HABA-based ionic liquid matrices for UV-MALDI-MS analysis of heparin and heparan sulfate oligosaccharides

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Polysulfated carbohydrates such as heparin (HP) and heparan sulfate (HS) are not easily amenable to usual ultraviolet matrix-assisted laser desorption/ionization-mass spectrometry (UV-MALDI)-MS analysis due to the thermal lability of their O- and N-SO3 moieties, and their poor ionization efficiency with common crystalline matrices. Recently, ionic liquid matrices showed considerable advantages over conventional matrices for MALDI-MS of acidic compounds. Two new ionic liquid matrices (ILMs) based on the combination of 2-(4-hydroxyphenylazo)benzoic acid (HABA) with 1,1,3,3-tetramethylguanidine and spermine were evaluated in the study herein. Both ILMs were successfully applied to the analysis of synthetic heparin oligosaccharides of well-characterized structures as well as to heparan sulfate-derived oligosaccharides from enzymatic depolymerization. HABA-based ILMs showed improved signal-to-noise ratio as well as a decrease of fragmentation/desulfuration processes and cation exchange. Sulfated oligosaccharides were detected with higher sensitivity than usual crystalline matrices, and their intact fully O- and N-sulfated species [M-Na]+ were easily observed on mass spectra. MALDI-MS characterization of challenging analytes such as heparan octasaccharide carrying 8-O and 4 N-sulfo groups, and heparin octadecasulfated dodecasaccharide was successfully achieved.

Keywords: heparan sulfate/heparin/ionic liquid matrices/MALDI-MS/sulfated oligosaccharide

Introduction

Glycosaminoglycans (GAGs), expressed at the cell surface and in the extra-cellular matrix, mediate cell–cell and cell–matrix interactions at the origin of a variety of physiological and pathological functions such as in embryonic development, cell growth and differentiation, homeostasis, inflammatory response, tumor growth, and microbial infection (Bishop et al. 2007). The problematic GAGs representative heparan sulfate (HS) is a highly anionic, linear polysaccharide made of about 20–200 disaccharides repeating units being consisted of an uronic acid and a glucosamine substituted with sulfate groups in various positions. During the HS biosynthesis, D-glucuronic acid (GlcA) can be epimerized at the C-5 position to give L-iduronic acid (IdoA), and both of them may be sulfated at the C-2 position, while the glucosamine residues may be O-sulfated (at the C-6 and more rarely at the C-3 position) and N-sulfated or N-acetylated. In addition to this variable pattern of sulfate substitution, clusters of N-sulfated disaccharides rich in IdoA and O-sulfo groups organize the polysaccharide chain in highly sulfated domains (NS) usually 3–8 disaccharides repeating units, flanked of intermediate sulfated domains made of N-acetylated and N-sulfated disaccharides (NA/NS domains) and N-acetylated domains lacking sulfated residues (NA). It results in a highly heterogeneous molecular chain exhibiting variable length, sulfation pattern, and domain organization, thus making HS as one of the most challenging biopolymer as regards structural analysis and functional assessment (Sasisekharan et al. 2006).

During the last decade, mass spectrometry (MS) has been widely recognized as a powerful and highly sensitive method for the structural analysis of carbohydrates, and decisive progresses are expected using recent MS developments for deciphering the huge informational content of HS (Zaia 2004). The relatively soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) have been mostly employed for the characterization of oligosaccharides (Zaia 2004). Although negative ESI-MS leads to a sensitive detection of HS oligosaccharides, the resulting mass spectra often display complex peak patterns due to sample heterogeneity and obvious cation exchange of the carboxylate and sulfate groups with Na+, K+ and to a lesser extent Li+, leading to a difficult interpretation of the molecular mass information. On the other hand, MALDI-MS yields mainly to monocharged ions, thus simplifying the structural elucidation. However, GAGs-based oligosaccharides are rarely analyzed by MALDI-MS because of the labile feature of the sulfate group, poor ionization efficiency with the matrices commonly used, and pronounced exchanges between proton and alkali metal cations. Fragmentation of the GAGs-derived oligosaccharides through the loss of SO3 is thus frequently observed, leading to the loss of structural information as regards the distribution of O- and N-SO3Na moieties. Being more labile than O-sulfo, N-sulfo groups are extremely important to be determined, as N-sulfation is a prerequisite for further epimerization and O-sulfation during the biosynthesis of HS (Safaiyan et al. 2000), and are a key event in generation of the NS domains involved in the binding of target protein ligands (Kreuger et al. 2006).

