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Analysis of Glycosaminoglycans Using Mass Spectrometry

Gregory O. Staples and Joseph Zaia*

Center for Biomedical Mass Spectrometry, Dept. of Biochemistry, Boston University School of Medicine

Abstract

The glycosaminoglycans (GAGs) are linear polysaccharides expressed on animal cell surfaces and in extracellular matrices. Their biosynthesis is under complex control and confers a domain structure that is essential to their ability to bind to protein partners. Key to understanding the functions of GAGs are methods to determine accurately and rapidly patterns of sulfation, acetylation and uronic acid epimerization that correlate with protein binding or other biological activities. Mass spectrometry (MS) is particularly suitable for the analysis of GAGs for biomedical purposes. Using modern ionization techniques it is possible to accurately determine molecular weights of GAG oligosaccharides and their distributions within a mixture. Methods for direct interfacing with liquid chromatography have been developed to permit online mass spectrometric analysis of GAGs. New tandem mass spectrometric methods for fine structure determination of GAGs are emerging. This review summarizes MS-based approaches for analysis of GAGs, including tissue extraction and chromatographic methods compatible with LC/MS and tandem MS.

Keywords

Mass spectrometry; glycosaminoglycan; proteoglycan; heparin; heparan sulfate; chondroitin sulfate; dermatan sulfate; hyaluronan; keratan sulfate

Introduction

Interest in the structure of GAGs is driven by their biology relevant to human health. The expression of GAG classes is required for embryogenesis (1) and for normal functioning of nearly every adult physiological system (2). GAGs are expressed on cell surfaces and in extracellular matrices in a spatially and temporally regulated manner so as to modulate the functions of the proteins to which they are attached (3). It is now appreciated that GAG expression plays important roles in development (1, 4, 5), pathogenesis (6, 7), anticoagulation (8, 9), metastasis (10–13), homeostasis (14), and angiogenesis (15). Key to the understanding of the diverse glycobiology of the GAG classes is the ability to determine their structures related to normal and disease phenotypes. Mass spectrometric methods have been developing rapidly over the past few years, to the point that large scale glycomics

*Corresponding author: Center for Biomedical Mass Spectrometry, 670 Albany St., Rm. 509, Boston, MA, 02118, USA, (v) 1-617-638-6762, (f) 1-617-638-6761, jzaia@bu.edu.

experiments are now possible. This review summarizes MS-based analytical methods for GAGs and their applications in biomedicine.

Glycosaminoglycan structure

Hyaluronan

As shown in Figure 1, hyaluronan (HA) consists of repeating disaccharide units of $[4\text{GlcA}\beta 1-3\text{GlcNAc}\beta 1-]_n$ and serves important structural roles in extracellular matrices. It is unique among animal carbohydrates because it is biosynthesized by extrusion from the plasma membrane (16). Unlike other GAG classes, it is not covalently linked to core proteins during biosynthesis. A number of extracellular matrix proteins contain lectin domains that bind HA. As a result, HA serves as a molecular tether that organizes arrays of proteins at the cell surface. Intact HA polymers range up to several megadaltons in size, and cleavage products are found in a number of biological contexts. Thus, molecular weight determination is a key aspect of analysis of HA structure. HA may also become covalently attached to proteins in the blood serum related to several diseases (17, 18) and analysis of such complexes is thus of interest.

Keratan sulfate

As shown in Figure 1, keratan sulfate (KS) consists of repeating disaccharide units of $[3\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-]_n$ that may be sulfated at the 6*O*-positions of Gal and GlcNAc. KS may be viewed as a sulfated neo-*N*-acetylactosamine extension that occurs on either *N*-linked or *O*-linked glycan classes (19). KS proteoglycans in extracellular matrices serve in roles related to tissue hydration and collagen fibril structure.

