Spectroscopy 18 (2004) 185–201
IOS Press

A structural analysis of heparin-like glycosaminoglycans using MALDI-TOF mass spectrometry

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Abstract. Mass spectrometry (MS) techniques have spear-headed the field of proteomics. Recently, MS has been used to structurally analyse carbohydrates. The heparin/heparan sulfate-like glycosaminoglycans (HLGAGs) present a special set of difficulties for structural analysis because they are highly sulfated and heterogeneous. We have used a matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-MS) technique in which heparin fragments are non-covalently bound to basic peptides of a known mass, so as to limit in-source desulfation and hence afford an accurate mass. We examined a range of different sized fragments with varying degrees of sulfation. The potential of combining the MALDI-MS technique with enzymatic digestion to obtain saccharide sequence information on heparin fragments was explored. A disaccharide analysis greatly assists in determining a sequence from MALDI-MS data. Enzymatic digestion followed by MALDI-MS allows structural data on heparin fragments too large for direct MALDI-MS to be obtained. We demonstrate that synthetic sulfated oligosaccharides can also be analysed by MALDI-MS. There are advantages and limitations with this methodology, but until superior MS techniques become readily accessible to biomedical scientists the MALDI-MS method provides a means to structurally analyse HLGAG fragments that have therapeutic potential because of their ability to bind to and functionally regulate a host of clinically important proteins.

1. Introduction

Glycosaminoglycans (GAGs) are linear co-polymers consisting of disaccharide units of a uronic acid and a hexosamine. Although heparin, a highly sulfated GAG, has been in clinical use as an anti-coagulant for decades it is only in the last decade that the wide range of biological activities that involve heparin or the heparin-like GAG (HLGAG), heparan sulfate, have been realised. In part, the lack of appreciation of the importance of these molecules has arisen because of a lack of understanding of the ways in which quite subtle changes in GAG structure can affect the extent to which they bind proteins.

Heparins and heparan sulfates are very diverse; being polydisperse mixtures of structurally related chains. Heparin and heparan sulfates are all attached to a core protein during their biosynthesis. Synthesis initiates through the assembly of a linkage tetrasaccharide, GlcUAGalGalXyl on serine residues in the

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proteoglycan core protein. This initiating reaction occurs at specific sites on the core protein defined by a motif of serine–glycine flanked by one or more acidic amino acids [8]. The addition of an α1,4-linked GlcNAc to the tetrasaccharide commits the chain to a HLGAG structure. Polymerization then takes place with the alternating addition of GlcUAβ1,4 and GlcNAcα1,4 residues. As the chain polymerizes it undergoes a series of modifications that include N-deacetylation, N-sulfation, epimerization of the GlcUA to IdoUA and O-sulfation at various positions [8].

In nature, heparan sulfates are covalently linked to a core protein and are part of a proteoglycan. Heparan sulfate proteoglycans are abundant in living organisms, being components of the extracellular matrix as well as being associated with the cell surfaces of most animal cells either as integral membrane components or by binding to cell surface proteins [8,9]. Heparin is found exclusively in mast cell secretory granules, and is released when mast cells respond to external stimuli [5]. Hence, heparan sulfates are likely to be the physiological ligands of many heparin-binding proteins. Recent genetic experiments have provided compelling evidence that the assembly of normal heparan sulfate structures is essential for the complete development of animal embryos. Experiments done in both *Drosophila* and mice indicate that mutations in the enzymes involved in the biosynthesis of heparan sulfate chains result in marked abnormalities [10,20]. Collectively the data indicate that certain critical stages of development require specific heparan sulfate structures. However, to date only certain abundant or biologically specific sequences have been structurally characterised. A major technical difficulty is that endogenous heparan sulfate is available in very small quantities and is structurally heterogeneous. Heparin is readily available and therefore is a useful alternative GAG to use to develop structural analysis methodologies that may be applied to heparan sulfate. Heparin-like GAG sequences of physiological and pharmacological relevance will only be discovered when efficient methods that use minimal material and are applicable to all HLGAG sequences are developed.

The demonstration that a specific pentasaccharide sequence within heparin is responsible for the activation of the protease inhibitor, antithrombin III [2,15] has stimulated efforts to define other biologically active motifs within heparin. The benefits of mass spectrometry to the structural characterization of biomolecules, which have been witnessed in other areas of biology, have not been readily transferred to the area of heparin-like glycosaminoglycans. The introduction of a complexation technique by Biemann, Sasisekharan and colleagues has enabled some of the throughput and sensitivity features of MALDI MS to be extended to GAG analysis [7,11,12,19,23]. In this technique, a cationic peptide, typically an arginine–glycine repeat is mixed with the MALDI matrix compound and the GAG oligosaccharide. The analytes are observed as an ion-pair with the peptide, allowing the mass of the oligosaccharide to be deduced by deducting the mass of the peptide. This method overcomes much of the in-source desulfation and metal adduct formation that occurs when HLGAGs are analyzed by negative-ion MALDI and is more sensitive by an order of magnitude. A particularly powerful extension of the method is the ability to perform chemical and enzymatic digestion of oligosaccharides and directly obtain spectra of the products, without cleanup, thus allowing determination of the sequence of the constituent disaccharides. We describe procedures used in our laboratory to obtain mass spectra of HLGAGs and emphasize some limitations of the technique. For a more general introduction to mass spectrometry, readers are referred to an earlier article in this series [21].

The use of nanospray sources appears to have overcome the desulfation and sensitivity issues of earlier attempts with electrospray ionization (ESI) mass spectrometry analysis of GAG oligosaccharides [18] and offers an alternative to the MALDI technique described herein. However, the ubiquity of suitable MALDI mass spectrometers, coupled with the ease of sample preparation and analysis, even for those with little experience with MS, will make the MALDI technique attractive for many researchers.

2. Structure of HLGAGS

The building blocks of HLGAGs can be considered as disaccharides consisting of an uronic acid residue (either glucuronic or iduronic acid) glycosidically linked to an *N*-acetylglucosamine residue. Complexity is introduced by various degrees of sulfation at specific sites. Most common are O-sulfation at O-2 of the uronic acid, O-6 of the glucosamine, and substitution of N-sulfate for N-acetyl (Fig. 1). Less common modifications include sulfation at O-3 of the glucosamine and the occurrence of unsubstituted glucosamine residues.

