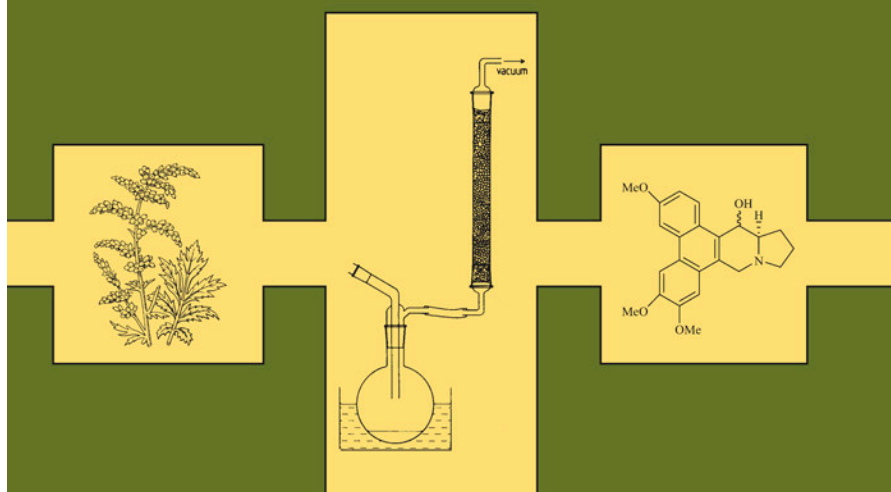




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Atta-ur-Rahman, FRS
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Edited by

Atta-ur-Rahman, FRS

*International Center for Chemical and Biological Sciences,
(H.E.J. Research Institute of Chemistry),
University of Karachi, Karachi 75270, Pakistan*



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HYALURONIC ACID: ITS FUNCTION AND DEGRADATION IN *IN VIVO* SYSTEMS

GRIGORIJ KOGAN¹, LADISLAV ŠOLTÉS², ROBERT STERN³,
JÜRGEN SCHILLER⁴, RANIERO MENDICHI⁵

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*Institute of Medical Physics and Biophysics, Faculty of Medicine,
University of Leipzig, 04107 Leipzig, Germany*

⁵*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 molecular 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. For experimental purposes, commercial HA samples are mostly of bacterial origin (gram-positive streptococci) or isolated from rooster combs, while in the human organism it is distributed in the skin, vitreous humor of the eye, umbilical cords, cartilage, and synovial fluid. In the latter, HA serves to maintain its viscoelastic properties required for lubrication of the joint. When hyaluronan is degraded by the action of free radicals or certain enzymes, synovial fluid loses its lubricating properties, which leads to increased wear of the joint and results in arthritic pain.

HA is also used as a diagnostic marker for many diseases including cancer, rheumatoid arthritis, and liver pathologies. 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. The observed reduction of the molecular mass of HA in the synovial fluid of patients suffering from rheumatic diseases led to *in vitro* studies of HA degradation by reactive oxidative species.

In this paper, the functional roles of hyaluronan in living organisms, 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.

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, Fig. (1) [1]. The structure of HA is very regular, the only minor deviation being the replacement of *N*-acetyl-D-glucosamine by deacetylated glucosamine residues.

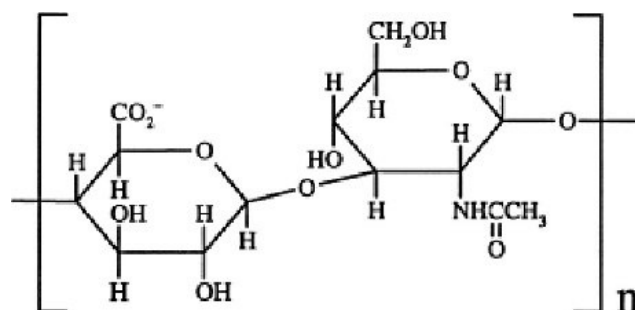


Fig. (1). Structure of the repeating unit of HA

HA belongs to a group of substances known as glycosaminoglycans (GAGs), being the simplest one among them, the only one not covalently associated with a core protein, not synthesized through the Golgi pathway, and the only non-sulfated one. The molecular mass of HA can reach as high as 10^5 - 10^7 Da and such high molar mass HA plays important physiological roles in living organism including maintenance of viscoelasticity of liquid connective tissues, such as synovial fluid 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 polymer can adopt a vast number of shapes, sizes, and configurations, whereas in the ECM under normal physiological conditions it is believed to exist as a random coiled molecule. It can

circulate freely, or be in a tissue-associated state. Due to its versatile biological properties and amazing viscoelastic properties, HA of both high and moderate or low molecular mass has found numerous applications in medical treatment and cosmetics preparations [3-5]. The current review will be focused on the occurrence and important biological functions of HA, as well as on the methods of its detection, characterization and some applications.

OCCURRENCE OF HA IN LIVING ORGANISMS AND ITS FUNCTIONS

Although HA occurs nearly ubiquitously (however in relatively small amounts) in the human body and in other vertebrates, the largest amounts of HA are found in the extracellular matrix of soft connective tissues [6]. Besides vertebrates, HA is also present in the capsules of some bacteria (e.g. strains of *Streptococci*), but 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. Some years later Carl Mörner continued this work by the investigation of the elemental composition of eye fluids with a special focus on the nitrogen and sulfur content [8].

Later, a more detailed investigation was performed by Karl Meyer, who summarized the results as follows [9]: "From the vitreous humor of cattle eyes a polysaccharide acid of high molecular mass has been obtained... As constituents there have been recognized a uronic acid, an amino sugar... It appears to be a substance unique in higher animals, and may be best compared with some of the specific polysaccharides of bacteria... We propose, for convenience, the name "hyaluronic acid", from hyaloid (vitreous) + 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 [10]. In the organism, HA occurs in many diverse forms, circulating freely, decorated with a variety of HA-binding proteins (hyaladherins), tissue-associated, intercolated into the ECM by electrostatic or covalent binding to other matrix molecules. It comprises a major portion of the intimate 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.

HA has also been assigned many functions: a space filler, an ion exchange filter, a medium for hydration, promoter of cell migration, regulator of the cell cycle, and a key to embryonic development, tissue repair, and regeneration. HA has size-specific functions such as those involved in inflammation, angiogenesis, promotion of wound healing, and induction of heat shock proteins. This suggests that the linear polymer fragments constitute an information-rich system. In this section, we review the occurrence and the variety of forms that the HA molecule can take *in vivo*, as well as provide a brief overview of the many functions attributed to this simple, unadorned, but 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]. The most relevant sources of HA are listed in Table (1).

Table 1. Occurrence of HA in Different Animal Tissues and Its Content

Tissue or body fluid	Concentration [$\mu\text{g/ml}$]	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 (synovial) fluid	1400-3600	The volume of the synovial fluid 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 vitreous body	140-340	HA concentration increases upon the maturation of this tissue.
Human dermis	200-500	Suggested as a "rejuvenating" agent in cosmetic dermatology [13].
Human epidermis	100	HA concentration is much higher around the cells that synthesize HA.
Rabbit brain	65	HA is supposed to reduce the probability of occurrence of brain tumors.
Rabbit muscle	27	HA is a major constituent in the pathological matrix that occludes the artery in coronary restenosis.
Human thoracic lymph	0.2-50	The low molecular 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 hyaluronidase.
Human serum	0.01-0.1	HA concentrations increase in serum from elderly people as well as in patients with R.A. and liver cirrhosis [14].

Below, we present the brief review of the occurrence of HA in some tissues and organs of the human body.

Localization of HA in the Organism

Inside-Outside

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 narrow confines of the ECM or the restricted volume of the intercellular space. However, when HA undergoes aqueous extraction from major sources, such as from the mucoid layer of the rooster comb, from joint fluid, the perivascular space of 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*, and little is known about the state of the HA within tight tissue spaces. 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 the hyaladherins [16,17]. These constitute a wide range of proteins and glycoproteins, the majority of which are members of the link superfamily of proteins [18]. The 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. Assigning specific isoforms with particular functions has not yet been accomplished. 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 hyalactans [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. This is an important structure that has not been well studied. 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 [36-39]. 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 [40,41] 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 [42].

Biosynthesis and the question of re-uptake of HA present a conundrum. 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 [43]. 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 in liver and kidney diseases, particularly in liver or kidney failure (for review see [44]).

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 [45]. The HA concentration in the circulation of cancer patients is also often elevated [46].

Fetal Tissues

HA is also prominent in embryogenesis. It helps to maintain the undifferentiated state, and removal of such by hyaluronidases is essential for the onset of differentiation [14]. There are very high levels of HA in the fetal circulation [47], as well as in amniotic fluid [48] 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 [49], 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 [50]. 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 (≈ 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 [51] 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 traumatic 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 this review.

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 [52]. The attachment is stabilized by a link protein that binds both aggrecan and hyaluronan [53]. A link protein-aggrecan complex occupies 24-30 monosaccharide residues on the HA chain [54]. 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

Synovial fluid 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. Though it has been reported that the ECM of brain tissue is relatively resistant to invasion by tumors of non-neuronal origin [55], it is known clinically that the brain is often invaded by metastases from other sites. Metastatic spread from carcinomas is in fact the most common form of brain tumors. These presumably invade by the hematogenous route.

HA in Cancer Tissue

The levels of HA surrounding tumor cells correlate with tumor aggressiveness [56]. 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 [57]. 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 [58]. Interaction of HA with cancer cell surface receptors enhances tumor cell survival and invasiveness [59,60]. It is clear that the interaction of HA with the malignant process occurs at multiple levels.

The 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 [61].

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 [62].

The fetal circulation contains high levels of HA, as do fetal tissues, and amniotic fluid [48]. 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 [34,35,63]. 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 G₀ 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), termed 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 [64].

It is commonly accepted that without the PG/HA interaction, the PGs would not be retained within cartilage. Since the prime 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 [65]. The interaction with collagen is, however, primarily mediated by the keratan and chondroitin sulfate of the individual PGs.

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 [66], and immunosuppressive [67,68], to intermediate-sized, comprising 25-50 disaccharide fragments, that are inflammatory [69], immunostimulatory, and highly angiogenic [70], to decasaccharides that compete with larger fragments for receptors [53], and to even smaller tetrasaccharides that are anti-apoptotic and inducers of heat shock proteins [71]. These low molecular 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.

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 are the instruments of choice to characterize the macroscopic properties of HA solutions [72]. The shock absorbing properties of synovial fluids 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 [73]. 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 [74]. Of recent interest is the observation that RHAMM may substitute for CD44 when the latter has undergone genetic deletion [75]. 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 [76].

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 [77] 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 [78].

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) [79] that play an important role in tissue destruction during inflammation.

BIOSYNTHESIS OF HA IN LIVING ORGANISMS AND *IN VITRO*

It was a very important discovery made by Clarris and Fraser that some cells (e.g. synovial cells) are covered by a hyaluronidase-sensitive coat [80]. Accordingly, different cell lines have variable ability to synthesize HA.

A very simplified schema of the biosynthesis of the individual glycosaminoglycans is shown in Fig. (2): Hyaluronan synthases synthesize large linear polymers containing the repeating disaccharide building block of HA by alternate addition of D-glucuronic acid and *N*-acetyl-D-glucosamine to the growing chain using their respective "activated" nucleotide sugars (UDP-glucuronic acid and UDP-*N*-acetyl-D-glucosamine) as substrates [81]. At least three different types of hyaluronan synthases are known to exist in the vertebrates.

The biosynthesis of HA is quite remarkable because it is the only glycosaminoglycan in vertebrates that is not synthesized in the Golgi apparatus, that is not sulfated, and that is not attached to a core protein to form a proteoglycan. HA is synthesized in the cytoplasm, on the inner surface of the cell surface membrane [82] and is extruded through that membrane into the extracellular space, sugars being added constantly onto the end of the molecule. There remains some controversy as to whether addition is to the reducing [83] or to the non-reducing [84] end of the molecules. Convincing arguments have been made for each of these two possibilities. In fibroblast cultures, the rate of HA biosynthesis is regulated in part by cell density, and hence by the proliferative state of the cell. At low cell densities, HA biosynthesis occurs at a high rate, and cell motility and cell proliferation are high, as well. On the other hand,

at high cell densities, cell proliferation is low and HA biosynthesis is shut down [85].

