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Review

Dedicated to Prof. Bogdan C. Simionescu on the occasion of his 75th anniversary

STRATEGIES OF HYALURONAN CHEMICAL MODIFICATIONS FOR BIOMEDICAL APPLICATIONS

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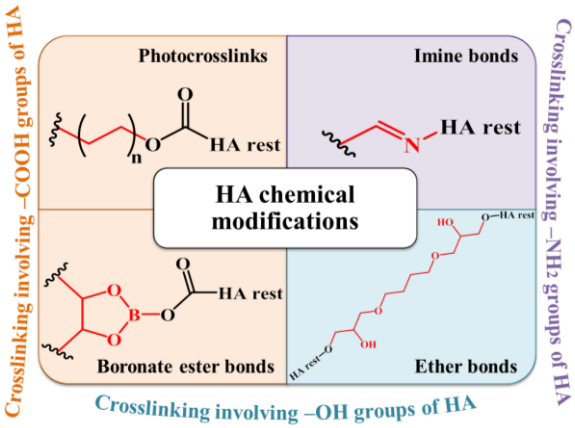
Hyaluronan (HA), a non-branched and multifunctional glycosaminoglycan, attracted interest by its breadth of biological roles, despite its structural simplicity. HA was considered a versatile building block to develop new biomaterials for more and more diverse and ingenious biomedical applications, such as scaffolds for tissue engineering, including cell and bioactive molecules, regenerative medicine, coatings and carriers used in imagistic investigations or therapy. The challenge of selective chemical reactions comes from HA limited solubility in organic solvents and sensitivity to enzymatic, mechanic and thermal degradation. All functional groups of HA can be modified by conjugation or covalent crosslinking with synthetic or natural compounds. All these modifications intent to improve the mechanical, rheological, swelling and controlled degradation properties of native HA in related biomaterials.



INTRODUCTION

HA glycosaminoglycan represents a primary component of extracellular matrix (ECM), a dynamic 3-dimensional network that provides structural support for cells and tissues. Hyaluronan as term was firstly introduced by Endre Balazs[1] in 1986 and was intended to include the different forms (hyaluronic acid, sodium hyaluronate). The physical and chemical characteristics of HA are unique, distinguishing this polysaccharide from the other glycosaminoglycans.

HA represents the largest linear unbranched and single non-sulfated glycosaminoglycan. The chemical structure of HA consists of alternating disaccharide repeating units of of β-D-(1-3) glucuronic acid (GlcA) and β-D-(1-4)-N-acetylglucosamine (GlcNAc). At the human body physiological pH at about 7.4, most carboxyl groups are deprotonated, and HA exists as a polyanion, in which the carboxyl group of the GlcA are negatively charged. The negative charge of HA is balanced with different cations present in the biological fluids (*e.g.*, Na+, K+, Ca2+, Mg2+). Moreover, HA can chelate different metal ions (*e.g.*

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iron and copper), responsible to generate harmful reactive oxygen species (ROS) that may cause cells components damage and finally cell death.[2] Therefore, HA is considered radical scavenger and cell protector.[3]

HA unique activities are related to specific features of its different molecular sizes. HA is generally synthesized under normal health conditions as a high-molecular mass biopolymer ranging from 1 000 to 6 000 kDa, with anti-inflammatory, anti-angiogenic, anti-proliferative, and immunosuppressive activity.[4] Moreover, it was shown to get involved in other important biological processes, such as space-filling, facilitates wound healing[5], being involved in ovulation, embryogenesis, and tissue regeneration.[6] On contrary, low molecular masses of HA were reported to induce the expression of pro-inflammatory cytokines and chemokines, and growth factors, associated with pathological conditions (*e.g.* oxidative stress, inflammation, cancer).[7]

Native HA exhibit impressive hydration capacity. It was experimentally concluded that only 2-fold increase in molecular mass or in concentration of HA, results in a 10-fold increase in bulk viscosity. The most distinctive properties of HA solutions are both their viscous and elastic non-Newtonian behavior. The rheology of HA in aqueous solutions was shown to be affected by concentration, the average molecular mass, ionic strength, pH, temperature and shear rate.[8]

It was revealed by computations and X-Ray measurements the random coil conformation of HA, where the equatorial side chains form polar hydrophilic patches, while the axial hydrogen atoms form non-polar hydrophobic patches (Fig. 1). As consequence, the longer the HA molecule, the more extensive and coherent the network even at low HA concentration.[9] HA molecular structure is stabilized by intermolecular hydrogen bonds between the acetamide group of GlcNAc and the carboxyl group of GlcA and, also through physical interactions between hydrophobic patches.[10]