Preparation of HLGAG oligosaccharides is typically achieved by either partial enzymatic (heparinase) digestion or nitrous acid depolymerization. The former has the advantage of introducing unsaturation at C-4 of the uronic acid (Fig. 1) and hence a useful chromophore for chromatographic separations. The mass of the oligosaccharides can be calculated according to Eq. (1). This equation is easily altered to account for modifications at the reducing terminus (e.g., by either derivatization or the use of nitrous acid generated oligosaccharides, Table 1) and the last two terms reduce to zero for oligosaccharides produced by heparinase digestion.

Table 1
Mass values for reducing terminus (*RT*) and non-reducing terminus modifications (*NRT*) in Eq. (1)

Modification	Variable
Unsaturation at non-reducing terminus	$NRT = -1.01$
Reducing terminus	$RT = 1.01$
C-4 hydroxyl at non-reducing terminus	$NRT = 17.01$
Loss of unsaturated uronic acid (e.g., by glycuronidase)	$NRT = -159.12$
–GalGalXyl–OH (linkage region)	$RT = 457.41$
Reduced	$RT = 3.01$
Anhydromannose	$RT = -58.06$
Anhydromannitol	$RT = -56.05$
Hydrazone or glycosylamine ($RNHNH_2$ or RNH_2) conjugate	$RT = (RNHNH_2 \text{ or } RNH_2)_{mass} - 17.01$
Reduced hydrazone or reductive amination conjugate	$RT = (RNHNH_2 \text{ or } RNH_2)_{mass} - 15.0$

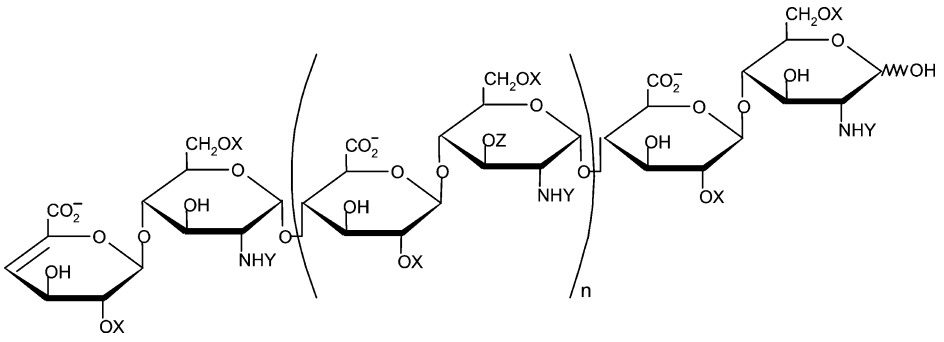

Fig. 1. Schematic of the structure of HLGAGs produced by heparinase action. Oligosaccharides derived from within a heparin chain will have an even numbered DP and contain an unsaturated uronic acid residue at the non-reducing terminus and a glucosamine residue at the reducing terminus. The uronic acid residue may be either iduronic or glucuronic acid. In this figure and generally throughout this review, no attempt is made to distinguish between these two epimers. X = SO_3^- or H (less common); Y = Ac or SO_3^-; Z = H or SO_3^- (less common); $n = 0, 1$, etc.

$$Mass = \big((DP/2) \times 379.32\big) + (p \times 80.06) + (n \times 38.03) + RT + NRT, \quad (1)$$

where:

DP = degree of polymerization,
p = no. of O-sulfates,
n = no. of N-sulfates,
RT = mass difference by substitution for OH at reducing terminus,
NRT = mass difference by substitution at unsaturation of non-reducing terminus
($RT = +1.01$ and $NRT = -1.01$ for oligosaccharides produced by heparinase and adhering to the structure in Fig. 1).

The mass of each potential DP6 oligosaccharide structure is listed in Table 2. The number of different structures with a particular mass can be calculated from Eq. (2) and is shown in Table 3. This analysis overestimates the degree of complexity that is likely to be observed, but nonetheless, alludes to the inherent redundancy of a particular mass.

$$\binom{n}{k} \times \binom{p}{l} = \frac{N!}{n!(N-n)!} \times \frac{P!}{p!(P-p)!}, \quad (2)$$

where

p = no. of O-sulfates,
P = maximum number of O-sulfates = $3 \times DP/2$,
n = no. of N-sulfates,
N = number of glucosamine residues = $DP/2$.

It is apparent from Eq. (1), that the smallest mass difference between oligosaccharides (of the same DP) is 4 Da. Thus, it is possible to distinguish the number of N- and O-sulfates and DP of a particular oligosaccharide if a mass accuracy of 2 Da can be achieved, which is well within the specifications of modern MALDI mass spectrometers. In practice, the DP has already been predetermined with a high degree of confidence.

Table 2
The mass (Da) of each of the potential DP6 isomers corresponding to the structure shown in Fig. 1

No. of O-sulfates (p)	No. of N-sulfates (n)			
	0	1	2	3
0	1137.96	1175.99	1214.02	1252.04
1	1218.03	1256.05	1294.08	1332.11
2	1298.09	1336.12	1374.14	1412.17
3	1378.16	1416.18	1454.21	1492.24
4	1458.22	1496.25	1534.27	1572.30
5	1538.28	1576.31	1614.34	1652.36
6	1618.35	1656.37	1694.40	1732.43
7	1698.41	1736.44	1774.47	1812.49
8	1778.48	1816.50	1854.53	1892.56
9	1858.54	1896.57	1934.59	1972.62

Table 3
The number of possible sequences corresponding to the structure shown in Fig. 1 for each mass listed in Table 1. The total number of sequences is 4096

No. of O-sulfates (p)	No. of N-sulfates (n)			
	0	1	2	3
0	1	3	3	1
1	9	27	27	9
2	36	108	108	36
3	84	252	252	84
4	126	378	378	126
5	126	378	378	126
6	84	252	252	84
7	36	108	108	36
8	9	27	27	9
9	1	3	3	1

3. MS of HLGAGs

3.1. Sample preparation: Preparation of pure oligosaccharides

For reliable interpretation of the spectra, the highest purity oligosaccharides are of paramount importance. Typically, a heparin digest is separated into size fractions by size-exclusion chromatography [22,24] (an example chromatogram is shown in Fig. 2A). The size fractions, which contain an even DP number, are then fractionated further by strong anion exchange (SAX) chromatography. In many instances, we have found it necessary to resort to 2-dimensional SAX to obtain pure oligosaccharides. Polymeric stationary phase media are stable over a wide range of pH, enabling the second dimension separation to be run at a different pH, which causes changes in chromatographic selectivity (Fig. 2B). Separations at pH 3 and pH 7.5 have proven useful. The order of separation, i.e. pH 3 and then 7.5 or *vice versa* does not appear to be critical. The different separation at the second pH appears to offer a vastly superior method than re-chromatography under the same conditions. Separations at higher pH, although feasible, may lead to degradation.