Although some details of the biosynthesis of HA have still to be clarified, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGF- β) and other cytokines, e.g. interleukin-1 (IL-1) are known to activate the synthesis of HA in the cells [86].

The HA polymer is so large and space-occupying that it is extruded while it is being synthesized, permitting unrestrained polymer growth. If the polymer were not being extruded as it was being synthesized, and instead remained intracellular, great havoc would ensue for the cell. Extrusion from the cell occurs perhaps through an ATP-binding cassette (ABC) transporter system [87,88] related to the multi-drug resistance transporters.

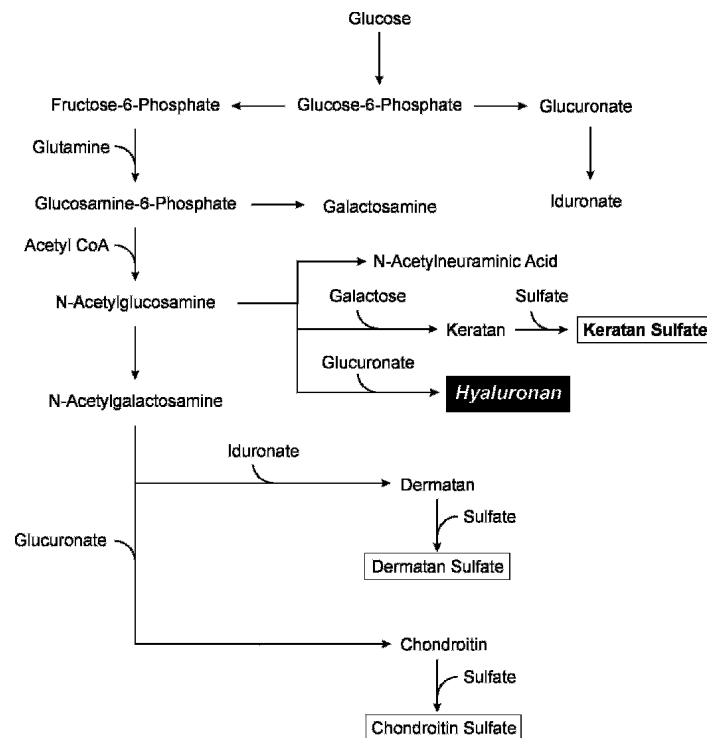


Fig. (2). Scheme of the biosynthesis of the most relevant GAGs

The Bacterial HA Synthases

A single enzyme is now recognized as being able to synthesize HA. This enzyme is a member of an enzyme family, integral membrane dual-headed glycosyl transferases that utilize alternatively the two UDP-activated sugar substrates, UDP-glucuronic acid, and UDP-*N*-acetyl-D-glucosamine. The enzymes that synthesize HA, the HA synthase (HAS) enzymes were extremely difficult to solubilize and characterize. The first description of an HA synthase enzyme activity was from a Group A *Streptococcus pyogenes*, in 1959 [89]. The cloning of a synthase from these same bacteria [90,91] and its successful expression [92] did not occur until 60 years after the first description of HA [9]. The bacterial enzymes appear to add sugars strictly to the reducing end [93].

A unique HAS enzyme occurs in the virus that infects an algae *Pasteurella multocida* (pmHAS) that is quite different from all other HA synthases [94]. This is in a class by itself and will not be considered further.

The Vertebrate HA Synthases

There are three to four HAS genes in the vertebrate genome, expressing enzymes with different properties exhibiting different rates of HA synthesis, as well as producing oligomers of different lengths, *HAS-1*, *-2* and *-3* [95]. The frog, *Xenopus laevis*, has apparently four such genes [95]. In vertebrates, the K_M is similar for HAS2 and HAS3, but considerably higher for HAS1. Furthermore, HAS1 and HAS2 produce relatively large forms of HA, (2×10^5 - 2×10^6 kDa), while HAS3 produces polymers of only half that size (10^5 - 10^6 kDa) [96]. Because of the versatile roles of HA in biology, some of which are size-dependent, it is reasonable to assume that some of the controls of such functions may be through expression and use of these different HAS isozymes.

In the human, the three HAS genes occur on three different chromosomes, but are very similar in size [97]:

<i>Has1</i>	19q13.3	578 aa
<i>Has2</i>	8q24.12	552 aa
<i>Has3</i>	16q22.1	553 aa

Interestingly, single or double knock-out mice for the *Has1* or the *Has3* genes are viable and fertile, and have no apparent phenotype, while the *Has2* knock-out mouse is an embryonic lethal. Among the many abnormalities is the need for HAS2 for normal cardiac development, particularly the cardiac cushion [98].

Genome structural analyses indicate these all evolved from a common ancestral gene. Structural similarities between species as widely separated as frogs and primates suggest that the HAS genes arose very early in vertebrate evolution. Remarkably, the vertebrate sequence has approximately 30% identity with the bacterial HA synthase, suggesting an even more ancient common ancestral gene. Sequence homology with chitin and cellulose synthase genes indicates that a common β -chain saccharide-synthesizing enzyme may have existed very early in evolution [99].

A Multiprotein Complex

A large number of other functions are required for the successful production of the HA polymer, each perhaps supported by a different protein. These might include binding sites for each of the two nucleotide sugar moieties, two separate glycosyl transferases activities, binding sites for anchoring or orienting the growing HA chain, and a ratchet-like transfer reaction that would promote extrusion of the growing polymer one sugar at a time. This is reflected in a multiprotein complex that was described for HA synthesis two decades ago [63,100], a result that was received with much skepticism at the time.

The Hyaluronasome

A putative multi-protein complex, membrane-associated in vertebrate cells for the synthesis, regulation, and degradation of HA is invoked, termed the "hyaluronasome."

This may have been the complex observed by Mian [63,100]. This mini-organelle may parallel the easily observed glycogen granules in liver and muscle cells [101]. This bead-like structure constitutes a functional unit containing substrate, the enzymes required for synthesis and catabolism, and all the proteins required for regulation of its metabolism. These proteins associate and dissociate according to the needs of the cell at the moment, regulation taking place as a result of allosteric interactions, and signal transduction pathways that activate or inactivate the pathways. There may be parallels between the α -linked and the strictly β -linked polysaccharides.

Preliminary evidence for the existence of such an organelle comes from a variety of sources. Treatment of cells with low concentrations of hyaluronidase stimulates anomalous levels of HA synthesis, suggesting that some feedback mechanisms exist that instruct the cells in the status of HA metabolism [102,103]. Treating cells with even higher concentrations of hyaluronidase stimulates levels of expression of the predominant HA receptor, CD44 [104,105].

Levels of HA deposition respond to the various physiological states of the cell. In cultures of normal animal cells, growth phase [34], and degrees of confluence are inversely related to HA levels in both fibroblasts [106] and keratinocytes [38]. HA has been correlated with the onset of mitosis and detachment from the substratum [35], calcium concentrations [107], anoxia and lactate levels [108], viral transformation [109], and serum stimulation [110,111].

Thus, there appears to be an interlocking directorate with dynamic reciprocity for the various components of HA deposition that responds to the immediate needs of the cell. A tightly associated complex, termed the hyaluronasome, that contains HA receptors, the HA synthases, hyaluronidases, their respective inhibitors, exo- β -glycosidases, as well as a spectrum of HA-binding proteins may constitute such an organelle.

Tissue-specific Expression of the HA Synthases

The HAS genes appear to be, in part, tissue and cell specific. HAS1 maintains low basal levels of HA synthesis. HAS2 is involved in embryonic and especially in cardiac cushion morphogenesis, and is associated with cell migration and invasion, cell proliferation, and with angiogenesis during development. HAS2 is also widely distributed in normal adult tissues. HAS3 appears to favor the malignant phenotype.

HA is a major molecule in joint fluid, and plays a major role in that tissue in joint motion and maintenance of normal joint homeostasis. In inflammatory diseases of the joint such as rheumatoid arthritis (RA), and in degenerative joint disease such as osteoarthritis (OA), the levels and average molecular size of HA chains are much reduced. Expressions of both HAS1 and 2 are decreased, while the hyaluronidases are increased in the various forms of arthritis, compared to control, with a concomitant decrease in average molecular sizes [112].

In the ovum cumulus complex, HAS2 mRNA is expressed when HA synthesis begins to rise. HAS2 has the ability to be stimulated by a number of cytokines and growth factors, including PDGF-BB in mesothelial cells. Glucocorticoids suppress HAS2 mRNA in dermal fibroblasts which contribute to the total body steroid effect. A similar suppression occurs in osteosarcoma cells [113]. IL-1 β and TGF- β inhibit HAS2 mRNA in osteosarcoma cells and in chondrocytes [114].

Regulation of HAS Enzymes

Little is known about regulation of the three different HAS enzymes. Experiments with HAS genes are usually conducted with transfection models in cultured cells. It may be difficult to extrapolate such results into the *in vivo* situation. Nonetheless, an overview of recent experiments in HAS control mechanisms will be reviewed here.

The vertebrate HAS enzymes, even though they catalyze the same reaction, may be differentially regulated because each produces a polymer of different size [99,115]. They have distinct expression patterns that may be controlled in part by various growth factors and cytokines [96,114,116-118].

The association of HA with edema and inflammation suggests that investigations of inflammatory disorders and anti-inflammatory drugs would provide a reasonable point of departure. Comparing the three isoforms of HAS enzymes in synovial fibroblasts in patients with rheumatoid arthritis and osteoarthritis, HAS1 mRNA is up-regulated by TGF- β , and HAS3 is up-regulated by IL-1 β and TNF- α , while HAS2 remains unchanged [119]. Induction of HAS2 in vascular smooth muscle cells occurs with prostaglandins [120]. During cervical ripening prior to parturition, HAS2 transcripts increase, while after delivery, transcripts of hyaluronidase-1 and -2 mRNAs increase [121]. It is apparent that levels of HA deposition participate critically in the process of delivery.

Compound K is a major component of the ginsenosides, the active ingredients of ginseng. There are many effects attributed to the ginsenosides, including anti-aging, anti-oxidant, and anti-inflammatory activities. In micro-array analysis of cultured cells, Compound K increases HAS2 mRNA in a time- and dose-dependent manner, and increases HA deposition in dermis and epidermis when applied to nude mouse skin [122].

HA Synthesis and Cancer

It is well established that HA participates in various phases of cancer, in growth, invasion, and metastatic spread. HA is an intrinsic component of the ECM, and disruption of that matrix leads to aberrant regulation of cell physiology. Extra- and intracellular signaling, dysregulation of proliferation, growth, and cytoskeleton organization, and abnormal production or retention of growth factors can lead to malignant transformation or enhance progression. The contribution of the HAS enzymes to such events has not been as yet elucidated.

An HA-rich matrix often correlates with a worse clinical prognosis. Metastatic potency is often enhanced in animal model systems when tumor cells are transfected with HAS genes. Inhibition of HAS gene expression with anti-HAS constructs reduces tumor adhesion and malignant potential [123,124].

In the course of cancer development, cells undergo changes that resemble epithelial to mesenchymal transitions that occur during embryology. Stimulation of HA synthesis is sufficient in some model systems to promote such transitions in apparently normal epithelial cell cultures. This transition can occur by transfection and over-expression of HAS2 [125].

There are many anomalies and conundrums in HA metabolism in relation to cancer biology and tumor progression. Some of these are now clarified by the observation that expression of Hyal1, a putative tumor suppressor gene product, can function as either a tumor promoter or a tumor suppressor, based on a dose-dependent effect [126]. Similarly, low levels of HAS2 expression over a narrow range stimulates tumor growth in transformed cells, while high levels of HAS2 expression inhibit tumor growth [118]. All these observations can be reduced to an argument that levels of HA deposition are able to either promote or suppress tumorigenicity in a complex dose-dependent fashion, with opposite results obtained at low and high doses.

