate ($\dot{\gamma}$) even for very dilute solutions. Unfortunately, the shear rate range of the usual viscometers used for the [η] measurements, both off-line (i.e. Ubbelohde viscometer: 1200-1500 s^{-1}) and on-line to a SEC system (i.e. DV: 2500-3000 s^{-1}) are too high for measuring the HA viscosity. As a consequence, at measuring [η] of HA very low shear rate viscometers (rotational or capillary) are required [177].

There is big theoretical and practical interest for the scaling relationship $[\eta] = f(M)$ for HA. Theoretically, the Mark-Houwink-Sakurada (MHS) plot for HA could be obtained by using an on-line viscometer combined with an SEC system applied to an appropriate number of broad MMD HA samples. Unfortunately, the $\dot{\gamma}$ range of on-line DV detectors is too high for HA even at very low flow rates. To obtain a reliable MHS plot for HA for an extended range of molar masses, Mendichi et. al. [177] used a modified on-line SCV detector with low flow rate and very low concentration of the HA samples. Figure 8 shows the MHS plot for HA obtained from a SEC-MALS-SCV system, constructed by gathering data of nine high and UHMM samples. Evidently, the MHS plot for HA is very unusual because it is absolutely non-linear. The right axis of Figure 8 reveals the instantaneous value of the slope *a* of the MHS plot. The slope *a* ranges approximately from 1.06 for relatively low molar masses (M < 100 kDa) to about 0.6 for UHMM (M > 1000 kDa). The slope *a* for the intermediate range of

molar masses is about 0.78. However, it is important to note that the variation of the slope *a* is continuous, denoting strong dependence of the HA stiffness on the chain length [177].

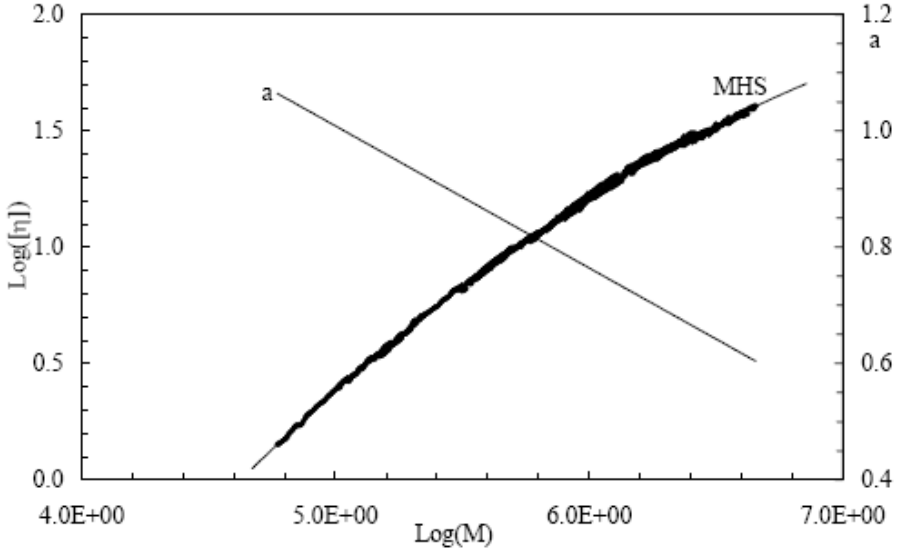

Figure 8. MHS plot, [η] = *f*(M), obtained from a SEC-MALS-SCV system and the superimposition of nine high and UHMM HA samples.

A similar result for the MHS plot for HA was obtained by Waters and Leiske [182], in spite of the fact that this group uses a relatively high shear rate on-line DV detector. Evidently, the marked curvature of the MHS plot for HA is unquestionable. Furthermore, the slope *a* for the low molar masses range (*a* = 1.06: M < 100 kDa) and intermediate high molar masses range (*a* = 0.78: 100 kDa < M < 1000 kDa) are in agreement with several results published previously [177]. Some problems concern the extreme UHMM range (M > 1000 kDa) with the relative slope $a \approx 0.6$. The question is: are the obtained [η] values of UHMM HA fractions reliable? Or are they rather underestimated as a consequence of the relatively high shear rates of the on-line Visc detectors? Probably, at this time it is necessary to perform further studies using very low shear rates, rotational viscometers, and UHMM HA samples to resolve this intriguing question.