The use of matrix additives (Mohr et al. 1995; Ohara et al. 2009), co-matrices (Dai et al. 1997) or complexes with...
Ionic liquid matrices for heparin oligosaccharide UV-MALDI-MS

![Structure of heparin-based oligosaccharides](https://academic.oup.com/glycob/article-abstract/20/2/224/2900202)

**Fig. 1.** Structure of heparin-based oligosaccharides used in this study. Trisulfated disaccharide (Dis-IS), hexasulfated tetrasaccharide (T11), and dodecasulfated octasaccharide (O11).

Basic peptides (Juhasz and Biemann 1994) have been previously reported to help in ionization and suppression of fragmentation of highly acidic oligosaccharides. More recently, room-temperature ionic liquids have generated a tremendous interest in various analytical chemistry applications (Anderson et al. 2006), particularly as constitutive of ionic liquid matrices (ILMs) for MALDI-MS of acidic compounds (Tholey 2006). ILMs are made of usual acidic crystalline MALDI matrices mixed with organic bases such as aliphatic or aromatic amines. Compared to classic solid matrices, ILMs allow an easy dissolution of the analyte and a homogenous spotting of the matrix-analyte mixture. ILMs have been first applied to the MALDI-MS of DNA oligomers, neutral glycans, protein, and phosphopeptides, for which ILMs led to high intensity of ion peaks, better reproducibility from spot-to-spot, and lower detection limits than conventional crystalline matrices (Armstrong et al. 2001; Carda-Broch et al. 2003; Zabet-Moghaddam et al. 2004). Lately ILMs have been successfully applied to the MALDI-MS in positive ionization mode of the sulfated carbohydrates such as octasulfated sucrose, octasulfated pentasaccharide Arixtra (Laremore et al. 2006), dermatan sulfate, and chondroitin sulfate oligosaccharides ranging from tetra- (dp4) to deca-saccharides (dp10) (Laremore et al. 2007). More recently, negative ionization MALDI-MS using tetramethylguanidinium salt of α-cyano-4-hydroxycinnaminic acid as ILM has been reported for carrageenan tetra- to dodeca-saccharides confirming that ILMs allow a high sensitivity detection and an efficient suppression of the sulfate dissociation (Fukuyama et al. 2008). However, very few data are still available about the use of ILMs for the MALDI-MS of oligosaccharides being both O- and N-sulfated such as in HS and heparin considered as a highly sulfated mast cell HS (Tissot et al. 2007). In the study herein, we report the implementation of new ILMs that are based on the combination of the acidic crystalline 2-(4-hydroxyphenylazo)benzoic acid (HABA) with 1,1,3,3-tetramethylguanidine (TMG) or spermine (Sp), and their application to the MALDI-MS analysis of O- and N-sulfated oligosaccharides derived from heparin and HS. For that purpose, a library of synthetic heparin tetrasaccharides and one octasaccharide exhibiting controlled structures and various O- and N-sulfate patterns was for the first time analyzed by MALDI-MS. The method was showed also well suited for the MS detection of natural HS and heparin oligosaccharides from tetra- (dp4) to octa-saccharides (dp8) generated by enzymatic depolymerization of the parent material. Finally using HABA/TMG2 ILM, we succeeded to detect in negative MALDI-MS a fully sulfated synthetic heparin dodecasaccharide carrying 12 O- and 6 N-sulfo groups, i.e. a sulfate content and a length matching well with the NS domain features governing the specificity of protein binding of HS.
Results and discussion