Different HAS isozymes may also be utilized at various stages of malignant progression [118]. Initial studies are also being reported relating specific HAS expression profiles to particular tumors. Pleural mesotheliomas are the most representative of the HA producing malignancies. HAS1 expression is significantly higher in mesotheliomas, compared to pulmonary adenocarcinomas, lesions that can be difficult to distinguish [127].

In mesenchymal precursor cells, HAS1 mRNA is up-regulated in the bone marrow of multiple myeloma patients [128]. HAS1 mRNA is increased 20-fold over normal donor controls. Six-fold greater production of HA is documented in such cells.

Another layer of complexity appears to be emerging in the relationship between the HAS genes and malignancy. Splice variants for HAS1 are also related to poor outcome in malignant myeloma. Aberrant intronic splicing of HAS1, resulting in three novel splice variants are detected in such patients, one of which correlates with reduced survival [129].

Inhibitors of HA Synthesis

Leflunomide (Arava) is an anti-inflammatory medication used to treat the symptoms of RA. Leflunomide suppresses HA release from the synovial cells that line the joint capsule in a dose-dependent manner [130]. Leflunomide specifically suppresses induction of HAS1. HAS2 and HAS3, which are constitutively expressed in synovial cells, are not affected. Leflunomide is a specific NF- κ B inhibitor, but does not affect HAS1 in this manner. Rather, leflunomide functions here by suppressing tyrosine kinases.

Inhibitors of HA synthesis have valuable therapeutic potential in the prevention of cancer invasion and metastasis. A novel inhibitor of HA synthesis has been identified, 4-methylumbelliferone (4-MU). However, this inhibitor does not have a direct effect on the HAS enzymes. Rather, it is glucuronidation of the inhibitor that depletes UDP-glucuronyl transferase stocks, required for the formation of UDP-glucuronic acid. Thus, it is substrate depletion that appears to be the mechanism of action. The inhibitor is effective in both vertebrate and bacterial systems [131]. 4-MU has been shown to inhibit liver metastases of melanoma following oral administration in mice [132].

Natural inhibitors of HA deposition have also been encountered. Naturally occurring antisense mRNAs to HAS2 inhibit HA synthesis and suppress cell proliferation. Computer searches have predicted over 2000 such sense-antisense RNA pairs, which are highly conserved between species. This may take up a large part of the genome, and appear to play a role in regulating expression, including transcription, RNA editing, post-transcriptional and translational effects [133].

Thus, even though a large number of HAS gene products have now been identified, the field is still in its relative infancy. Full characterization of the various human isozymes has not been achieved, and their aberrant expressions in disease states remain to be defined. The mechanisms, by which these enzymes are controlled, and the growth factors and cytokines that regulate these isozymes, and the inhibitors that modulate activity are only beginning to be outlined. Much information will become available as targeted mutagenesis, particularly for the *Has2* gene, is studied. This appears to be the key gene in mammalian biosynthesis of HA [134].

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 [135,136]. 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 & 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 [137,138]. 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 so-called “link proteins”. The presence of “frozen”/crystalline 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 [139] and subsequent change of their properties. Hygroscopicity of dry HA is another complicating factor, when the solution with a precisely defined concentration of HA is required. 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 immuno-stimulatory. Therefore, recently Camenisch and McDonald [140] 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 visco-supplementation. 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 [141]:

- 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 pharyngeal 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. [142]).

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 molecular 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 molecular mass of HA as well as on its concentration: When the concentration increases, the motion of the molecular segments becomes more restricted [143]. 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 [144].

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 [141] or [145], 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 [49]. 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 [146].

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 [147]. Restylane[®], produced by the Medicis Corp. (Scottsdale, AZ, USA) is prominent among such HA-based injectable materials [148]. 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.

Another major source of HA and the principle one for commercial purposes, is in the mucoid layer of the rooster comb. The synthesis of HA within the comb is under testosterone control

[149]. In mammals, swelling of the perineal area of the aroused female is attributed to HA accumulation, and is under control of estrogens. The "sex skin" of the baboon is a variant of this phenomenon.

During pregnancy, HA concentrations in the human cervix are very low, but increase rapidly at the onset of labor. HA, with its affinity for water molecules, loosens and separates cervical cells through tissue hydration. Thus HA plays an important role in cervical ripening, being involved in the regulation of cervical tissue water content. Modulation of the HA content of the cervix using various growth factors and chemokines have the potential for stimulating or facilitating smooth delivery. On the other hand, the potential exists for delaying parturition in women susceptible to preterm delivery.

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 from the previous 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 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 [150]. 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.

Otolaryngology

HA preparations with various viscoelastic properties promote healing of perforated tympanic membranes [151,152]. Films of HA esters, such as HYAFF[®] provided by Fidia, Inc. (Abano Terme, Italy) are used in ear and sinus surgery. These preparations promote wound healing of the tympanic membrane, facilitate re-epithelialization, 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. Hyaln B slurries injected into vocal cords produce no inflammatory reactions, and the material continues to be present even after one year [153].

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 [154]. Low molecular 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 [155].

Surgery and Wound Healing

High molecular size HA preparations, applied topically, promote healing of fresh skin wounds [156]. They also promote the healing of venous leg ulcers [157] and are useful in the management of chronic wounds [158].

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 [159].

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 [160]. 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* [161].

Urology

Hylan B is used in a bulking procedure for urinary sphincter muscles and as a treatment modality for certain kinds of urinary incontinence [162]. It is also used in combination with dextran sulfate for the treatment of children with vesico-ureteral reflux [163].

Future Directions

There is an emerging field of HA-derived therapeutics that depends on combination with proteins, other glycosaminoglycans, or using HA oligomers of defined size. Lower molecular mass HA fragments of defined size represent an information-rich system that can be used for specific purposes. Protein combinations, or inclusion within liposomes, augmented by targeting mechanisms such as CD44, or other HA receptors are still in their infancy (see [164]). However, precise targeting of such agents, including a new generation of anticancer therapeutics, offers a great promise. This, together with tissue engineering, indicate an exciting future for this field.

HA CATABOLISM IN LIVING ORGANISMS AND UNDER *IN VITRO* CONDITIONS

The HA of the vertebrate body turns over extremely rapidly, with a $t_{1/2}$ of 2-5 min in the bloodstream [165], and within days in tissues. The mechanisms, by which this occurs and how these reactions are regulated are not understood. The degradation of HA in the body occurs in a stepwise process [166] through three separate pathways, however details until recently have defied explication.

There are approximately 15 g of hyaluronan in the 70 kg individual, of which 5 g are cycled daily through the three pathways [167,168]. The relative contribution of each of these pathways to total turnover can only be estimated. A description of these three separate pathways is presented below.

Tissue and Whole Organism Turnover of HA

The primary pathway for HA turnover occurs at the tissue and whole organism level, and accounts for 85% of total catabolism. HA is released from tissue matrix, drained mostly into the lymphatics, with some residual into the bloodstream, with final steps of elimination occurring in liver, kidney, and possibly spleen. This pathway involves unique receptors such as HA Receptor for

Endocytosis (HARE) [169] and Lymphatic Vessel Endothelial HA receptor (LYVE-1) [170]. When the hepatic or renal arteries are ligated, levels of circulating HA rise immediately, underscoring the importance of this pathway [171].

Local Cellular Turnover of HA

The local turnover of HA at the cellular level includes binding, internalization, and degradation, which is relatively rapid, varying from hours to days. This degradation of HA occurs by a series of coordinated enzymatic reactions, constituting a catabolic pathway. Rapid measurement of such hyaluronidase (Hyal) activities has only recently become available. In addition, these somatic Hyals are present at exceedingly low concentrations. In human serum, e.g., Hyal1, the only hyaluronidase that occurs in serum, is present at the concentration 60 ng/ml [172]. These somatic enzymes have high specific activities, however are unstable during purification, unless detergents and protease inhibitors are constantly present during their isolation. Many of such difficulties have now been overcome, and much information is now available on the content and function of these enzymes.

The catabolic scheme, formulated only recently [173,174], proposes that the high molar mass HA polymer is cleaved in a series of controlled enzymatic scissions, in which the product of one reaction becomes the substrate for the subsequent reaction. These successive enzymatic reactions generate oligomers of ever-decreasing sizes. It may be reasonable to assume that these very enzymes are responsible for the supply and maintenance of size-specific oligomers. However, there is no evidence to date that the hyaluronidases perform such a function.

In the human genome, there are six known genes coding for the Hyals: *hyal1*, *hyal2*, and *hyal3*, *hyal4*, and *PH-20/Spam1*, as well as a pseudogene *Phyal1* that is transcribed but not translated. The first three Hyal genes, *hyal1-3*, are tightly clustered together on chromosome 3p21.3, and the latter three, *hyal4*, *PH-20/Spam1*, and *Phyal1*, are clustered similarly on chromosome 7q31.3 [175,176].

An original gene coding for a hyaluronidase-like enzyme may have appeared, followed by two duplication events, and then *en masse* duplication of this triad, generating the six genes. These events seem to have occurred before the appearance of modern mammals.

Hyal-1 and Hyal-2 are the two major Hyals in somatic tissues of vertebrates. Hyal-2 degrades high molar mass HA to an approximately 20 kDa product (\approx 50 disaccharide units), whereas Hyal-1 can degrade high molar mass HA to smaller products, predominantly to tetrasaccharides. Hyal-3 protein product is expressed, but the precise nature of its activity is still being investigated (B. Triggs-Raine, personal communication). Human PH-20 or SPAM1 (SPerm Adhesion Molecule 1) required for fertilization, is associated with testes, and facilitates penetration of sperm through the cumulus mass in its passage to the ovum. In some species, but not in human, an additional duplication event has occurred, yielding four genes at the 7q31.3 locus. The gene coding for PH-20 appears to have undergone a duplication, which may explain why the PH-20 knock-out mice are fertile [177]. Thus, there are seven *hyal* genes in some mammalian species. It is also reasonable to suggest that the Hyal genes in vertebrates are continuing to evolve.

Degradation of HA in somatic cells begins when extracellular high molar mass HA polymers of the ECM are tethered to the cell surface through the combined action of Hyal2 and the predominant HA receptor, CD44 [38]. Receptors other than CD44 may be involved, perhaps on a tissue-specific basis. Binding occurs with the assistance of Na⁺-H⁺ exchanger 1 (NHE1) [178]. The polymer is cleaved by Hyal2 to intermediate-sized polymers of approximately 20 kDa (50 disaccharides). These are highly angiogenic, immunostimulatory, and inflammatory. Fragmentation continues through the action of acid-active Hyal1 generating ultimately the small HA oligomers that can induce heat shock proteins and are anti-apoptotic [71].

There are two lysosomal exoglycosidases that cleave HA termini from the non-reducing termini, one saccharide at a time, β -

glucuronidase and β -*N*-acetylglucosaminidase. The monosaccharidic products, glucuronic acid and a glucosamine derivative, are released from lysosomes to cytoplasm and become available for other metabolic cycles.

Free Radical Scission of HA Chains Contribute to HA Turnover

Scission of HA can occur by free radicals under oxidative conditions, and is promoted by transition metal ions [179-181]. The exact proportion of total HA turnover contributed by free-radical scission in the vertebrate organism is not known.

Free radicals and hyaluronidases may have their activities coordinated under certain pathologic situations. A burst of myeloperoxidase activity occurs early in inflammation, when polymorphonuclear leukocytes (PMNL) are the predominant cells. These inflammatory cells, which are involved early in the course of infection and in wound healing, also generate superoxide radicals ($O_2^{\bullet-}$), and hence H_2O_2 , by way of an oxidative burst. Reaction of myeloperoxidase with H_2O_2 in the presence of chloride ions generates HOCl (hypochlorous acid). Exposure of HA to this mixture causes fragmentation. This process is inhibited by superoxide dismutase, metal ion chelators, and by albumin. Albumin is also an HA-binding protein, which may function as a protective mechanism for bound HA. The damage to HA induced by HOCl and by $O_2^{\bullet-}$ may be of significance at sites of inflammation, where both oxidants are generated. The high molecular size HA, which is anti-inflammatory, is cleaved to fragments of smaller size that possess inflammatory and angiogenic activities.

