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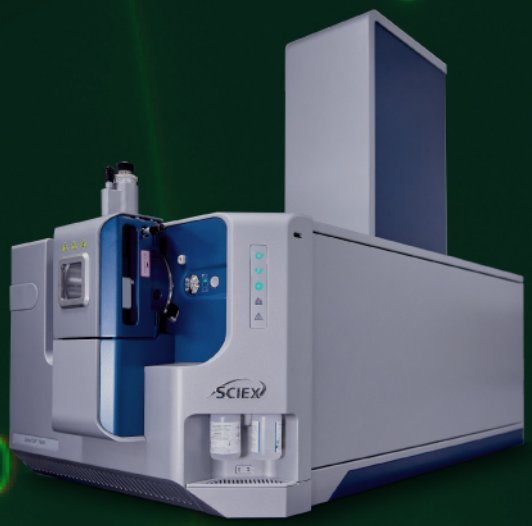

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REVIEW ARTICLE

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State-of-the-art glycosaminoglycan characterization

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Abstract

Glycosaminoglycans (GAGs) are heterogeneous acidic polysaccharides involved in a range of biological functions. They have a significant influence on the regulation of cellular processes and the development of various diseases and infections. To fully understand the functional roles that GAGs play in mammalian systems, including disease processes, it is essential to understand their structural features. Despite having a linear structure and a repetitive disaccharide backbone, their structural analysis is challenging and requires elaborate preparative and analytical techniques. In particular, the extent to which GAGs are sulfated, as well as variation in sulfate position across the entire oligosaccharide or on individual monosaccharides, represents a major obstacle. Here, we summarize the current state-of-the-art methodologies used for GAG sample preparation and analysis, discussing in detail liquid chromatograpy and mass spectrometry-based approaches, including advanced ion activation methods, ion mobility separations and infrared action spectroscopy of mass-selected species.

KEYWORDS

gas-phase spectroscopy, glycosaminoglycans, infrared spectroscopy, ion mobility spectrometry, liquid chromatography, mass spectrometry

1 | INTRODUCTION

Cell to cell communication is an essential process for multicellular organisms that facilitates their development, organogenesis, organism homeostasis, and tissue repair. The varied and abundant collection of polysaccharides and glycans on the surface of animal cells participate in cell–cell and cell–matrix interactions and also play a central role in mediating communication between cells. An important family of polysaccharides are glycosaminoglycans (GAGs), which are an essential part of the extracellular matrix (ECM). GAGs provide the ECM with the necessary flexiblity and elasticity to bring about these important and complex networks of cellular interaction (Mattson et al., 2016). GAGs coat the cell surface through a covalent linkage to protein cores, forming sulfated proteoglycans, including syndecans (transmembrane), glypicans (GPI; glycosylphosphatidylinositol anchored), and perlecan in the extracellular matrix. Generally, each proteoglycan core can carry between one and four polysaccharide chains (Pomin & Mulloy, 2018). Among the different proteoglycans expressed by mammals, serglycin, which is usually build up from heparin (Hep) chains, is the dominating species in granules of hematopoietic lineage cells. Its structural and functional characteristics are very dynamic and change depending on biological context (Kolset & Pejler, 2011).

Within the ECM network, GAGs play important roles as cellular sensors, transport regulators and relay chemo-mechanical signals from the ECM via cell–matrix connections (Antonio & Iozzo, 2005;

Ricard-Blum, 2017) to maintain fundamental functions in development (Dhoot et al., 2001; Hwang et al., 2003), pathogenesis (Shukla et al., 1999), anticoagulation (Casu et al., 2004; Shriver et al., 2000), angiogenesis (Huang et al., 2014; Raman et al., 2011), and metastasis (Sanderson, 2001).

The ECM is subject to a constant remodeling process that controls homeostasis and cell development (Theocharis et al., 2016). GAG length and the number of chains attached to a single proteoglycan core differ depending on tissue distribution. The sulfate patterns are modified by sulfatases to generate new GAG structures that can result in considerable changes in proteoglycan properties, providing the flexibity required to react to different biological needs (Frese et al., 2009; Hammond et al., 2014). A good example is the extracellular heparinase, which has been defined as a multitasking protein. It catalyzes the side chain trimming of heparan sulfate (HS) proteoglycans and contributes to the ECM remodeling process. However, in pathological conditions like inflammation, tumor growth, or fibrosis, the enzyme is overexpressed (Masola et al., 2018). Extracellular sulfatases, especially the 6-*O*-HS endosulfatase (Sulf) enzymes have regulatory functions in extracellular signaling, in the control of tumor growth and angiogenesis. They can remove 6-*O*-sulfate groups from nonreducing-terminal GlcN residues of HS, and Hep chains (Ai et al., 2005).

GAG complexity is further increased by the nontemplate-controlled biosynthesis and structural editing of GAG chains, which comprises several enzymes with tissue-specific isoforms (Sasarman et al., 2016; T. Carlsson & Kjellén 2012; Carlsson et al., 2008; Chen et al., 2018; Deligny et al., 2016; Filipek-Górniok et al., 2013; Kreuger & Kjellén, 2012; Uyama et al., 2007). Therefore, there is a great need for competent and sensitive analytical methods to characterize GAG structures, identify the broad range of protein–GAG interactions and understand the corresponding diversity of biological functions. Regardless of the complexity of existing analyses, a single approach is not sufficient to characterize the enormous structural diversity that is characteristic for GAGs. An integration of all analysis data from various orthogonal methods such as liquid chromatography (LC), mass spectrometry (MS), ion mobility (IM), and infrared spectroscopy (IR) is usually necessary.

2 | STRUCTURE OF GLYCOSAMINOGLYCAN FAMILIES

The GAG family includes hyaluronic acid (HA), heparin (Hep), HS, chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate (KS) (Figure 1). HA is a non-sulfated polymer that consists of repeating β-1,4-D-GlcA and β-1,3-N-GlcNAc units (Gupta et al., 2019). It has the highest polymerization among all GAGs, thus the longest chain of the GAG families (Larrañeta et al., 2018). HA has high viscoelasticity, high water absorption, and high biocompatibility, functioning as a lubricant in the ECM and mechanical stabilizer (Kogan et al., 2008). It maintains the water balance as a flow resistance-regulator (Larrañeta et al., 2018).

Hep and HS are some of the most acidic biopolymers found in nature (Jones, Beni, Limtiaco, et al., 2011). They are composed of a characteristic repeat of β-1,4-uronic acid (UA) and β-1,4-glucosamine (GlcN) units. The UA residue can be either α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA) and is unsubstituted or sulfated at the 2-*O* position. The GlcN residue can either be

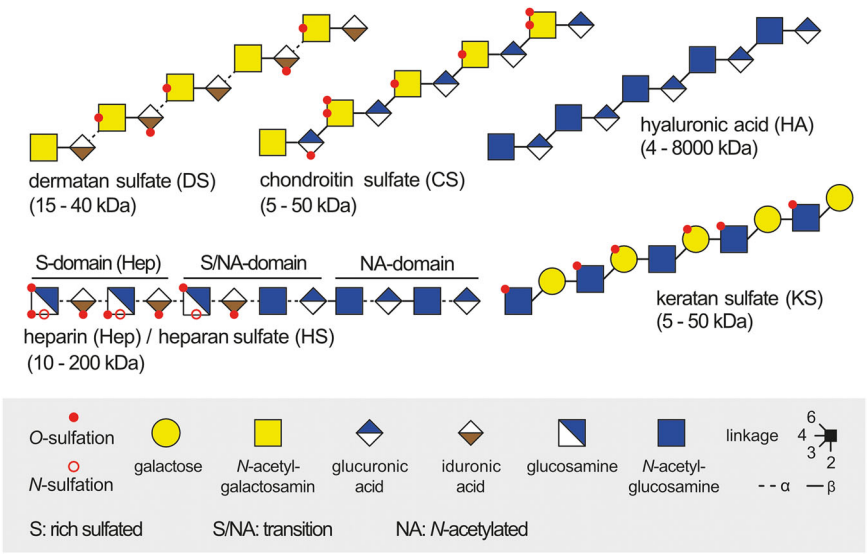

FIGURE 1 Representation of the five types of glycosaminoglycan structures. Possible sulfation presence, location (2S, 3S, 4S, or 6S) and linkages are indicated. The domain organization of Hep/HS is defined. S, rich sulfated; S/NA, transition; NA, *N*-acetylated [Color figure can be viewed at wileyonlinelibrary.com]

unmodified (GlcN), *N*-sulfated (GlcNS) or *N*-acetylated (GlcNAc) (Shriver et al., 2000, 2012). Variable patterns of *O*-sulfation can reside at the carbon 3-*O* (Esko & Lindahl, 2001; Li & Kusche-Gullberg, 2016; Thacker et al., 2014) and carbon 6-*O* positions (Jones, Beni, Limtiaco, et al., 2011; Wang et al., 2002). The heterogeneity of Hep results from variable sulfation patterns and the presence of hexuronic acid epimers, which is predominantly IdoA in Hep (Hagner-McWirther et al., 2000). In contrast to HS, Hep shows no domain organization and has a higher number of sulfates (~2.7 per disaccharide) (Dulaney & Huang, 2012) groups. In addition, Hep can be shorter than HS and has a polydisperse mixture of chains with different molecular weights (Taylor et al., 2019). HS is around 50–200 disaccharide units in length and contains an overall lower sulfation level of 1–2 sulfates per disaccharide, as well as the UA group being predominately GlcA. HS has three main regions, the *N*-acetylated domains (NA), transition domains with alternating *N*-acetylated/*N*-sulfated residues (NA/NS domains); and the fully *N*-sulfated domains (S domains) (Bame et al., 2000; Lyon & Gallagher, 1998). These separate domains are thought to be important for molecular function and also allow more structural variation to improve specificity of interactions with binding proteins.

CS chains are composed of alternating 1,4-linked β-D-GlcA and 1,3-linked *N*-acetyl galactosamine (GalNAc) units (Lamari & Karamanos, 2006). Various subtypes of CS exist, according to different sulfation patterns. CS-A is predominantly 4-sulfated on GalNAc residues (Hang Wang et al., 2008), and CS-C is mostly 6-sulfated on GalNAc (Nakano et al., 2010). CS-B is also called dermatan sulfate (DS) and has 2-sulfated α-L-IdoA units rather than β-D-GlcA. DS GalNAc units are predominantly 4-sulfated (Trowbridge & Gallo, 2002).

Keratan sulfate (KS) is composed of alternating 3-linked β-D-Gal and 4-linked β-D-GlcNAc units (Caterson & Melrose, 2018). It is the only GAG, which is not composed of UA. KS disaccharides can both be 6-sulfated, although sulfation at GlcNAc occurs more often (Funderburgh, 2002). These separate GAG familes are thought to be important for molecular functions and also allow more structural variation to improve specificity of interactions among binding proteins.

