Analysis of glycosaminoglycan-derived, precolumn, 2-aminoacridone–labeled disaccharides with LC-fluorescence and LC-MS detection

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Glycosaminoglycans (GAGs) possess considerable heterogeneity in average molecular mass, molecular mass range, disaccharide composition and content and position of sulfo groups. Despite recent technological advances in the analysis of GAGs, the determination of GAG disaccharide composition still remains challenging and provides key information required for understanding GAG function. Analysis of GAG-derived disaccharides relies on enzymatic treatment, providing one of the most practical and quantitative approaches for compositional mapping. Tagging the reducing end of disaccharides with an aromatic fluorescent label affords stable derivatives with properties that enable improved detection and resolution. HPLC with on-line electrospray ionization mass spectrometry (ESI-MS) offers a relatively soft ionization method for detection and characterization of sulfated oligosaccharides. GAGs obtained from tissues, biological fluids or cells are treated with various enzymes to obtain disaccharides that are fluorescently labeled with 2-aminoacridone (AMAC) and resolved by different LC systems for high-sensitivity detection by fluorescence, and then they are unambiguously characterized by MS. The preparation and labeling of GAG-derived disaccharides can be performed in ~1–2 d, and subsequent HPLC separation and on-line fluorescence detection and ESI-MS analysis takes another 1–2 h.

INTRODUCTION

Complex glycans—heteropolysaccharides and oligosaccharides—are ubiquitous bioactive (macro)molecular components of all organisms and the primary constituents of all cells and of the extracellular environment[1]. Glycosylation and the covalent attachment of glycans to proteins to generate glycoproteins (GPs) and proteoglycans (PGs; an important subset of GPs and the focus of this protocol), are complex post-translational modifications that are important in generating extensive phenotypes that afford functional diversity from limited genotypes[2]. In biosynthesis, glycans can be divided into two categories: linear polysaccharides, which consist of repeating disaccharide sequences and which are characterized by their extremely heterogeneous structure and physicochemical properties, known as GAGs, and the branched oligosaccharides of GPs and glycolipid glycans, generally consisting of 3–10 glycosidically linked monosaccharide units[3]. Over the past 30 years, there has been growth in the understanding of the structure and function of complex glycans and their derivatives along with increased knowledge of the capacity of genotypes to modulate glycan structures for the development of particular phenotypes[4]. The discipline of glycobiology has introduced the field of glycomics, analogous to the fields of genomics and proteomics, for the characterization of glycan structure and function in biological systems[5].

With the exception of a few heavily glycosylated mucins and some viral envelope GPs, the carbohydrate components of GPs generally represent a small percentage of their overall weight compared with the protein. In contrast, the protein core of PGs is post-translationally modified with anionic sulfated GAGs that comprise most of the mass of these macromolcules[6,7]. PGs are ubiquitous components of the extracellular matrix (ECM), but they are also present on cell surfaces and in basement membranes. PGs are able to interact with several proteins and cellular or extracellular components and to modulate multiple biological processes[8–11]. As a consequence, understanding the biological significance of these macromolecules and of their noncovalent interacting partners and their structure-function relationship should provide important information for possible therapeutic intervention[12]. However, owing to their structural complexity, the characterization of the structure-function relationship of PGs represents a challenging task as a result of enormous structural diversity[13,14].

GAGs are linear, negatively charged heteropolysaccharides classified into four families on the basis of their structure: hyaluronan (HA), keratan sulfate (KS), chondroitin sulfate (CS)/dermatan sulfate (DS) and heparin (Hep)/heparan sulfate (HS)[15,16]. With the exception of KS, GAGs are made up of repeating disaccharide units that consist of a hexuronic acid residue (for example, D-glucuronic acid (GlcA) and/or its C5-epimer L-iduronic acid (IdoA)) and a hexosamine residue (for example, D-glucosamine (GlcN) or D-galactosamine (GalN)). KS possesses D-galactose instead of a hexuronic acid residue, making it intractable by the methods described here, so that it is not considered further in this protocol.

HA, the simplest GAG, is mainly a component of ECM with a very high molecular mass (generally >1,000 kDa), and it consists of repeating disaccharide units of D-*N*-acetyl-glucosamine (GlcNAc) and GlcA[17] (see **Table 1** for GAG disaccharide structures). HA is not further modified and is not linked to a core protein; thus, it is not a PG.

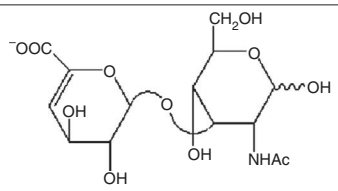
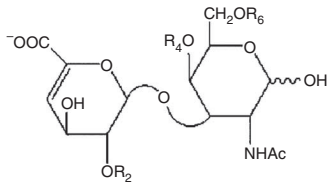
Naturally occurring CS/DS GAGs are biosynthesized as PGs, *O*-linked to many different core proteins, and they are widely distributed in animal tissues, having a molecular mass of ~20–50 kDa

(ref. 18). Initially biosynthesized with a repeating disaccharide unit of GlcA β(1→3) GalNAc (where Ac is acetyl)[15,18], the chondroitin backbone is subject to considerable modifications, including the introduction of sulfo groups at C4 or/and C6 (and rarely at C3) of the GalNAc residue and at C2 of the GlcA or IdoA residue[18–20], formed through GlcA epimerization in DS (**Table 1**). As a result of the biosynthetic processes, CS/DS chains with different levels of polymerization, charge density and disaccharide patterns, and the presence of specific oligosaccharide domains, are biosynthesized affording macromolecules of differing composition and sequence found in different cell types, tissues and animal species. CS/DS GAGs are implicated in major biological functions, including central nervous system development, wound repair, infection, signal transduction and morphogens, in addition to their conventional structural roles (for more complete and specialized works, see refs. 19–22).

Hep and HS GAGs, possessing the most complex and heterogeneous GAG structures, have molecular masses of 10–30 kDa, and they are localized on the cell surface, inside the cells or secreted into the ECM. Their disaccharide structures are based on either IdoA or GlcA, both of which can be modified with 2-*O*-sulfo groups; these uronic acids (UAs) are linked 1→4 to *N*-sulfo or *N*-acetyl GlcN residues that can be substituted with 3-*O*-sulfo and/or 6-*O*-sulfo groups[1,9,23] (**Table 1**). HS is biosynthesized as a PG *O*-linked to a number of different core proteins, whereas Hep is *O*-linked only to serglycin. The major repeating unit in HS is the nonsulfated disaccharide GlcA-(1→4)-GlcNAc, and, in contrast, Hep primarily comprises a trisulfated disaccharide IdoA-2S-(1→4)-GlcNS,6S. The extensive number of potential post-translational modifications in HS and Hep introduces a high level of structural variability dependent on species and individual organism, organ, tissue, cell type, environment conditions and developmental stage[24], which produces a large number of possible disaccharides and oligosaccharide sequences[9,13,14,23–26]. Both HS and Hep are key macromolecules involved in general cell functions, in angiogenesis and in host-pathogen interactions[9,15,27].

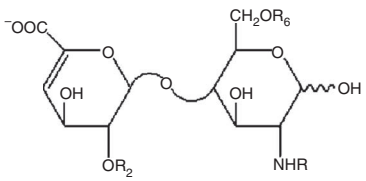
The recent explosion in the field of glycomics has led to an increased understanding of GAG structure and function, as well as to the discovery of novel active agents having nutraceutical (a functional food or dietary supplement) and pharmaceutical

TABLE 1 | Formulas, structure and mass of HA/CS/DS/HS/Hep disaccharides tagged with AMAC and the assignment of found ions in the mass spectra.

Disaccharide (Code)	Structure				Theoretical m.m.	MW-AMAC	Observed ions (charge)	Assignment
								
	HA							
0SHA	ΔUA-GlcNAc				379.1	573.1	571.5 (–1)	$[M-H]^-$
								
	CS/DS							
		R2	**R4**	**R6**				
0SCS (D0a0)	ΔUA-GalNAc	H	H	H	379.1	573.1	571.5 (–1)	$[M-H]^-$
2SCS (D2a0)	ΔUA2S-GalNAc	$SO3^-$	H	H	459.1	653.1	651.4 (–1)	$[M-H]^-$
6SCS (D0a6)	ΔUA-GalNAc6S	H	H	$SO3^-$	459.1	653.1	651.4 (–1)	$[M-H]^-$
4SCS (D0a4)	ΔUA-GalNAc4S	H	$SO3^-$	H	459.1	653.1	651.4 (–1)	$[M-H]^-$
2,6diSCS (D2a6)	ΔUA2S-GalNAc6S	$SO3^-$	H	$SO3^-$	539.0	733.0	731.4 (–1)	$[M-H]^-$
							753.2 (–1)	$[M+Na-2H]^-$
							365.1 (–2)	$[M-2H]^{2-}$

(continued)

TABLE 1 | Formulas, structure and mass of HA/CS/DS/HS/Hep disaccharides tagged with AMAC and the assignment of found ions in the mass spectra (continued).

