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# Hyaluronan: Preparation, Structure, Properties, and Applications†

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## Contents



## 1. Introduction

Hyaluronan (HA, Figure 1), a high molecular weight biopolysaccharide, was discovered by Meyer and Palmer in 1934 in the vitreous humor of cattle eyes.[1] HA is a member of a group of similar polysaccharides that have been termed "connective tissue polysaccharides", "mucopolysaccharides", or "glycosaminoglycans". These polysaccharides include chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin.[2]

† This paper is dedicated to Prof. Dr. A. Lauwers who has inspired us with his scientific approach, his honesty, and his human warmth.

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Lubomír Lapčík, Jr. was born in Bratislava, Slovakia, in 1963. He studied physical chemistry at the Faculty of Chemical Technology of the Slovak Technical University in Bratislava, where he received his M.S. degree in 1986. In 1991, he received his Ph.D. degree in physical chemistry from the same university with a thesis titled "Hyaluronan – Structure, Properties and Applications" (scientific mentors, Prof. V. Kellö and Prof. A. Staško). In 1995, he became Associate Professor of Physical Chemistry of the Faculty of Chemistry, Technical University of Brno, Czech Republic, where he is currently lecturing on colloidal and surface chemistry and chemical kinetics. In 1991–1992, he was granted a postdoctoral fellowship under Prof. T. G. M. van de Ven and Prof. D. Argyropoulos in the Pulp and Paper Research Center, McGill University, Montreal, Canada, where he worked on problems associated with the stability of colloidal suspensions and solid-state $^{13}C$ CP MAS NMR spectroscopy. In 1994, he spent six months as a visiting scientist on the Faculty of Pharmaceutical Sciences in Ghent, Belgium, where he worked on problems of photochemical cross-linking of hyaluronan under Prof. J. Demeester, Prof. A. Lauwers, and Prof. D. De Keukeleire. He is a member of the American Chemical Society, the Spectroscopic Society of Jan Marcus Marci, and the American Water Works Association. His research activities have resulted in 23 scientific publications and numerous poster and oral presentations at conferences. His current scientific interest is focused on thin layer plasmachemical modification and the characterization of oriented solid biopolymer surfaces.

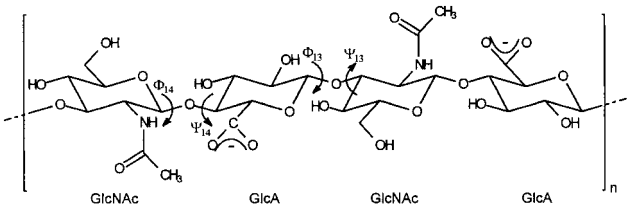


**Figure 1.** A tetrasaccharide from a HA chain. The torsion angles of the glycosidic linkages (Φ and Ψ) are defined in the text. (Reproduced from ref 4. Copyright 1994 American Chemical Society.)

HA is a linear, unbranched polymer. By chemical and enzymatic methods, Meyer and co-workers found HA to be composed of a repeating disaccharide that consists of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) linked by a $\beta$ 1-4 glycosidic bond.[3] The disaccharides are linked by $\beta$ 1-3 bonds to form the HA chain.

In addition to its presence in the vitreous body, HA occurs in many living substrata such as the extracellular matrix and synovial fluids.[5−8] The isolation, purification, and identification of nearly pure HA has been the center of scientific interest for many decades. The procedure developed by Balazs was the first industrially applied extraction method for the isolation and purification of pharmaceutical grade HA.[9] Umbilical cords and rooster combs were frozen

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Stefaan De Smedt was born in Geraardsbergen, Belgium, in 1967. He studied pharmacy at the University of Ghent, Belgium, and received his M.S. degree in pharmaceutical sciences in 1990. As a scholar of the Belgian Institute for the Encouragement of Scientific Research in Industry and Agriculture, he enrolled in a Ph.D. program at the University of Ghent, under the direction of Prof. J. Demeester. He studied rheology at the Catholic University of Leuven. He received the Scott Blair Biorheology Award in 1993–1995 for his work on the structural characterization of hyaluronan solutions. To study diffusion phenomena in polymer solutions, he collaborated with Prof. Y. Engelborghs at the Laboratory of Biomolecular Dynamics of the Catholic University of Leuven. In 1995, he joined the pharmaceutical development group of Janssen Research Foundation. Since 1997 he has been a postdoctoral fellow of F.W.O.-Vlaanderen at the Laboratory of General Biochemistry and Physical Pharmacy of the University of Ghent. He is a member of the Controlled Release Society, the European Federation for Pharmaceutical Sciences, the Belgian Society for Pharmaceutical Sciences, the European Society of Rheology, the Belgian Biophysical Society, and the Polymer Networks Group. He is a consultant to the *Journal of Controlled Release* and to *Pharmaceutical Research*. His current research interests include the mobility and interactions of macromolecular drugs in pharmaceutical polymer matrixes and biological polymer systems.

to destroy the cell membranes, and HA was extracted with water and precipitated in organic solvents such as, e.g., ethanol, chloroform, or cetylpyridinium chloride. After purification of the extract, 0.5% protein impurities remained, and the yield was 0.9 grams of HA per kilogram of the original material. Other

Joseph Demeester was born in Ghent, Belgium, in 1951. He received a M.S. degree in pharmaceutical sciences from the University of Ghent in 1974 and earned a Ph.D. degree in pharmaceutical sciences in 1980 under Prof. A. Lauwers. He became a professor at the same university in 1989 in the Laboratory of General Biochemistry and Physical Pharmacy. He was a laureate of the Belgian Royal Academy of Sciences in 1980 and first laureate of the Travel Grant of the Ministry of Education in 1981. He did postdoctoral research on light scattering and rheology at the Institute of Physical Chemistry of the University of Graz, Austria, with Prof. J. Schurz in 1985. He is a member of many scientific organizations, including the Biochemical Society, and has been vice-president of the Belgian Biophysical Society since 1994. In 1994, he became president of the International Center for Standards of the International Pharmaceutical Federation. His current research interests include the study of the action mechanism of different polymer-degrading pharmaceutical enzymes such as hyaluronidases, proteases, and cellulases, the characterization of polymers such as hyaluronates and proteoglycans, and the controlled delivery of macromolecular drugs using biodegradable polymer hydrogels combined with enzymes. He is married to Riet Debruyne and has four children.

Peter Chabreček was born in Raková, Slovakia, in 1955. He studied organic chemistry at the Comenius University in Bratislava where he received his M.S. degree in 1981. In 1986, he received his Ph.D. degree at the same university with a thesis titled "Synthesis and Studies of Benzothiazole Derivatives". In 1987–1988, he worked at the Research Institute of Preventive Medicine in Bratislava on the analytical characterization of pesticide residues and metabolites. In 1989, he joined the research group of Prof. A. Blažej at the Institute of Biotechnology, Slovak Technical University, Bratislava, where he worked on the isolation, modification, and characterization of biopolymers. From April 1992 to June 1996, he was a postdoctoral fellow at the Central Research Laboratories of Ciba-Geigy in Basel, Switzerland. His work there focused on the surface modification and characterization of polymeric materials for biological use, primarily for contact lenses. Currently, he is working for CSIRO, Australia, as a visiting scientist. His research activities have resulted in 18 scientific publications, 20 patents, and numerous conference presentations.

isolation and purification methods have been described by Galatík et al., Šoltés et al., and Della Valle et al. [10−12]

The bacterial production of HA by *Streptococcus equi*[13] and *Streptococcus zooepidemicus*[14] enabled it to be produced in larger quantities than could be achieved with the extraction methods. HA produced by *S. equi* has a lower molecular weight (MW) than does HA produced by *S. zooepidemicus*, which has a MW of about 1.8 to $2 \times 10^6$ Da with a yield of around 4 grams of HA per liter of the cultivated solution. At present, HA from various sources, with different degrees of purity and molecular weights, is available for medical applications (section 5). The main impurities, depending on the source and purification method, are bacterial endotoxines, chondroitin sulfates, proteins, nucleic acids, sodium chloride, and heavy metals. Water is usually present between 5 and 10% in the very hygroscopic powder or fibrous aggregate.

No official requirements for HA used in pharmaceutical applications have as yet been established. It is hoped this review will serve the scientific committees that are developing pharmaceutical monographs. Attention will have to be paid to the development of worldwide accepted physicochemical methods to identify HA. Since the first conventional infrared (IR) spectroscopic measurements on HA,[15] little attention has been given to the use of IR spectroscopy for its identification, although later studies showed that Fourier transform IR spectroscopy might be a useful way to do this.[16−18] In addition to identification methods, generally accepted physicochemical methods will be necessary to characterize the macromolecular properties of HA batches. Although, ideally, the complete molecular weight distribution should be determined, the characterization of the macromolecular properties of HA batches might become possible with a standardized determination of the intrinsic viscosity.[19]

It is beyond the scope of this paper to review the extensive clinical and biological research that has been conducted on HA. The main aim here is to describe the chemical and physicochemical features of this unique polysaccharide. Due to the medical interest in this polymer, this publication also reviews the physico-pharmaceutical and medically applied HA research. By this "double view" on HA we hope to create closer links between the fundamental and the application-oriented HA research of the future. This might reveal new perspectives[20] for this biopolymer, which is still expensive but, due to its exceptional hydrodynamic properties and its biocompatibility, hardly replaceable by other polymers. A secondary aim of this paper is to describe the main topics in the chemical research on HA derivatives. This review considers the promising future of HA applications and how it may be based on chemical derivatives of HA.

## 2. Macromolecular Character

### 2.1. Polyelectrolyte Properties and Conformation

The importance of the conformation and the interactions of HA in solution led to basic research on this polymer in these areas. In the 1940s, Blix and Snellman studied the size and shape of HA chains

from vitreous humor by streaming birefringence.[21] They observed that HA chains were polydisperse molecules with a long "particle length". In the early 1950s, Ogston and Stainer described how HA in solution behaved hydrodynamically like a large solvated sphere containing a thousand times more water than organic material.[22−24] Despite the very simple structure of the repeating disaccharide (Figure 1) and about 60 years of intensive research on the properties of HA solutions, the conformation of HA in solution has been very difficult to determine. As described in this section, the conformation and the interactions of HA in the dissolved state are still controversial.

### 2.1.1. Polyelectrolyte Properties

A typical polyelectrolyte pattern of viscosity was pointed out by Balazs and Laurent in the 1950s.[25] Upon complete ionization of the carboxylic groups within D-glucuronic acid, the charges are about 1 nm from each other. These charges are influenced by the ionic strength and pH of the environment and, in turn, influence the shape of the chains and their interactions with surrounding molecules. In 1957, Laurent compared static light scattering and viscosity results of sodium hyaluronate in water and cetylpyridinium hyaluronate in methanol.[26] He showed that the radius of gyration, which was 200 nm in the former solution and 120 nm in the latter, depends on the solvent, and he argued that the decrease of the radius of gyration in cetylpyridinium hyaluronate was due to a collapse of the chain as the charges become neutralized. Cleland showed that HA chains contract with increasing ionic strength and decreasing pH, which indicates their polyelectrolyte behavior.[27] More recently, Fouissac et al. and Hayashi et al. studied the influence of the ionic content on the radius of gyration and the persistence length of HA with different molecular weights.[28,29] Fouissac et al. showed the electrostatic expansion of HA chains could be well described within the framework of Odijk's model[30] by assuming a wormlike chain. However, Hayashi et al. indicated that the electrostatic contribution to the persistence length at a lower salt concentration is much larger than would be predicted from Odijk's model. In the presence of salts, the dissociation constant ($K$) of D-glucuronic acid on HA increases linearly as a function of the degree of ionization ($\alpha$).[31] While the p$K$ of the polymer, as obtained by extrapolation to $\alpha = 0$, was estimated to be 2.9, the p$K$ of the monomer D-glucuronic acid is 3.23.[32] The difference in p$K$ was attributed to effects of substitution at carbon 4.

