

High-performance liquid chromatography-mass spectrometry for mapping and sequencing glycosaminoglycan-derived oligosaccharides

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Glycosaminoglycans (GAGs) have proven to be very difficult to analyze and characterize because of their high negative charge density, polydispersity and sequence heterogeneity. As the specificity of the interactions between GAGs and proteins results from the structure of these polysaccharides, an understanding of GAG structure is essential for developing a structure–activity relationship. Electrospray ionization (ESI) mass spectrometry (MS) is particularly promising for the analysis of oligosaccharides chemically or enzymatically generated by GAGs because of its relatively soft ionization capacity. Furthermore, on-line high-performance liquid chromatography (HPLC)-MS greatly enhances the characterization of complex mixtures of GAG-derived oligosaccharides, providing important structural information and affording their disaccharide composition. A detailed protocol for producing oligosaccharides from various GAGs, using controlled, specific enzymatic or chemical depolymerization, is presented, together with their HPLC separation, using volatile reversed-phase ion-pairing reagents and on-line ESI-MS structural identification. This analysis provides an oligosaccharide map together with sequence information from a reading frame beginning at the nonreducing end of the GAG chains. The preparation of oligosaccharides can be carried out in 10 h, with subsequent HPLC analysis in 1–2 h and HPLC-MS analysis taking another 2 h.

INTRODUCTION

During the past 25 years, there has been a growth of understanding of post-translational modifications, particularly glycosylation¹, of the importance of glycan–protein interactions on biological activity^{2,3} and how genotype influences glycan structure and the development of phenotype⁴. Although new technologies for high-throughput measurements of gene expression, protein biosynthesis and protein–protein interactions have resulted in major advances in the fields of genomics and proteomics, the field of glycomics is just beginning to be explored^{2,3}. Over half of the human proteome is posttranslationally modified through glycosylation¹. Proteoglycans and glycoproteins have important roles in controlling the extracellular events that are critical in establishing phenotype. The key components of the extracellular matrix in all eukaryotic cells are proteoglycans⁵, consisting of a core protein to which a number of polysaccharide chains, called glycosaminoglycans (GAGs), are attached (see **Fig. 1** showing the main classes of GAGs)⁶. GAGs have critical roles in a number of biological processes, including anticoagulation, development, angiogenesis, axonal growth, cancer progression and microbial pathogenesis (see reviews in refs. 7–12).

In addition to their physiological (and pathophysiological) role, these natural complex polysaccharides are active biological and pharmaceutical agents, and several GAG-based drugs are clinically used, whereas others are advancing through clinical trials¹³. The development of GAG-based therapeutics has involved five distinct strategies that are particularly well illustrated in the development of heparin (HP)-based therapeutics. The first approach is the extraction and isolation of natural polysaccharides. Further chemical modification and/or controlled degradation/depolymerization of the backbone structure, followed by purification, has resulted

in a number of GAG-derived products, including the widely used class of therapeutic agents, namely, the low-molecular-weight HPs¹⁴. A second manufacturing strategy involves a complete chemical synthesis, and is exemplified by the HP pentasaccharide drug, Arixtra, used for thrombotic indications¹⁵. A third strategy uses bacterial fermentation to prepare the sulfated polysaccharide backbones of GAGs that are then either chemically or enzymatically modified and sulfonated to obtain products. The chemoenzymatic synthesis of a ‘bioengineered’ HP and its scale-up and evaluation are currently underway^{15–18}. The preparation of ‘bioengineered’ GAGs relies on bacterial polysaccharides from *Escherichia coli*, including defructosylated capsular polysaccharide K4 (chondroitin, CH)¹⁹ or capsular polysaccharide K5 (heparosan, HN)²⁰, or hyaluronan, CH or HN from *Pasteurella multocida*²¹. A fourth approach involves the full enzymatic synthesis of GAG in an artificial Golgi organelle, as has been recently shown in a simple prototype microfluidic reactor²². A fifth strategy, the metabolic engineering of cells to produce GAGs by fermentation, is in its nascent stages of investigation²³.

The fundamental biological, pathological, pharmacological and therapeutic roles of GAGs have challenged researchers to devise new ways to prepare these critical polysaccharides and devise novel methods to decode their fine complex structure, which is needed to establish structure–activity relationships. Solving the structure of GAGs is complicated as their biosynthesis is not template driven, and also because it involves multiple enzymes having tissue-specific isoforms and takes place in the Golgi, a poorly understood organelle^{24,25}. This results in sequence heterogeneity (types and positioning of disaccharide units) and polydispersity ($M_w/M_n > 1$). Furthermore, pharmacologically and biologically

Figure 1 | Different classes of GAGs and their disaccharide units. GAGs containing uronic acid residues can be grouped into three categories: (1) HA or hyaluronan, (2) CS/DS/CH/K4 and (3) HS/HP/HN. They are biosynthesized as polysaccharides of repeating disaccharides with *N*-acetylhexosamine, *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc) as one of the sugars. The alternating sugar is glucuronic acid (GlcA). HA is not modified further, whereas the other classes may be further modified by (1) the addition of *O*-sulfo groups on various hydroxyl groups, (2) the 5-epimerization of some GlcA residues to form iduronic acid (IdoA) residues (DS, HS and HP) and (3) the possible replacement of the *N*-acetyl groups of glucosamine residues with *N*-sulfo (HS and HP). UA corresponds to either GlcA or IdoA.

important (poly)oligosaccharides often contain rare, difficult to detect sequences that are responsible for their activity²⁶. The poly-anionic nature of these polysaccharides poses additional challenges for their separation and sensitive analysis, which can often require their chemical derivatization²⁷. High-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been used in combination with nuclear magnetic resonance (NMR) and mass spectrometry (MS) to solve many GAG-derived oligosaccharide structures (see reviews in refs. 2,26,28–31). NMR spectroscopy is limited by the necessity to obtain relatively large amounts of pure sample and matrix-assisted laser desorption/ionization, whereas a powerful technique also requires a pure sample and can be sufficiently energetic to damage the analyte.

A successful combination of controlled enzymatic or chemical depolymerization, separation, detection and spectral analysis provides the critical advantage for establishing the GAG structure. The direct analysis of intact GAGs is difficult because of their relatively high molecular mass and polydispersity. As a consequence, controlled enzymatic or chemical depolymerization is often required to lower their molecular weight and simplify these polydisperse mixtures before analysis^{32,33}. Although enzymatic depolymerization offers advantages based on enzyme specificity, chemical depolymerization using reagents with high chemoselectivity is also quite valuable^{32,33}. Electrospray ionization (ESI)-MS can be interfaced with HPLC and thus offers the possibility to analyze mixtures of GAG-derived oligosaccharides. On-line analysis eliminates the time-consuming fraction collection step, and greatly enhances the analysis and structural characterization of complex mixtures of GAG-derived oligosaccharides. ESI-MS is able to produce very gentle ionization and it can be easily connected to on-line liquid-phase analytical separation techniques, such as HPLC and CE. Strong anion exchange (SAX)-HPLC, although conventionally used to separate (di)oligosaccharides generally produced by enzymatically treated GAGs³⁴, is difficult to interface with ESI-MS, as it uses mobile phases containing high concentrations of nonvolatile salts required to elute the highly charged analytes. Standard reversed-phase ion-pairing (RPIP)-HPLC, although providing excellent chromatographic resolution, relies on high concentrations of nonvolatile quaternary ammonium salts that are retained in the mass spectrometer, making it incompatible for use with ESI-MS²⁶. Volatile ion-pairing reagents, such as volatile primary, secondary and tertiary amines³⁵, post-column addition of sheath liquid and the splitting of the eluent flow³⁶, have been applied to overcome these problems to develop an on-line GAG-derived oligosaccharide analysis and improve their

GAGs	Disaccharide unit	UA	Modifications
HP/HS		IdoA/GlcA	X: SO ₃ ⁻ or H Y: SO ₃ ⁻ or Ac or H
CS/DS		IdoA/GlcA	X: SO ₃ ⁻ or H
HA		GlcA	No modifications
HN		GlcA	No modifications
CH/K4		GlcA	X: H or fructose

characterization. An on-line RPIP-HPLC-ESI-MS protocol is presented for the separation and structural identification of GAG-derived oligosaccharides, prepared through controlled enzymatic depolymerization, a method in which exposure time to a specific enzyme or enzymes is reproducibly limited so as to prepare oligosaccharide products still containing enzyme-susceptible linkages²⁶. This protocol provides disaccharide composition, oligosaccharide mapping and sequence information from a reading frame beginning at the nonreducing end of the polysaccharide chain. Detailed protocols are provided for the analysis of GAGs purified from animal and bacterial sources.