Disaccharide (Code)	Structure				Theoretical m.m.	MW-AMAC	Observed ions (charge)	Assignment
4,6diSCS (D0a10)	ΔUA-GalNAc4S6S	H	SO_3^-	SO_3^-	539.0	733.0	731.4 (–1)	$[M-H]^-$
							753.2 (–1)	$[M+Na-2H]^-$
							365.1 (–2)	$[M-2H]^{2-}$
2,4diSCS (D2a4)	ΔUA2S-GalNAc4S	SO_3^-	SO_3^-	H	539.0	733.0	731.4 (–1)	$[M-H]^-$
							753.2 (–1)	$[M+Na-2H]^-$
							365.1 (–2)	$[M-2H]^{2-}$
2,4,6triSCS (D2a10)	ΔUA2S-GalNAc4S6S	SO_3^-	SO_3^-	SO_3^-	619.0	813.0	811.4 (–1)	$[M-H]^-$
							833.2 (–1)	$[M+Na-2H]^-$
							731.4(–1)	$[M-SO3-H]^-$
							404.7 (–2)	$[M-2H]^{2-}$
							365.1 (–2)	$[M-SO3-2H]^2$
								
		HS/Hep						
		R2	**R6**	**R**				
0SHS (D0A0)	ΔUA-GlcNAc	H	H	Ac	379.1	573.1	571.5 (–1)	$[M-H]^-$
NSHS (D0S0)	ΔUA-GlcNS	H	H	SO_3^-	417.1	611.1	609.5 (–1)	$[M-H]^-$
6SHS (D0A6)	ΔUA-GlcNAc6S	H	SO_3^-	Ac	459.1	653.1	651.5 (–1)	$[M-H]^-$
2SHS (D2A0)	ΔUA2S-GlcNAc	SO_3^-	H	Ac	459.1	653.1	651.4 (–1)	$[M-H]^-$
N,6diSHS (D0S6)	ΔUA-GlcNS6S	H	SO_3^-	SO_3^-	497.0	691.0	689.4 (–1)	$[M-H]^-$
							343.9 (–2)	$[M-2H]^{2-}$
N,2diSHS (D2S0)	ΔUA2S-GlcNS	SO_3^-	H	SO_3^-	497.0	691.0	689.4 (–1)	$[M-H]^-$
							343.9 (–2)	$[M-2H]^{2-}$
2,6diSHS (D2A6)	ΔUA2S-GlcNAc6S	SO_3^-	SO_3^-	Ac	539.0	733.0	731.4 (–1)	$[M-H]^-$
							753.2 (–1)	$[M+Na-2H]^-$
							365.0 (–2)	$[M-2H]^{2-}$
N,2,6triSHS (D2S6)	ΔUA2S-GlcNS6S	SO_3^-	SO_3^-	SO_3^-	576.9	770.9	769.4 (–1)	$[M-H]^-$
							791.2 (–1)	$[M+Na-2H]^-$
							383.9 (–2)	$[M-2H]^{2-}$

A descriptive name for each disaccharide is provided where N, 2, 4 and 6 correspond to the location of sulfo groups (S) and CS and HS designate the GAG family. A code, corresponding to each disaccharide, is provided in parentheses (see ref. 85). The abbreviated structure of each disaccharide is presented where ΔUA (D), Δ4,5-unsaturated uronic acid; Glc, glucosamine; Gal, galactosamine; Ac, acetyl group; S, sulfo group; m.m., molecular mass.

applications[3,12,20]. HA is largely used for supplementation of impaired synovial fluid in arthritic patients, applied after cataract surgery, as a filler in soft-tissue surgery, as a device in several surgical procedures and in tissue engineering; furthermore, it is applied to damaged tissue because of its capacity to promote wound healing[28,29]. Hep has proven to be useful in the treatment of a variety of disorders owing to its anticoagulant and antithrombotic activities[30], although the high heterogeneity of its structure has led to several side effects[31]. Thus, several new Hep derivatives have been developed to circumvent these deleterious side effects and retain its therapeutic capacity and provide more predictable pharmacological action[25]. Various low-molecular-weight (LMW)[23], ultra-LMW[32] and bioengineered[33] Heps have been investigated. Moreover, novel HS mimetics have been designed to modulate tumor progression and metastasis[34]. CS is currently used as a nutraceutical, and it is recommended by the European League against Rheumatism (EULAR) as a drug in Europe in the treatment of osteoarthritis[35]. DS is under evaluation for possible application in parasitic and viral infections, in regenerative medicine and in antitumor and anticoagulant therapy[20]. Finally, chemical and chemoenzymatic syntheses of various GAG oligosaccharides have been reported[36].

Decoding the fine chemical structure of GAGs is a key step in establishing the structure-activity relationship required for understanding the biochemical basis of GAG protein interaction, clarifying the biological and therapeutic roles of GAGs, and integrating information at the molecular, cellular, tissue and organism levels. As a consequence, analytical procedures are required that can accurately, reproducibly and robustly determine the structure and/or sequence of these complex glycans to ensure the quality and safety of GAG-based drugs and nutraceuticals.

Owing to the remarkably high structural information content of GAGs, various modern analytical techniques are required to determine their fine structure and sequence. These methods include HPLC, capillary electrophoresis (CE), gel permeation chromatography, PAGE and NMR[37–45].

A common approach for the structural analysis of polydisperse and heterogeneous GAGs is to perform a partial or complete depolymerization to produce a range of oligosaccharide fragments for oligosaccharide mapping[46] or constituent disaccharides for compositional analysis. Exhaustive enzymatic digestion using bacterial lyases[47–49] converts these macromolecules to their disaccharide building blocks with a 4,5-unsaturated UA residue at their nonreducing ends, which are then easily separated and quantified by other analytical techniques[39]. Hydrophilic, anionic disaccharides are not retained on reverse-phase (RP)-HPLC columns. By labeling these disaccharides with a hydrophobic fluorophore, their chromatographic properties in RP-HPLC can be improved, and their detection sensitivity can be enhanced[50,51]. Several highly sensitive analytical methods have involved labeling GAG-derived disaccharides. Labeling has relied on reductive amination with a reducing agent, such as 2-aminoacridone (AMAC), 2-aminobenzamide (2-AB) or 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY) fluorophores. These labeled disaccharides are then analyzed by a variety of methods including PAGE, CE with laser-induced fluorescence (LIF) detection, strong anion exchange (SAX)-HPLC or RP-HPLC coupled with on-line fluorescence detection (FD) or MS[52–64]. Among the modern analytical techniques, HPLC and ultraperformance LC (UPLC) separation combined with MS and fluorescence detection has brought the most progress in the field of glycomics because of the flexibility, sensitivity and the unambiguous fine structural information provided by LC-MS[39,43] (**Table 2**).

Compositional mapping of GAGs relies on their accurate and reproducible disaccharide composition after specific enzymatic treatment to produce unsaturated (and saturated) disaccharides. Moreover, along with a structural characterization, very sensitive analytical approaches are required, in particular for biological samples, such as fluids and cells, which produce small amounts of complex polysaccharides and related disaccharides. In this procedure, these dual requirements are met by using HPLC separation

TABLE 2 | Overview of various liquid chromatography separation on-line with MS for GAGs di(oligo)saccharides quantification and characterization.

Year	Technique	Application	Treatment	Main features	Reference
1993	Reverse-phase ion pairing (RPIP)-PIP-HPLC-ESI-MS	Hep oligosaccharides	Exhaustive enzymatic treatment	Di(oligo)saccharide composition of Hep	86
2001	Amine-hydrophobic interaction LC (HILIC)-ESI-MS	CS/DS/HS disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of CS/DS/HS	87,88
2001,2006	Size exclusion chromatography (SEC)-HPLC-ESI-MS	CS/DS oligosaccharides	Controlled enzymatic treatment	Compositional mapping of CS/DS oligosaccharides	89,90
2002	Capillary RPIP-HPLC-ESI-time of flight (TOF)-MS	HS disaccharides	Controlled enzymatic treatment	Disaccharide composition of HS. Oligosaccharide composition of Heparosan up to 40-mer	91
2003	RP-HPLC-ESI-MS	HA oligosaccharides	Controlled enzymatic treatment	Oligosaccharide composition of HA	92

(continued)

TABLE 2 | Overview of various liquid chromatography separation on-line with MS for GAGs di(oligo)saccharides quantification and characterization (continued).

Year	Technique	Application	Treatment	Main features	Reference
2004–2010	RPIP-HPLC-ESI-MS	Mapping and sequencing of GAGs-derived oligosaccharides	Controlled enzymatic treatment	Sequencing of oligosaccharides derived from Hep, CS, DS, Heparosan, *E. coli* K4 polysaccharide	46,82, 93–97
2004	SEC-HPLC-ESI-MS	Hep oligosaccharides	None	Oligosaccharide composition of LMW-Hep	98
2005	Graphitized carbon-HPLC-ESI-MS	CS/DS disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of CS/DS	99
2005	Graphitized carbon-HPLC-ESI-MS	GAGs disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of various GAGs	100
2006	RPIP-HPLC-ESI-MS	Hep oligosaccharides	None	Oligosaccharide composition of Hep	101
2007	RPIP-HPLC-ESI-MS	Hep oligosaccharides	Controlled enzymatic treatment	Determination of the action pattern of heparanase	102
2007,2010	RPIP-HPLC-ESI-MS	HA oligosaccharides	Controlled enzymatic treatment	Compositional mapping of HA oligosaccharides up to 40-mer	46,103
2008	Amide-HILIC-ESI-MS	Hep oligosaccharides	Controlled enzymatic treatment	Compositional mapping of Hep oligosaccharides	104
2008	Amide-HILIC-ESI-MS	CS/DS oligosaccharides	Controlled enzymatic treatment	Compositional mapping of CS/DS oligosaccharides	105
2008	RPIP-UPLC-ESI-Q-TOF-MS	Hep/HS disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of Hep/HS samples	80
2009	RPIP-UPLC-ESI-Q-TOF-MS	Hep oligosaccharides	None	Characterization of the molecular profile of LMW-Hep	106
2009	RPIP-Mf-HPLC-ESI-MS	Hep/HS disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of Hep/HS	107
2009	RPIP-HPLC-ESI-MS	CS oligosaccharides	Controlled enzymatic treatment	Determination of the action pattern of various CS lyases	108
2010	RP-HPLC-ESI-MS	HA/CS/DS disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of various HA/CS/DS	56
2010	RPIP-UPLC-ESI-MS	Hep disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of Hep samples	109
2010	RP-HPLC-ESI-MS	Hep/HS disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of various Hep/HS	55
2011	RPIP-UPLC-ESI-MS	Hep disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of Hep samples	110
2012	RPIP-UHPLC-ESI-Q-TOF-MS	Hep disaccharides	Exhaustive enzymatic treatment	Disaccharide composition of Hep samples	111
2013	HILIC-FT-ESI-MS	LMW-Hep profiling	None	Top-down analysis of LMW-Hep samples	112

FT, Fourier transform; Mf, Microflow.

