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Coupling Liquid Chromatography and Tandem Mass Spectrometry to Electrophoresis for In-Depth Analysis of Glycosaminoglycan Drugs: Heparin and the Multicomponent Sulodexide

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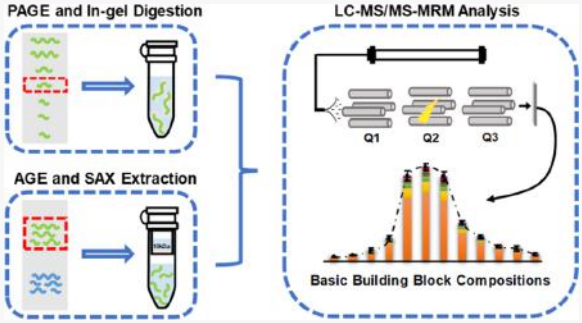
ABSTRACT: Glycosaminoglycans (GAGs) contribute to the treatment of many human diseases, especially in the field of thrombosis, because of their anticoagulant activity. GAGs interrupt the coagulation process by interacting with multiple coagulation factors through defined sequences within their linear and negatively charged chains, which are not fully elucidated. Numerous methods have been developed to characterize the structure of pharmaceutical GAGs, including *intravenously* or *subcutaneously* administered heparin and orally administered sulodexide. However, most currently available methods only focus on the oligosaccharide portion or analyze the whole mixture because longer-chain polysaccharides are extremely difficult to resolve by chromatographic separation. We have established two novel electrophoresis−mass spectrometry methods to provide a panoramic view of the structures of pharmaceutical GAGs. In the first method, an in-gel digestion procedure was developed to recover GAGs from the polyacrylamide gels, while in the second method, a strong anion exchange ultrafiltration procedure was developed to extract multiple GAG species from the agarose gels. Both procedures are compatible with liquid chromatography−tandem mass spectrometry, and structural information, such as disaccharide composition and chain length, can be revealed for each GAG fraction. The applications of these two methods on analysis of two different GAG drugs, heparin and sulodexide, were demonstrated. The current study offers the first robust tool to directly elucidate the structure of larger GAG chains with more biological importance rather than obtaining a vague picture of all chains as a mixture, which is fundamental for better understanding the structure−activity relationship and quality control of the GAG drugs.



INTRODUCTION

Glycosaminoglycans (GAGs) are a family of highly negatively charged polysaccharides consisting of repeating disaccharide units of a hexuronic acid residue or a galactose residue linked to a hexosamine residue. GAGs can be divided into several subfamilies, including chondroitin sulfate (CS)/dermatan sulfate (DS), heparin/heparan sulfate (HS), keratan sulfate, and hyaluronic acid, based on the structures of their basic disaccharides and sulfo group substitution patterns. GAGs play essential roles in many biological processes, including cell signaling, recognition, migration, and adhesion.[1,2] They are also involved in the occurrence and development of a number of human diseases, such as tumors, inflammation, and infectious diseases.[3−5] GAGs possess a wide range of biological activities including anti-inflammatory, anticoagulant, and antitumor metastasis and controlling angiogenesis.[6−8] In fact, GAG-based drugs have been widely used in the clinic for decades.[9,10] In addition, efforts to develop novel GAG drugs to treat a variety of acute or chronic diseases are continuously ongoing worldwide.[7,11]

Heparin and sulodexide are two representative GAG drugs. Both are anticoagulants that can be used to prevent clot formation, although their active pharmaceutical ingredients and drug formulas are different. The structures of heparin and sulodexide are shown in Figure 1. Heparin contains a single GAG species, with the linear sequence of a repeated glucuronic acid residue (GlcA) or iduronic residue (IdoA) alternating 1,4-linked to an *N*-glucosamine residue (GlcN). The hexuronic acid residue can be modified with a 2-*O*-sulfo group, and GlcN

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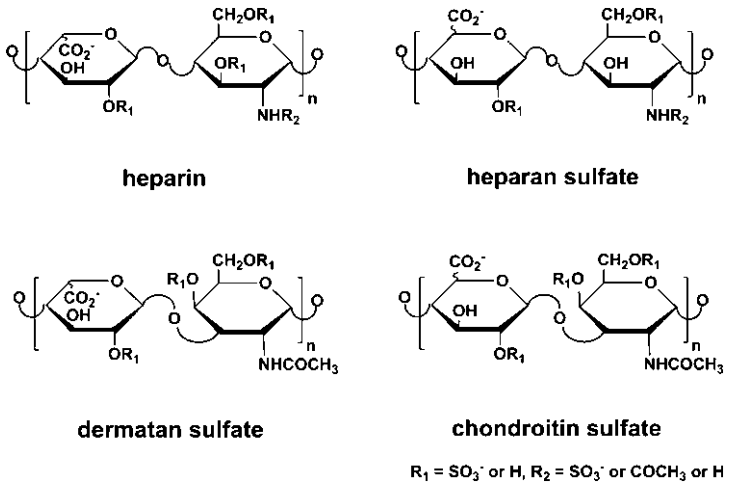

Figure 1. Structure of pharmaceutical GAGs. Heparin consists of a single GAG species, while sulodexide contains both heparin/HS and DS.