Chondroitin/dermatan sulfate

As shown in Figure 1, chondroitin sulfate (CS) consists of repeating units of $[4\text{GlcA}\beta 1-3\text{GalNAc}\beta 1-]_n$. The chains are attached to Ser via the tetrasaccharide linker $\text{GlcA}\beta 1-3\text{Gal}\beta 1-3\text{Gal}\beta 1-4\text{Xyl}\beta 1-$. Sulfation may occur at the C2 position of GlcA, and the C4 and C6 positions of GalNAc. CS with a high content of 4*O*-sulfated GalNAc is known as CS type A (CSA), that with a high content of 6*O*-sulfated GalNAc as CS type C (CSC). Dermatan sulfate (DS) is a sub-class of CS in which GlcA has been enzymatically epimerized during biosynthesis to IdoA and is also known as CS type B (CSB). The extent to which uronic acid residues are epimerized to form DS-like sequences varies among different tissues. IdoA residues are often found adjacent to a 4*O*-sulfated GalNAc residue. CS is abundant in cartilage and other extracellular matrices where it provides swelling pressure necessary for viscoelasticity (20). CS/DS also interacts with growth factors, including the fibroblast growth factor (FGF) family and others, modulating their bioavailability for signaling (21–23). The sulfation patterns of CS/DS change during development and with progression of osteoarthritis (24).

Heparan sulfate

As shown in Figure 1, heparan sulfate (HS) is biosynthesized as a repeating unit of $[4\text{GlcA}\beta 1-4\text{GlcNAc}\alpha 1-]_n$ that is subsequently modified by a series of enzymes in the Golgi apparatus. The chains are linked to Ser residues via the same tetrasaccharide linker as

CS/DS. The chain is first subjected to the activity of *N*-deacetylase/*N*-sulfotransferases that replace a subset of Ac groups with sulfate groups. The extent of these modifications defines the overall domain structure that characterizes HS chains in a given biological environment. Domains containing *N*-sulfated GlcN may be acted upon by a uronic acid epimerase and a series of *O*-sulfotransferases. Mature chains may be modified at the 2*O*-position of IdoA, the 6*O*- and 3*O*-positions of GlcNS/GlcNAc. The structure of HS consists of a regulated domain structure over which is superimposed a degree of heterogeneity. This gives rise to cell-type and temporally regulated diversity of HS structure and function (25). Heparin is a variant of HS, which has [4IdoA2S α 1-4GlcNS6S α 1-]_n, where S = sulfate, as the most abundant repeating disaccharide unit.

HS chains modify proteoglycans found on cell surfaces (for example syndecans and glypicans) and in extracellular matrices (for example perlecan). They bind many families of growth factors and growth factor receptors (26) and serve as cell surface co-receptors (27). The wide variety of growth factors to which HS binds and the importance of these interactions in developmental and adult physiological processes drives the need for effective analytical methods for analysis of this compound class.

Extraction of GAGs from tissue for mass spectrometric analysis

Because the structure and functions of GAGs are regulated in a spatial and temporal manner, it is essential to be able to extract GAGs from small quantities of biological tissue. Methods used for extraction of GAGs from tissue that are appropriate for chromatographic analysis (28, 29) need to be validated because they may produce unacceptable chemical noise background when mass spectrometry is used. Therefore, tissue extraction methods demonstrated to be appropriate for mass spectrometric analysis will be summarized here.

Classically, GAGs are extracted from tissue using guanidine hydrochloride and other chaotropic agents in combination with non-specific proteases (28, 30). The bond between the Xyl residue of the tetrasaccharide linker and Ser residue of the protein backbone is typically cleaved using alkaline β -elimination under reducing conditions, so as to prevent oligosaccharide peeling (31). Following release, the GAG chains are often enriched using anion exchange chromatography or with solvent precipitation.

For extraction of CS/DS from connective tissue, a procedure employing papain digestion, alkaline borohydride release, solid phase extraction, and ethanol precipitation has been found to eliminate contaminants causing high mass spectral background (32, 33). HS may be extracted for mass spectral analysis from homogenized and pronase digested tissue using anion exchange and size exclusion cartridges (34). KS oligosaccharides have been analyzed directly from frozen tissue sections by outlining a region with a hydrophobic pen and digesting the area with keratanases before subsequent ESI MS analysis (35, 36).

