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Hyaluronan: Preparation, Structure, Properties, and Applications†

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Contents

† 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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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]

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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.

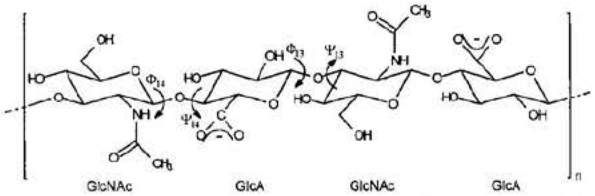

Figure 1. A tetrasaccharide from a HA chain. The torsion angles of the glycosidic linkages (Φ and Ψ) are defined in the text. (Reproduced from ref 4. Copyright 1994 American Chemical Society.)

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 β 1-4 glycosidic bond.[3] The disaccharides are linked by β 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 × 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 (α).[31] While the pK of the polymer, as obtained by extrapolation to $\alpha = 0$, was estimated to be 2.9, the pK of the monomer D-glucuronic acid is 3.23.[32] The difference in pK 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

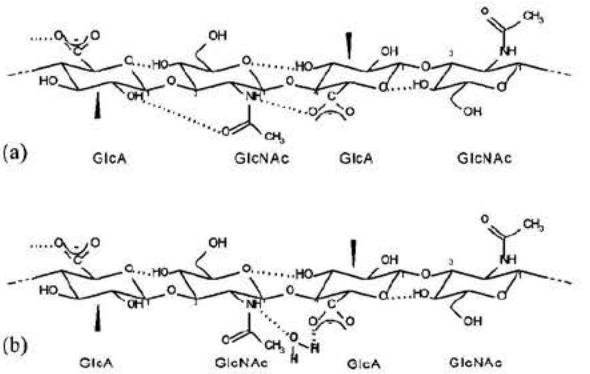

Figure 2. Secondary structure of HA in DMSO (a) and in DMSO containing water (b) as proposed by Heatley et al. The dotted lines indicate hydrogen bonds. Arrows indicate the glycol groups which are resistant to periodate oxidation (see text). (Reproduced with permission from ref 53. Copyright 1988 The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

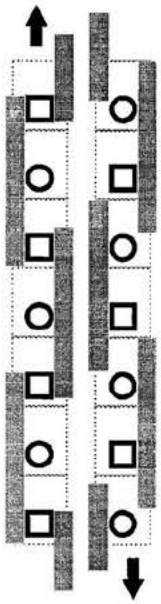

Figure 3. Scheme of a possible duplex formation between two HA chains.[46,47] The two participating single HA helices are antiparallel to each other. The dotted lines delineate each sugar unit, the circles represent acetamido, and squares represent carboxylate groups. The gray dotted bars are the hydrophobic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

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