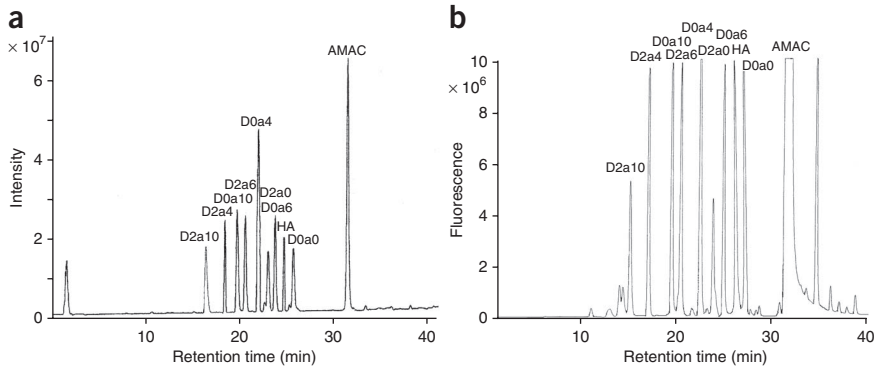


Figure 1 | Chromatogram of HA/CS/DS unsaturated disaccharides fluorotagged with AMAC, separated on an X-Bridge RP18 column. (**a**,**b**) On-line detection by ESI-MS in negative ion mode (**a**) and by FD (**b**). Nomenclature of GAG disaccharides according to Lawrence *et al.*[85] has been adopted. See **Table 1** for disaccharide structure. HA = 0SHA. Modified with permission from ref. 58.

followed on-line by highly sensitive FD coupled with unambiguous structural characterization by ESI-MS. This goal is achieved through two main key procedures: the exhaustive enzyme-catalyzed depolymerization of GAGs that produce a mixture of constituent disaccharides, and their further derivatization with a fluorescent group: AMAC. The derived fluorotagged disaccharides are separated by RP-HPLC and revealed with high sensitivity by a fluorescence detector and characterized by MS.

The exhaustive complete enzymatic depolymerization of GAGs must be obtained and accurately evaluated for a reproducible compositional disaccharide mapping. Standards should be used to confirm the extent of the enzymatic degradation along with the produced disaccharide separation; this can be done by common laboratory analytical approaches such as measuring absorbance at 232 nm by spectrophotometric assay and strong-anion-HPLC[46,65] or by performing RP ion-pairing HPLC separation and UV detection at 232 nm (refs. 46,66,67).

AMAC is a hydrophobic molecule with fluorescent properties; it is used as a labeling reagent for analysis of neutral and charged simple and complex saccharides[56,68,69]. The reducing ends of GAG-derived disaccharides that are exposed by enzyme cleavage are conveniently derivatized with the AMAC amino group by reductive amination. The Schiff base produced is reduced by using sodium cyanoborohydride. The resulting AMAC derivatives possess a strong UV absorbance, high-sensitivity fluorescence and new hydrophobic properties, which make their chromatographic separation easier and which eliminate the need for ion-pairing reagents. As a consequence, common HPLC solutions, such as ammonium acetate and ammonium formate, as well as solvents compatible with FD and MS may be used, all of which may be used to produce high-sensitivity detection and simple spectra.

In this procedure, chondroitin lyase ABC is used to totally depolymerize HA, CS and DS to constituent disaccharides. This enzyme is able to depolymerize HA along with the galactosaminoglycans CS and DS through a random, endolytic, β-eliminative mechanism[70], cleaving the glycosidic linkage between an *N*-acetyl-4-*O*-sulfo,6-*O*-sulfo (or 4-OH and/or 6-OH) GalNAc residue and a 2-*O*-sulfo GlcA/IdoA (or 2-OH) unit producing a C4-C5 ΔUA residue at the nonreducing end of the products (**Table 1**). After fluorotagging with AMAC, HA/CS/DS disaccharides can be separated by RP-HPLC and detected by FD and ESI-MS (**Fig. 1**). Excellent separation among the various HA, CS and DS unsaturated disaccharides is achieved, and related observed ions are found in good agreement with theoretical molecular mass values (**Fig. 2** and **Table 1**). In particular, isomeric nonsulfated HA and CS or DS disaccharides, such as monosulfated and disulfated CS or DS species, are distinctly separated by their retention times and unambiguously identified by their main ions and related sodium adducts. In general, simple mass spectra are obtained with no multiply charged ions even if major ions are observed that correspond to desulfonated species that are related to highly sulfated disaccharides (**Fig. 2** and **Table 1**).

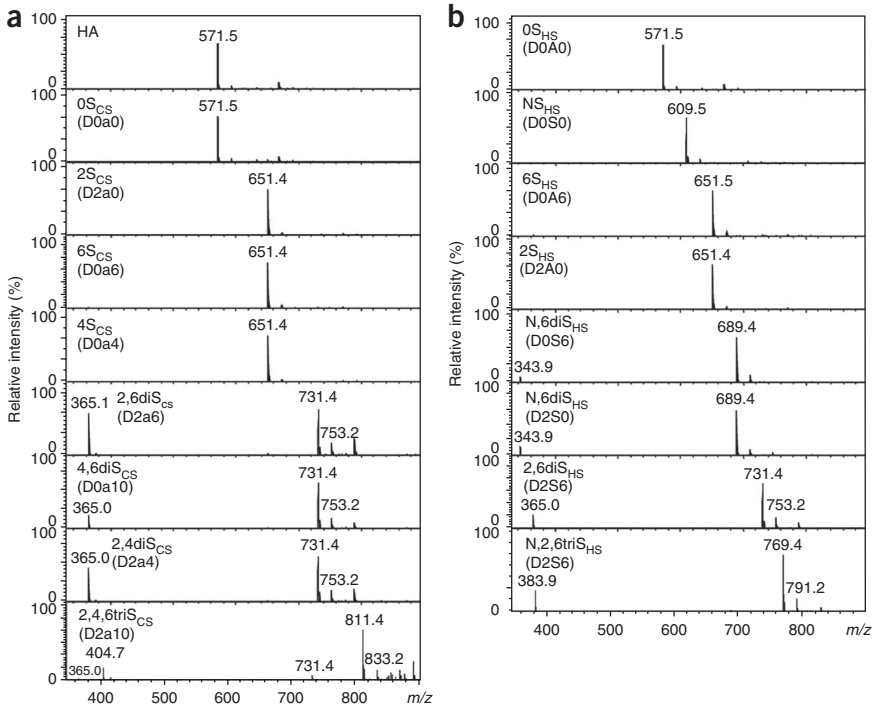


Figure 2 | ESI-MS spectra in the negative mode of HA/CS/DS and HS/hep disaccharides fluorotagged with AMAC. See **Table 1** for disaccharide structure and ion assignment. (**a**) HA/CS/DS-fluorotagged disaccharide standards. (**b**) HS/Hep-fluorotagged disaccharide standards. After optimization of the ESI source conditions, only the two trisulfated disaccharides, N,2,6triS$_{HS}$ (D2S6) and 2,4,6triS$_{CS}$ (D2a10), lose a small percentage of SO_3 groups (~3%). Other sulfated disaccharides show no SO_3 loss. HA = 0SHA.