In essence, other separation modes can be substituted, but due to the high mass redundancy of HLGAGs, efforts to establish the purity of an oligosaccharide beyond a single separation are encouraged. Regardless of the method for fractionation of oligosaccharides, involatile salts should be removed. This can be readily achieved with Sephadex G-25 for DP 6 or greater, whereas smaller saccharides are more efficiently desalted with Bio-Gel P-2. When large volume fractions are collected, either by wide bore columns or pooling of multiple injections it is advisable to concentrate the oligosaccharides by rechromatography prior to desalting. Due to the strong retention of the sulfated oligosaccharides on SAX media, it is sufficient to dilute to less than 0.3–0.4 M NaCl and reapply directly to a suitable column [3]. Either the same column or anion exchange cartridges with syringe adapters are suitable. After washing, the oligosaccharides are eluted in a small volume of 2.5 M NaCl with step wise gradients.

3.2. Procedure: Materials

1. Synthetic peptide, $(Arg–Gly)_{19}–Arg$, RP-HPLC purified, $>95\%$. Some vendors may not undertake the synthesis of this peptide. We have purchased from Auspep. Prepare a stock solution at 0.5 mM and store at $-20°C$. Small aliquots (5–10 μl) can be frozen for at least 3 months at $-20°C$.

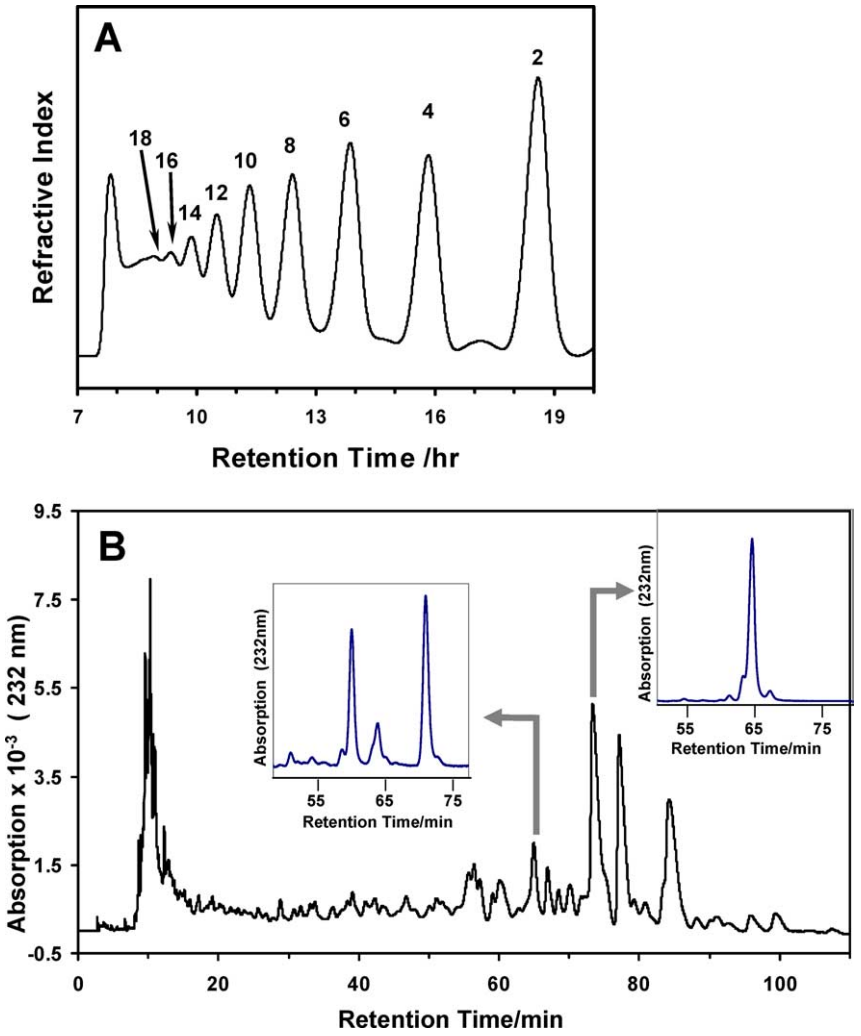

Fig. 2. A: Size-exclusion chromatography of heparin fragments digested with heparinase I. The DP of each peak is indicated. B: Two-dimensional anion exchange chromatography of a DP 10 pool (produced by partial depolymerization of heparin with heparinase I) using a Dionex Propac PA1 column (250 × 4.6 mm). The major chromatogram shows the first separation at pH 7.5 (10 mM Tris buffer). The insets show rechromatography of the indicated fractions at pH 3 (10 mM phosphoric acid). In each instance gradients of increasing NaCl concentration were used. Total gradient time was 100 minutes for the first separation and 60 minutes for the second.

2. Anion exchange resin, hydroxide form (Biorad). Store at 4°C.
3. Caffeic acid (3,4-dihydroxycinnamic acid) matrix (Fluka). Prepare fresh solutions at 10 mg/ml in 50% acetonitrile.

3.3. Protocol

1. Maintain all solutions in an ice bath.
2. Working concentration of the peptide solution is 50 μM. Dilute an aliquot of the stock solution and add 20–50 mg of the anion exchange resin (e.g., that which adheres to the outside of a 200 μl pipette tip). Mix thoroughly and centrifuge briefly to settle the beads.