The timed activity of PMNL within the first 24 h after wound infliction may be coordinated with the hyaluronidases. Such an orderly scission of HA chains can generate the inflammatory fragments required in the subsequent steps of inflammation and wound repair.

There is a vast literature describing the scission of HA by free radicals. A major challenge to the field would be studies that integrate free radical scission of HA with the physiological situations, using cultured cells or cell-free extracts. A more extensive description of HA scission by free radicals is presented elsewhere in this review.

Severe Stress and Regulation of HA Catabolism

Under conditions of severe stress, such as extensive blood loss, burns [182], massive trauma or surgery, shock, and septicemia [183], HA levels in the circulation increase dramatically. The HA may function as an emergency molecule, a naturally occurring volume expander to prevent or delay circulatory collapse.

HA content also increases in association with aggressive malignancies (for reviews, see [176,184]). How the three pathways interact with each other under these conditions is of major importance. Measuring HA or hyaluronidase levels in the blood stream are now simple and straightforward procedures, but are rarely performed at clinical laboratory tests. Tight regulation of catabolism is crucial for modulating steady state levels, important for normal homeostasis, as well as for embryonic development, wound healing, regeneration, and repair.

The interplay between these three individual catabolic pathways and with the HA synthetic reactions that result in such wide excursions of HA levels remains unknown. The clinical settings, particularly situations of severe stress, are valuable experiments in biology that should not be overlooked.

Further Analyses of the Vertebrate Hyaluronidase Enzyme Family

The vertebrate hyaluronidases, previously neglected enzymes [185], are all endo- β -*N*-acetylhexosaminidases employing substrate hydrolysis as their mechanism of action. These hydrolases have, until recently, been difficult to assay. Turbidometric and

viscometry-based assays require large amounts of enzymes, unsuitable for purification procedures.

A number of assays for the hydrolase-type of Hyals were developed in the last decade that facilitated their characterization. These include microtiter-based ELISA-like assays, in which a highly specific HA-binding protein substitutes for the antibody component [186]. Biotinylated HA bound to microtiter plates is subjected to Hyal activity, and the remaining HA quantified by an avidin-enzyme color reaction [107]. HA substrate gel zymography procedures were also formulated that facilitated additional studies of these enzymes [187].

The first vertebrate hyaluronidase activity to be identified was derived from a testicular extract [188], active at neutral pH. A similar enzyme, but active at acidic pH, was later documented in human serum [189]. Another thirty years passed before sufficient purification was achieved for sequence analysis [172].

Isoforms of Vertebrate Hyaluronidases

The hyaluronidase enzymes from vertebrate tissues occur in a number of isoforms. The biological significance of these isoforms is not known, nor is it understood how they participate in the catabolic scheme.

Hyal1 occurs as a 54 kDa form in plasma, while this and a processed 49 kDa isoform occur in urine [190], and in some tissue extracts. This latter isoform is the result of two endoproteolytic cleavages that remove 99 amino acids from the proximity of the carboxy terminus. The resulting protein chains are presumably held together by disulfide linkages.

Three of the hyaluronidases from vertebrates are GPI-anchored, namely Hyal2, Hyal4, and PH-20. Processed forms of Hyal2 and PH-20 have been detected, with removal of the GPI-membrane anchor, thus releasing the enzyme and generating a freely soluble form. This has been well documented for PH-20 [191,192]. Splice variants of Hyal1 and Hyal3 mRNAs have also been documented

[193], adding another layer of complexity to these enzyme isoforms.

Other Hyaluronidases in Biology

Bacterial Hyaluronidases

The Hyals from bacteria have been thoroughly characterized [194]. They are eliminases (lyases) that generate a double bond during the reaction, which is easily monitored by spectrophotometry. It is a common misconception that the bacterial Hyals have absolute specificity for HA. This is not entirely correct. Both bacterial and vertebrate enzymes degrade chondroitin (Ch) and chondroitin sulfate (ChS) [195], though some bacterial Hyals, such as the one from *Streptomyces hyalurolyticus*, often used as a research reagent, are however specific for HA.

Organisms Containing Endo- β -glucuronidase Type Hyaluronidases

There are hyaluronidases that function as endo- β -glucuronidases that cleave the (1 \rightarrow 3)- β glycosidic bonds. However, no sequence data on these enzymes is yet available. These enzymes are characteristic of annelids such as leeches *e.g.*, *Herudo medicinalis* [196-198], and certain crustaceans [199]. They utilize the hydrolysis mechanism, and thus resemble the vertebrate enzymes more closely than the prokaryotic hyaluronidases.

Fungal Hyaluronidases

Several species of fungi have been reported to contain hyaluronidases [200-202]. However, without extensive characterization, and in the absence of sequence data, it is difficult to evaluate these enzymes. There is no evidence that fungi contain HA, nor that they are able to uptake this GAG. The hyaluronidases may, however, be virulence factors for these organisms, similar to their function in some bacteria. Considering that opportunistic infections caused by *Candida* and other fungal organisms are on the increase with the use of wide-spectrum antibiotics, anti-cancer

drugs, immunosuppressive agents, and with the AIDS epidemic, these putative hyaluronidases should be studied more extensively.

Venom Hyaluronidases

Hyaluronidase activity is present in the venoms of a surprisingly wide variety of organisms. These include bees, wasps, hornets, spiders, scorpions, as well as certain species of fish, snakes, and lizards [203,204]. Some of these venoms, particularly those from snakes, have stretches of sequence with 36% identity with testicular hyaluronidase, PH-20. Interestingly, these hyaluronidases have absolute specificity for HA [205]. Hyaluronidase in venoms may act to facilitate penetration of other venom active ingredients. However, other evolutionary selective advantages of venom hyaluronidases may exist that have not yet been identified.

Enzymatic Mechanisms

Degradation of HA is performed by the vertebrate Hyals utilizing a hydrolase mechanism, while the bacterial enzymes use a lyase mechanism. Both classes of enzymes, however, are endo- β -*N*-acetylhexosaminidases that cleave the (1 \rightarrow 4)- β glycosidic bonds. Both groups of enzymes, hydrolases and lyases, have to a limited degree, the ability to degrade Ch and ChS. However this occurs at a much slower rate. Chondroitins are glycosaminoglycans that differ from HA only in one hydroxyl position at the C-4 atom, due to which the *N*-acetyl-D-glucosamine of HA becomes the *N*-acetyl-D-galactosamine of Ch.

Many of the vertebrate Hyals also have trans-glycosidase activities, with the ability to cross-link chains of HA [206], and the potential ability to cross-link chains of HA with ChS or Ch. The precise enzymatic mechanism of the process is not known, nor whether the reaction has biological significance.

The term "hyaluronidase" was introduced by Karl Meyer [207] to denote the enzymes that degrade predominantly HA. The enzymes were originally classified into three distinct classes, utilizing a scheme based only on the biochemical analyses of the

reaction products. The genetic and sequence data were not available at the time. The scheme is as follows:

- 1) The vertebrate β -endoglycosidase hydrolases, that are (1 \rightarrow 4)- β -*N*-acetylglycosaminidases (EC 3.2.1.35)
- 2) The bacterial β -endoglycosidase eliminases that also are (1 \rightarrow 4)- β -*N*-acetylglycosaminidases (EC 4.2.99.1)
- 3) The leech and crustacean hyaluronidases that are (1 \rightarrow 3)- β -glucuronidases (EC 3.2.1.36)

We now know that Meyer's classification scheme was remarkably accurate, and no modification is required. This is a tribute to biochemists such as Karl Meyer who worked in that golden age of carbohydrate chemistry and enzymology.

DEGRADATION OF HYALURONIC ACID BY ROS IN INFLAMMATORY DISEASES

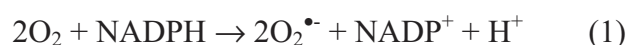
Many diseases are accompanied by inflammatory processes and in some of them the degradation of high molar mass HA is also involved. Such processes include, for instance, osteoarthritis (cartilage), wound healing (skin), asthma (airways), and urological malignancy. Usually the affected patient will complain 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 [208].

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 [209].

Neutrophils [210] are accumulated in the synovial fluid of the inflamed joints in large amounts [211], although the prime reasons for the accumulation and activation of neutrophils to generate ROS are not yet completely clarified [212]. The increased oxygen consumption by neutrophilic granulocytes upon stimulation under

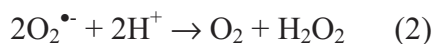
inflammatory conditions is commonly termed "respiratory burst" [213].

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 [214]:

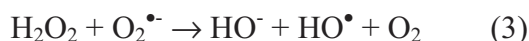


Although this is the most relevant pathway, other cellular sources for $O_2^{\bullet-}$ are the mitochondria in stressed cells, the generation of met-hemoglobin, and the reduction of oxygen by quinone radicals or by oxidized glutathione radicals ($GSSG^{\bullet-}$). The generation of $O_2^{\bullet-}$ 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:



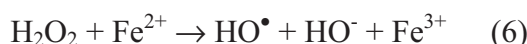
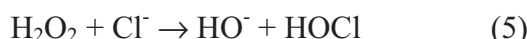
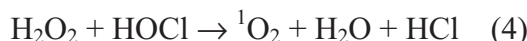
Since $O_2^{\bullet-}$ as well as H_2O_2 are simultaneously present, they are often assumed to react with each other:



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 [64]. These compounds are, however, deleterious in the presence of traces of transition metals, especially Fe^{2+} [215]. Accordingly, hydrogen peroxide is the starting material

for other ROS, including singlet oxygen (4), hypochlorous acid (5) and hydroxyl radicals (6) [216]:



The latter reaction (6) is the so-called "Fenton" reaction that is well known for over 100 years but still poses many mysteries [217]. 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 [64].

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" (RNS) [218]. The reader interested in details of these ROS and RNS is referred to a recent review by Šoltés *et al.* [219].

A highly simplified schema of the generation of individual ROS and RNS is shown in Fig. (3):

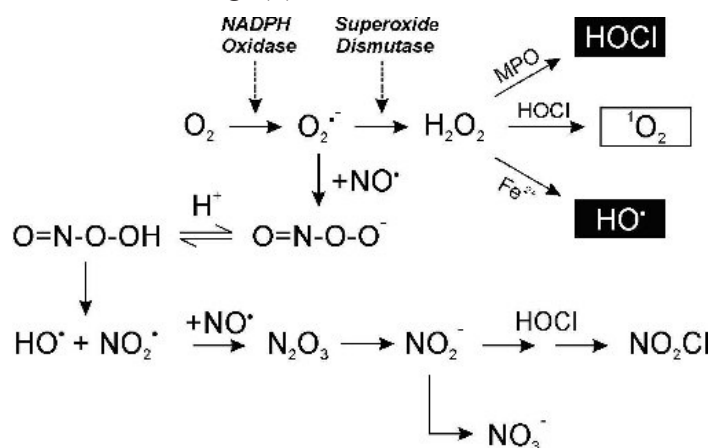


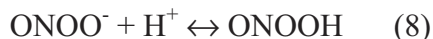
Fig. (3). Scheme of ROS and RNS generation under *in vivo* conditions. Please note that this is a very simplified summary of potential chemical reactions that does not take into consideration the different locations of enzymes and their substrates

Since its discovery, NO^\bullet has become one of the most frequently investigated biomolecules, because it functions as a second messenger molecule [218]. NO^\bullet possesses regulatory functions as endothelium-derived relaxation factor by interacting with the iron-containing prosthetic group of guanylate cyclase [220]. NO^\bullet is generated under *in vivo* conditions by the enzyme NO^\bullet synthase (NOS), of which several forms are known to exist [221]. 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 [222] (a survey of the half-life of many ROS and RNS is provided in [223]), 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 $\text{O}_2^{\bullet-}$ ($k = 3.7 \times 10^5 \text{ l mol}^{-1} \text{ s}^{-1}$) [224], peroxynitrite is formed.