MATERIALS

REAGENTS

- Chondroitin lyase ABC (50–250 units mg^{-1} protein, chondroitinase ABC, BSA-free, obtained from *Proteus vulgaris*, Enzyme Commission (EC) no. 4. 2.2.4; Sigma-Aldrich, cat. no. C3667). The enzyme can be assayed to confirm activity by using previously described methods[71] ▲ **CRITICAL** Store the enzyme at −80 °C for up to 6 months, and avoid repeated freeze-thaw cycles.
- Hep lyase I (heparinase I containing 0.2% (wt/vol) BSA from *Flavobacterium heparinum*, EC no. 4.2.2.7; from Iduron, Paterson Institute for Cancer Research, University of Manchester, cat. no. HEP-ENZ I). The enzyme can be assayed to confirm activity by using previously described methods[71] ▲ **CRITICAL** Store it at −80 °C for up to 6 months and avoid repeated freeze-thaw cycles.
- Hep lyase II (heparinase II containing 0.2% (wt/vol) BSA from *F. heparinum*, from Iduron, Paterson Institute for Cancer Research, University of Manchester, cat. no. HEP-ENZ II). The enzyme can be assayed to confirm activity by using previously described methods[71] ▲ **CRITICAL** Store it at −80 °C for up to 6 months and avoid repeated freeze-thaw cycles.
- Hep lyase III (heparinase III containing 0.2% (wt/vol) BSA from *F. heparinum*, EC no. 4.2.2.8; from Iduron, Paterson Institute for Cancer Research, University of Manchester, cat. no. HEP-ENZ III). The enzyme can be assayed to confirm activity by using previously described methods[71] ▲ **CRITICAL** Store it at −80 °C for up to 6 months and avoid repeated freeze-thaw cycles.
- Rooster comb HA (sodium salt of HA; Sigma-Aldrich, cat. no. H5388) ▲ **CRITICAL** Store the acid at −20 °C.
- Bovine trachea CS (sodium salt of CS; Sigma-Aldrich, cat. no. 27042) ▲ **CRITICAL** Store it at 2–8 °C.
- Skate CS (disulfated CS from *Raja porasa* cartilage may be extracted and purified according to Box 1 in ref. 46; http://www.nature.com/nprot/journal/v5/n6/box/nprot.2010.48_BX1.html). Alternatively, shark cartilage CS may be used (sodium salt of disulfated CS; Sigma-Aldrich, cat. no. C4384) ▲ **CRITICAL** Store it at 2–8 °C.
- Porcine intestinal mucosa DS (sodium salt of DS; Sigma-Aldrich, cat. no. C3788) ▲ **CRITICAL** Store it at 2–8 °C.
- Beef spleen HS (HS from beef spleen may be extracted and purified according to Box 1 in ref. 46; http://www.nature.com/nprot/journal/v5/n6/box/nprot.2010.48_BX1.html). Alternatively, bovine kidney HS may be used (sodium salt of HS; Sigma-Aldrich, cat. no. H7640) ▲ **CRITICAL** Store it at 2–8 °C.
- Bovine intestinal mucosa heparin (Hep from bovine intestinal mucosa may be extracted and purified according to Box 1 in ref. 46; http://www.nature.com/nprot/journal/v5/n6/box/nprot.2010.48_BX1.html). Alternatively, porcine intestinal mucosa Hep may be used (sodium salt of Hep; Sigma-Aldrich, cat. no. H3393) ▲ **CRITICAL** Store it at 2–8 °C.
- Fast-moving heparin (the fast-moving heparin (FM-Hep) component of Hep may be prepared according to refs. 72,73). Alternatively, FM-Hep may be used (sodium salt of HS FM fraction from porcine intestinal mucosa; Sigma-Aldrich, cat. no. H0519) ▲ **CRITICAL** Store it at 2–8 °C.
- Unfractionated Hep formulation (UHF; Calciparin, 12,500 UI/0.5 ml; Italfarmaco) ▲ **CRITICAL** Store it at 2–8 °C.
- Low-molecular-weight Hep (LMW-Hep; dalteparin from Fragmin, 2,500 UI/0.2 ml; Pfizer) ▲ **CRITICAL** Store it at 2–8 °C.
- Low-molecular-weight Hep (LMW-Hep; enoxaparin from Clexane, 4,000 UI/0.4 ml; Sanofi Aventis) ▲ **CRITICAL** Store it at 2–8 °C.
- Unsaturated HA disaccharide standard (**Table 1**) (sodium salt of unsaturated HA disaccharide; Sigma-Aldrich, cat. no. H9649) ▲ **CRITICAL** Store it at −20 °C.
- Unsaturated CS/DS disaccharide standards (**Table 1**) (sodium salt of 8 unsaturated CS/DS disaccharides; Iduron, cat. nos. CD001– CD008) ▲ **CRITICAL** Store standards at −20 °C.
- Unsaturated Hep/HS disaccharide standards (**Table 1**) (sodium salt of 12 unsaturated Hep/HS disaccharides; Iduron, cat. nos. HD001–HD013) ▲ **CRITICAL** Store standards at −20 °C.
- QAE Sephadex A-25 (GE Healthcare, cat. no. 17-0190-03).
- Acetic acid (≥99.7%, glacial; Sigma-Aldrich, cat. no. 320099) ! **CAUTION** This product is harmful if inhaled, if ingested or if it comes in contact with skin. It is an eye irritant. ▲ **CRITICAL** Store it at room temperature (20–24 °C).
- Acetonitrile LC-MS Chromasolv (≥99.9%; Sigma-Aldrich, cat. no. 34967) ! **CAUTION** This product is flammable; it is harmful if inhaled, ingested or if it comes in contact with skin. It is an irritant for eyes.
- AMAC, >98% (Sigma-Aldrich, cat. no. 06627) ▲ **CRITICAL** Store it at 2–8 °C and protect it from light.
- Ammonium acetate (99.9%; Sigma-Aldrich, cat. no. 372331) ▲ **CRITICAL** Store it at 2–8 °C.
- BSA, ≥98.0% (Sigma-Aldrich, cat. no. A7906) ▲ **CRITICAL** Store it at 2–8 °C.
- $NaBH_3CN$, ≥95.0% (Sigma-Aldrich, cat. no. 71435) ▲ **CRITICAL** Store it in a desiccator. ! **CAUTION** This product can release flammable hydrogen gas; it is toxic if inhaled, ingested or if it comes in contact with skin. It is an eye irritant.
- DMSO, ≥99.5% (Sigma-Aldrich, cat. no. D4540) ! **CAUTION** This product is flammable; it is toxic if inhaled, ingested or if it comes in contact with skin. It is an irritant for eyes.
- Methanol, LC-MS Chromanorm, ≥99.9% (VWR BDH, cat. no. 83638.320) ! **CAUTION** This product is flammable; it is toxic if inhaled, ingested or if it comes in contact with skin. It is an irritant for eyes.
- Ultrapure water was obtained with a Milli-Q system (Millipore). All other reagents, of the purest grade available, were from Sigma-Aldrich.

EQUIPMENT

- HPLC and UPLC systems composed of a minimum of a binary gradient pump
- On-line vacuum degasser ▲ **CRITICAL** An on-line degasser is essential to avoid the formation of air bubbles in mixed mobile phases comprising water and organic solvents and to maintain constant flow.
- High-sensitivity on-line high-performance fluorescence detector
- X-Bridge Shield RP18 column (3.5 µm, 4.6 × 100 mm; Waters, cat. no. 186003044)
- Discovery C18 column (5 µm, 4.6 × 250 mm; Supelco, cat. no. 504971)
- Acquity UPLC ethylene bridged hybrid (BEH) C18 column (1.7 µm, 2.1 × 150 mm; Waters, cat. no. 176000861)
- ESI mass spectra from Agilent 1100 series Classic or VL G2445A LC/mass selective detector (MSD) trap (Agilent Technologies)
- Injection Hamilton syringe (50 µl; Sigma-Aldrich, cat. no. 20736)
- Benchtop centrifuge (MicroCentrifugette 4214; ALC International, cat. no. 11172002)
- Microcon YM-3 filters (Ultracel molecular mass cutoff of 3 kDa; Amicon, cat. no. UFC5003BK)
- Microcon YM-10 filters (Ultracel molecular mass cutoff of 10 kDa; Amicon, cat. no. UFC5010BK)
- Ultrafree-MC centrifugal filters (Durapore-PVDF 0.45 µm; Millipore, cat. no. UFC30HV00)
- Vivapure Mini Q H spin column (Sartorius Stedium Biotech, cat. no. VS-IX01QH24)
- Syringe driven filter (Millex-GV 0.22 µm PVDF 33 mm, nonsterile; Millipore, cat. no. SLGV033NS)

REAGENT SETUP

Sample preparation Various GAGs to be used as standards may be purchased from Sigma-Aldrich and Iduron. Extraction and purification protocols for different tissue GAGs to be analyzed and characterized are available in specific scientific articles and monographs (see Box 1 in ref. 46 and bibliography thereof; http://www.nature.com/nprot/journal/v5/n6/box/nprot.2010.48_BX1.html). Owing to the high sensitivity and low limit of detection of the present procedure, we have included examples for extraction of GAGs from human plasma[56,74] (**Box 1**) and cells[75–77] (**Box 2**). These protocols may also be applied to other samples with a low GAG content, including biological fluids such as cell culture medium, as well as to ECM. The extracted GAGs can be analyzed immediately or stored frozen in solid form or in aqueous solution at −20 °C. ▲ **CRITICAL** All purified GAG samples should be free from contaminants and salts. After extraction from biological fluids or tissues, purification protocols should provide at least one solid-phase extraction step on an anion exchange microcolumn, a precipitation step using organic solvents, one desalting step by dialysis, a centrifugal membrane or desalting gel chromatography step, and a filtration step using a 0.2-µm membrane filter or centrifugation to remove particulates. Samples are dried or lyophilized before analysis. ▲ **CRITICAL** Before analysis, dissolve purified GAGs in ultrapure water. A preliminary quantitative step should be performed for further dilution procedures. To this end, the carbazole micro-assay in 96-well plates[78] (**Box 3**) may be useful.

Box 1 | Protocol to extract and purify GAGs from a biological fluid (human plasma or culture medium) ● TIMING ~2 d

! CAUTION Animal and human blood can contain viruses that may be hazardous, and it should be handled with gloves and disposed of as appropriate for biological waste. Collection and use of animal and human blood also requires appropriate accreditation of facilities and approval of collection protocols and compliance with ethical guidelines; for human donors, informed consent is required.

1. Freeze-dry 200 µl of plasma or culture medium.
2. Suspend the lyophilized sample in 500 µl of water and proteolyze it at 55 °C with 10 µl of a nonspecific protease such as actinase E (20 mg/ml) for 18 h.
3. Boil the sample for 10 min.
4. Centrifuge the sample at 10,000*g* for 10 min at 4 °C.
5. Suspend the pellet in 200 µl of water and centrifuge it at 10,000*g* for 10 min at 4 °C.
6. Collect together the supernatants and apply to a micro-column (0.5 cm × 1 cm) packed with QAE Sephadex A-25 anion-exchange resin equilibrated with distilled water. Wash the column with 1 ml of water and 1 ml of 0.2 M NaCl solution, and then release the polysaccharides with 1 ml of 2 M NaCl solution.
7. Filter the recovered solution on Microcon YM-3 filters at 10,000*g* for 60 min. Recover the retentate in 200 µl of water.
8. Add 4 volumes of ethanol and store it at 4 °C overnight.

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

9. Recover the precipitated GAG mixture by centrifugation at 5,000*g* for 20 min at 4 °C.
10. Suspend the pellet in 200 µl of water and filter it on Ultrafree-MC 0.45-µm centrifugal filters at 12,000*g* for 5 min at 4 °C.
11. Freeze-dry the filtrate solution.
12. Plasmatic CS (and possible DS, largely present in specific pathological conditions such as mucopolysaccharidsoses) can be recovered by removing HS (and Hep) by treating the sample exhaustively with Hep lyases I and III, followed by recovery using ethanol precipitation. After treatment with Hep lyases I and III, add 4 volumes of ethanol and store the sample at 4 °C overnight. Recover the precipitated CS (and DS) by centrifugation at 5,000*g* for 20 min at 4 °C. Suspend the pellet in 200 µl of water and filter it on Ultrafree-MC 0.45-µm centrifugal filters at 12,000*g* for 5 min. Freeze-dry the CS filtrate solution.
13. Plasmatic HS (and Hep) can be specifically recovered by removing CS (and possible DS) by treating the sample exhaustively with chondroitin lyase ABC, followed by recovery using ethanol precipitation. After chondroitin lyase ABC treatment, add 4 volumes of ethanol and store the sample at 4 °C overnight. Recover the precipitated HS (and Hep) by centrifugation at 5,000*g* for 20 min. Suspend the pellet in 200 µl of water and filter it on Ultrafree-MC 0.45-µm centrifugal filters at 12,000*g* for 5 min at 4 °C. Freeze-dry the HS filtrate solution.
14. A preliminary quantitative step should be performed by using the high-throughput and sensitive carbazole micro-assay in 96-well plates[78] (**Box 3**).

■ **PAUSE POINT** Samples may be stored at room temperature in controlled humidity before further enzymatic treatment.

Chondroitin lyase dissolving buffer (0.01% (wt/vol) BSA aqueous solution) Dissolve 0.01 g of BSA in 100 ml of ultrapure water. ▲ **CRITICAL** Always freshly prepare this solution each day and keep it at 2–8 °C before use.