may be *N*-acetylated (GlcNAc), *N*-sulfated (GlcNS), 3-*O*-sulfated (GlcN3S), and/or 6-*O*-sulfated (GlcN6S). Pharmaceutical heparin is isolated from the porcine intestinal mucosa, with a typical molecular weight (MW) of approximately 15 kDa. It is usually referred to as unfractionated heparin, in contrast to its various depolymerized forms, that is, low-MW heparins (LMWHs). The MWs of LMWHs are reduced to 4−7 kDa by either chemical or enzymatic depolymerization processes to enhance the bioavailability and reduce the risk of side effects. Heparin and LMWHs are usually given by *intravenous* or *subcutaneous* injection, while sulodexide is an orally active anticoagulant. The structure of sulodexide is even more complicated than heparin as it contains at least two GAG components, heparin/HS and DS.[12] HS possesses a similar backbone structure to heparin, but the major disaccharide in HS is GlcAGlcNAc, while the disaccharide IdoA2SGlcNS6S is dominant in heparin. The repeating disaccharides in DS are mainly an IdoA residue 1 → 3 linked to a 2-acetamido-2-deoxy-galactopyranosyl 4-sulfate (GalNAc4S) residue. Sulodexide is reportedly composed of 80% heparin/HS and 20% DS, with MWs of approximately 7 kDa for the heparin/HS fraction and 25 kDa for the DS fraction.[12,13] The structures of heparin/HS and DS have been characterized by removing other components using specific GAG lyases.[14] However, the overall profile of unmanipulated sulodexide has not been previously reported.

In-depth structural characterization of GAGs is essential to understanding their biological activities, which usually take effect through their interactions with proteins. From the aspect of GAG drugs, advanced analytical tools that can reveal the detailed structures of GAG molecules are necessary to ensure their efficacy and safety. Because GAG chains are polysaccharides with complicated and heterogeneous structures, not to mention that sulodexide is a multicomponent GAG drug, traditional analytical techniques can usually only provide superficial or limited structural information. In recent years, both the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have emphasized the need to develop and apply novel analytical tools to better understand the structures of GAG drugs.[15] Two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy is a powerful technique for resolving GAG monosaccharide composition and substitution. However, it requires a relatively large quantity and high-purity samples.[16] It also shows overlap issues when analyzing the multicomponent sulodexide. Different liquid chromatography (LC) methods have been used to separate GAGs based on their different properties. For example, strong-anion-exchange (SAX) chromatography[17] and reversed-phase (RP) ion pairing chromatography[18] are based on charge, size exclusion chromatography (SEC)[19] is based on molecular size, and hydrophilic interaction chromatography (HILIC)[20] is based on hydrophilicity. Unfortunately, most LC methods are more suitable to separate GAG disaccharides or oligosaccharides and, thus, provide poor resolution when dealing with GAG polysaccharides. In addition, the detection of GAGs eluted from LC separation is also an issue because of the lack of a chromophore. Similarly, mass spectrometry (MS) is capable of elucidating the structure and sequence of GAG oligosaccharides, but direct analysis of complicated intact GAGs is usually not feasible.[17,21]

Gel electrophoresis, including polyacrylamide gel electrophoresis (PAGE) and agarose gel electrophoresis (AGE), offers a broader applicable MW range when analyzing GAGs.[22−24] Gel electrophoresis not only separates GAG oligosaccharides but also offers a much better resolution for GAG polysaccharides compared to LC methods. Therefore, the full distribution profile of GAGs can be obtained. However, traditional gel electrophoresis only visualizes GAGs with Alcian blue or silver staining, while further identification and structural characterization of the bands are difficult. Unlike protein analysis, in which in-gel digestion and MS identification of the bands from the gels have become routine procedures,[25] a similar strategy has never been reported as applied to analyze GAGs. In this study, we aimed to establish a novel gel electrophoresis, in-gel digestion or extraction and the LC−tandem mass spectrometry (MS/MS) method for the unbiased analysis of complicated GAGs. Unfractionated heparin and sulodexide are used to represent single-component and multicomponent GAGs, respectively. This study provides a point of view that is different from any previously developed analytical technique. Furthermore, a diffusion-ordered spectroscopy (DOSY) NMR method was also developed in the current study to simultaneously analyze the heparin/HS and DS components in sulodexide as a parallel analysis. The

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complete structure of sulodexide, one of the most complex biomacromolecule drugs, was successfully elucidated.

EXPERIMENTAL SECTION

Materials. Heparin and LMWH (enoxaparin reference standard) were obtained from the United States Pharmacopoeia. The sulodexide injection (manufactured by Alfa Wassermann, Italy) was obtained from Qilu Hospital of Shandong University (Jinan, China). Heparin lyase I, II, and III were purchased from Aglyco (Beijing, China). Chondroitinase ABC was purchased from Sigma-Aldrich (St. Louis, MO, USA). Heparin disaccharide standards, CS/DS disaccharide standards, and a synthetic disaccharide (ΔUA2SGlcNCOEt6S, ΔIP) were purchased from Iduron (Manchester, UK), and their structures are presented in the Supporting Information, Figure S-1. Heparin and DS oligosaccharides with degrees of polymerization (dp) from 4 to 12 were prepared in-house to serve as reference standards for electrophoresis analysis (Supporting Information, Figure S-2). All reagents and chemicals were of high-performance LC (HPLC) grade or electrophoresis grade.

PAGE Analysis. PAGE analysis was performed on a vertical 20 × 20 cm gel. The resolving gel was 22% total acrylamide and contained 2% *N*,*N*′-methylene-bis-acrylamide, 20% acrylamide, and 15% sucrose. The stacking gel was 0.25% *N*,*N*′-methylene-bis-acrylamide and 4.75% acrylamide prepared in a resolving buffer (0.1 M Tris, 0.1 M boric acid, and 0.01 M disodium ethylenediaminetetraacetic acid; pH 6.3). Electrophoresis was performed at 350 V for 1 h and then at 420 V for 4 h.

The gel was stained with 0.5% Alcian blue for 15 min, followed by silver staining (1 g/L $AgNO_3$ solution). The staining was terminated by the addition of 10% acetic acid aqueous solution (v/v).