3. Mix 10 μl of 50 μM peptide supernatant with 90 μl of caffeic acid matrix solution and place 9 μl aliquots into vials/centrifuge tubes. Larger volumes can be prepared by adhering to the 1 : 9 ratio.
4. Dilute peptide mass calibration standards in caffeic acid solution (with or without peptide added) and spot onto the plate. Some plate designs allow the calibration mixture to be placed between the designated sample positions, although manual sample positioning is required during acquisition.
5. Quality control samples: Sucrose octasulfate (Toronto Research Chemicals, Carbomer) and sulfated cyclodextrins (Sigma, Carbomer) are useful test substrates/quality control samples that can be included with every batch. Prepare each quality control substance at two concentrations, e.g., 5 μM and 50 μM.
6. Add 1 μl of either oligosaccharide solution (5–100 μM) or quality control sample to a tube containing peptide/matrix mix. Vortex, centrifuge briefly and spot 1 μl onto a MALDI plate. Allow to air dry.
7. Occasionally, better crystallization and spectrum are achieved with a more dilute solution of the oligosaccharide, so prepare a 2- or 3-fold dilution of the oligosaccharide and add 1 μl of this diluted solution to another vial containing peptide/matrix mix, vortex and spot 1 μl onto a MALDI plate (Note B). A 2nd dilution may also be prepared, if desired.
8. Acquire spectra. We used a PerSeptive Biosystems (Applied Biosystems, Melbourne, Australia) Voyager reflectron time-of-flight instrument fitted with a 337 nm nitrogen laser. Delayed extraction was used to increase resolution (22 kV, grid at 93%, guide wire at 0.15%, pulse delay 150 ns, low mass gate at 2000, 50 shots averaged).

Notes

A. Calibration of the mass spectrometer is most readily achieved with peptide mass calibration standards, which are available from a variety of vendors. Typically, these cover the range up to $\sim$5 kDa.
B. Small diffuse crystals are best for sample acquisition. Long needles rarely yield useful spectra.

3.4. Examples and limitations

The technique was validated using a DP 4 heparin fragment known to be the common repeating tri-sulfated unit of heparin $(UA2SGlcNS6S)_2$. The observed mass of the fragment, determined after subtraction of the mass of the protonated peptide, is 1153.6 Da which is very close to the theoretical mass of 1154.9 Da and there is good signal to noise ratio (Fig. 3A). Figure 3B shows the spectrum of a DP 10, in which the deduced mass is 2 Da in error of a saccharide bearing 4 O-sulfates and 4 N-sulfates. Some de-sulfation of the fragment is also evident as a secondary peak appearing 80 Da smaller than the parent. Such mass errors are not uncommon with this particular MALDI technique and can compromise the ability to discern the degree of sulfation. To reduce the potential for incorrect assignment of the degree of sulfation we routinely subtract the observed mass of the peptide from that of the complex and reject spectra in which the observed mass of the peptide is more than 2 Da from the theoretical mass. The DP 10 in question eluted in a region of the chromatogram surrounded by oligosaccharides of higher degree of sulfation and prompting suspicion in the tentative assignment. We will subsequently demonstrate that this oligosaccharide contains an unusual modification.

The structural complexity of a pool of DP 6 heparin fragments obtained by heparinase digestion is readily observed using this MALDI technique (Fig. 3C). Although it is possible to assign structural information to the main peaks, it cannot be discounted that a small amount of desulfation has occurred within the mass spectrometer and contributes to some of this complexity. Some of the peaks have two

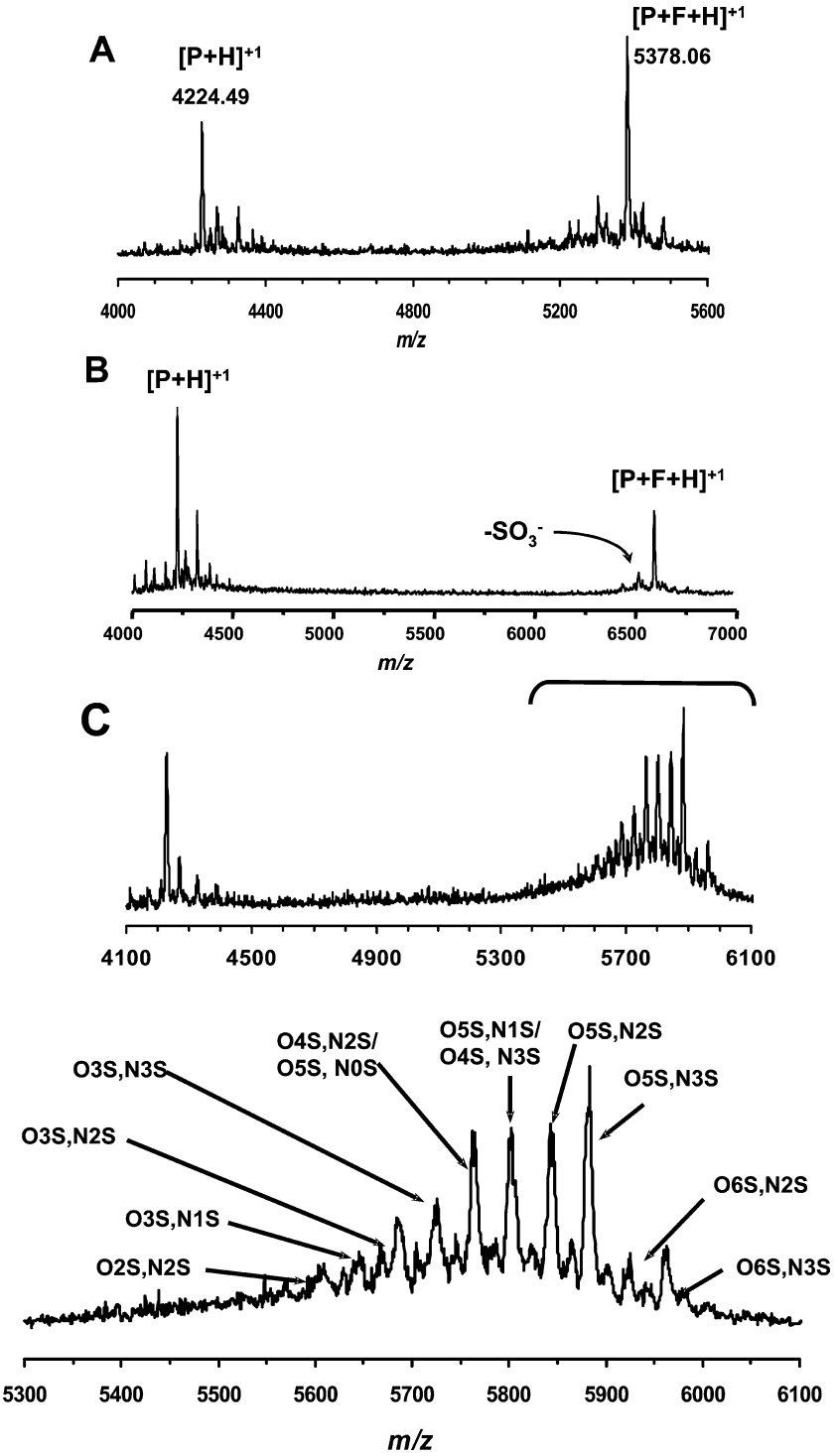

Fig. 3. Spectra of A: DP 4 $(UA2SGlcNS,6S)_2$; B: DP 10, measured mass 2367 Da, theoretical for 4 *O*-sulfates and 4 *N*-sulfates is 2369, desulfation is indicated, and C: DP 6 pool (produced by partial depolymerization of heparin with heparinase I), the region under the bracket has been expanded and is shown as the bottom spectrum.