High-performance liquid chromatography-MS and CE-MS are limited to mixtures of GAG and GAG-derived oligosaccharides that can be sufficiently well resolved by HPLC or CE and that can be analyzed by soft-ionization MS. Complex multicomponent mixtures, such as intact GAGs, can be difficult to fractionate into individual components, even using high-resolution chromatography or electrophoresis. Analytes having a high molecular mass, such as intact HP, or a high level of negative charge, such as persulfonated GAG oligosaccharides, often fail to yield mass spectra. Finally, analytes having sensitive functional groups such as labile *O*-acetyl groups or labile *O*- or *N*-sulfo groups can fragment even under soft ionization methods, making it difficult to obtain interpretable mass spectra. Such problematic GAG and GAG-derived oligosaccharides might be best examined by chemical, enzymatic or spectroscopic methods such as high-field NMR spectrometry.

MATERIALS

REAGENTS

- Heparinase I (200–600 U mg⁻¹ solid, heparin lyase I obtained from *Flavobacterium heparinum*, EC 4.2.2.7; Sigma-Aldrich, cat. no. H2519) ▲ **CRITICAL** Store at –20 °C.
- Heparinase III (200–600 U mg⁻¹ solid, heparin lyase III obtained from *F. heparinum*, EC 4.2.2.8; Sigma-Aldrich, cat. no. H8891) ▲ **CRITICAL** The enzyme solution maintained at pH 6–7 is stable for a week at –20 °C.
- Chondroitinase ABC (50–250 U mg⁻¹ protein, chondroitin ABC lyase obtained from *Proteus vulgaris*, EC 4.2.2.4; Sigma-Aldrich, cat. no. C3667) ▲ **CRITICAL** Store at –20 °C.
- Chondroitinase C (20 U mg⁻¹ solid, chondroitin C lyase obtained from *F. heparinum*; Sigma-Aldrich, cat. no. C0954) ▲ **CRITICAL** Store at –20 °C.
- Hyaluronidase (300–1,000 U mg⁻¹ solid, obtained from bovine testes, EC 3.2.1.35; Sigma-Aldrich, cat. no. H3757) ▲ **CRITICAL** Store at –20 °C.
- QAE Sephadex A-25 (GE Healthcare and Amersham Biosciences, cat. no. 17-0190-03).
- Bovine lung heparin (sodium salt, ≥140 USP U mg⁻¹; Sigma-Aldrich, cat. no. H4898) ▲ **CRITICAL** Store at <25 °C.
- Chondroitinase B (100–300 U mg⁻¹ solid, chondroitin lyase B obtained from *F. heparinum*; Sigma-Aldrich, cat. no. C8058).
- Bovine trachea chondroitin 4-sulfate (sodium salt of chondroitin sulfate (CS) A; Sigma-Aldrich, cat. no. 27042) ▲ **CRITICAL** Store at 2–8 °C.
- Porcine intestinal heparin sulfate (sodium salt of heparin sulfate (HS); Celsus Laboratories, cat. no. HO-3105) ▲ **CRITICAL** Store at 2–8 °C.
- Porcine intestinal mucosa dermatan sulfate (sodium salt of dermatan sulfate (DS); Sigma-Aldrich, cat. no. C3788) ▲ **CRITICAL** Store at 2–8 °C.
- Rooster comb hyaluronic acid (sodium salt of hyaluronic acid (HA); Sigma-Aldrich, cat. no. H5388) ▲ **CRITICAL** Store at –20 °C.
- Acetic acid (≥99.7% (wt/wt), glacial; Sigma-Aldrich, cat. no. 320099) ▲ **CRITICAL** Store at room temperature ! **CAUTION** This product is harmful if inhaled, ingested or if it comes in contact with skin. It is also an irritant for the eyes.
- Acetonitrile LC-MS Chromasolv (≥99.9%; Sigma-Aldrich, cat. no. 34967) ▲ **CRITICAL** Store at room temperature ! **CAUTION** This product is flammable, harmful if inhaled, ingested or if it comes in contact with skin. It is also an irritant for the eyes.
- Ammonium acetate (99.99%; Sigma-Aldrich, cat. no. 372331) ▲ **CRITICAL** Store at 2–8 °C.

- Tributylamine (≥99.5%; Sigma-Aldrich, cat. no. 90781) ▲ **CRITICAL** Store at room temperature. ! **CAUTION** This product is harmful if inhaled, ingested or if it comes in contact with skin.
- Bovine serum albumin (BSA, ≥98.0%; Sigma-Aldrich, cat. no. A7906) ▲ **CRITICAL** Store at 2–8 °C.
- Ultrapure water was obtained using a Milli-Q system (Millipore).
- All other reagents, of the purest grade available, were from Sigma-Aldrich.

EQUIPMENT

- The HPLC system is composed of a minimum of a binary gradient pump ▲ **CRITICAL** The HPLC system used in SAX-HPLC separation with ultraviolet (UV) detection must be made of corrosion-resistant titanium or polyarylether etherketone for use with eluents containing NaCl.
- On-line vacuum degasser.
- On-line multiple wavelength UV detector.
- Discovery C18 column (5 μm, 4.6 × 250 mm; Supelco, cat. no. 504971).
- Gemini C18 column (3 μm, 4.6 × 150 mm; Phenomenex, cat. no. 00F-4439-E0).
- Spherisorb 5-SAX (trimethylammonioethyl groups SiCH₂CH₂CH₂N⁺(CH₃)₃ in Cl⁻ form, 5 μm, 4.6 × 150 mm; Phenomenex, cat. no. 00F-4149-E0).
- ESI mass spectra from Agilent 1100 series Classic or VL G2445A LC/MSD trap (Agilent Technologies).
- Injection Hamilton syringe (100 μl; Sigma-Aldrich, cat. no. S-9766).
- Bench top centrifuge (MicroCentrifugette 4214, ALC International, cat. no. 11172002).

REAGENT SETUP

Sample preparation

- Different GAGs, such as HA, CS, DS, HS, HP, and bacterial polysaccharides, CH and HN, used as standards, may be purchased from Sigma-Aldrich, Seikagaku, Glycoscience (<http://www.amsbio.com/>) and Iduron.
- Extraction and purification protocols for various GAGs are available in specific scientific articles and monographs^{37–41}. We have included examples for extraction of animal GAG from a tissue sample (**Box 1**) and a bacterial GAG (K4 polysaccharide) from *E. coli* Ul-41 cells (**Box 2**)^{42–44}.
- Appropriate standards for the samples being analyzed should be prepared at the outset of the procedure. For example, in the analysis of a tissue or bacterial CH sample, standards might include CS, DS and CH. Furthermore, although analyses by research studies having limited amounts of sample can

BOX 1 | EXTRACTIVE PROTOCOL TO RECOVER AN ANIMAL GAG FROM A TISSUE SAMPLE

The following detailed procedure provides an example for isolation of GAGs from a tissue samples^{39–41}.