In-Gel Digestion of PAGE-Separated GAGs. Gel strips were evenly divided into equal parts and excised. Each part was further cut into small 1 × 1 mm pieces and transferred to a centrifuge tube. The gel pieces were washed with water and then washed with 50% acetonitrile aqueous solution twice. Pure acetonitrile was used to completely shrink and dehydrate the gel pieces. After vacufuge drying, a solution containing 20 mIU each of heparinase I, II, and III was used to reswell the gel pieces. In-gel digestion was carried out at 37 °C for 24 h. The digests were extracted by vortexing and sonicating the gel pieces in 50% acetonitrile aqueous solution three times, performing a final wash with pure acetonitrile, and drying using a vacufuge.

AGE Analysis. AGE analysis was performed on a horizontal gel (12 × 6 cm) according to a previously reported procedure.[26] Briefly, the sample was loaded into a 0.5% agarose gel and subjected to electrophoresis through two steps. The first step was running in 0.04 M barium acetate buffer (pH 5.8) and the second step was in 0.05 M 1,2-diaminopropane (pH 3.0). Both steps lasted for 1 h at a voltage of 120 V. The gel was visualized in 0.2% toluidine blue solution (water/ethanol/acetic acid at 49:50:1, v/v/v).

Extraction and Digestion of AGE-Separated GAGs. The gel strip was cut into the corresponding segments based on the separation. The segments were then melted to a liquid by heating to 80 °C and then passed through preheated Vivapure SAX spin columns by centrifuging at 60 °C. The spin columns were washed three times with 0.2 M NaCl, and the GAGs were released from the spin columns using a 2.5 M NaCl solution. The eluates were desalted using 10 kDa MW cutoff (MWCO) ultracentrifugal filters and then lyophilized.

The desalted GAGs were digested with either a cocktail of heparin lyase I, II, and III (30 mIU each) or chondroitinase ABC (100 mIU) in a 10 kDa MWCO ultracentrifugal device at 37 °C for 24 h. The digested building blocks of GAGs were obtained by centrifugal filtration and then lyophilized.

LC−MS/MS Multiple Reaction Monitoring Analysis. LC−MS/MS multiple reaction monitoring (MRM) was used to further characterize the GAG building blocks recovered from the PAGE and AGE analyses. A system consisting of a Fisher Scientific UltiMate 3000 ultraperformance LC coupled to a Fisher Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer was used to perform the analysis. Two separation methods were applied. The 2-aminoacridone (AMAC) labeling and RP LC method was performed on a Fisher Scientific BDS Hypersil C18 column (2.1 × 150 mm, 3 μm) using our previously reported conditions.[27] A Click XIon HILIC column (2.1 × 150 mm, 5 μm, 100 Å, Acchrom Technologies, Beijing, China) was used to separate unlabeled disaccharides. Mobile phase A was 5 mM ammonium acetate in water, and mobile phase B was 5 mM ammonium acetate in 98% acetonitrile aqueous solution (v/v). The gradient was 85% B for 5 min, 85−80% B over 15 min, 80−70% B over 60 min, 70−65% B over 15 min, 65−20% B over 10 min, and 20% B for 5 min. The flow rate was 250 μL/min.

The MRM mode was used for the MS/MS analysis. The MRM transitions are listed in Table S-1 in the Supporting Information. The Thermo Xcalibur software was used to process the MS data. The synthetic disaccharide ΔIP was used as an internal standard to eliminate the run-to-run differences.

NMR Analysis. All NMR spectra were recorded at 298 K on a Bruker AVIII 600 MHz spectrometer equipped with a cryoprobe. DOSY spectra were processed using Dynamics Center 2.6. Heteronuclear single-quantum coherence (HSQC) spectra were processed in TopSpin 3.6.2.

RESULTS AND DISCUSSION

Electrophoresis Analysis of Heparin and Sulodexide. Heparin and sulodexide were first subjected to electrophoresis on a polyacrylamide gel and visualized by silver staining (Figure 2a). LMWH was used as the MW ladder. The in-house-prepared heparin oligosaccharides from dp4 to dp12 were used as reference standards to designate the bands in the LMWH ladder. Larger saccharide chains in LMWH as well as in heparin and sulodexide became smeared because of their high complexity and heterogeneity. The profiles of heparin and sulodexide were distinguishable from one another in the PAGE analysis. Heparin showed a continuous distribution, and the bands completely disappeared when the sample was treated with a cocktail of heparinase I, II, and III (data not shown), indicating that it consists of single GAG species. In contrast, sulodexide exhibited a discrete distribution pattern. Selective enzymatic treatments, including heparinases, which specifically digest heparin and HS, and chondroitinase ABC, which degrades both CS and DS, were used to reduce the complexity of sulodexide. Lanes 3 and 4 were the remaining polysaccharides after heparinase and chondroitinase digestion, respectively. This suggests that there are two GAG components in sulodexide, a DS component (lane 3) and a heparin/HS component (lane 4). The heparin/HS and DS distributions overlapped to a great extent in the PAGE analysis,

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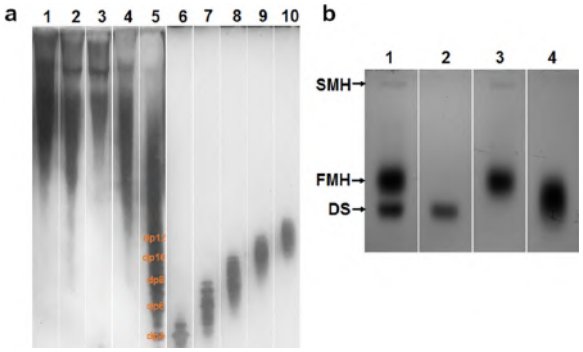

Figure 2. Electrophoresis analysis of heparin and sulodexide. (a) PAGE analysis. Lane 1, heparin; lane 2, sulodexide; lane 3, sulodexide treated with heparinase I, II, and III; lane 4, sulodexide treated with chondroitinase ABC; lane 5, LMWH reference standard; lane 6−10, reference standard heparin oligosaccharides dp4 to dp12. (b) AGE analysis. Lane 1, sulodexide; lane 2, sulodexide treated with heparinase I, II, and III; lane 3, sulodexide treated with chondroitinase ABC; lane 4, LMWH reference standard.

and this also showed that the heparin/HS component in sulodexide is more polydisperse than its DS component.