Analysis of GAGs using MALDI mass spectrometry

Highly sulfated GAG oligosaccharides were shown in the 1990s to produce weak signals and abundant losses of SO₃ due to in-source dissociation (37, 38). It was shown at that time that pairing of GAG oligosaccharides with a basic peptide enables detection of a complex

without losses of SO₃, thus allowing the determination of the oligosaccharide molecular weight. This approach was used in a series of biochemical studies on HS (9, 39–42) and formed the basis of a method for sequencing the oligosaccharides using a series of enzymatic and chemical modifications (43). Subsequent effort has focused on development of MALDI matrix conditions sufficient to improve the signal strength and eliminate the in-source dissociation problem without the need to add basic peptides. A number of studies have been published toward these ends, with improvements in signal but with the same problems of dissociative losses of sulfate groups during the ionization process (44–48). The ion abundances of CS disaccharides produced by exhaustive enzymatic depolymerization have been used in a quantitative assay in which a linear response is observed over a range of 4–40 pmol of disaccharide on the MALDI target. Derivatization of KS oligosaccharides with pyrenebutyric acid hydrazide has been used to increase their hydrophobicity and detectability using MALDI-TOF MS. Hyaluronan degree of polymerization (dp) 4–24 have been characterized using MALDI-TOF in a study that found that the strongest signals were obtained when the oligosaccharides were methyl esterified (49). Additionally, MALDI has been used to quantify hyaluronan oligosaccharides with a reported linear response between 40 fmol and 800 fmol (50). MALDI-TOF MS has also been used to characterize monodisperse hyaluronan oligomers synthesized using an immobilized enzyme reactor (51).

Analysis of GAGs using ESI mass spectrometry

ESI is well suited to analysis of GAGs due to the soft nature of the ionization process (52). As a result, GAGs may be ionized directly using negative polarity ESI without adding ion pairs to stabilize sulfate groups. It is generally advised that investigators use a standard molecule, such as the commercially available octasulfated pentasaccharide Arixtra, to test the source conditions of the mass spectrometer. Arixtra is representative of the most highly sulfated GAG oligosaccharides, and it may be considered an effective performance test for ion source settings. Once source conditions are set correctly, the extent of sulfate losses that occur during ionization will be minimal.

GAG disaccharide analysis

Direct infusion ESI has been used to quantify GAG disaccharide mixtures, with collisional activated dissociation (CAD) tandem MS used to differentiate positional isomers (53–56). Sample quantities may be minimized using a nano-ESI interface, such as has recently been demonstrated for CS and DS derived from neural tissue (57). A reversed phase ion pairing (RPIP) LC/MS system (*vide infra*) has been used to quantify reductively aminated GAG disaccharides (58, 59). This approach utilizes a stable isotope labeled variant of the reductive amination tag to insure accuracy of quantification. A size exclusion chromatography (SEC) LC/MS platform has been used to quantify HS disaccharides extracted from tissue with on-line tandem MS to resolve structural isomers (34). This approach quantifies saturated disaccharides derived from the non-reducing end of the parent HS chain and thus enables determination of average chain length. A set of ¹³C/¹⁵N-labeled HS disaccharides has been synthesized and used as internal standards for quantitative analysis using LC/MS (60).

GAG oligosaccharide analysis

Partial depolymerization of GAG chains using chemicals or enzymes is an effective means of producing oligosaccharides that are amenable to MS analysis. A number of bacterial GAG polysaccharide lyases (61) are available commercially and are widely used. The digestion products of these enzymes are mixtures that contain a complex distribution of oligosaccharides, necessitating the use of separations prior to MS analysis. Several LC approaches have been developed for this purpose, as summarized below and in Table 1.

Reversed phase LC/MS—The hydrophobicity of GAG saccharides may be increased to enable their binding to reversed phase columns using a variety of reducing end labels. Generally speaking, as the size of the glycan increases, the interaction with the reversed phase matrix will decrease. As a result, this approach is most applicable to relatively short GAG oligomers. Derivatization with 1-phenyl-3-methyl pyrazolone (PMP) has been used for LC/MS of GAG metabolites from biological fluids (62, 63). After chemical modification, the samples are solvent extracted and analyzed using off-line or on-line ESI MS (64). The advantage to this method is the minimal sample manipulation required in order to quantify GAG metabolites. Derivatization with PMP is also an effective means of derivatization of HS and DS oligosaccharides from urine of mucopolysaccharidosis patients (65). This approach was used to develop multiple reaction monitoring methods for quantification of PMP-oligosaccharides. The LC/MS-MRM data allowed a cohort of patient urine samples to be discriminated from those of healthy control subjects.