### 2.1.2. Conformation

Although light scattering[33] and intrinsic viscosity[34] experiments in the 1950s and 1960s suggested that HA chains in solution have an expanded "*somewhat stiff*" random coil structure, Cleland showed that the size of HA varies with pH and salt concentration as would be expected for a *flexible* polyelectrolyte.[27] In the 1970s, 2-, 3-, and 4-fold (both single and double) helical conformations of HA in the solid state were discovered from X-ray diffraction.[35−39] It was also shown that the helical form of HA in the solid state

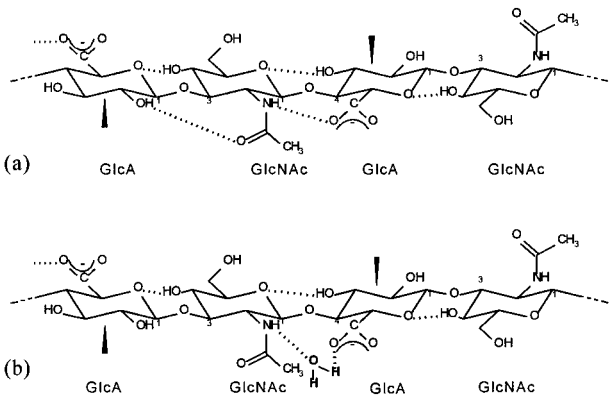


**Figure 2.** Secondary structure of HA in DMSO (a) and in DMSO containing water (b) as proposed by Heatley et al. The dotted lines indicate hydrogen bonds. Arrows indicate the glycol groups which are resistant to periodate oxidation (see text). (Reproduced with permission from ref 53. Copyright 1988 The Biochemical Society.)

depends on the counterion type, pH, temperature, and extent of hydration. The conformation of HA in the solid state was reviewed by Arnott et al.[40] The helical conformation of HA in the solid state raised the question whether any ordered form at all could exist under hydrated conditions. As discussed below, various conformations have been proposed.

By nuclear magnetic relaxation (NMR) measurements on HA solutions, Darke et al. identified two types of residues in HA chains.[41] The relationship between the relaxation times and conformational mobility showed that there are two types of domain with different mobility. While one had the mobility of a flexible polymer, the other was so stiff that it had to contain cooperative structure. The stiff part represented 55−70% of the HA structure, and this proportion was not altered by changes in ionic strength or temperature, by addition of a denaturant such as urea, or by moderate changes in pH. Therefore, they suggested that the stiff chain segments differed from the flexible chain segments by minor covalent features. According to Darke et al. the stiff segments were composed of at least 60 disaccharide units. This was questioned by Mathews and Decker.[42] From viscosity data they showed that a significant degree of stiffness still exists in HA chains, even after reduction of the chain composition from 2500 to less than 60 disaccharide units.

In the 1980s, Scott and colleagues continued to study intensively the conformational properties of dissolved HA oligomers by NMR which provided a physical proof of the existence of a structure that had been predicted from space-filling molecular models[43] and computer simulations[44] some years before. The HA conformation in solution was considered as an ordered structure in which each disaccharide unit is twisted through 180° compared with those ahead and behind in the chain. A 2-fold single helix was proposed as two turns bring back the original orientation.[45−48] In dimethylsulfoxide (DMSO) Scott showed that there were hydrogen bonds between adjacent sugar units (Figure 2).[45] The NMR work also showed evidence for the results of Scott and Tigwell[43] on the periodate oxidation of HA in solution. These experiments showed the glycol group in the

glucuronate residues (Figure 2) is oxidized 50−100 times more slowly than the glycol group in similar glycosaminoglycans. A stable conformation that involves hydrogen bonds between the carboxylate, acetamido, and hydroxyl groups was postulated for the periodate resistance of dissolved HA. They also suggested that the extended hydrogen-bonded arrays down both "sides" of the HA chains result in considerable rigidity of the polymer, which agreed with the earlier observations[33,34,41] that HA in aqueous solutions behaves like a rather stiff polymer. It could also explain the dramatic reversible decrease of the viscosity of alkaline HA solutions[42] as being due to the disruption of hydrogen bonds when participating protonated groups ionize and lose their H atoms. Alkali-induced ionization of hydroxyl groups in HA was also proposed by Welti et al. and Bociek et al. based on $^{1}H$ and $^{13}C$ NMR spectra.[49,50] The same view was offered by Ghosh et al. from static light scattering experiments performed to study alkali-induced conformational contraction of HA chains.[51]

NMR results reported by Cowman et al. on low molecular weight HA in *water* strongly indicated that the acetamide group was wrongly oriented to allow a hydrogen bond between the amide proton and the carboxyl group of the adjacent uronic acid subunit.[52] Some years later, Scott and co-workers observed that the secondary structure of HA, as established in "dry" DMSO (Figure 2), does change upon the addition of water.[53] They found evidence for the replacement of the hydrogen bond between the amide proton and the carboxyl group by a single water molecule bridging both groups (Figure 2). From further investigations using molecular models of HA fragments, Scott's group revealed that in HA fragments lacking water bridges two conformations are sterically possible having the same type of hydrogen bonding but differing in dihedral conformational angles near acetamido, glycol, and carboxylate groups bound by hydrogen bonds.[54] Molecular models of HA secondary structures containing water bridges revealed that such bridges can join the acetamido and carboxylate groups in four ways which are sterically different.

Besides extended hydrogen-bonded arrays, Scott and colleagues also observed large hydrophobic regions, of about eight CH groups, on alternate sides of the single HA helices.[45,48,53] Computer simulations and energy calculations confirmed that the HA 2-fold single helices in solutions may be energetically and sterically capable of extensive duplex formation driven by interactions between the hydrophobic "patches" of the HA chains (Figure 3).[46,47] In a later study, molecular models revealed that hydrophobic contacts are possible only between HA chains lacking water bridges in the secondary structure.[54] The hydrophobic patches were postulated not only to stabilize duplex formation but also to be a basis of the network-forming and laterally aggregating behavior of HA.[46] It was also suggested that they were the basis of HA interactions with lipid membranes and proteins (section 2.2.3). From $^{1}H$ NMR spectroscopy, gel permeation chromatography (GPC), and multiangle laser light scattering, Ghosh et al. observed that phospholipids such as dipalmitoyl phosphatidylcholine (DPC) bind to HA.[55] They suggested that DPC binding occurs by competition for the hydrophobic centers along the HA chains, as proposed by Scott et al.[53]

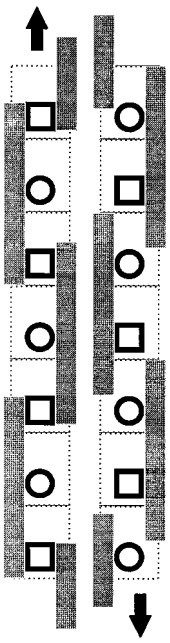

**Figure 3.** Scheme of a possible duplex formation between two HA chains.[46,47] The two participating single HA helices are antiparallel to each other. The dotted lines delineate each sugar unit, the circles represent acetamido, and squares represent carboxylate groups. The gray dotted bars are the hydrophobic patches stretching along three sugar units on alternate sides of the polymer chains.

Scott et al. showed that electrostatic repulsion between the negative charges may be countered not only by hydrophobic interactions but also by hydrogen bonding between the HA chains.[54] While most polar groups form intramolecular hydrogen bonds, two groups, namely the hydroxymethyl and the oxygen atom of the carboxylate group, are free (Figure 2). These groups could mediate intermolecular hydrophilic interactions in assemblies containing large numbers of HA molecules. Molecular modeling showed that hydrogen bonds between hydroxymethyl and carboxylate groups are possible only between antiparallel HA chains.[54] Each disaccharide residue can form two hydrogen bonds, so that bonds on one side of the HA molecule alternate with analogous bonds on the other side. Such hydrogen bonds can join antiparallel HA molecules into sheets which are planar or curved. Based on hydrophilic and hydrophobic interactions, Scott's group proposed that several kinds of lateral contact may exist between such sheets which may result in the formation of highly ordered structures.

In the 1990s, NMR work on the repeating disaccharide of HA,[56,57] HA oligomers,[58] and high molecular weight HA[59,60] continued. The NMR results on HA oligosaccharides reported by Toffanin et al. did not suggest a significant role for cooperative hydrogen bonding involving the acetamido group in the determination of the HA conformation in water.[58] From $^{13}C$ NMR experiments, Cowman and co-workers confirmed evidence of significant conformational

differences between HA in the solid state and dissolved HA.[59,60] Changes in the NMR spectra, upon dissolution of HA, were partially attributed to a change in the orientation of the acetamide group, possibly accompanied by a rotation about the $\beta$ 1-3 and $\beta$ 1-4 glycosidic linkages. In aqueous media, each disaccharide showed two intramolecular hydrogen bonds. Their data did not provide direct support for a stable water-mediated hydrogen bond across the $\beta$ 1-4 linkage as was observed by Scott and co-workers.[53] Cowman et al. also observed that greater conformational changes occur in the $\beta$ 1-3 linkage than in the $\beta$ 1-4 one.[60] These conclusions disagreed with Holmbeck et al., who studied the glycosidic angles of HA octasaccharides and found that the $\beta$ 1-4 linkage shows a greater flexibility than the $\beta$ 1-3 linkage.[4] On the basis of nuclear Overhauser enhancement (NOE) NMR combined with molecular modeling, they proposed that the conformation of an octasaccharide of HA in aqueous solutions is defined by the torsion angles of the glycosidic linkages and by the orientation of the side groups. $\phi_{13} = 46°$ and $\Psi_{13} = 24°$, which are the interglycosidic angles of the $\beta$ 1-3 linkage (Figure 1), were obtained from restrained molecular dynamics calculations. For the $\beta$ 1-4 linkage, two sets of values were consistent with the NOE restraints ($\phi_{14} = 24°$ and $\Psi_{14} = -53°$ *or* $\phi_{14} = 48°$ and $\Psi_{14} = 8°$).

