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The Detailed Structural Characterization of Chemically Modified Glycosaminoglycans is Absolutely Essential to Explain Potential Biological Effects

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The extracellular matrix (ECM) is a complex, highly organized tissue that is omnipresent in all vertebrates. Although the ECM is studied since many years by biochemical and biophysical methods [1] from the viewpoint of basic sciences, the increasing interest in ECM is nowadays coming from the considerable medical relevance of the ECM [2] and the increasing relevance of "regenerative medicine" [3]. In addition to obvious injuries such as skin burns, bone fractures, or mechanical cartilage injuries, many ECM-related diseases are also accompanied by inflammatory processes. Physicians define the "cardinal" symptoms of inflammation as the occurrence of pain, swelling, redness, heat, and loss of tissue function. From a more (bio) chemical view point, however, inflammatory processes are initiated by the infiltration of typical inflammation cells such as macrophages or neutrophils: these cells generate upon stimulation "reactive oxygen species" (ROS) such as hydroxyl radicals (HO•) or hypochlorous acid (HOCl) in addition to the release of a multitude of proteolytic enzymes such as elastase or collagenase which are all capable of degrading the different components of the ECM [4]. Despite the significant socioeconomic relevance [4], there is so far no perfect cure of ECM-related diseases!

The ECM is a water-rich tissue and water constitutes about 80% of its wet weight. The solid material of the ECM is represented by different collagen types and carbohydrates, the glycosaminoglycans (GAGs) [5] which will be in the focus of this editorial. GAGs are basically alternating co-polymers of either glucuronic acid or galactose and N-acetylglucosamine or N-acetylgalactosamine, which may be sulfated in various positions [6]. A survey of the different physiologically-relevant GAGs is shown in Figure 1.

Hyaluronan (HA) is the only non-sulfated GAG which has many different biomedical applications [7] and is a particularly useful agent to improve the viscoelastic properties of the joint (synovial) fluids from patients suffering from arthritis. While keratansulfate (KS) is only rarely used for biomedical purposes due to its considerable price, chondroitinsulfate (CS) which is much less expensive in comparison to KS is widely used as "biological" to treat musculoskeletal diseases - although it is not yet clear whether the application of CS has a protective effect [8]. An even higher medical interest is attracted by heparin (HE) which is widely used as an anticoagulant due to its high negative charge density realized by the high number of sulfate residues. It is nowadays well known that great care upon the preparation and application of heparin is required: during the recent "heparin contamination crisis" it became evident that severe adverse events (even deaths) occurred when patients were treated with heparin that contained oversulfated CS [9]. Therefore, it is important to note that rather similar GAGs may result in completely different effects!

The detailed analysis of (chemically modified) GAGs is one extremely important issue because not only the overall sulfate content but as well the sulfation pattern [10] is likely to have a significant impact on the biological effects of the related GAGs. Sometimes, the "same" GAGs are discussed to induce different effects in cell cultures and/or animal experiments: as far as we can say this problem is not stemming from the biological part of the related study but arises from an insufficient characterization of the used GAGs! In other words: different GAGs were actually used. Therefore, careful and detailed GAG analysis is the crucial point of this kind of investigations and a survey of frequently used methods to analyze GAGs is given in Figure 2.

There are many problems related to GAG characterization and GAG analysis is much more difficult than it seems [11]. On the one hand, GAGs are polysaccharides with non-uniform polymer repeating units where the distribution as well as the position (normally at 4- or 6- position of the glucose-/galactosamine or the 2-position of the uronic acid) of the sulfate residues is not homogeneous. According to our best knowledge there is not a single method available which allows the detailed characterization of all structural aspects of the intact GAG polysaccharides [5]. Although there are many (chromatographic or electrophoretic) techniques available which allow the estimation of the molecular weight as well as its distribution and methods which enable the determination of the overall sulfate contents (for instance, elementary analysis), there is not a single method available which allows the differentiation of potential isomers within the polysaccharide [6]. Although high-field nuclear magnetic resonance (NMR) spectroscopy may give rough information about the positions of the sulfate residues, the achievable spectral resolution is not high enough to enable detailed assignments [5]. The same is true for other spectroscopic methods such as infrared (IR) spectroscopy [12].