HA/CS/DS 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 of ultrapure water. Adjust the pH of the solution to 8.0 by using 0.1 M sodium hydroxide (as necessary), and make up the volume to 100 ml with ultrapure water. ▲ **CRITICAL** Store the solution at −20 °C for up to 1 year. Stock aliquots should be prepared.

Chondroitin lyase stock aliquots Dissolve chondroitin lyase in 0.01% (wt/vol) BSA aqueous solution at a concentration of 0.25 units per ml. Store the aliquots at 2–8 °C before use. ▲ **CRITICAL** Aliquots desiccated at −20 °C remain active for at least 6 months.

Box 2 | Protocol to extract and purify GAGs from cells (also useful for ECM) ● TIMING ~ 2 d

1. Centrifuge the cell medium of suspended cultured cells at 1,000*g* for 15 min at 4 °C. Adherent cells can be scraped from a plate with a rubber policeman, and both ECM and cells can be suspended for recovery by centrifugation at 1,000*g* for 15 min.

▲ **CRITICAL STEP** Low centrifuge *g*-forces are crucial to avoid breaking the cells.

2. Wash the recovered cell pellet twice in cold PBS at 4 °C and centrifuge it at 1,000*g* for 15 min at 4 °C.
3. Suspend the cell pellet sample in 750 µl of water and proteolyze it at 55 °C with 250 µl of a nonspecific protease such as actinase E (20 mg/ml, final actinase E concentration is 5 mg/ml) for 18 h.
4. Remove the particulates from the proteolysis solutions by passing through a Millex 0.22-µm syringe-driven filter.
5. Remove the peptides by centrifuging five times in a Microcon YM-10 at 10,000*g* for 15 min at 4 °C. Recover the retenate in 200 µl of water.
6. Freeze-dry the retenate solution.
7. Dissolved the freeze-dried sample by using 300 µl of 8 M urea and 2% (wt/vol) CHAPS buffer.
8. Equilibrate a Vivapure Mini Q H spin column with 8 M urea and 2% (wt/vol) CHAPS buffer. Load the sample and wash the column with 3 column-volumes of 200 mM aqueous NaCl solution.
9. Release GAGs by washing the column with 3 column-volumes of 16% (wt/vol) NaCl solution.

(continued)

Box 2 | (continued)

10. Filter the eluted solution three times on Microcon YM-3 filters at 10,000*g* for 20 min at 4 °C. Recover the retentate in 200 µl of water.
11. Freeze-dry the solution.
12. Cellular HA, CS and DS can be recovered by removing HS by treating the sample exhaustively with Hep lyase III, followed by recovery using ethanol precipitation. After Hep lyase III treatment, add 4 volumes of ethanol and store it at 4 °C overnight. Recover the precipitated HA, CS and DS by centrifugation at 5,000*g* for 20 min at 4 °C. Suspend the pellet in 200 µl of water, and filter it on Ultrafree-MC 0.45-µm centrifugal filters at 12,000*g* for 5 min at 4 °C. Freeze-dry the CS filtrate solution.
13. Cellular HS can be specifically recovered by removing HA, CS and DS by treating the sample exhaustively with chondroitin lyase ABC, followed by recovery using ethanol precipitation. After chondroitin lyase ABC treatment, add 4 volumes of ethanol and store the sample at 4 °C overnight. Recover the precipitated HS by centrifugation at 5,000*g* for 20 min. Suspend the pellet in 200 µl of water and filter it on Ultrafree-MC 0.45-µm centrifugal filters at 12,000*g* for 5 min at 4 °C. Freeze-dry the HS filtrate solution.
14. A preliminary quantitative step should be performed by using the high-throughput and carbazole micro-assay (**Box 3**) in 96-well plates[78].
15. If data on the linkage regions of CS or DS (i.e., where the GAG chain is linked to core peptide) are required, a mild β-elimination step is required[108]. Material recovered in step 11 is treated with 0.5 M aqueous LiOH at 4 °C for 24 h under an inert atmosphere (argon or nitrogen). After neutralization with 0.5 M HCl, peptides and salts are removed as described in steps 4–6, and the sample can then be processed through steps 12 and 13.

■ **PAUSE POINT** Samples may be stored at room temperature in controlled humidity before further enzymatic treatment.

Hep lyase 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 of ultrapure water. Adjust the pH of the solution to 7.5 by using 0.1 M HCl or 0.1 M sodium hydroxide (as necessary) and make up the volume to 100 ml with ultrapure water. ▲ **CRITICAL** Always freshly prepare this solution each day and keep it at 2–8 °C before use.

Hep/HS 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 and bring the volume to 90 ml with ultrapure water. Adjust the pH of the solution to 7.0 by using 0.1 M HCl or 0.1 M sodium hydroxide (as necessary), and make up the volume to 100 ml with ultrapure water. ▲ **CRITICAL** Store the solution at 2–8 °C for up to 1 month. Stock aliquots should be prepared.

Hep lyase stock aliquots Dissolve Hep lyase I, II 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 unit per ml for Hep lyase I and 0.5 units per ml for Hep lyase II and III (see above for the preparation of Hep lyase dissolving buffer). ▲ **CRITICAL** Aliquots should be kept frozen at −80 °C for medium- or long-term storage. Aliquots desiccated at −20 °C remain active for at least 6 months.

AMAC solution (0.1 M AMAC in glacial acetic acid-DMSO 3:17 (vol/vol) solution) for AMAC derivatization Dissolve 21.0 mg of AMAC in 1 ml of glacial acetic acid:DMSO 3:17 (vol/vol) solution. Gently mix and centrifuge at 1,000*g* for 5 min at room temperature. Store the solution at 2–8 °C protected from light before use. ▲ **CRITICAL** Aliquots desiccated at 2–8 °C and protected from light remain active for at least 1 month.

$NaBH_3CN$ solution (1 M $NaBH_3CN$) Dissolve 62.2 mg of $NaBH_3CN$ in 1 ml of ultrapure water for AMAC derivatization. Gently mix. ▲ **CRITICAL** Always freshly prepare this solution each day and keep it at 2–8 °C before use.

HPLC solvent A (60 mM ammonium acetate, pH 5.6) for on-line FD-MS detection for GAG disaccharides Dissolve 2.31 g of ammonium acetate in 400 ml of ultrapure water. Mix the solution for about 30 min and adjust the pH to 5.6 by using glacial acetic acid. Make up to 500 ml with ultrapure water. Degas the solvent. ▲ **CRITICAL** Carefully check the pH, as different pH values can alter elution times and result in overlapping of disaccharide species. ▲ **CRITICAL** Aqueous solvents may be stored at 2–8 °C for up to 1 week for further HPLC separations.

HPLC solvent B (methanol or acetonitrile) for on-line FD-MS detection for GAG disaccharide Degas the solvent. ! **CAUTION** Organic solvents should only be stored in an explosion-proof chamber.

Box 3 | The carbazole micro-assay ● TIMING ~40 min

Additional materials

Reagent A (25 mM sodium tetraborate in sulfuric acid). Dissolve 0.95 g of sodium tetraborate decahydrate in 2 ml of hot water and add 98 ml of ice-cold concentrated sulfuric acid carefully with stirring.
▲ **CRITICAL** Solution stored at 2–8 °C remains active for at least 1 month.
! **CAUTION** This solution is highly toxic if inhaled, ingested or if it comes in contact with skin and eyes.
▲ **CRITICAL** Store the solution at 2–8 °C for up to 1 month.
Reagent B (0.125% wt/vol carbazole in absolute ethanol). Dissolve 125 mg of carbazole (recrystallized from ethanol) in 100 ml of absolute ethanol to give a stable reagent.
▲ **CRITICAL** Store the solution at 2–8 °C for up to 1 month.
1. Calibration curve for the carbazole assay can be prepared by using bovine kidney HS or bovine trachea CS as standard. Add, separately, aliquots of 25 µl of HS or CS (containing 0, 0.5, 1.0, 1.5, 2.0 and 2.5 µg) to a 96-well microtiter plate in a refrigerated chamber.
2. Add aliquots of 25 µl of GAGs sample (diluted within 0–2.5 µg) to the microtiter plate in the refrigerated chamber.
3. Add 150 µl of ice-cold Reagent A to the microtiter plate with mixing and cooling in the refrigerated chamber.
4. Heat the mixture at 100 °C for 10 min in an oven (temperature setting at 100 °C).
5. Cool the reaction rapidly in the refrigerated chamber.
6. Add 5 µl of Reagent B and mix well.
7. Reheat at 100 °C for 15 min in the oven.
8. Cool the reaction rapidly in the refrigerated chamber.
9. Determine the absorbance at 525 nm with a 96-well microtiter plate reader.

UPLC solvent A (80 mM ammonium acetate) for on-line ESI-MS detection of GAG disaccharides Dissolve 3.08 g of ammonium acetate in 500 ml of ultrapure water. Mix it for about 30 min and degas the solvent. ▲ **CRITICAL** Aqueous solvents may be stored at 2–8 °C for further UPLC separations not more than about 1 week.

UPLC solvent B (methanol) for on-line ESI-MS detection of GAG disaccharides Degas the solvent. ! **CAUTION** Organic solvents should only be stored in an explosion-proof chamber.

EQUIPMENT SETUP

HPLC or UPLC system requirements An HPLC or UPLC system capable of mixing a ternary gradient is necessary for FD and MS detection. A Rheodyne (or similar) injection system capable of loading and injecting up to 50 µl of sample should also be used together with appropriate data acquisition, control and analysis software. A fully automatic sample injection system enabling great productivity and the highest possible level of precision would be desired. For FD, an on-line high-sensitivity FD system is required with a wide wavelength range both for excitation and emission (from 220 to 700 nm) with proven stability and a signal-to-noise ratio better than 350:1 for the Raman water peak, and it should be able to provide maximum stray light suppression for unrivaled detection sensitivity. For MS acquisition, we suggest an ESI source, such as an Agilent Technologies LC/MSD Trap Classic or a VL mass spectrometer possessing an atmospheric pressure ionization (API)-ESI interface with an orthogonal nebulizer, a single split-flow turbomolecular high-vacuum pump and a resolution of <2 p.p.m. *m/z*.