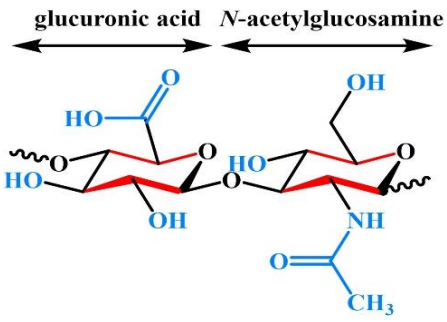

Fig. 1 – Hydrophylic (blue) and hydrophobic (red) patches in HA chain.

In highly concentrated HA solutions, the chains form a meshwork like structure, as self-associate to each other, as well as by steric interactions.[11] The part of the meshwork structure aligns when a continuous force is applied in a certain direction. The unique higher viscosity is due to both electrostatic and hydrophobic interactions in aqueous solution of high molecular masses molecular chains of HA, even in concentration as low as 1 mg/ml.[12,13]

1. BIOLOGICAL FUNCTIONS OF HA

Despite apparently simple in structure, HA can modulate multiple physical and physiological functions, strongly influenced by its relative chain length, size distribution and concentration[14], regulated by the dynamic balance between HA biosynthesis (controlled by three synthases) and degradation (by several hyaluronidases).[15] The concentration of HA differs with type of tissue, age, and disease condition.[16] Generally, HA amount of an average human adult is estimated to15 g of HA throughout entire body, with approximately one third of this amount turned over daily.[17]

HA in physiological fluids exhibit osmotic buffering capability, modulating the water content in extracellular spaces, due to specific non-ideal osmotic pressure behavior. In soft connective tissues, HA of very high molecular mass forms entangled networks, that control tissue hydration and water transport, contributing to fluid viscosity, supporting viscoelasticity lubrication and shock absorbance (*e.g.* in synovial joints fluid, eye vitreous body, tear fluid).[18] In other fluids (*e.g.* blood, saliva, and urine) HA have lower molecular masses.[19]

Hard tissues (*e.g.* like cartilage and skin), are associated with HA of high molecular masses, non-covalently assembled into a network, that ensures, beside hydration, a three-dimensional support for cells. Moreover, in the extracellular space, HA typically bound to matrix proteins. Hardingham and Muir[20] discovered for the first time the specific binding between HA and proteins in cartilage as proteoglycans (aggrecan). Later, other extracellular proteins with affinity for HA have been discovered – hyaladherins.[21] Hence, HA bind in proteoglycans (*e.g.* aggrecan and versican) provides structural integrity and proper hydration of cartilage and other tissues.[22]

In several pathological conditions the structure and organization of the ECM is compromised and low molecular masses of HA is elevated (*e.g.* synovial joints disease, liver fibrosis, diabetes,

myocardial damage, some types of cancers). Interestingly, HA plasmatic levels have been reported to be used as a biomarker to estimate tissue damage.[23-25] Studies shown the mechanism HA activate cell signaling cascades and modulate cell responses in damaged tissue, by direct interaction with specific cell membrane receptors.[26] Pathological conditions in tissues may alter HA synthesis leading to an increased production of small HA fragments, by both enzymatic and non-enzymatic paths.[27] Endogenous HA can be degraded by specific enzymes hyaluronidases and ROS, inducing inflammatory, immuno-stimulatory and angiogenic responses (*e.g.* myocardial infarction, arthritis, or transplant rejection).[28] A decrease of molecular mass of HA in synovial fluid by ROS generated during inflammation processes, is found in osteoarthritis pathology.[29]

2. BIOMEDICAL APPLICATIONS OF HA

The healing capacity of the human body can be limited in some cases and, as consequence, the external aid of foreign materials could facilitate regeneration, such are implantable engineered biomaterials capable to specifically interact with a targeted biological system. One type of such biomaterials is HA-based, clinically put into service of medical products for over decades.[30]