Many papers are indicating that soft ionization mass spectrometry (MS) techniques such as electrospray ionization (ESI) or matrix-assisted laser desorption and ionization (MALDI) are convenient methods to obtain the required detailed information of GAG composition [13]. Unfortunately, MS is exclusively applicable to GAG oligosaccharides - ideally the disaccharides - which are normally generated from the GAG polysaccharides by enzymatic digestion [5].

The most widely used enzymes are bacterial or testicular hyaluronidases as well as bacterial chondroitinases, in the majority of cases the ABC type from *proteus vulgaris*. The latter enzyme digests chondroitin-4-(CS-A), chondroitin-6-(CS-C) as well as dermatan sulfate (CS-B) under generation of an unsaturated disaccharide. The individual disaccharides (carrying a different number of sulfate residues

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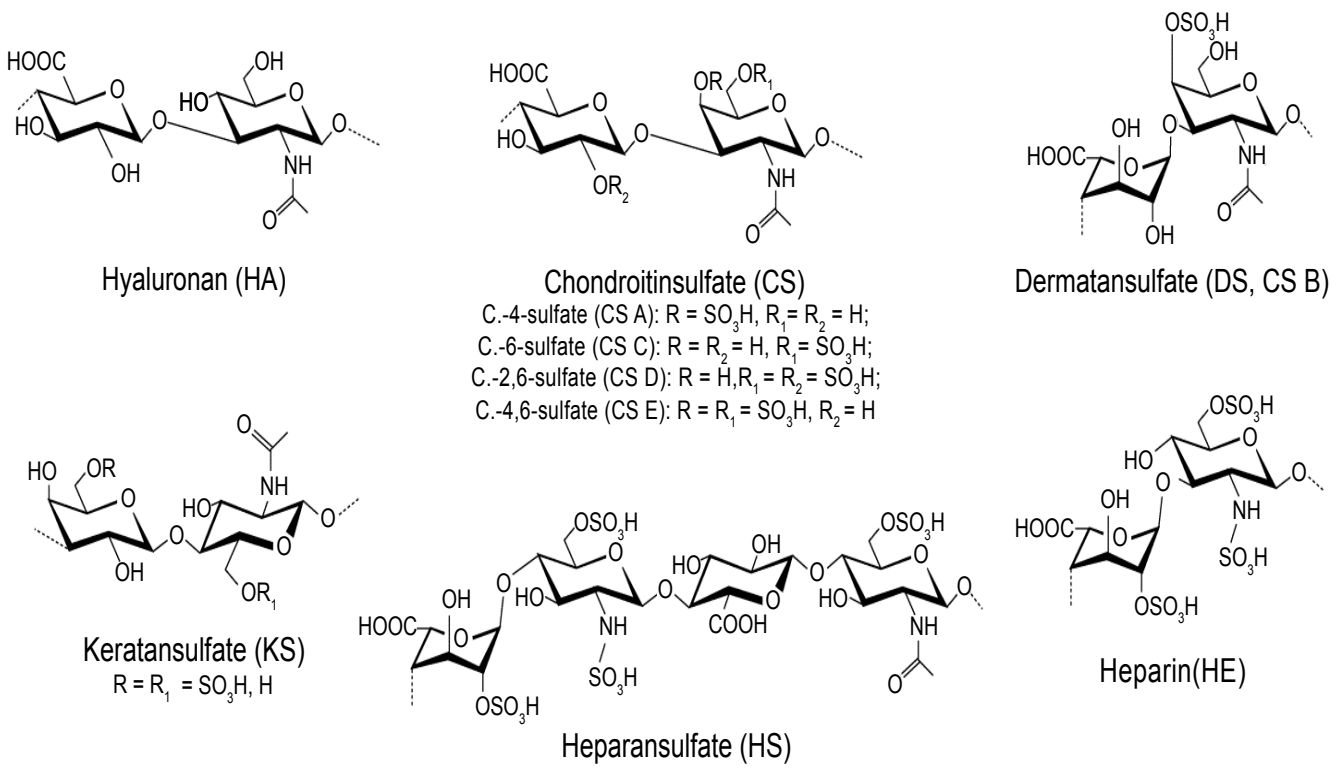