Rheology

Rheology is a powerful method for the characterization of HA properties. In particular, rotational rheometers are particularly suitable in studying the rheological properties of HA. In such rheometers, different geometries (cone/plate, plate/plate, and concentric cylinders) are applied to concentrated, semi-diluted, and diluted solutions. A typical rheometric test performed on a HA solution is the so-called "flow curve". In such a test, the dynamic viscosity (η) is measured as a function of the shear rate ($\dot{\gamma}$) at constant strain (shear rate or stress sweep). From the flow curve, the Newtonian dynamic viscosity (η_o), first plateau, and the critical shear rate ($\dot{\gamma}_c$), onset of non-Newtonian flow, could be determined. It is well

known that the η_o parameter is correlated by a scaling law with the molar mass of the sample, and knowing $\dot{\gamma}_c$, it is possible to calculate the longest relaxation time ($\lambda=1/\dot{\gamma}_c$) of the polymer.

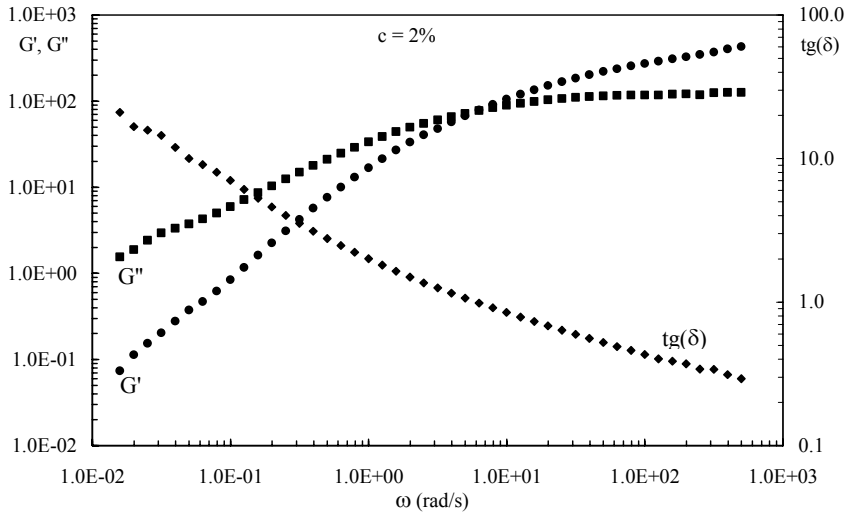

Figure 9. Frequency sweep (G', G", tg(δ) vs. the frequency ω) for an HA sample at 20 °C: M_w = 1350 kDa, D = 1.6, c = 2%.

The flow curves can be established for different concentrations and different molar masses of HA samples, and at different temperatures for a better insight into the molecular properties of polymers. Moreover, rotational rheometers can be used in dynamic oscillatory mode, frequency sweep, to assess the elastic G' module as well as the viscous G" module and the correlated phase angle δ, as a function of the frequency ω. G' and G" allow to study the viscoelastic behaviour of HA macromolecules. Figure 9 shows the frequency sweep curves (G', G", and tg(δ) vs. the frequency ω) for an HA sample (M_w = 1350 kDa, polydispersity index D=1.6, concentration c = 2%) at 20 °C.

It is necessary to note that in performing HA rheology, also the material of the rheometer (of the parts in contact with the HA solution) is of importance. Indeed, high-molar-mass HA samples easily degrade in the presence of metals in a solid state or in a form of dissolved cations. Recently, Stankovská *et al*. [183] have applied the method of rotational viscometry for HA degradation studies. Authors strongly recommend the use of an inert material, as Teflon or similar, for the parts of the rheometer in contact with the HA solution.

