RESEARCH PAPER

Efficient recovery of glycosaminoglycan oligosaccharides from polyacrylamide gel electrophoresis combined with mass spectrometry analysis

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Abstract To promote efficient separation and structural analysis of glycosaminoglycan oligosaccharides, we developed a straightforward method that combined gel electrophoresis and mass spectrometry (MS). Potential limitations of this approach (e.g., low extraction yields and weak compatibility with MS) were resolved by developing an active extraction procedure that yielded a quantitative amount of sulfated oligosaccharides from excised gel bands. The compatibility of obtained oligosaccharides for subsequent MS analysis was ensured using a single, simple clean-up step on a mixed C18/graphite carbon solid-phase column that was fully effective for polymerization degrees ranging from di- to dodecasaccharides. The reported combination of carbohydrates-polyacrylamide gel electrophoresis with MS was successfully applied to glucosamino- (heparin) and galactosamino- (dermantan sulfate) glycans, demonstrating the potential of our method for structural analysis of bioactive sulfated carbohydrates extracted from biological matrices.

Keywords C-PAGE · Gel extraction · Glycosaminoglycan · Mass spectrometry

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Introduction

Glycosaminoglycans (GAGs) are highly anionic linear polysaccharides found in the mammalian extracellular matrix and cell surfaces, where they are involved in many biological processes such as embryonic development, cell proliferation, and differentiation [1, 2], as well as responses to infectious disease [3]. Most GAG functions are mediated by the binding to target proteins (e.g., growth factors, cytokines and chemokines) through specific recognition of structural determinants such as the monosaccharide sequence and sulfation pattern. GAG chains have variable lengths and complex sulfation patterns and domain organizations, making these carbohydrates one of the most challenging biomacromolecules to study regarding their structural analysis and functional assessment [4, 5]. There is thus a strong demand in glycobiology for efficient separation methods and for tools to perform high-resolution structural analysis. For the structural analysis of carbohydrates, mass spectrometry (MS) is widely recognized as a powerful and highly sensitive method and definite progress has been made in deciphering the vast structural diversity of GAGs [6–12]. MS analysis of GAGs can be successfully combined with upstream separation methods to reduce the complexity of samples and remove interfering substances from the biological matrix. Liquid chromatography (LC) is one of the most widespread separation methods used for GAGs by reversed-phase chromatography either with ion pairing [13, 14] or after derivatization of oligosaccharides with a hydrophobic compound [15–19] and, more recently, porous graphite carbon [20–22] and hydrophilic interaction phases [23–26]. LC methods can be coupled with MS (LC/MS) for high-throughput analysis, but the required MS-compatible elution conditions are not always those that are optimal for separation. In addition, the mechanisms of chromotagraphic retention of these highly anionic and heterogeneous

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carbohydrates are not yet fully understood [12]. Therefore, separation mechanisms that depend on the content and distribution of sulfate groups as well as on saccharide sequences and glycosidic linkages are difficult to comprehend and predict for GAGs. In addition, given their high molecular weight, full-length GAGs are not amenable to direct MS or LC-MS analysis, and their structural characterization requires depolymerisation into smaller building blocks [20–22, 27–34]. As a result, GAG structural studies using LC and LC-MS generally focus on disaccharide composition although highly sulfated disaccharides are difficult to separate, and they raise a number of issues, such as saccharide derivatization that is required by certain LC methods using reversed stationary phases [19]. In comparison, electrophoretic separation methods have been less widely explored for GAG analysis and in glycomics in general [35], despite their importance in the development of proteomics in the past decade. Electrophoresis—either capillary or gel—has unsurpassed resolving power, with the resolution of a wide range of polymerization degrees regardless of sulfate content. Carbohydrate-polyacrylamide gel electrophoresis (C-PAGE) using a high acrylamide/bis-acrylamide percentage has already afforded high resolution of GAG-derived sulfated oligosaccharides from complex biological fluids [36–39]. Despite the high resolving power and easy implementation of C-PAGE, few studies have explored its combination with MS for the structural analysis of GAG oligosaccharides. One reason is the need for efficient recovery of separated oligosaccharides from the gel and, moreover, under conditions that are compatible with MS analysis. In this study, we report a method for GAG extraction from C-PAGE gels that recovers a quantitative amount of GAG oligosaccharides for subsequent MS analysis without resorting to complex capillary electrophoresis-mass spectrometry methods [40–42] or specific devices such as continuous-elution electrophoresis [43]. This simple PAGE method in combination with active extraction from the gel allowed us to successfully carry out MS structural characterization of heparin and dermatan sulfate oligosaccharides in enzymatic depolymerization reaction mixtures.

Materials and methods

Reagents and materials

2-(4-Hydroxyphenylazo)benzoic acid (HABA), 1,1,3,3-tetramethylguanidine (TMG), Trizma® base, ammonium persulfate, glycerol, acetonitrile (ACN), Alcian blue 8G×, N,N,N,N-tetramethylethylenediamine (TEMED), 1,9-dimethylmethylene blue (DMMB), phosphate buffer saline (PBS), calcium and sodium chloride, hydrochloric acid, trifluoroacetic acid (TFA), glycine, chondroitin sulfate-B (dermatane sulfate, DS) from porcine intestinal mucosa, and

chondroitinase ABC (EC 4.2.2.4) were purchased from Sigma-Aldrich Co. (Lyon, France). The acrylamide solution (29:1 acrylamide/bis-acrylamide (40 %; w/v)) was purchased from Roth (Karlsruhe, Germany). Heparin was purchased from Celsus Laboratories Inc. (Cincinnati, OH, USA). Heparinase I (EC 4.2.2.7) was purchased from Grampian Enzymes (Orkney, UK). 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, USA) was used. Microcuves (UVette®) were purchased from Eppendorf (Hamburg, Germany).

Oligosaccharides from enzymatic GAG depolymerisation

Porcine mucosal heparin (100 mg) was digested with heparinase I (500 mU/mL) in 1 mL of 10 mM PBS 3 mM $CaCl_2$ buffer, pH 7.3, for 6 h at 25 °C. The enzymatic reaction was stopped by heating to 100 °C for 5 min. Digestion products were then size-separated using a Bio-Gel P-10 column (Bio-Rad, Hercules, CA, USA) (100 × 3.2 cm) equilibrated with 0.25 M NaCl, and run at 0.4 mL/min. Eluted material, detected by refractometry (differential refractometer RI71, Merck), consisted of a graded series of size-uniform oligosaccharides resolved from disaccharide (dp2) to dodecasaccharide (dp12). To ensure size homogeneity, only the top peaks of each fraction were pooled, and each isolated fraction was rechromatographed on a Superdex peptide HR10/30 gel filtration column (GE Healthcare Life sciences, Velizy-Villacoublay, France) (10 × 300 cm) equilibrated with 0.1 mM NaCl, and run at 0.4 mL/min and detected by UV absorbance at λ = 232 nm.

Depolymerization of dermatan sulfate was carried out as described elsewhere [44]. Briefly, chondroitin sulfate-B (1 mg) was digested with chondroitinase ABC (40 mU) in 0.13 mL of 20 mM Tris-HCl buffer, pH 7.2 for 1 h at 37 °C. The enzymatic reaction was stopped by heating the digestion products to 100 °C for 5 min.