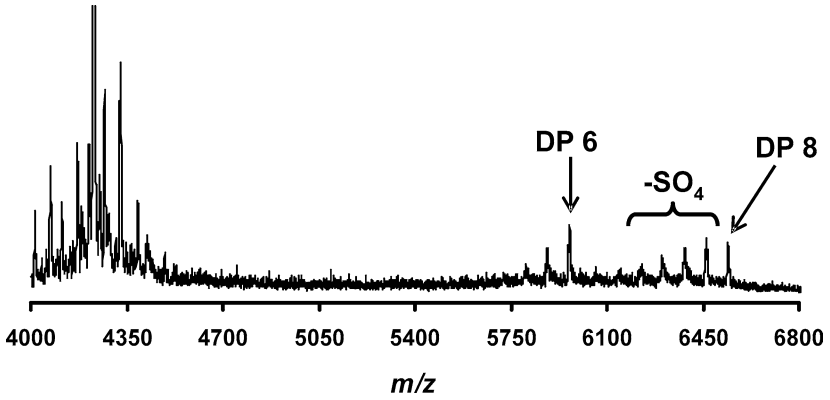

Fig. 4. Spectrum of a mixture of the DP 6, (δ-UA2SGlcNS6S) $(UA2SGlcNS6S)_2$ and DP 8 (δ-UA2SGlcNS6S) $(UA2SGlcNS6S)_3$. The concentration of the DP 6 was 3.3 μM and the DP 8 25 μM.

structures assigned. These peaks are quite broad and as the two structures differ by only 4 Da they are not resolved in the mixture. Clearly, the more species present the lower the apparent resolution. A HPLC SAX separation of the DP 6 pool and re-analysis by MALDI of the fractions indicated significant structural heterogeneity that could not be attributed to loss of sulfates within the mass spectrometer (data not shown).

There appears to be an upper size limit for obtaining a spectrum of heparin oligosaccharides, for we have been limited to a DP 10 containing 13 sulfates, although we have obtained spectra for DP 12 oligosaccharides containing fewer sulfates. Related to this feature are the differences in sensitivity observed for oligosaccharides of differing size. Although we do not contend that this MALDI technique is quantitative, the effect of size on the quality of spectra can be visualized by comparing the signal intensities of mixtures of oligosaccharides, as is shown in Fig. 4. For the oligosaccharides of DP 6 and DP 8 containing the same repeating trisaccharide structure, similar signal intensities occur when the DP6 is at approximately 1/8th the concentration of the DP 8. Moreover, the degree of de-sulfation induced within the source of the MALDI is far more pronounced with the DP 8. This effect is most pertinent when performing sequencing, for which the goal is to generate mixtures of different fragments, as discussed below.

4. HLGAG analogues

An adjunct to the use of HLGAGs as mediators of the biological activity of a particular protein is the application of carbohydrate analogues, which are typically homo-oligosaccharides (e.g., maltodextrins and dextrans) that have been chemically sulfated. It is possible to obtain spectra of such highly sulfated species, even though the number of sulfates exceeds that of the largest HLGAG observed. We show this for γ-cyclodextrin sulfate (Fig. 5A), for which it is possible to observe species corresponding to 16 sulfates. However, it is difficult to validate the technique and hence comment upon whether the distribution evident in the spectrum of γ-cyclodextrin reflects that of the sample or desulfation within the MALDI leads to an underestimate of the degree of sulfation. Fractionation of such mixtures is not straightforward [4,26] and other spectroscopic techniques (e.g., NMR) cannot clarify the degree of sulfation. An average degree of sulfation obtained from elemental analysis is of limited value except in cases in which complete sulfation is achieved. The difficulty in obtaining complete chemical sulfation of these larger

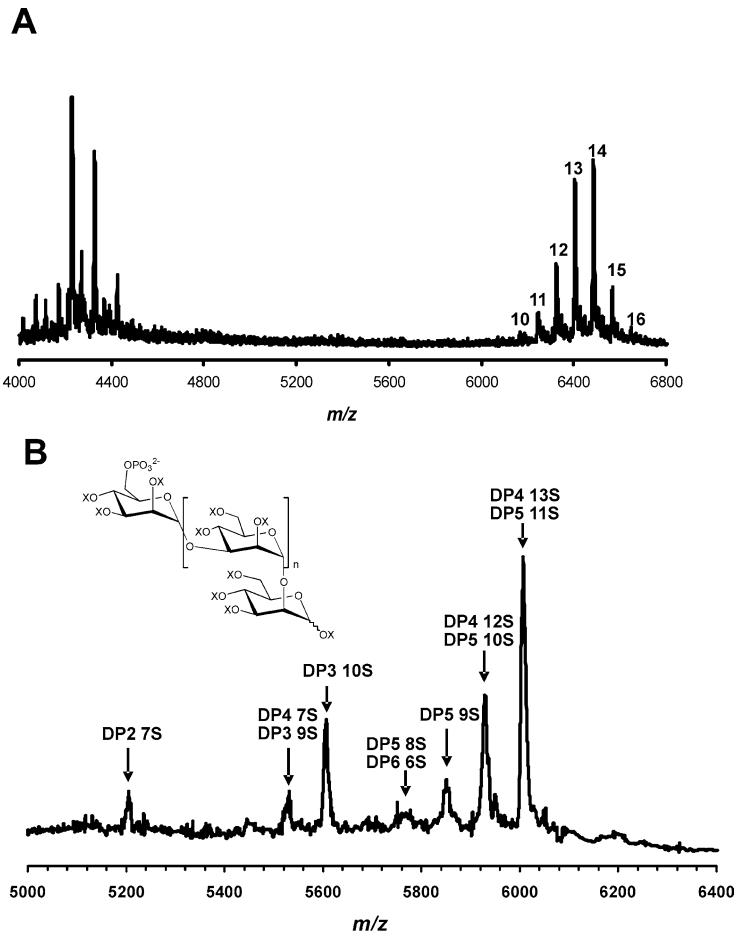

Fig. 5. Spectrum of A: γ-cyclodextrin sulfate and B: the mixture of sulfated, monophosphorylated mannose oligosaccharides. The insert shows the structure of these mannose oligosaccharides. The numbers in panel A correspond to the number of sulfates. X = H or SO_3^-; $n = 0$–4, but 60% of $n = 3$ and 30% of $n = 2$.

