Heparin Characterization: Challenges and Solutions

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Abstract

Although heparin is an important and widely prescribed pharmaceutical anticoagulant, its high degree of sequence microheterogeneity and size polydispersity make molecular-level characterization challenging. Unlike nucleic acids and proteins that are biosynthesized through template-driven assembly processes, heparin and the related glycosaminoglycan heparan sulfate are actively remodeled during biosynthesis through a series of enzymatic reactions that lead to variable levels of *O*- and *N*-sulfonation and uronic acid epimers. As summarized in this review, heparin sequence information is determined through a bottom-up approach that relies on depolymerization reactions, size- and charge-based separations, and sensitive mass spectrometric and nuclear magnetic resonance experiments to determine the structural identity of component oligosaccharides. The structure-elucidation process, along with its challenges and opportunities for future analytical improvements, is reviewed and illustrated for a heparin-derived hexasaccharide.

1. INTRODUCTION

1.1. Heparin Structure

GAG: glycosaminoglycan

HS: heparan sulfate

Heparin, a member of the glycosaminoglycan (GAG) family, which includes chondroitin sulfate, keratan sulfate, dermatan sulfate (DS), and heparan sulfate (HS), is a highly sulfated, linear polysaccharide. Composed of variously sulfonated hexuronic acid (1→4) D-glucosamine-repeating disaccharide building blocks, heparin is the most acidic biopolymer in nature. The uronic acid residue of heparin may be either α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA) and can be unsubstituted or sulfonated at the 2-*O* position. The glucosamine residue may be unmodified (GlcN), *N*-sulfonated (GlcNS), or *N*-acetylated (GlcNAc), with variable patterns of *O*-sulfonation at the 3-*O* and 6-*O* positions (**Figure 1**).

The microheterogeneity of heparin results from variable patterns of sulfonation and the presence of hexuronic acid epimers (1, 2). Heparin is biosynthesized in a multistep process that involves multiple enzymes in the endoplasmic reticulum and the Golgi apparatus of the mast cells of connective tissues. The specific modifications introduced by these enzymes are illustrated in **Figure 2*a***. Although this process appears to occur in a specific and orderly manner, its regulation is not yet well understood. The structurally related HS proteoglycans are expressed and secreted by mammalian cells and are strategically located on cell surfaces and in the extracellular matrix (3, 4). Unlike HS, heparin lacks domain organization and possesses a higher percentage of IdoA residues and sulfonate groups (~2.5 per disaccharide). Because the biosynthesis of heparin is not template driven, it is a polydisperse mixture that contains chains of different molecular weights.

1.2. Heparin's Biological Significance

Heparin and HS influence numerous physiological (5) and pathophysiological processes (6), including organo- (7), morpho- (8), angio- (9), and tumorigenesis (10); growth control (11); cell adhesion (12); inflammation (13); neural development and regeneration (14, 15); and hemostasis (16). Cell-surface HS proteoglycans also act as adhesion receptors for many viral and bacterial pathogens, concentrating them on cell surfaces and increasing the pathogen's ability to bind to secondary receptors responsible for internalization (17–19).

Heparin is one of the oldest drugs in widespread clinical use. It is also one of the few currently used pharmaceutical agents derived from animal sources (namely porcine intestine). Heparin mediates its biological functions through electrostatic interactions with basic amino acid residues

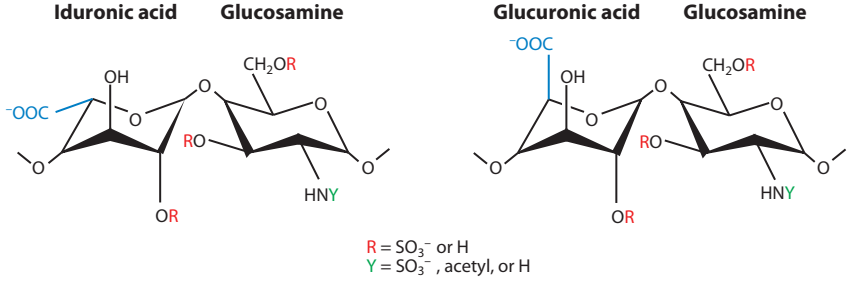


Figure 1

Structures of the possible disaccharide subunits of heparin showing the substitution sites on the uronic acid and glucosamine residues and the orientation of the carboxylate moiety in the iduronic and glucuronic acid epimers.