Mass Spectrometry

As has been described above, the most efficient method for the molar mass characterization of HA is the application of an LS detector in both off-line and in on-line mode used together with a SEC or F-FFF systems. However, there are situations, in which LS is not adequate for the molar mass characterization of HA. Typically, for low-molar-mass HA samples (oligomers, fragments, digestion or in general degradation products), LS is not

suitable due to a very low amplitude LS signal. In fact, it is well known that the LS signal depends on the molar mass, concentration, and $(dn/dc)^2$ of the polymeric sample. Consequently, for low-molar-mass HA samples, considering that the dn/dc parameter is a constant, the concentration must be increased in order to obtain an adequate signal-to-noise ratio. In many cases, however, concentrated HA solutions could be unsuitable for practical use, and it is thus better to apply an alternative characterization method. Moreover, the accuracy of the LS technique in determining the molar mass of macromolecules is about 3% when used under optimized conditions. In many cases, however, determination of the molar mass needs to be more accurate. Typically, for the samples of degradation fragments, higher accuracy up to one mass unit per the molar mass of the whole sample or per molar mass of the repeating unit of the macromolecule, is required. In such important cases, only the MS technique is adequate. In particular, two well-known soft ionization MS techniques, namely electro-spray ionization (ESI-MS) and matrix-assisted laser desorption ionization - time of flight (MALDI-TOF), are of particular interest for the molar mass characterization of HA biopolymer.

ESI-MS is a soft ionization technique [184] that allows to transfer ions from solution to the gas phase with little or no fragmentation at all. ESI introduces desolvated ions into the high vacuum environment required for MS from an atmospheric pressure stream of droplets of polar molecules in a mixed aqueous/organic solvent. Typically, the stream of droplets is generated by passing the output of a syringe pump or the eluent from an HPLC system through a fine stainless steel tip held at a high voltage. The ions produced in the ESI interface are analyzed in an MS detector, e.g. a triple quadrupole one. ESI-MS also offers the possibility of trapping and identifying short-lived intermediates, since ionic species in a solution are significantly attenuated when the ions pass into the gas phase. The limitations of ESI-MS are the need for relatively high purity samples and a poor tolerance for salts, buffers, and detergents. Furthermore, it is well known that ions produced in ESI-MS sources are multi-charged. This inconvenience makes the ESI-MS technique incompatible with polydisperse polymers. In practice, the ESI-MS technique is successfully applied for the characterization of a big variety of HA digestion products.

Considering the previously described limits of ESI-MS, MALDI-TOF is more efficient in determining the molar mass of native or derivatized low-molar-mass HA samples [184,185]. MALDI-TOF is a relatively novel MS technique, in which a co-precipitate of a UV-light absorbing matrix and a macromolecular sample is irradiated by a nanosecond laser pulse. Most of the laser energy is absorbed by the matrix, which prevents fragmentation of the macromolecules. In fact, MALDI-TOF is a soft ionization MS technique, practically avoiding fragmentation of the parent macromolecules as compared to other conventional MS techniques, and in contrast to the ESI-MS technique that produces mono-charged macromolecular ions: typically $[M+H]^+$, $[M-H]^-$, or $[M+Me]^+$, (where Me denotes a metal as for example Na or K present in the used matrix). The ionized macromolecules are accelerated in an electric field and enter the time-of-flight detector. During the flight in the detector tube, different macromolecules are separated according to their mass to charge ratio (m/z) and reach the detector at different times. Along with the synthetic polymers, the MALDI-TOF technique is used for the characterization of biomolecules, such as proteins, peptides, oligo- and polysaccharides, oligonucleotides, which molar masses range approximately between 100 and 400 kDa. As any other MS technique, MALDI-TOF is a very

sensitive method, which allows the detection of very low quantities of the samples (up to 10^{-15} to 10^{-18} mol) with an accuracy better than 0.1%.

MALDI-TOF was successfully utilized for determination of the molar mass of low-molar-mass HA samples [186]. The MALDI-TOF spectrum obtained by the authors clearly showed the major differences between the peaks corresponding to a mass of the disaccharide repeating unit of HA (378 Da), while the minor peaks correlated to the molar masses of the two monosaccharide components of HA [186].