MALDI-TOF/MS analysis

MALDI-TOF/MS experiments were performed using a Perspective Biosystems Voyager-DE Pro STR mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with a nitrogen UV laser (λ = 337 nm) pulsed at a 20-Hz frequency. MS analyses were performed in 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 (2500–2800 arbitrary unit) to avoid fragmentation and sulfo group losses, to maximize resolution (pulse width, 3 ns) and to obtain a maximal analyte signal with minimal matrix interference. Time delay between laser pulse and ion

extraction was set to 250 ns for analyte dp ≤ 4 or 300 ns for dp ≥ 6. Typically, mass spectra were obtained by accumulation of 500–1000 laser shots for each analysis and processed using Data Explorer 4.0 software (Applied Biosystems).

The ionic liquid matrix (ILM) HABA/TMG$_2$ was prepared as previously described [9]. Briefly, HABA was mixed with TMG 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, the ILM was left in vacuum overnight. ILM was then prepared at a concentration of 70–90 mg/mL. Further dilution to 9 mg/mL in methanol, and addition of 100 μM aqueous NaCl to prevent excessive desulfation upon the MALDI processing, was achieved extemporaneously to MS analysis.

Samples for MALDI-TOF/MS analysis were prepared by mixing the oligosaccharide solution and the ILM in a 1:1 volume ratio. Then, 1 μL of the mixture was next deposited on a mirror-polished stainless steel MALDI target and allowed to dry for 5 min at room temperature and atmospheric pressure.

NanoESI-mass spectrometry of gel extracted heparin oligosaccharides

The static nanoESI-MS experiments were carried out using a LTQ-Orbitrap XL from ThermoScientific (San Jose, CA, USA) and operated in negative ionization mode, with a spray voltage at −1.1 to −1.2 kV. One microliter of each sample was continuously infused through a fused silica tip with a 360-μm outer diameter, 20 μm inner diameter with a nominal tip end inner diameter of 10 ± 1.0 μm (Pico-tip, FS360-50-15-CE-20-C10.5, New Objective, Woburn, MA, USA) which provided a consistent signal during 10 min. Applied voltages were −35 and −120 V for the ion transfer capillary and the tube lens, respectively. The ion transfer capillary was held at 200 °C. Resolution was set to 30,000 (at m/z 400) for all studies, and the m/z ranges were set to 150–2000 in profile mode and in the normal mass range. The automatic gain control (AGC) allowed accumulating up to 1.10^6 ions for FTMS scans with a maximum injection time set to 500 ms and with 1 μscan acquired.

Carbohydrate-polyacrylamide gel electrophoresis (C-PAGE)

C-PAGE was performed in polyacrylamide gels (8.3 × 7.3 cm; thickness = 1 mm) composed of acrylamide/bis-acrylamide in a 100 mM Tris-HCl buffer, pH 7.8 at 6 and 27 % for stacking and resolving gels, respectively. For analysis, 8–10 μL of aqueous solution of oligosaccharide samples (≥1 μg) was loaded into wells. Gels were run in a Mini-PROTEAN® tetracell system (Bio-Rad) by applying a constant voltage of 250 V supplied by a voltage generator (Bio-Rad) for 40 min and using 40 mM Tris, 40 mM acetic acid, pH 7.8 as running buffer. Gels were stained with 0.5 % (w/v) Alcian blue in 98/2 % (v/v) H$_2$O/acetic acid for 1 h at room temperature. A destaining step was performed in water for at least 2 h at room temperature.

Oligosaccharide extraction from gel electrophoresis

Bands containing GAG oligosaccharides were excised and cut into small pieces (ca. 1 × 1 mm). The gel pieces were immersed in 500 μL of water in micro-centrifuge tubes, frozen at −80 °C for 30 min, and then thawed at 25 °C for 15 min. This freeze-thaw cycle was repeated twice, and the water fraction was collected thereafter. The gel pieces were then sonicated in 500 μL of water for 10 min at 60 °C. The aqueous fraction was again collected and 500 μL of ACN was added for 5 min at 25 °C. Sonication and water-ACN exchange were repeated four times. Gel pieces were once again sonicated in 500 μL of water for 10 min at 60 °C. Aqueous fractions were collected separately or pooled and freeze-dried for desalting and MS analyses.

Desalting of extracted oligosaccharides by solid-phase extraction (SPE)

Extracted oligosaccharides were desalted using a C18 + Carbon-SPE TopTip cartridge (Glygen, Columbia, MD, USA) as previously described by Wei et al. [21]. Briefly, the TopTip cartridge was conditioned twice with 50 μL of 80/20/0.1 % (v/v/v) ACN/H$_2$O/TFA followed by 3 × 50 μL of water. Extracted oligosaccharides were solubilized in 50 μL of water and centrifuged at 5000 rpm for 5 min to pellet residual acrylamide particles, and then sample was loaded onto a TopTip module. The TopTip was washed five times with 50 μL of pure water. The GAG oligosaccharides were eluted with 3× 50 μL 20/80 % (v/v) ACN/H$_2$O and 50 μL 40/60/0.05 % (v/v/v) ACN/H$_2$O/TFA. Elution was performed by centrifugation at 3500 rpm for 1 min. Each elution fraction was pooled, freeze-dried, and dissolved in water either in 8 μL for gel electrophoresis or in 10 μL for MS analysis and 1,9-dimethylmethylene blue assay.

Oligosaccharides quantification

Sulfated oligosaccharide quantification was performed using DMMB assay as previously described [45] with minor modifications. Briefly, 16 mg DMMB, 3.07 g of sodium chloride, and 2.37 g of glycine were dissolved in 95 mL HCl 0.1 M and completed to 1 L with water, pH 3.0 adjusted with NaOH. The obtained solution was stable for at least 4 months when stored at room temperature in darkness. One hundred twenty-five microliters of DMMB solution was directly added to 5 μL

of aqueous oligosaccharide samples. Absorbance at λ = 535 nm was measured in a Cary 50 UV-visible spectrophotometer (Varian, Walnut Creek, CA). Heparin quantities were determined based on a calibration curve established with heparin dp6 and dp10 standard solutions from 1 to 4 μg/mL.

Results and discussion

The electrophoretic separation and the extraction of GAG oligosaccharides reported in this study were successfully carried out using mini format vertical gels (8 cm). Heparin oligosaccharides ranging from di- (dp2) to decasaccharides (dp10) were well resolved in a 27 % polyacrylamide gel (not shown). For direct MS analysis of gel-separated oligosaccharides, a quantitative yield of salt-free samples is required. To meet these requirements, we designed an active extraction procedure for gel pieces based on three freeze (−80 °C)/thaw cycles
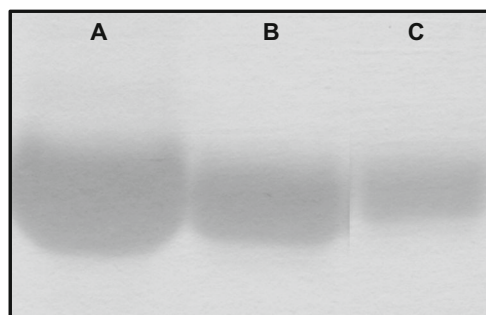

