

## **Impending challenges in glycosaminoglycan characterization**

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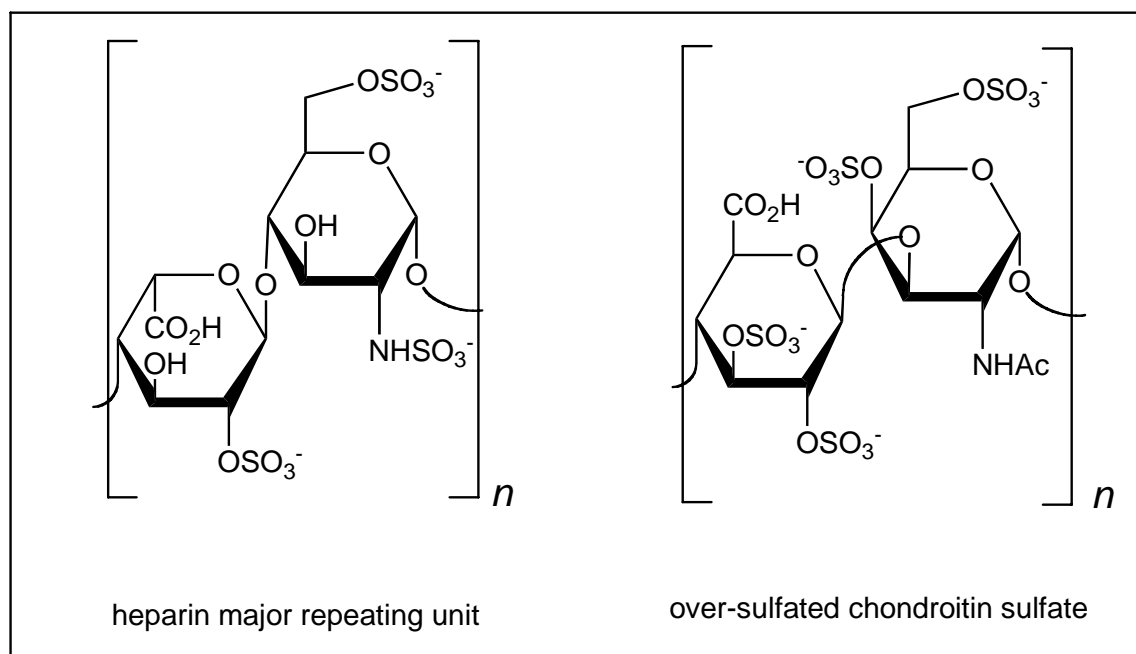
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Glycosaminoglycans (GAGs) are major components of the extracellular matrix surrounding many mammalian cell types; they also exist in cell free suspension where they serve a variety of roles in modifying enzyme function and dictating physical properties of essential biological fluids (Linhardt and Toida 2004). Structurally, they are long, unbranched, negatively charged, polydisperse polysaccharides (the most abundant polysaccharides in the body) made up of repeating disaccharide units. The disaccharide units are generally composed of a hexosamine (*N*-acetylgalactosamine or *N*-acetyl (sulfo) glucosamine) alternating with an uronic acid (such as glucuronate or iduronate). Superimposed on the polysaccharide backbone are complex patterns of amido (*N*) or ester (*O*)-linked sulfo group substitutions. There are various differences in saccharide units, chain length and sulfation patterns between the different classes. These subtle differences create great structural diversity within the GAGs, which underpins their functional diversity, and presents an enormous challenge for structure elucidation of these complex molecules. However, significant advances have been made in analytical technologies in recent years, and this trend looks set to continue in the future.

The challenges in characterization of these structurally diverse macromolecules were highlighted recently because of the contamination of a world-wide supply of raw material for the preparation of clinical of heparin and low molecular weight heparins that resulted in several deaths and the withdrawal of products from the market by major manufacturers (World Health Organization alert #118) (Guerrini *et al.* 2008). Heparin is a highly sulfated excreted polymer made up of primarily alternating *N*-sulfolucosamine units and iduronic or glucuronic acid (Figure 1).