! **CAUTION** Animal tissues can contain viruses and prions that may be hazardous and hence should be handled with gloves and disposed off as appropriate for biological waste.

1. Wash the tissue in cold phosphate-buffered saline (PBS) at 4 °C and cut tissue into small pieces, freeze dry and grind dry tissue into powder.
2. Remove fat by extracting the tissues with three solvent mixtures: chloroform:methanol (2:1, 1:1 and 1:2 (vol:vol)), each left overnight at room temperature, and dry defatted tissue in a fume hood.
3. Suspend defatted sample (5–10% (wt/vol)) in water and proteolyze at 55 °C with 10% (wt/wt) of a nonspecific protease such as actinase E (20 mg ml⁻¹) for 18 h.
4. Make up the supernatant to contain 8 M urea and 2% (wt/vol) CHAPS buffer, and remove any insoluble residue by centrifugation.
5. Equilibrate a Vivapure Maxi Q SAX spin column with 8 M urea and 2% (wt/vol) CHAPS buffer at pH 8.3, load the sample and wash the column with three column volumes of 200 mM aqueous NaCl.
6. Release GAGs by washing the column with one column volume of 16% (wt/vol) NaCl.
7. Perform methanol precipitation using 80% (vol/vol) methanol. For example, to one volume of GAG solution in 16% (wt/vol) NaCl, add four volumes of methanol and incubate overnight at 4 °C.

! **CAUTION** Organic and aqueous organic solvents should only be stored in an explosion-proof freezer.

8. Recover the precipitated GAG mixture by centrifugation (5,000g for 30 min).
9. HP and HS can be recovered by removing CS and DS by treating the sample exhaustively with chondroitinase ABC; CS can be recovered by removing HP, HS and DS by treating the sample exhaustively with heparinase I and III and chondroitinase B; and DS can be recovered by removing HP, HS and CS by treating the sample exhaustively with heparinase I and III and chondroitinase AC, followed by recovery using methanol precipitation (described above).

BOX 2 | EXTRACTIVE PROTOCOL TO RECOVER A BACTERIAL GAG PROVIDED FOR K4 POLYSACCHARIDE FROM *E. COLI* UL-41 CELLS

! CAUTION *E. coli* strains producing capsular polysaccharides can be pathogenic, and should be handled under appropriate controls, and cells should be autoclaved before disposal.

1. After culturing, centrifuge the cell medium at 4,000g for 30 min to remove cells and add five volumes of acetone to precipitate the polysaccharide fraction from the supernatant at $-20\text{ }^{\circ}\text{C}$ overnight.

! CAUTION Organic and aqueous organic solvents should only be stored in an explosion-proof freezer.

2. Centrifuge at 5,000g for 15 min and dry the recovered precipitate at $60\text{ }^{\circ}\text{C}$ for 12 h. Dissolve the dried precipitate in 5 ml of 50-mM NaCl by mixing.

3. Centrifuge at 10,000g for 10 min and apply the supernatant to a column (1 × 20 cm) packed with QAE Sephadex A-25 anion-exchange resin equilibrated with 50-mM NaCl solution.

4. Elute the polysaccharide by means of a linear gradient of NaCl from 50 mM to 2 M from 0 to 150 min by generic low-pressure liquid chromatography at a flow rate of 1 ml min^{-1} . Collect fractions of 2 ml and detect polysaccharides by UV monitoring at 214 nm and by borate-carbazole assay for uronic acids⁵³.

5. Dialyze the collected fractions corresponding to polysaccharide(s) species against double distilled water and freeze dry.

be performed in singlicate, it is advisable to analyze independent replicate ($n = 3-5$) samples and standards to determine statistical significance.

- Purified GAGs can be analyzed immediately or stored frozen in solid form or in aqueous solution at $<20\text{ }^{\circ}\text{C}$ and should be free from contaminants and salts. **▲ CRITICAL** All GAG samples should have been purified using at least one anion exchange step; one precipitation step using organic solvents, i.e., acetone ethanol or methanol; one desalting step, i.e., dialysis, centrifugal membrane or desalting gel chromatography; and one step to remove particulates, i.e., filtration through a $0.2\text{-}\mu\text{m}$ membrane filter or by centrifugation. Samples are dried or lyophilized before analysis.
- Before analysis, dissolve purified GAGs in ultrapure water to obtain a final concentration of 10 mg ml^{-1} .

Heparinase dissolving buffer (20-mM Tris-HCl (pH 7.5), 50-mM NaCl, 4-mM CaCl_2 and 0.01% (wt/vol) BSA) Dissolve 0.24 g of Tris-HCl, 0.044 g of CaCl_2 , 0.29 g of NaCl and 0.01 g of BSA in 90-ml ultrapure water. Adjust the pH of the solution to 7.5 using 0.1-M hydrochloric acid or 0.1-M sodium hydroxide (as necessary) and make up the volume to 100 ml with ultrapure water. Store at $-4\text{ }^{\circ}\text{C}$ before use, within 1 week of preparation.

HP/HS/HN digestion buffer solution (50-mM sodium phosphate buffer (pH 7.0)) Add 0.60 g of NaH_2PO_4 and 0.71 g of Na_2HPO_4 to 90 ml of ultrapure water. Adjust the pH of the solution to 7.0 using 0.1-M hydrochloric acid or 0.1-M sodium hydroxide (as necessary) and make up the volume to 100 ml with ultrapure water. Store at $-4\text{ }^{\circ}\text{C}$ before use.

Heparinase stock aliquots Dissolve heparinase I or III in 20-mM Tris-HCl (pH 7.5), 50-mM NaCl, 4-mM CaCl_2 and 0.01% (wt/vol) BSA at 1 U ml^{-1} (see above for the preparation of heparinase dissolving buffer). Aliquots should be kept frozen at $-80\text{ }^{\circ}\text{C}$ for medium- or long-term storage. The heparinase III solution at pH 6–7 is stable for a week at $-20\text{ }^{\circ}\text{C}$.

Chondroitinase dissolving buffer (0.01% (wt/vol) BSA aqueous solution) Dissolve 0.01 g of BSA in 100-ml ultrapure water. Store at $-4\text{ }^{\circ}\text{C}$ before use. **CS/DS/HA/CH digestion buffer solution (50-mM Tris (pH 8.0), with 60-mM sodium acetate)** Dissolve 0.61 g of Tris-HCl and 0.49 g of sodium acetate in 90 ml ultrapure water. Adjust the pH of the solution to 8.0 using 0.1-M sodium hydroxide (as necessary) and make up the volume to 100 ml with ultrapure water. Store at $-20\text{ }^{\circ}\text{C}$ before use.

Chondroitinase stock aliquots Dissolve chondroitinases in 0.01% (wt/vol) BSA aqueous solution at 0.4 U ml^{-1} . Store aliquots at $-4\text{ }^{\circ}\text{C}$ before use. Aliquots desiccated at $-20\text{ }^{\circ}\text{C}$ remain active for at least 6 months.

Hyaluronidase dissolving buffer (100-mM sodium acetate, 150-mM NaCl, 0.01% (wt/vol) BSA (pH 5.2)) Dissolve 0.82 g of sodium acetate, 0.88 g of NaCl and 0.01 g of BSA in 90 ml ultrapure water. Adjust the pH of the solution to 5.2 using 1-M acetic acid solution and make up the volume to 100 ml with ultrapure water. This solution should be always freshly prepared immediately before use.

HA digestion buffer solution (100-mM sodium acetate (pH 5.2) containing 150-mM NaCl) Dissolve 0.82 g of sodium acetate and 0.88 g of NaCl in

80-ml ultrapure water. Adjust the pH of the solution to 5.2 using 1-M acetic acid solution and make up to 100 ml with ultrapure water. This solution should be always freshly prepared immediately before use.