This compound is in equilibrium with its corresponding acid, the pK_a value of which is 6.8 [225]:



Peroxynitrous acid, however, is not stable and decomposes to yield HO^\bullet and NO_2^\bullet [224]. 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 of up to 4 mmol/l can be detected in synovial fluids of the patients suffering from RA [226].

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• radicals that represent extremely reactive species.

Reaction of HA with O₂•⁻

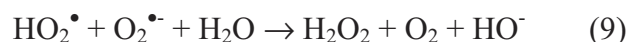
In many papers the term "superoxide" (HOO•) is used simultaneously with "superoxide anion radicals" (O₂•⁻). However, this is under the physiological conditions incorrect: The pK_a value of this acid-base equilibrium is 4.8 [227] and, therefore, there is only a very small contribution of HOO• at physiological 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 O₂•⁻ 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 [228]. However, not a single carbohydrate is mentioned in this comprehensive survey since no reaction could have been observed [229].

Although often suggested, it is nowadays clear that neither O₂•⁻ nor HOO• are able to react with carbohydrates as such. However, there is one recent study showing that O₂•⁻ plays an important role in the decomposition of the initial products generated by the reaction of other ROS, e.g. HOCl, with glycosaminoglycans [230]. This will be discussed below in more detail.

The "toxicity" of O₂•⁻ is exerted primarily by its penetration to important sites, where it is converted into further, more reactive oxygen species. When a reaction between O₂•⁻ and carbohydrates was observed, this was nearly exclusively the case when complex biomaterials, for instance cartilage [231] or body fluids, for instance joint (synovial) fluid [232], 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 [233]. 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 O₂•⁻. 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 $\text{O}_2^{\bullet-}$ is responsible for the observed deleterious effects.

Additionally, the recombination of two $\text{O}_2^{\bullet-}$ molecules (9) occurs much faster ($k = 8.3 \times 10^5 \text{ l mol}^{-1} \text{ s}^{-1}$) than other reactions.



Reaction of HA with H_2O_2

Pure hydrogen peroxide, similarly to $\text{O}_2^{\bullet-}$ is not capable of reacting with carbohydrates. Even if there are indications of the opposite [234], 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 [181,235,236].

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 reaction (6), i.e. *via* the Fe^{2+} ion-induced decomposition of hydrogen peroxide [237]. Because details of the Fenton reaction still remain unknown [217], reactions of HO^\bullet radicals are primarily investigated using radicals generated under *in vitro* conditions by water radiolysis [238]. Under these conditions, the kind and the yield of the generated radicals can be carefully controlled [239].

HO^\bullet radicals react in a diffusion-controlled way ($k \approx 6 \times 10^9 \text{ l mol}^{-1} \text{ s}^{-1}$) with virtually all compounds containing C-H groups under the abstraction of one hydrogen (H^\bullet) radical [240] leading to the generation of the corresponding alkyl radical.

The reaction between HO^\bullet and even simple carbohydrates is very complex [240]. 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 [240]. 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 peroxy 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 [240].

The second order rate constant of the initial reaction between the HO^\bullet radicals and HA is about one order of magnitude ($k \approx 7 \times 10^8 \text{ l mol}^{-1} \text{ s}^{-1}$) smaller in comparison to that of the reaction with glucose [179]. 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 [241], as well as in aqueous solution. The individual reaction steps summarized in Fig. (4) are quite similar to those occurring with the participation of glucose:

- a) Abstraction of one H radical from a C-H group under formation of the corresponding alkyl radical,
- b) Addition of molecular oxygen to the alkyl radical under generation of the corresponding peroxy radical,
- c) Elimination of O_2^\bullet ,
- d) Cleavage of the intermediate radicals under generation of the corresponding carbonyl compound.

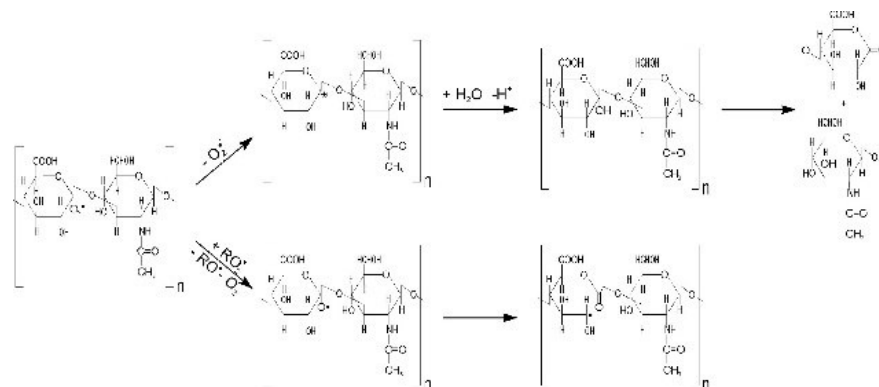


Fig. (4). Fragmentation patterns of HA under the influence of hydroxyl radicals (upon γ -irradiation)

The individual events are summarized in Fig. (4). Since the decrease of the molecular mass of HA is the prime effect of the action of HO^\bullet radicals, while functional groups are retained, methods enabling determination of the molecular mass were primarily applied in order to study the HO^\bullet radical-induced depolymerization of HA. For instance, Šoltés and coworkers used viscosimetry in order to study the effects of H_2O_2 and Cu^{2+} on HA solutions [242] 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 and enables the detection of even very small changes of the molecular mass. For instance, viscometry was also successfully used to study the (much weaker) effects of ozone and sunlight on HA solutions [243].

Changes of the molecular mass of HA could also be monitored by a number of further methods. For instance, size exclusion chromatography/multi-angle laser light scattering (SEC-MALLS) [244] and high performance liquid chromatography (HPLC) [215] 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. In Fig. (5), the influence of different doses of γ -irradiation on a solution of high molar mass HA was studied by ^1H NMR:

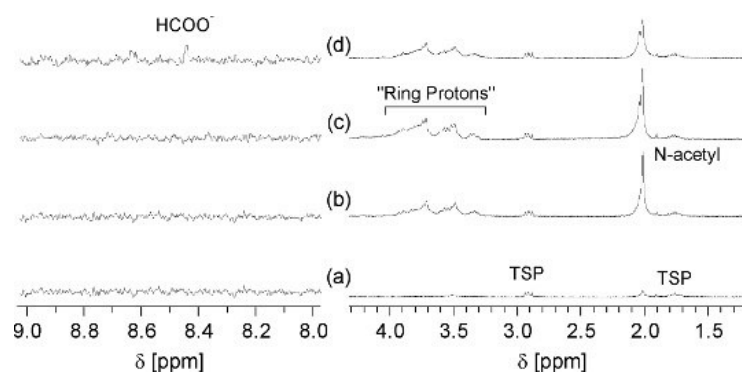


Fig. (5). ^1H NMR spectra of aqueous solutions of HA after exposition to irradiation from a ^{60}Co source. The following doses were applied: (a) no irradiation, (b) 5.15 kGy, (c) 10.3 kGy and (d) 20.6 kGy. TSP, Trimethylsilyl-propionic acid (internal standard). Adapted from [245]

Since NMR represents a kind of a "mobility filter", less mobile, rigid molecules such as HA with molecular masses in the MDa range are not detectable at all [245]. The lower the molecular mass of the compounds under investigation is, the more efficiently they are detected by using the NMR technique [245]. 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 [245]. 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 [240]. Therefore, it is evident that at high irradiation doses - in addition to the reduction of the molecular mass - fragmentation of the pyranose ring systems also occurs [245].

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 [246]: Subsequent to γ -irradiation, increased peak intensities of the *N*-acetyl groups of HA (at about 2.04 ppm) could be clearly monitored [247]. 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 synovial fluid. 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 [248].

Due to the quite similar structure of HA and other GAGs, e.g. ChS, clear distinction between both species can hardly be established by ^1H NMR. This is, however, possible by using ^{13}C NMR that is characterized by higher resolution than ^1H NMR [249]. 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 [250].

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 [251]: 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 [252].

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 [248].

This was demonstrated using the individual component monosaccharides of HA by measuring their consumption of HOCl [253,254]. 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 [255]. The reaction between HOCl and glucosamine may even be used for the quantitative determination of amino sugars [256].

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 $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$ system, on the other hand, on the solutions of HA [257]. 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 [257].

Using ^1H NMR spectroscopy [258] and a few years later also ^{13}C NMR [259], Schiller *et al.* are 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 are just transient products that decompose 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 the generation of acetate, on the one hand, and formate, on the other hand, enables the differentiation between the effects induced by HOCl and HO• radicals, respectively. It has been also shown that the acetate content is a potent marker of the MPO activity in the synovial fluid from patients with RA [259] 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 [254]: 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 molecular mass of the HA chain.

These results were extended a few years later by the same authors [260]: It was shown that the initially generated *N*-centered radicals undergo 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, undergo pH-independent β-scission reactions that result in glycosidic bond cleavage [260].

The stability of the initially generated *N*-chloroamide remains so far controversial. On the one hand, *N*-chloroamides are assumed to be "transient products" [258], while on the other hand, they were described to represent "long-lived species" [230]. This obvious discrepancy may result from two different reasons. First, this might result from the different experimental methods that were used: Schiller *et al.* [258] uses NMR spectroscopy, while Rees *et al.* [230] applies the 5-thio-2-nitrobenzoic acid (TNB) assay [261] to monitor the stability of the *N*-chloroamides. It is not yet clear, whether that

assay is capable of differentiating *N*-chloroamides and *N*-chloroamines that are generated by the cleavage of the initially generated *N*-chloroamides. Secondly, different experimental conditions may also contribute to these differences because, for instance, the presence of small amounts of transition metals also affects the stability of the *N*-chloroamides. Nevertheless, the observation that HOCl and $O_2^{\bullet-}$ act synergistically to induce fragmentation of ChS and HA is a very interesting fact that will stimulate further research.

Another study investigated the final products of the degradation of HA [262]: 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 Fig. (6).

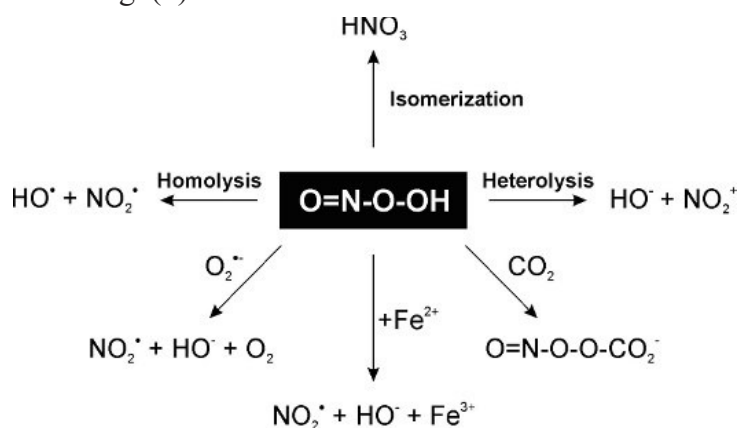


Fig. (6). Scheme of the potential reactions of peroxynitrite under generation of further harmful species

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 [263]. A more detailed investigation using spectroscopic methods as well as mass spectrometry (MS) has been published recently [264]: 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 [265] as well as ChS [266]. 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 ($^1\text{O}_2$) 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 $^1\text{O}_2$.

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 [267]. 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 [268].

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 molecular mass M_n (from osmometry), weight-average molecular mass M_w (from light scattering), z-average molecular mass M_z (from sedimentation), intrinsic viscosity $[\eta]$ and viscosity-average molecular 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 molecular 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 [269,270]: 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 [138,270-273]: 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 Schieronì [138] reported a successful SEC fractionation of medium, high, and also UHMM HA samples with the molecular 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 [272]. In the first part of the sub-chapter only separation methods for high molar mass HA samples will be considered. The separation methods suitable for low molar mass HA oligomers and fragments will be described in the last part of the sub-chapter dealing with the detection of HA degradation products. Due to their importance and predominant application in practice, only SEC and briefly F-FFF and IEC fractionation methods will be described.