Reversed phase ion pairing (RPIP LC/MS)—RPIP (66) entails addition of millimolar concentrations of an amine to the LC mobile phase to cause pairing of alkyl ammonium cations with the negatively charged GAG oligosaccharides so as to facilitate their interaction with a stationary reversed phase column (67). The amine reagent must be sufficiently volatile to be compatible with ESI MS while providing sufficient hydrophobicity for interaction with the stationary reversed phase. Dibutylamine has been shown to be effective for pairing with GAG oligosaccharides, and unsulfated heparosan oligomers up to dp40 were detected (68). An RPIP LC/MS system using tributylamine was effective for separating heparin oligosaccharides from dp2–20, clearly demonstrating the high chromatographic resolution of this approach (69). An RPIP LC/MS method using tripropylamine was used to separate a partially depolymerized heparin oligosaccharide mixture with >200 components (70). In this case, an on-line ion suppressor was used to remove the amine prior to the MS ion source. Recently, a microflow RPIP system was developed for quantification of HS disaccharides in which isotopically enriched disaccharides were used as internal standards (60). Ultra performance RPIP LC/MS has also been applied to HS disaccharide analysis and the results show analysis times as short as 5 min and chromatographic resolution of disaccharide anomers was observed (71, 72). The RPIP LC/MS approach has been used to characterize GAGs expressed in animal species throughout the evolutionary tree (58, 73). A set of detailed protocols for extraction of GAGs from animal tissue, bacterial cells, enzymatic depolymerization methods, and RPIP LC/MS has recently been published (74).

Hydrophilic interaction chromatography (HILIC) LC/MS—HILIC entails use of a polar stationary phase and a mobile phase gradient in which water serves as the elutropic

solvent (75). Capillary scale LC/MS using an amide-silica stationary phase has been demonstrated for *N*- and *O*-linked glycans, glycopeptides, and GAGs (32, 76–78). The typical mobile phase uses an ammonium formate modifier that is MS compatible. HILIC LC/MS has been used in the analysis of CS/DS oligosaccharides from connective tissue (32) and antithrombin-binding heparin hexamers (79). This chromatography mode has also been adapted for use with a chip-based LC/MS interface (80). Improved negative ion ESI performance was observed using an acetonitrile makeup flow (81), permitting quantification of heparin oligosaccharides up to dp18 in size.

Size exclusion chromatography—SEC has the advantage of its universal separation mechanism and is therefore applicable to GAGs on account of their free solubility in aqueous solvents. SEC LC/MS has been used to analyze mixtures of partially depolymerized CS oligosaccharides, enabling detection of oligosaccharides up to dp14 (82). This approach was also used in combination with on-line tandem MS for determination of sulfation and epimerization states for CS/DS extracted from connective tissue (83, 84). The sensitivity of SEC is limited because this chromatography mode cannot be scaled down. Despite this, it was possible to produce data for 10 microgram quantities of CS/DS extracted from tissue. An SEC LC/MS platform has been used for LC/MS and LC/tandem MS of HS disaccharides (34). An SEC LC/MS system can be fitted with an on-line ion suppressor to reduce chemical noise background from ammonium salts. This approach has been used for the analysis of low molecular weight heparins of biomedical interest (85).

Graphitized carbon chromatography (GCC) LC/MS—GCC produces very high resolution chromatographic separation for glycans and can withstand a wide range of pH, chemical, and physical conditions (86). The retention mechanism results from a combination of the polarizability of the graphitized carbon in interacting with polar analytes and its ability to bind planar molecules. Retention of oligosaccharides increases with molecular weight and the number of acidic residues. GCC is well suited for use in LC/MS (87) due to the relatively low concentration of additives required for effective chromatography and has been adapted for nanoscale LC/MS (88, 89). GCC necessitates reduction of released glycans to prevent chromatographic splitting of reducing end anomers.