In general, GAG chains vary considerably and this structural heterogeneity is due in large part to extensive sulfation. The occurrence of GAGs in the ECM and at cell surfaces contribute to multiple biomedical processes, interactions with extracellular proteins and various pathophysiological events. The high structural diversity characteristic of GAGs and diverse biological functions is a result of their complex, nontemplate-driven biosynthesis that has been described in detail elsewhere (Bishop et al., 2007; Sarrazin et al., 2011; Soares da Costa et al., 2017). Therefore, these aspects are not specifically addressed here.

3 | FUNCTIONS OF GLYCOSAMINOGLYCANS

Biological functions mediated by GAGs are done so through several structural properties such as composition, molecular weight, the type of glycosidic linkage, sulfation, and carboxylation. The protein core sequence mainly determines the location and the number of GAGs (cell membrane, secreted, or in ECM), while proteoglycan interactions with other molecules are largely mediated by GAGs. Sulfated GAGs are usually responsible for extracellular signaling and protein interactions, including cell and tissue development. The main drivers of these functions are electrostatic interactions, which regulate protein folding and recruitment or exclusion of other biomolecules (Gandhi & Mancera, 2008; Kjellén & Lindahl, 2018). Apart from GAG-dependent interactions, proteoglycans have a large number of different structural protein modules within their respective protein cores which mediate a variety of additional binding interactions (Iozzo & Schaefer, 2015). For example, the α-granule proteoglycan serglycin fullfils many functions in cargo packaging, cargo release, the decondensation and swelling of α-granules, receptor shedding and platelet activation (Chanzu et al., 2021; Kolset & Pejler, 2011).

Sulfation patterns have a strong influence on numerous aspects of cellular interactions (Hiroko Habuchi et al., 2004) and different disorders are linked to changes in the sulfation pattern (Soares da Costa et al., 2017). Various receptors are immobilized on the cell surface by GAGs and actively restrict the movement of bound proteins to one-dimension in three-dimensional space to create protein gradients next to the site of secretion and form a protective barrier around them (Clark et al., 2013; Wei et al., 2020). The ionic interactions between the carboxyl- and sulfate groups from GAGs and amino acid residues of neighboring proteins contribute to the formation of GAG-protein complexes (Smock & Meijers, 2018; Vallet et al., 2020). These multivalent interactions contribute to the protection of proteins from degradation or conformational change, modulate activation or deactivation of proteins, and mediate the creation of GAG–protein clusters at the cell surface (Ziegler & Seelig, 2008).

Arguably, the most prominent example of a GAG-protein interaction is the anticoagulant activity of Hep (Gray et al., 2012). The function of Hep as an anticoagulant

is based on its interaction with the protein antithrombin (AT-III), an inhibitor of thrombin (Li et al., 2004). The binding of a specific pentasaccharide within Hep to AT-III causes its allosteric activation and induces a change of the AT-III conformation which results in the stimulation of Factor IXa and inhibition of Factor Xa (Atha et al., 1985; Casu & Lindahl, 2001; Goldsmith & Mottonen, 1994; Hofmeister et al., 1991; Lindahl et al., 1980, 1983; Olson, 2002; Petitou et al., 2003; Riesenfeld et al., 1981).

Additionally, GAGs regulate the function and plasticity of synapses by mediating the adaption of neurons to changing environments (Saied-Santiago & Bülow, 2018). CS is the most abundant GAG in the central nervous system and contributes to brain and spinal cord development (Djerbal et al., 2017). HS and CS bound to proteoglycans (PGs) maintain the state of the central nervous system by regulating and changing synapse interactions (Mencio et al., 2020; Rhodes & Fawcett, 2004). The absence of these GAGs is associated with diseases like Alzheimer's (DeWitt et al., 1993), epilepsy (Yutsudo & Kitagawa, 2015), and schizophrenia (Pantazopoulos et al., 2015).

CSPGs, KSPGs, and DSPGs, are the main components of the cartilage ECM and function in the generation of osmotic pressure to withstand compressive loads and the activation of chondrocytes by specific interactions with growth factor proteins (Gao et al., 2014; Horkay, 2012).

4 | LABORATORY METHODS FOR GLYCOSAMINOGLYCAN PREPARATION

A general workflow of GAG isolation from tissues includes several purification steps, followed by depolymerization into oligo- or disaccharides and subsequent analysis. In the following section, details on the methods required for GAG extraction and analysis are described.

4.1 | Extraction methods

Hep was the first GAG to be extracted. This is in large part due to being identified to have anticoagulant activity and therefore considerable thereapeutic potential. As a result of the successful pharmaceutical application as an anticoagulant, extraction methods for Hep have been continuously developed for nearly a century. In 1933 Charles and Scott isolated and purified Hep using an alkaline method following ethanol precipation, protease digestion and a second ethanol precipation (Charles & Scott, 1936). Acetone extraction was introduced, followed by protein digestion and further acetone precipation (Freeman et al. 1957). Phase extraction methods utilising chloroform-methanol, followed by protein digestion (trypsin and papain), alkaline treatment, acetone precipation, anion exchange chromatography and ethanol precipation were established (Volpi, 1999). Most recently, phenol, guanidine, and chloroform were used for GAG extraction followed by weak anion exchange and enzymatic digestion to remove proteins, DNA, RNA, and glycans. Subsequently, GAGs were purified using a final step of weak anion exchange chromatography (Guimond et al., 2009).

These methods involve the extraction of lipids and membrane components using organic solvents and the removal of the PG from the GAG chain either using alkaline buffers to induce β-elimination of the GAG chains or proteolytic digestion of the protein. Removal of existing proteins, peptides, DNA, RNA, and small molecules was performed either by precipation with organic solvents and salts or anion exchange chromatography. All GAG families have applied a combination of these extraction methods. Hep can be fractionated by its high charge, however, HS, CS, DS, and KS are isolated individually through enzymatic digestion of the GAG chains.

4.2 | Depolymerization methods

GAG chains are long polymers and therefore too complex and heterogenous to be analysed in their intact form that derives meaningful structural information. However, the bioactive motifs within the GAG chain usually range from a few disaccharides to dodecamers (Townley & Bülow, 2018). Therefore, chemical- or enzymatic digestion of the intact GAG chain into smaller oligosaccharides provides a useful approach to study biologically relevant structures and activity relationships on targeted sections of the GAG.

Hep is clinically used as an anticoagulant. As a full-length chain, it has greater ability to bind a larger number of protein complexes, which might potentially lead to unintended side effects. For clinical applications, Hep is usually chemically or enzymatically cleaved into low molecular weight heparins (LMWHs), which provides fewer side effects, as a result of having few protein interactions (Frydman, 1996). All currently established methods to depolymerize Hep are shown in Figure 2. The LMWH enoxaparin is generated by the depolymerization of Hep by esterification with benzyl chloride and alkaline hydrolysis, whereas tinzaparin is generated by enzymatic digestion. The LMWHs dalteparin and reviparin are generated by partial nitrous acid depolymerization of Hep (Baytas & Linhardt, 2020).

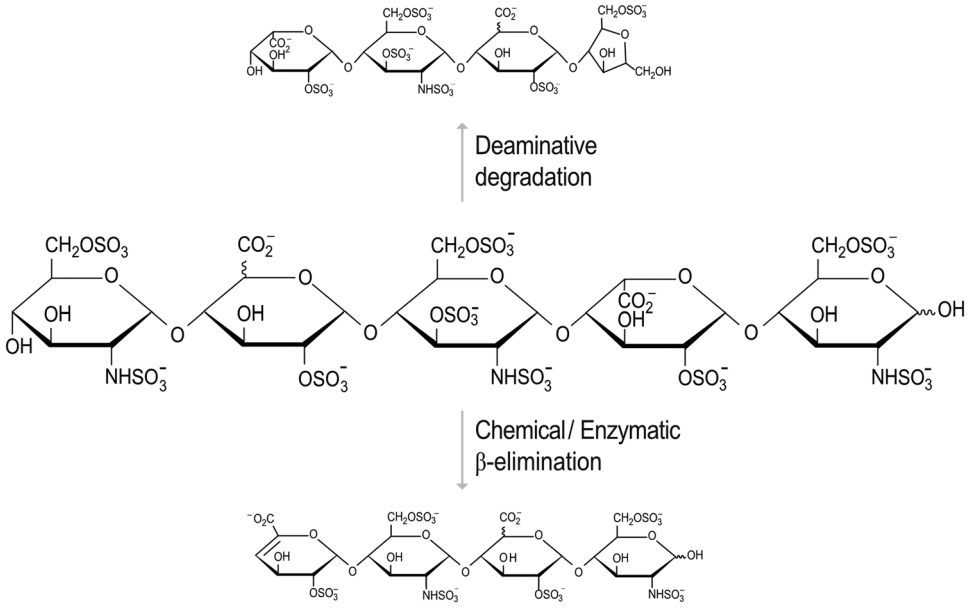

FIGURE 2 Depolymerization methods of Hep to produce low molecular weight heparins. The centered Hep chain can be depolymerized by deaminative degradation or by chemical/enzymatical β-elimination

4.2.1 | Enzymatic digestion

Many GAG degrading enzymes have been identified and are either endo- or exo-lytic lyases or hydrolases (Ernst et al. 1995). Lyases cleave by an eliminative mechanism to produce a 4,5-unsaturated uronic acid at the non-reducing chain terminus (Linhardt et al., 2006; Maruyama et al., 2009). Hydrolases cleave by adding water equivalents to glycosidic bonds creating saturated cleavage products (Davies & Henrissat, 1995; Maruyama et al., 2009). In this article the focus is set on lyases which create unsaturated uronic acid and therefore enable UV detection of generated products.

As all mammal organisms turnover GAG chains and edit them based on cellular requirements, bacteria have numerous GAG-degrading enzymes which they utilise in pathogensis. Enzymatic depolymerization is often used in disaccharide analysis methods, for example, for quantification of disaccharide units between tissues providing important data on the natural variation of the GAG structure (Alonge et al., 2019; Kuiper & Sharma, 2015; Li et al., 2017; Saad & Leary, 2003, 2005; Song et al., 2020; Turiák et al., 2018). The possibility to analyze GAG structure variations has led to comparisons by compositional analyzes of different GAG chains from different organs in different species, and disease states (Saad & Leary, 2003; Skidmore et al., 2010; Wei et al., 2011; Zaia & Costello, 2001).

Enzymatic degradation of GAG chains using bacterial lyases results in a 4, 5-unsaturated double bond on the uronosyl residue, a chromophore that absorbs at a wavelength of 232 nm (Linhardt, 2001). Hep and HS can be depolymerized from polysaccharides into disaccharides using heparinases I, II, and III (Lohse & Linhardt, 1992; Wu et al., 2014). Heparinase I cleaves the polymer chain between GlcNS(±6S)α1-4IdoA(2S). Heparinase II is less specific and has a broad range of activity, it cleaves the GAG chain between GlcN residues which can be *N*-sulfated or *N*-acetylated and 2-*O*-sulfated IdoA, unsubstituted IdoA or GlcA (GlcNR(±6S)α1-4GlcA/IdoA). Heparinase III cleaves at sites between GlcNac or GlcNS and IdoA, which can be either 2-*O*-sulfated, unsubstitued or GlcA (Desai et al., 1993; Wei et al., 2005).