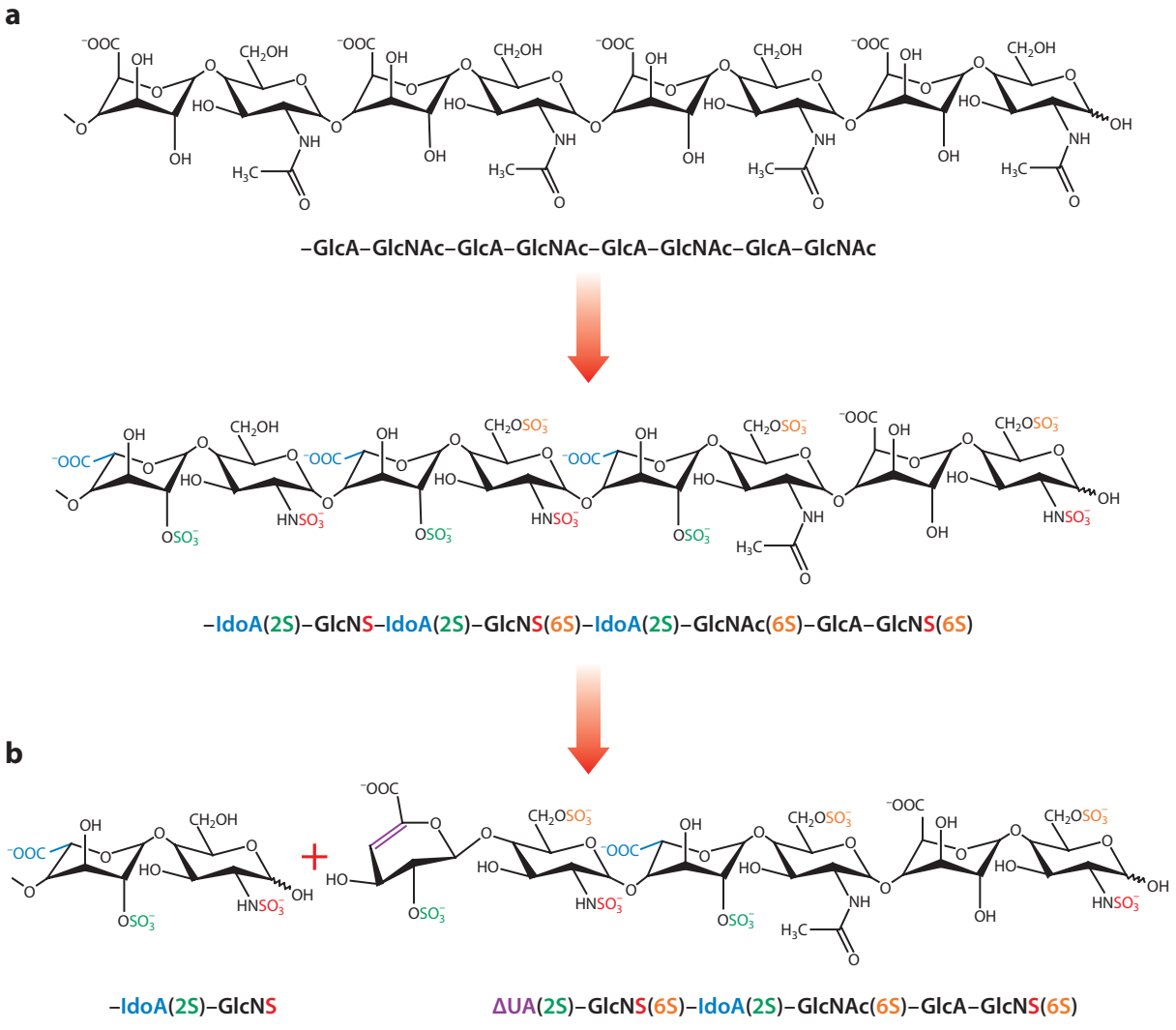


Figure 2

(*a*) Schematic illustration of the origin of heparin's microheterogeneous structure. The final structure of heparin is the result of incomplete sequential modifications of the $[GlcA\text{-}(1,4)\text{-}GlcNAc]_n$ polymer by *N*-deacetylase/*N*-sulfotransferase-, C5 epimerase-, and 2-*O*- and 6-*O*-sulfotransferase-catalyzed reactions. (*b*) Heparinase I depolymerization of heparin results in smaller oligosaccharides that contain an unsaturated uronate residue at the nonreducing end of the cleaved chain. Abbreviations: GlcA, D-glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine.

of target proteins (1). The anticoagulant activity of heparin arises primarily through its interactions with the serine protease inhibitor antithrombin III. Binding of a specific heparin pentasaccharide sequence initiates a conformational change in antithrombin III that increases the flexibility of its reactive site loop and, as a result, its binding affinity for thrombin and other coagulation-cascade proteases. Heparin is widely used in hemodialysis and in the initial treatment of venous thrombosis, pulmonary embolism, and acute coronary syndrome. A major drawback of heparin administration resides in the low predictability of coagulation parameters and the concomitant risk of potential

bleeding. However, the most serious side effect is heparin-induced thrombocytopenia (low platelet count). To address these risks and limitations, low-molecular-weight heparins (LMWHs) were introduced into clinical use (20). LMWHs are manufactured from unfractionated heparin by controlled depolymerization. Further success of the clinical application of LMWHs resides in their enhanced subcutaneous bioavailability and improved pharmacokinetics.

NMR: nuclear magnetic resonance

1.3. Challenges of Studying Heparin

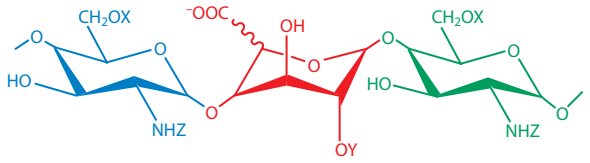
A more detailed knowledge of heparin and HS structure is required to develop a better understanding of the mechanisms by which they mediate so many biological processes. Analytical methods that provide molecular-level structural characterization are also critical for securing the quality and safety of heparin drugs. The adulteration of pharmaceutical heparin in late 2007 and early 2008 drew attention to the need for improvements to the analytical methods used for the rapid identification of GAGs and their potential impurities (21). The difficulty in heparin and HS analysis resides in their high negative-charge density, polydispersity, and microheterogeneity. The molecular-level characterization of heparin and HS requires techniques that can distinguish minor differences in structure, for example, positional isomers and epimers. These biologically derived samples are often available in limited quantities that are obtained by labor-intensive isolation protocols; therefore, sensitive characterization methods are essential. Furthermore, trace contaminants that are similar in structure and character to heparin are difficult to separate and identify without selective and sophisticated techniques (22). The complexity of these samples necessitates orthogonal methods to accurately detect and identify heparin oligosaccharides (23). Application of stable and radioisotopes can also advance the structural elucidation of heparin and HS (24), and hyphenated techniques can play a critical role in this effort (25).

2. DEPOLYMERIZATION AND SPECIFIC LABELING REACTIONS

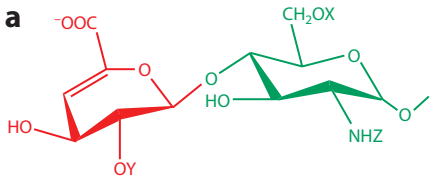
Due to heparin and HS's high degree of structural diversity, most methods used to characterize them utilize a bottom-up approach, whereby the intact polysaccharide chains are chemically or enzymatically depolymerized to smaller oligosaccharides prior to analysis (26). In an exhaustive digestion, heparin and HS are reduced to their disaccharide building blocks, which allows compositional analysis. Studies using larger oligosaccharides, which are more biologically relevant, are conducted on samples obtained by partial depolymerization. In these studies, the digested oligosaccharides are separated into size-uniform fractions, which are further resolved to individual oligosaccharides by subsequent charge-based separation. The purified oligosaccharides can then be characterized through the use of mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR).