Negative ion GCC LC/MS has been used to analyze enzymatic digests of GAGs including hyaluronan, KS, heparin and HS (90). For this work, the GCC column was operated at relatively high pH using an ammonium bicarbonate modifier. A similar LC/MS approach was used to analyze CS released by in-gel polysaccharide lyase digestion of aggrecan samples (91, 92). The GCC stage allowed separation of isomeric disaccharides and tandem MS was used to assign sulfation positions. An acidic mobile phase has been used for LC/MS disaccharide analysis of CS disaccharides from lung tissue and bronchoalveolar lavage fluid (93). No reduction was used and the chromatography separated the anomeric forms with identification of sulfation positions based on retention times.

Multidimensional LC separations combined with MS—Analysis of GAG structural isomers is of considerable interest, owing to the prevalence of isomeric mixtures in biological systems. HS chains are expressed in domains of high and low degree of sulfation, respectively. Heparin lyase III deolymers the low degree of sulfation domains while

leaving the high degree of sulfation domains intact. An HS samples was partially depolymerized using polysaccharide heparin lyase III, subjected to SEC and then to high resolution strong anion exchange (SAX) chromatography (94). MS analysis showed the presence of the same hexasaccharide composition in two different SAX fractions, indicating the existence of two structural isomers. The tandem mass spectra of the two fractions showed differences in terms of product ion abundances for a common set of m/z values. However, losses of SO_3 were observed in the tandem mass spectra, making direct assignment of the positions of sulfation not possible. The two fractions were the analyzed using ion mobility MS, showing distinct mobility arrival time distributions. These results were consistent with the conclusion that the two structural isomers had differing shapes in the gas phase that were resolved by mobility analysis. One dimensional proton nuclear magnetic resonance analysis of the two fractions was consistent with the presence of two hexamers differing only by C5-epimerization at one position. These results illustrate the value of a mobility dimension for resolving GAG oligosaccharide structural isomers in MS studies.

Capillary electrophoresis (CE)—The acidity of GAGs makes them attractive candidates for analysis by CE/MS (95, 96). A forward polarity CE method has been used to separate hyaluronan oligosaccharides using a polyacrylamide coated fused silica capillary with negative polarity ESI MS detection (97). The MS detection enabled identification of the hyaluronan oligosaccharides, many of which had similar migration times. A reversed polarity CE method has been used to separate HS disaccharides with negative polarity ESI MS detection (98). This approach is advantageous because the MS dimension enables identification of disaccharides for which no electrophoretic standards exist. An on-line sheathless forward polarity CE method has been used to analyze CS/DS oligosaccharide mixtures (99–101). Using this system, it was possible to acquire on-line tandem MS of a CS/DS dp18 oligosaccharide and to assign the sulfation pattern. A forward polarity CE method has also been used for on-line CE/MS of heparin oligosaccharides (102). Frontal analysis capillary electrophoresis entails continuous electrokinetic injection of a mixture of a protein and oligosaccharide and has been used to detect complexes between antithrombin and a synthetic heparin oligosaccharide (103).

Reducing end tags for GAG MS

The addition of a reducing end tag may be employed to add a chromophore or fluorophore to a GAG oligosaccharide to facilitate optical detection, as a hydrophobic group to facilitate chromatographic separation, and/or as a mass tag to enable mass spectrometric quantification (104). Anthranilic acid in two stable isotope forms (d_0/d_4) has been used to enable glycomics of CS/DS in connective tissue using HILIC LC/MS (32, 83, 84). For these studies, the light form of the tag was used to label a standard CS/DS oligosaccharide mixture as an internal instrument performance control. A tetraplex (d_0, d_4, d_8, d_{12}) reductive amination tag has been developed to enable simultaneous MS analysis of four samples (105). Results were shown for MS of CS, heparin, and *N*-linked glycans (106). A tag based on stable isotopes of aniline ($^{12}\text{C}_6/^{13}\text{C}_6$) has been used with RPIP LC/MS in a study of evolutionary differences in GAG structure based on disaccharide profiles (58). This tag has

the advantage of a mass shift (6 Da) and of carbon backbone labeling to minimize chromatographic resolution of heavy and light forms.