Fig. 1 Carbohydrate-polyacrylamide gel electrophoresis of extracted heparin hexasaccharides. (A) 10 μg of heparin hexasaccharide marker. Heparin hexasaccharides extracted from (B) stained and (C) unstained gel bands

followed by five cycles of shrinkage in acetonitrile (ACN; 25 °C)/expansion in water (60 °C). This procedure was first implemented using well-defined heparin oligosaccharides dp6 and dp10. First, 10 μg of each oligosaccharide was separated in a 27 % polyacrylamide gel, and then the resulting gel bands
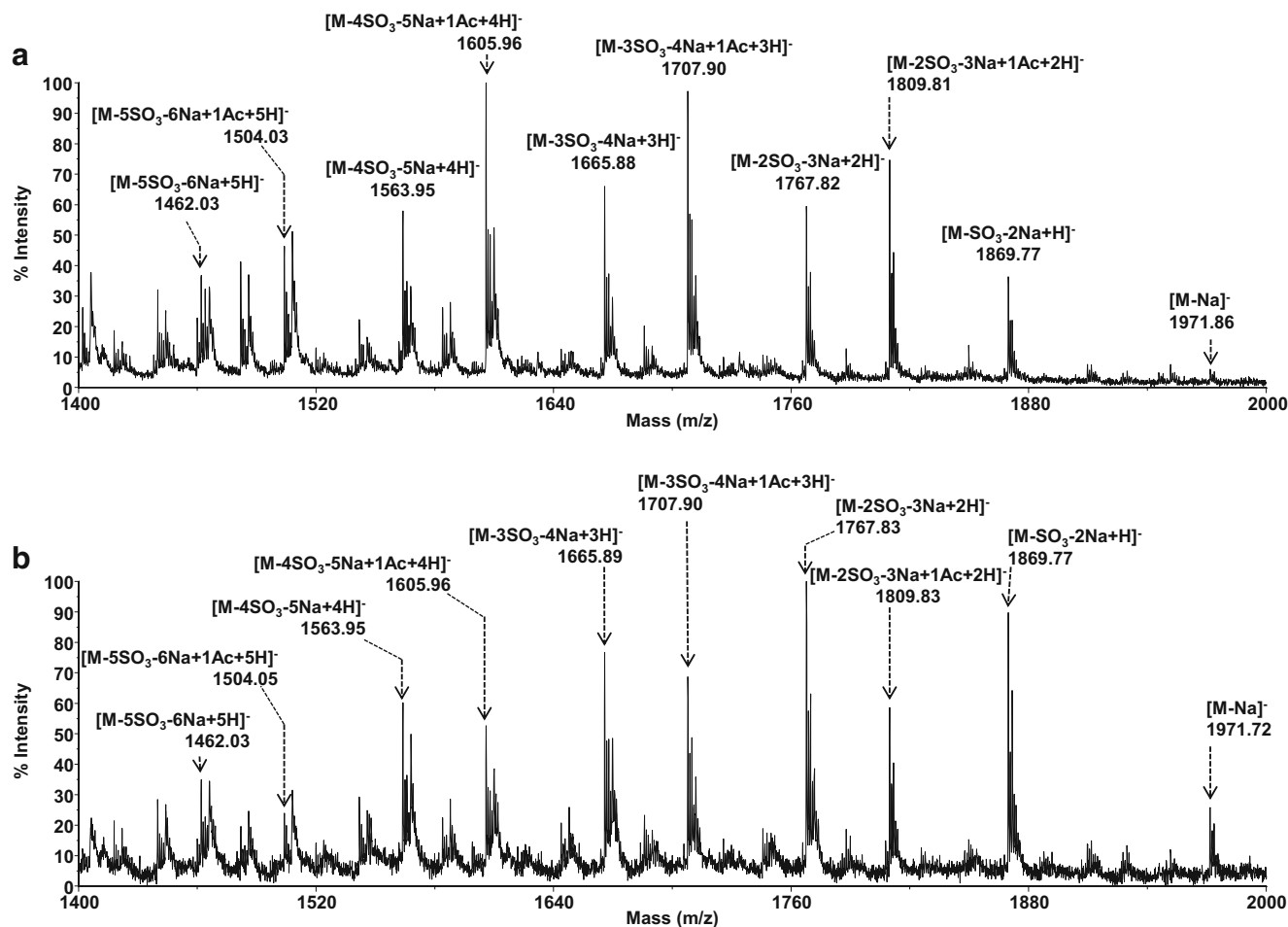

Fig. 2 Negative ion reflector MALDI mass spectra of heparin hexasaccharides after gel extraction and solid-phase desalting. A Heparin hexasaccharides in 250 mM NaCl desalted on a mixed C18/graphite carbon solid phase. B Heparin hexasaccharides after in-gel extraction and desalting; 25 pmol was spotted with 9 mg/mL HABA/TMG$_2$ doped with 100 μM NaCl. M is the molecular ion corresponding to the fully sodiated sulfated hexasaccharide (DP6, 9 S, 12 Na, M = 1994.70 Da); 0.5 μL of desalted sample was mixed with 9 mg/mL HABA/TMG$_2$ doped with 100 μM NaCl
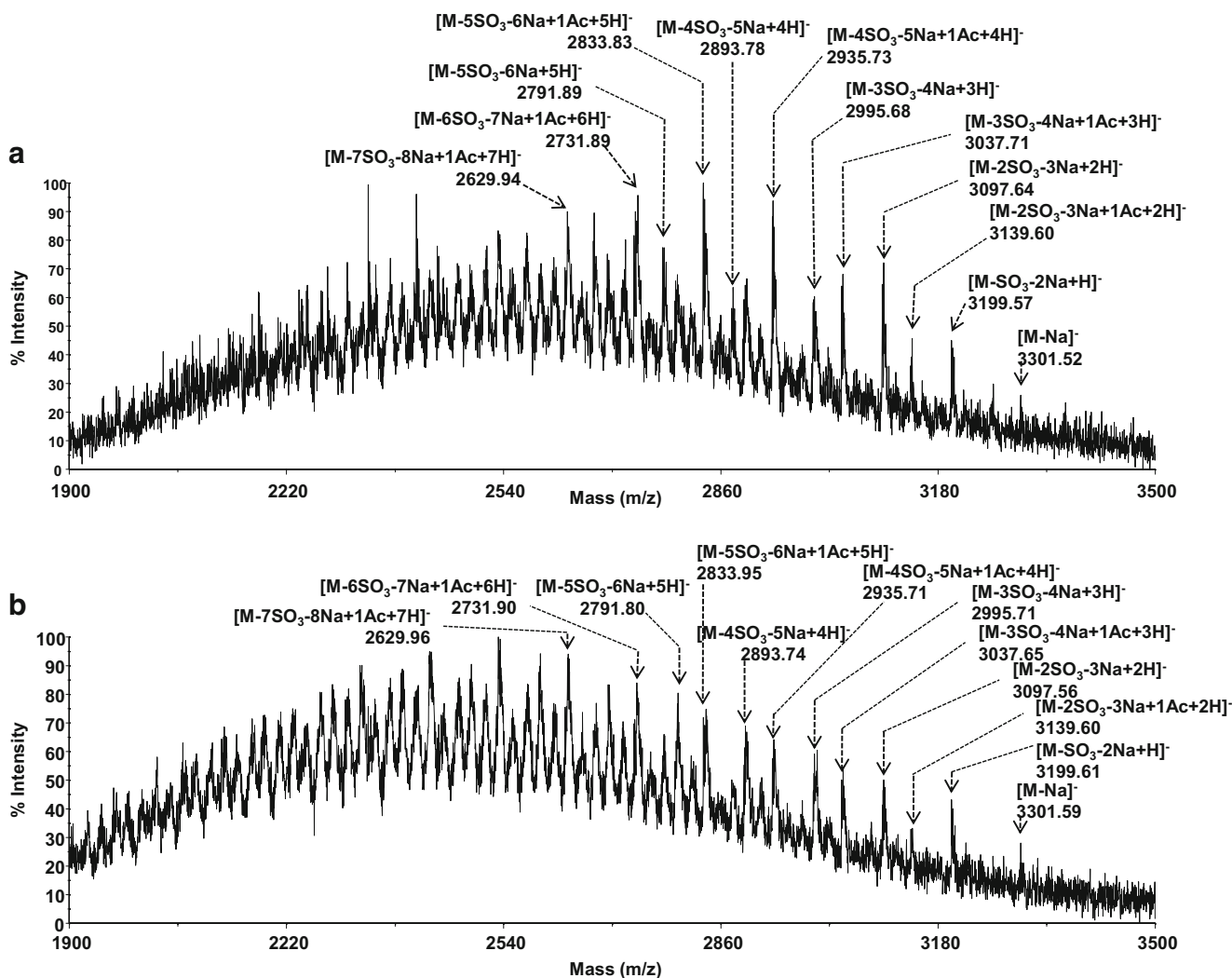
Fig. 3 Negative ion linear MALDI mass spectra of heparin decasaccharides after gel extraction and solid-phase desalting. **A** Heparin decasaccharides in 250 mM NaCl desalted on a mixed C18-graphite carbon solid phase. **B** Heparin decasaccharides after in-gel extraction and desalting; 60 pmol was spotted with 9 mg/mL HABA/TMG$_2$ doped with 100 μM NaCl. M is the molecular ion corresponding to the fully sodiated sulfated decasaccharide (DP10, 15 S, 20 Na, M = 3324.50 Da); 0.5 μL of desalted sample was mixed with 9 mg/mL HABA/TMG$_2$ doped with 100 μM NaCl