2.1. Enzymatic Digestion

Exhaustive digestions of heparin and HS are typically carried out by use of a cocktail containing the enzymes heparinase I, II, and III to selectively cleave the biopolymer at glucosamine (1→4) uronic acid glycosidic bonds. The enzymatic reaction inserts a double bond at the nonreducing end of each cleaved chain to create an ultraviolet (UV) chromophore that absorbs at a wavelength of 232 nm (e.g., **Figure 2*b***), thereby facilitating detection (27). Such heparin lyase enzymes, produced by *Flavobacterium heparinium*, are highly specific to heparin and HS and are classified according to their substrate specificity (28). The various heparinase specificities and the structures of the resulting cleavage products are illustrated in **Figure 3*a***. Heparinase I cleaves the polymer

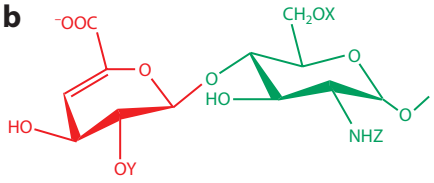


a



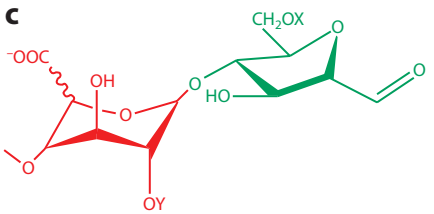
Enzymatic		
Heparinase I[a]	**Heparinase II[b]**	**Heparinase III[b]**
X = SO_3^- or H	X = SO_3^- or H	X = SO_3^- or H
Y = SO_3^-	Y = SO_3^- or H	Y = H
Z = SO_3^-	Z = SO_3^- or Ac	Z = SO_3^- or Ac
[a]Hexuronic acid must be IdoA.		
[b]Hexuronic acid can be GlcA or IdoA.		

b



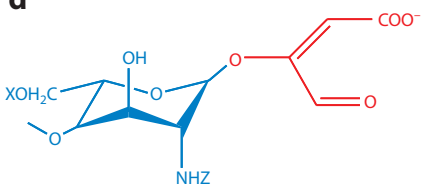
β–Elimination	
Alkyl halide	**Alkyl alcohol**
X = SO_3^- or H	X = SO_3^- or H
Y = H	Y = SO_3^- or H
Z = SO_3^-, Ac, or H	Z = SO_3^-, Ac, or H

c



Reductive deamination	
pH 1.5	**pH 4**
X = SO_3^- or H	X = SO_3^- or H
Y = SO_3^- or H	Y = SO_3^- or H
Z = SO_3^-	Z = H

d



Oxidation
X = SO_3^- or H
Y = H
Z = SO_3^- or Ac

Figure 3

The depolymerization specificities and products of the (*a*) enzymatic, (*b*) β-elimination, (*c*) reductive deamination, and (*d*) oxidation reactions of heparin and heparan sulfate based on the substitutions at positions X, Y, and Z.

chain between GlcNS and 2-*O*-sulfonated IdoA residues—the most common substitution motif in most forms of intact heparin. Heparinase II is less specific; it cleaves between glucosamine residues that can be *N*-sulfonated or *N*-acetylated and 2-*O*-sulfonated IdoA, unsubstituted IdoA, or GlcA residues (28). Heparinase III cleaves specifically at sites between *N*-acetylated or *N*-sulfonated glucosamine and 2-*O*-unsubstituted IdoA or GlcA. Because the disaccharide GlcNAc(1→4)GlcA is commonly found in HS, heparinase III is often used for HS digestions.

2.2. Chemical Depolymerization

Chemical depolymerization of heparin and HS oligosaccharides can occur by several means: β-elimination, reductive deamination, and oxidation. The specificities and reaction products are shown in **Figure 3**. β-Elimination (**Figure 3*b***) mimics enzymatic cleavage through a chemical reaction that introduces a double bond at the nonreducing end of each cleaved oligosaccharide. The depolymerization is carried out through a two-step reaction in which the carboxylate group on the C5 carbon of the nonreducing-end hexuronic acid is reacted with a benzyl halide to form an ester. Then, a strong base extracts the proton at the C5 position of the nonreducing-end uronic acid, resulting in the formation of the double bond between C4 and C5. The benzyl ester is then eliminated through hydrolysis in the basic solution (29). Reductive deamination (**Figure 3*c***) is typically performed with nitrous acid; the specificity of cleavage is determined by the solution pH. If the pH is at or below 1.5, cleavage occurs at GlcNS residues, whereas if the pH is 4.0, cleavage occurs at *N*-unsubstituted GlcN. To cleave GlcNAc glycosidic bonds via reductive deamination, samples need to be first deacetylated and then treated with nitrous acid at pH 4.0. Reductive deamination alters the structure of the glucosamine, thereby producing a 2,5-anhydro-D-mannose residue at the reducing end of the cleaved oligosaccharide (**Figure 3*c***) (30). Oxidative depolymerization (**Figure 3*d***) is performed with a combination of hydrogen peroxide and divalent copper or iron ions (31). This reaction requires vicinal diols at the C2 and C3 positions of the hexuronic acid residue and results in cleavage and subsequent formation of an oxidized fragment of the hexuronic acid (32).

Except for cleavage products created by β-elimination, which introduces a double bond at the nonreducing end of each cleaved oligosaccharide, the products of chemical depolymerization lack UV-active functional groups for sensitive detection by UV absorbance. The detection of heparin-derived oligosaccharides produced through reductive deamination or oxidative cleavage can be enhanced by specific labeling to introduce a chromophore, fluorophore, or radiolabel. A common labeling strategy is modification of the carboxylate groups of the uronic acid residues. By use of carbodiimide reagents, amine-containing chromophores or fluorophores can be conjugated to the oligosaccharide; however, because each disaccharide contains a carboxylate group, incomplete derivatization can complicate the separation and quantitation of larger oligosaccharides. An alternative approach attaches the label to the reducing end of the oligosaccharide. There is only one reducing end on any heparin-derived oligosaccharide, regardless of its length or composition, so quantitation via this approach is more straightforward. Amine-containing labels can be conjugated to the C1 carbon of the reducing-end residue by incubating the oligosaccharide with excess label under slightly acidic conditions in the presence of a mild reducing agent such as sodium cyanoborohydride (33). Oligosaccharides produced through reductive deamination can also be conjugated to a hydrazide-containing label to form a Schiff base (34). Another labeling scheme involves the reduction of the C1 carbonyl of the terminal anhydromanose residue that is produced by reductive deamination with tritiated borohydride (35). This reaction introduces a sensitive radiolabel but requires the handling and disposal of radioactive material (36).