The enzymatic depolymerization of CS/DS is made possible by various chondroitinases (CSases), yielding di- and small oligosaccharides (Hettiaratchi et al., 2020; Kasinathan et al., 2016; Yamagata et al., 1968). These include CSase ABC I, CSase ABC II, CSase AC, and CSase B. The chondroitinase family of enzymes is named following the type of chondroitin sulfate chain it can digest. Therefore, chondroitinase A is able to degrade CS-A (GlcA (β1-3)-GalNAc4S), whereas chondroitinase C digests CS-C (GlcA (β1-3)-GalNAc6S) and chondroitinase B digests DS (IdoA (β1-3)-GalNAc4S).

HA can also be depolymerized by bacterial lyases; *Streptococcus pneumoniae* hyaluronate lyase is a bacterial enzyme which specifically cleaves the β-(1→4) linkage in HA and CS. It belongs to the family of β-endoglycosidases and functions by β-elimination with introduction of an unsaturated bond (Jedrzejas et al., 2002; Li et al., 2000). The mammalian hyaluronidase is a hydrolase enzyme that

can digest HA and CS (Bilong et al., 2021; Kaneiwa et al., 2010).

4.2.2 | Chemical depolymerization of GAGs

Chemical depolymerization methods of GAGs are very versatile and can mainly occur by two principles: β-elimination and reductive deamination. The process of β-elimination mimics the bacterial lyase depolymerization through a chemical two-step reaction, which introduces a double bond at the nonreducing ends of each cleaved GAG fragment. The carboxylate group on the C5 carbon of the nonreducing end is benzylated with benzyl chloride. During the reaction, the proton at the C5 position on the nonreducing end is abstracted by a strong base, a double bond between C4 and C5 is formed and the glycosidic bond is cleaved. After this reaction step, the benzyl ester is eliminated through basic hydrolysis (Jones, Beni, Limtiaco, et al., 2011).

Reductive deamination at GlcNS residues is achieved by using nitrous acid or isoamyl nitrite. The deamination process results in an unstable nitrososulfamide, which immediately loses nitrogen and sulfate. A carbocation at the C-2 position of the saccharide is generated (Conrad 2001). The depolymerization by reductive deamination alters the GlcN structure by producing 2,5-anhydro-D-mannose residues at the reducing ends of the fragment (Shively & Conrad, 1976). Deamination with nitrous acid keeps the original GlcA/IdoA unaffected. There is no loss of information regarding the stereochemistry of the hexuronic acid. However, information about *N*-sulfation and *N*-acetylation is lost. The process of deamination can be controlled by adjusting the pH, reaction temperature, and duration (Bienkowski & Conrad, 1985).

Enzymatic and chemical depolymerization reactions both produce similar oligosaccharide products. At completion, heparinase I and III produce oligosaccharide mixtures whereas heparinase II produces disaccharides. These enzymes have also been identified to digest chemically modified Hep and HS at a reduced efficiency (Shriver et al., 1998). Hep cleavage by chemical processes can degrade GAGs into oligosaccharides by time inhibition of the reaction. Otherwise the GAG chain is digested to the smallest GAG unit. Most relevant bioactive motifs range from tetra- to deca-saccharides (Miller et al., 2020), but also binding interactions with trisaccharides have been demonstrated. A trisaccharide motif in HS containing 2-*O*-sulfated IdoA and 6-*O*-desulfated GlcN was found to bind to fibroblast growth factor 1 (FGF-1), and a hexasaccharide from HS with a single 2-*O*-sulfated IdoA binds to FGF-2 (Kreuger et al. 2001). A pentasaccharide sequence with 2-*O*-sulfated IdoA and 6-*O*-desulfated GlcN from Hep also strongly binds FGF-2 thereby enhancing the binding affinity to FGF receptors (Maccarana et al., 1994; Miller et al., 2014).

4.3 | UV- and fluorescent-labeling

Depolymerization of GAGs by bacterial lyases or β-elimination leads to the formation of unsaturated pyranose rings, which absorb at 232 nm and can therefore be detected with common UV detectors such as those coupled to HPLC systems (Alkrad et al., 2003; Chandarajoti et al., 2016). Other strategies are fluorescent labeling of GAGs with fluorophores, which can be excited and detected at specific wavelengths with a fluorescence detector. The fluorophores 2-aminoacridone (AMAC) (Chang et al., 2012; Kitagawa et al., 1995), procainamide (ProA) (Antia et al., 2018) and Bodipy-FL-hydrazide (Skidmore et al., 2010) are suitable for GAG analysis. The derivatization takes place selectively and exclusively at the reducing end with the formation of a Schiff base. Labelling with fluorophores has enabled fmol sensitivity and detection of subtle changes in GAG disaccharides among biological samples.

4.4 | Production of glycosaminoglycan-derived standards

The identification and quantification of biological GAG samples requires the availability of GAG standards with known concentrations. GAG standards and unknown samples can be separated by HPLC enabling the GAG type and its modifications to be assigned based on the comparison with specific retention times of known standard. Comparisons of peak areas can then be used for quantification. In most of the cases, Hep is used as basis for the production and preparation of standards. It is purified from pig intestines and di- and oligosaccharides are generated from the precursor material (Lee et al., 2020). The preparation of GAG chains into oligosaccharides and disaccharides can be carried out chemoenzymatically or via chemical synthesis (Yates et al., 1996). Generated cleavage products can be chemically modified, which significantly increases the yield of disaccharides with a defined chemical structure and defined modification. For example, the disaccharide UA2S-GlcNS6S, which is often found in Hep (Nagamine et al., 2012), enables the production of various sulfated disaccharides by chemical cleavage of sulfate groups.

In general, GAG standards can be produced using various mechanisms. One approach follows the purely

chemical synthesis of the sugar building blocks based on repetitive steps of protection, activation, coupling, and deprotection. There are several modular approaches for the synthesis of HS oligosaccharides, each of which makes use of selectively protected disaccharide building blocks and corresponding glycosyl donors. The principle outcome was that di- and oligosaccharide libraries were created with which both structural and biological studies on the inhibition of BACE-1 protease (Arungundram et al., 2009) and substrate specificities of the sulfate sulfotransferase 3-OST3a (Nguyen et al., 2012) were examined. In addition, several selectively protected tetrasaccharides with regioselective *O*- and *N*-sulfation and desulfation were assembled and used to prepare a library of 47 HS oligosaccharides and to construct a HS microarray. The generated tetrasaccharides included 12 differently sulfated derivatives (Zong et al., 2017). The chemical synthesis of HS fragments was greatly simplified by the introduction of aminopentyl linkers protected by benzyloxycarbonyl groups. The linker was modified by a perfluorodecyl tag, which enabled the purification of highly polar intermediates by fluorous solid phase extraction (Zong et al., 2013). Additionally, GlcA donors were found to give high yields of coupling products after protection of the C-2 hydroxyl group with a 4-acetoxy-2,2-dimethyl butanoyl- or levulinoyl ester and the C-4 hydroxyl modified with a selectively removable 2-methylnaphthyl ether (Dhamale et al., 2014). In another approach GlcN residues were modified by different patterns of *N*-acetyl and *N*-sulfate moieties using azido- or trifluoromethylphenyl-methanimine-modified glycosyl donors. Together with the orthogonal hydroxyl protecting groups levulinic ester, thexyldimethylsilyl ether, allyloxycarbonate, and 9-fluorenylmethyl carbonate, different *O*-sulfation modification patterns were constructed (Sun et al., 2020). Recently, a modular synthetic approach providing structurally diverse HS oligosaccharides with and without 3-OS was carried out. With this approach 27 hexasaccharides were used to create a glycan microarray used to examine binding affinities of HS-binding proteins (Chopra et al., 2021).

Recent advances in synthesis include chemical derivatization methods with enzyme-catalyzed reactions for the assembly of di- or oligosaccharides (Wang et al., 2021; Zhang et al., 2017). With the enzymatic component of synthesis glycosylation, epimerase, and sulfation reactions with high stereo- and regio-selectivity can be performed without the need for repetitive protection and deprotection steps resulting in bioactive GAG chains (Wang et al., 2021; Xu et al., 2012). In comparison to chemical synthesis, chemo-enzymatic methods provide exceptional regioselectivity, shorten the required reaction time and result in significantly higher product yields (Chappell & Liu, 2013). However, the chemoenzymatic workflow requires deep understanding of enzyme specificity as well as the application of chemically synthesized donors and acceptors to perform target-based synthesis of GAG chains (Dickinson et al., 2014). Chemoenzymatic synthesis employs enzyme catalysts and different precursor structures, for example, uridine 5′-diphosphosphate-sugar donors, sulfate donors, acceptors, and oxazoline precursors and it is generally divided into two different approaches. The semisynthetic approach uses naturally occurring polysaccharides and therefore, the control over polymer size and composition is lost. The second approach employs synthases and UDP-sugars and the GAG chain synthesis reaction is carried out in either step-wise elongation or in a synchronized polymerization reaction both resulting in more defined products with narrow size distributions (DeAngelis et al., 2013).

For the chemoenzymatic synthesis of poly- and oligosaccharides the HS polymerase catalyzes the synthesis of the disaccharide backbone consisting of repeating units of GlcA and GlcNAc. More modifications are performed by the sulfotransferases and epimerases *N*-deacetylase, *N*-sulfotransferase, C_5-epimerase, 2-*O*-sulfotransferase, 6-*O*-sulfotransferase, and 3-*O*-sulfotransferase (Xu et al., 2011). The HS backbone was also chemically synthesized and further modified by enzymatical approaches employing *N*- and *O*-sulfotransferases and the C_5-epimerase resulting in oligosaccharides with different sulfation and epimerization patterns (Lu et al., 2018). Subsequently, oligosaccharides can be regioselectively functionalized by azido acids offering highly reactive intermediate structures (Zhang et al., 2020). Additionally, fluorous tags facilitate their purification. A series of *N*- and *O*-sulfated HS oligosaccharides were successfully synthesized and purified employing this technique (Cai et al., 2014). Additionally, the synthesis of a biotinylated heparosan hexasaccharide was improved by modifying the Hep backbone with a *N*-trifluoroacetylglucosamine residue during a one-pot multienzyme strategy (Wu et al., 2015). Targeted chemo-enzymatic synthesis of HS oligosaccharides was performed employing the HS 6-*O*-sulfotransferase (Yi et al., 2020), HS 3-*O*-sulfotransferase (Dhurandhare et al., 2020) and 2-*O*-sulfotransferase (Hsieh et al., 2014) which transfer sulfates to the corresponding hydroxyl group position. This type of synthesis is carried out to generate HS di-, tetra-, and hexasaccharide standards with defined biological activities. The 6-*O*-sulfotransferase has been additionally engineered to achieve fine control of the 6-*O*-sulfation (Yi et al., 2020).