Crystalline matrix versus ionic liquid matrices

Carrying N- and O-sulfo groups and being a predominant “building block” in heparin, the disaccharide Dis-IS IdoA(2-OSO$_3$)-GlcNSO$_3$(6-OSO$_3$) (Figure 1) was used as a model compound to evaluate several ionic liquid matrices. Usual crystalline matrices were ineffective to yield any detected ions from this disaccharide in negative ionization MALDI-MS (not shown). The use of additive in the crystalline matrix was previously reported to be beneficial, such as the THAP/Nor matrix (Tissot et al. 2007). Nevertheless, the intact species of the disaccharide Dis-IS (8 pmol/spot) observed in a protonated form at $m/z$ 575.97 was barely detected using THAP/Nor (Figure 3A). Abundant desulfated species were also detected, as evidenced by the mass shift of 80 mass units at $m/z$ 496.03 and $m/z$ 416.10. In addition to SO$_3$ losses, this matrix led to inhomogeneous intra- and inter-deposits impairing reproducible analyses, and it afforded a low sensitivity requiring at least 50 pmol/spot. For comparison and using the same laser tuning, the recently described ionic liquid matrix CHCA/TMG$_2$ (Laremore et al. 2006, 2007), led to a more sensitive detection of Dis-IS and an improved signal-to-noise ratio (Figure 3B). Under these conditions, the intact Dis-IS was detected as sodiated ions at $m/z$ 641.91 and $m/z$ 619.93. However, CHCA/TMG$_2$ did not suppress desulfation, as shown by ions at $m/z$ 518.00 and $m/z$ 416.07 corresponding to the loss of one and two SO$_3$Na groups, respectively.

HABA-based ionic matrices improved the detection sensitivity as...
Fig. 3. Negative ion reflector MALDI mass spectra of Dis-IS (8 pmol/spot) with the following matrices (A) THAP/Nor 80/20, (B) CHCA/TMG2, (C) HABA/TMG2, and (D) HABA/Sp2. Matrix clusters are denoted by closed circles. Acquisition was achieved with an accelerating potential of −20 kV and a grid percentage of 70%. An extraction delay of 150 ns was applied. Analytes were detected by randomly shots on the spot and signal acquired was an average of 300 shots.
A HABA/TMG2

- [M-5SO3-8Na+7H]⁻ 813.20
- [M-5SO3-7Na+6H]⁻ 835.18
- [M-4SO3-6Na+5H]⁻ 857.21
- [M-4SO3-5Na+4H]⁻ 959.13
- [M-4SO3-5Na+4H]⁻ 975.08
- [M-3SO3-4Na+3H]⁻ 1061.06
- [M-3SO3-4Na+3H]⁻ 1077.03
- [M-2SO3-3Na+2H]⁻ 1162.99
- [M-2SO3-3Na+2H]⁻ 1264.94
- [M-1SO3-2Na+H]⁻ 1382.82

B HABA/Sp2

- [M-5SO3-8Na+7H]⁻ 813.18
- [M-5SO3-7Na+6H]⁻ 835.17
- [M-4SO3-6Na+5H]⁻ 857.24
- [M-4SO3-5Na+4H]⁻ 959.16
- [M-4SO3-5Na+4H]⁻ 977.09
- [M-3SO3-4Na+3H]⁻ 1061.09
- [M-1SO3-2Na+H]⁻ 1163.01
- [M-1SO3-2Na+H]⁻ 1264.94
- [M-2Na+K]⁻ 1386.87

Fig. 4. Negative ion reflector MALDI mass spectra of synthetic tetrasaccharide T11 at 12.5 and 0.5 pmol/spot with (A) HABA/TMG2 and (B) HABA/Sp2, respectively. Analysis conditions were the same as in Figure 3, except that the number of shots that was 400.

75 fmol/spot of disaccharide Dis-IS was enough for the detection of the [M-Na]⁻ ion (not shown). Spermine is a highly basic polycation which has been previously described as effective to achieve analyses of acidic carbohydrates, glycoconjugates (Mechref and Novotny 1998), and oligonucleotides (Terrier et al. 2007; Asara and Allison 1999; Distler and Allison 2002) through an efficient competition with cations on anionic groups. This property could lead to a reduced cation exchange as observed with HABA/Sp2 (Muddiman et al. 1996). It is assumed that the improvement in signal-to-noise ratios was likely due to a better transfer of Na⁺/K⁺ rather than H⁺ from the HABA matrix to analyte during the desorption/ionization process of carbohydrates (Juhasz et al. 1993).