3. SEPARATION OF HEPARIN-DERIVED OLIGOSACCHARIDES

Because heparin depolymerization reactions produce a complex mixture of variously substituted di- (or larger) oligosaccharides, it is usually necessary to incorporate one or more separation steps into the analysis. If the goal of an experiment is characterization of the disaccharide composition of a heparin sample, an exhaustive digestion must be used to reduce the biopolymer to its component disaccharides. Although direct analysis of heparin digests can be performed with MS (37–40),

most studies perform compositional analysis with a separation method such as capillary electrophoresis (CE) or high-performance liquid chromatography (HPLC) to resolve the individual disaccharides. For samples that are depolymerized through enzymatic or β-elimination reactions, compositional analysis has been simplified by the commercial availability of authentic disaccharide standards.

CE: capillary electrophoresis

SEC: size-exclusion chromatography

Frequently, the experimental goal is isolation and characterization of larger heparin oligosaccharides, often as part of a study to explore their protein-binding properties or biological activity. In such cases, digestion is quenched before all the heparin is reduced to its component disaccharides. The analysis of the resulting mixture is complicated by the presence of variously sized oligosaccharides consisting of a diverse set of positional and configurational isomers. For such samples, a single separation cannot adequately resolve the individual components, and two or more orthogonal separation approaches may be employed.

3.1. Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) is generally the first step in the analysis of a partially depolymerized heparin sample. This step resolves the mixture of heparin-derived oligosaccharides into size-uniform fractions (41, 42). **Figure 4** shows the results of the preparative-scale SEC separation of a porcine intestinal mucosa heparin sample that was partially digested with heparinase I. This SEC separation, conducted with a Bio-Gel P10 fine gel packed into a 3 cm × 200 cm column, resolved the component oligosaccharides up to the hexadecasaccharide peak. Because of the specificity of the enzymatic reaction, primarily even-numbered oligomers are produced, although small quantities of trisaccharides have also been reported (42).

Preparative SEC can be carried out on the scale ranging from 100 mg to 1 g, which allows the resolution of size-uniform fractions for studies of heparin-protein interactions or for subsequent separation to yield purified single-component oligosaccharides. A common approach involves collection of the preparative SEC eluates in volumes of a few milliliters to define each peak in the chromatogram. Upon completion of the separation, the collected fractions that correspond to a given oligomer size are pooled for subsequent separation according to charge. However, SEC peaks are not necessarily homogeneous, and the practice of pooling can make it more difficult to resolve trace components in the secondary separation (42, 43). Preparative-scale SEC separations generally take on the order of days to complete and, therefore, are not useful for the rapid characterization of heparin. In contrast, analytical SEC separations require only a few hours to achieve the same size fractionation by use of microgram quantities of material, which makes this method amenable to analysis of heparin and HS samples that are available only in limited amounts. These characteristics make analytical SEC a useful tool for rapid heparin analysis, especially when coupled directly to a secondary separation or a highly selective detection method such as MS (42, 44–46).

3.2. Capillary Electrophoresis and Polyacrylamide Gel Electrophoresis

CE is a useful and increasingly employed method for the separation of many types of carbohydrates (47, 48). It is especially amenable to the separation of heparin and HS oligosaccharides due to their high negative charges. CE separations of heparin oligosaccharides are most effective in reversed-polarity mode (43). Reversed-polarity separations use acidic buffers in the pH range of 3.5 to 4.0 to reduce electroosmotic flow. This pH range also provides the optimum resolution of heparin positional and configurational isomers, given that subtle structural variations can produce small differences in the pK_a values of the hexuronic acid carboxylate moieties, thereby aiding in their resolution (43).

CE has been used for disaccharide resolution and quantitation in the compositional analysis of heparin and HS (49, 50); however, the poor reproducibility of CE can make its routine use challenging (50). CE methods have also been extended to the analysis of larger oligosaccharides (43); such methods include a technique developed specifically for the determination of therapeutically important LMWH samples (51). **Figure 4** shows an example of a CE electropherogram measured for a SEC hexasaccharide fraction. Although this electropherogram contains many peaks, some component hexasaccharides remain unresolved. CE can fairly easily resolve all of the heparin-derived disaccharide standards, including positional isomers; however, sample complexity increases rapidly with increasing chain length. For example, if all common sulfonate positional isomers and uronic acid epimers were present, a sample composed of only hexasaccharides with eight sulfonate groups could theoretically contain 48 unique hexasaccharides having the same net charge—an analytical challenge even for a high-resolution separation method such as CE.

SAX-HPLC: strong anion exchange high-performance liquid chromatography

RPIP-HPLC: reversed-phase ion-pair high-performance liquid chromatography

Polyacrylamide gel electrophoresis (PAGE), which predates CE methods, has also been used to separate heparin oligosaccharides. PAGE is a useful means of characterizing the size distribution of heparin digest samples (52–54) as well as for analysis of LMWHs (55). However, PAGE is not used for heparin-compositional analysis because the resolution of individual disaccharides is relatively poor. In addition, this method is not easily amenable to detection by MS, which is necessary for molecular-level characterization of larger oligosaccharides.

3.3. Anion Exchange Chromatography

HPLC can offer a robust approach for the separation and analysis of heparin and HS oligosaccharides. Strong anion exchange (SAX)-HPLC is often used for the separation of GAG oligosaccharides, especially those derived from heparin and HS (41, 53, 56, 57). As with other separation techniques, analytical SAX columns provide the highest resolution, whereas preparative-scale columns have greater sample capacity. Preparative or semipreparative SAX separations allow the injection of larger quantities and the isolation of purified component oligosaccharides for subsequent characterization experiments. Because of the complexity of heparin digest samples, a common protocol involves the injection of size-uniform SEC fractions onto the SAX column. The semipreparative SAX-HPLC separation of a heparin-derived SEC hexasaccharide fraction (**Figure 4**) is better able to resolve the individual hexasaccharide components than either CE or reversed-phase ion-pair (RPIP)-HPLC (discussed in Section 3.5). This improved resolution comes with a cost; the SAX separation requires much longer analysis times than either CE or RPIP-HPLC. Another disadvantage of SAX-HPLC is the high–ionic strength mobile phase (e.g., 2 M NaCl) required for the elution of highly charged heparin oligosaccharides. As a result, SAX

