Towards GAG glycomics: Analysis of highly sulfated heparins by MALDI-TOF mass spectrometry

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Glycomics is a developing field that provides structural information on complex populations of glycans isolated from tissues, cells and organs. Strategies employing matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) are central to glycomic analysis. Current MALDI-based glycomic strategies are capable of efficiently analyzing glycoprotein and glycosphingolipid glycans, however, little attention has been paid to devising glycomic methodologies suited to the analysis of glycosaminoglycans (GAGs). They are long (up to 200 residues) linear glycans composed of repeating disaccharide units of alternating hexuronic acid (HexA) and D-glucosamine (GlcN) residues (Gallagher et al. 1990; Turnbull et al. 2001). The heparan sulfate (HS) family, including heparin, is one of the most studied GAG types. It is expressed on core proteins in the extracellular matrix and on the surface of almost all mammalian cell types. All the residues are linked by (1→4) bonds. HS chains present a rather organized structure which is determined by the repertoire of enzymes present in each cell type (Gallagher 2001; Esko and Selleck 2002; Murphy et al. 2004). As a consequence of these enzymatic modifications, each GlcN residue can be either N-acetylated (NAc) or N-sulfated (NS), and both can be further O-sulfated (6S). D-Glucuronic residues can be epimerized to L-iduronic residues, and both can be 2-O-sulfated (2S), even if L-iduronic residues are preferred substrates (Rabenstein 2002). Heparin is a highly sulfated HS analogue, which contains a high level of 2-O-sulfated (6S) and GlcNS(6S) residues. A key feature of heparin/HS synthesis is that not all available substrate sites are acted upon by each enzyme, which creates a high degree of structural heterogeneity (Gallagher et al. 1990; Turnbull et al. 2001). However, it is now believed that the modification steps are occurring in discrete regions of the polysaccharide chain, thus creating domains where some of the modifications are more concentrated than anywhere else on the chain (Gallagher 2001).

Because of the great variety of possible permutations arising from the modifications described above, the sequencing of GAGs is still a difficult and not yet routine task, despite the considerable progress that has been made over the past five years (Liu et al. 2002; Saad and Leary 2003; Thanawiroon and Linhardt 2003; Thanawiroon et al. 2004; Saad et al. 2005). The structural elucidation of GAGs has been further complicated by their intractability to some of the key analytical tools developed for glycomic studies, for example matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) (Dell and Morris 2001), which allows N- and O-glycan profiles to be obtained from small quantities of biological material. Until now, MALDI MS has rarely been successfully applied to the analysis of heavily negatively charged glycans such as GAGs. The low extent of desorption induced by the laser for samples containing a large number of acidic groups, together with the facile loss of sulfate moieties in the MALDI source have been major challenges.

Over the past 10 years, considerable efforts have allowed the development of methodologies enabling the precise analysis of tiny quantities of complex mixtures of N- or O-glycans present in a biological sample (Dell and Morris 2001; Haslam et al. 2001). Recently, an equivalent method has been adapted for glycosphingolipid systematic structural analysis (Parry et al. 2007). However, these advances do not encompass all classes of glycans. Indeed, there is currently no glycomic approach for the structural analysis of glycosaminoglycans (GAGs). They are long (up to 200 residues) linear glycans composed of repeating disaccharide units of alternating hexuronic acid (HexA) and D-glucosamine (GlcN) residues (Gallagher et al. 1990; Turnbull et al. 2001). The heparan sulfate (HS) family, including heparin, is one of the most studied GAG types. It is expressed on core proteins in the extracellular matrix and on the surface of almost all mammalian cell types. All the residues are linked by (1→4) bonds. HS chains present a rather organized structure which is determined by the repertoire of enzymes present in each cell type (Gallagher 2001; Esko and Selleck 2002; Murphy et al. 2004). As a consequence of these enzymatic modifications, each GlcN residue can be either N-acetylated (NAc) or N-sulfated (NS), and both can be further O-sulfated (6S). D-Glucuronic residues can be epimerized to L-iduronic residues, and both can be 2-O-sulfated (2S), even if L-iduronic residues are preferred substrates (Rabenstein 2002). Heparin is a highly sulfated HS analogue, which contains a high level of 2-O-sulfated (6S) and GlcNS(6S) residues. A key feature of heparin/HS synthesis is that not all available substrate sites are acted upon by each enzyme, which creates a high degree of structural heterogeneity (Gallagher et al. 1990; Turnbull et al. 2001). However, it is now believed that the modification steps are occurring in discrete regions of the polysaccharide chain, thus creating domains where some of the modifications are more concentrated than anywhere else on the chain (Gallagher 2001).