**Synthetic heparin tetra- and octa-saccharides**

Synthetic heparin repeating region tetra- and octa-saccharides with the sulfation degree varying from 3 to 12, including 1 N-sulfo group per disaccharide unit (Figure 1) were analyzed using HABA/TMG2 and HABA/Sp2 ILMs. Results obtained with the highly sulfated tetrasaccharide T11 are shown in Figure 4 (see supplementary figure S1 for the other tetrasaccharides). 12.5 pmol of T11/spot was needed to efficiently detect the intact sulfated tetrasaccharide as a [M-Na]⁻ ion at m/z 1366.87 in HABA/TMG2 (Figure 4A), while 50 pmol/spot was required in CHCA/TMG2 (not shown). The sensitivity was further improved in HABA/Sp2, which allowed the detection of the [M-Na]⁻ intact ion with only 0.5 pmol/spot of T11 (Figure 4B). In addition, the use of HABA/Sp2 led to a suppression of the Na⁺/K⁺ exchanges and an increase in signal to noise (S/N) by a factor 5 for the [M-Na]⁻ intact ion peak. Nevertheless, partial SO3 losses still occurred with both ILMs.

Carrying 8 O- and 4 N-sulfo groups, the synthetic heparin repeating region octasaccharide O11 represented a more challenging analyte as MALDI efficiency fallen with increasing molecular weight and negative charges (Laremore et al. 2007). Nevertheless, HABA/TMG2 and HABA/Sp2 ILMs allowed the detection of the ion corresponding to the fully sulfated intact octasaccharide [M-Na]⁻ at m/z 2696.7 (Figure 5A and B). The absolute intensity of ions was enhanced by a factor 4 with the HABA-based matrices compared to CHCA/TMG2 at the same laser power (not shown). The use of spermine as a pairing agent yielded a lower background noise, reduction of cation exchange and narrower peaks than with TMG. The mass resolution determined at 50% of the peak height at m/z 2696.6 was higher with spermine (m/Δm = 8659) than that with TMG (m/Δm = 3319), as already reported by Vandell and Limbach (1999) when polyamines were used for anionic biomolecules analysis. It is finally worth mentioning that the most intense peak detected at m/z 2288.9 corresponded to the octasaccharide having lost four SO3 groups ([M-4SO3-5Na+4H]⁻). Given to the dissociation energy values for N-S and O-S bonds, i.e. 467 and 518 kJ/mol...
respectively (Lide 2006–2007). N-Sulfate appears as the most labile group. It can thus be assumed that the successive ions detected at m/z 2594.7, 2492.8, 2390.8, and 2288.9 corresponded to the successive loss of the four N-sulfo groups, while the following series of ions (peaks at m/z 2187.0, 2085.0, 1983.1, 1881.1, 1779.1, 1677.1, and 1575.1) could be attributed to the loss of the more stable O-sulfo groups. This peak distribution may be a key feature to distinguish O- and N-sulfo groups in sulfated carbohydrate.

Heparin-derived oligosaccharides from enzymatic depolymerization

Tetra- and octa-saccharide fractions from heparin (HP dp4, HP dp8) were obtained by depolymerization with heparin lyase and size fractionation. Five main ion clusters were distinguished in MALDI-MS spectra of the tetrasaccharide fraction obtained in both matrices HABA/TMG₂ (Figure 6A) and HABA/Sp₂ (Figure 6B). They corresponded to tetrasaccharides with different sulfate contents from two to six sulfate groups. Although slight desulfation upon MS might contribute to this sulfate heterogeneity, the obtained spectra more likely indicated that the enzymatic fraction contained a mixture of various tetrasaccharides as observed by strong anion-exchange HPLC analysis (not shown). The highest sulfated species appearing at m/z 1306.64 was attributed to a hexasulfated tetrasaccharide (dp4,6S,8Na, Figure 6) similar to the above-mentioned synthetic tetrasaccharide T₁₁, i.e. with 4-O and 2-N sulfate groups. The most abundant species were represented by the tetrasulfated tetrasaccharide. The tetrasaccharides were mainly detected with an unsaturated Δ⁴,⁵-uronic acid at their nonreducing ends as expected from the use of a lyase for depolymerization. Meanwhile, it is worth mentioning that low intensity signals corresponding to saturated forms appeared for each tetrasaccharide species at m/z 1324.64, 1222.72, and 1121.01. The presence of these saturated oligosaccharides was likely due to residual hydrolase activity that often contaminates lyase preparation (Linhardt et al. 1987).

Compared to HABA/TMG₂, HABA/Sp₂ led to a slight increase (+10%) in the relative intensity of the [M-Na]⁻ peaks and a lower background noise (−25%).