were excised from unstained gels or from gels stained with Alcian blue. The ACN and water fractions obtained upon extraction from the 27 % polyacrylamide gel were pooled separately, and their oligosaccharide contents were controlled by an additional gel electrophoresis (Fig. 1). Most of the initially loaded dp6 and dp10 oligosaccharides were recovered in the water fraction and not in the ACN fraction. The comparison of the aqueous fractions either from stained or from unstained gels showed that the staining step had only a slight effect on recovery yield (Fig. 1). Compared with previously reported extraction methods based on passive release from crushed gel pieces [39], the ACN/water extraction under mild heating and sonication, and including freeze/thaw and shrinkage/expansion cycles, led to a highly quantitative recovery of 80 % of in-gel-separated oligosaccharides. To limit

interference in MS analysis and ensure enhanced detection of the extracted oligosaccharides, the aqueous fraction was then cleaned up to remove salts and contaminants left over from the gel matrix. Previous reports indicate that filtration of sulfated heparin disaccharides on centrifuge microcolumns packed with graphite carbon and a C18 solid-phase extraction sorbent is a fast, simple, and inexpensive clean-up method [21]. According to our results, this mixed-phase extraction method was also suitable for higher polymerization degrees ranging from tetra- to dodecasaccharides with successive use of 20/80 % ACN/water, 60/40 % ACN/water, and 0.05 % TFA as the eluent system. For example, 10 μg of a mixture of sulfated hexasaccharides from heparin in 250 mM NaCl was desalted and purified on a mixed C18/graphite carbon cartridge, and then analyzed by matrix-assisted laser
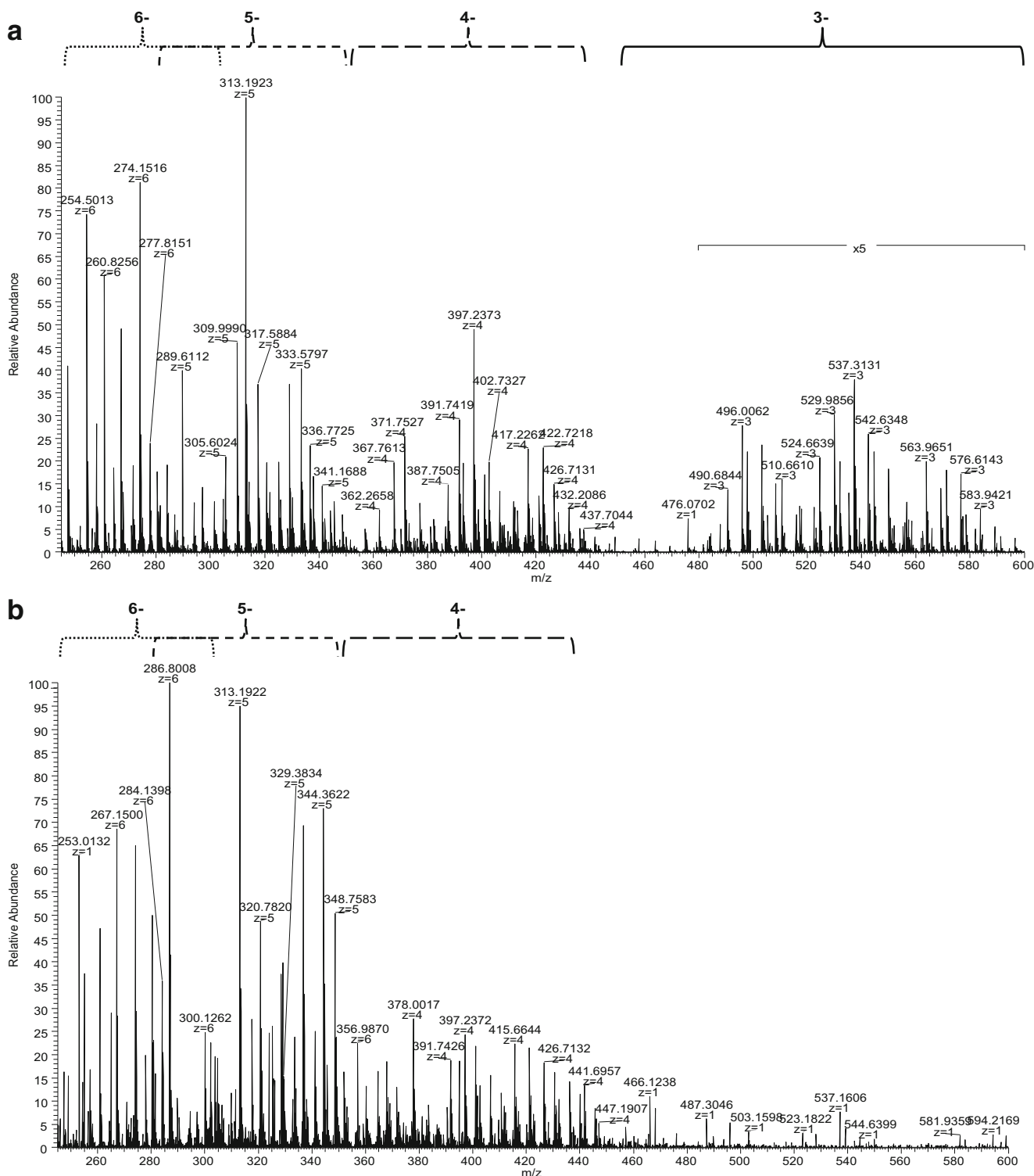
Fig. 4 Negative ion nanoESI full mass spectra of heparin hexasaccharides after **A** desalting step on a mixed C18/graphite carbon solid phase, and **B** in-gel extraction and desalting steps. Five hundred picomoles in 1 μL was infused

desorption-ionization/time-of-flight (MALDI-TOF)/MS in negative ionization mode. The MS spectrum (Fig. 2A) showed a well-defined peak distribution assigned to sodiated hexasaccharides, with sulfate contents ranging from the fully