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 not simple but, on the contrary, 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 Schieronì [138]. 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. In general, a column set composed of two aqueous columns with larger particle size and larger pore size are suitable for the SEC fractionation HA. Fig (7) shows the raw signals (MALS 90°, single-capillary viscometer SCV and differential refractive index DRI detectors) of a high molar mass HA sample with $M_w = 652$ kDa and $D = 2.1$. The HA sample was successfully fractionated with a SEC system using the following experimental procedure: two polymeric TSKgel PW columns (G6000-G5000) from Tosoh Bioscience (Stuttgart, Germany), 0.15 M NaCl as the mobile phase, temperature 37 °C, 0.4 ml/min flow rate, 100 μ l of injection volume and 0.2 mg/ml of sample concentration. The SEC-MALS-SCV chromatographic system is the most powerful method in the molecular characterization of HA. The SEC-MALS-SCV system is equipped with three on-line detectors: a multi-angle laser light scattering, a single capillary viscometer and a differential refractometer as concentration detector.

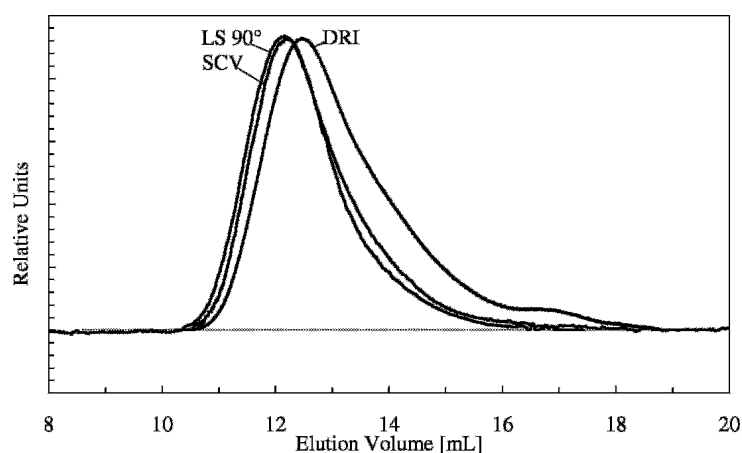


Fig. (7). Raw signals from a SEC-MALS-SCV chromatographic system (LS 90°, SCV, DRI on-line detectors) for a high molar mass HA sample ($M_w = 652$ kDa, $D = 2.1$)

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 [274]. 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.

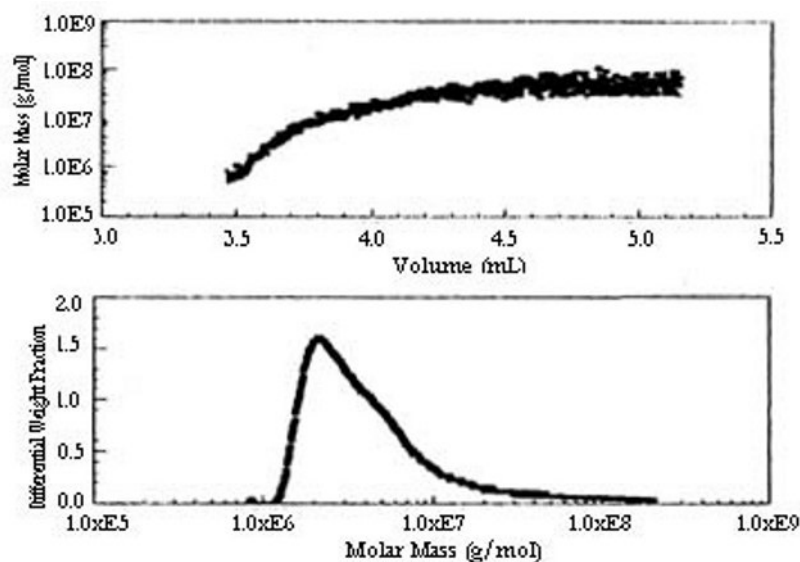


Fig. (8). $M = f(V)$ experimental function (upper panel) and MMD (lower panel) of a Hylan HA sample from a F-FFF-MALS system. Reprinted with permission of the publisher from [270]

The F-FFF fractionation was successfully applied to high and UHMM HA samples [271]. Fig. (8) shows the $M = f(V)$ experimental function (where V denotes the elution volume) and the MMD of a HA sample (Hylan from Biomatrix, Ridgefield, NJ, USA) obtained by a F-FFF system. Hylan is a soluble slightly cross-linked HA with an exceptional ultra-high molar mass (about 10 MDa or more) and broad MMD (D about 4.5 [272]). 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 [273]. 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 a LS and/or viscometers.

MMD of HA from 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 [138,275]. 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 [276]. 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 [274].

Determination of Molar Mass, Size and Conformation of HA from 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 analysed 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.

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

The principal equations for elastic LS have been reviewed recently [270] and will be only summarized herein. In brief, following the Zimm theory [277], 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:

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

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, $\theta > 0^\circ$, and $R(\theta)$ in absence of interference, $\theta = 0^\circ$.

$$P(\theta) \equiv \frac{R(\theta)}{R(\theta = 0^\circ)} \quad (\text{III})$$

$P(\theta)$ is very important because it allows to estimate the size of the macromolecules. Debye [278] found that the reciprocal of $P(\theta)$, in a certain range, could be approximated by the following equation:

$$P(\theta)^{-1} = 1 + \frac{1}{3} \cdot \mu^2 \cdot \langle s^2 \rangle \quad (\text{IV})$$

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.

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

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 $\langle s^2 \rangle^{1/2}$ 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 $\Delta R(\theta = 0^\circ)$ cannot be estimated directly due to the interference with the primary incident light. Thus, at measuring $\Delta R(\theta = 0^\circ)$, two different strategies may be used corresponding to two different LS instruments: low-angle laser LS (LALLS) and multi-angle laser LS (MALLS). A LALLS photometer measures $R(\theta)$ at a scattering angle as low as possible at assumption that this value corresponds to $\Delta R(\theta = 0^\circ)$. On the contrary, a MALLS photometer measures $R(\theta)$ in a wide range of angles by means of an array of photodiodes, and $\Delta R(\theta = 0^\circ)$ 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 [270]. 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 R_g can be obtained, while from the on-line Visc detector one can calculate $[\eta]$. Fig. (9) 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 [270,279].

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 Fig. (10). Starting from the initial MMD, the molecular weight 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 ($R_g = f(V)$ determined from MALS and $[\eta] = f(V)$ from Visc), the respective distributions (RGD and IVD) and averages ($R_{g,n}$, $R_{g,w}$, $R_{g,z}$, and $[\eta]_n$, $[\eta]_w$, $[\eta]_z$) can be obtained [279].

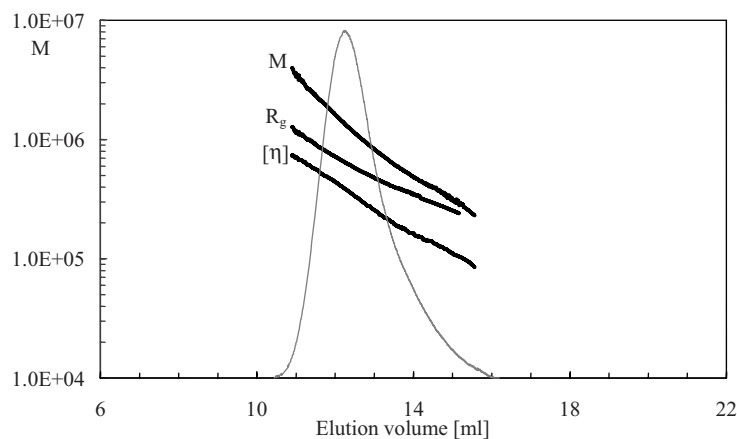


Fig. (9). $M = f(V)$, $R_g = f(V)$ and $[\eta] = f(V)$ experimental dependencies obtained from a SEC-MALS-SCV system for a high molar mass HA sample ($M_w = 1314$ kDa, $D = 1.6$)

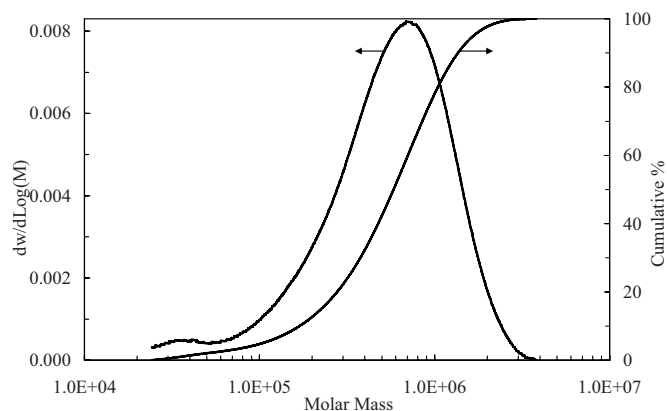


Fig. (10). Differential and cumulative MMD for a high molar mass HA sample ($M_w = 652$ kDa, $D = 2.1$) estimated using a SEC-MALS system. Arrows indicate assignment of the curves to y axes

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 R_{g_i} values for each fraction of a sample, it is possible to obtain the $R_g = f(M)$ 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 Fig. (11). 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 [275].

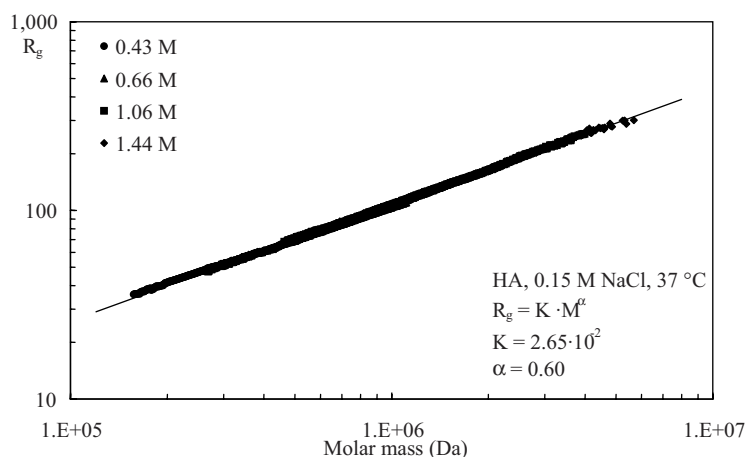


Fig. (11). Conformation plot, $R_g = f(M)$, from a SEC-MALS system, obtained from the superimposition of the data of four high molar mass HA samples

Intrinsic Viscosity and Mark-Houwink-Sakurada Plot from Viscometry

Measurement of viscosity $[\eta]$ 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 $[\eta]$ measurements, both off-line (i.e. Ubbelohde viscometer: $1200\text{--}1500\text{ s}^{-1}$) and on-line to a SEC system (i.e. DV: $2500\text{--}3000\text{ s}^{-1}$) are too high for measuring the HA

viscosity. As a consequence, at measuring $[\eta]$ of HA very low shear rate viscometers (rotational or capillary) are required [275].

Fig. (12) illustrates measurement of $[\eta]$ for a UHMM HA sample by an off-line Bishop multi-bulb capillary viscometer. In a single test using the three bulbs, the obtained apparent (incorrect for non-Newtonian flow) $\dot{\gamma}$ values were approximately equal 725 s^{-1} , 422 s^{-1} , and 187 s^{-1} . The influence of $\dot{\gamma}$ on the reduced viscosity η_{sp}/c value for each concentration is evident. Consequently, the $[\eta]$ value for this UHMM HA sample ($[\eta] = 26.64 \text{ dl/g}$) was obtained using a double extrapolation: 1) $\dot{\gamma} \rightarrow 0$; 2) $c \rightarrow 0$.