## 2.2. Hydrodynamic Behavior and Interactions

### *2.2.1. Hydrodynamic Behavior*

The high viscosity of HA solutions and its importance in synovial fluid as well as in many medical applications have for years stimulated the investigations on the hydrodynamic properties and molecular interactions in HA solutions. The hydrodynamic behavior of HA solutions is so exceptional that most other polymer solutions fail to duplicate its viscoelastic behavior.[61]

Early studies on the rheological properties of HA solutions and synovial fluid,[22−24,62−66] which were reviewed by Balazs in 1974,[67] showed that the viscosity is a complex function of polymer and salt concentration, pH and shear rate. With increasing shear rate, even at low shear rates and low concentrations ($c$), HA solutions exhibit a shear thinning behavior (Figure 4): the molecules deform and align in the streamlines of flow, which results in a decrease in the viscosity. This behavior occurs when pushing HA solutions through medical needles, for example, which makes them easy to handle.[68]

Recently, Rinaudo's group studied the hydrodynamic behavior of HA solutions by focusing on the relation between the zero shear rate viscosity ($\eta_0$) and the concentration and molecular weight of bacterial HA.[69,70] Bacterial HA was used to avoid protein-mediated aggregation of dissolved HA chains, which might occur if animal HA were used. They observed a greater dependency of $\eta_0$ on the molecular weight; $\eta_0 \sim MW^4$ was found instead of $\eta_0 \sim MW^{3.4}$ as would be expected for flexible polymers. They also stressed that the critical concentration where the chains start to entangle was about 10 times lower than values

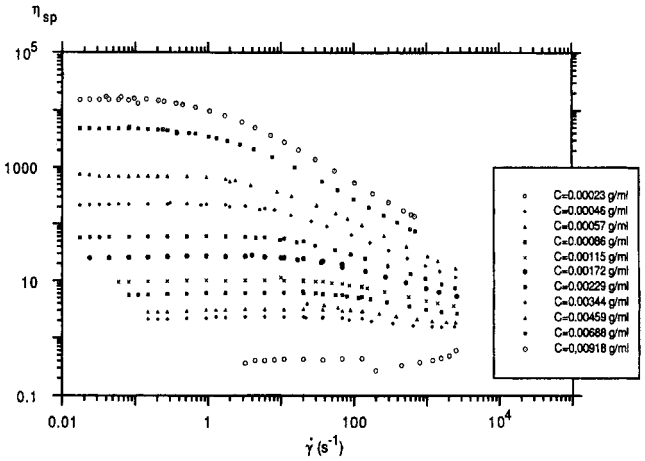


**Figure 4.** Influence of shear rate ($\dot{\gamma}$) on the specific viscosity ($\eta_{sp}$) of HA solutions at different polymer concentrations. (Reproduced from ref 69. Copyright 1993 American Chemical Society.)

found for flexible chains. Both phenomena were attributed to the stiffness of the HA chains. The dependency of $\eta_0$ on the molecular weight and concentration differed slightly from the results of Wik et al.,[71] who used animal HA. Rinaudo and coworkers attributed this to a higher amount of protein in the animal HA. Experiments using bacterial HA "contaminated" with added proteins were not performed and would be useful for confirming this position.

The hydrodynamic and rheological behavior of HA mixtures composed of chains with different molecular weights was also studied.[72] This study arose from difficulties in obtaining bacterial HA with the same MW from different fermentations, which in turn prevents the preparation of bacterial HA solutions having a reproducible viscosity. Given the viscosity, the HA concentration, and the solvent conditions, Rinaudo et al. studied how to mix HA batches with different MW.[72] They showed that the viscosity for HA mixtures at a given ionic strength and temperature is a function of the overlap parameter $c[\eta]$, $[\eta]$ being the intrinsic viscosity of the HA mixture which can be estimated from the additivity law

$$[\eta] = \sum \omega_i [\eta]_i \qquad (1)$$

where $i$ represents the HA species with intrinsic viscosity $[\eta]_i$ and $\omega_i$ is the mass fraction of species $i$ in the mixture.

### *2.2.2. Interactions between HA Chains*

As represented in section 2.1.2, in the past decade important features were discovered on the interactions between HA chains from NMR experiments and molecular modeling. Other views which were postulated on the nature of the interactions between dissolved HA chains, which strongly influence the hydrodynamic properties of HA solutions, are represented in this section.

In an early study on the viscoelastic properties of HA solutions, Gibbs et al. found that at pH 2.5 HA solutions showed an entirely different dynamic rheological behavior than at pH 1.5 and pH 7.[64] At pH

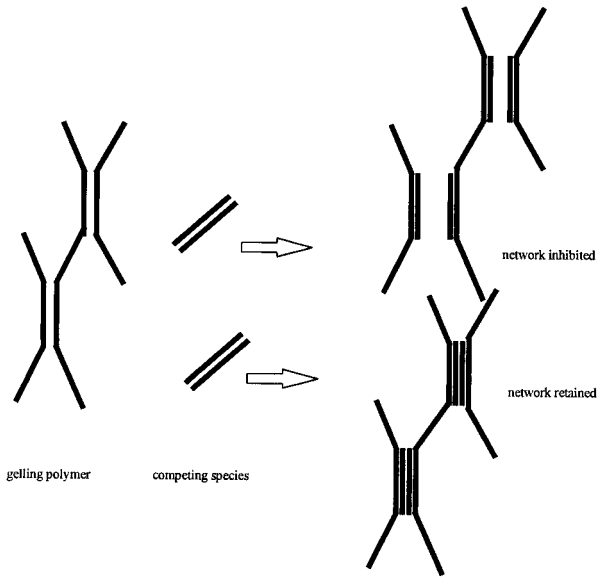


**Figure 5.** Schematic representation of competitive inhibition of network formation as proposed by Morris et al.[73,74]

2.5, the HA solutions were more elastic and exhibited a "paste-like" character on gentle shaking or stirring, while the reduced curves obtained for the solutions at a different pH were very similar in shape. They suggested that the paste-like behavior was not due to the introduction of covalent intermolecular cross-links nor to entanglement coupling between HA molecules. Rather, they attributed it to a pronounced stiffening of the HA chains. They assumed that there is a critical balance at pH 2.5 between the repulsive forces (provided by the still-ionized carboxyl groups) and the attractive (electrostatic or hydrogen bonds mediated) interactions operating between the molecular chain elements.

In the late 1970s, specific interactions between HA chains were proposed by Morris and co-workers to explain the rheological behavior of HA solutions.[73,74] Intermolecular associations in HA solutions were investigated with a competitive inhibition approach (Figure 5) using oscillatory measurements of dynamic viscosity. HA solutions under physiological conditions of pH and ionic strength showed coupling behavior typical of a transient polymer network. Upon the addition of an equal concentration of HA segments (~60 disaccharide units), the transient network behavior was lost, and the rheological properties of the HA solutions closely approximated those typical of isolated chains. No inhibition was observed in very short chain segments (~4 disaccharide residues) or with longer segments (~400 disaccharides). It was suggested that HA chains whose lengths permit them to paticipate in only one intermolecular junction (which does not give rise to a network structure) interfere with the network formation by occupying binding sites on the longer chains. They suggested that HA chains interact by the formation of specific cooperative junctions analogous to those characterized for plant structural polysaccharides but with substantially shorter lifetimes. More recently, Nishinari's group did not observe these phenomena.[75] As in the study of Welsh et al.,[74] they investigated the viscoelastic effect of the addition of an equal

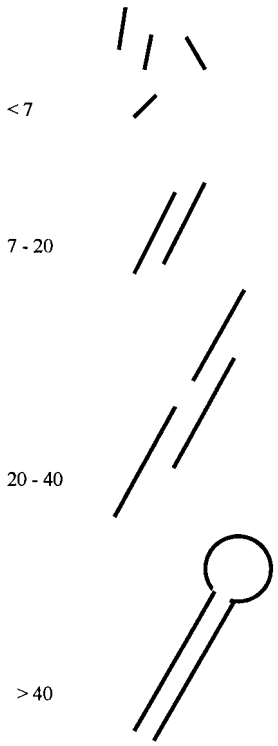


**Figure 6.** Model of Turner et al. for self-association of HA segments as a function of the length of the segments.[77]

concentration of HA segments consisting of 3−600 disaccharide units to high molecular weight HA solutions. In solutions of HA in distilled water, the addition of HA segments composed of less than 65 disaccharide units lowered the moduli, while longer chain segments increased the moduli. In physiological saline, the decrease of the moduli was not observed, while longer chain segments increased the moduli. The same group showed that when sugars such as galactose, glucose, and fructose were added to HA solutions, both the elastic as well as the viscous modulus was increased.[76] This was attributed to the creation of hydrogen bonds strengthening the transient network and to the immobilization of water molecules by hydration of the sugars, which might increase the "effective" HA concentration. They concluded that the influence of the addition of HA chain segments on the rheological properties of HA solutions results from the superposition of a chain length effect, a salt effect, and a sugar effect.

The influence of the HA chain length on self-association was studied by Turner et al. (Figure 6).[77] From capillary viscosimetry, light scattering, and circular dichroism (CD) spectroscopy they indicated concentration-dependent intermolecular associations of HA chains. Their measurements showed that HA fragments containing less than approximately seven disaccharides do not undergo self-association. HA fragments of approximately 7−20 disaccharides were proposed to exist in a monomer−dimer equilibrium, while HA fragments of 20−35 disaccharides showed aggregation beyond the dimer level. HA fragments of approximately 35 or more disaccharides were thought to be capable of associating intramolecularly with the formation of "hairpins".

Aggregation of HA chains was also observed by Ribitsch et al., who studied unpurified umbilical cord HA solutions by static light scattering and viscosity measurements.[78] It was observed that the light scattering curves develop an oscillatory fine structure on a time scale of days, indicating aggregates of HA. The oscillation in the scattering curves was called "multimerisates". They disappeared in HA solutions of high ionic strength. Such "microgels" had already been observed earlier by Schurz et al. and were described as "dense microgel-like particles".[79]

Dynamic light scattering measurements on HA solutions revealed unspecific entanglements in combination with specific chain association, to be present in HA solutions. For moderately concentrated HA solutions Terbojevich et al. showed that the HA concentration, where $Kc/R_0$ exhibits a maximum, was inversely related to the molecular weight,[80] where $K$ is the optical constant and $R_0$ is the modified Rayleigh ratio at zero angle. The maximum concentration was always higher than the critical "coil overlap concentration" for entanglements. The proposed explanation of this maximum was the onset of concentration-dependent specific, chain−chain association phenomena that intensify entanglement coupling. Reed et al. showed evidence for entanglements from dynamic and static light scattering.[32] These results showed that, at low ionic strength in the pH range between 3 and 9, high molecular weight HA chains form highly polydisperse entanglements that may be stabilized by hydrogen bonds or other noncovalent forces. Also De Smedt et al.[81] showed indications for the presence of an "entanglement-based" network in HA solutions as the concentration dependence of the zero shear rate viscosity and the elastic plateau modulus was measured as predicted by the blob model of de Gennes,[82] which is valid for entangled polymers solutions.

Additional information on interactions in HA solutions came from electron microscopy (EM). Using rotary shadowing, EM, and computer simulations, Scott et al. concluded that an extensive and coherent network is formed especially for high molecular weight HA, even at low HA concentration (<1 $\mu$g/mL).[46] Thick uniform lateral aggregates of a diameter extending for hundreds of nanometers were visible. The longer the HA molecule, the more branching is possible and the more extensive and coherent the network. While these EM observations agreed with results reported by Gross,[83] who performed the first EM study on HA in 1948 and who observed "anastomosing fibrous processes", they disagreed with the results of Fessler et al., who visualized single HA chains but found little evidence of HA branching.[84]

The influence of counterions on interactions between HA molecules is another important aspect of the understanding of the physical properties of HA solutions. Sheehan et al. showed that some physical properties of HA solutions changed with the presence of $Na^+$, $K^+$, and $Ca^{2+}$ ions.[85] In NaCl and $CaCl_2$, aggregates of HA chains persist into dilute solutions but not in KCl. Contrary to the results of Sheehan et al., Månsson et al. showed with low-angle laser light scattering that there was no difference in MW of sodium and potassium hyaluronate in 0.2 M NaCl solution.[86] CD[87,88] and electron spin resonance (ESR) experiments[89] showed that complexes between HA chains and copper ions are achieved via the carboxyl groups of the chains. These associations clearly changed the rheological properties of the HA solutions.[89,90] Similar associations were observed between HA chains and zinc and cobalt.[91]

**Table 1. Mesh Size in HA Solutions**

| | mesh size (nm)[a] | | | |
|---|---|---|---|---|
| HA concn (%) | a | b | c | d |
| 3.0 | $>4 \pm 0.5$ | | | |
| 2.0 | $6 \pm 0.5$ | | | |
| 1.4 | $11 \pm 0.5$ | $13 \pm 2$ | $10 \pm 1$ | $20 \pm 3$ |
| 0.8 | $30 \pm 0.5$ | $18 \pm 2$ | $14 \pm 2$ | $27 \pm 4$ |

[a] Group a was measured by ESR using PAA probes. Groups b, c, and d were measured by FRAP using FITC-dex probes with molecular weight 71 (b), 148 (c), and 487 kDa (d). Reproduced from ref 96. Copyright 1994 American Chemical Society.