Because PAGE separates GAGs mainly based on their MWs and cannot resolve heparin/HS and DS components with overlapping molecular sizes, AGE was applied to analyze this multicomponent GAG drug. In AGE, agarose is used as a supporting medium, in which the negatively charged GAGs migrate in a different way from that in an acrylamide gel. The AGE analysis results for sulodexide are shown in Figure 2b. The following three fractions were observed for sulodexide (lane 1): a faint and narrow band appearing at the top of the AGE gel, an intense and broad band located in the middle of the gel, and another intense band below the most intense band. Again, selected GAG lyases were used to identify which GAG category each fraction belonged to. After heparinase treatment, only the band at the lowest position was still present (lane 2), while chondroitinase removed this band but left the other two fractions (lane 3). This was revealed that the bottom band was DS, while the two bands above it were heparin/HS GAGs with distinct structures and properties, which are referred to as fast-moving heparin/HS (FMH) and slow-moving heparin/HS (SMH). The three fractions were extracted separately from the agarose gel and characterized using LC−MS/MS in the following experiments. The LMWH in lane 4 migrated slightly slower than DS, which has much higher MW, suggesting that the MW was not the determining factor for GAG electrophoretic mobility in the AGE analysis. This was also proved by the AGE analysis of heparin and the DS oligosaccharide reference standard (Figure S-3 in the Supporting Information).

Coupling PAGE to LC−MS/MS Analysis with In-Gel Digestion. Blotting is traditionally used to transfer GAGs from electrophoresis gels onto nylon or nitrocellulose membranes, and then, GAGs are released by soaking the corresponding membrane stripes in solution.[28,29] However, GAGs diffuse relatively fast on the membrane, leading to reduced separation resolution and low recovery. Furthermore, the blotting procedure is too cumbersome to be used by ordinary experimental operators. In the current study, we designed a new workflow coupling electrophoresis to LC−MS/MS analysis for GAGs, which is presented in Figure 3. Two different strategies were taken to recover GAGs from the PAGE and AGE gels. For the PAGE analysis, procedures similar to protein in-gel digestion were used. The gel bands containing GAG polysaccharides were individually excised from the gel and then cut into small pieces. Aqueous acetonitrile was used to repeatedly dehydrate the gel pieces to remove buffer salts and other impurities. The gel pieces were then completely dehydrated by acetonitrile and rehydrated with an aqueous buffer containing a cocktail of heparinase I, II, and III, which in-gel digests GAG polysaccharides to their basic disaccharide building blocks. The GAG disaccharides were extracted from the gel pieces by dehydrating the gel pieces again using acetonitrile. However, the in-gel digestion of the DS component of sulodexide was unsuccessful, likely because of the size of chondroitinase ABC being too large (an MW of approximately 120 kDa, according to the information from the vendor) to enter the PAGE gel during the rehydration step. Therefore, the in-gel digestion procedure is recommended for use to recover heparin or HS for future LC−MS/MS analysis.

Coupling AGE to LC−MS/MS Analysis with SAX Ultrafiltration. Because PAGE cannot resolve the heparin/HS and DS components of sulodexide, AGE offers an

Figure 3. Analytical workflow for the electrophoresis−MS analysis of GAGs.

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Anal. Chem. 2021, 93, 1433−1442