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Introduction

Glycomics, which aims to define the entire set of glycans present in a defined system whether it be an individual cell type, tissue or whole organism, has considerably benefited from the development and the optimization of mass spectrometry (MS) analytical tools (Dell 1987; Dell et al. 1991; Reason et al. 1991).

Keywords: glycomics/glycosaminoglycans/ionic liquid/MALDI mass spectrometry
limitations of this technology for GAG analysis (Juhasz and Bie mann 1995). The most convenient technique used for the analysis of such compounds is generally Electrospray tandem mass spectrometry (ES-MS/MS) (Saad et al. 2005). In this method, the ionization process is more efficient for heavily charged molecules and it is softer, thus preventing loss of the sulfate groups in the MS experiment. However, ES-MS is not readily adapted for high throughput analyses which would support the development of glycomic strategies for GAGs.

Despite the poor ionization observed, a few attempts to develop MALDI analysis of GAGs or GAG-related compounds have emerged over the past years (Erra-Balsells et al. 2000; Fukuyama et al. 2002; Antonopoulos et al. 2005), the most promising one being the use of ionic liquids (Laremore et al. 2006, 2007), which have been successfully applied to study chondroitin sulfate oligosaccharides (Laremore et al. 2007). However, their potential for glycomic investigations of the more heavily sulfated HS family has not been explored. In this paper, we describe MALDI strategies that have been optimized for the analysis of highly sulfated oligosaccharides and are potentially well suited to high throughput glycomic analysis of GAGs derived from cells and tissues. We demonstrate the potential of crystalline matrices, such as norharmane and 2′,4′,6′-trihydroxyacetophenone (THAP), as well as ionic liquids for the detection of fully sulfated molecular species by MALDI-time-of-flight (TOF) MS. Heparin-derived oligosaccharides as large as decasaccharide carrying up to 13 sulfate groups have been successfully analyzed by MALDI-MS for the first time. Moreover, we show that MALDI-TOF/TOF MS/MS can be employed to provide structural information using either crystalline or liquid matrices.

Results

Analyzes of three isomeric disaccharides by MALDI-TOF MS using crystalline and ionic liquid matrices

Because MALDI-MS of purified heparin-derived oligosaccharides is poorly documented and MS/MS analysis using MALDI-TOF/TOF instrumentation has not previously been described, we chose to start our study with some of the representative disaccharide constituents of the HS family. The purpose here was to assess the usefulness of two different types of matrices, crystalline and liquid, as well as to determine the type of structural information which could be obtained from MS/MS spectra acquired with each of them. Three unsaturated disaccharides, ΔIIS [ΔHexA-GlcNS(6S)], ΔIII IS [ΔHexA(2S)-GlcNS] and ΔIH [ΔHexA(2S)-GlcN(6S)] were analyzed by MALDI-TOF and MALDI-TOF/TOF mass spectrometry using either a crystalline matrix composed of 20% of THAP and 80% of norharmane (TN) or the ionic liquid ImCHCA (see the Materials and methods section for details). Figure 1 shows the spectra obtained for ΔIIS in TN (Figure 1A) and in ImCHCA (Figure 1B). Depending on the matrix used, the molecular ion is either fully sodiated (m/z 540 in ImCHCA) partially sodiated (m/z 518 in both spectra) or fully protonated (m/z 496 in both spectra). The ionic liquid results in a higher rate of sodiation whereas the main species detected using the crystalline matrix is mainly protonated.

![Fig. 1. MALDI-TOF MS spectra of the heparin-derived disaccharide ΔIIS ΔHexA-GlcNS(6S) obtained in (A) a mix of THAP and norharmane or in (B) the ionic liquid ImCHCA. Peaks labelled with an asterisk correspond to adducts of matrix molecules.](image-url)
Nevertheless, both present a certain amount of desulfation (m/z 416) which is low in the ionic liquid spectrum and rather high in the crystalline matrix spectrum (Figure 1A). As the importance of the sulfate group’s stability upon MS/MS experiments is an important factor determining the quality of the structural information which can be potentially obtained by tandem MS, we performed MALDI TOF/TOF MS/MS experiments on each of the three isomeric disaccharides in each of the two matrices.