### 2.2.3. Interactions between HA Chains and Other Macromolecules

*2.2.3.1. Nonspecific Interactions.* In the 1960s and 1970s, a series of studies were conducted on the interactions between connective tissue polysaccharides and other macromolecules. They were reviewed by Comper and Laurent in 1978.[92] In these studies, Laurent et al. investigated the transport of globular particles with diameters ranging from 4.7 to 365 nm through HA solutions and the use of HA in separating macromolecules in the ultracentrifuge.[93,94] The retarded diffusion observed was interpreted as a macromolecular sieving effect of the polysaccharide. Transport studies in HA solutions were later extended to investigate the diffusion of linear polymers.[95] Unlike globular particles of equal hydrodynamic dimensions, the linear polymers were less retarded in the HA network. These results suggested that asymmetric particles and coiled molecules preferentially penetrate the HA network by "end-on" movements, as this offered fewer obstacles than would a random movement of whole hydrodynamic units. De Smedt et al. investigated whether information on the network structure in the "sieving" HA solutions could be obtained from the reduced mobility of macromolecules.[96] With fluorescence recovery after photobleaching (FRAP), they measured the retarded diffusion of fluorescently labeled dextrans (FITC-dex) with different molecular weights in HA solutions. These measurements allowed them to estimate the average mesh size of the HA network (see Table 1). Shenoy et al. measured the mesh size in HA networks by ESR. Poly(acrylic acid) (PAA) chains of different size were used as macromolecular probes.[97] When the macomolecular probe was smaller than the mesh size as calculated from the FRAP experiments,[96] the rotational correlation times in the HA solutions equaled the rotational correlation times in the solvent. For larger macromolecular probes, the rotational correlation times differed, indicating a sterical hindrance by the HA network. The mesh size

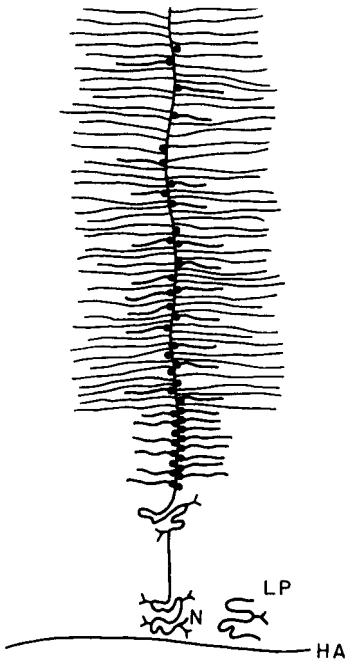


**Figure 7.** Schematic drawing of interactions between HA and cartilage proteoglycan. LP is the link protein. The globular HA binding region G1 is at the N-terminal end of the core protein. (Reproduced with permission from ref 103. Copyright 1977 John Wiley & Sons, Inc.)

calculated from ESR experiments agreed closely with the values calculated from FRAP measurements (Table 1).

*2.2.3.2. Specific Interactions.* Many of the physiological and cell biological functions of HA, such as its role in cartilage, cell aggregation, cell migration, and cell differentiation, are based on specific interactions of HA with matrix proteins and cell surface HA receptor proteins.[7] Toole suggested the name hyaldherins for proteins that have homologous HA binding regions.[98] It is beyond the scope of this review to report in detail on the specific interactions in the biological functions of HA. Excellent reviews have been published by Turley,[99] Laurent,[7] Stamenkovic et al.,[100] and Knudson et al.[101] This section reviews the major discoveries in this field.

In 1972, Hardingham and Muir discovered that HA specifically interacts with the cartilage proteoglycan aggrecan.[102] Proteoglycan aggregate formation involves a specific link-protein-mediated interaction between proteoglycans and HA (Figure 7).[103] The proteoglycan core protein has two globular regions (G1 and G2) at its N-terminal end, G1 being the HA binding region.[104,105] It has been shown that peptides of the link protein show sequence homology with the HA binding region on the proteoglycan.[106] The complete amino acid sequence of link protein showed that the C-terminal half of the molecule contains two loops, which have considerable homology with each other.[107−109] In addition to the specific interactions between HA and aggrecan, highly specific interactions between HA and many other macromolecules such as the cartilage proteoglycan versican,[110] the human brain protein hyaluronectin,[111] interleukin 1$\beta$,[112] and the $\alpha$3 chain of type IV collagen[113] have been reported.

Besides specific interactions with matrix hyaldherins, HA shows specific interactions with cell surface molecules. Although there are likely to be several distinct cell surface HA binding proteins, only the cell adhesion molecule CD44 and the receptor for HA-mediated mobility, which are discussed below, have been characterized at the molecular level.

Encouraged by early observations that a low concentration of HA could aggregate certain cells,[114] Underhill and colleagues investigated Simian virus transformed 3T3 cells and discovered a HA binding glycoprotein receptor with a MW of 85 kDa.[115−117] In 1989, it was reported that CD44 receptors, which are broadly distributed cell surface glycoproteins,[98,118] show homology with the cartilage proteins that bind HA.[118,119] It also became clear that CD44 receptors bind HA.[120,121] Underhill and colleagues[122] also showed that the CD44 receptor was identical to the HA binding glycoprotein receptor from the 3T3 cells they had discovered some years before.[115−117] Studies on CD44 showed that the CD44 molecule may participate in lymphocyte activation,[123] cell−cell adhesion,[124] and interactions between cells and the extracellular matrix.[125,126]

Turley isolated a cell-associated HA binding protein from locomoting chick fibroblasts that colocates on the cell membrane with actine filaments.[127,128] It is a large complex of proteins (MW = $1-2 \times 10^6$ Da) that includes a HA binding site and a protein kinase. This protein is concentrated in the lamellae of migrating fibroblasts but is lost from the cell surface as cell locomotion slows. Upon binding to the protein, HA induces metabolic changes in the cells, such as stimulation of the protein kinase activity. Using *ras*-transformed cells, Turley et al. showed that an increased expression of the HA binding protein significantly stimulated locomotion.[129] This seemed to depend on the presence of extracellular HA as antibodies against the HA binding protein, and digestion of HA by hyaluronidase blocked the stimulatory effect. This HA binding fibroblast protein was called RHAMM, an acronym for the receptor for HA-mediated mobility. In contrast to CD44, RHAMM does not display significant homology to the link protein HA binding domain.[130]

Although HA occurs as a high molecular weight polymer, numerous studies have established that only short oligosaccharides are necessary for recognition and binding to hyaldherins.[97] Hascall and Heinegard[131] showed that decasaccharides are necessary for binding by aggrecan core protein and link protein while, more recently, Underhill et al.[132] showed that hexasaccharides are long enough to bind to the CD44 cell receptors.

## 2.3. Molecular Weight

### *2.3.1. Studies on the Average Molecular Weight and Molecular Weight Distribution*

The MW of HA samples, which ranges from $10^4$ to $10^7$ Da, depends on the HA source and on the isolation and purification procedures used. The molecular weight distribution (MWD) of HA was studied by GPC using agar gels[133−135] and porous

silicate fillings.[136] In these studies, the highest resolved MW was less than $10^6$ Da. Often used separation columns are TSK G6000 PW, TSK G5000 PW,[137−141] and Shodex OH Pak 800pR columns.[141] A good resolution for separation of HA fragments could also be obtained by Fractogel TSK HW-65S and HW-75S as well as by Separan HEMA-S1000 columns.[136,142] GPC has also been used for measuring the changes of the MWD of HA during enzymatic hydrolysis.[143] When comparing MW values of HA determined by GPC, one has to be aware of the lack of columns that separate high molecular weight ($>10^7$ Da) fractions. Therefore MW and MWD cannot be determined accurately. There is an urgent need to develop column packing materials with pore sizes that allow the penetration of the very large HA molecules. Moreover, the commercial availability of HA molecular weight standards would significantly improve the determination of MW and MWD by GPC.

In addition to GPC, many different absolute methods to determine the MW, such as sedimentation,[22−24] light scattering,[32,144] osmometry, and the combination of sedimentation and diffusion experiments,[34,145] have been used. The determination of the molecular weight by light scattering requires the refractive index increment (d$n$/d$c$) of the HA solutions. The d$n$/d$c$ values as determined by various authors ranged between 0.140 and 0.183.[27,34,79,144,146,147] Discrepancies in d$n$/d$c$ values were mainly caused by impurities in the HA batches, errors in measuring HA concentrations, and faulty calibration of the differential refractometers. Since the square of d$n$/d$c$ is used in light scattering equations to determine the MW, large errors in the MW were introduced. The determination of MW by analytical centrifugation requires the partial specific volume ($\nu$) of HA. The oldest value ($\nu$ = 0.66 mL g$^{-1}$) was determined by Varga by pycnometry.[148] A similar value (0.653 mL g$^{-1}$) was given by Silpananta et al.[149] from density gradient centrifugations while Cleland[150] found 0.557 mL g$^{-1}$. Wik[71] determined $\nu$ as a function of the NaCl concentration and found 0.47 mL g$^{-1}$ at 0.001 M NaCl, an increase to 0.56 mL g$^{-1}$ at 0.05 M NaCl. $\nu$ remained almost 0.56 mL g$^{-1}$ with further increases of the NaCl concentration to 0.25 M.

The viscosity average molecular weight ($M_v$) of HA has often been determined from its intrinsic viscosity with the Mark−Houwink equation:

$$[\eta] = KM_v^a \qquad (2)$$

It has been shown that $a$ and $K$ depend on the ionic strength[28,69,79,151,152] and the pH[153] of the HA solutions. As for most polysaccharides, the logarithmic dependence of [$\eta$] versus $M_v$ is not linear in a wide range of HA molecular weights as the parameters of the Mark−Houwink equation change.[154,155] For MW < $10^6$ Da, values for $K$ and $a$ of 0.029 and 0.80, respectively, were reported, while for MW > $10^6$ Da, $K$ and $a$ equaled 0.397 and 0.601. Fouissac et al. reported the dependence of the Mark−Houwink exponent of HA on the ionic strength.[28,69]

Another study to determine the MWD of HA was based on electrophoresis using a 0.5% agarose gel and a cationic dye to detect HA.[154] For calibration, high molecular weight HA samples, characterized by light scattering ($0.68 \times 10^6$ Da < MW < $4.6 \times 10^6$ Da), were used. This method allowed separation of very high molecular weight samples (up to at least $6 \times 10^6$ Da).