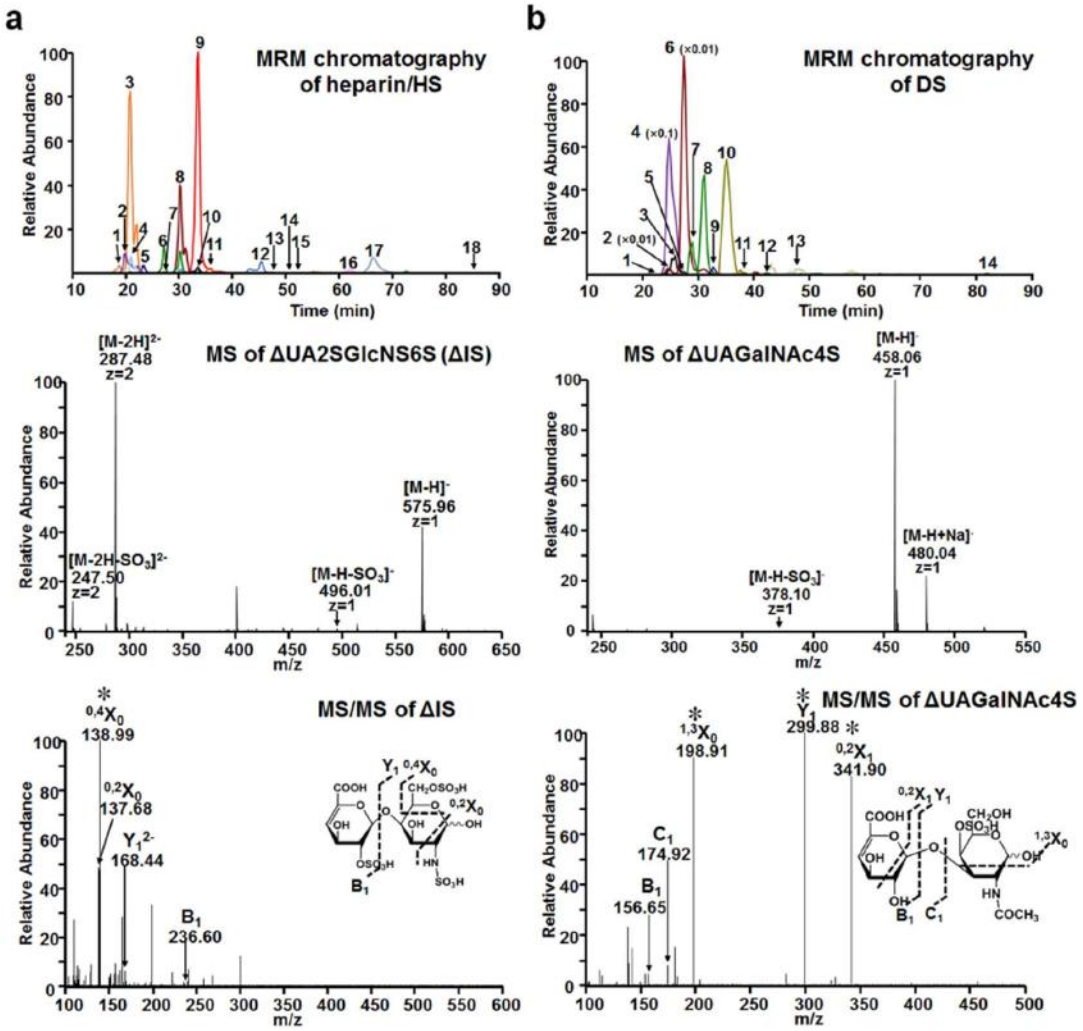

Figure 4. LC−MS/MS MRM chromatogram and example MS and MS/MS spectra. (a) Heparin/HS disaccharides. (b) DS disaccharides. Product ions marked with * were selected for MRM transitions.

alternative way to separate this multicomponent GAG drug. However, in-gel digestion of GAGs was impractical because an agarose gel cannot be dehydrated by acetonitrile. The texture of an agarose gel is different from that of a polyacrylamide gel. Agarose is a linear polysaccharide consisting of alternating 1,3-β-D-galactose and 1,4-α-L-3,6-anhydro-L-galactopyranose, which form a double-helix structure to constitute the agarose gel scaffold. The double-helix structure is held firmly by agarose polysaccharide hydroxyl bonds and is not accessible to acetonitrile, which makes it difficult to dehydrate the agarose gel. The advantage of AGE gels is that they easily melt when heated at a relatively low temperature. The three fractions of sulodexide separated on the AGE gel are excised and melted to liquid. Then, a SAX ultrafiltration procedure was developed to extract the negatively charged GAGs (Figure 3). Because sulodexide consists of both heparin/HS and DS GAGs, heparinases and chondroitinase ABC were both used to conduct the in-solution digestion after extraction. Then, heparin/HS disaccharides and DS disaccharides were separately recovered and analyzed by LC−MS/MS.

Establishment of LC−MS/MS-MRM Methods. Because GAG electrophoresis is a very sensitive analysis and only a few micrograms of the sample are loaded, it requires that the following LC−MS/MS detection is also of high sensitivity. We have previously reported two LC−MS/MS methods for the ultrahigh sensitivity analysis of GAG disaccharides.[10,27] Both methods take advantage of the triple quadrupole mass spectrometry MRM mode to target and quantify all GAG disaccharides in the multiplex. The difference is that GAG disaccharides were labeled by AMAC and separated on a RP C18 column in one method, while they were directly separated on a HILIC column in the other method. AMAC labeling enhanced the specificity of the analysis, while the HILIC method is more convenient. These two LC−MS/MS MRM methods are interchangeable during analysis of PAGE- and AGE-separated GAGs. The ionization and fragmentation conditions were optimized to prevent in source loss of fragile sulfo groups and to provide sufficient product ions for deducing the disaccharide structures. The LC separation chromatograms as well as example MS and tandem MS

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Anal. Chem. 2021, 93, 1433−1442

spectra are presented in Figure 4. Assignments of tandem mass spectra for all other heparin disaccharides and DS disaccharides are presented in the Supporting Information Figures S-4 and S-5, respectively. Several terminal disaccharides from the nonreducing end (NRE) of heparin (Figure 5)

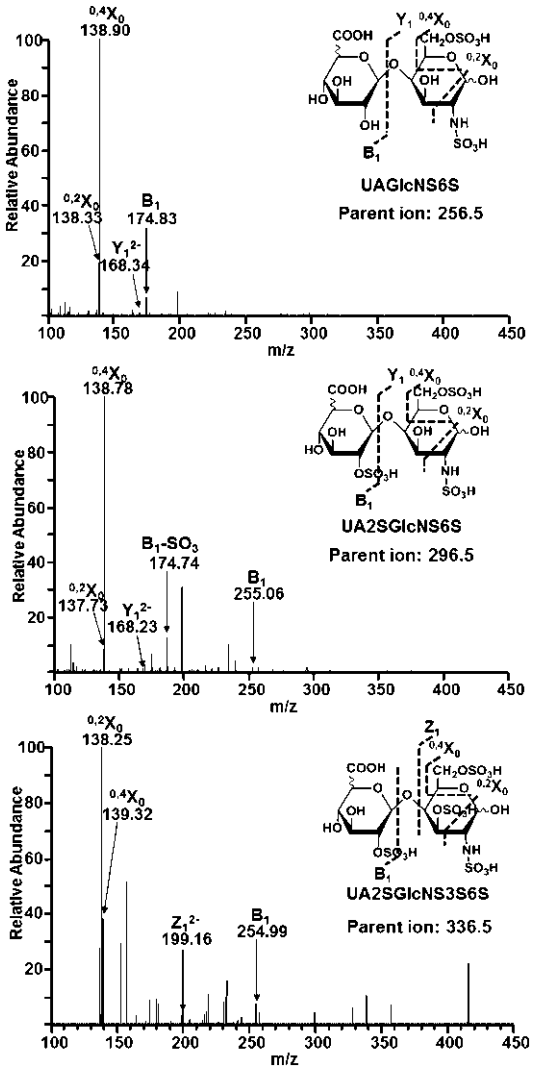

Figure 5. MS/MS spectra and structures of heparin NRE disaccharides.