The three disaccharides were either dissolved in TN or in ImCHCA matrices and were analyzed by MALDI-TOF/TOF MS/MS in negative mode, using the same energy for the three parent ions (see the Materials and methods section). The [M-H]− species were selected for collisional activation and Figure 2 shows the resulting spectra. In order to facilitate the description of the results, we will first focus on the spectra shown in Figure 2A, B and C corresponding to the MS/MS data obtained in crystalline matrix for the m/z 496 peak of ΔIIS, ΔIIIS and ΔIH, respectively. These spectra are consistent with the data obtained in ES-MS/MS (data not shown) on these three disaccharides. As expected, the main fragment ion obtained for each of the three disaccharides corresponds to the loss of a sulfate group (m/z 416). Concomitant loss of water gives the signal at m/z 398. A few characteristic ions generally found in ES-spectra are also present in our MALDI MS/MS data. For example, the peak at m/z 59 only detected in the spectrum of ΔIH, is characteristic of an un-substituted nitrogen atom on the glucosamine residue (see spectrum annotations, nomenclature according to Domon and Costello 1988), ions at m/z 138 and m/z 139, corresponding to

![Fig. 2. MALDI-TOF/TOF MS/MS spectra of the three isomeric heparin-derived disaccharide ΔIIS ΔHexA-GlcNS(6S), ΔIIIS ΔHexA(2S)-GlcNS and ΔIH ΔHexA(2S)-GlcN(6S) using either a mix of THAP and norharmane (TN) or the ionic liquid ImCHCA. Data obtained using TN as matrix for ΔIIS, ΔIIIS and ΔIH are shown in (A), (B) and (C), respectively. Data obtained using ImCHCA as matrix for ΔIIS, ΔIIIS and ΔIH are shown in (D), (E) and (F), respectively. Peaks labelled with an asterisk correspond to fragments of matrix molecules.]
MALDI-MS analyzes of tetra- and hexasaccharides from heparin

Heparin tetra- and hexasaccharides were prepared as described in the Materials and methods section. Data obtained for two representative fractions (dp4, dp6) are presented here. The two fractions were first analyzed by ES-MS to establish their likely content. The ES-MS profiles indicated that the main species were dp4 carrying six sulfate groups and dp6 carrying nine sulfate groups (data not shown). The MALDI-MS spectra obtained for each sample in the two matrices are shown in Figure 3. Figure 3A and B gives the results obtained for the dp4 fraction. In the spectrum acquired using the ionic liquid matrix, the most intense peaks observed in our spectrum correspond to two consecutive exchanges of sodium atoms for hydrogen atoms as represented by peaks at m/z 979, 957, related to peak at m/z 1001 or peaks at m/z 877, 855 related to peak m/z 899. These de-sodiated ions form the main series of peaks at m/z 899, 1059, 957, 855 and 753, the last corresponding to a dp4 carrying a single sulfate group. In principle, ions with low levels of sulfate could be derived from genuine constituents having low levels of sulfation, rather than being products of in-source desulfation of the hexasulfated tetrasaccharide. However, it has been shown occurring upon MALDI ionization (Laremore et al. 2006). The most intense peaks observed in our spectrum correspond to two consecutive exchanges of sodium atoms for hydrogen atoms as represented by peaks at m/z 979, 957, related to peak at m/z 1001 or peaks at m/z 877, 855 related to peak m/z 899. These de-sodiated ions form the main series of peaks at m/z 1059, 957, 855 and 753, the last corresponding to a dp4 carrying a single sulfate group. In principle, ions with low levels of sulfate could be derived from genuine constituents having low levels of sulfation, rather than being products of in-source desulfation of the hexasulfated tetrasaccharide. However, it has been shown

0.2X or 1.3X0 and 0.4X0 or 1.3X1, respectively, are characteristic of either sulfated GlcNS (m/z 138), and 6-O sulfated GlcN(6S) or 2-O sulfated HexA (m/z 139) (Saad and Leary 2003). We also observed ions at m/z 237 and 157, corresponding to the B1 and [B1-SO3] ions of ΔIIIS and ΔIH and ions at m/z 338 and 258 correspond to the Y2 and [Y2-SO3] ions of ΔIIS, the ion at m/z 338 being specific for the GlcNS(6S) residue (Saad and Leary 2003). All these ions have been fully described in ES-MS/MS experiments and are used to unequivocally identify each of these isomeric disaccharides (Saad and Leary 2003; Saad et al. 2005).