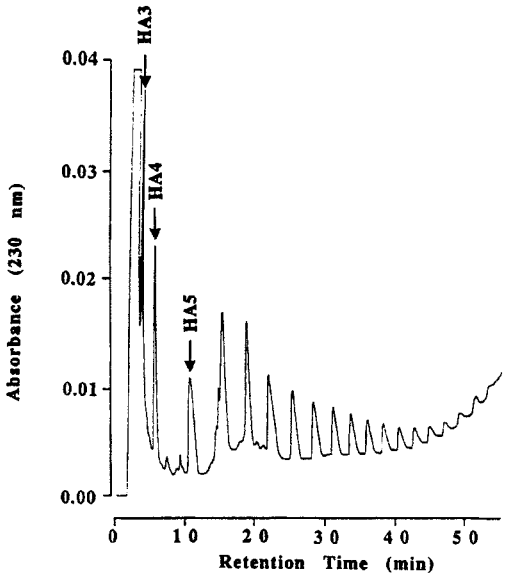


**Figure 8.** Separation of HA oligomers up to 20 repeating disaccharide units by the use of anion-exchange HPLC. The oligomers were produced by enzymatic digestion. Peaks indicated were tested with HA standards of 3 (HA3), 4 (HA4), and 5 (HA5) repeating units. (Reproduced with permission from ref 156. Copyright 1993 Elsevier Science.)

### 2.3.2. Separation of HA Oligosaccharides

The separation of HA oligosaccharides was investigated in the course of NMR studies on the solution conformation of HA fragments (section 2.1.2). It has also been a major topic in projects on the characterization of digestion products as obtained by enzymatic degradation (section 3.4). Moreover, the analytical effort to separate HA oligosaccharides was driven by extensive investigations on the biological role of HA fragments (section 2.2.3.2), which require HA oligosaccharides of precisely defined MW.

A short overview of separation methods for HA oligosaccharides was represented by Holmbeck et al.[156] For many years, HA fragments were separated by GPC, while the detection of the peaks was based, for example, on the analysis of the uronic acids.[157] Although GPC was capable of separating HA oligomers up to 23 disaccharide units, it was time consuming. High-performance liquid chromatographic (HPLC) methods, including normal- and reversed-phase ion-pair and weak-anion exchange, were used for the separation and characterization of HA oligosaccharides.[156,158−164] The largest fragment that these HPLC methods were able to separate was six disaccharide units. More recently, by means of high-performance anion-exchange chromatography with pellicular anion-exchange resin and UV detection, Holmbeck et al.[156] separated HA oligosaccharides up to 20 repeating disaccharides (Figure 8) while Price et al.[165] showed that up to 16 HA disaccharide units can be characterized by electronspray-ionization

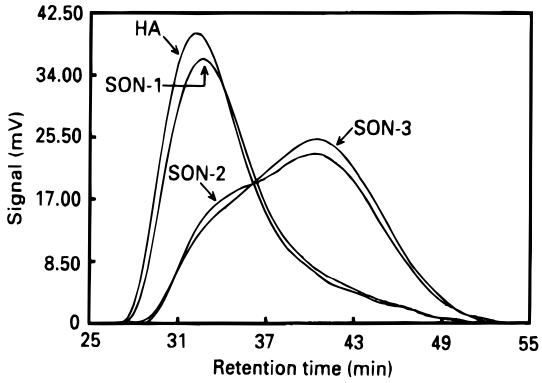


**Figure 9.** Gel permeation chromatograms of HA degraded by sonification during 0 (HA), 15 (SON-1), 45 (SON-2), and 60 (SON-3) min.[174] (Reproduced with permission from ref 174. Copyright 1995 The Biochemical Society.)

mass spectrometry and high-performance anion-exchange chromatography with pulsed amperometric detection.

The development of gel electrophoresis methods allowed the analysis of oligosaccharides between 7 and 250 disaccharide units.[166,167] Capillary zone electrophoresis was used for the separation of HA, chondroitin sulfate, and dermatan sulfate oligosaccharides.[168,169]

## *3. Degradation*

### 3.1. Ultrasonic Degradation

HA solutions are degraded by ultrasonic treatment. The mechanism and kinetics are not yet well understood. A linear relationship between the reciprocal value of the square of the MW of HA and the reaction time has been observed.[136,170−172] In another study, this linear relationship was not observed but the reciprocal value of the molecular weight in function of time was linear.[173] Sonification appears to degrade HA in a nonrandom fashion that results in a constant bimodal molecular weight distribution (Figure 9).[174] It was suggested that high molecular weight HA chains are degraded more slowly than low molecular weight chains. Despite the use of different HA samples and different ultrasound energies, several research groups agree that ultrasound does not allow complete degradation of HA chains into monomers.[171,172,174,175] The degradation process results in HA chains with molecular weight higher than 10 kDa.[175]

### 3.2. pH-Dependent and Thermal Degradation

Light scattering analysis has shown that HA solutions in acid media degrade randomly.[176] Also at an alkaline pH random chain scission occurs. As explained in section 2.1.2, at alkaline pH, hydrogen bonds which take part in the structural organization of HA chains are destroyed, which results in a large loss of the intrinsic stiffness and the formation of a more compact, flexible random coil.[51] Even in distilled water, rheological measurements indicate that sodium hyaluronate solutions retrograde: with increasing temperature the viscosity decreases in function of time and exponentially in function of temperature.[177] Other studies have also reported on the thermal degradation of HA solutions.[155,171,178] ESR studies on the attack of hydroxyl radicals as a function of the pH of the HA solutions have shown evidence of a random abstraction of hydrogen atoms at all the C−H bonds of HA except at the *N*-acetyl side chain and at C-2 of GlcNAc.[179] It was found that the majority of the glucuronic acid derived radicals undergo rapid base- and acid-catalyzed rearrangements while those from *N*-acetylglucosamine do not.

### 3.3. Degradation by Free Radicals

Free radical depolymerization of HA has been observed with the freeze-drying of sodium hyaluronate.[180] While repeated freeze−thaw cycles do not influence the MW of HA, freeze-drying remarkably causes a rapid decrease of MW during the first cycle while additional depolymerization occurs during the next cycles. Free radical scavengers are able to protect HA during lyophilization.

At higher temperatures and in the presence of thiols or transition metals, HA degradation derives from metal-mediated production of reactive hydroxyl radicals.[179,181−186] The degradation of HA by $Fe^{2+}$ is based on the oxygen-mediated oxidation to $Fe^{3+}$, thereby generating the reactive superoxide radical-anion ($O_2^{\bullet-}$):

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$$

As a reducing agent $O_2^{\bullet-}$ regenerates $Fe^{2+}$, and as an oxydizing agent $H_2O_2$ is produced via reaction with a hydroperoxide ($HO_2^{\bullet}$):

$$O_2^{\bullet-} + H^+ \rightarrow HO_2^{\bullet}$$

$$HO_2^{\bullet} + H^+ + O_2^{\bullet-} \rightarrow H_2O_2 + O_2$$

According to the reactions described above, a low pH promotes formation of $H_2O_2$ and subsequent reaction with excess $Fe^{2+}$ leads to the formation of hydroxyl radicals ($OH^{\bullet}$; Haber-Weiss reaction):[187, 188]

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

The effect of enzymatically generated oxygen radicals (hydroxyl radicals, superoxide anion radicals) on HA degradation in synovial fluids has been studied.[189−192] Free radicals randomly cleave the side groups from the HA chains, which dramatically affects the tertiary structure. After a longer exposure time, main chain scissions also occur. Free radical scavengers, such as 2,6-diisopropylphenol, effectively stabilize against hydroxyl radicals.[193]

The influence of irradiation on the degradation of HA,[194−196] especially in connection with eye pathologies,[142,197] has long been a main focus of HA research. UV irradiation reduces the viscosity of HA solutions.[25,142] The intervention of reactive oxygen radicals and singlet oxygen in connection with UV irradiation, led to destruction of the interglycosidic bonds in addition to decarboxylation. UV irradiation-mediated degradation of HA has been confirmed.[142,198,199]

Irradiation of HA solutions in an ozone−air atmosphere decreases the viscosity. Eye irritation by photochemical smog and increased exposure to sunlight can be explained by HA degradation.[200] HA degradation tends to increase the number of molecules in the limited volume of the vitreous body and this phenomenon might trigger a buildup of intraocular pressure thereby leading to a destruction of the tertiary and quaternary structures of HA adducts with collagen.[201] Ueno et al. showed, both in vitro and in vivo, that the vitreous body liquefies upon irradiation by visible light in the presence of photosensitizers, like methylene blue and riboflavin.[142]

The influence of varying radicals was evaluated by Andley et al. using specific trapping agents of $OH^{\bullet}$ and $O_2^{\bullet -}$ radicals and singlet oxygen.[192] They found that HA degradation was stimulated by singlet oxygen following change of the tertiary and quaternary structures of HA.[202] UV irradiation of HA solutions in the presence of 1-anthracene sulfonic acid results in degradation of HA chains with a loss of elastic properties.[202] The degradation was attributed to the formation of a singlet oxygen reaction product of anthracene.[203]

HA radicals, generated by UV irradiation, were identified by ESR spectroscopy.[142,201,204] The results were in agreement with the ESR spectra, observed for HA degradation induced by hydroxyl radicals.[179]

As explained in section 2.1.2, oxidative degradation of HA can be caused by periodic acid,[43,186,205] due to destruction of the C-2 and C-3 glycol groups present in HA (Figure 2). However, an alternative mechanism may rupture the main polymer chain.[205]

### 3.4. Enzymatic Degradation

The most prevalent degradation of HA in biological environments is catalyzed by various enzymes called "hyaluronidases" or "hyases".[206,207] According to the degradation mechanism, hyaluronidases are divided into three groups. Hyaluronate-4-glycanohydrolase hydrolyzes the *β*-*N*-acetyl-glucosamine bonds to yield even numbered oligosaccharides with glucuronic acid residues at the nonreducing end. To this main hyase group belong testicular, lysosomal, and venom hyase. Other hyases are hyaluronate-3-glycanohydrolase and hyaluronate lyase, which are also called leech hyase and bacterial hyase, respectively. Similar to acid hydrolysis, HA is enzymatically hydrolyzed by hyaluronidase by a random degradation mechanism.[143,174]

## *4. Chemical Derivatization of HA*

### 4.1. Hylans

As shown in section 6.2, considerable attention has been focused on studying cross-linked derivatives of HA, the hylans.[208] In this review, only the most relevant synthetic approaches to synthesize hylans are described.

In early studies on derivatization of HA, Laurent used 1,2,3,4-diepoxybutane to cross-link HA chains.[209] A general method to cross-link HA chains is based on the reaction of formaldehyde (or other agents, such as dimethylurea, dimethylolethyleneurea, ethylene oxide, polyaziridine, or polyisocyanate) and HA at a neutral pH, which involves polysaccharide hydroxyl groups and protein amino or imino groups.[210] The protein links of the HA chains are reflected by the jelly behavior of the solution. Under proper reaction conditions, two to eight HA molecules are connected. The MW of hylans, prepared by this method, varies between $8 \times 10^6$ and $24 \times 10^6$ Da. The protein content is from 0.4 to 0.8%.

Another method used the reaction between divinyl sulfone (DVS) and the hydroxyl groups of HA.[211,212] DVS reacts readily with HA in aqueous alkaline solutions at room temperature to provide cross-linked HA gels. The reaction is very fast, and strong gels are obtained within minutes.

The need to remove unreacted chemicals from cross-linked HA gels for medical applications initiated research on photochemical cross-linking. Photocurable HA was synthesized by Matsuda et al. using several approaches.[213] Hydroxyl or carboxyl groups of the polymer were esterified with the photoreactive compound. Consequently, photocurable HA was irradiated by UV light using wavelengths between 260 and 400 nm. Photoreactive chromophores that react with HA hydroxyl groups are cinnamic acids (Figure 10a), 1-carboxyalkyl substituted uracils (Figure 10b), and 7-carboxyalkoxyl-substituted coumarins (Figure 10c). Photoreactive chromophores that react with the HA carboxyl groups are 1,2-*O*-ethanothymine (Figure 10d) and derivatives of 1,2-*O*-ethanouracil (Figure 10e−h).