Usually, cleaved disaccharides are first purified by size exclusion chromatography (SEC) and then separated

with strong anion exchangers (Liu et al., 2019; Victor et al., 2009; Ziegler & Zaia, 2006). Subsequently, several sample preparation and fractionation steps are required (Powell et al., 2010). The purification steps of Hep from raw mucosal material results in waste by-products that are less sulfated and show less biological activity. The purified by-products are an economical source of structurally similar Hep and HS polysaccharides and enable a cheaper production of disaccharide standards (Taylor et al., 2019). Quantification of unknown samples must be carried out under consideration of different commercial enzymes, which were used for the production of disaccharide standards. The yield of enzymatically produced standards varies and depends on enzyme activity. For this reason, multiple analyses should always be carried out using enzymes from the same supplier.

5 | LIQUID CHROMATOGRAPHIC SEPARATION OF GLYCOSAMINOGLYCANS

LC is one of the most suitable methods for the isolation of GAG oligosaccharides. Often SEC is used after GAG depolymerization to separate oligosaccharide products of varying chain length. To achieve greater level of purity, weak and strong anion-exchange (SAX), reversed-phase (RP) and ion pairing (RP-IP) chromatography, porous graphitized carbon (PGC) and hydrophilic interaction chromatography (HILIC) have proven to be valuable (Figure 3).

5.1 | Size-exclusion chromatography

SEC is a chromatographic method in which macromolecules are separated based on their hydrodynamic volume and is characteristically robust, reproducible and universally practical across laboratories (Liu, 2015). Hydrodynamic volume is a measure of size for a given molecule and, at a constant density, also molecular weight. SEC depends on the ability of molecules to enter the pores of the stationary phase. The most important parameter is the molecular size of the analytes; small analytes can access pores more readily than large analytes. Therefore, larger molecules move faster through the column. Analytes are eluted in the order of decreasing molecular size. Further unwanted retention may arise from electrostatic interactions with the stationary phase, which can be minimized by using mobile phases with high ionic strength (Brusotti et al. 2018). The column characteristics must be considered carefully in terms of sample capacity, resolution, and separation effectivity. Large columns are more suitable for high-resolution separations and characterization of small sample qantities (Harrowing & Chaudhuri, 2003). There

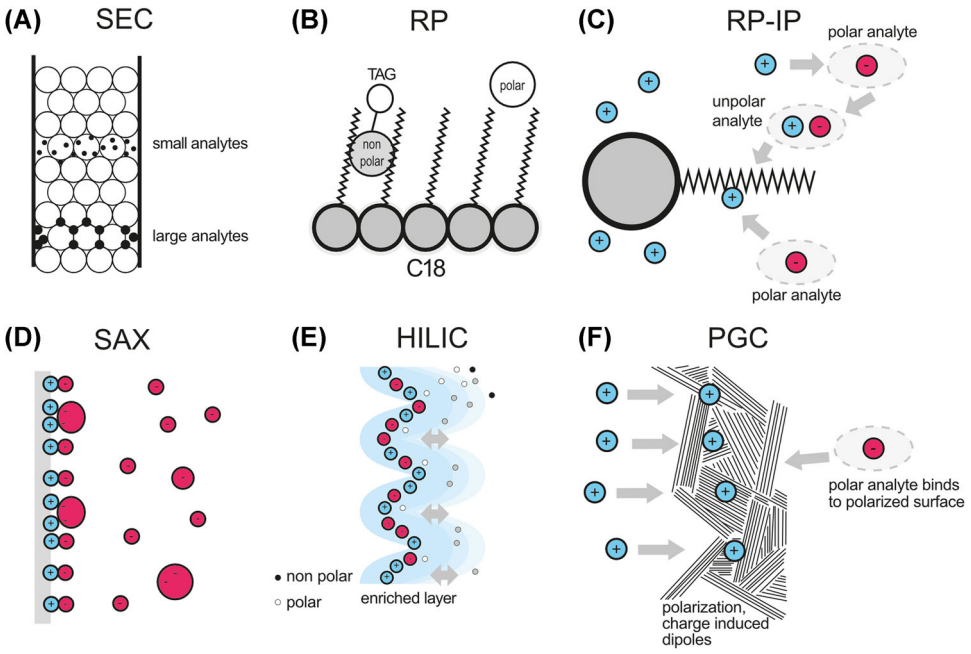

FIGURE 3 Overview of suitable chromatography methods for GAG purification or analysis. (A) Size exclusion chromatography, (B) reversed-phase chromatography, (C) reversed-phase chromatography with ion pairing, (D) strong anion exchange chromatography, (E) hydrophilic interaction chromatography, and (F) porous graphitic carbon chromatography [Color figure can be viewed at wileyonlinelibrary.com]

are various size exclusion experiments in which depolymerized Hep was separated and fractionated according to the size of its fragments (Chuang et al., 2001; Rice et al., 1985; Wang et al., 2012; Zhang et al., 2013; Ziegler & Zaia, 2006). Minimizing sample complexity via separation of a long GAG chain is crucial for further characterization of the sulfation level or isomerization.

5.2 | Anion-exchange chromatography

GAGs are negatively charged polysaccharides and therefore highly suitable to be separated using anion exchange techniques, as shown previously for intact GAG chains as well as chemically or enzymatically depolymerized oligo- and disaccharides (Linhardt, Rice, Kim, Engelken, et al., 1988; Linhardt, Rice, Kim, Lohse, et al., 1988; Pervin et al., 1995; Shastri et al., 2013). Strong anion exchange (SAX) is the approved method for FDA-approved quality control of pharmeutical Hep (Beni et al., 2011; Guerrini et al., 2009; Jones, Beni, Limtiaco, et al., 2011; Keire et al., 2010, 2011; Ye et al., 2013). Anion exchange chromatography separates analytes according to charge, with the stationary phase containing many positively–charged functional groups, such as diethylaminoethyl (DEAE) groups for weak anion exchange or a quarternary amines for strong anion exchange. The analyte of interest is retained under low ionic conditions, where a molecule of higher ionic strength displaces the anionic counter ions (typically chloride or phosphate). The elution of the molecules is achieved by increasing the ionic strength of the elution buffer over a gradient until displacement of the analyte occurs. The major limitation of SAX is that the number of negative charges (sulfates/carboxylates) on different sugar structures may be identical, in which case these structures elute simultaneously. For small saccharides (e.g., disaccharides), there is a significant degree of separation based on the presence of 2OS, 6OS, and NS groups, but as the oligosaccharide becomes longer this resolution decreases. DEAE is often applied to full length GAG chains, whereas strong anion exchange (SAX) is applied to oligosaccharides ranging from 2 to 20 monosaccharide units (Chuang et al., 2001; Rice et al., 1985). The majority of anion exchange columns used for GAGs are commericially available. However, there are also non-commercial columns that have been developed as a result of the complexicity of GAGs to achieve pure oligosaccharides (Miller et al., 2016; Mourier & Viskov, 2004; Mourier et al., 2015). Alternatives to conventional SAX columns are C8 or C18 columns derivatized with cetyltrimethylammonium salts (CTA-SAX). This derivatization results in a SAX stationary phase with different amounts of coating, which allows for the separation of isomeric structures that cannot be separated using commerical columns (Miller et al., 2016; Mourier & Viskov, 2004; Mourier et al., 2015). A combination of offline MS-compatible SEC, SAX and CTA-SAX can isolate pure structures, using volatile buffers to minimize sample loss (Miller et al., 2016). The CTA-SAX columns provide excellent resolution for oligosaccharides purified from other methods such as SEC or conventional SAX. However, these are based on a C18 or C8 matrix which can also interact with proteins and tagged GAG structures. Conventional SAX columns on the other hand are made from silica beads and are suitable for fluorescent labels and enzymes from GAG digestion protocols (Guimond et al., 2009; Kitagawa et al., 1995; Skidmore et al., 2006, 2009; Yamada et al., 2007).

5.3 | Reversed-phase ion pairing chromatography

Reversed-phase stationary phases are covalently bound alkyl- or aromatic ligands which provide a hydrophobic column surface. The solutes are usually dissolved in polar mobile phases and interact with the stationary phase according to their hydrophobicity. The elution is performed by decreasing the polarity of the mobile phase using organic solvents (Žuvela et al., 2019). Reversed-phase ion pairing (RP-IP) chromatography is performed on di- and oligosaccharides as full-length GAG chains, when complexed with an ion pairing reagent, are either retained on the stationary phase or are not sufficiently resolved (Jones, Beni, & Larive, 2011; Karamanos et al., 1997; Li et al., 2014b; Thanawiroon & Linhardt, 2003; Thanawiroon et al., 2004; Toyoda et al., 1999).

Understanding the mechanism of reversed-phase ion pairing for GAG separation revolves around two theories: (1) the negative charge of the sulfate group interacting with the amine on the ion pairing reagent and a neutral structure interacting with the stationary phase and (2) the ion pairing reagent coating the stationary phase and the negative sulfate of the oligosaccharide binding to the amine group within the ion pairing reagent in an anion exchange chromatography manner. None of the theories have yet to be proven unambiguously and in reality it may well be a combination of both (Jones, Beni, & Larive, 2011; Karamanos et al., 1997). RP-IP chromatography provides high resolution separation for di- and oligosaccharides and complete separation of disaccharides for HS and Hep has been achieved (Galeotti & Volpi, 2013; Jones, Beni, Limtiaco, et al., 2011; Karamanos et al., 1997; Korir et al., 2008; Toyoda et al., 1999; Vongchan et al., 2005; Xu et al., 2015). In addition, RP-IP is compatible with LC-MS, albeit with the challenge that each sulfate groups on the GAG oligosaccharide can complex with an ion pairing reagent, so a minimum number of complexed sulfate

or carboxylate groups would be one and the maximum number would be all sulfate and carboxylates in the saccharide are complexed with an ion pairing reagent (Doneanu et al., 2009; Henriksen et al., 2006; Langeslay et al., 2013; Li et al., 2014a; Thanawiroon et al., 2004). RP-IP chromatography also suffers from signal suppression during electrospray ionization (ESI) as a result of the ion pairing reagent (Doneanu et al., 2009). Despite this perceived shortcoming, the resolution and LC-MS compability makes RP-IP an excellent method within the GAG toolbox. RP-IP chromatography with each disaccharide coupled to a tag is the most common method for off-line and on-line analysis.

5.4 | Hydrophilic interaction chromatography

HILIC is a chromatographic method that uses stationary phases consisting of bare silica, zwitterionic functional groups, for example, sulfobetaines or amide-groups. For HILIC, aprotic organic mobile phases are used, which contain at least 2% water and forms a water-enriched layer immobilized at the surface of the stationary phase. The separation mechanism is based on a distribution of the analyte molecules between the water layer and the mobile phase. Therefore, predominantly polar analytes are retained in the water-enriched layer at the stationary phase and are eluted after increasing the aqueous buffer content in the mobile phase (Alpert 1990; Buszewski & Noga, 2012). HILIC can be directly coupled to MS due to the high organic content in the mobile phase which supports efficient evaporation, thereby increasing sensitivity and minimizing ion suppression (Dreyfuss et al., 2011; Hitchcock et al., 2008; Naimy et al., 2008; Naimy et al., 2010; Shi & Zaia, 2009; Staples et al., 2009). Additionally, HILIC-MS is highly sensitive and routinely used for the analysis of GAG disaccharides (Antia et al., 2018; Gill et al., 2013; Tóth et al., 2020) and GAG oligosaccharides (Li, Zhang, et al., 2012; Liu et al., 2019; Wu et al., 2019). As a final point, the use of stationary phases with sub-2 micron particles has further increased the resolution and speed of GAG analysis (Ouyang et al., 2016).