With regard to the octasaccharide fraction, the MALDI spectrum in HABA/TMG₂ showed numerous peaks distributed along a wide bell-shape curve, which revealed the heterogeneity of this oligosaccharide fraction (Figure 7). Nevertheless, most of the peaks could be assigned to sodiated octasaccharides with a sulfate content ranging from 12 sulfate groups at m/z 2636.59 (dp8,12S,16Na) to three sulfate groups at m/z 1718.63 for the unsaturated form and at m/z 2654.65 and 1736.71 for their respective saturated forms (italic). Interestingly, N-acetylated octasaccharides were also detected, with N-acetyl group (Ac) per oligosaccharide for the peak series (asterisk) from m/z 2474.74 to 1759.08 and corresponding to deca- to tri-sulfated octasaccharides respectively. A similar series was observed for the saturated form (underlined asterisk). An additional broad peaks series (triangle) was observed from approximately m/z 2515 to approximately 1700 (with an interval of 102 mass units), but was not enough resolved to determine the oligosaccharides structure.

Surprisingly, the MALDI spectrum in HABA/Sp₂ showed a dramatic reduction in the overall ion signal (not shown). The
lower proton affinity of spermine (1005 kJ/mol) (Simmons and Limbach 1998) compared to TMG (1032 kJ/mol) (Hunter and Lias 1998) may induce a higher protonation of the sulfated oligosaccharides and consequently a diminished molecular ion stability and extended fragmentation.

**Heparan sulfate octasaccharide fraction from enzymatic depolymerization**

MALDI-MS analysis of heparan sulfate octasaccharides (HS dp8) issued from enzymatic depolymerization was performed in HABA/TMG2 ILM (Figure 8). The spectrum was dominated by two intense peak series (dotted lines), one attributed to an octasaccharide with one N-acetyl group, with a sulfate content ranging from seven sulfate groups at m/z 2168.87 (dp8,7S,11Na,1Ac) to one sulfate group at m/z 1557.32, and the second corresponding to an octasaccharide with two N-acetyl groups with a sulfate content ranging from six sulfate groups at m/z 2108.95 (dp8,6S,10Na,2Ac) to one sulfate group at m/z 1497.38. Both oligosaccharides were detected with unsaturated reducing ends, but their corresponding saturated forms were also present under minor ions (triangle and open circle series, respectively). Finally, an additional broad peaks series (asterisk) was observed from approximately m/z 2150 to approximately 1540 (with interval of 102 mass units). Peaks in the high mass range were not enough resolved to delineate the oligosaccharides structure. However, in the low mass range, well-resolved isotopic profiles were detected and corresponded to an unsaturated octasaccharide with three N-acetyl groups with a sulfate content ranging from three sulfate groups at m/z 1844.92 (dp8,3S,7Na,3Ac) to one sulfate group at m/z 1642.20.

The lower sulfate content and the more numerous N-acetylated residues observed here with HS oligosaccharides compared to HP oligosaccharides were consistent with the partial N-deacetylation/N-sulfation of GlcNac units during the biosynthesis of HS (Lindahl et al. 1998). Indeed 40–50% of the GlcNac residues were converted to N-sulfoglucosamine (Murphy et al. 2004), and HS showed an average of 0.2–0.7 sulfate/disaccharide (Rabenstein 2002). In these HS and HP samples from natural sources, the presence of multiple species could induce a decrease in the desorption/ionization efficiency of a given analyte due to competition with the other compounds.
peaks shifts of 102 mass units (loss of SO₃Na), 16 (Na/K exchange), and 22 (Na/K exchange). The main detected species were assigned to partially desulfated dodecasaccharide such as at m/z 2872.89 and 2856.39 corresponding to a [M-11SO₃⁻-19Na⁺+2K⁺+16H⁺]⁻ (asterisk, Figure 9) and [M-11SO₃⁻-18Na⁺+K⁺+16H⁺]⁻ (underlined asterisk, Figure 9) respectively. To the best of our knowledge, this is the longer and most N- and O-sulfated oligosaccharide detected by MALDI-MS, despite the K⁺/Na⁺/H⁺ exchanges and the likely lower desorption/ionization efficiency due to the strong anionic character of this sulfated dodecasaccharide.