MS/MS analyzes of the sodiated parent ions (m/z 540) observed using the ionic liquid matrix are shown in Figure 2D, E and F for ΔIIS, ΔIIIS and ΔIH, respectively. Spectrum in Figure 2F shows a rather unusual profile with the main fragment ion corresponding to exchange of a proton for a sodium ion. This is different from the behavior of ΔIIIS and ΔIIS, where desulfation is the favored pathway. It may suggest that under our conditions, the desulfation is more likely to occur first at the nitrogen atom rather than at the oxygen atom. This would be consistent with the values of the enthalpy of gaseous species SO and SN which have been reported as being of 521.7 and 464 kJ mol⁻¹, respectively (Kerr 2000), indicating that N-sulfation requires less energy to be lost than O-sulfation. In earlier studies, fast atom bombardment (FAB)-MS analyzes of three disaccharides from heparin and HS also showed that desulfation upon ionization was occurring at a higher rate in NS containing molecules (Li et al. 1995). Another difference with the spectra obtained in the crystalline matrix is the presence of fragment ions that are rarely found in ES-MS/MS analyzes (Saad and Leary 2003; Naggar et al. 2004) although they have been observed in FAB-MS/MS experiments (Li et al. 1995). For example, the peak at m/z 319, corresponding to a 2,4A₂ fragment ions, and the peak at m/z 277 corresponding to [C₁-Na-H] ions, are both characteristic of a ΔHexA(2S) present in the spectra of ΔIIIS and/or ΔIH (see Figure 2), and were not observed in the spectra obtained in the crystalline matrix.

Fig. 3. MALDI-TOF MS spectra of representative dp4 and dp6 heparin-derived fractions. (A) Spectrum of the dp4 fraction acquired using ImCHCA as matrix. (B) Spectrum of the dp4 fraction acquired using norhambrane as matrix. (C) Spectrum of the dp6 fraction acquired using ImCHCA as matrix. (D) Spectrum of the dp6 fraction acquired using norhambrane as matrix.
previously that the analysis of two structurally defined and purified synthetic sulfated oligosaccharides using an ionic liquid led to a similar bell-shape profile (Laremore et al. 2006). Moreover, careful analysis of the ES-MS spectrum obtained for the dp4 fraction, confirmed that under-sulfated contaminants were minor. Therefore, we conclude that the poorly sulfated tetrasccharides detected in our MALDI spectra predominantly arise from in-source desulfation rather than from heterogeneity of sulfation levels in the dp4 fraction.

Some of the most intense peaks are present as a doublet separated by 6 mu (e.g. \( m/z \) 1307 and 1301, \( m/z \) 1205 and 1199, \( m/z \) 1183 and 1177). This can be explained by the presence of potassium ions in the matrix or in the sample preparation as the difference of 6 mu is consistent with the exchange of two sodium ions for one proton and one potassium ion.

When compared to the profile obtained in norharmane (Figure 3B), it is clear that the crystalline matrix promotes desulfation, as the main ions observed correspond to the de-sodiated, desulfated forms at \( m/z \) 753, 855, 957 and 1059. The information about the size of the chain as well as the number of sulfate groups obtained using ionic liquid is consistent with the ES data, demonstrating the utility of sodiation for the preservation of labile groups on the glycosidic backbone. Without the information collected from the spectrum acquired in ionic liquid, the unambiguous interpretation of the spectrum acquired in norharmane would have been more difficult.

The two spectra (Figure 3C and D) obtained upon analysis of the hexasaccharide exhibit the same patterns as those observed for the dp4 oligosaccharide. Our ES-MS analyzes indicated that the dp6 carries nine sulfate groups (data not shown) and this was confirmed by the MALDI experiment. The molecular ion carrying 11 sodium atoms is detected in the ionic liquid at \( m/z \) 1972 (Figure 3C), and is accompanied by major ions consistent with consecutive losses of NaSO\(_3\) (peaks at \( m/z \) 1870, 1768, 1666, 1564) or exchanges of sodium for hydrogen (peaks at \( m/z \) 1848, 1746, 1644, 1622, 1542, 1520, 1454). Additionally a cluster of ions is observed above the molecular ion (\( m/z \) 3142) which is consistent with adducts of sodiated CHCA. From our previous observations on the MALDI profiles obtained for the dp4 and the dp6 oligosaccharides, the presence of these peaks suggests that the peak at \( m/z \) 3142 is the biggest intact molecular ion present in this sample. We computed all possible decasaccharide structures comprising between 0 and 5 N-acetyl groups and 0 to 15 sulfates, taking into account that all the negative groups but one would be sodiated. Only one composition, a dp10 carrying one N-acetyl group and 13 sulfate groups, matched the observed molecular ion at \( m/z \) 3142. To confirm the length and the assignment of a single N-acetyl group, we analyzed the sample in the crystalline matrix. A series of peaks separated by 80 mu was observed which