5.5 | Porous graphitic carbon chromatography

PGC chromatography has the benefit of combining high resolution and enhanced stability against extreme pH and many physicochemical conditions. The retention mechanism is based on a combination of the polarizability and high binding capacity of the stationary phase. It depends on interactions between polar moieties of the solutes and the induced dipoles at the planar surface of the PGC phase and is responsible for the increased retention for polar compounds. The retention of oligosaccharides increases with the acidity and the molecular weight of the analyte. Therefore, PGC is sensitive to small differences in the electron distribution of the analytes, which explains the high selectivity observed in the separation of isomers (Bapiro et al., 2016; Pereira, 2008). PGC chromatography of highly sulfated oligosaccharides has to be carried out very carefully because very strong retention and even irreversible binding to the stationary phase is possible. However, it was demonstrated that protonated tetrasaccharides up to decasaccharides can be successfully eluted from commercial PGC columns (Miller et al. 2017). Highly sulfated GAGs larger than decasaccharides in buffers compatible for mass spectrometry keep difficult to be removed from PGC surfaces.

Since the concentration of additives is usually low, no purification procedures are necessary after the chromatography step, making a coupling to MS straightforward, even in negative ion mode (Ashwood et al., 2019). PGC LC-MS with negative ion polarity was used to analyze enzymatically depolymerized GAGs, for example, HA, HP, HS, and KS (Huang et al., 2011; Karlsson et al., 2005; Wei et al., 2011, 2013). A similar approach was used to analyze lyase-digested CS from aggrecan after gel electrophoresis (Estrella et al., 2007). A combination of PGC and tandem MS was shown to be efficient in characterizing disaccharide isomers including a position-specific determination of sulfate groups (Miller et al., 2016).

6 | ANALYSIS OF GLYCOSAMINOGLYCANS WITH MASS SPECTROMETRY

MS is a highly versatile method to determine the composition of disaccharides, the molecular weight of larger oligosaccharides, the type of functional groups, and, to a certain extent the GAG sequence. The acquisition of sequence-specific information is also possible with traditional gel electrophoresis and blotting techniques using different reducing end and nonreducing end labeling strategies (van Kuppevelt et al., 2017), albeit at the cost of longer analysis time and a lower informational content than MS. MS, especially in combination with LC (LC-MS), is therefore arguably the best currently available tool for structural analysis of GAGs.

MS analysis is a crucial step in identifying positions of functional groups on GAGs. A major issue is the unintented loss of sulfate modifications (Zaia, 2004). In the gas-phase, sulfate groups are highly labile and readily

lost in the form of neutral SO_3 (McClellan et al., 2002). The reaction is catalyzed by protons and therefore the deprotonation of sulfate groups or adduct formation with cations are efficient ways to prevent this undesired process (Naggar et al., 2004; Shi et al., 2012). A study of counter ions for ESI mass spectrometry analyzes of sucrose octasulfate showed substantial fragmentation as a result of sulfate loss. However, quaternary ammonium and phosphonium salts can stabilize the sulfate groups and yield excellent ESI spectra in the positive ion mode (Gunay et al., 2003).

Sulfate loss not only complicates the interpretation of mass spectra, but importantly it also leads to the loss of essential information regarding the number and position of sulfation. Therefore, it is crucial to employ gentle ionization techniques and source conditions to minimize the activation of fragile GAG ions upon their transfer into the gas-phase (Leach et al., 2017). It is not suprising that after the advent of ESI and matrix-assisted laser desorption ionization (MALDI), many different approaches have been tested regarding their utility for GAG analysis (Saad & Leary, 2003; Zaia, 2004, 2009; ZaZaia & Costello, 2003, 2001). With careful and adapted optimization of instrument parameters, sulfated polysaccharides can be measured in negative ion mode and also in positive ion mode under some conditions (Lemmnitzer et al., 2021) without in-source fragmentation of the sulfate moieties (Desaire & Leary, 2000; Naggar et al., 2004). Isomeric Hep disaccharides could also be determined by isotope labeling and ion trap tandem mass spectrometry (Saad & Leary, 2004).

Another way to circumvent sulfate losses and achieve identification of GAG isomers is the chemical derivatization of sulfation sites. Here, synthetic HS tetrasaccharides are first reduced by sodium borohydride and then completely permethylated and desulfated (Huang et al., 2016). The permethylation protects non-sulfated groups and can additionally help in the assignment of sulfation sites. The original sites of sulfation are chemically derivatized with trideuteroacetyl groups. Consequently, the derivatized tetrasaccharides are sufficiently hydrophobic for retention on a C18 RPLC column and can be analyzed by MS without undesired loss of sulfate groups (Huang et al., 2016). A similar derivatization of HS oligosaccharides with propionyl groups was also reported (Liang et al., 2018). The synthetic approach resulted in comparable derivatization efficiencies and comparable sequencing results (Liang et al., 2018; Liu et al., 2020). Another general approach to minimize sulfate losses is the use of ion suppressors during LC-MS experiments. This removes cations from the mobile phase thereby maximizing and stabilizing the charge of the GAGs (Staples & Zaia, 2011).

7 | ION ACTIVATION METHODS IN TANDEM MS

A large variety of ion activation methods are available to generate fragments in tandem MS experiments. However, in the context of GAGs, only very few provide a sufficiently diagnostic fragmentation pattern. The most widely used techniques are collision-induced dissociation (CID) (Johnson & Carlson, 2015; Kailemia et al., 2012), electron detachment dissociation (EDD) (Wolff, Laremore, Aslam, et al., 2008) and negative electron transfer dissociation (NETD) (Wolff et al., 2010). Information on the composition and structure of the polysaccharide can be derived from the fragmentation of glycosidic bonds, whereas information from cross-ring cleavage is necessary to determine the position of sulfation at carbon-2,3,4, and 6, as well as *N*-sulfation and *N*-acetylation (Kailemia et al., 2015; Eugen et al., 2011). Especially for the latter, an interpretation of the spectra is often complicated and requires expert knowledge for unambiguous assignments. Reproducible and automated high-throughput processes, which are supported by data interpretation software, are particularly desirable (Chiu et al., 2015, 2017; SaDamerell et al., 2012; Duan & Amster, 2018; Hogan et al., 2018; Hong et al., 2017; Hu et al., 2017; Ly et al., 2010; Maxwell et al., 2012; Saad & Leary 2005), albeit challenging to achieve (Duan & Amster, 2018; Hogan et al., 2018). The use of activation methods is crucial for GAG analysis and their characteristic features are described below.

7.1 | Collision induced dissociation

With CID experiments ions are accelerated by an electric field against a neutral gas such as nitrogen or argon at approximately 10^{-3} mbar, resulting in multiple collisions of each ion with the buffer gas. Between each collision event, there is sufficient time for internal vibrational redistribution of the energy, which effectively leads to slow heating of the ions and eventually cleavage of the weakest covalent bonds. The generated fragments are subsequently directed to the mass analyzer where their m/z is measured. To reliably identify all structural details and functional groups on a given precursor ion, the presence of diagnostic fragments is required. Many groups successfully applied CID for the sequencing of GAGs (Guo & Reinhold, 2019; Huang et al., 2016; Johnson & Carlson, 2015; Kailemia et al., 2012, 2013; Liang et al., 2018; Naggar et al. 2004; Saad & Leary 2005; Zaia et al., 2007) however, the informational content of the fragment spectra strongly depends on the degree of sulfation. Similar to source activation, sulfate loss is the

predominant fragmentation outcome observed in CID. This is particularly disadvantageous for the analysis of highly sulfated GAGs in which sulfates are easily lost even at low collisional activation (Jean-Yves Salpin, 2017; McClellan et al., 2002; Zaia & Costello, 2001). It was demonstrated that sulfate losses can be reduced by deprotonation of -OSO_3H groups (Zaia & Costello 2003) or through chemical derivatization using permethylation (Huang et al., 2016). The exchange of H^+ with metal cations can also stabilize sulfate groups, which in turn increases the informational content of the CID fragment spectra (Medzihradszky et al., 2007; Shi et al., 2012). Multiple consecutive CID fragmentation steps can be used in sequential MS (MS^n) experiments to obtain some further structural information (Flangea et al., 2009; Gill et al., 2013; Staples & Zaia, 2011).

Despite the disadvantageous fragmentation behaviour with CID, it has been successfully used for GAG sequencing, for example, in the case of extracted bikunin CS (Ly et al., 2011). Several isomers were identified based on the intensity of different glycosidic bond fragments in the CID MS/MS spectrum. Additionally, the relative abundance of the detected B, *X* and *Y* ions enabled the differentiation between CS and DS (Domon & Costello, 1988; Mirgorodskaya et al., 2018). A significant disadvantage of this method is that the ratio of specific peak intensities is not a universal parameter and strongly depends on the experimental conditions. The relative peak intensities therefore have to be determined using GAG standards before unknown structures can be identified.

7.2 | Negative electron transfer dissociation

Electron transfer dissociation (ETD) is a common technique for the fragmentation of peptides. ETD requires highly charged ions and is therefore limited to ionization by electrospray. With ETD the dissolved sample is first ionized using ESI in positive ion polarity and precursor ions formed in the gas-phase are trapped in an ion trap. Then, the precursor cations react with radical anions to form an unstable cation radical. This unstable radical dissociates into two fragments, typically c- and z-type ions. The cleavages occur randomly, depending on the position in the sequence at which the radical is formed (Leach et al., 2017; Wolff et al., 2010). For GAG analysis, the most desired fragmentation approach is one in which the dissociation of the precursor is accomplished at low energy to minimize sulfate loss (Hu et al., 2017). The technique that meets these requirements best is negative electron transfer dissociation (NETD). In NETD the loss of an electron from the analyte anion is caused by the interaction with a reagent cation. Consequently, the electron deficient anion radical undergoes internal rearrangement and dissociates into fragments (Lermyte et al., 2018). While ETD is mainly used for the fragmentation of polycations (Brodbelt, 2016), NETD is primarily suitable for polyanionic species such as highly sulfated GAGs (Wu et al., 2018).

Tandem MS of GAGs using NETD was first carried out using a linear quadrupole and ion trap instrument, but the modest resolving power and low mass accuracy limited the ability to identify highly charged structures (Leach et al., 2017). In later approaches, NETD was applied to GAGs using fourier transform ion cyclotron resonance mass spectrometry (FTICR) (Leach et al., 2011). Additionally, NETD experiments have been applied in distinguishing CS and DS providing low degrees of sulfate losses, high resolution and adaption to different instrument types (Leach et al., 2011; Wolff et al., 2010; Wu et al., 2019). It generates structurally informative fragments on sulfated HS oligosaccharides that facilitates the assignment of 3-*O*-sulfation on synthetic HS isomers (Wu et al., 2018).