oligosaccharides ensures that there will be a spread of undersulfated species, which presumably would follow a Poisson distribution. A spectrum of a mixture of phosphosulfomannans [4] differing both in degrees of sulfation and DP is shown in Fig. 5B. The structure of the oligosaccharides shown in the Fig. 5B insert. This spectrum highlights some of the limitations of the technique. The mass of a neutral hexose unit is 162 Da, only 2 Da different from the mass of 2 sulfate residues. Thus, for example, it is not possible to resolve a DP 5 from a DP 4 with 2 more sulfates in a mixture of the two. Furthermore, due to the mass accuracy that is routinely achieved, it is not always possible to discriminate between these two possibilities, even if the sample is pure. Many of the peaks in Fig. 5B have therefore been assigned two possible structures.

5. Sequencing

The measurement of the mass of the oligosaccharide enables the number of N- and O-sulfates and DP to be determined. Due to the high mass redundancy of HLGAGs, little else can be deduced about

the structure with this information alone. The substitution pattern of the sulfates along an oligosaccharide is equivalent to determination of the sequence of the constituent disaccharides. It then becomes evident that the disaccharide composition becomes an immensely powerful piece of information with the capacity to dramatically simplify the number of candidate sequences. For example, there are 64350 sequences containing a DP 10 with 8 O-sulfates and 3 N-sulfates, but this reduces to at most 120 sequences if the disaccharide composition is known. If the sequence contains two copies of a disaccharide then the number of sequences reduces even further, e.g., for the composition (U2SGlcNAc)(U2SGlcNAc6S)(U2SGlcNS)(U2SGlcNS6S)$_2$, there are only 24 sequences.

Determination of disaccharide composition can be accomplished by a variety of analytical methods. In essence, each of these methods consists of an exhaustive digestion (by either chemical or enzymatic means) with subsequent chromatographic or electrophoretic separation and analysis. An advantage of enzymatic digestion is that authentic standards are available for identification and quantification of the components. Heparinase digestion based methods cannot distinguish between iduronic and glucuronic acid and the enzymes do not cleave adjacent to uncommon modifications such as 3-O-sulfated glucosamine residues [5]. Nitrous acid digestion can overcome both these limitations, but disaccharide standards are not available and the separation of all possible components is challenging. The latter is compounded by the fact that only 90% of the products will be the desired 2-anhydromannose disaccharides and the remainder will be ring contraction products. The choice of separation and detection methodology will most likely be governed by the desired sensitivity (i.e. sample consumption) as well as the equipment, skills and experience present, in a particular laboratory.

The sequence of the component disaccharides can then be deduced by partial digestion of the oligosaccharide with analysis of the products by MALDI. For spotting the samples on the MALDI sample plate a seeded crystallization technique is used, which is more tolerant of complex buffer systems.

5.1. Preparation of heparinases

Heparinases must be prepared in a MALDI compatible buffer and it is necessary to remove the BSA, which is added to many commercial preparations to aid stability. BSA can be efficiently removed by passage over an anion exchange column or cartridge using ethanolamine acetate as eluent. The sample may be dissolved in 10–20 mM Tris buffer pH 7.5 containing less than 30 mM NaCl. The BSA is retained and heparinase elutes in the void. Prior to use, block the column with an injection of BSA and equilibrate with the low ionic strength buffer (check buffer contents). The heparinase fraction is then adjusted to the composition of the MALDI sequencing buffer. As it may be necessary to fine tune the working concentration of enzyme after an initial trial, dilute a portion to approximately 100 nM in MALDI sequencing buffer by using the specifications in Table 4. We have observed heparinases prepared in this buffer system to be active after 5 months storage at -20°C.

Table 4
The properties of heparin lyases

Enzyme	Mol. Wt (kDa)	Activity[1,2] (IU/mg)	Activity of 100 nM soln
Heparinase I	43	∼110	0.47 IU/ml
Heparinase II	85	∼20	0.17 IU/ml
Heparinase III	73	∼50	0.36 IU/ml

[1]The activity shown is an example, which is representative of commercial lots. Users should consult manufacturer specifications of each batch.
[2]One international unit (IU) is equivalent to 600 Sigma units.

5.2. Procedure: Preparation of a seeded plate (adapted from [25])

1. Prepare a fresh solution of caffeic acid at 20 mg/ml in 50% acetonitrile.
2. Spot 1 μl at all positions on the sample plate, or as many positions as desired (seeded and unseeded, or conventionally prepared samples can be analyzed on the same plate).
3. Allow to air dry (thoroughly).
4. Place a microscope slide or similar glass plate on the dried crystals. Force the slide down with a large eraser or thumb and twist the slide to crush the matrix crystals.
5. Remove the slide and brush with a tissue to remove loosely adhered crystals to leave a thin coating of powdered matrix at each sample position.

5.3. Sample preparation

1. Prepare all matrices and standards as described in points 1–6 for direct analysis, except place 4.5 μl caffeic acid/peptide mix into tubes.
2. Mass calibration for analyses of seeded samples should be performed with standards and quality control samples spotted on seeded wells.
3. During use, maintain the heparinases at -20°C by use of a cryo box.
4. To 5 μl sample in a small centrifuge tube, add 1 μl of heparinase. Mix thoroughly (vortex by gentle flicking) and briefly spin to settle the contents.
5. At predetermined time points, e.g. 0.5, 3, 10, 30 minutes withdraw a 0.5 ml aliquot and mix with 4.5 μl of caffeic acid/peptide mix.
6. Spot 1 μl onto a seeded well and allow to dry for several minutes (the spot does not need to dry completely).
7. When several spots (e.g., other samples or time points) have been allowed to dry for a few minutes, aliquot 10 μl of water onto each spot. Blot each of the drops by placing the corner of a small piece of tissue at the edge of each drop. Add another 10 μl of water to each spot and blot each spot again. Allow the spots to dry completely.
8. Proceed with other samples.
9. Acquire spectra and analyze as before.