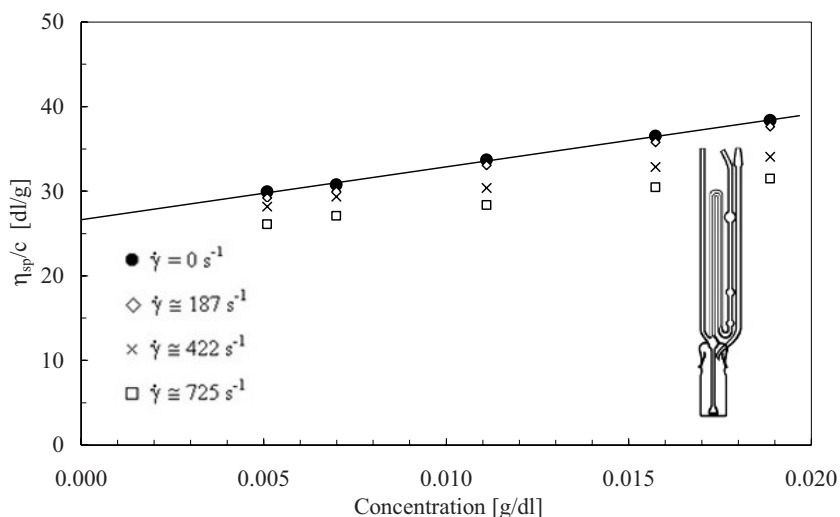


Fig. (12). $[\eta]$ of a UHMM HA sample measured by an off-line Bishop multi-bulb capillary viscometer (inset)

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.* [275] used a modified on-line SCV

detector with low flow rate and very low concentration of the HA samples. Fig. (13) 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 Fig. (13) 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 [275].

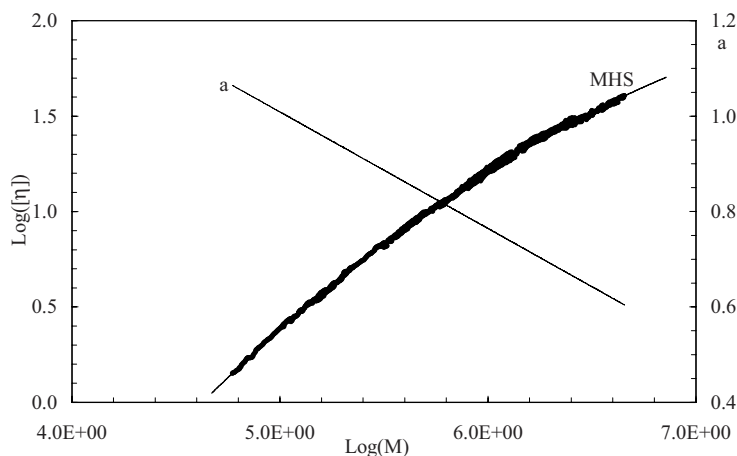


Fig. (13). MHS plot, $[\eta] = f(M)$, obtained from a SEC-MALS-SCV system and the superimposition of nine high and UHMM HA samples

A similar result for the MHS plot for HA was obtained by Waters and Leiske [280], 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 ($a = 0.78$: $100 \text{ kDa} < M < 1000 \text{ kDa}$) are in agreement with several results published previously [275]. Some problems concern the extreme

UHMM range ($M > 1000$ kDa) with the relative slope $a \approx 0.6$. The question is: are the obtained $[\eta]$ 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.

In many laboratories it is customary to calculate average molar mass M_v of an HA sample from the $[\eta]$ value by means of the well known MHS equation. The method is very popular because it involves a less expensive off-line viscometer. Unfortunately, the MHS coefficients for HA are not constant, and the MHS equation has to be used very cautiously and only in the first approximation. Taking into consideration this strong limitation, Mendichi *et al.* [275] evaluated the following MHS coefficients for HA in 0.15 M NaCl at 37 °C:

$$\begin{aligned} [\eta] &= 1.29 \cdot 10^{-5} M^{1.056} & \{M < 10^5 \text{ g/mol}\} \\ [\eta] &= 3.39 \cdot 10^{-4} M^{0.778} & \{10^5 \text{ g/mol} < M < 10^6 \text{ g/mol}\} \\ [\eta] &= 3.95 \cdot 10^{-3} M^{0.604} & \{M > 10^6 \text{ g/mol}\} \end{aligned}$$

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_0 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.

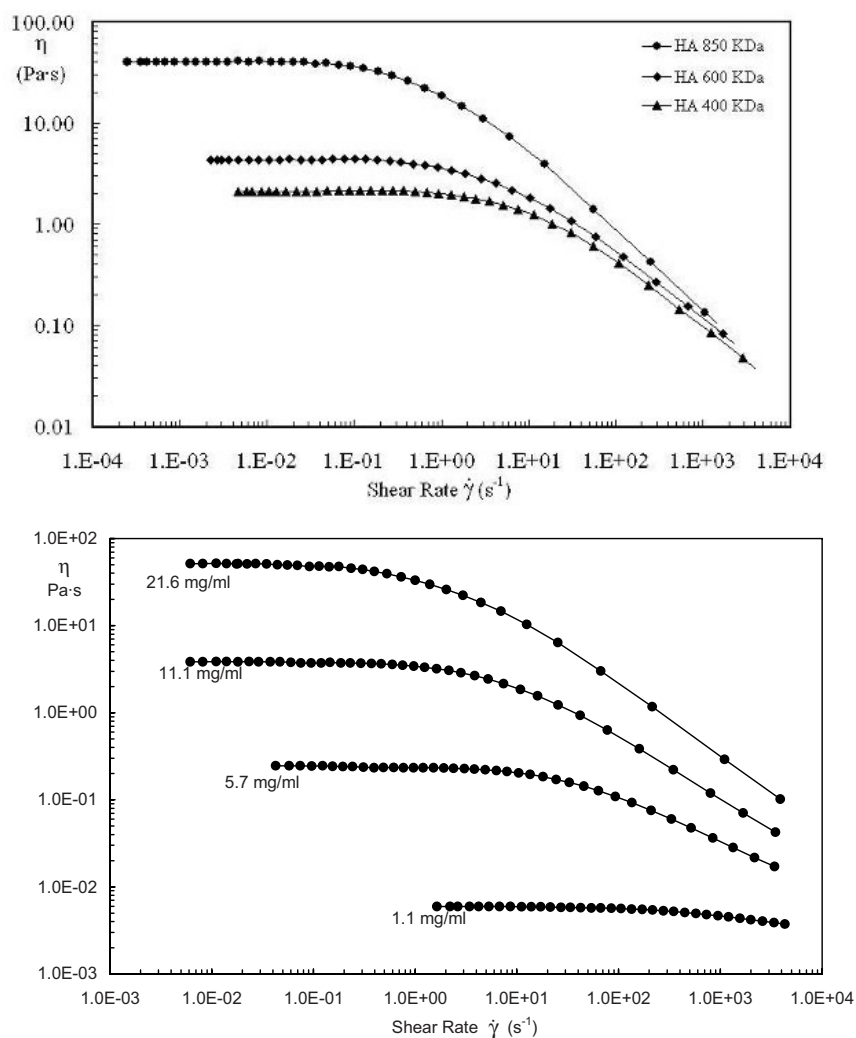


Fig. (14). Flow curves for HA at 20 °C. Left: three HA samples, $c \approx 1\%$, of different molar mass, $M_w = 850, 600$, and 400 kDa. Right: HA sample, $M_w = 1,350$ kDa, and four different concentrations: $c = 1.1, 5.7, 11.1$, and 21.6 mg/ml

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. Fig. (14) shows results of a series of rheological tests of HA polymers with different molar masses at different concentrations. Fig. (14, left panel) shows the flow curves for three different HA samples with the M_w values of 850 kDa, 600 kDa, and 400 kDa. Fig. (14, right panel) exhibits the flow curves for an HA sample at four different concentrations ranging from 0.11% to 2.16%. The flow curves are obtained by using an AR 2000 stress-controlled rheometer from TA Instruments (New Castle, DE, USA). A cone/plate geometry is used. The rotor was made of the acrylic material, 4 cm of diameter and 1° of cone angle. The measurements were performed at 20°C .

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. Fig. (15) shows the frequency sweep curves (G' , G'' , and $\text{tg}(\delta)$) vs. the frequency ω for an HA sample ($M_w=1350$ kDa, polydispersity index $D=1.6$, concentration $c = 2\%$) at 20°C .

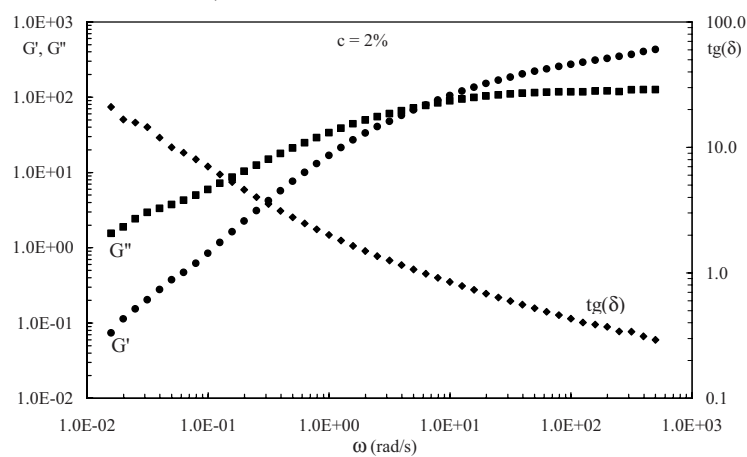


Fig. (15). Frequency sweep (G' , G'' , $\text{tg}(\delta)$) vs. the frequency ω for an HA sample at 20°C : $M_w = 1350$ kDa, $D = 1.6$, $c = 2\%$

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.* [281] 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 [282] 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 [282,283]. 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 [283]. Fig. (16) shows a MALDI-TOF spectrum of a HA fraction separated using an SEC system. The MALDI-TOF spectrum clearly shows the major mass differences between the peaks corresponding to a mass of the disaccharide repeating unit of HA, (378 Da), and the minor peaks correlated to the molar masses of the two monosaccharide components of HA.

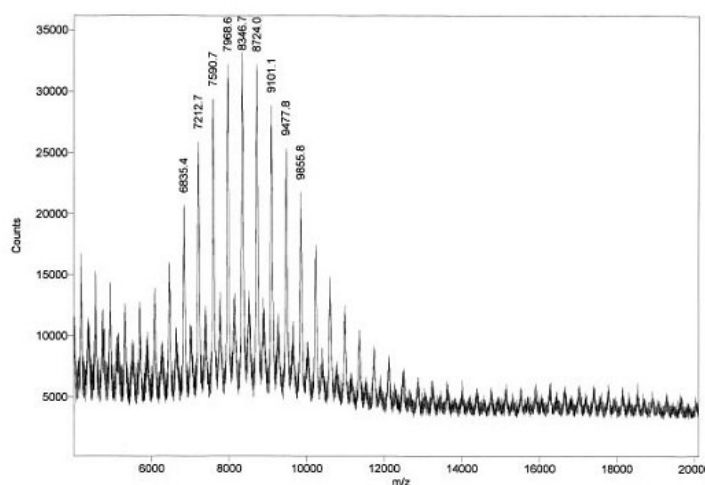


Fig. (16). Expanded view of a MALDI-TOF spectrum for a HA fraction separated using an SEC system. Reprinted with permission from [283]

It is well known that at present MALDI-TOF analysis of broad MMD polymers represents a difficult task [282]. 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: i) chemical (under acidic or alkaline conditions); ii) enzymatic (the enzyme most frequently used in HA degradation is hyaluronidase); iii) free-radicals cleavage; iv) thermal degradation; v) sonication; vi) irradiation; vii) 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 more simple and less expensive 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 molecular mass M_v calculated by means of the MHS equation.

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. Fig. (17) 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).