The idea of utilizing for the first time HA in human medicine, as aqueous viscous solutions, is dated back to 1960s for replacing vitreous of the eye.[31] Twenty years later, in 1980s, the ophthalmic device solution was first commercialized; since then, high molecular mass HA-based biomaterials have been applied in the fields of ophthalmology, dermatology, rheumatology etc., due to their special viscoelastic properties and hydration capacity.[32] The therapeutic HA aqueous solutions applications based on the viscous and elastic properties are viscosurgery (used in ophthalmic surgery), viscoaugmentation (to fill and augment the intercellular spaces of connective tissues), viscoseparation (to separate connective tissue surfaces), viscosupplementation (to supplement or replace biological fluids such as synovial fluid) and viscoprotection (to protect wounded tissue surfaces and promote the wound healing).[33]

Biomedical applications of HA-based biomaterials are limited by short turnover rate and limited mechanical properties of native HA. As the HA physiological life time usually last 48–72 h, chemical modifications are necessary to improve its persistence and mechanical performances. The abundance of HA in majority of tissues, together with its accessibility to eventual chemical modification, has made HA an attractive building block for a wide range of biomedical applications, including scaffolds for tissue engineering and regenerative medicine, coatings and carriers for drug delivery.[34] The mechanical strength, physical and chemical properties of the materials depend on the type and the degree of chemical modification.[35] The physical properties of HA-based biomaterials include its storage and loss modulus, compressive stress, compressive modulus, porosity and pores distribution, swelling rate, and degradation rate. The rheological behaviour of gel-like materials can be altered according to the chemical modification, e.g. the elasticity can be increased by sulphation.

3. HA CHEMICAL MODIFICATIONS STRATEGIES FOR BIOMEDICAL APPLICATIONS

HA chemical modifications strategies target mainly the functional sites of the biopolymer, the four different types of functional groups: carboxylic acid, hydroxyl, N-acetamide and reducing end group. All functionalities permit characteristic chemical reactions. Historically, HA was known for the chemical transformation of its hydroxyl groups, including ethers and ester formation, substitution, elimination. Recently, several processes also described transformation of carboxyl groups and deacetylated amino groups. The schematic representation of chemical modifications in HA chain is given in Fig. 2.

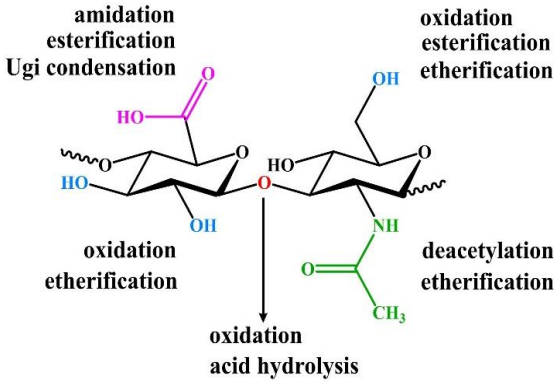

Fig. 2 – HA functional groups for chemical modifications: carboxylic acid (magenta), hydroxyl groups (blue) and, N-acetamide (green).

All these functional groups of HA can be modified to different products by two techniques,

based on the same chemical reactions, conjugation and crosslinking. Conjugation represents grafting of a monofunctional compound (*e.g.* bioactive molecules, marker molecules) onto one HA chain, by a single covalent bond. Crosslinking link together different chains of native or conjugated HA, by two or more covalent bonds.[36] HA crosslinking can be achieved either from native HA (direct crosslinking) or from its conjugates, by synthesis. Conjugation and crosslinking are performed for different purposes. Conjugation allows developing drug carrier systems with improved delivery, pro-drugs by linking active molecules to HA or for bioactive coatings. Crosslinking is intended to improve the mechanical, rheological and swelling properties of HA, to reduce its degradation rate and, obtaining derivatives with a longer persistence in the site of application.

As a particular aspect, native HA in aqueous or organic solvents could have a very high viscosity for homogeneous reaction conditions in term of complete solubility and good miscibility. In order to improve solution rheology, the controlled degradation of high molecular mass HA as starting materials prior to chemical modification reactions could often be an advantage.[37]

Modification of hydroxyl groups

It is not clear which of the hydroxyl groups is most reactive, though it is reasonable to assume that the reaction occurs firstly on the primary hydroxyl group. Over the years, different derivatives of HA have been obtained through reactions between the hydroxyl groups of HA and mono- or bi-functional reagents, such as ethers, hemiacetals, esters and carbamates. Some reactions were performed in water, while others, involving reagents sensitive to hydrolysis, require organic solvents (e.g. dimethylformamide or dimethylsulfoxide). However, some reactions in aqueous solutions are pH-sensitive and require for acidic or alkaline conditions, which have been shown to induce significant HA chain hydrolysis.[38]