5.4. Interpretation of spectra

The actions of the various heparinases are summarized in Fig. 7. Briefly, heparinase I will only cleave between two N-sulfated disaccharides if the uronic acid towards the reducing end is also 2-O-sulfated. Heparinase III will only cleave between two disaccharides if the same 2-O-sulfation is absent. Heparinase II is far more general and has both exolytic (processive from the non-reducing end) and endolytic activity, unlike heparinase I, which is predominantly exolytic. Although heparinase I preferentially cleaves iduronic residues and heparinases II and III prefer glucuronic residues, caution should be used in applying such criteria unless it can be demonstrated that it is possible to discriminate between the two epimers under the digestion conditions used.

The initial production of the oligosaccharides using a heparinase predicates the sulfation pattern at each end of the oligosaccharide, unless it is an oligosaccharide derived from chain termini. Thus, preparation of oligosaccharides by depolymerization with heparinase I will yield oligosaccharides bearing one of only two disaccharides at the non-reducing terminus (i.e. UA2SGlcNS6S or UA2SGlcNS) and one of the four N-sulfated disaccharides at the reducing terminus. This enhances interpretation of spectra,

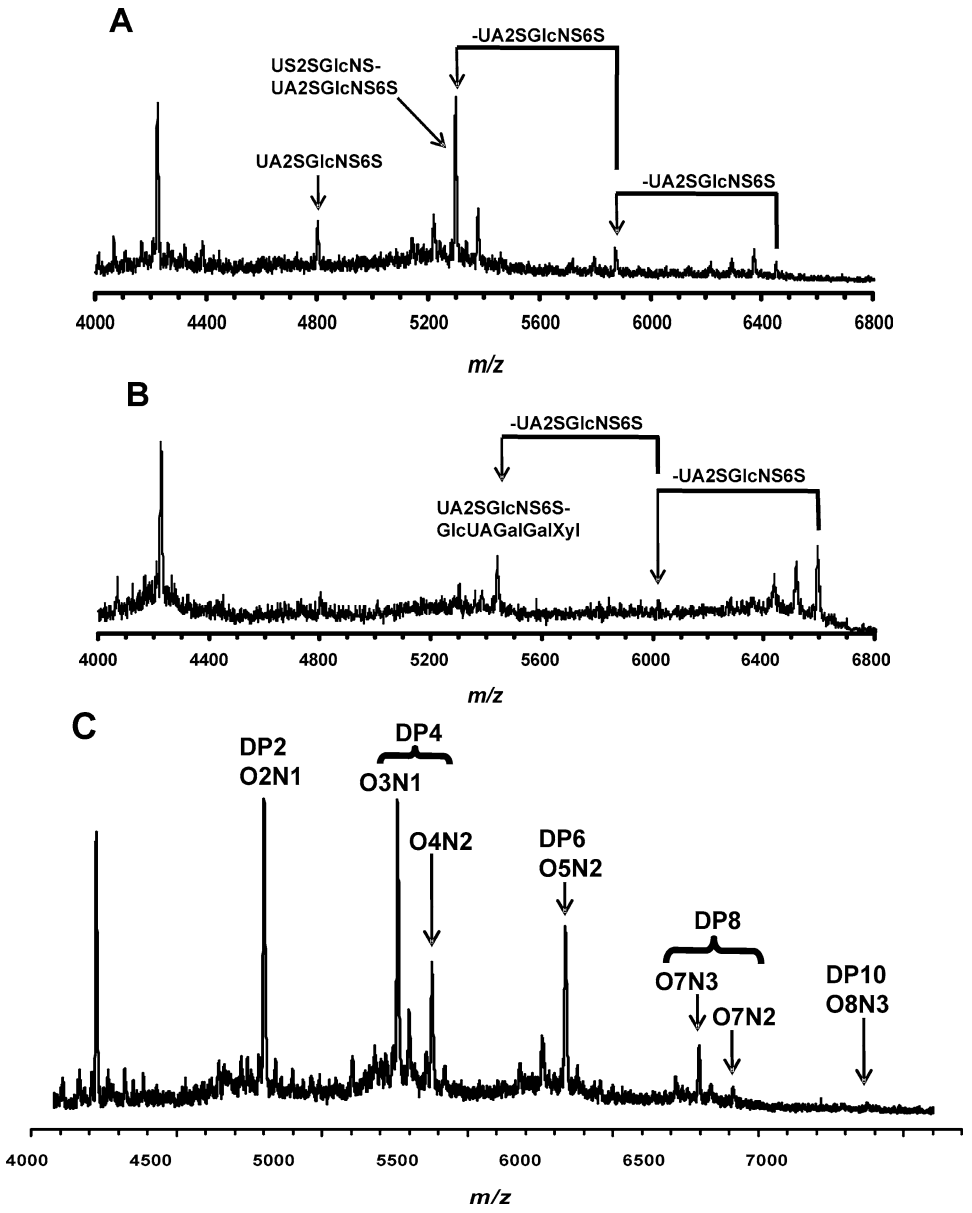

Fig. 6. Spectra of the heparinase digestion products of A: DP 8 containing the disaccharide composition UA2SGlcNS-$(UA2SGlcNS6S)_3$, B: DP 10 shown in Fig. 3B, containing a linkage region oligosaccharide and the trisulfated disaccharide and C: DP 12 digested with heparinase II. The number of N-sulfates and O-sulfates indicated by the mass of each peak is shown.

for it further constrains the possible sequences. Finally, since DP 4 substrates are cleaved only slowly by the heparinases little significance should be associated to the fact that they persist during digestion reactions.