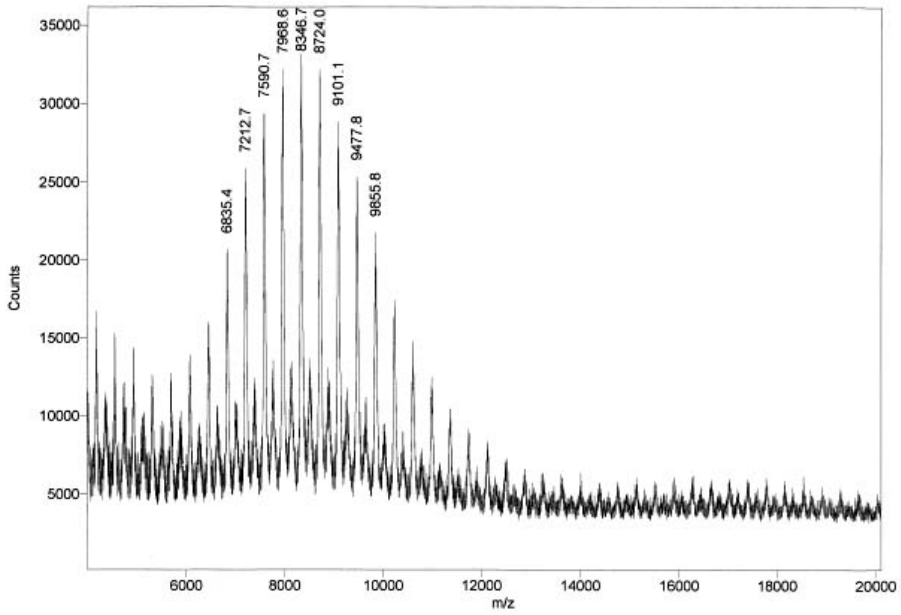

Figure 10. Expanded view of a MALDI-TOF spectrum for a HA fraction separated using a SEC system. Reprinted with permission from Elsevier from [186].

It is well known that at present MALDI-TOF analysis of broad MMD polymers represents a difficult task [184]. Typically, as the polydispersity of the sample increases approximately to above 1.2, the MALDI-TOF spectrum tends to contain only peaks in the low m/z range. This results from a strong competition between different macromolecular species. Thus, for polydisperse polymers a fractionation by SEC or other separation methods is required prior to a MALDI-TOF analysis in order to obtain narrow MMD fractions. Subsequently, the polymeric narrow MMD fractions could be analyzed off-line using a MALDI-TOF system.

METHODS OF DETECTION OF HA DEGRADATION PRODUCTS

High-molar-mass HA polymers play an important role as an essential structural component of the ECM. For its characterization, physical, molecular, conformational, and rheological properties of HA have been extensively studied in the past by many authors. However, in order to better understand the free radical degradation of HA, which plays an important role in many pathological states, to estimate kinetics of the degradation process, to

assess protection from degradation as well as interaction of HA with proteins, receptors, etc., low-molar-mass HA oligomers or fragments are very useful. HA can be degraded to smaller fragments up to very short, well defined in size oligomers by means of a big variety of different methods: a) chemical (under acidic or alkaline conditions); b) enzymatic (the enzyme most frequently used in HA degradation is hyaluronidase); c) free-radicals cleavage; d) thermal degradation; e) sonication; f) irradiation; g) strong physical stress induced for example by shearing. Various experimental methods could be used for detection of HA degradation products. In general, such methods could be subdivided into separation and characterization methods of fragments and oligomers of HA produced during the degradation process.

The simplest method for evaluation of the degradation kinetics of a high-molar-mass HA polymer is to measure changes in MMD, hydrodynamic or rheological properties. Changes in MMD of the HA could be measured by an SEC system equipped with an on-line LS detector. Using such a method, the complete MMD of the degraded HA samples can be obtained and certain information could be inferred on the type of degradation (scission).

A very convenient and inexpensive method of monitoring the HA degradation process is the use of an off-line capillary viscometer. In a previous section we have described the advantages and limitations of this method. Despite its limitations, off-line viscometry is an effective and quite sensitive method for the evaluation of the extent of HA degradation. Obviously, for the off-line viscometry, the parameter of interest in evaluating the degradation extent is η or the corresponding viscosity-average molar mass M_v calculated by means of the MHS equation.

