

MASS SPECTROMETRY FOR GLYCOFORM ANALYSIS OF GLYCOSAMINOGLYCANS: A NEW APPROACH TO AN OLD PROBLEM

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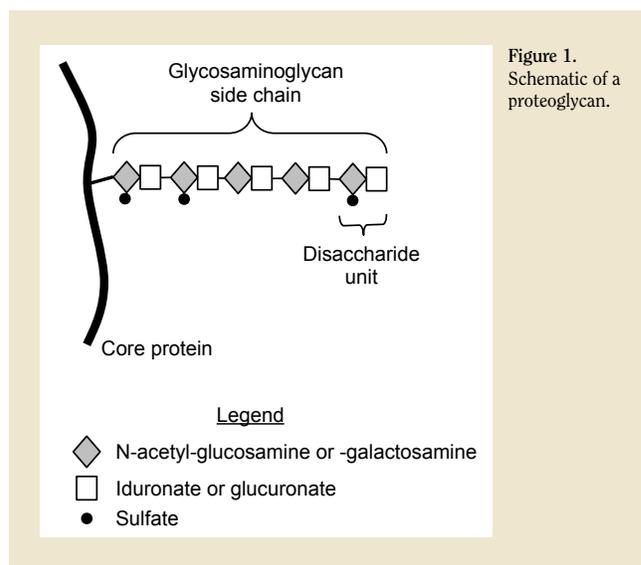
INTRODUCTION

Interplay between protein and carbohydrate components in the extracellular matrix sustains the unique biomechanical properties of cartilage. Long-chain sugars known as glycosaminoglycans (GAGs) are enmeshed within a collagen lattice. Water is attracted to the highly-6sulfated GAG chains and causes them to swell. Because GAGs are constrained within a collagen lattice, the swelling generates hydrostatic pressure that enables cartilage to bear loads. Loss of GAGs from the matrix, as well as changes in composition or sulfation, may set the stage for degeneration of cartilage tissue.

Technical constraints of current analytical methods limit our knowledge of GAG structures (glycoforms) in normal cartilage and the changes that occur with pathologic conditions. Most methods require complete digestion of GAGs into disaccharides prior to analysis. The abundance of different disaccharide forms can be measured, but information on their arrangement (patterning) is lost. New approaches are needed to obtain quantitative information on patterning and other structural variables. The objective of this interdisciplinary collaboration is to adapt novel, mass spectrometry-based platform technologies to analyze the fine structure of GAGs from human cartilage.

GLYCOSAMINOGLYCAN STRUCTURE

Proteoglycans are comprised of one or more GAGs attached to a protein core (Figure 1). Subtypes of GAGs such as keratan sulfate (KS) or chondroitin sulfate (CS) contain characteristic combinations of disaccharide units. Additional structural diversity in GAGs is generated by sulfate modification and epimerization of uronic acid within the disaccharides. Chondroitin sulfate and dermatan sulfate are closely related in structure; dermatan sulfate is distinguished by having disaccharide repeats that contain iduronic acid, rather than glucuronic acid. The components of disaccharides may be sulfated at different locations,



such as the 4 position in galactosamine (chondroitin 4-sulfate, or chondroitin sulfate type A) or at the 6 position (chondroitin 6-sulfate, or chondroitin sulfate type C).

Changes in cartilage GAG composition and sulfation occur during normal development and with disease. For example, the number of KS chains on aggrecan increases after the age of ~20 years^{1,2}, and there is a shift in the relative amounts of chondroitin 4-sulfate and chondroitin 6-sulfate³⁻⁵. Changes that occur in osteoarthritic (OA) cartilage may be distinct from aging, such as a decrease in KS⁶ and altered sulfation at terminal residues⁷. With current analytical methods that require complete digestion of GAGs into disaccharides, much of the information on structural variables is destroyed. That information could be retained, however, by using methods that are able to distinguish larger oligosaccharides (Figure 2).

A MASS SPECTRAL APPROACH TO ANALYZE GLYCOSAMINOGLYCAN STRUCTURE

In mass spectral analysis, different chemical structures display characteristic peaks of ion abundance (i.e., diagnostic ions). Specific structures can be identified and quantified by measuring the abundance of diagnostic ions. Several types of mass spectral approaches have been developed for analysis of biomolecules, including proteins and DNA.