7.3 | Other electron-based fragmentation methods

Another technique that is efficient in fragmenting multiply negatively charged ions like GAGs is electron detachment dissociation (EDD) (Agyekum et al., 2015; Leach et al., 2008; Oh et al., 2011; Wolff, Amster, et al., 2007; Wolff, Chi, et al., 2007; Wolff, Laremore, Busch, et al., 2008). EDD is based on the introduction of low-energy electrons to trapped gas-phase ions. An electron beam detaches an electron from a negatively charged precursor ion, which causes the formation of odd-electron species. The release of potential energy from the odd-electron results in fragmentation and cross-ring cleavages of the precursor ion. This information enables the characterization of posttranslational modifications and the primary sequence of proteins, peptides, carbohydrates and oligo-nucleotides (Adamson & Håkansson, 2007; Anusiewicz et al., 2005). EDD has also been shown to be highly valuable for studying GAGs—being successfully applied for identifying sulfation on GAG tetrasaccharides (Wolff, Amster, et al. 2007) and decasaccharides (Kailemia et al., 2013). EDD also enables the distinction between IdoA and GlcA present in tetrasaccharides (Wolff, Chi, et al., 2007) and distinguishing the CS/DS chains of bikunin (Chi et al., 2008). Additionally, EDD fragmentation allowed the assignment of C-5 stereochemistry in 2-*O*-sulfated uronic acid epimers

among ten synthetic HS tetrasaccharides (Agyekum et al., 2015). Lastely, a combination of IM-MS and EDD is possible and was used to separate and identify isomeric GAG gas-phase ions (Kailemia et al., 2014).

The fragmentation of singly charged ions with electronic excitation from a high-energy electron beam (>20 eV) is referred to as electron-induced dissociation (EID) (Jones et al., 2015). EID generates fragments on hexuronic acid residues with even- and odd-electron glycosidic and cross-ring products. Fragmentation of hexuronic acid residues by EID (and also EDD) are hypothesised to occur because they exhibit labile behavior under electronic excitation (Leach et al., 2012). Mass spectra recorded from EID fragmentation are similar for GlcA and IdoA in GAG epimers (Wolff, Laremore, Aslam, et al., 2008).

Although fragment-based analyses of GAGs is very promising and has been applied successfully on several occasions, it remains difficult to reliably identify isomers in the molecule at different sulfate positions. Sulfates are very labile, fragment easily and as a result it is unclear whether the detected sugar fragments have already been desulfated or whether they still carry sulfate groups. Detected cross-ring fragments in negative mode cannot be distinguished from one another because they are isomers. This makes it difficult to identify and determine the position of sulfate groups. Ion mobility spectroscopy (IMS) can fill this gap and delivers structure-specific data capable of differentiating isomers (Hofmann et al., 2015; Miller et al., 2020). Additionally, structure- and sequence-specific information may in the future be obtained from the UV photodissociation mass spectrometry (Brodbelt et al., 2020) which showed promising first results and recently became commercially available.

8 | METHODS AND APPLICATIONS OF GLYCOSAMINOGLYCAN ANALYSIS

8.1 | Disaccharide analysis

The analysis of GAG disaccharides is one of the most often used approaches for GAG characterization with several methods having been reported over the years. First, GAGs have to be effectively depolymerized to be able to completely resolve their structures. Either bacterial polysaccharide lyase enzymes or deaminative cleavage via nitrous acid are commonly used to generate disaccharides from GAG chains (Ernst et al., 1995; Sun et al., 2017). Many chromatography-based methods are generally applicable for disaccharide analysis and most of them utilize MS detection (Gill et al., 2013; Staples & Zaia, 2011), UV absorbance (Lu et al., 2010; Yang et al., 2012) or fluorescence (Lu et al., 2010; Volpi et al., 2014). The detection via fluorescence usually increases the sensitivity of GAG disaccharide analysis in comparison to UV detection (Yang et al., 2012). On the chromatography side, RP chromatography, RP-IP chromatography, SAX chromatography, PGC chromatography, and HILIC are the most common approaches in disaccharide analysis.

Many GAG monosaccharide and disaccharide analyzes were performed using RP chromatography. After derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) unique disaccharides were detected and a discrimination between Hep/HS, CS/DS, and HA was possible (Zhu et al., 2014). The separation and detection of HS disaccharides within a single run of 18 min was carried out using a selected ion recording precolumn RP derivatization with AMAC (Antia et al., 2017). The separation efficiency can be further increased by the addition of ion-pairing agents like tributylamine (Yang et al., 2011), *n*-pentylamine (Doneanu et al., 2009) or *n*-hexylamine (Solakyildirim et al., 2010). RP HPLC was expanded by using tetrabutylammonium bisulfate as an ion-pairing reagent. With this approach disaccharides and oligosaccharides were separated and quantified by UV detection without additional derivatization (Galeotti & Volpi, 2013). In recent years advanced MS technologies have been introduced resulting in disaccharide profiles that can be used as biomarkers in cancer diagnosis. For example, a novel LC-tandem MS approach utilizing dibutylamine with RP chromatography was developed that enabled the determination of previously unknown methylation and sulfation patterns on the nonreducing ends of CS/DS disaccharides from human breast carcinoma (Persson et al. 2018). Further, a coupled SAX IM-MS approach using ammonium bicarbonate as eluent was shown to be highly beneficial for the analysis of complex Hep/HS di- and oligo-saccharides. The use of ammonium bicarbonate buffer for GAG elution improved the resolution through both weaker dissociation and conformational coordination of the ammonium across the sulfate groups (Miller et al., 2016). Also, PGC chromatography yielded promising results for GAG disaccharide analysis (Karlsson et al., 2005). PGC-MS combined with gel electrophoresis and chemical release of digested GAG fragments resulted in the determination of di- and hexasaccharides from CS (Estrella et al., 2007). The high-resolving power afforded by PGC also led to the high-sensitive detection of oligosaccharide isomers (Miller et al., 2017).

In addition to SAX, PGC and RP applications, HILIC chromatography in combination with MS is also proficient in separating and detecting depolymerized GAGs. A

HILIC-ESI-Fourier transform-MS platform was developed to characterize commercially available LMWHs (Li, Zhang, et al., 2012). In this study the HILIC stationary phase relied on a cross-linked diol rather than amide chemistry and provided highly resolved chromatographic separation as well as stable and high efficiency ionization. The use of organic solvents, low backpressure and superior evaporation makes HILIC highly suitable for LC-MS. Rare disaccharide compositions of Hep, enoxaparin, and nadroparin have been successfully investigated using HILIC–quadrupole time-of-flight MS (Ouyang et al., 2016). Additionally, a HILIC-MS/MS fragmentation technique for analysis of LMWHs prepared by nitrous acid depolymerization was developed (Sun et al., 2017). For the analysis of GAG disaccharides derived from prostate cancer tissues, a weak anion exchange retention mechanism in combination with HILIC was shown to be useful (Turiák et al., 2018).

MS detection, and partly fluorescence detection, have been used for the quantitation of GAGs (Volpi et al., 2014; Yu et al., 2019). A pure LC-MS based quantification of 23 sulfated disaccharides from porcine cartilage and ligament was performed using selected reaction monitoring (Osago et al., 2014). In addition, isotope reductive amination tags were used for the quantitative analysis of Hep, LMWHs, and CS (Bowman & Zaia, 2010; Lattová & Perreault, 2013; Mangrum et al., 2017). Highly sulfated Hep isomers were also quantified by IMS using NETD (Wei et al., 2019).

8.2 | Analysis of glycosaminoglycan oligo- and polysaccharides

GAG sequencing is arguably fundamental to fully understand protein-GAG interactions, in particular in the context of developing new GAG therapeutics. Traditionally, MS-based analyzes of GAG oligo- and polysaccharides have been carried out following bottom-up and top-down approaches. In a bottom-up approach, GAGs are either chemically or enzymatically cleaved into smaller chains before chromatographic separation and MS analysis. Although bottom-up approaches are widely used and enable sensitive identification of di- and oligosaccharides, the associated sample preparation is time-consuming, and therefore challenging to implement in high-throughput analyzes (Li et al., 2014a; Santos et al., 2017). In top-down approaches, intact GAG polysaccharides are analyzed and provide sequence information of the GAG chain without the need of extensive sample preparation steps. However, high sensitivity, selectivity, and high resolution are required to get comprehensive results. Therefore, different MS techniques are generally used for GAG characterization in top-down approaches (Robu et al. 2018). The most significant limitation of these techniques remains sulfate loss during fragmentation. However, this phenomenon can be reduced by a combination of charge state modifications and metal ion adduction as the following examples of tandem MS approaches demonstrate.

Existing LC-MS approaches were applied towards small GAG chains like bikunin and LMWHs (Li, Ly, et al., 2012). Broad charge distributions and sulfate losses were found in experiments using LC-MS in negative mode with FTICR-MS. A targeted complexation of the sulfate groups with metal cations increases their stability and maximizes fragmentation (i.e., MS/MS) of the ring structures and glycosidic bonds (Chi et al., 2008). A combination of top-down and bottom-up techniques has proven to be the most sensible and promising approach, for example, by carrying out soft depolymerization to obtain long oligosaccharides or using capillary electrophoresis before MS analysis. Methods including both top-down and bottom-up approaches have been established by various research groups (Lin et al., 2017; Liu et al., 2017a, 2017b).

A comprehensive di- and oligo-saccharide analysis allows the quantification and profiling of GAG molecules, but a detailed structure determination remains challenging. Therefore, sequential chemical derivatization strategies, including permethylation, desulfation, and trideuteroperacetylation were applied (Huang et al., 2013, 2016; Liang et al., 2018). Derivatization at the original sulfation sites prevented information loss due to sulfate group loss and enabled discrimination between HS oligosaccharide sequences by glycosidic bond cleavages. Subsequently, derivatization techniques were combined with LC-MS/MS and resulted in a complete sequence determination of five synthetic GAG oligosaccharides (Huang et al., 2013).

In another tandem MS approach, the complete structural analysis of highly sulfated Hep and HS oligosaccharides was reported. Stabilisation of sulfate groups was achieved through the use of ion suppressors (Staples & Zaia, 2011) and complete deprotonation by Na^+/H^+ exchange or charging during the ESI process (Kailemia et al., 2013). The generation of deprotonated precursor ions was strongly facilitated by the addition of sodium hydroxide. This approach worked for several biological- and synthetic HS oligosaccharides with up to 12 saccharide subunits and up to 11 sulfate groups.