Tandem MS of GAGs

In order to specify the structures of GAG oligosaccharides it is desirable to produce information on (1) the number of sulfate and acetate groups per monosaccharide, (2) the positions of sulfates and acetates on the residues and (3) the positions of uronic acid epimers. For KS oligosaccharides, only the 6*O*- positions of Gal and GlcNAc can be sulfated, and thus the mass of the modified residue suffices. For CS/DS, sulfation is possible at the 4*O*- and/or 6*O*-positions of GalNAc in addition to the 2*O*-position of HexA. Thus, additional experiments are needed for determination of sulfation position for GalNAc. CAD dissociation of oligosaccharides derived from CS types A, B, and C, respectively produces distinct product ions, the abundances of which correlate with patterns of sulfation and epimerization (84, 107, 108). It is therefore possible to determine the percentages of the three types of oligosaccharides in a biological sample by comparing the CAD tandem MS product ion abundances with those of standards. CS/DS oligosaccharides are amenable to CAD tandem mass spectrometric analysis using negative ESI, as has been demonstrated by several groups (107, 109–111). Recently, a CS from decorin were digested separately with chondroitinase ACI so as to produce oligosaccharides with a high content of IdoA and with chondroitinase B so as to produce oligosaccharides with a high content of GlcA. Mult-stage MS was then used to determine the presence of over-, under- and regular-sulfated oligosaccharides (112). Oligosaccharides from dp10–14 derived from CS types A, B and C, respectively, have been analyzed using negative ion ESI MS³ (113). The results showed that the MS³ stage determined information on the epimerization of individual uronic acid residues beyond that obtained in the MS² stage.

For HS oligosaccharides, purified standards with defined patterns of sulfation, acetylation, and epimerization do not exist. Thus, it is very important to produce as much information as possible when analyzing HS samples from biological sources. The tendency of HS oligosaccharides to undergo undesirable losses of SO₃ during CAD tandem MS depends on the negative ion charge state (114–116). Thus, the abundances of product ions from glycosidic bond and cross-ring cleavages increase with the negative charge state. Unfortunately, charge-charge repulsion limits the ability to produce such charge states for highly sulfated HS oligosaccharides. The problem is exacerbated when using on-line LC/MS, where the charge states are not as high as observed using direct infusion (81). Pairing of GAG oligosaccharides with metal cations serves to reduce the extent to which losses of SO₃ are observed during CAD tandem MS (115). This effect is now being exploited for electron detachment dissociation (EDD) tandem MS of GAGs (see below).

Activated electron dissociation of negatively charged analytes in the form of EDD or negative electron transfer dissociation (nETD) has potential to produce highly informative glycosidic bond and cross-ring cleavages (117, 118). EDD of pairs of disulfated heparin tetramers differing by the C5-epimeric position of the third residue showed that ^{0,2}A₃, B₃ and B₃-CO₂ ions were present in the GlcA form and absent in the IdoA form (119). The authors proposed a radical fragmentation mechanism dependant on the C5 epimeric position.

Electron induced dissociation (EID) occurs as a result of electron irradiation of singly negatively charged precursor ions (120). When the same pair of heparin tetrasaccharides was subjected to EID, a series of abundant glycosidic bond and cross-ring cleavages was observed, but the spectra were the same for both epimers (121). These results indicate that electronic excitation and radical fragmentation are key to differentiating C5 epimers of GAGs. Recently, nETD in a commercial ion trap was shown to be capable of uronic acid epimers from heparin tetrasaccharides (122). The tetrasaccharides were chemically de-*N*-sulfated prior to analysis and the products analyzed by nETD were unsulfated.

Tandem mass spectra of GAGs are relatively complex owing to the relatively high charge states of the precursor ions resulting in product ions that may be present with more than one charge state. As a result, computational approaches for interpretation of GAG tandem mass spectra are of considerable interest. Product ion patterns resulting from multi-stage tandem MS of GAGs and disaccharide analysis data have been analyzed using the heparin oligosaccharide sequencing tool (HOST) (53). This algorithm calculates all possible sequences for a given GAG composition and eliminates those that are not consistent with the disaccharide analysis and MS compositional data, digestion enzyme activities, and tandem MS data. A software tool for analysis of GAG mass spectra has been developed based on the Glycoworkbench platform (123). This tool calculates GAG compositions and all possible tandem mass spectrometric product ions thereof to facilitate interpretation of the data.