and DS (Supporting Information, Figure S-5) were identified, which could be used to calculate the average MW for the polysaccharides. The average MW of these components can be calculated with the following equation

$$\overline{MW} = \frac{\sum A_{NREx} M_{NREx}^{\ 2}}{\sum A_{NREx} M_{NREx}} + \frac{\sum A_{BBi} M_{BBi}^{\ 2}}{\sum A_{BBi} M_{BBi}} \times \frac{\sum A_{BBi}}{\sum A_{NREx}} + M_{RE}$$

where A_{NREx} is the area of the NRE disaccharide peak x, M_{NREx} is the MW of the NRE disaccharide x, A_{BBi} is the area of the backbone building block peak i, M_{BBi} is the MW of the backbone building block i, and M_{RE} is the MW of the linkage region at the reducing end (RE).

LC−MS/MS MRM Analysis of PAGE-Separated Heparin. The principles of PAGE analysis of GAGs and sodium dodecyl sulfate (SDS)-PAGE analysis of proteins are very similar. In the SDS-PAGE analysis, proteins are linearized to polypeptides using reducing reagents and denaturing detergents. The polypeptides run toward the positive electrode, driven by the negative charges provided by SDS. The electrophoretic mobilities of proteins depend on their MWs, which are usually calculated with appropriate MW markers. The GAG polysaccharides are linear and carry negative charges. Thus, they run to the positive electrode without the need of SDS. However, there are two questions yet to be answered for the behavior of GAGs in the PAGE analysis. The first is whether the linear range of GAG MWs is inversely proportional to their migration distances. Because the dispersity of GAG oligosaccharides increases with their sizes, available GAG markers with defined MWs are usually not larger than a dp of 20, which is not able to cover the full range of heparin polysaccharides. In the approach developed herein, the MW of the GAGs from each gel band can be calculated directly based on their disaccharide composition. The disaccharides detected by LC−MS/MS MRM analysis can be sorted into two groups, saturated disaccharides from the original NRE of heparin and unsaturated disaccharides derived from the middle of the heparin backbone when cleaved by heparinases. The RE of heparin, which is a unique linkage tetrasaccharide linked to a serine residue, is not detected because it lacks an aldehyde group for AMAC labeling. The ratio of saturated to unsaturated disaccharides determines the MW of the corresponding heparin chains because each heparin chain possesses only one NRE. The MWs ranged from 3.9 to 46.8 kDa, with an average MW of 14.4 kDa calculated from the MRM composition data. A good correlation relationship between log MWs and migration distance was observed (Figure 6a). The second question is whether the sulfation substitution variation of GAGs affects their electrophoretic mobility. The composition of disaccharides, including 3 NRE-saturated disaccharides and 8 common unsaturated heparin disaccharides, was determined for 12 of 20 gel zones, as presented in Figure 6b. No disaccharide was detected in the remaining eight zones. The disaccharide compositions were found to be very close in all 12 gel zones, indicating that the heparin polysaccharides were not separated based on their sulfation compositions. To investigate the possible sample degradation during the relatively complicated procedure prior to the MS analysis, the same heparin sample was also directly analyzed by LC−MS/MS, and its disaccharide composition was comparable to the accumulated disaccharide composition from the PAGE−MS analysis (Supporting Information, Table S-2), indicating that the procedure was amiable to keep the GAG structure intact.

The LC−MS/MS MRM results have proven that the electrophoretic mobility of GAGs in the PAGE analysis is solely dependent on their molecular sizes, which can be presented as a linear regression equation of logarithms of GAG MWs inversely proportional to their migration distance on PAGE. It also indicated that heparin was a continuously spreading polysaccharide with uniform disaccharide compositions among different MW segments.

LC−MS/MS MRM Analysis of AGE-Separated Sulodexide. AGE was originally used to analyze nucleic acids and

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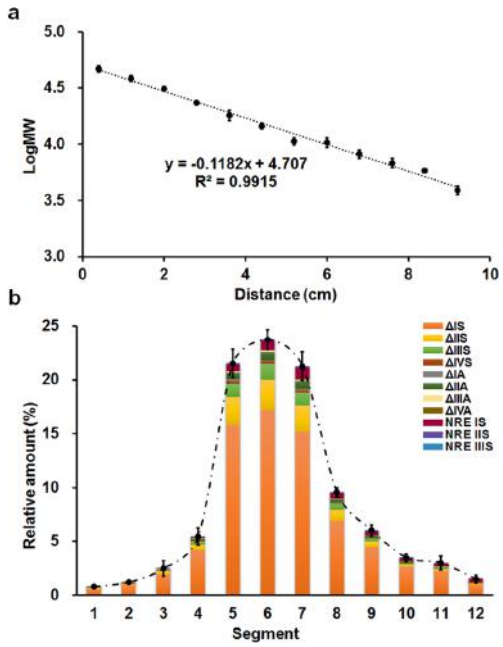

Figure 6. PAGE−MS analysis of heparin. (a) Linear relationship between the migration distance and MW. (b) Basic building block compositions of segments with different MW ranges.

was later adapted to analyze GAGs. However, GAGs exhibit different migrating behaviors from nucleic acids in AGE analysis. The driving force for nucleic acids during electrophoresis to the positive electrode relies on the negative charges on their backbones. Because the number of negative charges carried by nucleic acid molecules is proportional to their chain length, their migration distance in AGE analysis shows a linear relationship to the logarithm of their MWs. In the case of GAGs, the electrophoretic mobility is not solely dependent on MWs. As shown in Figure 2b, the higher-MW DS moved faster than the lower-MW LMWH, although LMWH is known to be more negatively charged than DS. Therefore, the MWs of GAGs in the AGE analysis cannot be estimated using a MW marker. Overall, the AGE analysis is powerful for resolving different GAG components, but it provides very limited structural information, such as MW, identity, and composition.

