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Orbitrap mass spectrometry characterization of hybrid chondroitin/dermatan sulfate hexasaccharide domains expressed in brain

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ABSTRACT

In the central nervous system, chondroitin/dermatan sulfate (CS/DS) glycosaminoglycans (GAGs) modulate neurotrophic effects and glial cell maturation during brain development. Previous reports revealed that GAG composition could be responsible for CS/DS activities in brain. In this work, for the structural characterization of DS- and CS-rich domains in hybrid GAG chains extracted from neural tissue, we have developed an advanced approach based on high-resolution mass spectrometry (MS) using nanoelectrospray ionization Orbitrap in the negative ion mode. Our high-resolution MS and multistage MS approach was developed and applied to hexasaccharides obtained from 4- and 14-week-old mouse brains by GAG digestion with chondroitin B and in parallel with AC I lyase. The expression of DS- and CS-rich domains in the two tissues was assessed comparatively. The analyses indicated an age-related structural variability of the CS/DS motifs. The older brain was found to contain more structures and a higher sulfation of DS-rich regions, whereas the younger brain was found to be characterized by a higher sulfation of CS-rich regions. By multistage MS using collision-induced dissociation, we also demonstrated the incidence in mouse brain of an atypical [4,5-Δ-GlcAGalNAc(IdoAGalNAc)$_2$], presenting a bisulfated CS disaccharide formed by 3-*O*-sulfate-4,5-Δ-GlcA and 6-*O*-sulfate-GalNAc moieties.

Glycosaminoglycans (GAGs),[1] the major components of the extracellular matrix, are responsible for many biological activities of proteoglycans (PGs) and play an important role in tissue development, maturation, and various diseases [1,2].

Chondroitin sulfate (CS) and dermatan sulfate (DS) GAGs are *O*-covalently attached to a wide diversity of PG proteins via a xylose-galactose-galactose (Xyl-Gal-Gal) trisaccharide linkage [3]. CS chain contains a repetitive glucuronic acid (GlcA) β1–3 *N*-acetylgalactosamine (GalNAc) disaccharide units joined by β1–4 glycosidic bond, whereas DS chain is composed of a similar structure with GlcA epimerized to iduronic acid (IdoA). In biological tissues, CS and DS are often found as hybrid structures, where DS might be interspersed with CS domains as disaccharide isolated units or could form longer DS clusters [4]. In the central nervous system (CNS), CS/DS PGs modulate neurotrophic [5] glial cell maturation during brain development, cellular behavior, and extracellular matrix organization [6–8]. Several studies showed that the composition of the hybrid molecule, as well as the distribution and position of the sulfate along the chain, could be responsible for CS/DS biological activities in brain [8]. Sulfation occurs at GalNAc and/or IdoA/GlcA in various combinations; their modification during chain elongation by specific sulfotransferases produces characteristic patterns and considerable structural diversity in the CNS [9]. In this context, the analysis of CS/DS oligosaccharide motifs in brain and identification of over-, regularly, and undersulfated alternating patterns became the focus of research in the field.

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[1] *Abbreviations used:* GAG, glycosaminoglycan; PG, proteoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; GlcA, glucuronic acid; GalNAc, *N*-acetylgalactosamine; IdoA, iduronic acid; CNS, central nervous system; MS, mass spectrometry; ESI, electrospray ionization; MS^n, multistage MS; FTICR, Fourier transform ion cyclotron resonance; CID, collision-induced dissociation; EDD, electron detachment dissociation; NETD, negative electron transfer dissociation; MS/MS, tandem MS; SEC, size exclusion chromatography; HCT, high-capacity ion trap; QTOF, quadrupole time-of-flight; UV, ultraviolet; DDA, data-dependent acquisition.

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During the past decade, sophisticated mass spectrometry (MS) techniques and related hyphenated methods, as well as several bioinformatics platforms for the interpretation of mass spectra, were developed, optimized, and introduced in glycosaminoglycomics [10–14]. The feasibility of electrospray ionization (ESI) MS, and multistage MS (MS^n) was demonstrated for the analysis of CS/DS hexasaccharide motifs in human decorin [15] as well as for the successful characterization of regularly and irregularly sulfated domains [16,17]. High mass accuracy and high resolution were provided by Fourier transform ion cyclotron resonance (FTICR) instruments to detect GAG species and to distinguish ions with close m/z values [18]. For detailed structural analysis of CS/DS, collision-induced dissociation (CID) in multiple stages, electron detachment dissociation (EDD), negative electron transfer dissociation (NETD), and infrared multiphoton dissociation (IRMPD) emerged lately as the most powerful fragmentation techniques [15,16,18–22]. EDD and NETD performed on modern FTICR mass spectrometers [19–22] were shown to provide a detailed GAG structural analysis in a single tandem MS (MS/MS) experiment due to the formation of cross-ring cleavage ions diagnostic for regularly sulfated hexa-, octa-, and decasaccharides. Separation methods for characterization of single GAG components in complex mixtures such as liquid chromatography (LC) [17,23], size exclusion chromatography (SEC) [24], and capillary electrophoresis (CE) [25,26] were also employed in combination with MS and MS/MS by CID. For separation, sequencing, conformational analysis, and isomeric differentiation, more recently ion mobility mass spectrometry was also developed and successfully introduced in GAG analysis [27–29].