sulfated form comprising nine sulfate groups (ΔDP6, 9 S, 12 Na) at m/z 1971.86 ([M-Na]$^-$) to the form with four sulfate groups at m/z 1462.03 ([M-5SO$_3$-6Na + 5H]$^-$), and to mono-acetylated hexasaccharides (ΔDP6, 8 S, 1 Ac, 11 Na), with
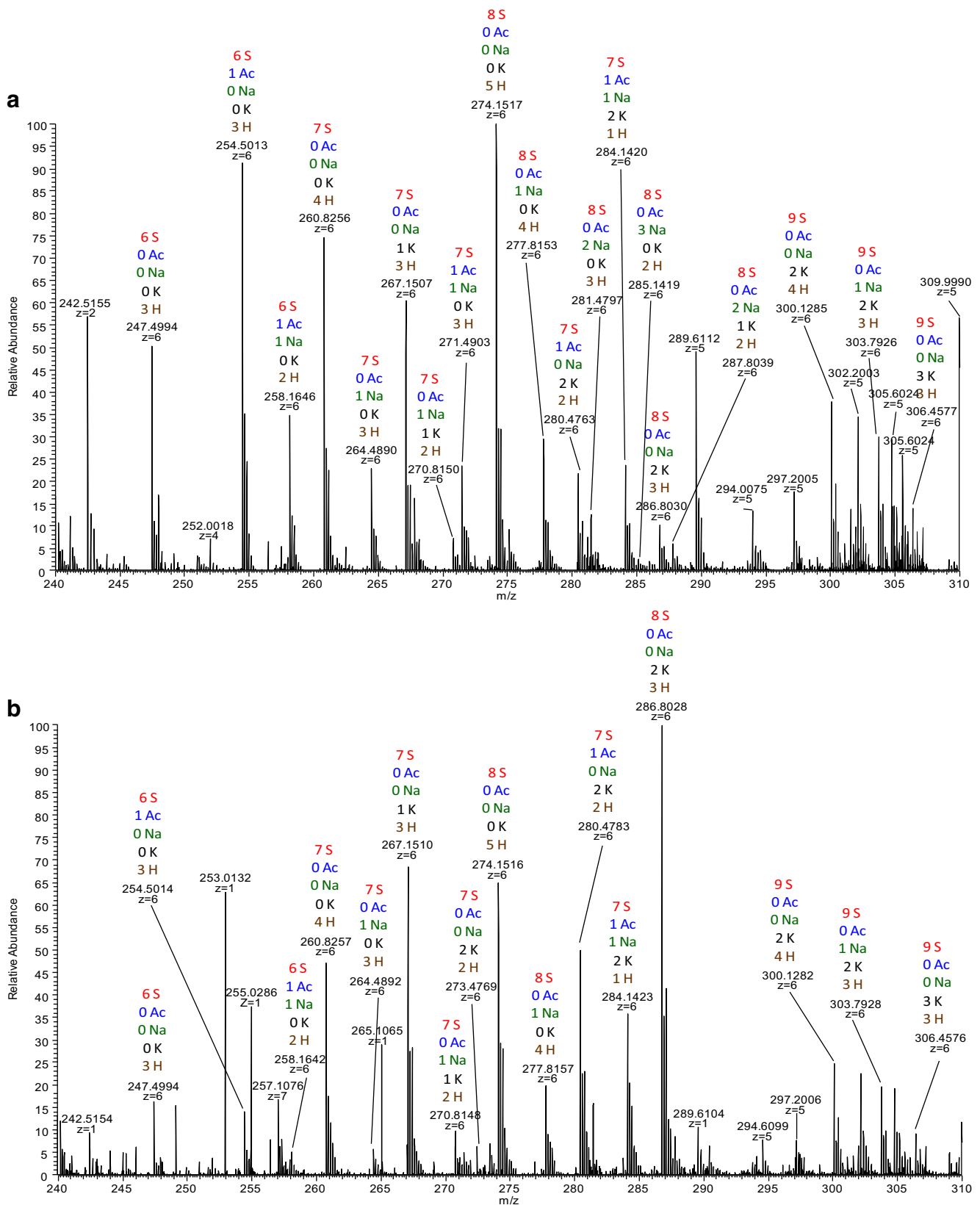
Fig. 5 Negative ion nanoESI mass spectra of charge state 6- of heparin hexasaccharide after **A** desalting step on a mixed C18/graphite carbon solid phase, and **B** in-gel extraction and desalting steps. Five hundred picomoles in 1 μL was infused

Fig. 6 Carbohydrate-polyacrylamide gel electrophoresis of enzymatic depolymerization products of **A** heparin and **B** dermatan sulfate. Boxed gel bands correspond to oligosaccharides extracted from the gel prior to MS analysis. HP-DP10, HP-DP8, and HP-DP6 are heparin oligosaccharide size markers
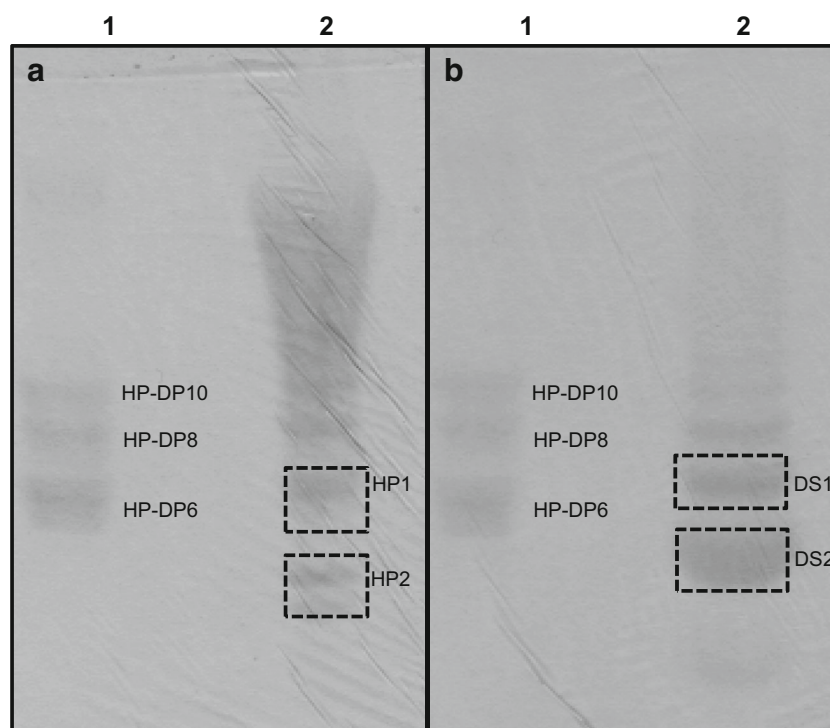
sulfate contents ranging from seven sulfate groups at m/z 1809.81 ([M-2SO$_3$-3Na + 1Ac + 2H]$^-$) to four sulfate groups at m/z 1504.03 ([M-5SO$_3$-6Na + 1Ac + 5H]$^-$). The detected structures were in agreement with the expected hexasaccharides produced by enzyme depolymerization of heparin, thus indicating that the desalting step on a mixed C18/graphite carbon solid-phase did not alter the composition or the structure of the loaded oligosaccharides. Heparin deca- and dodecasaccharides were also successfully desalted and analyzed by MS (Fig. 3A), showing that desalting on mixed C18/graphite carbon solid-phase can be used on a wide range of polymerization degrees.