![Fig. 4. MALDI-TOF MS spectra of the heparin-derived dp10 fraction. MS spectra are shown in (A) for InChICA and in (B) for norharmane. Dashed arrows represent the loss of 102 mu corresponding to the replacement of a NaSO\(_3\) group by a proton and dotted arrows represent the matrix adducts observed with the highest sulfated species (+211 mu). The cluster of satellite ions is due to different levels of salt adducts.](image)
suggested that the acidic groups present were protonated, not sodiated (Figure 4B). When computing the possible structures for the peak at \( m/z \) 2047, we found that this peak was likely to correspond to a dp10 carrying four sulfate groups (i.e. having lost nine sulfate groups during the ionization process using this matrix) and one N-acetyl group, which is consistent with the results obtained in ionic liquid. The most abundant peak at \( m/z \) 1727 corresponds to a dp10 carrying one acetyl group and no sulfate group. The MALDI profile also shows another interesting peak at \( m/z \) 1685. This peak could correspond to a dp10 oligosaccharide carrying no acetyl group and no sulfate group.

**MALDI-TOF/TOF MS/MS analysis of heparin-derived tetrasaccharide and decasaccharide**

Our MALDI-TOF-TOF MS/MS experiments on variously sulfated disaccharides gave some structurally informative fragment ions. To explore whether this technology could be usefully applied to larger and more heavily sulfated oligosaccharides, we carried out two different types of experiments. In the first instance, we focused on identifying the positions of the sulfate groups in one of the dp4 heparin derived sample (MS data shown Figure 3A and B). In an initial experiment, we selected the intact molecular ion detected in the ionic liquid for collisional activation. Unfortunately, the spectrum observed did not contain structurally informative data, mainly because the major peak detected corresponded to the loss of a single NaSO₄ group and there was little additional fragmentation (data not shown). This result is not too surprising as the presence of a high number of sodium atoms (although really useful for preserving sulfate groups in the mass fingerprinting step) has been previously demonstrated as being the major cause for the lack of fragmentation of negatively charged molecules by ES-MS/MS (Gunay et al. 2003).

We reasoned that MS/MS analyzes on species carrying a lower content of sodium atoms and a lower content of sulfate groups might yield fragment ions additional to loss of sulfate. We therefore selected ions which carried from one to four sulfate groups (\( m/z \) 753, 855, 957, and 1059), i.e. species derived from loss of between five and two sulfate groups, respectively, during the ionization of the hexasulfated dp4 in the MS experiment. Our objectives were to document the MALDI-TOF/TOF behavior of the variously sulfated species and to explore whether collisional activation of partially desulfated components would yield data allowing assignments of the locations of substituents in the intact molecule. MS/MS experiments were carried out in either the crystalline matrix or the ionic liquid depending on which matrix gave the strongest signals at \( m/z \) 753, 855, 957 and 1059.

For each MS/MS spectrum, we focused our attention on signals whose abundance was 2% or more of the most abundant fragment ion which was assigned a value of 100%. Up to 40 fragment ions of this abundance were detected in the MS/MS spectra. We considered all possible isomeric forms when annotating these fragment ions. Many of the daughter ions can correspond to several structures, which seriously complicates the assignments. However, in order to limit the number of combinations to consider, we computed the masses of daughter ions which were produced by a maximum of two cleavages, among which one could be a cross-ring-type cleavage. When the parent ion carried sodium, we considered the daughter ions as potentially carrying an equivalent or lower number of sodium atoms. Since norharmane offers a better sensitivity for the less sulfated tetrasaccharides, we analyzed the monosulfated ions using this matrix. Figure 5 shows the MS/MS data obtained for parent ions at \( m/z \) 753 (monosulfated).

The MS/MS data obtained from the non-sodiated monosulfated tetrasaccharide were of very high quality (Figure 5). Most of the low-mass end ions detected in the MS/MS spectra of the disaccharides are clearly observable in this spectrum (compare Figure 2). Ions at \( m/z \) 282, 398, 574, 592 are characteristic of a non-sulfated terminal GlcN residue, with the ion at \( m/z \) 694 being characteristic of the absence of the sulfate group on the N-position of the terminal GlcN residue (see Table I for annotation). On the other hand, the set of ions at \( m/z \) 258, 240 and 222 are characteristic of sulfated GlcN residues, the last being specific to a terminal sulfated GlcN residue. Also, the high intensity of the peak at \( m/z \) 59, characteristic of a free amino group, as well as the low intensity of the peak at \( m/z \) 138 (diagnostic for NS groups) indicates that the majority of N-sulfate groups were lost during the ionization process occurring in the MS analysis. With respect to O-sulfation, it is more difficult to draw any conclusion on which of the 2-O- or 6-O-sulfate group is the most labile under our conditions. A single ion at \( m/z \) 237 is characteristic for 2-O-sulfation, the other sulfated.