The LC−MS/MS analysis offers an ideal complementary way to characterize GAGs separated by AGE analysis. Three sulodexide segments, SMH, FMH, and DS, were excised from the AGE gel, extracted using SAX spin columns, and exhaustively digested using heparinases and chondroitinase ABC. MRM transitions targeting all heparin/HS and CS/DS basic building block disaccharides and oligosaccharides were applied. The results are shown in Figure 7a. The identities of the three segments were confirmed as follows: the heparin major disaccharide ΔIS was found to be the most abundant species in the SMH and FMH digests, while the DS major disaccharide ΔUAGalNAc4S was dominant in the digest of the DS segment. For FMH, a total of 17 basic building blocks were identified and quantified. They were then sorted into four groups. The first group included eight common heparin disaccharides that are composed of the majority of the GAG backbone. The second group included three 3-*O*-sulfated tetrasaccharides, which were derived from the specific antithrombin-binding pentasaccharide sequence in heparin. They are the structural motifs responsible for the anticoagulant activity. The third group was three saturated disaccharides retained from the original NRE of GAG polysaccharide chains, from which the MWs of the GAG chains can be calculated. The last group was three unusual structures. Two of these structures contained a GalGalXyl trisaccharide with an oxidized serine residue, which is the linkage region between GAGs and proteins, and the other was an odd-numbered trisaccharide, likely a byproduct of the peeling reaction during the manufacturing process. The ratio of NRE disaccharides to middle disaccharides was 1:17, indicating an average chain length of approximately 40 monosaccharide units (calculated as 1 NRE disaccharide, 17 middle disaccharides, and 1 RE tetrasaccharide) and an average MW of 10.7 kDa (calculated based on the chain length and disaccharide composition) for the FMH. Fewer building blocks were detected for the SMH because it was present in very low abundance in sulodexide. Nevertheless, the structure of SMH was still covered to a great extent by characterizing its backbone (eight common disaccharides), NRE (three saturated disaccharides), RE (two linkage regions), and active motif (two 3-*O*-sulfated tetrasaccharides), and the average MW of the SMH segment was calculated as approximately 15.4 kDa. While the SMH is usually considered a heparin, it is controversial whether the FMH belonged to heparin or HS. Heparins are more highly sulfated than HS, especially at the GlcN residue *N*-position. Typically, heparin carries approximately 2.7 sulfo groups per disaccharide, while HS only carries approximately 1 or more sulfo groups per disaccharide.[30] The FMH and SMH were determined to have 2.1 and 2.7 sulfo groups per disaccharide, respectively, by the LC−MS/MS MRM disaccharide composition analysis. With respect to the MWs and disaccharide compositions, the SMH has a typical heparin structure, while the FMH should not be categorized to HS. Instead, it is an undersulfated heparin with a slightly lower MW.

The structure of DS is less variable than heparin as a total of 13 building blocks were detected by the LC−MS/MS analysis. The linkage structure at the RE of DS could carry one sulfo group, which is characteristic of the CS/DS family.[29] Again, based on the ratio of the NRE saturated to middle unsaturated disaccharides and the disaccharide composition, the MW and sulfation degree of DS in sulodexide were determined to be 19.9 kDa and 1.1 sulfo group per disaccharide, respectively. The sulfation patterns of heparin and DS components in sulodexide disclosed by the AGE and LC−MS/MS analyses were comparable with the results obtained by 2D HSQC NMR spectroscopy (Figure S-6 in the Supporting Information), which demonstrated the reliability of the new method established herein.

Moreover, the quantities of the three segments in sulodexide were obtained by summing up the corresponding building blocks, which are presented as percentages of approximately 70.0% for the FMH, 29.1% for the DS, and 0.9% for the SMH (Figure 7a). The result was basically consistent with the quantification results based on AGE gel staining (FMH 67.8%, DS 29.8%, and SMH 2.4%). Compared to the traditional gel permeation chromatography (GPC) method, which poorly separated heparin/HS and DS (Table S-3 in the Supporting Information), we provided more accurate and convincing MWs and quantity information for each GAG component in sulodexide. Indeed, the combination of AGE analysis and LC−MS/MS MRM analysis provided the most detailed and

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Anal. Chem. 2021, 93, 1433−1442