Hyaluronidase stock aliquots Immediately before use, prepare a solution containing $2,500\text{ U ml}^{-1}$ of hyaluronidase in enzyme-dissolving buffer. No enzyme aliquots should be prepared and stored, and the hyaluronidase solution should always be freshly prepared immediately before use.

Preparation of HPLC solvents (SAX separation and UV detection)

- **Solvent A (50 mM aqueous NaCl):** Dissolve 2.92 g of sodium chloride in 1 liter of ultrapure water. Adjust the pH to 3.5 using 1-M hydrochloric acid and filter and degas the solvent.
- **Solvent B (1.2 M aqueous NaCl):** Dissolve 70.13 g of NaCl in 1 liter of ultrapure water. Adjust the pH to 3.5 using 1-M hydrochloric acid and filter and degas the solvent in the same manner as carried out for solvent A.

Preparation of HPLC solvents (on-line ESI-MS detection)

- **Solvent C (water/acetonitrile (80:20), 15 mM tributylamine and 50 mM ammonium acetate (pH 7.0)):** Dissolve 1.93 g of ammonium acetate and 1.79 g of tributylamine in 100-ml acetonitrile and 350 ml of ultrapure water. Mix for about 30 min and adjust the pH to 7.0 using glacial acetic acid. Make up the volume to 500 ml with ultrapure water. Degas the solvent.
- **Solvent D (water/acetonitrile (35:65), 15 mM tributylamine and 50 mM ammonium acetate (pH 7.0)):** Dissolve 1.93 g of ammonium acetate and 1.79 g of tributylamine in 325 ml of acetonitrile and 100 ml of ultrapure water. Mix for about 30 min and adjust the pH to 7.0 using glacial acetic acid. Make up the volume to 500 ml with ultrapure water. Degas the solvent.

▲ CRITICAL Carefully check the pH, as different pH values can alter elution time periods and result in overlapping of oligosaccharides species.

▲ CRITICAL Solvents may be stored at $-4\text{ }^{\circ}\text{C}$ for further HPLC separations for not more than about 1 week. **! CAUTION** Organic solvents and aqueous organic solvent mixtures should only be stored in an explosion-proof refrigerator.

EQUIPMENT SETUP

HPLC system requirements An HPLC system capable of mixing a binary gradient is necessary for both UV and MS detection. The system should be capable of delivering a flow rate of at least 2 ml min^{-1} for UV and MS detection. A Rheodyne (or similar) injection system capable of loading and injecting up to $100\text{ }\mu\text{l}$ of sample should also be used, together with appropriate data acquisition, control and analysis software. For UV detection, an on-line multiple wavelength UV absorbance detector is required with the detection wavelength held constant at $\lambda_{\text{abs}} = 232$ or 214 nm . For MS acquisition, an ESI source of Agilent Technologies LC/MSD Trap Classic or VL mass spectrometer possessing an API-ESI interface with orthogonal nebulizer, a single split-flow turbomolecular high-vacuum pump and a resolution of $<2\text{ }\mu\text{m}$ is suggested.

HPLC column preparation

- **SAX-HPLC separation and UV detection.** Under the experimental conditions adopted, the preconditioning time has been reduced to 30 min. Column preconditioning is achieved by washing the column with isocratic solvent

A for 5 min, followed by a gradient wash from 100% solvent A to 100% solvent B at a flow rate of 1.2 ml min⁻¹ for 25 min. The column is then washed for 5 min with solvent A, after which the system is ready for sample separation.

For separation and UV detection, SAX columns (4 mm i.d., 150 mm length) provide an excellent resolution of both unsaturated and saturated oligosaccharides derived from the various GAGs. Peak symmetry is generally high, elution times are extremely consistent and stable baselines are achievable. These features facilitate an accurate identification of peaks. Column longevity is generally high, in particular when they are protected with suitable disposable guard column cartridges.

- **RPIP-HPLC separation and (UV)-ESI-MS detection.** Column preconditioning is achieved by washing the column with isocratic solvent C for 10 min, followed by a gradient separation to 100% solvent D, at a flow rate of 0.5 ml min⁻¹ for the Discovery C18 column or at 0.3 ml min⁻¹ for the Gemini C18 column for 40 min. After conditioning for 20 min in solvent C, the system is ready to perform sample separation.

For separation and (UV)-ESI-MS detection and characterization, these two columns provide excellent separation of oligosaccharides derived from enzymatic or chemical treatment of GAGs. Elution time periods are extremely consistent and stable baselines are achieved. Column longevity is generally high, in particular when they are protected with suitable disposable guard column cartridges.

ESI-MS equipment setup Optimization is generally performed by direct injection (6 μl min⁻¹, 1 mg min⁻¹) of trisulfated HP or monosulfated CS/DS disaccharide with mobile phase Solvent C at a flow rate of 0.5 ml min⁻¹. The electrospray interface is set in negative ionization mode with the skimmer potential at -19.7 V, the capillary exit at -48.4 V and a source of temperature of 350 °C to obtain maximum abundance of standard disaccharide ions in full scan spectra (200–2,200 Da, 10 full scans per second). Nitrogen is used as a drying (12 liter min⁻¹) and nebulizing gas

(4.14 bars). Total ion chromatograms and mass spectra are processed using Agilent Chemstation A.07. The software versions used are 4.0 LC/MSD trap control 5.0 and Data Analysis 2.2 (Agilent Technologies).

For MS simplification experiments, 5-mM tributylamine in acetonitrile is optimally used as post-column addition reagent directly infused into the ESI source at a flow rate of 6 ml min⁻¹ by using an Agilent syringe pump. The sample and the post-column addition reagent are both sprayed at the same time to ensure in-source mixing.

For the analysis of highly sulfated oligosaccharides, i.e., those derived from HP, the addition of acetonitrile as sheath liquid resulted in an improvement in ion intensity and spectral simplification, possibly due to weakening of the solvent–solute and ion–counter–ion interactions, producing the subsequent release of free analyte ions into the evaporation process. Furthermore, the most intense ion peaks of each oligosaccharide produced by cation adduction are always associated with the tributylamine adduct. As a consequence, the post-column addition of a low concentration (5 mM) of tributylamine ion-pairing reagent in acetonitrile provided the most simplified spectra, in particular, by suppressing Na⁺/NH₄⁺ adduction without influencing the charge state reduction. However, a substantial reduction in ion intensity of about fivefold is generally observed.

A major advantage of post-column addition of tributylamine/acetonitrile is the disappearance of other cation adduct peaks, i.e., Na⁺, K⁺ or NH₄⁺, resulting in an essential reduction in ‘chemical noise,’ producing simplified and interpretable spectra. In fact, the optimized ESI mass spectra are successfully simplified, in particular, by showing the elimination of cationic adducts for small HP oligosaccharides, disaccharide–hexasaccharide (2–6-mers), and a significant decrease in the relative abundance of sodiated (Na⁺) and ammoniated (NH₄⁺) adducts for larger oligosaccharides, 8–14-mers. However, the presence of tributylamine may result in peak doubling, presumably through the resolution of the α- and β-anomeric forms of the individual oligosaccharides.

PROCEDURE

Enzyme treatment and production of GAG-derived oligosaccharides ● TIMING ~10 h

1| The generation of GAG-derived oligosaccharides relies on the partial controlled enzymatic depolymerization of GAG chains by using specific enzymes. Oligosaccharides of various sizes and structures may be produced depending on the structure of the substrate, the enzyme specificity and the site of action, i.e., at the nonreducing end resulting in a saturated oligosaccharide or at internal chain sites affording an unsaturated oligosaccharide through the introduction of a carbon–carbon double bond between C-4 and C-5 of the terminal uronic acid residue. The resulting oligosaccharides are separated and structurally characterized by RPIP-HPLC-ESI-MS. Set up the reactions for HP/HS treatment with heparinase I (option A), HS/HN treatment with heparinase III (option B), CS/DS/CH/K4 treatment with chondroitinase ABC (or chondroitinase C)(option C) and/or HA treatment with hyaluronidase (option D). Multiple samples in singlicate or replicate ($n = 3-5$) can be treated with a single option or with all three options if the GAG composition is not known beforehand. It is also often useful to include various GAG standards in amounts bracketing the concentrations of GAGs in the samples, as measured by carbazole assay.