Within the last twenty years, approaches based on FTICR-MS have been applied, providing detailed structural information of GAG sequences (Laremore et al., 2010). At the MS^1 level, a mass accuracy of 1 ppm was achieved (Russell et al., 2002). FTICR-MS

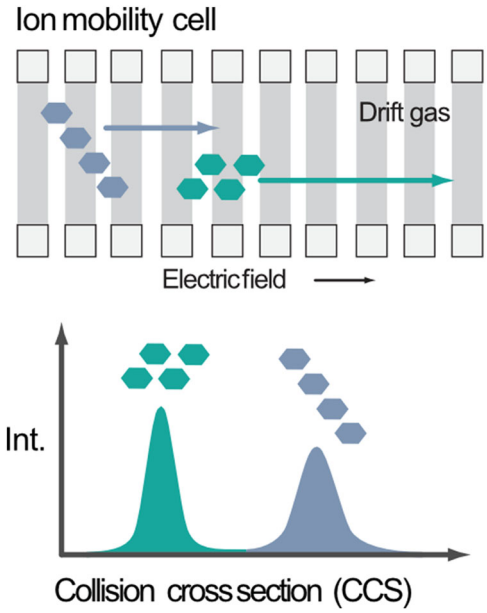

FIGURE 4 Principle of IMS. Ions are separated according to their size, shape, and charge. Gas-phase ions are guided by an electric field and collide with drift gas ions in the cell. Larger ions (blue) experience more collisions with the gas and have longer drift times compared to smaller (green) ions. IMS, ion mobility spectrometry [Color figure can be viewed at wileyonlinelibrary.com]

measurements have allowed the assignment of composition, the determination of chain length, and the number, and type of modifications. The number of saccharides and the sulfation degree of GAGs from bikunin were investigated using FTICR-MS, which was the first complete sequencing of bikunin GAG chains (Chi et al., 2008).

Recently, the sequence motifs of DS chains from decorin and depolymerized CS and DS fragments with different sulfation patterns were investigated using a combination of SEC, SAX, gel electrophoresis, and tandem MS (Yu et al., 2017). In addition, the sequence of an *N*-unsubstituted Hep/HS hexasaccharide was successfully determined. The sequencing approach included depolymerization of GAG chains by deaminative degradation, SEC and RP-IP LC-MS (Liang et al., 2015).

9 | ION MOBILITY-MASS SPECTROMETRY

MS is a great tool for biomolecule analysis, however isomer seperation remains an analytical challenge. One way to resolve this challenge is to fragment the species of interest using CID (Harvey, 2000; Harvey et al., 1997) or ETD (Han & Costello, 2011). Another strategy involves derivatization, for example, permethylation, followed by MS^n fragmentation in ion-trap instruments (Ashline et al., 2005; Huang et al., 2016; Liang et al., 2018; Sheeley & Reinhold, 1998; Viseux et al., 1998; Weiskopf et al., 1998). All these strategies have their advantages and disadvantages, but what all have in common is that their application is time-consuming and the data interpretation is complex. Therefore, it would be desirable to add an additional dimension to the commonly applied MS or LC-MS approaches to separate isomers without further structural modifications. IMS fulfills this requirement by providing an additional dimension of biomolecular separation and therefore structural information (Bohrer et al., 2008; Hofmann & Pagel, 2017; Kanu et al., 2008; Lapthorn et al., 2013). IMS involves separation of biomolecules by their charge, size, and shape. The analyte ions are guided by a weak electric field through a cell filled with inert neutral gas (He, N_2). Compact ions collide less frequently with the inert gas than larger ions and can traverse the cell faster (Figure 4) (Gabelica & Marklund, 2018; Hoffmann et al., 2017).

Over the last years, several IMS systems have become commercially available. They differ significantly in the type of electric field, duty cycle and the achieved IMS resolution. The first commercial instrument was the Waters Synapt HDMS (Waters MS-Technologies) which uses the traveling-wave ion mobility spectrometry (TWIMS) technique (Harvey et al., 2015). TWIMS instruments consist of a stacked-ring ion guide on to which a travelling voltage pulse is applied to propel the ions through the gas-filled IMS cell (Cumeras et al., 2015; Giles, 2013; Giles et al., 2010; Hoffmann et al., 2014). In the following years, other manufacturers followed with their own IMS instruments. The Agilent 6560 IM-TOF LC/MS (Agilent) instrument uses the drift tube ion mobility spectrometry (DTIMS), a traditional IMS technique that has been previoulsy used in home-built IMS instruments. In DTIMS a uniform electric field along the axis of the drift tube is used to transport the ions through the IMS cell (Cumeras et al., 2015). Another well established technique is the field asymmetric waveform ion mobility **s**pectrometry (FAIMS) which has been commercialized by various companies, for example, in the FAIMSPro Interface by Thermo Fisher (Thermo Fisher Scientifc). The general working principle of FAIMS is based on a strong asymmetric oscillating electric field (Hale et al., 2020). This provides exceptionally high resolution, albeit at the drawback that the mobility behaviour is difficult to predict and that ions might be unintentially activated—a problem of particular relevance in GAG analysis. Finally, Bruker Daltonics (Bremen, D) introduced several generations of TIMS-TOF instruments, which use rapped ion mobility

spectrometry (TIMS) technology. In TIMS ions are trapped and held stationary in a moving stream of gas until they are released from the TIMS analyzer according to their mobility (Michelmann et al., 2015; Ridgeway et al., 2018).

Except in FAIMS, the measured parameter in all IMS techniques is the drift time of the particular analyte ions. The drift time is an instrument-dependent value that is affected by multiple parameters such as IM gas pressure, temperature, and most importantly, the applied electric field. In many cases drift times can be converted into mobilites, which can subsequently be used to determine an instrument independent value—the rotational-averaged collision-cross section (CCS) (Dziekonski et al., 2018; Gabelica & Marklund, 2018; Pagel & Harvey, 2013). CCSs are inherrent molecular properties that relate to the average area of the molecule colliding with the drift gas and can therefore be used for structural classification. Analagous to e glucose units (GU) generated by dextran calibration in HILIC chromatography of glycans (Gautam et al., 2020), IMS-derived CCS values have the potential to be implemented as an additional search parameter for database analyzes of complex carbohydrates (Struwe et al., 2016).

The potential of IMS for the analysis of complex glycan mixtures is remarkable. IMS can be interfaced to different mass spectrometry platforms offering multi-dimensional separation (Delvaux et al., 2021), while retaining the major advantages of classical MS such as low sample consumption and short analysis time. Therefore, it is not surprising that IMS was previously applied succcesfully to separate and distinguish glycan isomers. For example, it was shown that synthetic oligosaccharide isomers can be successfully separated using TWIMS, despite only minor differences in their regio- and stereochemistry at a singly glycosidic bond (Hofmann et al., 2015). Furthermore, it was demonstrated that fragment-based approaches can identify fucosylated (Sastre Toraño et al., 2019) and sialylated linkages (Hofmann & Pagel, 2017; Hofmann et al., 2017; Lane et al., 2019) which can be used to determine characteristic features on milk oligosaccharides and complex *N*-glycans (Harvey et al., 2018b; Pagel & Harvey, 2013). The combination of mass measurement and IM-MS analysis also enabled the assignment and identification of isomeric glycopeptides and separation into different charge states (Creese & Cooper, 2012; Zhu et al., 2015). IM-MS can also be used to characterize *O*-glycan standards with subtle structural differences illustrating its potential in biological and structural studies (Zheng et al., 2016).

As illustrated by the few aformentioned examples, IMS has been extensively applied towards analysis of isomeric glycan mixtures (Harvey & Struwe, 2018; Harvey et al., 2018a; Jin et al., 2019). In contrast to *N*-glycans and small synthetic oligosaccharides, GAGs are much more challenging. Aside from from their vast structural complexity and polydispersity, the biggest obstacle is their highly labile nature as discussed above. Despite these challenging requirements, various combinations of IMS techniques and electron-based dissociation methods were used to characterize complex GAG mixtures. Six synthetically produced Hep/HS-like octasaccharide isomers were analyzed by TWIMS-MS and tandem mass spectrometry (Miller et al., 2015). The octasaccharides were isomeric with regard to GlcA or IdoA positioning. Using IM-MS, it was shown that structures including GlcA exhibited a more compact formation, whereas IdoA-containing oligosaccharides were more extended. Additionally, it was observed that the change from IdoA to GlcA in specific locations resulted in conformational distortions, which were also reflected by different spectra with unique sets of diagnostic fragment ions. Interestingly, a correlation was found between the formation of glycosidic product ions under low energy conditions and the GlcA group containing isomers. Ultilising the same collision energy for octasaccharide isomers containing IdoA and GlcA, the GlcA-isomers resulted in a higher ion intensity. The specific behaviour of GlcA groups enabled the complete sequencing of GlcA and IdoA positions in each of the four positions located in each octasaccharide structure (Miller et al., 2015).

These experiments revealed that small changes, present within large biopolymers can have a major impact on the structure, which in turn influences GAG function. It is therefore crucial to identify all strcutral details in bioactive GAGs. A first step in this direction was the recent emergence of "Shotgun" IM-MS Sequencing ($SIMMS_2$) (Miller et al., 2020). Here, HS oligosaccharides were fragmented in the IM-MS instrument and CCS values were determined for each fragment. Subsequently, the acquired data was matched against known values for 36 fully defined HS oligosaccharides up to decasaccharides. This database comparison permitted a precise sequence determination of validated standards and unknown, natural occurring GAG species including variants with rare but biologically relevant 3O-sulfate groups. This approach also allowed to elucidate structure–activity relationships by identifying two fibroblast growth factor inhibiting hexasaccharide structures from a HS oligosaccharide library screening (Miller et al., 2020).

Another study used IM-MS to identify conformational changes that occur in fully sulfated Hep octasaccharides after the successive addition of metal ions (Seo et al., 2011). Various metal ions induced conformational changes in Hep oligosaccharide structures.

Consequently, the interaction of Hep and Hep-binding proteins was altered and can result in a multitude of different biological functions (Kjellén & Lindahl, 2018; Peysselon & Ricard-Blum, 2014; Weiss et al., 2017).

Also, other IMS and IMS-based techniques have been successfully applied for GAG analysis. For example, FAIMS was combined with FTICR-MS and used to separate isomeric and isobaric GAG oligosaccharides before EDD fragmentation (Kailemia et al., 2014). Additionally, TIMS technology enables high resolution and high ion transmission, which showed great promise for separating GAG isomers (Wei et al., 2019). TIMS-NETD-MS/MS, has already been successfully used to characterize highly sulfated HP and HS oligosaccharides without loss of sulfate groups (Wei et al., 2019). Positional isomers can be determined by prior calibration with synthetic tetra- and hexa-saccharide standards, including sulfation positional isomers. In addition to direct GAG analyzes, IMS is often used in combination with native MS and CID to investigate protein GAG interactions (Zhao et al., 2015, 2017). Optimising IMS and MS parmaters is critical as even the smallest changes can significantly impact separation or ion stability (Song et al., 2020).

Despite the outstanding potential of IMS, not all isomers can be easily resolved and it is also not straightforward to predict the success (or failure) of a particular gas-phase separation. A comprehensive analysis of GAG oligosaccharides including all structural features usually requires the combination of several orthogonal techniques.

10 | NOVEL DEVELOPMENTS IN GAS-PHASE ION SPECTROSCOPY

Infrared (IR) ion spectroscopy is a powerful tool for the identification of functional groups in biomolecules. Classical IR spectroscopy measures the attenuation of the incident light and is broadly applied for the analysis of solutions and solids. On the other hand, performing similar absorption spectroscopy experiments on gas-phase ions is typically not possible. The concentration at which ions can be trapped in a mass spectrometer is determined by the space-charge limit and is usually several orders of magnitude below what would be required for classical spectroscopy. To circumvent this problem, action spectroscopy techniques are used. As the name implies, it measures an action, a response of molecules to resonant absorption of photons at a specific wavelength. The monitored action can range from the fragmentation of covalent bonds, to changes in electronic transitions or the dissociation of a weakly associated molecular tag (Oomens et al., 2006).