It is widely used as an anticoagulant in the treatment of acute coronary conditions. The nature of the contamination was eventually identified, but only as a result of the application of a number of advanced analytical techniques seldom if ever used in the quality control of this important biomedical product (Guerrini *et al.* 2008). At the height of efforts to complete this characterization, the authors of this commentary were scheduled to speak at a symposium hosted by the Complex Carbohydrate Research Center of the University of Georgia entitled “**Low Molecular Weight Heparins to Proteoglycans**”. A panel discussion was included at these

proceedings where it became possible to summarize the outcomes of this characterization and highlight methodologies that could impact GAG characterization in the future. We present the results of this discussion here.

The discussion began with a summary of the current understanding of the origin of the contamination, its identification, and suggestions of any new tests that might be adopted by the FDA and other agencies that would avoid future contaminations. Dr. Linhardt suggested that the FDA will probably revise the tests that are required on the starting material and suggested that criteria based on NMR and separation chemistry will likely be defined for testing the active pharmaceutical ingredient prior to its formulation into a drug product. The structure of the contaminant, identified in part through NMR methods, is an over-sulfated chondroitin sulfate, which comes from a chemically sulfonated cartilage-derived chondroitin sulfate, a less expensive feedstock than heparin. All sources of the heparin contamination, which ranged from 1-25%, have been traced to China. Over-sulfated chondroitin sulfate, a product of China, has no known applications. Over-sulfated chondroitin sulfate is produced in China for purposes that are not still clear.

The discussion then continued by addressing the challenges in GAG structural elucidation, and the feasibility of what may be called "GAGomics" using mass spectrometry. A useful observation is that it is probably not always necessary to characterize all the oligosaccharides in a GAG mixture. One can have a targeted approach by analyzing a pool of oligosaccharides after some kind of affinity chromatography to enrich for an active fraction, or a set of oligosaccharides separated and selected by bioassay screening (Thompson *et al.* 2007). It is now possible to separate and analyze GAG oligosaccharides using online ESI-MS/MS experiments using amide or reversed-phase ion-pairing chromatography, which should make it possible to characterize the structure of the oligosaccharides within the active fraction of interest (Naimy *et al.* 2008). Although the GAG oligosaccharides have great heterogeneity, it should still be feasible to obtain detailed structural information using both low-resolution mass spectrometry (ion-trap and Q-TOFs) (Saad *et al.* 2005) and the high-resolution (FTICR) mass spectrometers (Schenauer *et al.* 2007). New analytical methods that afford information on epimerization status and sulfation at certain positions are now becoming available and certainly will receive more attention in the future (Wolff *et al.* 2007). It is also important not to ignore other GAG products like low molecular weight heparins that pose challenges due to chemical modifications at their non-reducing end and 1,6-anhydro formation at their reducing terminus. The structural assignment and quantitation of these modifications probably requires orthogonal methods and could not be achieved by MS techniques alone. An attempt was made to estimate the size of oligosaccharide that is now within the reach of detailed analysis and project what size will be routinely accessible in the foreseeable future. The authors believe that tetrasaccharides (4 sugar units) are the size that is easily analyzed right now using both high resolution and low-resolution MS techniques. Tetrasaccharide mixtures containing dozens of different species can be analyzed. At the moment, higher resolution MS techniques are required for hexasaccharides and octasaccharides (6 and 8 sugar units, respectively); however, it is likely that such analyses will be more routinely accessible in the next two years.

A note of caution highlighted in the discussion was that focusing simply on analysis of purified or synthesized oligosaccharides may not be useful in all situations, because it appears that GAGs

are often active as mixtures and individual oligosaccharides may be unable to exhibit the same complex pharmacological activities as polycomponent drugs like heparin and low molecular weight heparins. This is the result of the heterogeneous nature of GAGs and their participation in many different types of complex interactions.

It was readily apparent from the discussion that there is reason to be optimistic about improved analysis of GAGs in the future. This view is based on considerable improvements in both NMR and mass spectrometry technologies in recent years. Also, significant advances have been made towards developing an understanding of the underlying biology of GAGS, and their structure-function relationships. This trend is expected to continue, permitting the challenging problem of GAGs characterization to be addressed, and opening a wealth of opportunities for exploiting this understanding in the pharma and biotech arenas.

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