Ether were formed by epoxide ring opening in the presence of the HA hydroxyl groups. Laurent, Hellsing, and Gelotte were the first to report HA crosslinking with 1,2,3,4-diepoxybutane as the crosslinking agent, reaction performed in strong alkaline conditions, at pH 13–14, at 50°C, within 2 h.[39] Twenty years later, Malson and Lindqvist patented the crosslinking of HA with butanediol-diglycidyl ether (BDDE).[40] HA-BDDE ether represents the most marketed HA derivative, as can be simple obtained in aqueous ambient, and can be degraded into non-cytotoxic fragments.[36] Ether derivatives of HA in alkaline aqueous solution have been also synthetized in the presence of other epoxides, like ethylene glycol-diglycidyl ether, polyglycerol polyglycidyl ether, epichlorohydrin and 1,2,7,8 diepoxyoctane. Other efficient methods to obtain ether derivatives of HA include divinyl sulfone (DVS) or ethylene sulphide in water.[41]

The hydroxyl groups of HA can be also esterified by reacting with anhydrides (e.g. octenyl succinic anhydride or methacrylic anhydride) under alkaline conditions. Alternatively, HA esterification by convertion to a salt soluble in DMSO can be achieved, to activate compounds such as acylchloride carboxylates. The synthetize of carbamate derivatives with high degrees of substitution can be driven by activation of HA hydroxyl groups to cyanate esters, and the subsequent reaction in basic water with amines, in a reaction of only 1 hour.[42,43]

Early experiments on the sulfation of HA were carried out in the 1950s,[44] with sulfuric acid or chlorosulfonic acid used as sulfating agents.[45] In spite of these drastic reaction conditions, a complete sulfation of all free OH groups did not result, but caused partial degradation of the HA polymer chain. More recent attempts, reagents as complexes of SO_3 with organic amines, especially triethylamine or amides like DMF were used, as relatively milder reagents, causing less HA chain degradation. Studies on regioselectivity of sulfation reaction proved that the primary hydroxyl groups are more reactive.[46] The sulfation of hydroxyl groups is of great importance due to the ability to bind growth factors, as it was shown for sulfated HA and TGF-β1. Moreover, the sulfation of HA has been shown to promote stem cell differentiation.[47]

Amidation of the vicinal hydroxyls groups of the uronic acid units in HA can be achieved in the presence of cyanogen bromide.[48] N-substituted carbamate bonds resulted after reaction between an activated HA cyanate ester intermediate with the amine, beside HA-isourea as secondary product. High degrees of substitution were achieved using the native HA sodium salt and a slight excess of reagents in water, only in one hour. However, the high pH necessary for reduction to occur decreases the molecular mass of the HA chain. This method allowed the obtaining of a wide range of HA derivatives, with tunable properties.[49]

The periodate oxidation, also called Malaprade oxidation, is acting on cis-diols, leading to the

cleavage of the sugar ring and forming corresponding carbonyl moieties.[50] The vicinal hydroxyls of HA can be modified by opening the sugar ring at the carbon-carbon bond the D-glucuronic acid moiety of HA to dialdehydes, in the presence of sodium periodate, that further can be functionalized with amino- or hydrazide- derivatives.[51] Studies indicated a decrease in molecular mass during reaction, from 1.3 MDa for native HA to 260 kDa for the resulting HA-aldehyde.[52] This method was previous utilized for grafting peptides at the aldehyde groups.[53]

Hemiacetal formation using glutaraldehyde for HA crosslinking was reported.[54] The authors noticed that the reaction take place in acetone-water solvent, but not in an ethanol-water, suggesting the inhibition of the crosslinking reaction could be affected by the competitive reaction with the hydroxyl groups of ethanol. Glutaraldehyde crosslinking was demonstrated to be unstable by Collins and Birkinshaw,[41] that stabilized the resulted hydrogel by neutralizing it through swelling in buffer. However, glutaraldehyde has the disadvantage of being toxic, requiring thorough purification of the final product.