HPLC/UPLC column preparation X-Bridge RP18 column preconditioning for GAG disaccharide separation is achieved by washing with isocratic 60 mM ammonium acetate (pH 5.6)/acetonitrile at a 98:2 ratio for 10 min, followed by a gradient separation to 100% ammonium acetate/acetonitrile at a 30:70 ratio for 50 min at a flow rate of 1 ml/min. Discovery C18 column preconditioning is obtained by washing with isocratic 60 mM ammonium acetate (pH 5.6)/methanol at a 95:5 ratio for 10 min, followed by a gradient separation to 100% ammonium acetate/methanol at a 50:50 ratio for 55 min at a flow rate of 1 ml/min. Acquity UPLC C18 column preconditioning is carried out by washing with isocratic 80 mM ammonium acetate/methanol at a 88:12 ratio for 5 min followed by linear gradients of 12–15% methanol from 5 to 15 min, 15%–30% methanol from 15 to 30 min and 30–100% methanol from 30 to 60 min at a flow rate of 0.1 ml/min. After reconditioning for 10 min in initial mobile-phase conditions, the systems are ready to perform sample separation.

For separation and FD-ESI-MS detection and characterization, these columns provide excellent separation of AMAC-fluorotagged disaccharides derived from enzymatic or chemical treatment of GAGs. Stable baselines are achieved and elution times are extremely consistent if a reconditioning period of ~10 min is performed between two consecutive runs. Column longevity is generally high, particularly when they are protected with suitable disposable guard column cartridges and samples are filtered on 0.45-µm filters.

ESI-MS equipment setup The ESI source was optimized over the MS range of unsaturated AMAC-labeled disaccharides from 572 to 811 *m/z*. The ESI source was tuned by using on-line direct injection of 0.1 ml of each unsaturated AMAC-labeled disaccharide standard (1 mg/ml). Finally, we determined to use ESI source parameters of N,6diS_{HS} (D0S6, *m/z* 689) as the optimized condition to detect all the disaccharides. Under these conditions, the loss of sulfo groups from triply sulfated disaccharides was <5%. Parameters such as capillary skimmer, cap exit and so on were tuned as the ESI source conditions (**Supplementary Fig. 1**).

The electrospray interface is set in negative ionization mode with a skimmer potential of −19.7V, a capillary exit −48.4 V and a source temperature of 350 °C to obtain maximum abundance of the standard disaccharide ions in full scan spectra (200–2,200 Da, ten full scans per s). Nitrogen is used as a drying (12 liters/min) and nebulizing gas (4.14 bars). Total ion chromatograms (TIC) and mass spectra were processed using Agilent Chemstation A.07. Software versions we used were LC/MSD trap control 5.0 and Data Analysis 2.2 (Agilent Technologies).

FD setup After column preconditioning and reconditioning for 10 min in initial mobile-phase conditions (see HPLC/UPLC columns preparation), the FD is generally ready to perform sample separation.

PROCEDURE

Enzyme treatment and production of GAG-derived disaccharides ● TIMING ~5–10 h

1| In contrast to GAG-derived oligosaccharides that are prepared on the basis of the partial controlled enzymatic degradation[46] (**Table 2**), the production of GAG-derived disaccharides relies on the exhaustive enzymatic depolymerization of the GAG chains using specific enzymes. Disaccharides of various structures bearing a different number of sulfo groups in various positions (**Table 1**) may be produced, depending on the substrate, the enzyme specificity and the site of action. The exhaustive action of GAG lyases[47–49] on GAGs catalyzes their β-eliminative cleavage to disaccharide (and oligosaccharides still resistant to their activity) products having a 4,5-unsaturated UA residue (ΔUA) at their nonreducing ends. The resulting disaccharides, before or after fluorescent tagging, can be separated by various analytical approaches. Set up the reactions for HA/CS/DS treatment with chondroitin lyase ABC (option A), HS/Hep treatment with a cocktail of Hep lyases (option B) or HA/CS/DS and HS/Hep with both chondroitin lyase ABC and Hep lyases (option C). Multiple replicate samples ($n \geq 3$) can be treated with a single or multiple options if the GAG composition is not known beforehand. It is very useful to include various GAG standards (**Table 1**) in amounts bracketing the concentrations of GAGs in the unknown samples, as measured by the carbazole assay[78], as such as in known concentration for an accurate evaluation of the enzymatic treatment owing to the necessity to have an exhaustive and reproducible GAG depolymerization to generate disaccharides. The biological starting material is the lyophilized biological fluid, cell or tissue extract, prepared as described in Box 1 in ref. 46 (http://www.nature.com/nprot/journal/v5/n6/box/nprot.2010.48_BX1.html) and in **Boxes 1** and **2** of this protocol.

(A) HA/CS/DS treatment

(i) Add 0.1 mg of HA and CS and DS standard (in 100 µl) to 700 µl of 50 mM Tris/60 mM sodium acetate (pH 8.0) in a 1.5-ml centrifuge tube. Dissolve the lyophilized sample in 800 µl of 50 mM Tris/60 mM sodium acetate (pH 8.0) in a 1.5-ml centrifuge tube.

(ii) Add 50 mU of chondroitin lyase ABC (in 200 µl) to the 1.5-ml centrifuge tube.

(B) HS/Hep treatment

(i) Add 0.1 mg of HS and Hep standard (in 100 µl) to 850 µl of 50 mM sodium phosphate buffer (pH 7.0) in a 1.5-ml centrifuge tube. Dissolve the lyophilized sample in 850 µl of 50 mM sodium phosphate buffer (pH 7.0) in a 1.5-ml centrifuge tube.

(ii) Add 10 mU of Hep lyase I (10 µl), 10 mU of Hep lyase II (20 µl) and 10 mU of Hep lyase III (20 µl) to the 1.5-ml centrifuge tube.

(C) Simultaneous HA/CS/DS and HS/Hep treatment

(i) Add 0.1 mg of each GAGs standard, HA and CS and DS and HS and Hep (in a total of 100 µl), to 200 µl of distilled water in a 1.5-ml centrifuge tube. Dissolve the lyophilized sample in 300 µl of distilled water in a 1.5-ml centrifuge tube.
(ii) Add 50 mU of chondroitin lyase ABC (200 µl) to the 1.5-ml centrifuge tube.
(iii) Incubate the reaction mixture in a water bath at 37 °C for 5 h.
(iv) Heat the reaction mixture in a boiling water bath for 10 min.
(v) Remove the denatured protein by centrifugation at 12,000*g* for 10 min at room temperature and recover the supernatant.
(vi) Add 450 µl of 50 mM sodium phosphate buffer (pH 7.0).
(vii) Add 10 mU of Hep lyase I (10 µl), 10 mU of Hep lyase II (20 µl) and 10 mU of Hep lyase III (20 µl).

2| Incubate the reaction mixture in a water bath at 37 °C for 5 h.

3| Heat the reaction mixture in a boiling water bath for 10 min to inactivate the enzyme and to stop the reaction.

4| Remove the denatured protein by centrifugation at 12,000*g* for 10 min at room temperature. Use the supernatant for derivatization with AMAC and disaccharide characterization by HPLC-FD-ESI-MS.
■ **PAUSE POINT** Samples may be stored at 4 °C for 1–2 d or at –20 °C for long-term storage before further AMAC derivatization. The samples generated via Step 1A, 1B and 1C are referred to as sample types A, B and C, respectively.

Fluorotagging GAG-derived disaccharides (and residual oligosaccharides) by AMAC ● TIMING ~9–10 h

5| Completely freeze-dry all of the solution before derivatization. About 5 h is sufficient enough to freeze-dry 1 ml of solution.
? TROUBLESHOOTING

6| Add 5 µl of 0.1 M AMAC in glacial acetic acid:DMSO 3:17 (vol/vol) solution per estimated lyophilized µg of disaccharide or oligosaccharide standard, or sample based on the carbazole micro-assay.

7| Incubate the above reaction solution at room temperature for 15 min.

8| Add 1 M $NaBH_3CN$ solution to the reaction solution.

9| Briefly centrifuge at 10,000*g* for 1 min at room temperature.

10| Incubate the reaction mixture at 45 °C for 4 h.

11| Make up the reaction solution to ~ 200 ng/µl with DMSO-ultrapure water at a 1:1 ratio (vol/vol).
? TROUBLESHOOTING

GAG disaccharide analysis and characterization by RP-HPLC on-line with FD and ESI-MS ● TIMING ~1–2 h

▲ **CRITICAL** Steps 13 and 14 describe how to set up the HPLC-MS instrument for analysis of the disaccharide standards and for the disaccharides derived from the samples (types A, B and C) generated in Step 3. Both disaccharide standards and disaccharides derived from the samples are separately derivatized with AMAC in Steps 6–11 by HPLC-FD-ESI-MS. After conditioning, the system is ready to perform sample separation (Steps 15–18).

12| Equilibrate the appropriate HPLC or UPLC column for 20 min with the corresponding ratio of ammonium acetate to acetonitrile/methanol, as shown in the table below:

	HA/CS/DS (sample type A)	HS/Hep (sample type B)	HA/CS/DS and HS/Hep (sample type C)
Column	C18 column X-Bridge Shield	Discovery C18	Acquity UPLC BEH C18 column
Ammonium acetate (vol%)	60 mM, pH 5.6, 98%	60 mM, pH 5.6, 95%	80 mM, 88%
Acetonitrile (vol%)	2%	5%	—
Methanol (vol%)	—	—	12%
Flow rate	1.0 ml/min	1.0 ml/min	0.1 ml/min

13| Equilibrate the electrospray interface on-line with HPLC (sample types A and B) by flushing it with conditioning solvents at the related flow rates for 30 min, as previously reported. Set the mass analyzer in negative ionization mode with the skimmer potential set at –43.2 V, capillary exit set at –120.4 V and temperature set at 350 °C in full-scan spectra (100–2,000 Da, ten full scans per s) with a maximum accumulation time of 300 ms and an ion charge control (ICC) target of 20,000. Set nitrogen at 12 liters/min and nebulizing gas at 4.14 bars. Equilibrate the electrospray interface on-line with UPLC (sample type C) by flushing with conditioning solvents at the related flow rates for 30 min. Set the mass analyzer in negative ionization mode with the skimmer potential set at –40.0 V, capillary exit set at –40.0 V and a temperature of 350 °C in full-scan spectra (150–1,200 Da, ten full scans/s). Set nitrogen at 8 liters min^{-1} and nebulizing gas at 2.76 bars.