This desalting method was then applied to purified fractions of heparin hexa- and decasaccharides previously extracted from a 27 % polyacrylamide electrophoresis gel. The MALDI mass spectrum obtained for 10 µg of hexasaccharides loaded on the gel exhibited a characteristic ion pattern for heparin oligosaccharides, comprising the fully sulfated hexasaccharide (ΔDP6, 9 S, 12 Na) and the mono-acetylated hexasaccharide (ΔDP6, 8 S, 1 Ac, 11 Na) (Fig. 2B). Further tested on extracted heparin decasaccharides, the method yielded an exploitable, albeit more complex, MS spectrum (Fig. 3B). Characteristic ion patterns for heparin decasaccharides revealed non-acetylated species with 10 to 15 sulfate groups at m/z 2791.80 ([M-5SO$_3$-6Na + 5H]$^-$) and m/z 3301.59 ([M-Na]$^-$), respectively. Mono-acetylated decasaccharides were also detected with sulfate contents ranging from 8 to 13 sulfate groups at m/z 2629.96 ([M-7SO$_3$-8Na + 1Ac + 7H]$^-$) and m/z 3139.60 ([M-2SO$_3$-3Na + 1Ac + 2H]$^-$), respectively. It is worth noting that the desalting and extraction procedure was also well suited for electrospray (ESI) MS analysis as shown by the obtained negative nanoESI mass spectra of extracted heparin hexasaccharide (Fig. 4). The resulting spectrum showed numerous peaks corresponding to hexasaccharide ions with charge state ranging from 3- to 6- for sample issued from only desalting step (Fig. 4A) and from 4- to 6- for sample resulting from the combined in gel extraction/desalting step (Fig. 4B). A focus on the charge state 6- evidenced almost the same main hexasaccharide species with various sulfate content as those observed on the MALDI spectra (Fig. 5), showing that the sulfate variability either pre-existing or resulting from the ionization process is not a specific issue of the MALDI method. Hence, nanoESI spectra revealed numerous peaks unambiguously assigned to hexasaccharides with six to nine sulfates (m/z 347.4994 to 306.4577/304.4576). Mono-N-acetylated forms were also detected, with oligosaccharides carrying exclusively six (m/z 254.5013 and m/z 254.5014 to m/z 258.1642 in Fig. 5A, B, respectively) and seven sulfate groups (m/z 271.4903 to m/z 284.1420 and m/z 271.4903 to m/z 284.1423 in Fig. 5A, B, respectively). In addition, several cation exchanges involving H/Na/K were also observed.
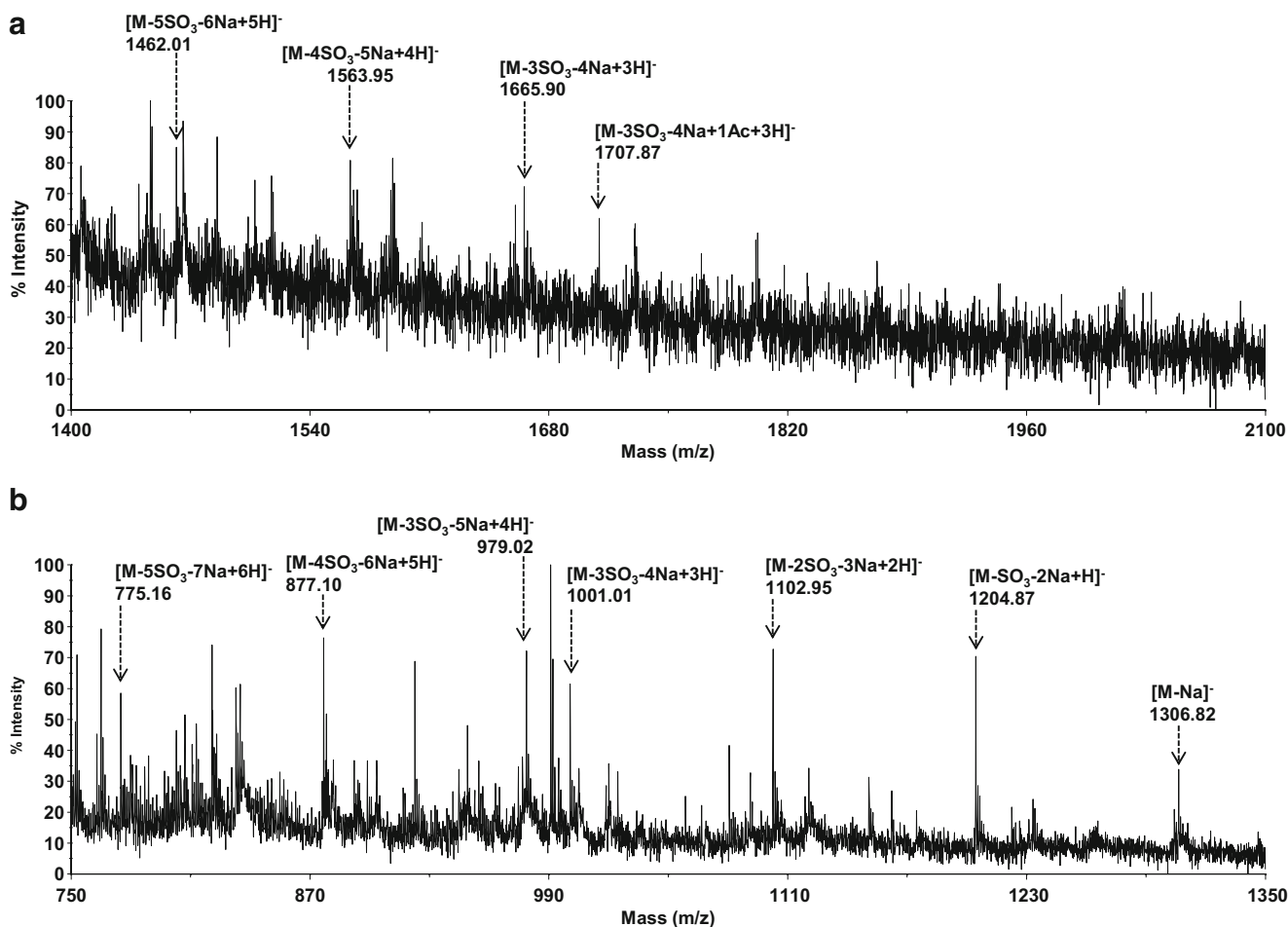
Fig. 7 Negative ion reflector MALDI-TOF/MS analysis of sulfated oligosaccharides produced by the enzyme depolymerization of heparin and extracted from carbohydrate-polyacrylamide gels. **A** Mass spectrum of heparin hexasaccharides extracted from the HP1 gel band; M is the molecular ion corresponding to the fully sodiated sulfated hexasaccharide (DP6, 9 S, 12 Na, M = 1994.70 Da). **B** Mass spectrum of heparin tetrasaccharides extracted from the HP2 gel band. M is the molecular ion corresponding to the fully sodiated sulfated tetrasaccharide (DP4, 6 S, 8 Na, M = 1329.8 Da); 0.5 μL of the desalted sample was mixed with 9 mg/mL HABA/TMG$_2$ doped with 100 μM NaCl

These results show that our combined approach involving gel extraction and a desalting procedure leads to successful analysis of highly sulfated oligosaccharides, after simple and efficient separation by gel electrophoresis.

Furthermore, 1 μg of oligosaccharides loaded on a gel led to lower peak intensities, with exploitable mass spectra for hexasaccharides, but not for decasaccharides. Therefore, 1 μg is thus the lowest amount that can be satisfactorily used for subsequent extraction and MS analysis. Nevertheless, this amount is of the same order of typical values of catabolic oligosaccharides that can be extracted from biological fluids.