![Fig. 5. MALDI-TOF/TOF MS/MS spectra of the dp4-related species at \( m/z \) 753 (MS data shown Figure 3A and B). Assignments are presented in Table S-1. A minor species at \( m/z \) 751 and fragment ions from this component are also present in the spectrum. This peak corresponds to a 1,5-A₄ cross ring fragment of a dp4. The fragment ion carries one sulfate group and two Na atoms. The complete peak list obtained from this spectrum has been checked against this fragment ion and only two characteristic ions have been identified at \( m/z \) 709 and 637, corresponding to cross ring fragment ions.](image-url)
Table I. Assignments of the characteristic peaks detected in the MS/MS data of the ion at m/z 753 acquired in norharmane

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The maximum error on the fragment mass was set to 0.1 mu. None of the ions shown carries any sodium atom. Fragment ions at m/z 282, 398, 574, 592 and 694 carry one sulfation whose location remains undetermined and could be on any of the possible positions indicated by letter X, Y or Z. S: sulfate group.

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As the intensity of the species containing a higher content of sulfate groups and sodium atoms is of sufficient quality in the MS spectrum acquired in norharmane (compare Figure 3A and B), we performed MS/MS analyzes on the di-, tri- and tetra-sulfated components arising from the ionic liquid-mediated ionization of hexasulfated dp4. Figure S-1 and Table S-I in the supplementary material show the results obtained for the disulfated tetrasaccharide. Similar to the monosulfated tetrasaccharide, many of the daughter ions obtained are not characteristic of precise sulfate group positions. Nevertheless, some of the fragment ions observed rule out particular isomeric structures. For example, the ion at m/z 558 cannot be obtained if the two remaining sulfate groups are located on the N-position of the terminal GlcN residue and on the 2-O-position of the unsaturated IdoA residue. In the same way, an ion at m/z 574 could not be produced if the terminal GlcN is both N- and 6-O-sulfated. Also, the ion at m/z 796 is indicative of a free amino group on the terminal GlcN residue, indicating a complete loss of this N-sulfate during the ionization process. This conclusion is corroborated by the very low abundance of the m/z 138 peak. A few ions are characteristic for sulfate position such as ions at m/z 222 (sulfate being on the terminal GlcN) and 319 (the unsaturated hexuronic acid being 2-O-sulfated).

Figure S-2 and Table S-II summarize the data obtained for the trisulfated di-sodiated tetrasaccharide (m/z 957). Once again, different degrees of information are provided. The first one concerns the precise position of sulfate groups as determined by ions at m/z 209, 259, 319, all characteristic of 2-O-sulfated fragments. The second level of information provided by ions such as m/z 413 and 481 concerns the juxtaposition of sulfates along the glycosidic backbone (see Table S-II). Finally, ions at m/z 824 and 898 indicate that the terminal GlcN is not disulfated, whereas another ion at m/z 875 suggests that the unsaturated hexuronic acid is not sulfated.

We performed the same type of analysis on the tetrasaccharide carrying four sulfate groups and three sodium atoms (ion at m/z 1059, Figure S-3, Table S-III). Although the fragmentation was efficient, the information obtained on the sulfate group positions is limited. Only 2-O sulfation was clearly identified with ions at m/z 259, 319, 337. Otherwise, there was a restricted number of peaks which could inform on the arrangements of two or more sulfates, the most interesting ones being the ions at m/z 778 and 800 which cannot be observed if the terminal GlcN is disulfated.

Finally, in order to explore the utility of MALDI-TOF/TOF MS/MS for defining the location of NAc residues in relatively large GAG fragments, we carried out collisional activation on m/z 1685 and 1727 in the MALDI spectrum of the purified dp10 oligosaccharide (see Figure 4B). The masses of these ions are consistent with a dp10 oligosaccharide having zero and one N-acetyl group, respectively. The data obtained are shown Figure 6A and B. The MS/MS spectrum of m/z 1727 (Figure 6A) unequivocally demonstrates the presence of a single acetyl group (fragment ions at m/z 1389, 1168, 715). Moreover, the complexity of the fragmentation suggests that there are at least two possible positions for this acetyl group (see annotations and schemes; Figure 6A). The ions originating from the upper structure (m/z 992, 1010) are more abundant than the ones characteristic of the second possible isomer (m/z 1034, 1228),
Fig. 6. MALDI-TOF/TOF MS/MS acquired in norharmane for 2 parent ions observed for the heparin-derived decasaccharide. (A) shows the spectrum obtained from the m/z 1727 parent ion. (B) shows the data obtained for the minor species at m/z 1685. Cartoons with assignments are shown on the top of each MS/MS spectrum. Ions at m/z 793, 951 and 1288 correspond to fragments produced by double cleavage (one cross ring and one glycosidic cleavage). None of these three ions is characteristic of a N-acetyl group position. ◀ unsaturated hexuronic acid, ▲ Glucosamine, ◁ hexuronic acid, ■ N-acetylgalactosamine.

thus suggesting that the upper structure is the dominant component. The MS/MS data obtained for the peak at m/z 1685 unequivocally demonstrate the presence of a dp10 backbone carrying no N-acetyl group (Figure 6B).