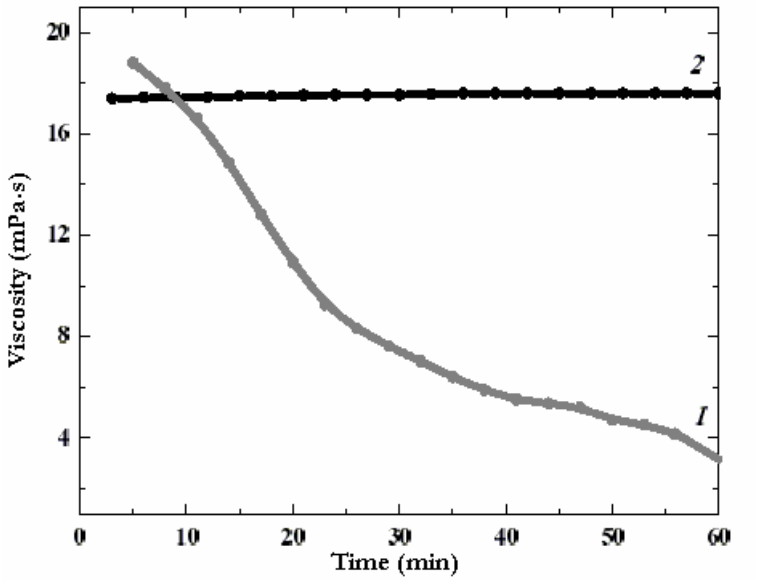

Figure 11. Kinetics of the decrease of η value of an HA solution (c = 0.3%) incubated with 3% H_2O_2 (curve 1) measured with a rotational viscometer. As a control, the viscosity-time profile of the intact HA is shown (curve 2).

A more sensitive method for evaluation of HA degradation is the use of a rheometer. Rheological parameters, such as η, G', and G" are very sensitive to HA degradation. All the

rheological properties are strongly correlated to the whole MMD of the HA sample. Furthermore, the degradation of the HA sample in solution can be followed directly in the instrument in the course of time. This fact is very important and advantageous for the rheological methods. Figure 11 shows the kinetics of a decrease of dynamic viscosity η at degradation of HA (c = 0.3%) when the sample is incubated with 3% H_2O_2 established using a rotational viscometer (curve 1). As a control, the viscosity-time dependence of the intact HA is also shown (curve 2).

Besides the decrease of the sample molar mass during the HA degradation, some changes in the chemical structure of the polymer also occur. As a result, low-molar-mass fragments are in general chemically modified with regard to the starting native high-molar-mass HA. Such modifications are usually due to extraction of hydrogen atoms, oxidation of functional groups, scission of chemical bonds, etc. Moreover, the end-group analysis of the fragments is also of interest and may provide important information on the character and extent of degradation. In an accurate analysis of HA fragmentation, such chemical modifications as well as the decrease of the molar mass of the fragments have to be detected by specific analytical methods.

Separation and Purification of HA Fragments

Different methods can be used for the separation and purification of low-molar-mass HA fragments and oligomers. Other than for analytical goals, separation and purification of HA fragments and oligomers is particularly important for their subsequent studies. Capila *et al.* [171] and Cowman and Mendichi [172] have recently reviewed the most important methods used for separation and purification of HA fragments. For simplicity, such methods can be classified as HPLC methods (that is, SEC, RPC, normal phase chromatography NPC, IEC) and electrophoretic methods (that is, gel and capillary electrophoresis).

HPLC Methods

HA oligomers can be separated and purified by SEC. It is well known that SEC is the most straightforward method among the molar mass dependent fractionations. Typically, the smaller fragments (from tetramer to 24-mer) can be individually resolved by SEC columns. Many other RPC methods were used in separation and purification of HA fragments and oligomers including post-column derivatization. Detailed description of such methods can be found in the literature [171].

Electrophoretic Methods

HA in solution is a charged polymer with a constant charge-to-mass ratio regardless of molar mass. Hence, electrophoretic methods are good candidates for separation of the HA fragments. Ideally, using such methods a molar mass based separation is anticipated. For this goal different gels were used: 1) cross-linked polyacrylamide gels of varying porosity

(different concentrations and degrees of cross-linking) were applied for the separation of the low-molar-mass HA fragments; 2) agarose gels were used for the separation of higher-molar-mass HA oligomers and polymers.

Polyacrylamide gel electrophoresis (PAGE) is the classical, probably the most frequently used, method for the separation of HA oligomers. The PAGE method was described in detail by Cowman and Mendichi [172]. Visualization of HA fragments is usually obtained by different staining methods. In general, using the PAGE technique for HA good separation of the individual bands up to about 40-mer can be obtained.