Figure 4

Flow chart of the steps for heparin and heparan sulfate analysis and characterization. Analysis normally begins with a size-exclusion chromatography (SEC) separation to fractionate digested heparin into size-uniform fractions. SEC is typically followed by an orthogonal separation method to isolate individual oligosaccharides for subsequent characterization. Shown are example reversed-phase ion-pair ultrahigh-performance liquid chromatography (RPIP-UPLC) and strong anion exchange high-performance liquid chromatography (SAX-HPLC) chromatograms and a capillary electrophoresis (CE) electropherogram for the hexasaccharide SEC fraction. For the structural characterization of the isolated oligosaccharide, a combination of mass spectrometry (MS) and nuclear magnetic resonance (NMR) is used. Shown are the MS and NMR spectra for a hexasaccharide isolated with SAX-HPLC (*peak indicated by the asterisk*): ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNAc(6S)-GlcA-GlcNS(6S); its structure is shown in **Figure 2*b***. The NMR resonance at 2.049 ppm indicates the presence of an *N*-acetyl-D-glucosamine residue. Abbreviations: ΔUA(2S), 2-*O*-sulfo-$\Delta^{4,5}$-uronic acid; GlcNS(6S), *N*-sulfo-6-*O*-sulfo-D-glucosamine; IdoA(2S), 2-*O*-sulfo-L-iduronic acid; GlcNAc(6S), *N*-acetyl-6-*O*-sulfo-D-glucosamine; GlcA, D-glucuronic acid.

is not easily amenable to detection by MS, and components isolated using this approach must be desalted prior to characterization by MS or NMR.

An alternative to SAX separations is weak anion exchange (WAX)-HPLC. The WAX column is packed with an amino-bonded stationary phase that interacts more weakly with anionic analytes than do the quaternary amine functionalized polymers used for SAX separations. As a result, WAX separations require less salt to elute highly charged analytes, making this method more compatible with hyphenated techniques such as HPLC-NMR. Limtiaco et al. (58) showed that WAX could be used to separate DS, heparin, and the semisynthetic oversulfated chondroitin sulfate (OSCS) through a displacement-based mechanism.

3.4. Hydrophilic Interaction Chromatography and Graphitized Carbon Separations

Hydrophilic interaction chromatography (HILIC) can also be used to separate heparin and HS oligosaccharides, as well as other GAGs (59, 60), although the separations reported thus far have not been effective in resolving isomeric oligosaccharides. The inability of HILIC separations to resolve disaccharide positional isomers limits this method's utility in compositional analysis.

The use of graphitized carbon columns for the HPLC separation of heparin disaccharides has also been reported (61). Although this method can resolve positional isomers, it offers poor resolution between the *N*-acetylated and *N*-sulfonated disaccharides. Although these disaccharides can be distinguished by MS, overlap between *N*-acetylated and *N*-sulfonated disaccharides makes it impossible to visualize the separation by UV absorbance alone.

3.5. Reversed-Phase Ion-Pair High-Performance Liquid Chromatography

RPIP-HPLC is an increasingly important method for the separation of heparin and HS oligosaccharides. The popularity of this approach stems from the widespread availability of reversed-phase HPLC columns and instruments as well as the ease of implementation of RPIP-HPLC with a variety of detection methods (e.g., UV, fluorescence, and MS). RPIP-HPLC is typically performed on octadecyl (C_{18}) columns with a lipophilic alkyl ammonium salt used as an ion-pairing reagent (IPR). The IPR aids in the retention and resolution of the charged analyte through electrostatic interactions. The transient analyte-IPR ion pair is relatively neutral and hydrophobic, which facilitates interactions with the hydrophobic stationary phase of the reversed-phase HPLC column (62–64). An important application of RPIP-HPLC is the full disaccharide compositional analysis of heparin and HS (65–68). With the incorporation of postcolumn fluorescent labeling (68), RPIP-HPLC can be used for the compositional analysis of samples that are isolated from biological tissues and available only in very limited quantities (69, 70).

Although the earliest separations used quaternary ammonium salts such as tetrabutylammonium, incorporation of more volatile reagents such as tributylamine has made RPIP-HPLC more amenable to detection by MS. Hyphenation of the two techniques greatly improves the detection sensitivity, compared with UV absorbance, and provides important structural information about the analytes (67, 71–74). Compared with RPIP-HPLC, SAX is better able to resolve complex mixtures of larger heparin-derived oligosaccharides, but because it requires a desalting step prior to mass spectrometric analysis, RPIP-HPLC is often preferred for rapid separation and analysis.

Smaller column-packing materials (e.g., 2 μm or less) can improve the speed and sensitivity of heparin disaccharide analysis using RPIP-HPLC (68). Ultrahigh-performance liquid chromatography (UPLC) separations allow the complete resolution of all possible heparin disaccharides, including positional isomers. Commercially available UPLC instruments utilize

1.7-μm-particle columns and proprietary mobile-phase pumps that can withstand the high pressures (up to 15,000 psi) needed to push mobile phase through the column. Korir et al. (75) reported an RPIP-UPLC separation that separates 11 commercially available heparin disaccharides in under 5 min with minimal sample preparation. Further work has improved upon this separation while also using the heparin disaccharides to probe the RPIP separation mechanism (76). The speed of analysis and high resolution provided by RPIP-UPLC separations make this method an excellent choice for the full compositional analysis of heparin and HS (75, 76). The RPIP-UPLC separation of the hexasaccharide SEC fraction lacks the resolution achieved through SAX-HPLC (**Figure 4**); however, RPIP-UPLC has a distinct advantage in terms of the ease with which it can be coupled to MS detection. This advantage is especially significant given the lack of commercially available standards for heparin-derived hexasaccharides.

ESI-MS: electrospray ionization mass spectrometry

4. MASS SPECTROMETRY

MS is a useful and sensitive technique that can provide oligosaccharide molecular weight, the degree of sulfonation and acetylation, monosaccharide composition, and under controlled experimental conditions, sequence. A more detailed discussion of the use of MS for the structural and compositional analysis of GAGs can be found in Reference 77. Most MS studies of heparin and HS utilize electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), although, as discussed in Section 4.3, the recently introduced method of electron detachment dissociation (EDD) shows great promise.