? TROUBLESHOOTING

(A) HP/HS treatment

- Add 1 mg of HP or HS (100 μl) to 700 μl of 50 mM sodium phosphate buffer (pH 7.0) in a 1.5-ml centrifuge tube.
- Add 0.2 mU of heparinase I (200 μl) to the 1.5-ml centrifuge tube.

(B) HS/HN treatment

- Add 1 mg of HS or HN (100 μl) to 700 μl of 50 mM sodium phosphate buffer (pH 7.0) in a 1.5-ml centrifuge tube.
- Add 0.2 mU of heparinase III (200 μl) to the 1.5-ml centrifuge tube.

(C) CS/DS/CH/K4/HA treatment

- Add 1 mg of CS, DS, CH, K4 or HA (100 μl) to 700 μl of 50 mM Tris or 60 mM sodium acetate (pH 8.0) in a 1.5-ml centrifuge tube.
- Add 0.2 mU of chondroitinase ABC (or chondroitinase C) (200 μl) to the 1.5-ml centrifuge tube.

(D) HA treatment

- Add 1 mg of HA (100 μl) to 700 μl of 100 mM sodium acetate containing 150 mM NaCl (pH 5.2) in a 1.5-ml centrifuge tube.
- Add 250 U of hyaluronidase (200 μl) to the 1.5-ml centrifuge tube.

PROTOCOL

- Incubate the reaction mixture in a water bath at 37 °C.
- Remove aliquots of 100 μl , 0.1 mg HA, at various time points for depolymerization analysis by SAX-HPLC-UV or RPIP-HPLC-UV.
- Heat the aliquots in a boiling water bath for 10 min to stop the reaction.
- Remove the denatured protein by centrifugation at 12,000g for 10 min at room temperature.

- Use the supernatant for oligosaccharide structural characterization by RPIP-HPLC-ESI-MS.

■ **PAUSE POINT** Samples may be stored at 4 °C for 1–2 d or at –20 °C for long-term storage before analysis.

Oligosaccharide analysis using HPLC and UV detection ● **TIMING** ~1–2 h

- Perform depolymerization analysis of the aliquots generated in Step 3 by SAX-HPLC-UV (option A) or RPIP-HPLC-UV (option B).

? TROUBLESHOOTING

(A) SAX-HPLC-UV

- Equilibrate the SAX column in solvent A for 5 min, followed by a gradient separation from 100% solvent A to 100% solvent B at a flow rate of 1.2 ml min⁻¹ for 25 min.
- After conditioning for 5 min in solvent A, the system is ready to perform sample separation.
- Inject the sample at a concentration of about 20- μg GAG oligosaccharide onto the injection loop using a Hamilton syringe. The amount of sample can be estimated from the amount of starting GAG determined by the carbazole assay or by dissolving the sample in 1 ml of water and determining its absorbance at 232 nm in a quartz cuvette with a 1-cm path length. An absorbance of 0.1 corresponds to about 20- μg GAG oligosaccharide.
- Elute the oligosaccharides with isocratic solvent A for 5 min, followed by a gradient separation from 100% solvent A to 100% solvent B over a 60-min time period at a flow rate of 1.2 ml min⁻¹. A flatter gradient can be used for samples with no (or low) sulfation, whereas a steeper gradient can be used for highly sulfated samples.
- Monitor the eluent using the on-line UV or UV/visible detector at $\lambda_{\text{abs}} = 232$ nm for unsaturated oligomers or at $\lambda_{\text{abs}} = 214$ nm for saturated species.
- Identify the oligosaccharide peaks and the extent of enzymatic treatment on the basis of the elution order, as indicated as an example in **Figure 2** or by using available standards.

(B) RPIP-HPLC-UV

- Equilibrate the C18 column(s) in solvent C for 20 min at a flow rate of 0.5 ml min⁻¹ (for the Discovery C18 column) or at 0.3 ml min⁻¹ (for the Gemini C18 column). After conditioning, the system is ready to perform sample separation.
- Inject the sample at a concentration of about 20- μg GAG onto the injection loop using a Hamilton syringe.
- Elute the oligosaccharides with a gradient separation from 100% solvent C to 100% solvent D over a 90-min time period at a flow rate of 0.5 ml min⁻¹ (for the Discovery C18 column) or at 0.3 ml min⁻¹ (for the Gemini C18 column).
- Monitor the eluent using the on-line UV or UV/visible detector at $\lambda_{\text{abs}} = 232$ nm for unsaturated oligosaccharides.
- Identify the oligosaccharide peaks and the extent of enzymatic treatment on the basis of the elution order, as indicated as an example in **Figure 3a** or by using available standards.

Oligosaccharide characterization by means of RPIP-HPLC-ESI-MS ● **TIMING** ~2 h

- Equilibrate the C18 column(s) in solvent C for 60 min at a flow rate of 0.5 ml min⁻¹ (for the Discovery C18 column) or at 0.3 ml min⁻¹ (for the Gemini C18 column). After conditioning, the system is ready to perform sample separation.
- Equilibrate the electrospray interface by flushing the solvent C on-line with HPLC for 60 min at a flow rate of 0.5 ml min⁻¹ (for the Discovery C18 column) or 0.3 ml min⁻¹ (for the Gemini C18 column). Set the mass analyzer in negative ionization mode with the skimmer potential at –19.7 V, the capillary exit at –48.4 V and a source of temperature of 350 °C in full scan spectra (200–2,200 Da, 10 full scans per second). Set nitrogen at 12 liter min⁻¹ and nebulizing gas at 4.14 bars.
- Inject the sample at a concentration of about 100–200 μg GAG onto the injection loop using a Hamilton syringe.

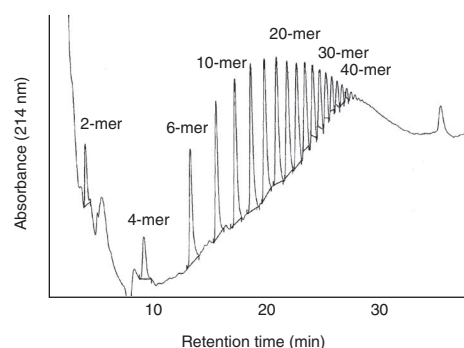
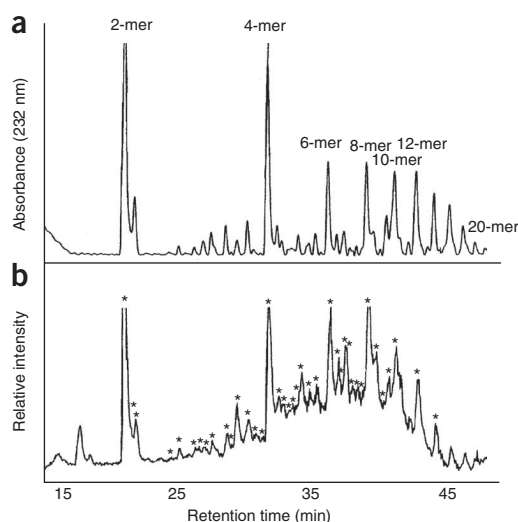


Figure 2 | SAX-HPLC of HA oligosaccharides produced by testicular hyaluronidase digestion detected at 214 nm. Modified from reference 47.