Infrared multiple photon dissociation (IRMPD) spectroscopy is a type of IR action spectroscopy in which the action is the dissociation or fragmentation after sequential absorption of multiple photons. After absorption of each individual photon, the photon energy is distributed throughout the molecule via intramolecular vibrational redistribution (IVR). As a result, the internal energy of the ion increases gradually, leading to hot ions which eventually dissociate into smaller fragments. Monitoring the fragmentation yield as a function of the wavelength using monochromatic light leads to an IR spectrum (Cismesia et al., 2018; Seo et al., 2017). Over the last years, tunable benchtop laser systems became readily available and IRMPD spectroscopy was applied to study of a broad rang of biomolecules in the gas-phase, including GAGs (Song et al., 2020). For example, GlcNAc3S and GlcNAc6S were studied by IRMPD spectroscopy and characteristic spectroscopic patterns for sulfation were identified through comparison to reference standards (Schindler et al., 2017). Furthermore, it was found that individual hexuronic acid epimers in HA tetrasaccharides can be distinguished from their unique gas-phase IR fingerprints. Similarly diagnostic vibrational spectra were reported for GalNAc4S and GalNAc6S (Renois-Predelus et al., 2018).

Despite its straightforward instrumentation and broad applicability, IRMPD spectroscopy suffers from peak broadening and red shifting of bands arising from the thermal activation of ions during multiple photon absorption (Oomens et al., 2006). Additionally, the conformational flexibility of larger oligosaccharides may lead to several coexisting conformers at room temperature, which absorb at different wavelengths. The resulting spectra are therefore usually broad and congested, which limits the application of IRMPD spectroscopy to smaller mono- and disaccharides (Mucha et al., 2019).

A technique to overcome the limitations of spectral congestion is cryogenic gas-phase IR spectroscopy. Here the conformational flexibility of ions is suppressed by cooling of the ions to ultracold temperatures. Further spectral broadening is prevented either using single-photonic activation or by cooling of the ions during the irradiation with multiple photons. The resulting consequences for spectral quality can be significant.

A highly powerful, but also technically elaborate technique in cryogenic gas-phase IR spectroscopy is based on the encapsulation of analyte ions in superfulidic helium nanodroplets (González Flórez et al., 2016). Here, ions are generated by nano ESI, selected according their m/z values in a quadrupole and acculumated in a cryogenic ion trap with a temperature of 90 K. Subsequently, ions are picked up by traversing superfluid helium nanodroplets and cooled down to their equilibrium

temperature at 0.4 K. The ions, embedded in helium nanodroplets, are then irradiated by IR photons from a tunable, narrow-bandwidth laser. Upon resonant absorption the ions are released from the nanodroplets and can be detected via time-of-flight analysis (Figure 5).

This technique was applied for characterizing a set of six synthetic trisaccharide isomers that only differed in the composition, connectivity or configuration at one particular glycosidic bond (Mucha et al., 2017). The obtained spectra were highly resolved with vibrational bands being only a few wavenumbers wide and diagnostic to minute structural details. This enabled the straightforward differentiation of all possible types of isomerism in glycans. Using the same technique, IR signatures of characteristic fragment ions were recorded and revealed structural details of gas-phase fucose migration in fucosylated glycans (Mucha et al., 2018). Further studies studies highlighted the unique role of a mobile proton in this migration process (Lettow et al., 2019). IR spectroscopy in helium nanodroplets was also successfully used to study GAG oligosaccharides up to pentasaccharides. Vibrational bands specific for sulfate groups were found to in a spectral range in which no other diagnostic vibrations occur (Lettow et al., 2020b). In a further study, HS tetrasaccharide diastereomers were analyzed and revealed a strong spectra-structure correlation arising from specific intramolecular ion interactions (Lettow et al., 2020a).

Other, more widely used techniques for cold-ion spectroscopy are based on cooling of the ions in cold-ion traps. To record an IR spectrum, these experiments typically monitor the dissociation of weakly bound, noninteracting, messenger tags upon irradiation with a tunable benchtop laser (Khanal et al., 2017; Roithová et al., 2016; Voronina et al., 2016). Messenger tags, for example, atoms or small molecules (N_2, H_2), form weakly bound ionic complexes with the analytes and result in a lower dissociation threshold of the system. The absolute temperature in these experiments is with 10–70 K, considerably higher than the subkelvin temperature in helium nanodroplets. However, the spectral quality and with that the diagnostic potential is almost identical. Using this technique it was, for example, possible to distinguish five singly sulfated GAG disaccharide isomers based on their unique vibrational fingerprints (Khanal et al., 2017).

11 | OUTLOOK

GAGs are a physiologically and pharmacologically relevant class of complex carbohydrates that are fundamental for a range of cellular processes. Their complex sulfation patterns and epimerization variants make their structural analysis exceedingly complex, especially compared to other glycoconjugates. Consequently, GAG sequencing requires the most sophisticated methods, both preparative and analytical,

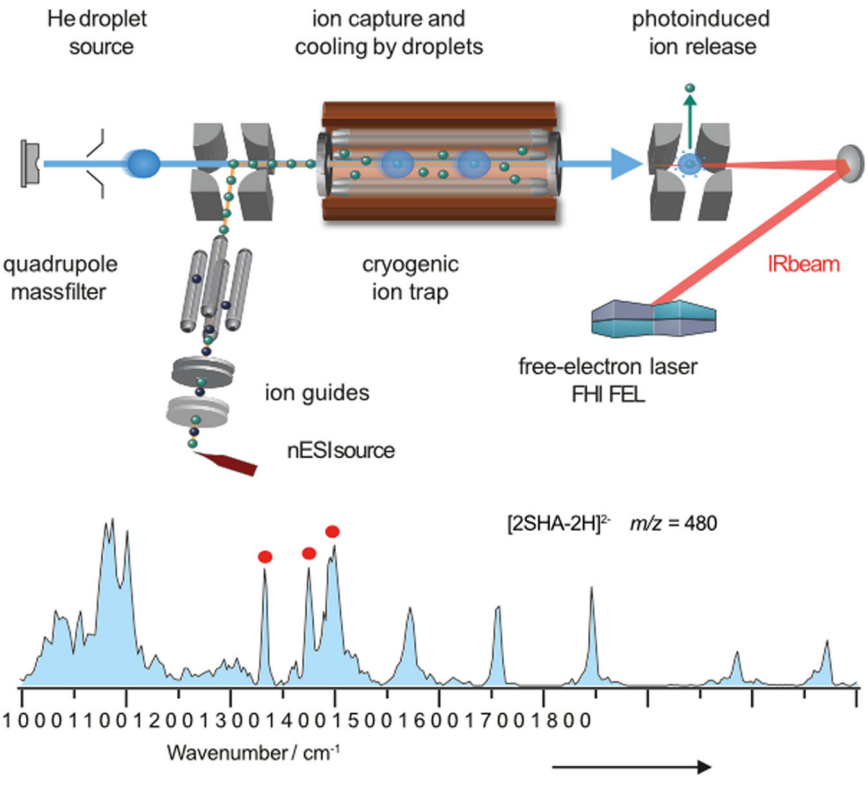

FIGURE 5 Schematic diagram of an IR-MS instrument. Fragment ions are accumulated in an ion trap and their mass-to-charge ratio is measured via time-of-flight analysis. Helium droplets pick up trapped ions, which are immediately cooled to 0.37 K. Subsequently, the droplets are irradiated with monochromatic, high-intensity IR radiation, for example, using a FEL. FEL, free-electron laser [Color figure can be viewed at wileyonlinelibrary.com]

to generate information-rich structural information. Suitable preparative steps are essential to achieve sufficient purity and concentration for a given analysis. Applied chromatographic techniques will continue to evolve and yet existing approaches, such as HILIC, SAX, and RP-IP chromatography, are poweful but also require specific adaptations and modifications to purify complex GAG mixtures. However, stand-alone chromatographic methods require large amounts of samples and involve either UV chromophores or fluorescence tags for detection. In addition, optimized depolymerization processes are necessary to generate a reproducible, average distribution of GAG oligosaccharides.

Chromatography coupled to mass spectrometry will likley remain the workhorse for GAG sequencing and novel dissociation methods, namely those that are electron-based, will propel the field. Similarly, IMS technology offers exceptionally high benefits and diverse possibilities, especially for the separation of isomers and the differentiation of analytes in general. Furthermore, emerging IR spectroscopy techniques, and in particular those working at cryogenic temperatures, enable an increased resolving power and nearly limitless possibilities to differentiate isomers and their fragments. Currently, gas-phase spectroscopy techniques require specialized light sources and sophisticated intstrumentation, which limits their application to a few labs worldwide. However, the development of user-friendly instruments and tunable benchtop laser systems is progressing rapidly. Gas-phase spectroscopy technology might therefore find a way into broader application in the future.

ACRONYMS

AMAC	2-Aminoacridon
CCS	collisional cross section
CID	collision induced dissociation
CS	chondroitin sulfate
CSPGs	chondroitin sulfate proteoglycans
CTA	cetyl-trimethyl-ammonia
DS	dermatan sulfate
DSPGs	dermatan sulfate proteoglycans
DTIMS	drift tube ion mobility spectrometry
ECD	electron capture dissociation
ECM	extracellular matrix
EDD	electron detachment dissociation
EID	electron induced dissociation
ESI	electrospray ionization
ETD	electron transfer dissociation
FAIMS	High-field asymmetric-waveform ion-mobility spectrometry
FTICR-MS	fourier transform ion cyclotron resonance mass spectrometry
GaGs	glycosaminoglycans
Gal	galactose
GalNAc	*N*-acetylgalactosamine
Glc	glucose
GlcA	glucuronic acid
GlcN	glucosamine
GlcNAc	*N*-acetylglucosamine
HA	hyaluronic acid
Hep	heparin
HILIC	hydrophilic interaction chromatography
HS	heparansulfate
IdoA	iduronic acid
IMS	ion mobility spectrometry
IM-MS	ion mobility mass spectrometry
IR	infrared
IRMPD	infrared multiple photon dissociation
KS	keratan sulfate
KSPGs	keratan sulfate proteoglycans
LC-MS	liquid chromatography mass spectrometry
LMWHs	low molecular weight heparins
MALDI	matrix assisted laser desorption ionization
Man	mannose
MS	mass spectrometry
NEDD	negative electron detachment dissociation
NETD	negative electron transfer dissociation
PGC	porous graphitic chromatography
PGs	proteoglycans
Proc	procainamide
RP-IP	reversed-phase ion pairing
SAX	strong anion exchange
SEC	size exclusion chromatography
SRM	selected reaction monitoring
TIMS	trapped ion mobility spectrometry
TWIMS	traveling wave ion mobility spectrometry
UA	uronic acid
Xyl	xylose

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