**Synthetic octadecasulfated dodecasaccharide**

MALDI-MS analysis of the longer and more challenging synthetic octadecasulfated dodecasaccharide (molecular weight of the fully sodiated form = 4095.56 g/mol) was attempted using an HABA/TMG₂ ionic matrix. Although the broad envelop displayed on the MALDI-MS spectrum (Figure 9), several ion distributions could be distinguished with the following peaks shifts of 102 mass units (loss of SO₃Na), 16 (Na⁺/K⁺ exchange), and 22 (Na⁺/H⁺ exchange). The main detected

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**Fig. 7.** Negative ion reflector MALDI mass spectra of octasaccharide mixture at 1 mg/mL issued of heparin depolymerization analyzed with the ionic liquid matrix HABA/TMG₂. MALDI conditions were the same as in Figure 3, except for the extraction delay that was turned to 300 nsec and the number of shots that was 800. M was the molecular ion corresponding to a fully sodiated dodecasulfated octasaccharide (dp8,12S,16Na) under unsaturated (plain notation) and saturated form (italic notation).

**Fig. 8.** Negative ion reflector MALDI mass spectrum of octasaccharides mixture at 5 mg/mL issued of heparan sulfate depolymerization analyzed at the ionic liquid matrix HABA/TMG₂. MALDI conditions were the same as in Figure 3, except for the extraction delay that was turned to 300 nsec and the number of shots that was 800. M was the molecular ion corresponding to a fully sodiated heptasulfated octasaccharide with 1 N-acetyl group (dp8,7S,11Na,1Ac, plain notation) under the unsaturated form and a fully sodiated hexasulfated octasaccharide with 2 N-acetyl groups (dp8,6S,10Na,2Ac, italic notation) under the unsaturated form.
Conclusion

Two new ILMs, HABA/TMG₂ and HABA/Sp₂, were synthesized and successfully applied to the analysis by MALDI-MS of heparin and HS. The use of HABA rather than CHCA favors the cationic attachment to the sulfated group of the oligosaccharides. The main consequence was a decreased rate of desulfation by limiting the rupture of the N–S and O–S bonds. This limited fragmentation/desulfation process was further reinforced by the use of TMG and spermine as counter-ions. Compared to TMG, spermine led to a reduced sodium/potassium exchange, increased signal-to-noise ratio and relative intensities, and improved peak shapes. Nevertheless, spermine was ineffective in our hand to allow the detection of sulfated oligosaccharides with dp ≥ 8. These specific features of the described HABA-based matrices associated with the well-known advantages of ILMs, i.e. homogeneity of spot, very small amount of analyte required, and the simple and cheaper sample preparation highlight interesting outlooks for high throughput analysis of complex glycosaminoglycan structures. Further enhancement are under current investigations such as the optimization of the analyte/matrix ratio and the extension to other organic bases as cationic counter ions for the analysis of O- and N-sulfated carbohydrates from various sources and the MALDI-MS monitoring of their enzymatic transformation.

Materials and methods

Materials

2-(4-hydroxyphenylazo)benzoic acid (HABA), α-cyano-4-hydroxycinnamic acid (CHCA), 2’,4’,6’-trihydroxyacetophenone (THAP), 1,1,3,3-tetramethylguanidine (TMG), norharmane (Nor), spermine (Sp), and disaccharide-IS (α-DUA-2S-[1→4]-GlcNS-6S, Dis-IS) were purchased from Sigma-Aldrich Co. (Saint Quentin Fallavier, France). Heparinase I (Heparin lyase I, EC 4.2.2.7) and heparinase III (EC 4.2.2.8) from Flavobacterium heparinum were purchased from Sigma-Aldrich Co. (Saint Quentin Fallavier, France). Other chemicals and reagents were obtained from commercial sources at the highest purity available. All solvents were analytical grade, and ultrapure water (Milli-Q, Millipore, Milford, MA) was used.

Synthetic heparin repeating region oligosaccharides

Heparin tetrasaccharides, octasaccharide (O₁₁) and dodecasaccharide in the sodium salt form were obtained by combinatorial (Dilhas et al. 2008) or conventional total synthesis as previously reported (Lubineau et al. 2004; Baleux et al. 2009).