Analysis of GAG-protein complexes

Mass spectrometry is emerging as a key technology in the analysis of GAG-protein complexes. The biological effects of GAG expression are mediated to a large degree by the protein molecules to which they bind (124). In the biological setting, proteins are likely to interact with the domains of GAG chains having appropriate densities and patterns of backbone modifications in terms of sulfate, acetate and uronic acid epimerization. The structures of these domains are likely to reflect a series of variants on a core structure. Thus, methods for analysis of GAG-protein interactions need to account for the likelihood that a series of structural variants will be observed.

An MS method has been developed to analyze GAG oligosaccharides that bind proteins of interest using a combination of reversed phase trapping and ultrafiltration (125). The method was used to analyze complexes between chemokines and heparin octasaccharides, yielding both the distribution of GAG oligosaccharides present and the protein-carbohydrate stoichiometries (126, 127). Tandem MS was also used to determine the pattern of heparin octasaccharide sulfation for structures that bound to chemokines (128). The approach of direct MS analysis of protein-oligosaccharide complexes has also been used to evaluate inhibitors of chemokine function. The binding data were used to determine dissociation constants between chemokines and small molecule inhibitors (129).

Studies on binding between chemokines, a heparin oligosaccharide, and a competitor demonstrate the state-of-the art for GAG-protein binding analysis. Chemokines function in inflammatory processes by inducing leukocytes to undergo chemotaxis and extravasation. Binding between chemokines and heparan sulfate is essential for formation chemokine

gradients. ESI MS has been used to study binding between heparin and heparan sulfate oligosaccharides (94, 125–127, 130, 131). Recently, ESI MS was used to probe competitive interactions between C-C motif chemokine 7 (CCL7), a peptide derived from chemokine receptor CCR2 and the synthetic heparin pentasaccharide Arixtra (see Figure 2 for structures) (132). The CCR2 peptide contains sulfated tyrosine residues. As shown by the ESI MS analysis (Figure 3), this peptide competes with the heparin pentasaccharide for binding to CCL7. The disulfated form of the peptide was shown to have a higher binding affinity than does the Arixtra pentasaccharide, as demonstrated by the increased abundance of the peak corresponding to CCL7+disulfated CCR2 peptide in Figure 3b. These results demonstrate the effectiveness of ESI MS for analysis of competitive inhibitors of GAG-protein binding.

ESI-MS has been used to analyze binding stoichiometries for mixtures of FGF1, FGF receptor (FGFR) and heparin dp24 oligosaccharides (133). The data demonstrate the formation of two FGF:FGFR complexes on a single heparin chain and have implications for binding between GAG chains and growth factors *in vivo*. Complexes between antithrombin (AT) and heparin oligosaccharides have also been studied using ESI MS (134). These studies showed the distribution of heparin dp6 that bind AT under conditions where a fraction of the protein remains free in solution, and thus all binding-competent dp6 compositions are observed. The approach was extended to include analysis of binding of a low molecular weight heparin preparation to AT. A combination of SEC and hydrophobic trapping was used to isolate heparin dp6 that bind specifically to AT (79). The data showed high affinity for dp6 with 8–9 sulfate groups and no acetate groups. The bound oligosaccharides had high activity for inhibiting Factor Xa cleavage of a synthetic substrate. Such a method has potential for analysis of GAG oligosaccharides that bind any soluble protein of interest.

Conclusions

The fact that GAG expression is essential for myriad aspects of human physiology drives the need for analytical methods development. These glycans are expressed in a spatially and temporally regulated and dynamic manner. As a result, methods rapid and sensitive enough for high throughput are needed. There are now effective methods for extracting GAGs from tissue for mass spectrometric analysis. These methods remain relatively labor intensive, and it may be possible for more rapid methods used for chromatographic analysis to be applied with MS (29).