An example of sequencing a DP 8 fragment that had been produced by depolymerization with heparinase I is shown in Fig. 6A. This fragment with a disaccharide composition of (UA2SGlcNS)(UA2SGlc-

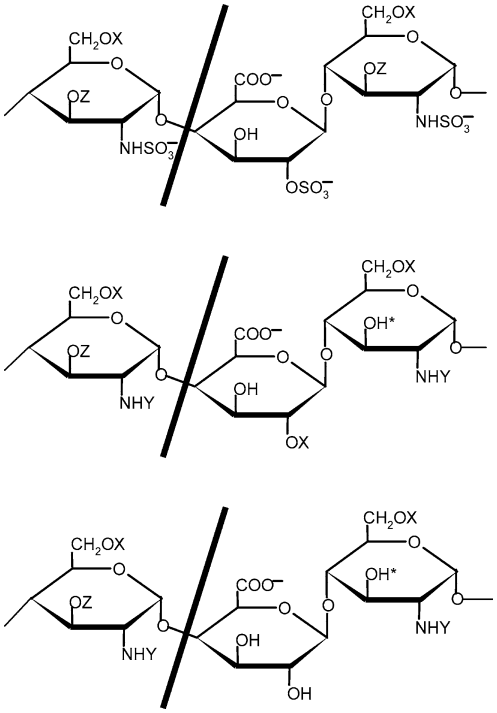

Fig. 7. The cleavage action of heparinases I, II and III, from top to bottom. In each instance, the glycosidic bond is cleaved and unsaturation is introduced at C-4 of the uronic acid residue. Note that sensitivity to heparinases II and III require that the C-3 OH of the glucosamine residue (marked with an asterisk) must be free; sulfation will prevent cleavage by these enzymes. X = SO_3^- or H (less common); Y = Ac or SO_3^-; Z = H or SO_3^- (less common).

NS6S)$_3$, was digested with heparinase I and re-analysed by MALDI. After 10 min of digestion the predominant peak is the tetrasaccharide UA2SGlcNS-UA2SGlcNS6S. Also evident are the parent and a DP 6, which has lost one UA2SGlcNS6S (Fig. 6A). The tetrasaccharide has resulted from the loss of two UA2SGlcNS6S units. Thus, at least one of the terminal disaccharides is UA2SGlcNS6S, however it is not possible to determine whether the other terminal disaccharide is UA2SGlcNS or UA2SGlcNS6S. The DP 10 fragment originally shown in Fig. 3B, was re-analysed after heparinase digestion and the spectrum is shown in Fig. 6B. Heparinase digestion cleaved two trisulfated disaccharides and the mass of the remaining large fragment exactly corresponded to one trisulfated disaccharide and the linkage tetrasaccharide (GlcUAGalGalXyl). Hence, the structure is (UA2SGlcNS6S)$_3$GlcUAGalGalXyl and not an oligosaccharide bearing 4 O-sulfates and 4 N-sulfates as suggested by the spectrum acquired by direct analysis.

The final example (Fig. 6C) is the application of this sequencing technique to the structural analysis of a DP 12 fragment that was too large to give spectra. A heparinase II digestion is shown. In the spectrum (Fig. 6C) there is clear evidence of peaks corresponding to DP 2, DP 4, DP 6, and DP 8 and when the spectrum is expanded a small DP 10 peak is evident. Structures can be assigned to all peaks. The disaccharide analysis indicated this DP 12 fragment was composed of UA2SGlcNS6S, UAGlcNS6S and UAGlcNAc6S. The DP 8 and DP 10 structures suggest the presence of two N-acetylated disaccharides and so collectively the data indicate that the parent DP 12 most likely has 10 O-sulfates and 4 N-sulfates.

A heparinase III digestion of this DP 12 would be diagnostic for the location of the non-2-*O*-sulfated disaccharides.

6. Summary

We have demonstrated that MALDI-MS technology is readily applicable to the analysis of HLGAGs. The deduction of considerable structural information from mass data is possible for HLGAGs because of their underlying structural regularity. The technique described here is one developed by Biemann, Sasisekharan and colleagues [7,11,12,19,23] in which HLGAG fragments are analysed as an ion-pair with a basic peptide. The method is quick and easy to perform, it uses very little analyte (0.5–5 pmol) and non-MS specialists can interpret the spectra. This is in marked contrast to many of the early attempts to use MS for HLGAG analysis, which were characterised by abundant multiple adducts of alkali cations, partial loss of sulfate groups and low sensitivity (relative to proteins and peptides) [1,6,13,16].

Nevertheless, a few limitations of this MALDI-MS method should be highlighted. It appears that the signal to noise ratio depends on the degree of sulfation and the size of the oligosaccharide. Spectra could not be obtained for heparin fragments larger than DP 10 and containing more than 13 sulfates, although it was possible to obtain spectra of some DP 12 fragments with less than 13 sulfates. In our hands, desulfation within the source of the MALDI is more pronounced with longer, more highly sulfated HLGAGs. We have shown here, and in an earlier publication, that spectra can be obtained with non-HLGAG saccharides that carry 16 sulfates [4]. Thus, it is a combination of the GAG backbone and the number of sulfates that is problematic.

An attractive aspect of the MALDI-MS method is that it is possible to digest HLGAG oligosaccharides and directly obtain spectra of the cleavage products. Mass data of the cleavage products together with knowledge of the disaccharide composition of the parent HLGAG fragment allows determination of the disaccharide sequence. In some instances cleaving the fragment with heparinases of different specificity is informative for locating particular structures within the sequence. Indeed, following enzymatic cleavage, sequence data can be obtained on HLGAG fragments too large to give spectra directly. For sequence analyses there is no substitute for fragment purity, but as the DP of the HLGAG fragments increases to 12 or beyond obtaining preparations of uniform structure becomes a challenge. Clearly, the MALDI-MS method is useful for visualising structural heterogeneity within a mixture/pool of HLGAG fragments of the same degree of polymerization. In this regard, we have found MALDI MS to be a valuable adjunct to separation methods to probe heterogeneity.

There is considerable interest in developing mass spectrometry methods applicable to analysis of HLGAGs. Recent publications document improvements in electrospray ionization mass spectrometry utilising both microscale and nano-electrospray methodologies for GAG analysis [14,18,27]. Although with these exciting new methods the HLGAG oligosaccharides are observed directly, the spectra are difficult for a non-specialist to interpret and in some cases specialised mass spectrometry hardware is required. As expected, nano-electrospray ionization MS and MALDI-MS produced comparable data when a heptasulfated heparan sulfate octasaccharide was analysed [17]. The ease of the MALDI method means that biomedical scientists can readily obtain structural information on HLGAG fragments that bind clinically relevant proteins. Proteins that bind to and are functionally regulated by HLGAGs include growth factors, chemokines, enzymes and virus receptors; hence, knowledge of the structure of the HLGAG binding motif may provide the basis for novel drugs. The MALDI-MS method is currently providing the means by which the specificity of HLGAG–protein interactions can be determined, and the potential for therapeutics based on HLGAG structures realised.

Acknowledgements

This work was funded by Meditech Research Limited, Melbourne, Victoria, Australia. Special thanks go to Dr Vito Ferro, Progen Industries Limited, Brisbane, Queensland, Australia, for the phosphosulfomannan preparation.

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