4.1. Negative-Ion Electrospray Ionization Mass Spectrometry

Because of the highly anionic nature of heparin and HS, negative-mode ESI-MS is an attractive choice for their analysis (78). Given the complexity of depolymerized heparin samples, another benefit of ESI-MS is the ease of hyphenation with separation techniques such as CE and HPLC. Often, however, negative-ion ESI-MS spectra of heparin oligosaccharides are complicated by the presence of sodium and potassium adducts. Careful desalting, very clean sample preparation and instrumental components, and the use of ammonium buffers can reduce adduction (40). An advantage of coupling RPIP separations with MS detection is that salt adducts can be further reduced because sodium and potassium ions are largely eluted in the void volume of the column, whereas heparin oligosaccharides are retained through their interaction with the IPR (75).

Another challenge in the characterization of heparin and HS oligosaccharides by use of MS is the prevalence of sulfate loss during ionization. Sulfate loss from disaccharides may be catalyzed by trace amounts of acid; thus, one must be careful to remove acid from the analyte solution and source before performing MS analysis (79). It is also important to choose ionization and ion-extraction conditions carefully, as sulfate loss can occur within the source region of the ESI interface. **Figure 4** shows the negative-ion ESI-TOF (time-of-flight) mass spectrum acquired for a heparin-derived hexasaccharide (**Figure 2*b***) that was isolated by SAX-HPLC and desalted prior to the RPIP-UPLC separation. As this mass spectrum shows, in addition to the doubly charged molecular ion peak, $[M\text{-}2H]^{2-}$, several related peaks including those for sulfate loss and adduction of the IPR TrBA (tributylamine) were also detected.

4.2. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

MALDI is a soft ionization method that enables relatively high throughput MS analysis on small quantities of sample. As in ESI-MS, a common problem encountered in the use of

MALDI-MS: matrix-assisted laser desorption/ionization mass spectrometry

MALDI-MS for analysis of heparin oligosaccharides is fragmentation, especially sulfate loss, during the ionization process. Early work showed that complexation of oligosaccharides by synthetic $(Arg\text{-}Gly)_n$ peptides or by the small basic protein angiogenin allowed the oligosaccharide to remain intact through the ionization process (80). The full oligosaccharide-peptide complex was detected by positive-ion MS, and the molecular weight of the oligosaccharide was calculated by subtraction. Later studies used a similar complexation approach through the addition of quaternary ammonium or phosphonium salts as a comatrix for MALDI-MS (81). The use of cesium salts to suppress sulfate loss when using MALDI-MS in an ionic liquid matrix has also been explored (82). Tissot et al. (83) tested the ionic liquid 1-methylimidazolium α-cyano-4-hydroxycinnamate as a MALDI matrix and successfully ionized heparin di-, tetra-, hexa-, octa-, and decasaccharides. Dihydroxybenzoic acid and norharmane are also effective MALDI matrices for the structural characterization of heparin and HS by MS (84). Clearly, MALDI-MS has great potential for the characterization of heparin and HS oligosaccharides.

4.3. Tandem Mass Spectrometry

The use of tandem MS (MS/MS) to determine both the structure and the sequence of oligosaccharides is an ongoing area of interest in GAG research. One limitation of this approach is the propensity of heparin oligosaccharides to fragment via sulfate loss instead of through more informative ring cleavages. Zaia & Costello (78) demonstrated that the degree of fragmentation of any oligosaccharide is highly dependent upon the charge state of the ion selected for collision-induced dissociation (CID). These authors showed that through careful selection of charge states and addition of calcium ions to stabilize the sulfonate groups, the degree and abundance of ring cleavage ions can be enhanced. Meissen et al. (85) recently reported that MS/MS with CID can be used to distinguish 3-*O*-sulfonation sites from 6-*O*-sulfonation sites in heparin oligosaccharides by inducing specific cross-ring cleavages.

Ultimately, the ability to use tandem MS to sequence GAG oligosaccharides requires the generation of unique and informative fragments for each monosaccharide. In addition to cleavage of glycosidic linkages, this process also requires numerous cross-ring cleavages to distinguish variable points of sulfonation and acetylation. MALDI-LIFT-TOF/TOF and ESI-CID-MS/MS were compared for the structural analysis of *N*-acetylheparosan (a biosynthetic heparin precursor) oligosaccharides (86). This study showed that MALDI-LIFT-TOF/TOF yielded much better cleavage specificity than did CID. More recently, the advancement of the MS/MS technique EDD has proven useful for creating unique cross-ring cleavages in heparin and HS. By using EDD with Fourier transform ion cyclotron resonance, Wolff et al. (87) showed that diagnostic fragment ions can be created that distinguish IdoA from GlcA in tetrasaccharides. Further advances in the use of EDD will probably lead to exciting new applications of MS/MS for the characterization of heparin and HS oligosaccharides.

5. NUCLEAR MAGNETIC RESONANCE

NMR spectroscopy is highly sensitive to minor variations in molecular structure, making it an important technique for heparin characterization. A simple ^{1}H NMR survey spectrum can both reveal the number of monosaccharide residues present and provide a tentative compositional analysis based on comparison between ^{1}H chemical shifts and reference data (2, 88, 89). Visual examination of the ^{1}H NMR spectrum (**Figure 4**) indicates that the hexasaccharide peak isolated by SAX-HPLC contains a single component that includes a GlcNAc residue, indicated by the singlet resonance at 2.049 ppm. Although this ^{1}H NMR survey spectrum provides important information

about sample purity and composition, complete structural characterization of the hexasaccharide requires two-dimensional NMR (53). With the powerful arsenal of two-dimensional experimental techniques available, NMR spectroscopy can be used to determine the sequence of the component monosaccharide residues and unambiguously determine sites of *N*-acetylation as well as of *N*- and *O*-sulfonation along the oligosaccharide chain. Most importantly, NMR spectroscopy can also specify the orientation of the anomeric linkage connecting the various disaccharide subunits and easily distinguishes IdoA and GlcA residues.

Because of its high level of microheterogeneity and polydisperse nature, the complete structural characterization of unfractionated heparin continues to be a major challenge. Therefore, NMR measurements are usually reserved for characterization of purified single oligosaccharides obtained by chemical or enzymatic depolymerization, followed by size- and charge-based separations. Interpretation of NMR spectral data is usually facilitated by molecular-weight and fragmentation information provided by MS.