Mass measurement serves to define the compositions of GAGs with respect to monosaccharides, sulfate and acetate groups. Such mass measurement may be carried out using either MALDI or ESI methods. MALDI MS is most effective when basic peptides are used to pair with the GAG oligosaccharide analytes to prevent dissociation to sulfate groups during the desorption/ionization process. GAG oligosaccharides may be analyzed directly using ESI MS with minimal sulfate losses during ionization. Several chromatography methods LC/MS are now used, including SEC, RP, RPIP, HILIC, and PGC (see Table 1). These methods are very effective. CE/MS may also be used but the interface with the mass spectrometer remains a challenge to robust performance. The heterogeneity inherent in

GAGs drives the use of multidimensional chromatography prior to MS analysis. The ion mobility dimension clearly shows great potential for resolving structural isomers in the gas phase to maximize the value of tandem mass spectra acquired thereafter for differentiating the isomers.

Tandem MS using collisional dissociation remain limited by the losses of sulfate observed. Such losses are least significant for CS/DS oligosaccharides in which there is approximately one sulfate group per disaccharide repeat. Sulfate loss is a significant problem for highly sulfated heparin and HS oligosaccharides. The most promising approaches for sequencing GAG oligosaccharides using tandem MS are EDD and nETD. These techniques are capable of distinguishing oligosaccharides differing only by a uronic acid epimers via specific product ions. The analysis requires extended instrument acquisition times and are most appropriate for analysis of highly purified samples.

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Abbreviations used

CS	chondroitin sulfate
DS	dermatan sulfate
ESI	electrospray ionization
EDD	electron detachment dissociation
ETD	electron transfer dissociation
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GAG	glycosaminoglycan
GCC	graphitized carbon chromatography
HA	hyaluronan
HILIC	hydrophilic interaction chromatography
HS	heparan sulfate
KS	keratan sulfate
LC	liquid chromatography
MALDI	matrix assisted laser desorption/ionization
MS	mass spectrometry
RP	reversed phase
RPIP	reversed phase ion pairing
SAX	strong anion exchange chromatography

SEC	size exclusion chromatography
TOF	time-of-flight

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Figure 1.

Structures of the glycosaminoglycan classes. The nascent polysaccharide repeats are given in parenthesis in the figure. Hyaluronan is expressed as an unmodified polysaccharide. The mature HS chains have some uronic acid residues epimerized to IdoA. Sulfation of HS may occur at the 2*O*-position of uronic acids and at the N-, 3*O*-, and 6*O*-positions of GlcN. CS chains may undergo epimerization to create IdoA residues. Sulfation may occur at the 2*O*-position of uronic acids, and the 4*O*- and 6*O*-positions of GalNAc residues. KS chains are sulfated at many 6*O*-positions of GlcNAc and 6*O*-positions of Gal residues.

Figure 2.
Structures for Arixtra (A) and disulfated CCR2 21–30 peptide (B)

Figure 3.

Mass spectra of C-C motif chemokine 7 (CCL7) and Arixtra with the addition of competing ligand, disulfated CCR2 21–30. The upper spectrum (A) represents a sample containing 10 μ M CCL7, 10 μ M Arixtra, and 1 μ M disulfated CCR2. The lower spectrum (B) represents a sample containing 10 μ M CCL7, 10 μ M Arixtra, and 10 μ M disulfated CCR2. Structures for Arixtra and disulfated CCR2 21–30 peptide are defined in Figure 2. © 2010, Elsevier, Inc. Used with permission.

Table 1

Summary of GAG LC/MS methods

Method	Advantages	Disadvantages	References
SEC	Very robust, non-adsorptive mechanism	Poor sensitivity and resolution, not scalable	(34, 82–85, 135)
Reversed phase	Robust, sensitive, scalable	Retention time decreases with glycan mass, polarity	(64, 65)
Reversed phase ion pairing	Sensitive, high resolution, scalable	Amines contaminate MS source	(58, 60, 68–70, 74)
Hydrophilic interaction chromatography	Robust, sensitive, predictable retention times, scalable	Low chromatographic resolution	(32, 79–81, 136)
Porous graphitized carbon	Sensitive, high resolution, chemical stability, scalable	Poisoning of GPC column by contaminants, recovery problems	(90, 91, 137)