The extraction method was then applied to more complex samples from crude reaction mixtures of sulfated oligosaccharides produced by enzymatic depolymerization of heparin and dermatan sulfate. Gel electrophoresis showed a continuum of oligosaccharides for both mixtures, indicating that the depolymerization reaction yielded a wide range of oligosaccharides of various polymerization degrees (Fig. 6). Oligosaccharides contained in the more resolved, lower gel bands were extracted and analyzed by MALDI-TOF/MS. Low intensity mass spectra were obtained in agreement with the weak gel staining, suggesting that the amount of extracted oligosaccharides was around the above-mentioned limit value (i.e., 1 μg). Nevertheless, species corresponding to heparin hexasaccharides could be detected in the HP1 band whose electrophoretic migration was close to the fully sulfated heparin hexasaccharide marker (Fig. 7A). The sodiated hexasaccharide ΔDP6 (9 S, 12 Na) was detected as a partially desulfated species at m/z 1462.01 ([M-5SO$_3$-6Na + 5H]$^-$), 1563.95 ([M-4SO$_3$-5Na + 4H]$^-$), and 1665.90 ([M-3SO$_3$-4Na + 3H]$^-$), whereas a weak peak at m/z 1787.87 ([M-3SO$_3$-4Na + 3H]$^-$) was putatively attributed to its mono-acetylated counterpart (ΔDP6, 8 S, 1 Ac, 11 Na). Oligosaccharides extracted from the HP2 band
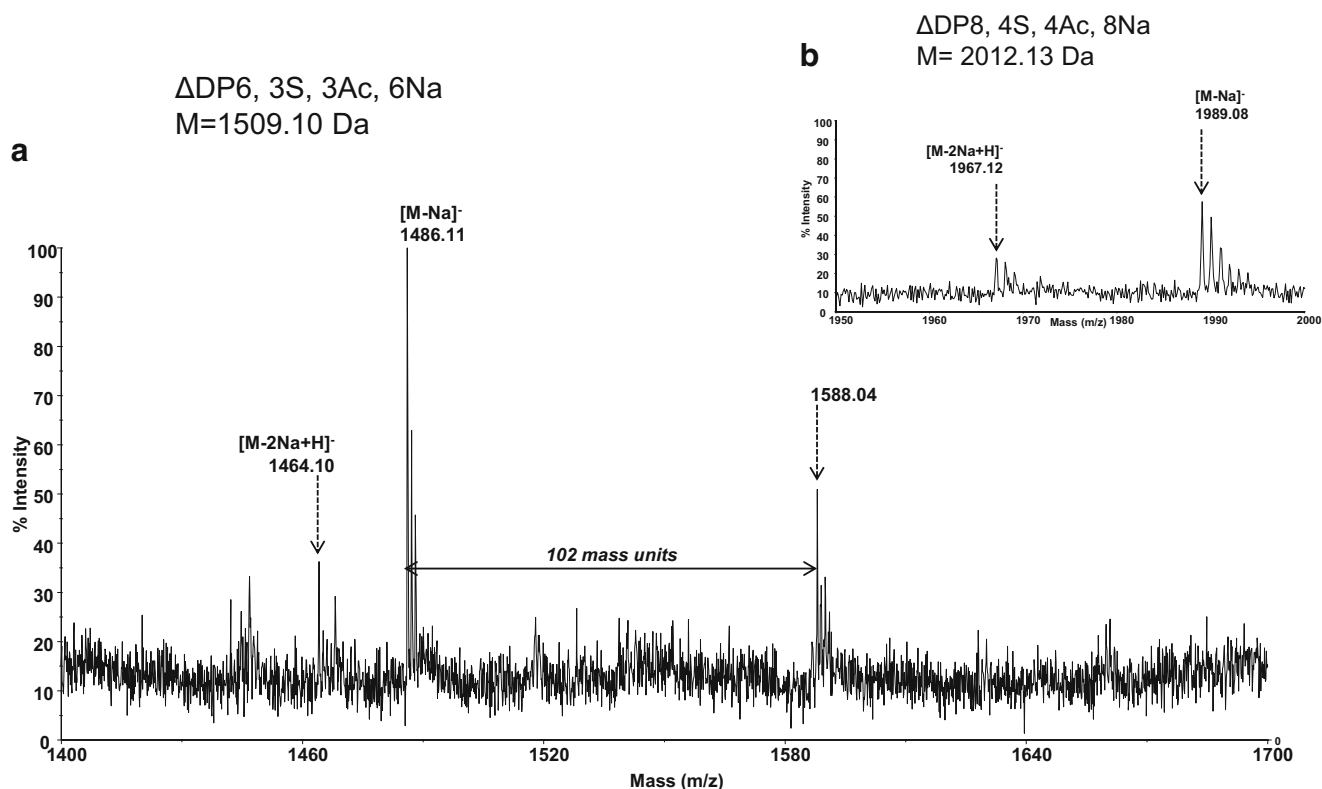
Fig. 8 Negative ion reflector MALDI-TOF/MS analysis of sulfated oligosaccharides produced by the enzyme depolymerization of dermatan sulfate and extracted from the DS1 gel band. **A** Mass spectrum of dermatan sulfate hexasaccharides. M is the molecular ion corresponding to the fully sodiated trisulfated hexasaccharide (DP6, 3 S, 3 Ac, 6 Na, M = 1509.10 Da). **B** Zoom on dermatan sulfate octasaccharides. M is the molecular ion corresponding to the fully sodiated tetrasulfated octasaccharide (DP8, 4 S, 4 Ac, 8 Na, M = 2012.13 Da); 0.5 μL of desalted sample was mixed with 9 mg/mL HABA/TMG$_2$ doped with 100 μM NaCl

yielded more intense ions in the MALDI-TOF spectrum corresponding to heparin sulfated tetrasaccharides ranging from the fully sulfated species (ΔDP4, 6 S, 8 Na) detected at m/z 1306.82 ([M-Na]$^-$) to the monosulfated species at m/z 775.16 ([M-5SO$_3$-7Na + 6H]$^-$) (Fig. 7B). This result indicates that heparin oligosaccharides were efficiently recovered from the complex reaction mixture following the gel extraction and clean-up (desalting) procedure. The hexa- and tetrasaccharides ions identified by MALDI-TOF/MS in the HP1 and HP2 bands closely matched those expected from the enzymatic depolymerization of heparin by heparinase I.