Several factors must be considered to ensure that satisfactory NMR spectra are acquired for heparin structural studies. Naturally, sample purity is an important consideration. The isolated oligosaccharide components should have a purity of greater than 80% to 90% to avoid complications in structural determinations. Additional so-called NMR-silent impurities must also be minimized. Although NMR is more salt tolerant than MS, high levels of salt in oligosaccharide samples isolated from SAX separations can make it impossible to properly tune and match the NMR probe, and a desalting step is usually required prior to analysis. As demonstrated by McIwen (90), heparin resonance line widths are severely affected by binding of trace paramagnetic impurities. Paramagnetic transition-metal ions, which may be present as production impurities or introduced during oligosaccharide isolation, can cause line broadening through paramagnetic relaxation enhancement. The addition of a small amount of deuterated EDTA (ethylenediaminetetraacetic acid) significantly improves the spectral quality for both unfractionated heparin (90) and LMWH, and it can also be helpful in the analysis of isolated oligosaccharides (89). Finally, if the oligosaccharide is isolated from a RPIP-HPLC separation, the sample will be contaminated by the IPR. Use of a volatile IPR such as ethylamine or removal of less volatile reagents by cation exchange can facilitate sample cleanup for NMR analysis (91).

5.1. Two-Dimensional Nuclear Magnetic Resonance Analysis of Heparin Oligosaccharides

Structural characterization of heparin-derived oligosaccharides is typically initiated by identification of the individual monosaccharide subunits in the oligosaccharide chain, using scalar couplings to provide through-bond connectivities. These connectivities can be obtained through a number of two-dimensional NMR experiments, including homonuclear correlation spectroscopy (COSY) and homonuclear total correlation spectroscopy (TOCSY) experiments, as well as heteronuclear single and multiple quantum coherence spectroscopy experiments (53, 92–94). Through the COSY spectrum (**Figure 5*a***), connections between coupled protons on adjacent carbon atoms within a monosaccharide ring can be identified. The well-resolved anomeric resonances and the H4 resonance of the $\Delta^{4,5}$UA residue of oligosaccharides produced by enzymatic cleavage or by β-elimination provide an entry point for analysis of the COSY spectrum. However, the limited ^{1}H NMR chemical shift dispersion of carbohydrates can make interpretation of the COSY data challenging, even for medium-sized oligosaccharides. Because the TOCSY experiment transfers ^{1}H scalar coupling information throughout a spin system, the TOCSY spectrum allows detection of the connectivity of all the protons within each monosaccharide residue through the anomeric resonances. The TOCSY spectrum in **Figure 5*b*** was used to assign resonances of a heparin-derived

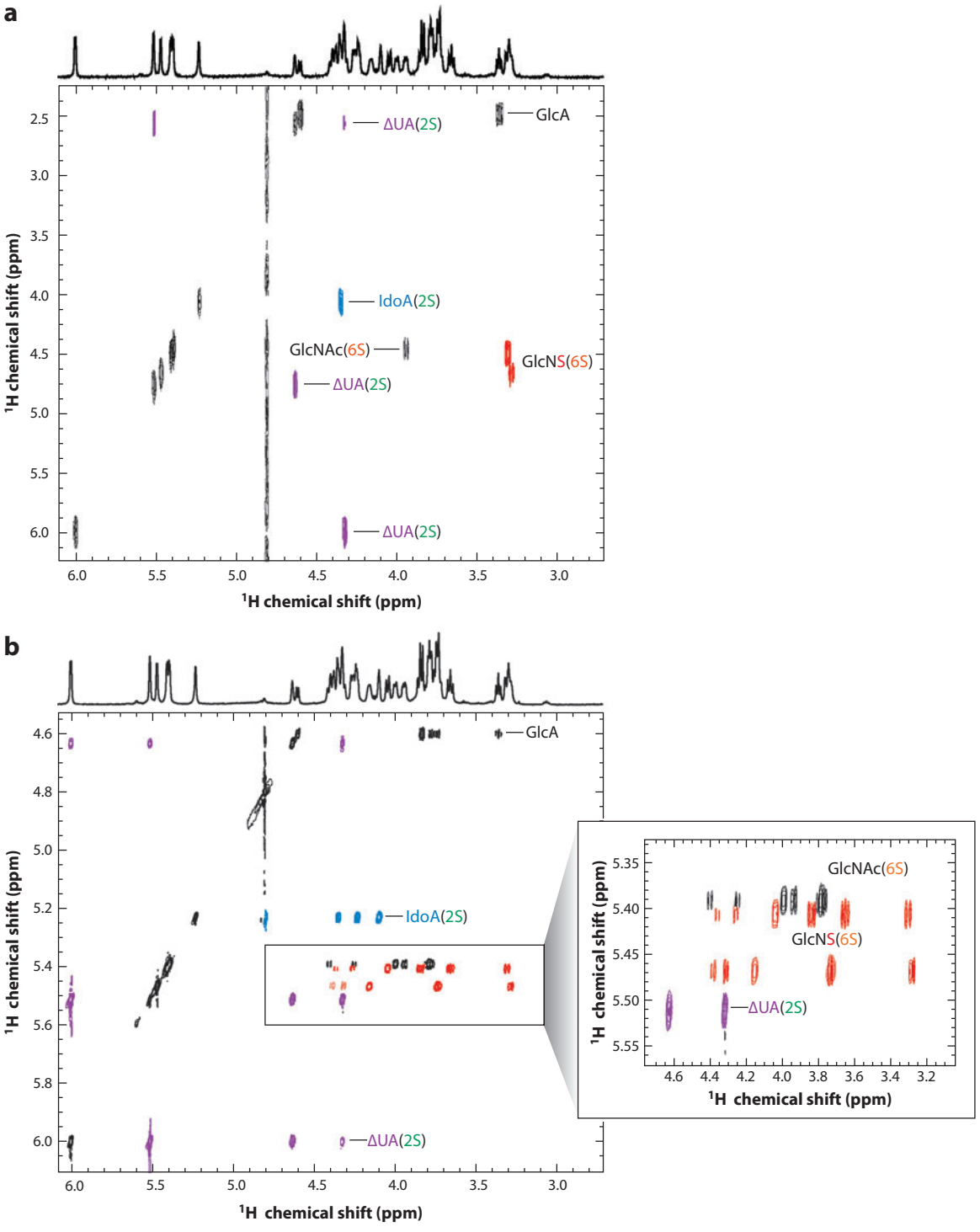


Figure 5

(*Continued*)