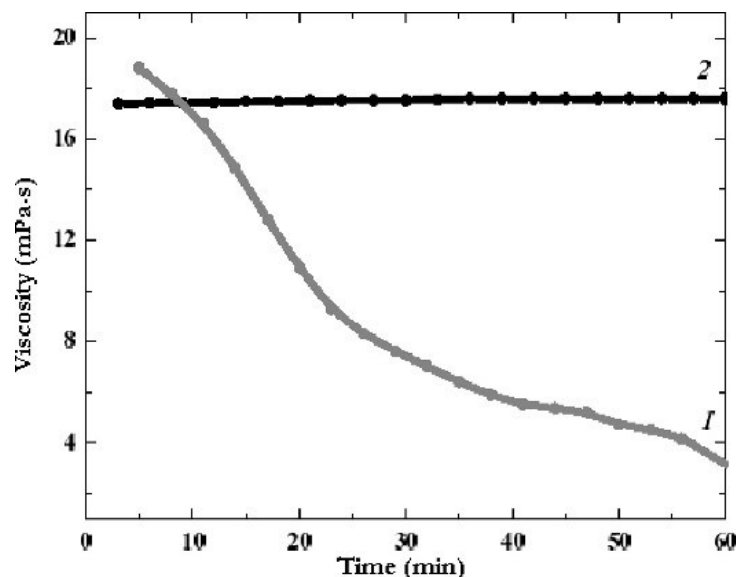


Fig. (17). Kinetics of the decrease of η value of an HA solution ($c = 0.3\%$) incubated with $3\% \text{H}_2\text{O}_2$ (curve 1) measured with a rotational viscometer. As a control, the viscosity-time profile of the intact HA is shown (curve 2)

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.* [269] and Cowman and Mendichi [270] 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. Fig. (18) shows the fractionation of HA oligomers using a Superdex 30 column (Pharmacia Biotech) and a 0.1 M NH_4HCO_3 mobile phase [284].

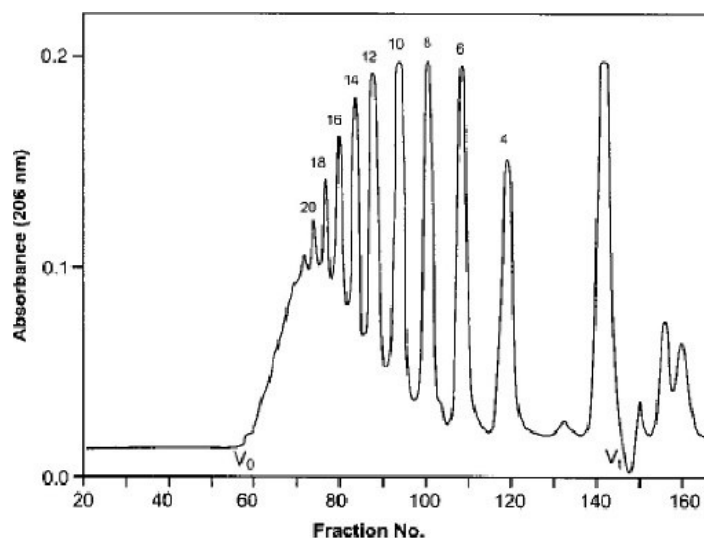


Fig. (18). Fractionation of HA oligomers by a Superdex 30 column eluted with M NH_4HCO_3 . Reprinted with permission from [284]

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 [269].

IEC is a highly sensitive method for the separation of HA fragments and oligomers. Fig. (19) shows an example of IEC fractionation using a YMC Amino column (250×4 mm) (YMC Europe GmbH, Dinslaken, Germany) [285]. A linear gradient of 16–800 mM NaH_2PO_4 , 1 ml/min, was run at 40 °C. Chromatograms of individual HA oligomers (panels a–i) are presented, as well as the chromatogram of a mixture of oligomers (panel j) obtained after the digestion with bovine testicular hyaluronidase. Molar mass of individual oligomers was established by the ESI-MS technique.

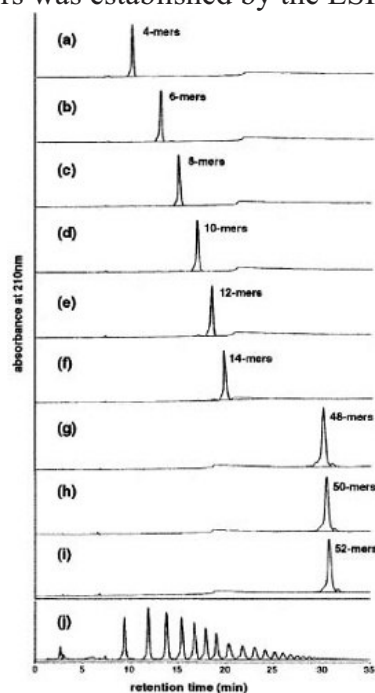


Fig. (19). IEC profiles of HA oligomers (panels a–i) and of a mixture of fragments after bovine testicular hyaluronidase digestion (panel j). Reprinted with permission from [285]

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 crosslinking) 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 [270]. 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 [270].

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 [269,270].

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. Fig. (20) shows negative mode ESI-MS spectra of some HA fragments: the spectra of: a) tetramer; b) 14-mer; and c) 52-mer are presented. The high informative content of the ESI-MS technique is evident.

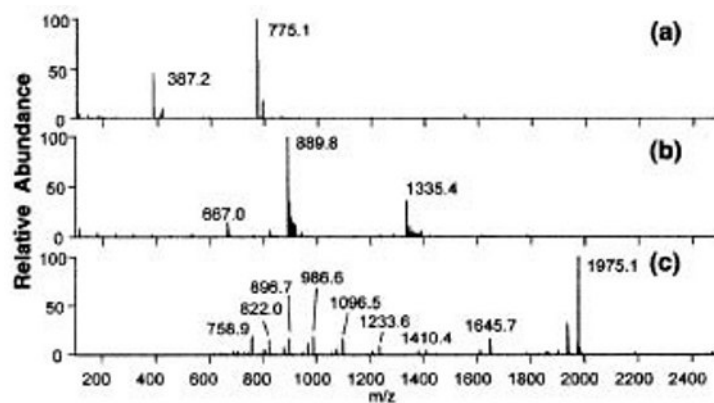


Fig. (20). Negative mode ESI-MS spectra of HA fragments: a) tetramer, b) 14-mer, c) 52-mer. Adapted with permission from [285]

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 [282]. Fig. (21) shows a negative mode MALDI-TOF spectrum of an HA fraction composed of three individual oligomers: 30-mer, 32-mer, and 34-mer [286]. In general, chemical structure, ionization, end groups, etc. are usually characterized by means of the MALDI-TOF detector [282].

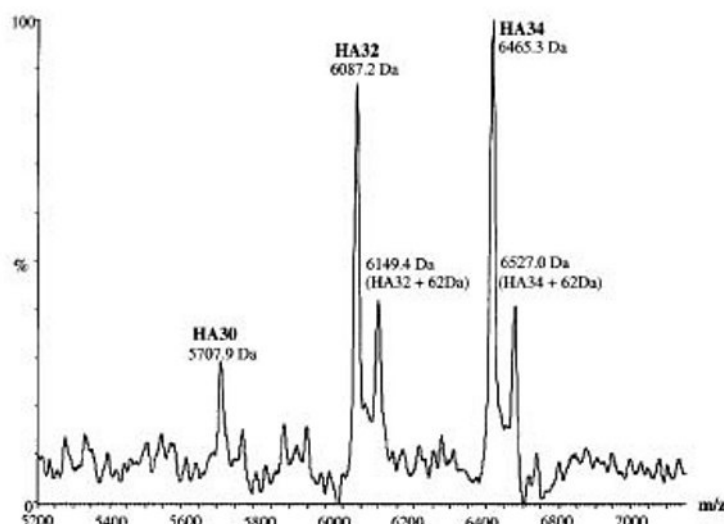


Fig. (21). Negative mode MALDI-TOF spectrum of HA fraction composed of 30-mer, 32-mer, and 34-mer. Reprinted with permission from [286]

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. The detailed description of the application of these two methodologies to the study of HA degradation was provided above in a subchapter “Degradation of Hyaluronic Acid by ROS in Inflammatory Diseases”. NMR spectroscopy was proved useful in determining purity and structure of the individual HA oligomers [287], whereas the $\cdot\text{OH}$ radical action on the HA polymer and on the two component monosaccharides was extensively investigated by means of ESR [288].

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*.

ABBREVIATIONS AND SYMBOLS

4-MU	=	4-Methylumbelliferone
ABC	=	ATP-Binding Cassette
CD	=	Cluster of Differentiation
CE	=	Capillary Electrophoresis
Ch	=	Chondroitin
ChS	=	Chondroitin Sulfate
D	=	Polydispersity Index, $D=M_w/M_n$
Da	=	Dalton
DRI	=	Differential Refractometer
DV	=	Differential Viscometer
ECM	=	Extracellular Matrix
EGF	=	Epidermal Growth Factor
ELISA	=	Enzyme-Linked Immunosorbent Assay
ESI-MS	=	Electrospray Ionization-Mass Spectrometry
ESR	=	Electron Spin Resonance
FACE	=	Fluorophore-Assisted Carbohydrate Electrophoresis
FDA	=	Food and Drug Administration
F-FFF	=	Flow-Field Flow Fractionation
GAG	=	Glycosaminoglycan
GlcA	=	Glucuronic Acid
GlcNAc	=	N-Acetylglucosamine
Gy	=	Gray (Unit of the Energy Dose: J/kg)
HA	=	Hyaluronan (Hyaluronic Acid)
HAF	=	Hyaluronan Fragment
HAS	=	Hyaluronan Synthase
HARE	=	HA Receptor for Endocytosis
HPLC	=	High Performance Liquid Chromatography
Hyal	=	Hyaluronidase

IEC	=	Ion-Exchange Chromatography
IL-1	=	Interleukin-1
k	=	Second Order Rate Constant
K_M	=	Michaelis Constant
KS	=	Keratan Sulfate
LALS	=	Low-Angle Light Scattering
LS	=	Light Scattering
LYVE-1	=	Lymphatic Vessel Endothelial HA receptor 1
M_n , M_w , M_v , M_z	=	Numeric-, Weight-, Viscosity-, z-Average Molar Masses
MALDI-TOF	=	Matrix-Assisted Laser Desorption and Ionization Time-of-Flight
MALS	=	Multi-Angle Light Scattering
MHS	=	Mark-Houwink-Sakurada Equation
MMD	=	Molecular Mass Distribution
MPO	=	Myeloperoxidase
MS	=	Mass Spectrometry
NADPH	=	Nicotinamide-Dinucleotide Phosphate
NF- κ B	=	Nuclear Factor- κ B
NMR	=	Nuclear Magnetic Resonance (Spectroscopy)
NPC	=	Normal Phase Chromatography
PAGE	=	Polyacrylamide Gel Electrophoresis
PDGF	=	Platelet-Derived Growth Factor
PG	=	Proteoglycan
pKa	=	Natural Logarithm of Acid/Base Equilibrium
PMNL	=	Polymorphonuclear Leukocyte(s)
ppm	=	Parts Per Million
R_g , R_h	=	Radius of Gyration, Hydrodynamic Radius
RA	=	Rheumatoid Arthritis
RHAMM	=	receptor for HA-mediated motility
RNS	=	Reactive Nitrogen Species
ROS	=	Reactive Oxygen Species
RPC	=	Reverse Phase Chromatography
SCV	=	Single Capillary Viscometer
SEC	=	Size-Exclusion Chromatography
SPAM1	=	Sperm Adhesion Molecule 1

TGF- β	=	Transforming Growth Factor β
TNB	=	5-Thio-2-nitrobenzoic Acid
TSP	=	Trimethylsilyl Propionic Acid
Visc	=	On-Line Viscometer
UDP	=	Uridine-5'-diphosphate
UHMM	=	Ultra-High Molar Mass
UV	=	Ultraviolet
δ	=	Phase Angle, $\tan(\delta)=G''/G'$
$\dot{\gamma}$	=	Shear Rate
$[\eta]$, IV	=	Intrinsic Viscosity
η , η_0	=	Dynamic Viscosity, Newtonian Dynamic Viscosity
G'	=	Elastic or Sstorage, or In-Phase Modulus
G''	=	Viscous or Loss, or Out-Phase Modulus

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