Discussion

We describe in this paper a comprehensive MALDI approach applied to natural GAG oligosaccharides. Oligosaccharides up to the dp10 level were successfully profiled by MALDI-TOF MS and partial structural information was obtained by MALDI-TOF/TOF MS/MS experiments. From the first MS/MS analysis of isomeric HS-disaccharides, we conclude that MALDI-TOF/TOF MS/MS spectra obtained in the ionic liquid have similarities to data obtained a decade ago on FAB instruments (Lamb et al. 1992; Ii et al. 1995), although MALDI has much higher sensitivity and yields better quality data. This observation has been confirmed by the analyzes of longer oligosaccharides. Our data show that GAG-derived oligosaccharides containing as many as 13 sulfate groups can be observed as intact molecular ions by MALDI MS in an ionic liquid matrix. The moderate desulfation observed using this matrix is likely to be due to the high content of sodium atoms, which stabilize the sulfate groups and partly prevent their elimination during the ionization process. Because the intact molecular species is observed in this matrix, the following important information can be deduced: the size of the oligosaccharide, the number of acetyl groups and the number of sulfate groups. Despite the high level of desulfation occurring in the crystalline matrix, this experiment is helpful for confirming the length of the backbone and the number of acetyl groups. Indeed, the main species detected using the crystalline matrix usually carry zero or one sulfate group and are generally free of any sodium. The number of combinations (dp, acetyl
group, one or zero sulfate group) is therefore far smaller, and it is easier to find a composition that matches the observed m/z ratio. Thus, experiments employing the two types of matrix are highly complementary.

This study shows the first MALDI-TOF/TOF MS/MS experiments performed on sulfated GAG-derived oligosaccharides. Our MS/MS analyses of four of the observed desulfated species in the MS spectra of hexasulfated dp4 indicate that although, as expected, N-sulfates are considerably more labile than O-sulfates, N-desulfation is not complete. Consequently, some fragment ions diagnostic of N- and O-sulfated residues are present even in components which have retained only one or two sulfates during the ionization process. Hence, a significant volume of useful information can be derived from the presence or absence of diagnostic fragment ions of the type observed in the MS/MS spectra of the isomeric disaccharides. It should be noted that manual assignments of all the possible structures attributable to the many fragment ions observed in each MS/MS spectrum is very time-consuming and is not practicable for future high throughput glycomic analyses. To overcome this bottleneck, we have developed a bioinformatics tool which assists the annotation of MALDI mass fingerprints and automatically attributes possible structures to daughter ions obtained by MALDI-MS/MS strategies (manuscript in preparation).

The efficiency of our MALDI methods has been tested using a previously sequenced heparin-derived decasaccharide. The results obtained in our study are in total agreement with the data obtained previously on the same fraction (Shriver et al. 2000). Moreover, our methodology has provided additional structural information concerning the glycosidic backbone by identifying two main isomeric positions for the N-acetyl group of the main species (m/z 3142, dp10 13 sulfate groups, 1 N-acetyl group) as well as providing new information on the minor species (at m/z 1685) mentioned but not identified in the initial study (Shriver et al. 2000). Importantly, the MALDI-TOF profiling as well as the multiple MALDI-TOF/TOF analyses were performed on a single spot of sample, containing only a few tens of picomoles of oligosaccharide.