Modifications of carboxyl groups

Amidation with carbodiimides in water is one of the most widely used modification of HA.[55] The most used carbodiimide is1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC), known for its water solubility. Danishefsky and Siskovic first converted the carboxyl groups of HA to amides.[56] Amidation with EDC has the advantage of not lead to cleavage of the HA chain and therefore maintains its high molecular mass related to its valuable viscoelastic properties. However, reagents need to be added into excess as EDC hydrolysis cannot be avoided. Amidation can be performed with 2-chloro-1-methylpyridinium iodide in dimethylformamide (DMF), to minimize the reagent hydrolysis.[57] When no amine is added to the reaction mixture, esterification takes place as the reagent-activated HA reacts with its own hydroxyl groups, resulting an ester crosslink gel between the HA chains, called auto-crosslinked gels. Della Valle patented this procedure, performed in dimethyl sulfoxide (DMSO).[58] The autocrosslinking is unique compared to other crosslinking techniques as no other bridge molecules are present between the crosslinked HA chains. This feature ensures that only the HA natural components of are released during its degradation in the body.

Ester formation using epoxides such as glycidyl methacrylate have been described to synthesize methacrylated HA, capable of further photocrosslinking reactions.[59-61] The reaction was performed in water, in the presence of triethylamine in excess, as a catalyst.

Ugi condensation for HA crosslinking, with diamines as a cross-linker to form diamide linkages have been described.[62-63] The reaction was performed in water, at acidic pH 3, in the presence of formaldehyde, cyclohexyl isocyanide and the diamine. However, the use of carcinogenic formaldehyde, requires specific handling. This method leads also to a secondary amide, adding a second pending group (cyclohexyl in this case).

Modification of N-acetyl groups

Deacetylation of the N-acetyl group of HA was performed to recover the corresponding amino group, which can further react with an acid, by the amidation methods. Deacetylation is usually performed in the presence of hydrazine sulfate, over five days at 55°C, which results in severe chain fragmentation. Bellini and Topai patented the amidation of HA by reaction of deacetylated amino-group of HA with an acid, previously activated with a carbodiimide, to the amide bond.[64] This method was used to crosslink HA with the alginic acid carboxylic groups.[65] Moreover, the deacetylated amino groups were reacted with the carboxylic groups of HA to obtain an auto-crosslinked hydrogel. HA deacetylation was possible using enzymes.[66]

Conjugation of reducing end group

Advanced applications (*e.g.*, microarrays, functional molecular and cellular assays, as well as biosensors) or biomaterial scaffolds require the attachment of HA to surfaces. As consequence, specific conjugation of active molecules to the reducing end of HA chain is desirable. The most frequently techniques used for HA functionalization are currently hydrazine[67] and oxime[68]. Other approaches uses reductive amination (*e.g.*, ethylendiamine dihydrochloride and sodium cyanoborohydride).[69] HA functionalization at the reducing end can also be achieved with a thiol group, enabling direct immobilization on metal surfaces and coupling to marker molecules as biotin. The reducing end group functionalization

can be achieved by treating HA with cysteamine and sodium cyanoborohydride as reductive agent.[70]

4. FUTURE PERSPECTIVES

Although HA possess a rich set of *in vivo* biological cues, the biophysical signals from HA-based biomaterials may dramatically differ depending on the fabrication method.[71,72] HA derivatives are capable to be developed as injectable products, implantable scaffolds, 3D hydrogel matrices encapsulating living cells and drug delivery systems, appropriate techniques for efficient, low-cost and safe modification of HA have to be continuously explored. Hence, efforts have been done to develop one-pot reactions, preferably proceed in aqueous ambient and, under mild conditions. Moreover, alternative approaches to efficiently HA modifications have been adapted, such as "click chemistry" syntheses, solvent-free methods, in situ and eventually photo-crosslinking of functionalized HA, etc.

Current strategies aim the *in situ* formation of HA-based networks with dynamic and reversible bonds, which can break down and reform, with or without external stimuli, with self-healing capacity. As a drawback, these HA networks may have poor mechanical properties or low stability. In order to overcome these shortcomings, networks can be double crosslinked, an initial physical crosslinking with fast gelation and reversible interactions, followed by a covalent crosslinking, that provides stability and improved mechanical properties.[73,74] These dynamic bonds confer adaptability, self-healing capacity, stress relaxation, or shear thinning properties to the biomaterial. Such features, adapted on HA chain, allow the biomaterial to flow, to be printed or injected under the appropriate shear forces and, once the force or deformation has stopped, to recover its macroscopic properties. These dynamic hydrogels attract nowadays special attention, due to their possible applications as in situ injectable biomaterials, with minimally invasive intervention.[75-77]

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