Dr. Zaia's group at the Mass Spectrometry Resource, Boston University School of Medicine proposed that a combination of liquid chromatography (LC) and tandem mass spectral analysis (MS/MS) could be used for direct analysis of oligosaccharides, to obtain patterning information without additional purifica-

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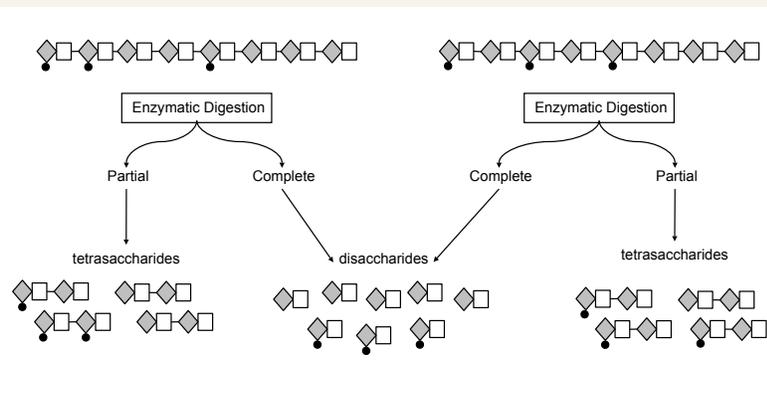


Figure 2. Partial digestion of glycosaminoglycans preserves structural information. In this example, two different GAGs subjected to complete digestion yield the same disaccharides. In contrast, partial digestion yields tetrasaccharides that are distinguished by the pattern of sulfated residues.

tion and digestion steps^{8,9}. The determination of chondroitin sulfate sulfation using disaccharide analysis is a well-established method. Determination of dermatan sulfate content, however, requires multiple steps separate from disaccharide analysis. Dr. Zaia's group has shown that analysis of tetramer oligosaccharides of cartilage CS using mass spectrometry determines sulfation and epimerization simultaneously. This approach was validated through a series of experiments measuring CS glycoforms in dermatan sulfates from porcine skin, decorins from three different tissues (articular cartilage, sclera, and cervix), and cartilage extracts^{8,10}. The next step was to apply this novel methodology to GAGs from tissue samples.

APPLICATION OF THE MASS SPECTRAL APPROACH TO CARTILAGE TISSUE

The biology of cartilage presents technical challenges for analysis of GAGs by any method. Regional variations in the tissue (especially with degeneration and disease) impede characterization within subtle or focal alterations. Analysis by LC-MS/MS is additionally challenging because of the amount and purity of starting material required. Articular cartilage excised from the shoulder joint of a juvenile calf was used in

a series of experiments to begin addressing these issues.

Initially, we attempted to use standard procedures to extract GAGs from intact tissue for mass spectral analysis. Cartilage was digested with papain and CS oligosaccharides were prepared by standard methods. Mass spectra of those samples, however, showed a high level of noise, and the CS diagnostic ion peak at 458 *m/z* was not discernable. In a series of optimization experiments¹¹, we successfully developed a modified sample workup procedure that produces high-quality analytical data on GAGs from cartilage tissue (Figure 3). Glycoform abundances in CS were then measured with that optimized sample workup. In a sample of articular cartilage from a young calf, 57.9%

of tetrasaccharides were chondroitin 4-sulfate-like, 40.1% were chondroitin 6-sulfate-like, and 2% were dermatan sulfate-like. To validate those results, capillary electrophoresis (CE) was used to measure disaccharides in the same sample. The measured amounts of 4-sulfated (57.5%), 6-sulfated (39.1%) and unsulfated disaccharides (3.4%) were in good agreement with the glycoform data obtained by LC-MS/MS.

GLYCOFORM ANALYSIS OF HUMAN CARTILAGE

Reproducibility of glycoform measurements with the LC-MS/MS platform was evaluated with human articular cartilage samples. Three explants of normal-appearing tissue were excised from a tibial plateau that was discarded during total knee arthroplasty for osteoarthritis. Triplicate aliquots of each papain-digested explant were subjected to the optimized sample workup procedure for mass spectral analysis. Measurement of CS glycoforms was highly reproducible and again showed good agreement with disaccharide compositions measured by CE (Table 1). The low abundance of chondroitin 4-sulfate glycoforms measured in those samples was consistent with published data for adult cartilage.

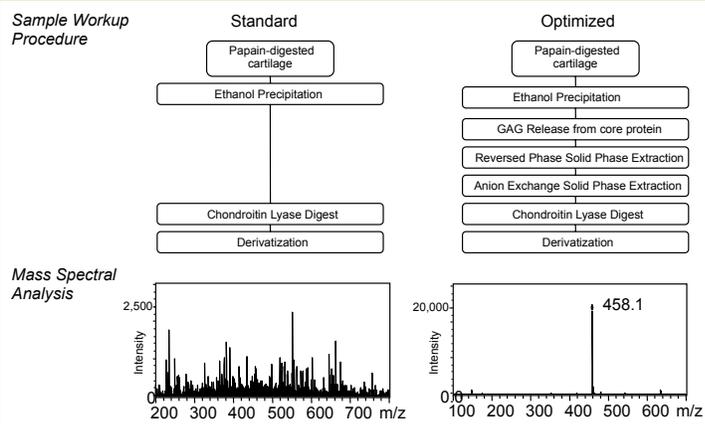


Figure 3. Improved sample workup procedure to prepare chondroitin sulfate from intact tissues for mass spectral analysis. Samples prepared with standard protocols have contaminants that mask the CS diagnostic ion peak at 458 *m/z*. Those contaminants are removed by additional steps in the optimized sample workup procedure.