Another study reported on combinations of poly(ethylene glycol) (PEG) oligoglycolylacrylates and HA.[214] Carbonyl diimidazole (CDI)-activated PEG monoacrylate was used to synthesize PEG-acrylate-HA. Acrylated HA was photopolymerized by UV irradiation using 2,2-dimethoxy-2-phenylacetophenone as initiator.

HA polyglycerol polyglycidyl ether (PGPGE**)** cross-linked gel matrixes were applied for visible light induced ocular drug delivery.[215] The cross-linking reaction proceeds at a basic pH and at a temperature of 60 °C, immediately after mixing HA with PGPGE. The observed gels were opaque with a water content of 99.5%.

### 4.2. Other HA Derivatives

Non-cross-linked HA derivatives were also studied. To synthesize carboxyl esters of HA, an original method based on the reaction between quarternary ammonium salts of HA and alkylating agents in aprotic solvents was proposed.[216−219] The reaction can be carried out with various alcohols. The degree of esterification varied betweeen 0 and 100%. The HA esters are insoluble in water, and their physicochemical properties differ from the original HA.

According to a patent by Balazs and Leshchiner, HA can be modified with synthetic polymers such as polyurethanes (aliphatic, aromatic, and araliphatic polyurethanes and polyesterurethanes), polyolefines (polyethylene, poly(vinyl chloride), poly(vinyl alcohol), and polyvinyl acetate), acrylic polymers, polyamides, polyesters, and polysiloxanes.[211] In another patent,

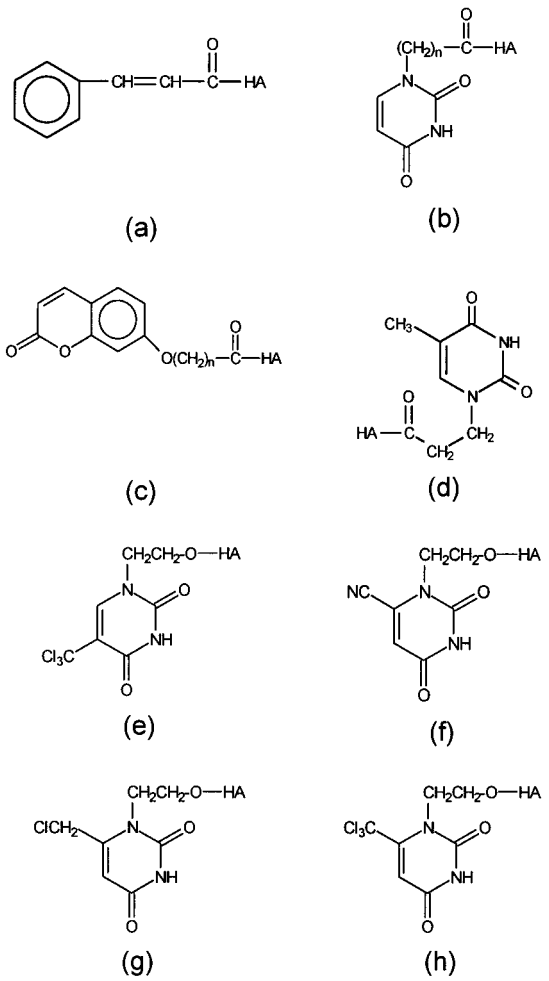


**Figure 10.** Photoreactive chromophores linked to HA for cross-linking: cinnamic acid (a), 1-carboxyalkyl substituted uracils (b), 7-carboxyalkoxyl-substituted coumarins (c), 1,2-*O*-ethanothymine (d), 1,2-*O*-ethano-5-trichloromethyluracil (e), 1,2-O-ethano-6-cyanouracil (f), 1,2-*O*-ethano-6-chloromethyluracil (g), and 1,2-*O*-ethano-6-trichloromethyluracil (h).[213]

the activation of the synthetic hydrophilic polymer by PEG derivatives is described.[220] HA needs to be deacetylated by basic hydrolysis to provide free amino groups which subsequently react with PEG. Reaction between HA and the synthetic polymer can even be performed after injection in situ.

According to a patent by Elwood and Riccarton, HA can be conjugated with chemotherapeutic or other physiologically active substances.[221] Conjugation proceeds via amino groups present in drugs such as antibiotics. Prior to the reaction with the drug, the HA chains must be activated via reaction between cyanogen bromide and the HA hydroxyl groups or by reaction between carbodiimides and the HA carboxyl groups. After reaction, the HA−drug complex is precipitated into pure ethanol.

For the clinical treatment of arthritis, antioxidant-grafted derivatives of HA were synthesized.[222] Grafting proceeds at least on one hydroxyl group of a HA chain by reaction of the methyltricaprylammonium salt of HA with the antioxidant 3,5-di-*tert*-butyl-4-hydroxybenzoyl chloride in polar aprotic solvents.

HA, functionalized with hydrazides (Figure 11), was prepared under mild reaction conditions.[223−225]

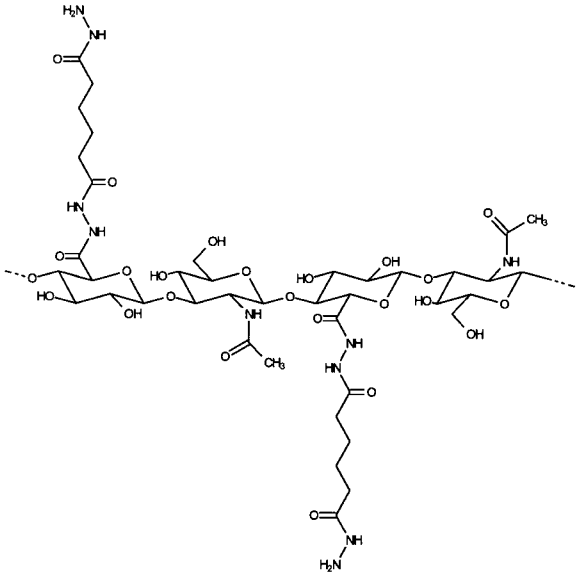

**Figure 11.** The disaccharide unit of HA functionalized with hydrazides.[223−225]

The presence of a pendant hydrazido group allows coupling with drugs and/or cross-linking.

Palmitoyl hyaluronates were used in the preparation of polysaccharide-coated liposomes applied in drug delivery.[226,227] This prevents destruction of liposomes by phospholipase. It was found that the length of the HA chains influences the final conformation when deposited on the surface of the liposomes. If the MW of the HA chains decreased, the palmitoyl hyaluronates showed better emulsification activity in water−oil mixtures.

## 5. Established Medical and Pharmaceutical Applications of HA

In 1994, Goa and Benfield published a detailed review on the pharmacology, the use of HA as a surgical aid in ophthalmology, and its therapeutic potential in joint disease and wound healing.[228] They especially focused on the current role of HA in cataract surgery and in the treatment of arthritis. Our aim here is to differentiate clearly between currently established medical and pharmaceutical applications (this section) and ongoing research on future applications (section 6), which was not discussed by Goa and Benfield. Although HA might open new perspectives for cosmetic applications, we do not discuss them here because scientific information on this subject is lacking.

The use of HA in ophthalmologic surgery has been described in several reviews.[229−231] The main physicochemical features that contribute to its success in ophthalmology are the semiflexible properties (section 2.1) of the biocompatible high molecular weight HA chains and the interactions between chains. Those physicochemical characteristics give rise to the unique viscoelastic properties of HA solutions. When stationary, the high viscosity of HA permits manipulation of ophthalmologic tissues and maintenance of the depth and shape of the anterior chamber. Its low viscosity at high shear rates (when forced through a cannula) facilitates its injection and removal. The

**Table 2. Some Hyaluronan Formulations on the European and American Market**

| product | source | *c* (%) | MW (Da) | commercialized by | indications |
|---|---|---|---|---|---|
| Amo Vitrax | | 3.0 | | Allergan | ophthalmology |
| Amvisc | rooster combs | 1.2 | $2 \times 10^6$ | Chiron | ophthalmology |
| Amvisc Plus | rooster combs | 1.6 | $1.5 \times 10^6$ | Chiron | ophthalmology[a,b] |
| Aquaderm | | | | Baker Cummins | dermatology[c] |
| ARTZ | rooster combs | 1.0 | $6-12 \times 10^5$ | Seikagaku | osteoarthritis |
| Bionect | | 0.2 | | Fidia | wound healing[d] |
| Connettivina | rooster combs | 0.2 | $0.9-1.6 \times 10^5$ | Biofarma | wound healing[d] |
| Cytistat | | | | Bioniche | urology[e] |
| Dormer211 | | | | Dormer Labs | dermatology[c] |
| Eyestil | | 0.5 | | Ophthapharma | ophthalmology[b] |
| Healon | rooster combs | 1.0 | $4 \times 10^6$ | Pharmacia | ophthalmology[a,b] |
| Healon GV | rooster combs | 1.4 | $5 \times 10^6$ | Pharmacia | ophthalmology |
| Healonid | rooster combs | | | Pharmacia | ophthalmology[b] |
| Hyalart | | 1.0 | | TRB | osteoarthritis |
| Hyalgan | rooster combs | 1.0 | $5-7.3 \times 10^5$ | Fidia | osteoarthritis |
| Hyalistil | | 0.2 | | Sifi | ophthalmology |
| Hylumed[i] | | 0.1−1 | | Genzyme | wound healing[f] |
| IAL | rooster combs | 1.0 | $9 \times 10^5$ | Fidia | ophthalmology |
| Ialugen | | 0.05 | | Ibsa | wound healing[g] |
| Jossalind | | 0.2 | | Jossa | wound healing[g] |
| Microvisc | | 1.0 | | Bohus Bio Tech | ophthalmology |
| Opegan | rooster combs | 1.0 | $1.5 \times 10^5$ | Seikagaku | ophthalmology[h] |
| Opelead | bacterial | 1.0 | $2.6 \times 10^6$ | Seikagaku | osteoarthritis |
| Ophthalin | | 1.0 | | Ciba-Geigy | ophthalmology[b] |
| Provisc | | | | Alcon | ophthalmology |
| Synvisc[j] | | 0.8 | | Biomatrix | osteoarthritis |
| Viscoat[k] | bacterial | 3.0 | $5 \times 10^5$ | Cilco | ophthalmology[b] |
| Vitrax | rooster combs | 3.0 | $7 \times 10^5$ | Allergan | ophthalmology |

[a] Viscoelastic HA solution used for the replacement of vitreous and aqueous humor. [b] Viscoelastic HA solution used for intraocular injection during eye surgery and/or as adjuvant to promote surgical tissue repair of the eye. [c] HA lotion and/or cream used as emollient to enhance the restorative functions of the skin. [d] HA ointment, cream, and dressings for wound healing. [e] HA solution used for temporary replacement of the glycosaminoglycan layer in the bladder. [f] Hylumed products are also used in ophthalmology, as soft tissue implants, and as surface coatings. [g] HA dressing for wound healing. [h] Viscoelastic HA solution used as surgical aid in artificial lens implantation. [i] Hylumed powder, solutions, gels, and films exist of low as well as high molecular weight HA. [j] Covalently cross-linked HA product. [k] Also contains chondroitin sulfate.

elasticity of HA solutions protects ocular cells from damage caused by contact with surgical instruments and implants. In ophthalmologic surgery, HA products have received approval as viscoelastic tools (Table 2). For the time being, the 1% HA solution as a viscoelastic adjunct in patients undergoing cataract surgery is currently the most widely used HA application.[228]