Heparin and heparan sulfate oligosaccharides from enzymatic depolymerization

Heparin oligosaccharides were prepared as described previously (Vives et al. 2002). Briefly, porcine mucosal heparin (10 g) was digested with heparinase I (8 mU/mL) in 150 mL of 0.1 mg/mL BSA, 2 mM CaCl₂, 50 mM NaCl, and 5 mM Tris buffer, pH 7.5, for 54 h at 25°C. The enzymatic reaction was stopped by heating the digest at 100°C for 5 min. Digestion products were then size-separated using a Bio-Gel P-10 column (Bio-Rad, Hercules, CA) (4.4 × 150 cm), equilibrated with 0.25 M NaCl, and run at 1 mL/min. Eluted material, detected by absorbance at 232 nm, consisted of a graded series of size-uniform oligosaccharides resolved from disaccharide (dp2) to octadecasaccharide (dp18). To ensure size homogeneity, only the top fractions of each peak were pooled, and each isolated fraction was re-chromatographed on a gel filtration column to further eliminate possible contamination. Samples were diazylated against distilled water, freeze dried, and quantified by a
colormetric assay (Bitter and Muir 1962) or weighted. Heparan sulfate-derived oligosaccharides were prepared similarly, starting from 10 g of HS instead of heparin, and using heparinase III (20 mU/mL) instead of heparinase I as a depolymerizing enzyme. Heparin and heparan sulfate octasaccharide mixtures were solubilized in water at 1 and 5 mg/mL, respectively.

**Preparation of the ionic liquid matrices (ILMs)**

Ionic liquid matrices used in this study (Figure 2A) were prepared as described by Armstrong et al. (2001). CHCA was mixed with TMG at a 1/2 molar ratio in methanol. HABA was mixed with TMG or Sp at a 1/2 molar ratio in methanol, and the obtained solution was then sonicated for 15 min at 40°C. After removal of methanol by centrifugal evaporation in a SpeedVac for 3 h at room temperature, ILMs were left in vacuum overnight. ILMs were then prepared at a concentration of 70–90 mg/mL in methanol for use as a matrix. Once prepared, methanol solutions of ILMs could be stored at 4°C up to 1 week and were used without further purification. CHCA-based ILM was solid at room temperature, while HABA-based ILMs were viscous liquids. THAP was dissolved in 80/20 (v/v) solution of acetonitrile/water at 20 mg/mL and Nor was prepared at 10 mg/mL in a 50/50 (v/v) solution of acetonitrile/water, and then both were mixed at a 20/80 THAP/Nor ratio.

**Samples preparation**

The concentration of aqueous tetrasaccharide solutions was determined by a modified uronic acid carbazole reaction (Bitter and Muir 1962), measuring the absorbance at 530 nm by a nanodrop 1000 spectrophotometer (Thermo-Electron, Waltham, MA). Sample concentrations used for MALDI-MS analyses ranged from 0.5 to 1 mg/mL. Samples for MALDI-MS analysis were prepared by mixing 0.5 to 1 μL of oligosaccharide solution and one volume of ionic liquid matrix. An amount of 0.25 to 1 μL of the mixture was deposited on a mirror polished stainless steel MALDI target and allowed to dry at room temperature and atmospheric pressure.

**Mass spectrometry**

MALDI-TOF/MS experiments were performed using a Perseptive Biosystems Voyager-DE Pro STR MALDI-TOF mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). This instrument was equipped with a nitrogen UV laser (λ = 337 nm) pulsed at a 20 Hz frequency. The mass spectrometer was operated in the negative ion reflector mode with an accelerating potential of ~20 kV and a grid percentage equal to 70%. Mass spectra were recorded with the laser intensity set just above the ionization threshold (2500–3400 in arbitrary units, on our instrument) to avoid fragmentation and sulfo group losses, to maximize the resolution (pulse width 3 ns) and to result in a strong analyte signal with minimal matrix interference. Time delay between laser pulse and ion extraction was set to 150 ns for anaLyte dp ≤ 4 or 300 ns for dp ≥ 8. Typically, mass spectra were obtained by accumulation of 200–800 laser shots for each analysis and processed using Data Explorer 4.0 software (Applied Biosystems).

**Supplementary data**

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

**Abbreviations**

CHCA, hydroxycinnamic acid; dp, degree of polymerization; ESI, electrospray; GAG, glycosaminoglycan; GlcA, d-glucuronic acid; HABA, 2-(4-hydroxyphenylazo)benzoic acid; HexA, hexuronic acid; HP, heparin; HS, heparan sulfate; IdoA, L-iduronic acid; ILM, ionic liquid matrix; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; NA, N-acetylated; Nor, Norharmane; NS, N-sulfated; Sp, Spermine; THAP, 2,4,6-trihydroxyacetophenone; TMG, 1,1,3,3-tetramethylguanidine; TOF, time-of-flight.

**References**