Figure 3 | RPIP-HPLC separation of HP unsaturated oligosaccharides obtained from controlled heparinase I depolymerization. **(a)** UV detection at 232 nm with the major peaks associated with oligosaccharides of selected sizes are shown. **(b)** Total ion chromatogram (TIC) in the negative ion mode using ESI-MS detection. Asterisks show all of the assigned peaks; see reference 26 for structures. UV detects the molar concentration of only unsaturated oligosaccharides and hence is particularly useful in quantification, whereas TIC detects all molecules with a peak intensity that is dependent on both molar concentration and ionization efficiency. This research was originally published in reference 26.



11 | Elute oligosaccharides with a gradient separation from 100% solvent C to solvent D over a 90-min time period at a flow rate of 0.5 ml min⁻¹ (for the Discovery C18 column) or at 0.3 ml min⁻¹ (for the Gemini C18 column).

12 | Monitor the eluent using the mass detector.

? TROUBLESHOOTING

● TIMING

Steps 1–6, Enzyme treatment and production of GAG oligosaccharides: ~10 h

Step 7, Oligosaccharide analysis using HPLC and UV detection: ~1–2 h

Steps 8–12, Oligosaccharide characterization by means of RPIP-HPLC-ESI-MS: ~2 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
7	No peaks (oligosaccharides) are observed in SAX- or RPIP-HPLC and UV detection	Enzyme is inactive	Check for enzyme activity on a GAG standard
		Insufficient enzymatic treatment	Extend the digestion time
	Baseline chromatogram in SAX- or RPIP-HPLC and UV detection is noisy and contains spurious peaks	Solvent inadequately degassed	Use an on-line vacuum degasser to the HPLC system
		Purity of reagents has been compromised	Reprepare fresh reagents and HPLC solvents
12	Overlapping of oligosaccharides species in RPIP-HPLC-ESI-MS	GAG sample was impure	Repurify GAG sample and treat again with lyase
		pH values of HPLC solvents are different than 7.0	Carefully check for pH of HPLC solvents. Reprepate fresh HPLC solvents
		Sample contains salt	Either repeat purification and depolymerization of GAG sample or desalt oligosaccharides
	Elution time periods change or become unstable from run to run in RPIP-HPLC-ESI-MS	The column has not been well conditioned from run to run	Reequilibrate the C18 column(s) in solvent C for 20 min at a flow rate of 0.5 ml min ⁻¹ (for the Discovery C18 column) or 0.3 ml min ⁻¹ (for the Gemini C18 column)
		No peaks are evident by mass spectrometry	Solvent flow is inadequate
		High voltages and spray chamber currents are off	Make sure the electrospray high voltages are switched on
		Drying gas flow is insufficient	Check the drying gas flow and temperature
		Pressure is too low	Make sure the fore and high-vacuum pressure are within normal ranges

ANTICIPATED RESULTS

GAG-derived oligosaccharide mapping and sequence analysis relies on the specific enzymatic degradation to produce unsaturated or saturated oligosaccharides. This is achieved through controlled, partial (20–40%), enzyme-catalyzed depolymerization of GAGs producing a mixture mainly composed of oligosaccharides ranging from disaccharides (2-mers) to oligosaccharides of >40-mers. SAX-HPLC (Fig. 2) or RPIP-HPLC separation and UV detection at 232 nm for unsaturated (Fig. 3a) or at 214 nm for saturated (Fig. 2) oligomers is used to confirm the extent of enzymatic degradation.

Extensive experiments have been undertaken to optimize the HPLC separation of both small (2-6-mers) and large oligosaccharides (10-30-mers with sulfo groups and 10-40-mers without sulfo groups)⁴⁵. RPIP-HPLC uses a MS-friendly mobile phase including volatile ion-pairing reagents, volatile inorganic salt and an organic modifier. When choosing the volatile ion-pairing reagent, compatibility with on-line MS and the capacity to improve column resolution should be considered. Suitable ion-pairing reagents should possess a low alkyl chain length affording the high volatility required for compatibility with MS and a longer alkyl chain length capable of the greater on-column retention of oligosaccharides associated with enhanced separation. To this aim, tributylamine provides optimal retention with MS compatibility. In addition, organic co-solvent, solvent pH, temperature, gradient profiles of volatile inorganic salt and various ion-pairing reagents needed to be optimized by using off-line RPIP-HPLC and UV detection, to obtain efficient oligosaccharide separation⁴⁵. Ammonium formate and ammonium acetate gradients were found to produce low peak intensities and complicated spectra because of ion suppression. However, RPIP-HPLC using an acetonitrile gradient at a fixed concentration (50 mM) of ammonium acetate (pH 7.0), and in the presence of 15-mM tributylamine afforded both high chromatographic resolution (Fig. 3a) and excellent MS analyses.

Heparin lyase I was used to prepare HP oligosaccharides²⁶. This enzyme is able to depolymerize HP through a random, endolytic, β-eliminative mechanism cleaving the glycosidic linkage between an *N*-sulfo-6-*O*-sulfo (or OH) glucosamine residue and a 2-*O*-sulfo iduronic acid unit producing a C4–C5 unsaturated uronate residue at the nonreducing end of the products. On the basis of a 30% completion of the heparinase-catalyzed depolymerization reaction, HP-derived oligosaccharides consisting primarily of repeating trisulfated disaccharide units (Fig. 1) were separated by RPIP-HPLC and detected by ESI-MS (Fig. 3b); some saturated oligosaccharides were also identified arising from the original nonreducing end of polysaccharide chains. The structural characterization of these saturated oligosaccharides provided sequence information from a reading frame that begins at the nonreducing terminus of the HP chain. Moreover, 16 nonreducing end oligosaccharides were observed and identified, having both an even and odd number of saccharide residues, most of which are not predicted on the basis of biosynthesis or known pathways of HP catabolism. Finally, molecular ions could be assigned for oligosaccharides as large as a tetradecasaccharide, and MS detection was also proved for oligosaccharides formed up to 30 saccharide units²⁶.

Dermatan sulfate was purified from marine clam *Scapharca inaequivalvis* and partially depolymerized by controlled digestion with chondroitin ABC lyase. Generated oligosaccharides were separated from 2- to 10-mer by on-line RPIP-HPLC-ESI-MS (Fig. 4)⁴⁶. DS oligosaccharides up to 10-mer were separated in ~40 min. The negative ESI-MS spectra of the peaks separated by RPIP-HPLC-ESI-MS are illustrated in Figure 5, showing ion species related to the various oligosaccharides produced by the enzyme treatment. The most prominently generated oligosaccharides were formed from the repeating monosulfated disaccharide (Fig. 1)

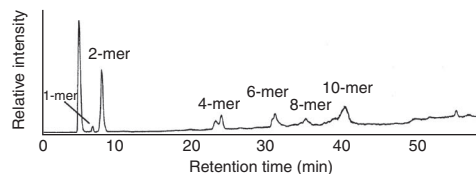


Figure 4 | Total ion chromatograms of *Scapharca inaequivalvis* DS oligosaccharides up to 10-mer in the negative ion mode obtained by partial treatment with chondroitin ABC lyase and separated by means of RPIP-HPLC. Modified from reference 46.