Under normal physiological conditions, HA pervades the surface of particular tissues and diffuses into the synovial space to lubricate the joints and to prevent mechanical damage by its shock-absorbing properties. Again, the viscoelastic properties are responsible for protecting, lubricating, and stabilizing cells and tissue layers while walking.[231] Due to the depolymerization of HA chains by reactive radicals that are generated during the inflammatory process of arthritis, the rheological properties of endogenous HA alters tremendously.[232,233] On the basis of the postulate that HA may benefit patients with arthritis by supplementing the lubricating characteristics of synovial fluid, HA is injected into the joints. However, more investigation is still required to determine conclusively the real extent to which these effects may occur in humans.[228]

Table 2 shows HA-based products available on the market. The underlying basis for its application in wound healing is the role of HA in tissue reconstruction.[234−236] During the first few days of tissue repair, endogeneous HA is the predominant glycosaminoglycan present in wounds and forms the template necessary for reconstruction following injury.[237] The repair of tympanic membranes by daily instillation of HA solutions locally in the ear is the most important wound-healing application.[238,239] However, as is the case for for the intraarticular HA injections, the healing effect of HA on tympanic membrane perforations and other wounds still has to be confirmed.[228]

## *6. Medical and Pharmaceutical Research toward Future Applications*

Since the 1980s both HA and its derivatives have been intensively explored for use as topical, injectable, and implantable vehicles for the controlled delivery of a variety of drugs. During the past decade, hundreds of patents on potential medical, pharmaceutical, and cosmetic applications were generated. It is beyond the scope of this review to present all this information. It is our intention mainly to present those findings that might really open new perspectives toward applications of HA and its derivatives. In 1991, Larsen et al. and Drobník published excellent reviews on studies on HA and its derivatives in relation to drug delivery.[240,241] This section of the paper will focus on studies that were performed since the publication of these reviews.

## 6.1. Perspectives for Future Applications of Nonmodified HA

### 6.1.1. Ophthalmology

Aqueous eye drops have the disadvantage that more than 75% of the solution applied is lost due to drainage within the first 2 min after instillation. Therefore, viscosity-enhancing polymers have been added to increase the ocular residence and subsequently to improve the bioavailability of the drug. Gurny et al. concluded that native HA solutions have the unique capability of prolonging the precorneal residence time of pilocarpine.[242] They suggested that HA interacts directly with the cell membranes of the corneal tissue. Ludwig et al. confirmed those results.[243] They showed that high molecular weight HA (Healon) prolongs the retention of fluorescein in the precorneal area of human eyes. They compared the HA solutions with equiviscous hydroxyethylcellulose solutions and found that both polymers improved significantly the residence of fluorescein in the tear film. However, HA solutions were much better tolerated. This was attributed to the more pronounced pseudoplastic properties of HA solutions (Figure 4): the viscosity of HA solutions drops dramatically at high shear rates during blinking. This causes less irritation and minor discomfort. Durrani et al. showed that high molecular weight HA (Healon) improved the ocular bioavailability of pilocarpine significantly more than did low molecular weight HA (MW = $3.2 \times 10^5$ and $1.34 \times 10^5$ Da).[244] This agreed with an earlier study that showed that a high molecular weight, as offered by Healon, is an essential requirement for a good performance of HA as an ophthalmic drug vehicle.[245] Other studies that reported the potentials of HA to increase the ocular bioavailability of drugs and to sustain the ocular drug release are described elsewhere.[246−248] Besides the typical rheological properties of the HA solutions, it was asked whether ocular bioadhesion of HA also plays a role in the enhanced ocular drug bioavailability. Saettone et al. investigated the mucoadhesive properties of HA solutions.[249] In an early study, they investigated the mucoadhesive properties in vitro of low (MW = $1.34 \times 10^5$ Da) and higher molecular weight HA (MW = $6.2 \times 10^5$ Da), using porcine gastric mucin. They detected good to excellent mucoadhesive properties of the HA solutions, especially for the high molecular weight HA, but stressed the limitations of their method for measuring bioadhesion. In a later study on rabbits, they investigated the influence of HA solutions on the ocular bioavailability of pilocarpine.[250] They performed experiments both in the presence and in the absence of precorneal mucin they removed by treating the eyes with *N*-acetylcysteine. If no precorneal mucin was present, the influence on the ocular bioavailability was significantly decreased, which indicates that a mucoadhesive interaction might be responsible for the influence of HA on the ocular drug bioavailability. Viscosimetric measurements confirmed the interaction between HA and the precorneal mucin. In a more recent study, Pritchard et al. concluded that HA is an excellent adhesive.[251] As did Saettone et al., they also performed their in vitro study with gastrointestinal mucus, which questions if information on ocular bioadhesion might be extrapolated.[249] Pritchard et al. used both low as well as high molecular weight HA (MW = $1.7 \times 10^5$ and $4 \times 10^6$ Da).[251] In opposition to the results of Saettone et al., high molecular weight HA seemed to show less adhesion than did low molecular weight HA. The literature on the bioadhesive properties of HA remains rather confusing. Test methods have to be improved in order to determine the potentials of HA toward ocular bioadhesion and bioadhesion in general.

### 6.1.2. Nasal Drug Delivery

In peptide delivery, it is well known that oral administration is often limited by the instability and poor absorption of peptides in the gastrointestinal tract. Biopharmaceutical research is being conducted to deliver peptides to the systemic circulation via the nasal canal. In 1993, Morimoto et al. showed that high molecular weight native HA (MW > $3 \times 10^5$ Da) moderately enhanced the nasal absorption of vasopressin in rats while lower molecular weight HA (MW = $5.5 \times 10^4$ Da) had no effect.[252] The mechanism of nasal absorption enhancement by native HA is unclear. This study also pointed out that native HA is not nasal ciliotoxic as it did not affect the nasal mucociliary beat frequency in rabbits. The industrial interest for the nasal use of native HA is apparent from the numerous patents on HA-based nasal formulations for delivery of vaccines,[253] local allergy inhibitors,[254] and peptides.[255]

### 6.1.3. Parenteral Drug Delivery

One main field in the pharmaceutical and medical research on native HA is its use as a carrier of drugs in parenteral administrations. Drobnik explained in detail why HA is so promising: it is safely metabolized in the lysozomes and its molecular structure provides suitable chemical groups for physicochemical interactions with drugs.[241] Moreover, HA is "invisible" to the immune system since it is a natural component of the body.

In the 1990s, much work, in vitro and in vivo, was undertaken to challenge the suitability of native HA as a drug carrier. The use of HA as a drug carrier has been considered, especially in the development of long delivering injections for peptides and proteins. Due to the short biological half-life, typical dosage regimens for therapeutic proteins involve daily intravenous or subcutaneous injections. Sustained release formulations based on HA were tested. Prisell et al. investigated native HA (MW = $5.8 \times 10^5$ Da) as a vehicle for peptide growth factors given subcutaneously.[256] From diffusion studies, they showed the diffusion rate of the peptide to be extremely low in HA solutions, which indicates an interaction between HA and the peptide. They also showed, in vitro and in rats, that the release rate of peptides is dependent on the HA concentration. This application was patented in 1993.[257] Meyer et al. published similar results with human recombinant granulocyte colony stimulating factor after subcutaneous injec-

tions of this peptide, in combination with HA, into hamsters.[258] Their data suggest that high molecular weight HA (MW > $4 \times 10^6$ Da) at relatively low concentration (<2%) is preferred for achieving sustained activity for 5 days. In a European patent application, Igari et al. described the use of HA for sustained release formulations of erythropoietin.[259]

Doherty et al. described a prolongation of lidocaine-induced epidural anesthesia in rabbits by medium molecular weight HA formulations.[260] They calculated the rate of drug absorption from the lidocaine−HA complex was decreased 4-fold relative to that of lidocaine solution without HA having the same viscosity.

#### 6.1.4. Lymphatic Drug Delivery

Earlier experiments observed that isotopically labeled HA moves from rabbit synovial fluid into lymph node sinuses.[261] After intravenous injection of a large amount of labeled HA in mice, HA was also observed in the lymph nodes.[262] These observations led to investigation of the potential of HA to direct drugs through lymphatic pathways, as the lymphatic system is a major participant in the dissemination of many malignant tumors. After injection of a drug into the skin or into the muscles, most drugs of low molecular weight diffuse rapidly through capillary walls into the blood stream and with very little to the lymph. By coupling the drug to HA, it might be easier to transport the drug to the lymph nodes. The lymphatic system as a potential route for delivery of drugs with HA was recently discussed by Fraser.[263]

#### 6.1.5. Dermatology

Due to the low permeability and lipophilic nature of human skin, very few drugs can be delivered transdermally to achieve therapeutic levels in the skin. In the past few years, promising results have been reported in the treatment of arthritic pain and basal cell carcinoma with dermatological HA-based formulations.[263,264] Especially successful results were observed with the nonsteroidal drug diclofenac.[265] From in vitro studies with human skin, Brown et al. showed diclofenac in a HA-based gel accumulates in the epidermis and remains present for days after a single topical dose.[266] The labeled HA activity was found to penetrate all layers of the skin, but most of the HA label was found to be retained in the epidermis. This observation was supported by the discovery of HA binding properties in the epidermal cell membranes.[267] Bennet et al. also compared the topical diclofenac delivery properties of HA solutions with a rheologically equivalent concentration of sodium carboxymethyl cellulose (NaCMC) and a weight equivalent concentration of chondroitin sulfate.[264] Neither NaCMC nor chondroitin sulfate exerted the controlled release effect seen for HA formulations. These results supported the hypothesis that the controlled depot effect of diclofenac is a characteristic specific to HA. However, by means of circular dichroism, near-infrared fluorescence, and capillary gel methods, Brown et al. surprisingly showed the absence of any interaction between diclofenac and HA.[268] These promising dermatological results suggest that HA could be used to enhance the localization of a number of possible drugs within the epidermis/dermis. Such an effect is an obvious advantage if the site of action lies within the skin layers (e.g., antifungal and antibacterial agents).

#### 6.1.6. Surgery

The prevention of postoperative formation of adhesions is often an issue. Topical applications of HA solutions have been shown to reduce adhesions after abdominal and orthopedic surgery.[269] Mitchell et al. also showed that postoperative pericardial adhesions were significantly less severe if 0.4% HA solutions were topically administered to animals during the time of the pericardiotomy.[270] The real mechanism by which HA solutions prevent adhesion formation is unknown. Mitchell et al. concluded that further studies are warranted on the mechanism by which these solutions prevent adhesion, their optimal dose and method of application, and documentation of their safe use in humans.

#### 6.1.7. Implants

Incidents of radiolucency and bioincompatibility of the currently available mammary implants, such as silicone gels, have prompted the search for alternatives. Lin et al. studied the use of HA as an alternative filler material.[271] They showed that HA-filled implants have softness comparable to that of silicone gel and are more radiolucent so there is better visualization of breast structures around the implant. Although no adverse reactions were observed during the first year after the implantation in animals, they pointed out that more studies are needed to determine the real potential of HA in this field.