A larger set of samples obtained from 5 donors (13 explants, weighing 16-60 mg) was analyzed to determine the variance of glycoform measurements. In that group of samples, the mean abundance of chondroitin 6-sulfate-like tetrasaccharides was 90.4% ± 3.2 and chondroitin 4-sulfate-like was 8.5% ± 3.1. Those quantities were similar to our other results with adult cartilage. Variance between triplicate aliquots that were analyzed for each sample was very low for chondroitin 6-sulfate-like tetrasaccharides (coefficient of variation = 1.9%). For chondroitin 4-sulfate like tetrasaccharides, variance was greater (21.5%) and was likely due to the low abundance of that glycoform. Nonetheless, these results show that the LC-MS/MS approach is a sensitive, accurate method to quantify glycoform structures from as little as 16 mg of cartilage tissue.

Explant No.	Glycoform Abundance (% of total)			Disaccharide Abundance (% of total)	
	Chondroitin 4-sulfate-like	Chondroitin 6-sulfate-like	Dermatan sulfate	4-sulfated disaccharides	6-sulfated disaccharides
1	6.3	93.1	0.7	8.7	91.3
2	6.1	92.8	1.2	9.9	90.2
3	6.3	93.2	0.6	9.0	91.0

Table 1. Measurement of chondroitin sulfate glycoforms (by mass spectral analysis) and disaccharides (by capillary electrophoresis) in replicate explants from adult human articular cartilage.

THE NEXT STEPS

This work demonstrates the feasibility of mass spectral approaches for glycoform analysis of GAGs. At this stage, the methodology is highly sensitive and requires just 10 µg of GAGs from each sample. That threshold is expected to decrease as more powerful instrumentation is developed. Another benefit that will come with enhanced sensitivity is the potential to quantify rare structures that are not detectable by other means. Ultimately, the goal is to develop a true “glycomic” approach for simultaneous analysis of multiple GAGs. These methods will

uncover new insights into the structure-function relationships of GAGs in cartilage.

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References

1. **Maroudas A, Bayliss MT, Venn MF.** Further studies on the composition of human femoral head cartilage. *Ann Rheum Dis* 1980;39(5):514-23.
2. **Venn MF.** Variation of chemical composition with age in human femoral head cartilage. *Ann Rheum Dis* 1978;37(2):168-74.
3. **Cheng F, Heinegard D, Fransson L, Bayliss M, Bielicki J, Hopwood J, Yoshida K.** Variations in the chondroitin sulfate-protein linkage region of aggrecans from bovine nasal and human articular cartilages. *J Biol Chem* 1996;271(45):28572-80.
4. **Lauder RM, Huckerby TN, Brown GM, Bayliss MT, Nieduszynski IA.** Age-related changes in the sulphation of the chondroitin sulphate linkage region from human articular cartilage aggrecan. *Biochem J* 2001;358(Pt 2):523-8.
5. **Plaas AH, Wong-Palms S, Roughley PJ, Midura RJ, Hascall VC.** Chemical and immunological assay of the nonreducing terminal residues of chondroitin sulfate from human aggrecan. *J Biol Chem* 1997;272(33):20603-10.
6. **Aigner T, Hemmel M, Neureiter D, Gebhard PM, Zeiler G, Kirchner T, McKenna L.** Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritis human articular knee cartilage: a study of proliferation, programmed cell death (apoptosis), and viability of chondrocytes in normal and osteoarthritic human knee cartilage. *Arthritis Rheum* 2001;44(6):1304-12.
7. **Plaas AH, West LA, Wong-Palms S, Nelson FR.** Glycosaminoglycan sulfation in human osteoarthritis. Disease-related alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J Biol Chem* 1998;273(20):12642-9.
8. **Hitchcock AM, Costello CE, Zaia J.** Glycoform quantification of chondroitin/dermatan sulfate using a liquid chromatography-tandem mass spectrometry platform. *Biochemistry* 2006;45(7):2350-61.
9. **Zaia J, Li XQ, Chan SY, Costello CE.** Tandem mass spectrometric strategies for determination of sulfation positions and uronic acid epimerization in chondroitin sulfate oligosaccharides. *J Am Soc Mass Spectrom* 2003;14(11):1270-81.
10. **Miller MJ, Costello CE, Malmstrom A, Zaia J.** A tandem mass spectrometric approach to determination of chondroitin/dermatan sulfate oligosaccharide glycoforms. *Glycobiology* 2006.
11. **Hitchcock AM, Shortkroff S, Yates KE, Costello CE, Zaia J.** Optimized extraction of glycosaminoglycans from cartilage for disease state glycomics.; 2006; Seattle, WA.