14| Inject GAG disaccharide standard at a concentration of about 0.2–0.5 µg, and then inject samples at a concentration of about 1–2 µg onto the injection loop by using a Hamilton syringe.

15| Elute the disaccharides with the appropriate gradient, as shown in the table below:

	CS/DS/HA (sample type A)	HS/hep (sample type B)	CS/DS/HA and HS/hep (sample type C)
Column	C18 column X-Bridge Shield	Discovery C18	Acquity UPLC BEH C18 column
Solvent A	60 mM ammonium acetate, pH 5.6	60 mM ammonium acetate, pH 5.6	80 mM ammonium acetate
Solvent B	Acetonitrile	Acetonitrile	Methanol
Equilibrate solution	2% solvent B	5% solvent B	12% solvent B
Gradient	2–30% solvent B over 50 min	5–50% solvent B over 65 min	12% solvent B for 5 min, 12–15% solvent B over 5–15 min, 15–30% solvent B over 15–30 min, 30–100% solvent B over 30–60 min

16| Monitor the eluent by using the on-line FD detector at excitation λ = 428 nm and emission λ = 525 nm; gain is set at 100 for unsaturated disaccharides derivatized with AMAC. Simultaneously monitor the eluent by using the on-line mass detector setup in series.

17| Identify the disaccharide peaks based upon the elution order and by using available standards and/or by means of ESI-MS characterization (see, for example, **Fig. 1**).

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
5	No absorbance is observed by spectrophotometric detection at 232 nm of treated GAG standard mixture in HCl 10 mM	Enzyme is inactive Insufficient enzymatic treatment	Check for enzyme activity on a GAG standard
	No peaks (disaccharides) are observed in SAX-HPLC or RPIP-HPLC and UV detection	No exhaustive activity	Extend digestion time Increase the enzyme(s) amount (units per GAG extract)

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
11	No peaks (disaccharides) are observed in RP-HPLC and fluorescence detection	AMAC reagent is insufficient	Increase the molarity of the AMAC reagent
		$NaBH_3CN$ reagent is insufficient	Increase the molarity of $NaBH_3CN$
		Derivatization conditions are not adequate	Set temperature at 45 °C for a minimum time treatment of 4 h
		GAG sample is not adequately lyophilized	Re-prepare GAG sample and re-lyophilize under optimum conditions Treat again with lyase(s) and AMAC derivatization
		GAG sample is impure	Re-purify the GAG sample and treat it again with lyase(s) and AMAC derivatization
17	Baseline chromatogram in HPLC and fluorescence detection is noisy and contains spurious peaks	Solvent inadequately degassed	Use an on-line vacuum degasser with the HPLC system
		Purity of reagents has been compromised	Re-prepare fresh reagents and HPLC solvents
		GAG sample is impure	Re-purify GAG sample and treat again with lyase(s)
	Overlapping of GAG disaccharides or free oligosaccharides in HPLC-ESI-MS	pH values of HPLC solvents are different than optimal	Careful check for pH of HPLC solvents. Reprepare fresh HPLC solvents
		Sample contains salt	Either repeat purification and depolymerization of GAG sample or desalt disaccharides Either repeat purification of free oligosaccharides in complex matrix or desalt extracted oligosaccharides
		Partial enzymatic digestion of material	Check enzyme activities using standard GAGs or an enzyme assay[65] Add more enzymes
	Elution times change or become unstable from run to run in HPLC-ESI-MS	The column has not been well conditioned from run to run	Re-equilibrate the column(s) in solvent A for 20 min at the indicated flow rate
	No peaks are evident by MS	Solvent flow is inadequate	Make sure the solvent is flowing from the needle Make sure that the divert valve setting is correct
		High voltages and spay 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 flow and high vacuum pressure are within normal ranges

● TIMING

Steps 1–4, enzyme treatment and production of GAG-derived disaccharides: ~5–10 h
Steps 5–11, fluorotagging GAG-derived disaccharides (and residual oligosaccharides) by AMAC: ~9–10 h
Steps 12–17, GAG disaccharide characterization by RP-HPLC-FD-ESI-MS: ~1–2 h
Box 1, protocol to extract and purify GAGs from a biological fluid (human plasma or culture medium): ~2 d
Box 2, Protocol to extract and purify GAGs from cells (also useful for ECM): ~2 d
Box 3, The carbazole micro-assay: ~40 min

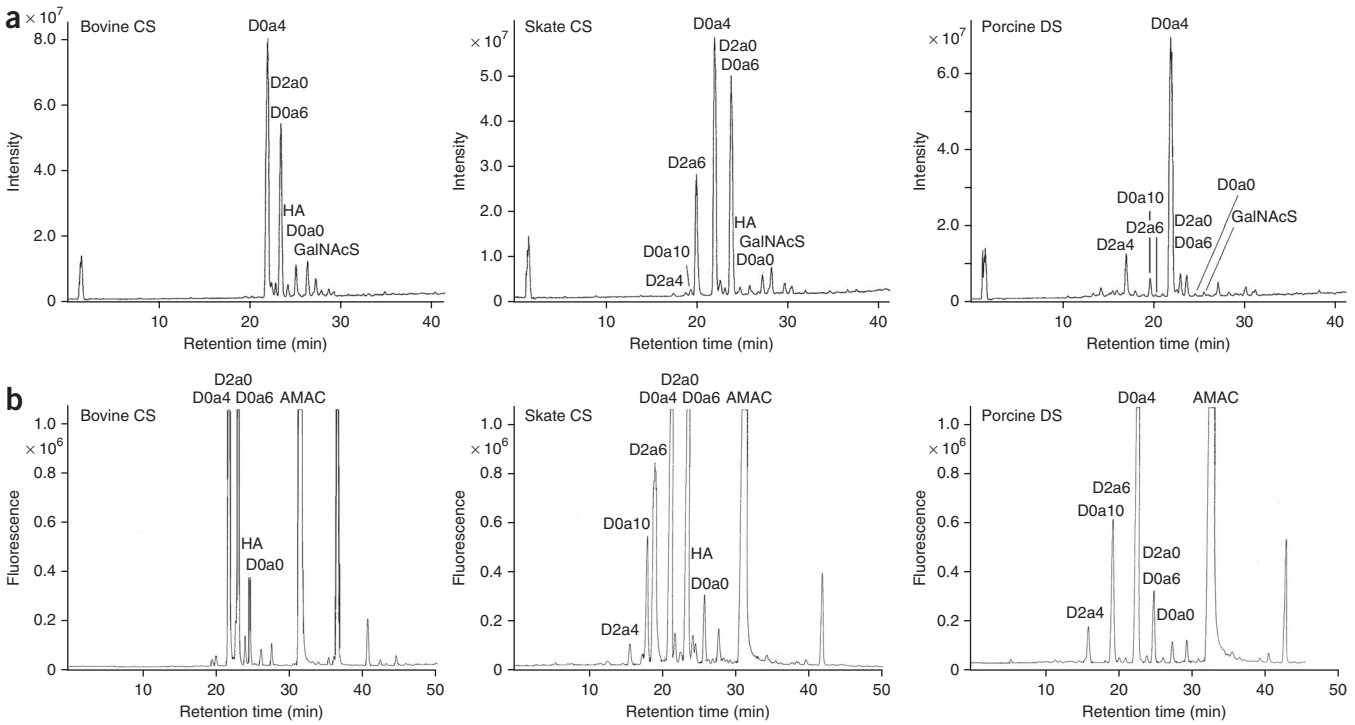


Figure 3 | Analysis of AMAC-tagged constituent disaccharides (and sulfated monosaccharide, GalNS) of CS from bovine, skate cartilage and DS from porcine mucosa obtained by exhaustive treatment with chondroitin ABC lyase and separated on an X-Bridge RP18 column. (**a**,**b**) TICs (**a**) and FD detection spectrum (**b**). See **Table 1** for disaccharide structure. HA = OSHA. Modified with permission from ref. 58.

ANTICIPATED RESULTS

Figure 3 shows results from an experiment in which CS from bovine and skate cartilage and DS from porcine mucosa were exhaustively digested with chondroitin ABC lyase and released disaccharides were derivatized with AMAC and separated by RP-HPLC-FD-ESI-MS[58]. Minor peaks can be unambiguously assigned to known disaccharides by MS, and other minor species, of variable intensities depending on samples, were observed to be common to all CS and DS species. In particular, by means of this analytical approach, it is possible to observe minor but substantial percentages of the peak labeled GalNAcS identified as the monosulfated monosaccharide located at the nonreducing end of CS/DS[58], purified from various sources and released by the action of chondroitin lyase. Furthermore, the unsaturated disaccharide of HA can be clearly identified by MS spectrum in CS samples. This is consistent with the results of previous studies and shows that it is a commonly occurring impurity[79]. The use of MS in identifying peaks is extremely important when analyzing GAGs in biological samples, as unknown peaks are commonly observed in fluorescence chromatograms (**Fig. 3**) and correct assignment can be performed by ESI connected on-line with the FD detector.

By this approach, consistent quantitative disaccharide compositions were obtained for CS and DS samples by comparing data obtained with FD and MS and data reported in the literature[58]. Endogenous CS in normal human plasma was further evaluated by FD and ESI-MS[58]. The application of MS detection (**Fig. 4a**) on-line with FD permitted the unambiguous determination of plasmatic CS disaccharides fluorotagged with AMAC, particularly for species present in low amounts, such as the $6S_{CS}$, which can easily mistaken for other low peaks (**Fig. 4b**).