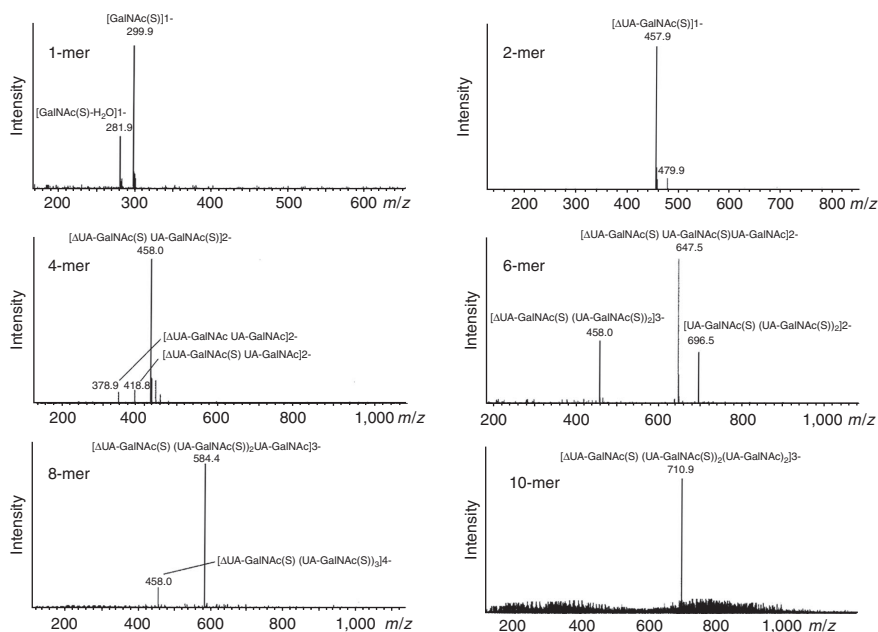


Figure 5 | The ESI-MS spectrum in the negative mode of each single *Scapharca inaequivalvis* DS oligosaccharide species separated by means of RPIP-HPLC from 1- to 10-mer. ΔUA, 4-deoxy-α-L-threohex-4-enopyranosyluronic acid; GalNAc, *N*-acetylgalactosamine; and S, sulfo. Modified from reference 46.



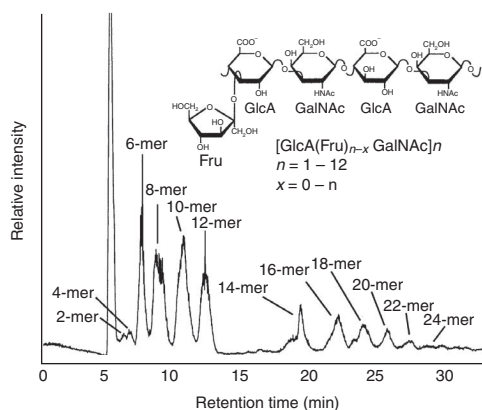


Figure 6 | Total ion chromatograms of K4 oligosaccharides up to 24-mers in negative ion mode separated by means of RPIP-HPLC. The inset shows the structure of the partially fructosylated *Escherichia coli* K4 polysaccharide (a tetrasaccharide fructosylated on a glucuronic acid residue is reported as an example). The tentative structure of the K4 oligosaccharidic sequences previously determined⁴³ is also illustrated. D-glucuronic acid is reported as GlcA, N-acetylgalactosamine as GalNAc and D-fructose as Fru.

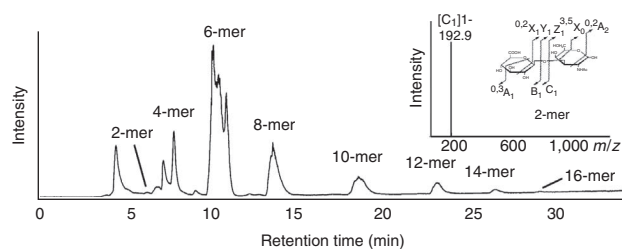


Figure 7 | Total ion chromatograms of unsulfated saturated chondroitin oligosaccharides up to 16-mers in negative ion mode separated by means of RPIP-HPLC. The inset shows the ESI-MS/MS spectra of the chondroitin disaccharide. The nomenclature for disaccharide fragmentation proposed by Domon and Costello⁵² is also illustrated. Fragment ions that contain a nonreducing terminus are labeled with uppercase letters from the beginning of the alphabet (A,B,C), and those that contain the reducing end of the oligosaccharide are labeled with letters from the end of the alphabet (X,Y,Z). Subscripts indicate the cleaved ions. A and X ions are produced by cleavage across the glycosidic ring, and are labeled by assigning a number to each ring bond and counting clockwise. Ions produced from cleavage of successive residues are labeled A_m , B_n and C_m , with $m = 1$ for the nonreducing end, and X_n , Y_n and Z_n , with $n = 1$ for the reducing-end residue. Modified from reference 42.

units; the structures of the major oligosaccharides, from 6- to 10-mer, confirmed the presence of ~20% of nonsulfated disaccharides. Furthermore, a minor but significant percentage of a monosaccharide of m/z 300, identified as monosulfated N-acetylgalactosamine and belonging to the DS nonreducing end, was observed. Furthermore, a saturated hexasaccharide derived from the nonreducing terminus of the intact DS polymer ending with a uronic acid residue was also characterized.

E. coli K4 polysaccharide was partially digested by using testicular hyaluronidase, and the generated saturated oligosaccharides were separated and characterized by on-line RPIP-HPLC-ESI-MS (**Fig. 6**)⁴³. Identification and structural information were obtained for oligosaccharides containing 2–24 monomers, from 2- to 24-mers. Smaller K4 species from 2- to 4-mers mainly showed $[M-H]^{-1}$ anions, whereas the 6- to 8-mers existed predominantly at the charge state of -2 . The K4 oligosaccharides from 10- to 14-mers were mainly characterized by a charge state of -3 , whereas oligomers from 16- to 20-mers were represented by $[M-4H]^{-4}$ anions. Finally, longer K4 oligosaccharides, from 22- to 24-mers, existed at the charge states of -4 and -5 and, for this last species, $[M-6H]^{-6}$ anions also appeared.

By using the same analytical approach, we showed that chondroitin C lyase was unable to function on fructosylated sequences inside a partially fructosylated *E. coli* K4 polymer⁴⁴. Chondroitin C lyase was able to produce different unsaturated oligosaccharides by functioning on an ~27% fructosylated K4 polymer. The on-line RPIP-HPLC-ESI-MS analytical approach identified the disaccharide nature of the main species produced by this lyase as Δ UA-N-acetylgalactosamine (GalNAc) (not shown). Furthermore, the nondigested sequences inside the *E. coli* polymer were characterized as oligosaccharides bearing a fructose for each glucuronic acid residue, corresponding to unsaturated fully fructosylated oligomers, from 4- to 10-mers.

A further study⁴² analyzed the complete LC-ESI-MS and tandem MS spectra in negative ion mode of saturated unsulfated CH oligosaccharides up to 16-mers (**Fig. 7**). A comparison was also carried out with HA oligomers differing only in the nature of the hexosamine unit. MS/MS of the CH disaccharide on the singly charged precursor at m/z 396.1 afforded a glycosidic cleavage C_1 product ion having m/z 192.9 (**Fig. 7**). In tetrasaccharide, C_2 (m/z 396.0) and C_3 (m/z 572.0) anions were produced by glycosidic cleavage, and a C_5 $[M-2H]^{-2}$ ion (m/z 475.1) was generated by the cleavage of hexasaccharide. Finally, a C_7 ion (m/z 664.6) having a charge state of -2 was produced from the octasaccharide, and the same fragmentation pattern of deprotonated oligomers was also observed for the largest oligosaccharides. As a consequence, saturated CH oligosaccharides larger than a tetrasaccharide and formed of x -mer units dissociate to almost exclusive form CX-1-type ions.