The MS analysis of oligosaccharides contained in the DS1 gel band from the dermatan sulfate depolymerization revealed the presence of trisulfated hexasaccharides (ΔDP6, 3 S, 3 Ac, 6 Na) detected at m/z 1464.10 ([M-2Na + H]$^-$) and m/z 1486.11 ([M-Na]$^-$) (Fig. 8A). This oligosaccharide was less sulfated than the above-described heparin hexasaccharide, in agreement with the lower sulfate content of dermatan sulfate [46–48]. The structure ΔUA-GalNAc4S-(UA-GalNAc4S)$_2$ was assigned to this hexasaccharide, consistent with the average composition of dermatan sulfate from porcine intestinal mucosa, composed of repetitive monosulfated disaccharide units (UA-GalNAc4S)$_n$ [24]. An additional hexasaccharide species was detected at m/z 1588.04, i.e., exhibiting an increment of 101.93 mass units corresponding to an additional sodiated sulfate group (-H/+SO$_3$Na). This hexasaccharide contained thus four sulfate groups, likely indicating the presence of a disulfated disaccharide unit UA-GalNAc4S6S in addition to two other monosulfated disaccharide units [24]. Unexpectedly, ions corresponding to tetrasulfated octasaccharides (ΔDP8, 4 S, 4 Ac, 8 Na) were also detected at a higher m/z range at m/z 1967.12 ([M-2Na + H]$^-$) and 1989.08 ([M-Na]$^-$) with the putative sequence ΔUA-GalNAc4S-(UA-GalNAc4S)$_3$ (Fig. 8B). MS analysis of the DS2 gel band revealed the presence of the disulfated tetrasaccharide (ΔDP4, 2 S, 2 Ac, 4 Na) at m/z 983.11 ([M-Na]$^-$) and minor monosulfated tetrasaccharide (ΔDP4, 1 S, 2 Ac, 4 Na) at m/z 859.23 ([M-SO$_3$-3Na + 2H]$^-$) (Fig. 9A). The ion at m/z 1085.05 corresponding to a trisulfated tetrasaccharide again indicated the presence of a disulfated disaccharide unit in dermatan sulfate. Lastly,
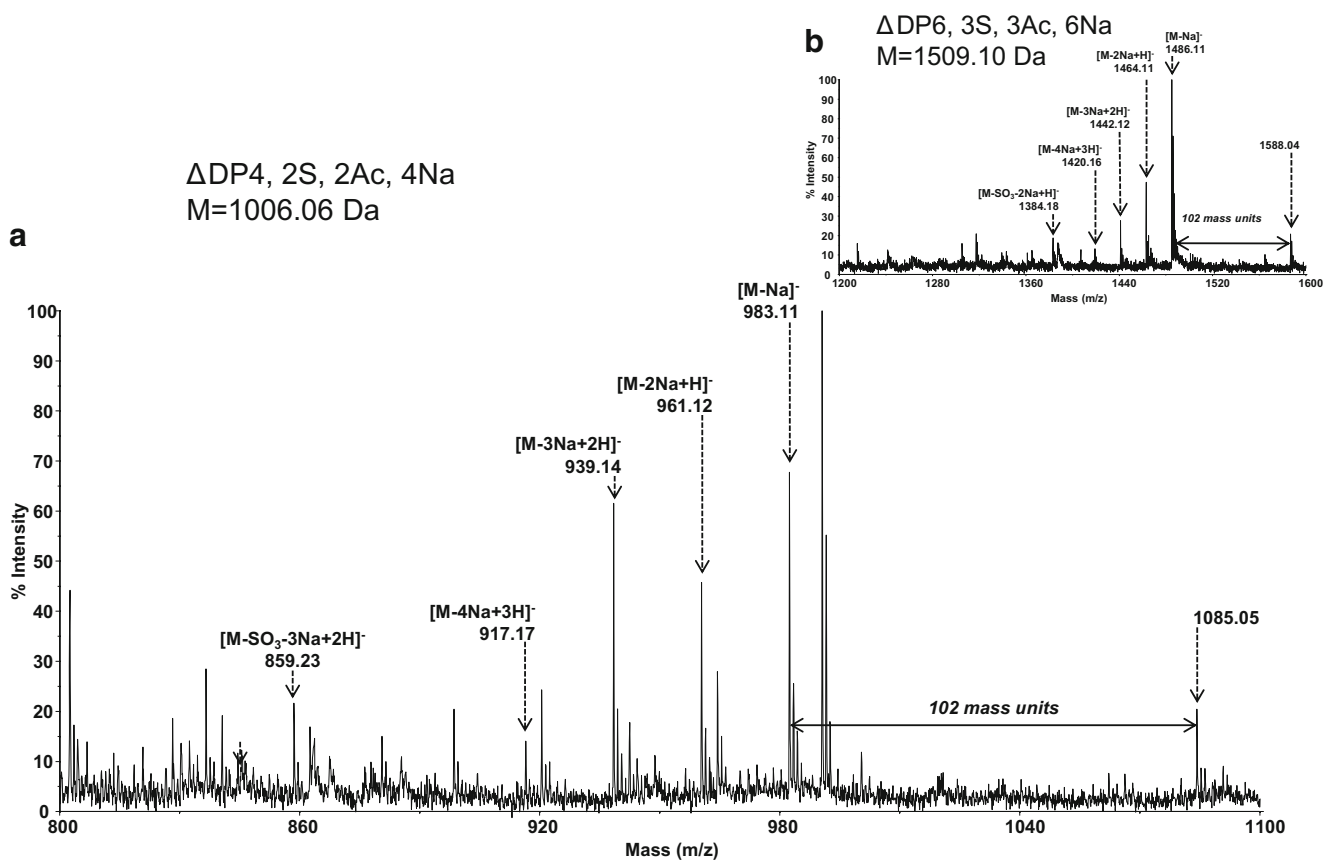
Fig. 9 Negative ion reflector MALDI-TOF/MS analysis of sulfated oligosaccharides produced by the enzyme depolymerization of dermatan sulfate and extracted from the DS2 gel band. **A** Mass spectrum of dermatan sulfate tetrasaccharides. M is the molecular ion corresponding to the fully sodiated trisulfated tetrasaccharide (DP4, 2 S, 2 Ac, 4 Na, M = 1006.06 Da). **B** Zoom on dermatan sulfate hexasaccharide (DP6, 3 S, 3 Ac, 6 Na, M = 1509.10 Da); 0.5 µL of desalted sample was mixed with 9 mg/mL HABA/TMG$_2$ doped with 100 µM NaCl

trisulfated hexasaccharides were also observed in this DS2 gel band at *m/z* 1464.11 ([M-2Na + H]$^-$) and *m/z* 1486.11 [M-Na]$^-$, as well as a minor tetrasulfated species at *m/z* 1588.04 (Fig. 9B). Applied to dermatan sulfate, the extraction procedure lead to the recovery and subsequent MS analysis of oligosaccharides of lower charge than heparin. In addition to the monosulfated disaccharide building block UA-GalNAc4S known as a main constitutive unit of dermatan sulfate from porcine intestinal mucosa, MS analysis showed the significant presence of more heavily sulfated disaccharide units in some detected oligosaccharides, at the average ratio of one disulfated disaccharide unit for every tetra- or hexasaccharide.

Conclusion

Similar to the well-established procedures in proteomics for peptide gel extraction and MS sequencing, an analogous strategy can be implemented for sulfated GAG oligosaccharides. We developed an in-gel (C-PAGE)

extraction method that successfully yields a quantitative amount of sulfated oligosaccharides after specific treatment of the excised gel bands. The obtained oligosaccharides were compatible with subsequent MS analysis after the application of a single, simple clean-up step on a mixed C18/graphite carbon solid-phase column. Instead of using multiple and successive strong anion-exchange and filtration steps, our procedure is more rapid: separation and MS analysis can be carried out within one working day, a much shorter time period compared with previously reported procedures [39, 49]. Our method provides sulfated oligosaccharides ready for MALDI-TOF/MS as well as for electrospray MS. C-PAGE is an easy, fast, and readily available separation technique that offers one of the highest levels of resolution for GAG oligosaccharides. Given the ease of the implementation of this extraction method, its combination with MS is an affordable and attractive alternative to more complex or costly analytical approaches. Our method thus offers effective means for practically any laboratory to carry out fine structural analysis on bioactive sulfated carbohydrates.

Compliance with ethical standards

Conflict of interest   The authors declare that they have no conflict of interest.

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