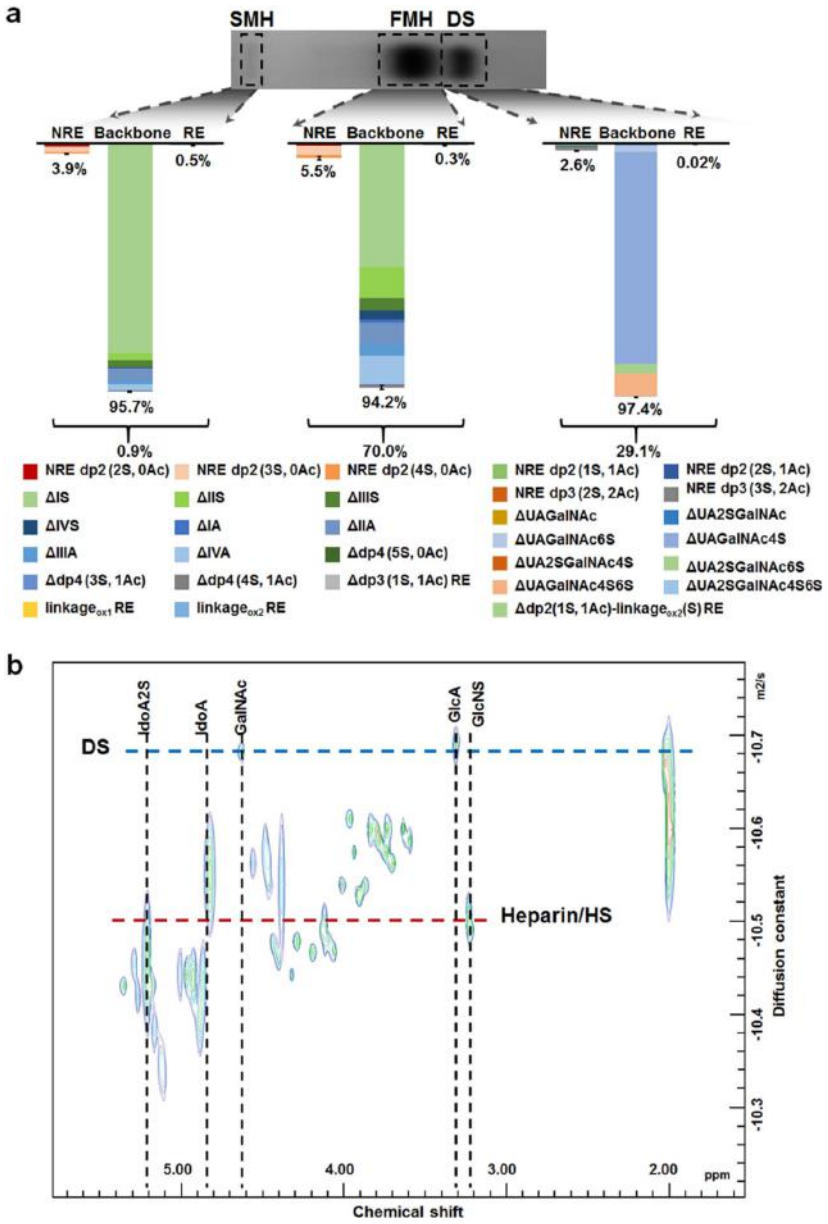

Figure 7. AGE−MS and DOSY NMR analysis of sulodexide. (a) Compositions of three GAG species in sulodexide revealed by AGE−MS analysis. (b) Heparin/HS and DS structures characterized by DOSY NMR spectroscopy.

comprehensive structural elucidation for the multicomponent sulodexide to date.

METHOD VALIDATION

The electrophoresis and LC−MS/MS MRM methods were validated by analyzing a series of samples ranging from 5 to 100 μg in triplicate and comparing the data to those obtained by direct digestion of GAGs with LC−MS/MS analysis. The validation results are shown in Figure S-7 in the Supporting Information. All R^2 values were greater than 0.98, and the slopes were greater than 0.8, confirming that these two methods are reliable and reproducible.

NMR Analysis of Sulodexide. DOSY NMR spectroscopy is capable of directly analyzing different compounds in a mixture based on their respective sizes and shapes.[31] It is a pseudo 2D spectrum with the chemical shift on the *x*-axis and the log (diffusion constant) on the *y*-axis. Components with different diffusion coefficients are displayed as different traces. To our knowledge, the analysis of sulodexide using DOSY NMR has never been reported.

Two groups of traces, corresponding to the heparin and DS components in sulodexide, were observed in the DOSY spectrum of sulodexide (Figure 7b), although some traces were overlapped. The GalNAc signals and GlcNS signals are characteristic signals for DS and heparin, respectively. Signals

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Anal. Chem. 2021, 93, 1433−1442

for GalNAc and GlcA belonging to DS were at a smaller diffusion coefficient, while signals for GlcNS and IdoA2S related to heparin were found at a relatively larger diffusion coefficient, suggesting that the DS component has a larger MW compared to heparin. Both DS and heparin contain the IdoA structure, which show its signals with unresolvable diffusion coefficients, indicating the overlap of MW distributions for DS and heparin components in sulodexide. By integrating characteristic peaks in the DOSY spectrum of sulodexide, the ratio of heparin to DS was 3:1. This is comparable to the result obtained by the electrophoresis−MS approach. However, the FMH and SMH were not differentiable in the DOSY NMR analysis.

CONCLUSIONS

Understanding the full structure of GAG drugs is essential for ensuring their drug safety and efficacy. However, most currently available methods are focused on building blocks or fragments of overall polysaccharides, leaving the high MW components not specifically characterized because of their poor resolvability using most chromatography techniques. In this study, we employed two different electrophoresis approaches to show the full distribution profiles of unfractionated heparin or separate individual species from the multicomponent sulodexide. An in-gel digestion procedure and SAX extraction procedure were established, which are suitable for the PAGE- and AGE-separated GAGs to facilitate the MS characterization of every segment in these polysaccharides. As GAGs usually require a specific length, with sizes as large as an octadecasaccharide or even longer, to interact with their target proteins, the electrophoresis−MS approach described herein provides the first robust tool to directly elucidate the structure of these relatively large chains with more biological importance rather than obtaining a vague picture of all chains as a mixture. Furthermore, the superior sensitivity of the electrophoresis−MS approach makes it a promising tool to analyze complex GAGs isolated from important biological samples. A panoramic view of these GAGs might be disclosed by the combination of AGE−MS analysis to simultaneously characterize different GAG species and PAGE−MS analysis to unbiasedly profile the more complicated HS.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c03330.

MRM parameters for detecting GAG building blocks; comparison of the disaccharide composition results obtained by the PAGE−MS method and direct LC−MS/MS analysis; GPC−multiangle laser light scattering analysis of sulodexide; structures of eight heparin disaccharide standards, eight CS/DS disaccharide standards, and a synthetic disaccharide; SEC separation and MS characterization of heparin and DS oligosaccharides; AGE analysis of heparin and DS oligosaccharide reference standards; MS and MS/MS spectra of heparin/HS disaccharides; MS and MS/MS spectra of DS disaccharides; 2D-HSQC-NMR analysis of heparin/HS and DS in sulodexide; and method validation (PDF)

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Notes

The authors declare no competing financial interest.

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