The performance of mass spectrometry in CS/DS research also increased significantly after the introduction of microfluidics for electrospray. Hence, NanoMate robot with automatic sample infusion by nanoESI through silicon chips was coupled to either high-capacity ion trap (HCT) [30] or quadrupole time-of-flight (QTOF) [24] instruments and applied for the characterization of CS/DS chains of various lengths and sulfation patterns. Other MS strategies for high mass accuracy and resolution combined with MS^n were successfully developed on desorption ESI (DESI)-LTQ-Orbitrap for hyaluronic acid, heparin, and heparin sulfate investigation [31].

In our previous study [32], using chip-based nanoESI MS technology, CS and DS disaccharides extracted from 4-week-old mouse brain were investigated. In this preliminary research, a new oversulfated [4,5-Δ-GlcA(3S)GalNAc(6S)] motif was discovered in neural tissue and thoroughly characterized by CID. Later, research related to CS/DS expression in brain was conducted toward structural analysis of CS/DS tetrasaccharides. The employed methodology, which included fully automated chip-based nanoESI HCT CID MS^2–MS^4 and QTOF MS [33], revealed two novel structural motifs in brain tissue: [4,5-Δ-GlcAGalNAc(4S)IdoA(2S,3S)GalNAc] and [4,5-Δ-GlcAGalNAcIdoA(2S,3S)GalNAc(4S)].

In the current study, we have extended the MS-based research to complex hybrid CS/DS hexasaccharide mixtures resulting after enzymatic treatment with B lyase and in parallel with AC I lyase. To elucidate the differences in the CS/DS expression induced by postnatal brain development, a comparative assay between 4- and 14-week-old mouse brains was carried out. The MS strategy employed screening and sequencing of the CS- and DS-rich hexamers by negative ion mode nanoESI Orbitrap MS and CID MS^2–MS^4. The results have shown that an age-related structural variability of the CS/DS motifs in the two investigated brains exists. Moreover, an uncommon [4,5-Δ-GlcA(3S)GalNAc(6S)$(IdoAGalNAc)_2$] species was identified, which substantiates the previous findings related to the occurrence of the oversulfated [4,5-Δ-GlcA(3S)GalNAc(6S)] motif in brain.

Materials and methods

Sampling of brain tissues

Fresh healthy whole brains from C57BL/6 wild-type mice were excised with a scalpel. Two cerebral tissues with a total mass $m = 1.09$ g from 4-week-old mouse and two whole brains with a total mass $m = 1.13$ g from 14-week-old mouse were prepared in parallel under the same experimental conditions. CNS samples were cut into small pieces and incubated overnight in 4 M guanidinium chloride buffer.

CS/DS extraction and purification

The CS/DS brain extraction and purification procedure followed the protocol described previously by us [34]. Briefly, the samples were centrifuged and the supernatant was dissolved in Tris/HCl buffer (pH 7.4) containing 150 mM NaCl and applied on a DEAE–anion exchange column (Pall Life Sciences, Dreieich, Germany). Intact PGs were eluted with Tris/HCl buffer (pH 7.4) containing 1 M NaCl and dialyzed against water in a 14,000-MWCO (molecular weight cutoff) membrane. Free intact GAG chains were released by β-elimination in 1 M $NaBH_4$/NaOH solution and applied again on DEAE–anion exchange column. CS/DS structures were purified by ethanol precipitation and digestion with HNO_2. Both CS/DS samples, derived from 4- and 14-week-old mouse, were depolymerized in parallel with two chondroitin lyases: AC I lyase and B lyase (Seikagaku Kogyo, Tokyo, Japan).

Chondroitin B lyase cleaves the linkage between GalNAc and L-IdoA, whereas chondroitin AC I lyase cleaves the linkage between GalNAc and D-GlcA, as demonstrated previously. Both enzymes belong to the class of lyases that cleave glycosidic bond by β-elimination reaction, following this strict specificity, which does not depend on the sulfation content and pattern. This mechanism involves the neutralization of the negative charge of the carboxylic acid group, followed by abstraction of the C5 proton (chiral center) and subsequent elimination of the C4 hydroxyl group by introducing a C4–C5 double bond. Due to the specificity of the lyases, the origin of the Δ-HexA in the oligosaccharides is known. Even though the stereochemistry is lost after the elimination, for better understanding of HexA origin, throughout this article iduronic acid- and glucuronic acid-derived Δ-HexA are denoted as Δ-IdoA and Δ-GlcA, respectively.

The oligosaccharide structures resulting after depolymerization were separated by SEC using a high-performance liquid chromatography (HPLC) 540+ instrument (Kontron Instruments HPLC system 500, Bio-Tek Kontron Instruments, Milan, Italy) equipped with a binary pump with high-pressure gradient mixer (Pump System 525) and ultraviolet (UV) diode array detector (Diode Array Detector 540). Separation was performed on a Superdex Peptide HR10/30 column (Amersham–Pharmacia, Freiburg, Germany) using 0.5 M NH_4HCO_3 solution as running and elution buffer at a flow rate of 0.5 ml/min. Hexasaccharide pools were collected under continuous UV detection at 232 nm. Although traces of octasaccharides were found in the final sample solution, a rechromatography of the collected fractions was not performed because additional purification steps might have led to sample loss.

Sample preparation for MS

Hexasaccharide fraction sample solutions obtained after SEC were dried to complete desiccation in a SpeedVac Concentrator (SPD 111V-230, Thermo Electron, Asheville, NC, USA) coupled to