RPIP-HPLC-ESI-MS was applied to the separation and characterization of oligosaccharides from 2- to 40-mers produced by hyaluronidase digestion of HA (**Fig. 8**)⁴⁷, obtaining complete identification and structural information for each oligomer species. A series of negatively charged species of various m/z ratios were observed with smaller HA oligosaccharides (2- to 4-mers) present mainly as $[M-H]^{-1}$ anions and the 6- to 10-mers existing predominantly at a charged

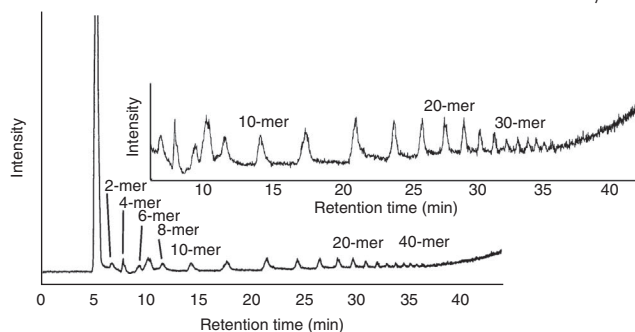


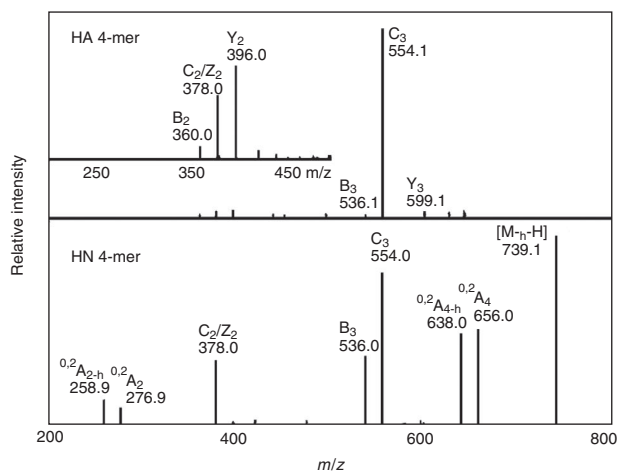
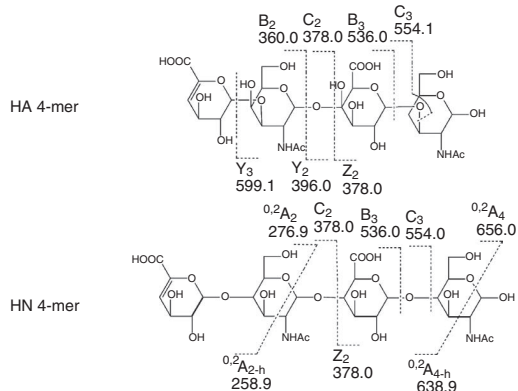
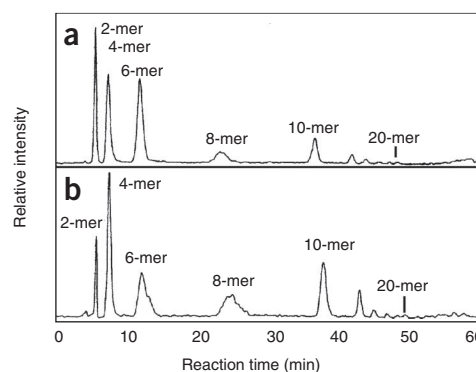
Figure 8 | Total ion chromatograms of HA-saturated oligosaccharides up to 40-mers in negative ion mode separated by means of RPIP-HPLC. The inset panel shows the chromatogram expanded in the region from 6 to 42 min. Modified from reference 47.

Figure 9 | Analysis of unsaturated oligosaccharides derived from nonsulfated HA and HN GAGs. Total ion chromatogram of (a) HA oligosaccharides and (b) HN oligosaccharides produced by enzymatic procedure and separated by RPIP-HPLC. Modified from reference 48.

state of -2 . The HA oligomers from 12- to 18-mers were mainly represented by $[M-3H]^{-3}$ anions; species from 20- to 28/30-mers were characterized by a charge state of -4 ; and oligosaccharides from 32- to 40-mers existed as $[M-5H]^{-5}$ anions (not shown).

In subsequent studies⁴⁸, RPIP-HPLC-ESI-MS and tandem MS were applied to structurally distinguish isobaric oligosaccharides enzymatically prepared from HA and HN (**Fig. 9**), having the linkage pattern between glucuronic acid and *N*-acetylglucosamine residues (in HA, 1→3 and in HN, 1→4) as unique difference. Tandem MS afforded spectra in which glycosidic cleavage fragment ions were observed for both series of oligosaccharides, but cross-ring cleavage ions $^{0,2}An$ and $^{0,2}An-h$ (n is even number) were detected only in *N*-acetylglucosamine residues of HN oligomers (**Fig. 10**). These specific cross-ring cleavage fragment ions are useful to definitively distinguish HA and HN oligosaccharides.

Heparin-derived oligosaccharides are among the most highly sulfated oligomer species, and their analysis clearly shows the potential of on-line RPIP-HPLC-ESI-MS. HN, an *E. coli*-derived unsulfated polysaccharide, has been analyzed by this method following its heparin lyase III treatment. Using a shallow acetonitrile gradient, structural information can be successfully obtained on HN-derived oligosaccharides as large as 20-mers, such as on nonsulfated polymers as HA and CH, showing the use of this method for bacterial GAGs. Other potential applications of this analytical approach might include the analysis of structurally related GAGs, including HS, DS, CS and keratan sulfate. Unlike HA, HN and CH, these GAGs contain sulfo groups but are considerably less sulfated than HP. Most importantly, these GAGs are structurally complex and are responsible for many physiological and pathophysiological activities⁶⁻¹¹. The detection of saturated oligosaccharides derived from the original nonreducing end of HP chains in this study suggests that it might be possible to obtain structural characterization and sequence information of HS using the nonreducing end as a reading. Midura *et al.*⁴⁹ have applied nonspectral methods to identify the nonreducing ends of CS chains.



This analytical approach may also be applied to determine the action pattern of different enzymes that can function on complex GAGs. RPIP-HPLC-ESI-MS is a powerful method for the analysis of products afforded through the digestion of GAGs by polysaccharide enzymes, as shown for chondroitin lyases⁵⁰. In fact, the product distribution can be profiled through HPLC, and the product structure can be characterized and identified by MS, and the action patterns of various enzymes can be confirmed. Furthermore, the application of this analytical approach also allows the observation of minor products arising from the natural sequence microheterogeneity of GAGs, such as nonsulfated, disulfated and trisulfated disaccharide sequences and oligosaccharides, originating from the nonreducing terminus of GAG chains. As a consequence, on-line RPIP-HPLC separation and MS characterization offer excellent tools for the identification of minor sequence heterogeneities, having a profound importance in understanding GAG biology.

The suitability of this method for the on-line structural characterization of oligomers produced by chemical degradation has also been evaluated using alginic acid⁵¹. Complete on-line separation and characterization procedures of fully saturated alginic acid oligosaccharides from monosaccharide

Figure 10 | MS/MS spectra of HA 4-mer, tetrasaccharide from HA and HN 4-mer, tetrasaccharide from HN. MS/MS spectra are labeled on the basis of the nomenclature of Domon and Costello⁵². Modified from reference 48.

to beyond 23-mers, obtained by a chemical depolymerization process, were compared with those of unsaturated aliginic acid oligosaccharides, monosaccharide to 10-mers, obtained by lyase-catalyzed depolymerization⁵¹. On-line RPIP-HPLC-ESI-MS enables the separation and simultaneous characterization of complex saturated oligomer mixtures obtained by chemical treatment without extensive sample purification.

Experiments on sulfated GAGs using the tandem MS capabilities of the ion-trap instrument afforded fragmentation, leading to the loss of sulfo groups. Additional MS/MS studies considering various experimental conditions will be necessary to obtain through-glycosidic and through-ring fragmentations and to determine the positioning of sulfo groups within oligosaccharides. The correct evaluation of sulfo groups in the nonreducing end of oligosaccharides by MS/MS will only require the determination of iduronic or glucuronic acid residues to understand the diversity of these structures in GAGs.

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AUTHOR CONTRIBUTIONS All authors contributed equally to this work. R.J.L. designed and developed the RPIP-HPLC-ESI-MS. R.J.L. and N.V. applied this methodology to the structural study of various complex natural polymers with respect to bacterial polysaccharides.

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