When HA samples contain only several disaccharide repeating units (from 10- to 12-mers), PAGE is not appropriate. An alternative gel electrophoretic procedure known as Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) is more suited for these cases. In FACE technique, the sample is derivatized with a fluorescent group at the reducing end-group prior to electrophoresis. The FACE electrophoretic method was also described in detail by Cowman and Mendichi [172].

Along with the gel electrophoresis, capillary electrophoresis (CE) was successfully applied for the separation of HA fragments. CE is a high-resolution, rapid, quantitative method suitable for low-molar-mass HA samples. Procedures for the fractionation and the characterization of HA oligomers are well established and described in detail [171,172].

Detection of HA Fragments and Oligomers

Many detectors can be used in on-line connection to a chromatographic system for detection of the eluted HA products. They include UV (single wavelength or diode array), refractive index, fluorescent, or amperometric detectors. However, if more detailed information is required, as for example chemical modifications of HA fragments in comparison to the native high-molar-mass HA, more sophisticated detectors must be used. In such cases, MS detection (MALDI-TOF and ESI-MS) is particularly suitable, which is able to provide big amount of important information. ESI-MS can be used on-line with a chromatographic system (HPLC, CE), whereas a MALDI-TOF detector has to be used only off-line, or after a fractionation. MALDI-TOF analysis requires preliminary preparation of the sample-matrix mixture and in general this procedure is performed off-line, even though certain semi-automatic evaporative interfaces are available.

The already described ESI-MS technique is an effective and sensitive method for the characterization of low-molar-mass HA fragments. Typically, ESI-MS is used on-line to a chromatographic system and many efficient interfaces have been developed. In applying this method, certain problems may arise if salts, buffers, and detergents are present in the mobile phase. Moreover, the technique is not applicable to the polydisperse samples. The ESI-MS spectrum could be interpreted only if several distinct HA fragments are present after a successful separation and are represented by individual peaks. This MS method is especially powerful for the analysis of low-molar-mass HA.

MALDI-TOF precludes many previously mentioned problems and limitations of the ESI-MS technique. However, it is important to note that MALDI-TOF and ESI-MS are complementary, not alternative, techniques. Depending of the analytical goal, either one of these two methods can be more effective. MALDI-TOF technique was described in detail in

the literature [184]. In general, chemical structure, ionization, end groups, etc. are usually characterized by means of the MALDI-TOF detector [184].

Despite the high informative content of MS techniques, it is often required to gain better insight into the chemical structure of HA fragments and into the process of their degradation by free radicals. To this goal NMR and ESR spectroscopies can be used. NMR spectroscopy was proved useful in determining purity and structure of the individual HA oligomers [185], whereas the $^{\bullet}OH$ radical action on the HA polymer and on the two component monosaccharides was extensively investigated by means of ESR [188].

CONCLUSION

In recent years, new biological roles have been ascribed to HA along with its essential function as a component of ECM. Due to its unique rheological properties, hyaluronan has found many applications in pharmacy and medicine. Since it has been reported that biological effects of HA depend on its molar mass, methods of analysis of the HA fragments, establishment of their size and composition have gained on importance. Precise characterization of HA preparations, assessment of their purity and structure will allow for better understanding of the biological function of HA as well as of the processes of its degradation in living organisms and *in vitro*. In the recent years, our research group has extensively investigated mechanisms of oxidative degradation of hyaluronan by reactive oxygen species, in particular in the presence of transition metals (and ascorbic acid), thus in vitro modeling the conditions occurring in the inflamed joint of the arthritic patients [119,136,137,189]. In the investigation of the degradation processes rotational viscometry [136], MALDI-TOF mass spectrometry and spin-trap Electron Paramagnetic Resonance [137,190] has been applied as well as attempt was made to distinguish HA fragments using chemiluminiscent and thermal analysis methods [155]. The presented chapter thus constitutes a compilation of the obtained results with an in-depth overview of the state-of-the-art in the area of investigation of hyaluronan – an amazing and versatile biopolymer, which scope of applications has only begun to be realized and implemented in practice.

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