Figure 1: Di- and tetrasaccharide repeating units of the most important natural GAGs. In dependence on the biological source of the GAGs, structural variations may occur to a minor extent (e.g. differences of the sulfation patterns) in low-sulfated GAGs (CS, DS, KS) but to a larger extent (variations of monosaccharides in the repeating unit) in high-sulfated HE and HS. The "free" acids are given in all cases although the occurrence of the corresponding (sodium) salts is much more probable at physiological conditions. Please note that the different commercially available CS preparations are sorted according to their sulfation patterns.

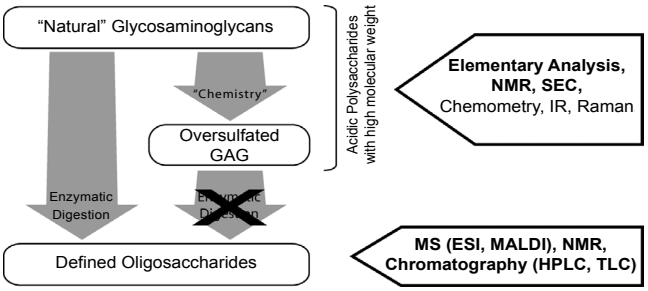

Figure 2: Survey of analytical methods which are typically used to characterize GAGs. Please note that not all the indicated methods are applicable to the poly- and larger oligosaccharides. However, the enzymatic digestion of the polysaccharides into defined oligosaccharides is very difficult if chemically modified (in particular, oversulfated) GAGs are of interest. "Chemometry" indicates methods which allow the determination of a dedicated chemical parameter, for instance, the number of reducing endgroups. Abbreviations: ESI, electrospray ionization; HPLC, high-performance liquid chromatography; IR, infrared; MALDI, matrix-assisted laser and ionization; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; SEC, size exclusion chromatography; TLC, thin-layer chromatography.

in different positions) can be easily separated by chromatographic methods and subsequently characterized by MS.

This approach works perfectly when natural GAGs with a moderate extent of sulfation are of interest: the *m/z* ratio (and, thus, the molecular weight) gives an information about the overall content of sulfate residues and the position of the sulfate residues can be conveniently determined by MS/MS (or when required MS^n) since some fragmentation products are exclusively detectable when the sulfate residue is located in a specific position [14]. In particular difficult cases and severe assignment problems, there are also enzymes (such as sulfatases) or even chemicals (such as N-methyl-N-(trimethylsilyl) trifluoroacetamide (MTSTFA)) available which allow the regioselective desulfation of GAG oligosaccharides [15].

Unfortunately, all enzymes required for the degradation of the polysaccharides are inhibited by oversulfated GAGs and are, thus, not capable of digesting chemically modified GAGs with an excess of sulfate residues and/or an unusual sulfation pattern. Although this lacking digestibility represents an important application advantage since the biological life time of these GAGs is prolonged at these conditions, this is a serious problem for the analysis of such chemically modified GAGs: even if GAGs can be also depolymerized by chemical agents such as moderately concentrated acids or ROS, chemical degradation is always accompanied by site reactions (particularly cleavage of the sulfate ester and/or the amide linkage) [16] which aggravate subsequent analysis. Although promising methods have been recently established to improve the digestibility of chemically modified GAGs [17] much more research interest should be paid - at least in our opinion - to this very important topic and particularly the development of enzymes capable of cleaving chemically modified GAGs into defined oligosaccharides. Otherwise, biological effects of chemically modified GAGs can be easily misinterpreted!

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