### 6.2. Perspectives for Future Applications of Modified HA

#### 6.2.1. Hylans

As explained in section 4.1, hylans are produced by chemically cross-linking HA chains. The resulting structures have either a higher molecular weight (hylan fluid) or form infinite molecular networks (hylan gels, membranes, and microspheres).[272] While the hylans exhibit biological compatibility similar to that of HA, the physical and rheological properties of hylans are significantly different from those of native HA.[272,273]

In 1991, Larsen et al. reviewed the use of hylans for drug delivery[240] and presented studies on hylans in combination with a series of drugs. Since a controlled release of the antibiotic gentamycin would be beneficial for ophthalmic and wound therapy, hylan fluids and hylan gels including gentamycin were developed.[240] Studies as a function of the ionic strength of the solvent clearly have shown that the ionic interactions between the cationic gentamicin and the anionic hylan are responsible for dramatically retarded release in vitro and in vivo. By the introduction of negatively charged sulfate groups on soluble hylan polymer, a prolonged release of betaxolol, which is used to manage the elevated intraocular

pressure associated with glaucoma, was achieved.[240] This indicates that the modification resulted in enhanced interactions between the drug and the hylan polymer.

In vitro assays also showed an 8-fold reduction in the levels of interferon released over 24 h and a reduced "burst" effect as compared to controls without hylan.[240] The sustained release of interferon from a hylan matrix was confirmed after subcutaneous administration in rats. Interactions between HA and proteins have been described elsewhere and might explain the slowed release of interferon from a hylan matrix. A hylan coating on polyvinylacetal (PVA) disks loaded with serotonin led to an increased loading dose and dramatically altered the release rates of serotonin.[240] It only took 3 days to release 90% of the serotonin from the uncoated PVA disks while it took more than 7 days for hylan-coated PVA disks. This system exemplifies the use of hylan in modifying the loading capacities and the release characteristics of polymers.

Hylan gels for embolization and drug delivery have been developed in which hylan gels form the matrix for thrombin.[240] At the same time, hylan gels are tissue compatible and provide the physical properties needed to be easily transported through small lumen catheters. Injection of this material intravascularly in rabbits resulted in occlusion of the blood vessel due to the combined action of thrombin release, which locally catalyzes the fibrin formation, and the physical obstruction by the viscoelastic hylan gel.

Inflammation responsive degradation of HA gels, synthesized by cross-linking HA chains with ethyleneglycol diglycidyl ether and polyglycerol polyglycidyl ether (section 4.1) were studied by Yui et al.[215,274,275] On the basis of the earlier observations of McCord[191] that hydroxyl radicals play a role in the degradation of HA in vivo and in vitro (section 3.3), Yui et al. expected that HA gels would be easily degraded after injection in an area where inflammatory reactions are occurring and where hydroxyl radicals are generated in abundance. They concluded that HA gels can be specifically degraded by hydroxyl radicals while the degradation proceeds via a surface reaction. They also showed the degradation of the gels by hyaluronidase was precluded by prevention of hyaluronidase access to the gel. They also showed the in vivo degradation of the HA gels was mainly due to acute inflammation and would not occur under normal healthy conditions. They envisaged these HA gels as a new family of implantable drug delivery systems that only degrade and release the entrapped drugs in response to an inflammatory reaction as in patients suffering from rheumatoid arthritis.

Larsen et al. evaluated the dermal and the immunologic compatibility after intradermal and subdermal injection of divinyl sulfone cross-linked HA (section 4.1) in mice and rabbits.[240] After injection of the hylan particles no significant dermal tissue reaction or antibody production was observed, which indicates that hylan is well tolerated.

Most of the conventional materials do not meet the demands required for both their surface and bulk properties when used as biomaterials. The principal disadvantage of high oxygen permeable silicone-based contact lenses is their nonpolar surface, which gives rise to the formation of lipid deposits and wettability problems. Even silicone−hydrogel extended wear contact lenses, which were claimed to be wettable because of the moderate water content, have not proven to be wettable in clinical testing. The ability of a surface to retain a continuous layer of an aqueous solution, such as a tear film, is determined mostly by the composition of the material surface. Wettability in the ocular environment, protein and lipid deposition, frictional comfort, and other properties all depend significantly on surface characteristics. Therefore, the silicone- or fluoropolymer-based contact lenses must be surface modified. Modification of the surface of contact lenses may occur by HA to render them biocompatible with the ocular environment. Chatelier et al. coated fluoropolymer-based contact lenses with a thin polymer film produced by plasma polymerization of methanol vapor.[276] After reaction with a solution of epichlorhydrin, the lenses were soaked in a solution of HA for 20 h. The lenses were then removed, washed, and dried. The air/water contact angles of the contact lenses were decreased as compared to untreated controls. In the same patent, the methanol-plasma-treated lenses also reacted with diisocyanate compounds generating very reactive isocyanate groups on lens surfaces. These reactive groups then reacted with biopolymers such as HA. The treated lenses showed improved hydrophilicity and water retention. Chabrecek and Lohmann used macrophotoinitiators, derived from reactive photoinitiators and HA molecules, for the photographting of HA on contact lens surfaces under UV irradiation.[277−281] The treated surfaces had considerably lower advancing and receding contact angles in comparison to untreated ones. The lenses with HA coating are usually further modified to minimize the charge effect of carboxylic groups on protein adsorption.

### *6.2.2. HA Esters*

Section 4 explained the synthesis of esters of HA. As those materials might be of great interest in clinical practice, especially for wound dressing and drug release, many medically and pharmaceutically oriented studies have been published on the subject since the early 1990s. From an application point of view, we will discuss two groups of HA esters: HA esterified with nontherapeutic alcohols and HA esterified with hydroxyl functions of therapeutic molecules.

*6.2.2.1. HA Esters and Nontherapeutic Alcohols.* Esterification of the carboxyl groups on HA (section 4.2) with nontherapeutic alcohols such as ethyl, propyl, benzyl, and dodecyl alcohol, gave rise to a new class of semisynthetic polymers. Dependent on the substituent and on the degree of esterification, they could be used to produce a whole series of medical and pharmaceutical products such as medical thread,[282] films,[283] microspheres,[284] pellets,[285] membranes,[286,287] corneal shields,[288] and implants.[289] The group of E. Topp at the University of Kansas studied in detail the permeability for solutes of ethyl and

benzyl esters of HA. In 1991, they showed small neutral and positively charged molecules, like vasopressin (MW = 1142 Da), insulin (MW = 6 kDa), and chlorpromazine (MW = 355 Da), easily penetrate films synthesized from fully esterified HA with ethyl alcohol.[285] Less permeability was obtained for negatively charged solutes such as sodium benzoate (MW = 144 Da), sodium fluorescein (MW = 376 Da), and fluorescent-labeled dextran (MW = 3.9 kDa). In a later study, slower diffusion in membranes of ethyl and benzyl esters of HA was observed, especially for larger peptides and proteins.[286] It seemed that solutes with molecular weights up to 15 kDa penetrated HA ethyl ester membranes while HA benzyl ester membranes were impermeable to solutes having molecular weights greater than 6 kDa. The diffusion of peptides and proteins in benzyl ester membranes was slower than in ethyl ester membranes. This was attributed to a lower degree of swelling of HA benzyl derivatives. They also showed that the logarithm of the diffusion coefficients was linearly related to the logarithm of the molecular weight and to the square of the radius of the peptides and the proteins. This agreed with the predictions of the free volume theory of Yasuda.[290,291] In 1995, the same group also investigated the influence of the hydrophilicity of neutral low molecular weight drugs and of the membrane hydration on the diffusion in membranes of HA benzyl esters.[287] The diffusivity was found to be a clear function of the membrane hydration for all the drugs and membranes studied. While a linear relationship between the logarithm of the diffusivity and the inverse of the membrane hydration was observed for hydrophilic drugs, as the free volume theory predicts, a nonlinear relationship was observed for hydrophobic drugs. They explained that this could be attributed to a second diffusion pathway involving the hydrophobic regions of the hydrated HA esters. While, due to a low partition coefficient, hydrophilic drugs only diffuse via the hydrophilic solvent regions that correspond to the free volume of the hydrated membrane, hydrophobic drugs show a parallel diffusion in both the hydrophilic as well as the hydrophobic regions. In a study using differential scanning calorimetry, these authors explained the hydration of HA ester membranes in detail and identified free and weakly bound water (freezable water) and nonfreezing strongly bound water.[218] They showed in hydrated HA benzyl ester membranes that there is a good correlation between the percent of freezable water and the permeability of various model compounds. This suggests the solutes may transport primarily in freezable water.

To explore the sustained release properties of HA ester matrixes, the release of chlorpromazine, which was physically incorporated into films of HA ethyl and HA benzyl esters, was investigated.[283] A complete release was observed to occur in less than 200 min. The rapid release was attributed to the high degree of hydration of the films. This observation suggested, as far as "small drugs" are concerned, that matrixes of HA esters would probably be most useful in applications where rapid drug release is desired or where prolonged release from a poorly hydrated region is preferred.

In 1989, Saettone et al. observed that the mucoadhesive properties of 75% esterified HA esters were similar to those of native HA.[249] Those encouraging results prompted investigation of HA esters for medical applications where bioadhesion could be an advantage. Richardson et al. studied the potentials of microspheres, consisting of partially and fully esterified HA with benzyl groups, for vaginal administration of calcitonin.[292,293] Although the promising in vivo pharmacokinetic profiles from this study were thought to be related to a close attachment of the microspheres to the vaginal epithelium, the bioadhesive properties of HA esters were questioned recently by Pritchard et al.[251] They evaluated the bioadhesion of HA ethyl and benzyl esters and concluded that esterification of HA results in some reduction of bioadhesive properties. In the work of Illum et al., who studied microspheres of HA esters as a nasal delivery system for insulin, the extent to which the bioadhesive properties of the HA esters were factors in the observed nasal absorption of insulin remained unclear.[294]

From in vitro studies on fibroblast cultures, Cortivo et al. concluded that fully esterified ethyl and propyl esters of HA seem to be nontoxic.[295] In a later study, Benedetti et al. showed that ethyl and benzyl esters of HA have a good biocompatibility in vivo after implantation in rats.[289] From the same study, they concluded that biodegradation varies according to both the type and the degree of esterification.

*6.2.2.2. Mixed Esters of HA, Nontherapeutic Alcohols, and Drugs.* As mentioned in section 6.2.2.1, the release of small drugs from matrixes of HA esters is rapid. To decrease the release rate of small drugs such as steroidal antiinflammatories from such matrixes, prodrugs of HA esters were synthesized. HA was partially esterified with a nontherapeutic alcohol while the other part of the carboxyl groups was esterified with the hydroxyl groups of a steroidal drug. Since the 1980s, the esterification of native HA with hydrocortisone and methylprednisolone has been considered.[296] It was thought that by covalently linking the steroidal drug to HA a biocompatible injectable sustained release complex for hydrocortisone/methylprednisolone could be developed that, at the same time, would overcome the well-known solubility limitations of the steroids. Due to different polymeric properties of HA esters as compared to those of native HA, not only aqueous injectables but also novel drug delivery devices such as films and microspheres containing covalently linked steroids could be studied. In the early 1990s, the drug release of HA ester microspheres with hydrocortisone[284] and methylprednisolone[297] physically dispersed into the matrix was compared to microspheres with the steroids chemically bound to the polymer backbone by an ester linkage. When the drug was physically dispersed, its in vitro release was essentially completed in 10 min (in the case of hydrocortisone). When the drug was covalently bound, the release was much slower, taking more than 100 h! A detailed study on the enzymatic and nonenzymatic hydrolysis

of hydrocortisone from native HA and HA esters was represented by Rajewski et al.[296] HA esters covalently linked to steroids were especially explored in order to evaluate their potential for ophthalmic drug delivery. Kyyrönen et al. studied the in vitro and in vivo release of microspheres of methylprednisolone linked to HA esters,[297] Bucolo et al. investigated the in vitro and in vivo release of corneal shields of HA esters linked to methylprednisolone, and Hume studied the ocular sustained delivery of prednisolone using films of HA benzyl esters.[209,298]

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