RP-HPLC is extremely efficient in the separation of structurally different HS- or Hep-derived unsaturated

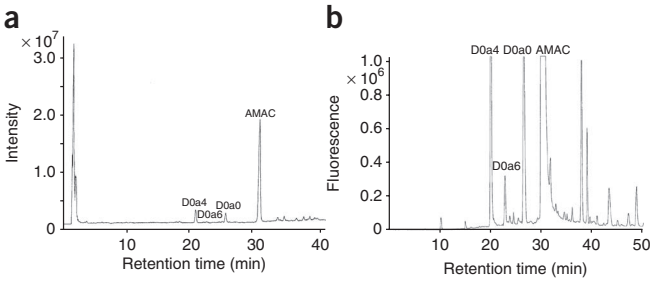


Figure 4 | Analysis of disaccharides from endogeneous healthy human plasma CS derivatized with AMAC, separated on an X-Bridge RP18 column. (**a**,**b**) TIC with detection using ESI-MS in negative mode (**a**) and detection by fluorescence measurement (**b**). See **Table 1** for disaccharide structure. Additional peaks (unlabeled) eluting after AMAC correspond to sample impurities, and they are unrelated to AMAC-labeled GAG disaccharides. Modified with permission from ref. 58.

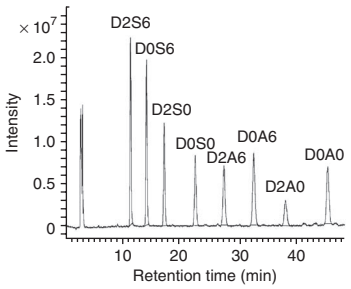


Figure 5 | TIC of Hep/HS unsaturated disaccharides derivatized with AMAC separated by a Discovery C18 column and detected by ESI-MS in negative ion mode. See **Table 1** for disaccharide structure. Modified with permission from ref. 55.

disaccharides fluorotagged with AMAC (**Table 1**), with ESI-MS showing a limit of detection of ~200–500 ng and FD showing a limit of detection ~50–100 times greater[55]. As is evident, very sharp peaks are obtained and full separation is observed in particular for N-sulfated disaccharides (**Fig. 5**) being species having free amino group very rare in HS and Hep[80]. Along with a very high sensitivity provided by FD, ESI-MS affords the mass of each disaccharide (**Fig. 2**), generally with the corresponding sodium adduct and limited desulfated products (**Table 1**).

In another experiment, a cocktail of Hep lyases, endowed with different specificity toward variously sulfated GlcNAc or GlcNS and GlcA or IdoA linkages, was used to totally degrade HS and Hep samples into their constituent disaccharides. Related compositional mapping was performed after their derivatization with AMAC. Disaccharides from HS and UHF, FM-Hep and commercial LMW-Hep samples were separated by RP-HPLC and detected by ESI-MS (**Fig. 6**). FD was also applied to increase the sensitivity of the analysis, but owing to the presence of several unknown peaks the correct assignment was obtained by using ESI connected on-line. This RP-HPLC analysis with FD revelation and ESI-MS acquisition was able to separate and identify the Hep-saturated trisulfated disaccharide with a retention time close to the unsaturated species arising from the nonreducing end of the polymer chains. As expected, the percentage of saturated trisulfated disaccharide was found to be inversely proportional to the molecular mass of the Hep samples, ~2.5% in UHF, ~5–8% in FM-Hep and ~15% in dalteparin[55]. Enoxaparin did not show the presence of saturated nonreducing ends owing to the β-eliminative depolymerization process that is able to introduce a double bond on the degraded chains.

A reliable method for the quantitative analysis of disaccharides arising from mixtures of HA, CS or DS and HSor Hep has been developed on the basis of the RP-UPLC separation and on-line FD-ESI-MS detection and quantification of AMAC derivatives to improve the GAG compositional mapping[54]. The challenge was to develop a single analytical method to concurrently determine all 17 GAG-derived disaccharides (**Table 1**), some of which were also structural isomers with the same mass and charge. The present analytical protocol provides multiple ways to separate and identify each disaccharide, such as using different retention times according to the presence of hydrophobic AMAC groups and mass, or using MS/MS, which can be used to distinguish between disaccharides of identical mass. Compositional analysis and quantification and characterization of Hep and HS by ESI ion-trap MS[81,82], based on volatile mobile-phase components compatible with the ion source and FD, is also able to provide high sensitivity detection. In fact, quantitative disaccharide compositional analysis is directly related to important GAG biological (and pharmacological) functions, as well as to an increased interest in complex polysaccharide structural characterization of cultured cells, culture medium, ECM, genetically engineered cells, and biological fluids and tissues from healthy, diseased and knockout organisms, thus making their detection and quantitative analysis very important. **Figure 7** illustrates the complete GAG disaccharide compositional mapping of three biological samples, a tissue sample (see Box 1 in ref. 46 for preparative protocol; http://www.nature.com/nprot/journal/v5/n6/box/nprot.2010.48_BX1.html), a biological fluid (**Box 1**) and a cell extract (**Box 2**),

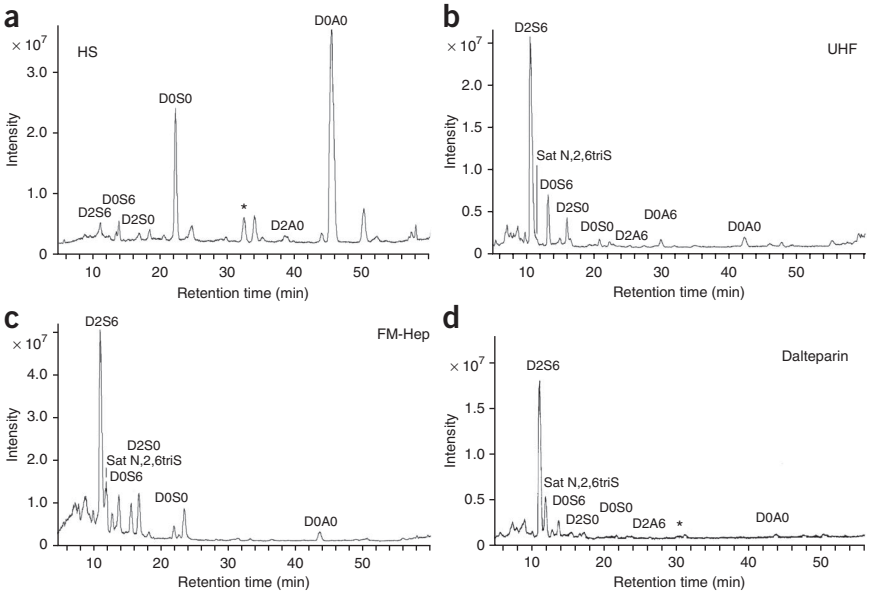


Figure 6 | TIC of disaccharides produced by the exhaustive action of Hep lyases on beef spleen HS (HS), beef mucosa Hep (UHF), fast-moving Hep (FM-Hep) and LMW-Hep dalteparin (Dalteparin) fluorotaggged with AMAC and separated on a Discovery C18 column and detected by ESI-MS. Sat N,2,6,triS corresponds to the saturated trisulfated disaccharide species. Asterisk (*) indicates an unidentified peak. See **Table 1** for disaccharide structure. Modified with permission from ref. 55.

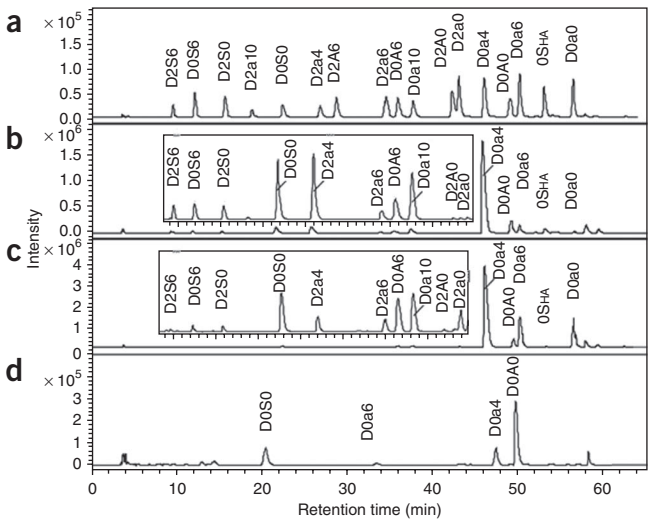


Figure 7 | EIC of AMAC-tagged disaccharide standards and from GAGs extracted from different biological sources. (**a**–**d**) Disaccharide standards (**a**), camel liver (**b**), camel urine (**c**) and CHO cells (**d**), generated by the treatment with a cocktail of lyases on Acquity UPLC BEH C18 column separation. See **Table 1** for disaccharide structure. Modified with permission from ref. 54.

performed by separation with RP-UPLC-MS of AMAC-derived disaccharides. Samples were directly injected onto LC-MS without the removal of excess AMAC reagent. By this approach, several issues were resolved, such as the difficulty in cross-comparison of analytical data obtained on different families of GAGs and time-consuming and complicated multiple sample recoveries. The use of $NaBH_3CN$ as a reductive reagent in the labeling step introduces sodium, and its complete removal would be complicated, requiring additional steps. Moreover, our experience has shown that there are benefits to sodiation, i.e., exchanging H^+ with metal Na^+ has been shown to stabilize sulfate groups and to increase the formation of sequence-informative fragment ions in MS/MS[83].

The present approach has the capacity to specifically characterize and determine the presence of important oligosaccharide species that are not revealed by conventional HPLC and UV detection, such as the sulfated monosaccharide located in the nonreducing CS and DS chains and the saturated trisulfated disaccharide belonging to the nonreducing end of HS and Hep. In addition, AMAC-tagged disaccharides are analyzed without any interference from excess fluorophore reagent, thus avoiding generally time-consuming sample-cleaning procedures. Finally, the detection limits of the separation of AMAC-derivatized molecules are improved by using FD[55,58,84]. Accordingly, we used both detectors coupled to HPLC with FD before ESI-MS acquisition to analyze GAG disaccharides extracted from biological samples in very low amounts. In fact, fluorescence measurement was able to detect endogenous disaccharides with high sensitivity, and ESI-MS was applied to unambiguously characterize disaccharide species from other peaks observed during fluorescence acquisition.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS All authors contributed equally to this work. N.V. designed and developed the on-line HPLC-FD-ESI-MS analysis of AMAC-tagged GAGs disaccharides. R.J.L. and B.Y. designed and developed the RP-HPLC-ESI-MS concurrent separation of multiple families of AMAC-tagged GAG disaccharides. F.G. applied this methodology to the structural study of various GAGs.

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