Hence, MALDI methodologies are sufficiently sensitive for the implementation alongside specific biological and chemical tools as part of an integrated glycomics strategy for high throughput profiling of the GAG content of cells and tissues. Based on our studies, we suggest that a suitable glycomic strategy should begin with MALDI-TOF mass fingerprinting of oligosaccharide mixtures derived from enzymatic or chemical digestion and purified using established chromatographic procedures (Gillingham, UK) unless otherwise indicated. Disaccharide fragments were purchased from Grampian Enzymes (Orkney, UK). Two sets of heparin oligosaccharides were prepared. For the first one, bovine lung heparin (Calbiochem, La Jolla, CA) was partially digested using heparinase I (IBEX, Montreal, Canada) in 100 mM Na acetate, 0.1 mM Ca acetate (pH 7) at 37°C for different time intervals before heating at 100°C for 2 min to terminate the reactions. Partial digests were fractionated according to hydrodynamic volume using gel filtration chromatography on a Superdex 30 column (3 × 200 cm) run at 0.5 mL/min in 0.5 M ammonium hydrogen carbonate using an AKTA purifier 10 FPLC system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) (Hussain et al. 2006). Fractions of 1 mL were collected. Size-defined fractions were further separated by strong anion exchange chromatography using a Propac PA1 column (9 × 250 m/M) (Dionex, Camberley, UK) and HPLC (Shimadzu, Milton Keynes, UK). BLH saccharides were eluted with a 0–1 M linear gradient of sodium chloride over 90 min. After each chromatography step, peak fractions were pooled and de-salted using HiPrep 26/10 desalting columns (GE Healthcare), eluting with water before lyophilization. For each chromatography step, elution was monitored at 232 nm (Hussain et al. 2006). The decasaccharide analyzed in this study was prepared according to previous protocols (Rhomberg et al. 1998; Shriver et al. 2000). The second set of dp4 and dp6 oligosaccharides were prepared by high-resolution gel filtration of a partial heparinase digest, as described in Goger et al. (2002).

Preparation of the matrices

The preparation of the ionic liquid has been described previously (Mank et al. 2004) and a few further modifications have been made as follows. Solutions of 200 mM of hydroxycinnamic acid (CHCA) were prepared in pure methanol. Equimolar amount of 1-methylimidazole was added at the CHCA solution. Solutions were vigorously mixed and sonicated for 5 min prior to being completely dried down under nitrogen stream. To achieve complete solvent removal, the dried matrix was further evaporated under vacuum for 1 h. The crystals obtained were weighed and pure methanol was added in order to prepared ionic liquid solution at a concentration of 70 mg/mL. Once prepared,
the solution were kept for a maximum of four days in the dark at 4°C. THAP monohydrate was dissolved in 80:20 (v:v) solution of acetonitrile: water at 20 mg/mL and norharmane was prepared at 10 mg/mL in a 50:50 (v:v) solution of acetonitrile and water. The two matrices were mixed at different ratios (v:v) and the most efficient resulting mix of matrix was used for each of the oligomers.

**MALDI-TOF and MALDI-TOF/TOF analysis of heparin oligosaccharides**

MALDI-TOF MS and MALDI-TOF/TOF MS/MS analyzes were performed using a 4800 MALDI TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA). MS experiments were acquired using the reflectron settings in the negative mode. MS/MS data were obtained using the 1 kV mode with argon or air as collision gas (CID cell gas pressure 3.5 × 10⁻⁶ torr). Samples were dissolved and diluted with water prior to being mixed with the matrices (1:1 volume ratio). The samples were dried under vacuum for 30 min.

**ESI-MS and MS/MS analysis of heparin oligosaccharides**

ESI-MS and MS/MS spectra were acquired using a quadrupole-TOF (Micromass, Manchester, UK) instrument. The heparin fractions were dissolved in methanol:water (35:65 volume ratio) to the concentration of 10 μM before loading into a nanospray capillary coated with a thin layer of gold/palladium, inner diameter 2 mm (Proxene, Odense, Denmark). A potential of 2.0 kV was applied to a nanoflow tip. The drying gas used was N₂, and when necessary, the collision gas was argon (with the collision gas pressure maintained at 10⁻⁴ mbar). Collision energies varied depending on the size of the oligosaccharide, typically between 5 and 20 eV.

**Supplementary material**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org.

**Funding**

Biotechnology and Biological Sciences Research Council (BBSRC); the Wellcome Trust, Cancer Research UK to J.G.; the Medical Research Council (MRC) and the Human Frontier Science Program to J.E.T.; A.D. is a BBSRC Professorial Fellowship. J.E.T. is a Senior Fellow from MRC; Research Councils UK to B.T., Y.A. and Z.Z.; Basic Technology Grant to A.D. and J.E.T. (GR/S79268).

**Acknowledgement**

We thank Prof. Robert Linhardt for provision of the purified heparin decasaccharide.

**Conflict of interest statement**

None declared.

**Abbreviations**

CHCA, hydroxycinnamic acid; ES, electrospray; FAB, fast atom bombardment; GAG, glycosaminoglycan; GlcN, D-glucosamine; HexA, hexuronic acid; HS, heparan sulfate; ImCHCA, α-cyano-4-hydroxycinnamate; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; mu, mass unit; NAc, N-acetylated; NS, N-sulfated; THAP, 2',4',6'-trihydroxyacetophenone; TOF, time-of-flight; TN, norharmane.

**References**