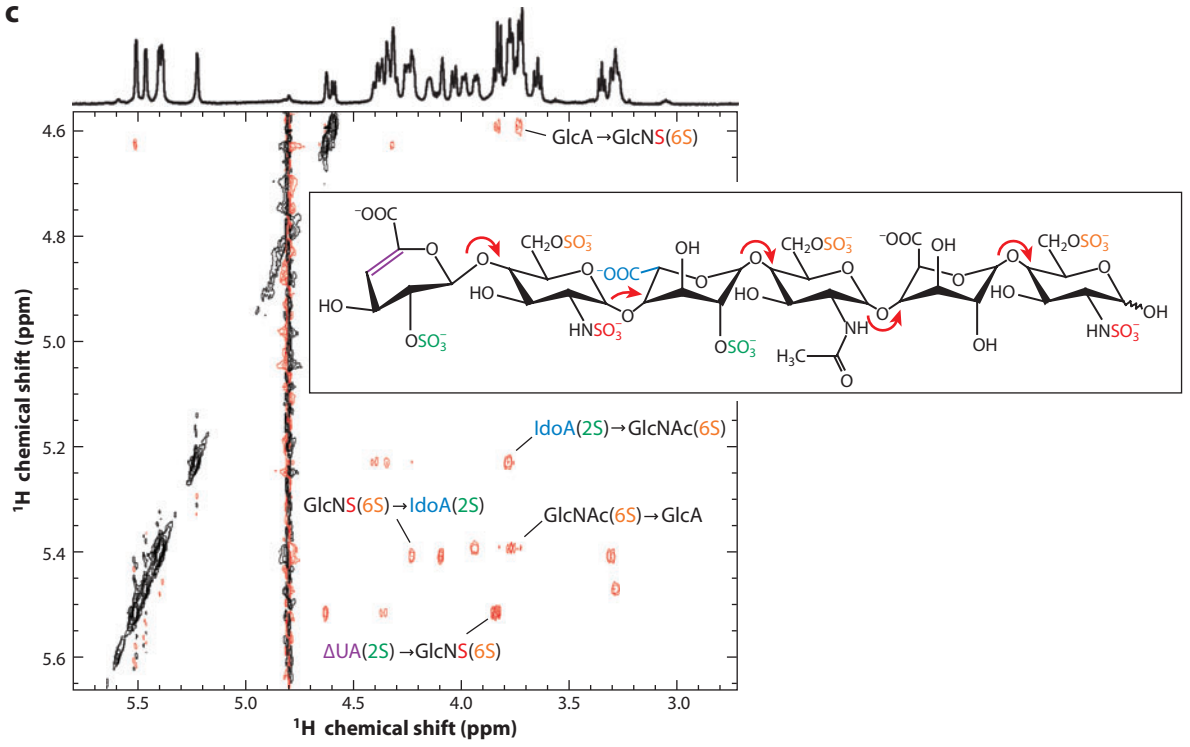


Figure 5

Two-dimensional nuclear magnetic resonance spectra obtained for the heparin-derived hexasaccharide ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNAc(6S)-GlcA-GlcNS(6S), isolated by strong anion exchange high-performance liquid chromatography. (*a*) Partial correlation spectroscopy (COSY) spectrum showing cross peaks to spin-spin-coupled protons on adjacent carbons. (*b*) Partial total correlation spectroscopy (TOCSY) spectrum showing cross peaks of the individual monosaccharide resonances to the corresponding well-resolved anomeric resonances. The region defined by the box is shown in the expansion. The COSY and TOCSY cross peaks are colored according to the following scheme: black, unmodified glucuronic acid (GlcA) and *N*-acetyl-6-*O*-sulfoglucosamine [GlcNAc(6S)]; violet, nonreducing-end 2-*O*-sulfo-hexuronic acid [ΔUA(2S)]; blue, 2-*O*-sulfo-iduronic acid [IdoA(2S)]; red, *N*-sulfo-6-*O*-sulfo-glucosamine [GlcNS(6S)] residues (see **Figure 2*b***). (*c*) Partial rotating-frame Overhauser effect spectroscopy spectrum showing the important peaks used to establish the sequence of the monosaccharide residues constituting the heparin-derived hexasaccharide.

hexasaccharide, whose structure is shown in **Figure 2*b***. Through comparison of the ^{1}H chemical shift data obtained from the TOCSY spectrum with reference data, the structural identities of the monosaccharide residues can be deduced. For complicated spectra with extensive overlap in the anomeric region of the ^{1}H NMR spectrum, the band-selective homonuclear-decoupled (BASHD)-TOCSY experiment can provide improved resolution (89). The heteronuclear single and multiple quantum coherence spectroscopy experiments provide additional information about the type, number, and position of C–H bonds and offer improved spectral resolution by taking advantage of the greater dispersion of the ^{13}C chemical shift dimension. The HEHAHA (Heteronuclear Hartmann Hahn) experiment, introduced by Bendiak et al. (95) and Jones & Bendiak (96), is an additional tool used to characterize oligosaccharide primary structure and to identify the positions of modification, especially those introduced by *O*-acetylation.

Following assignment of the resonances of the individual monosaccharide residues, the oligosaccharide sequence is determined through dipolar coupling information obtained via

rotating-frame Overhauser effect spectroscopy (ROESY). The ROESY spectrum can also distinguish IdoA and GlcA through unique interresidue cross peaks. In conjunction with the TOCSY results, the ROESY spectrum (**Figure 5*c***) was used to establish sequence information that conclusively determined the hexasaccharide structure. In the ROESY spectrum, cross peaks arise between resonances of the H1 and the H4 protons of adjacent residues connected via the glycosidic bond, which reveals the relative positions of the monosaccharide residues within the hexasaccharide chain. Again, in cases in which the spectrum is complicated by resonance overlap, the BASHD version of the ROESY experiment can provide enhanced resolution (89).

5.2. Nuclear Magnetic Resonance Diffusion Measurements

NMR diffusion measurements are useful for the analysis of complex mixtures, such as those obtained by heparin depolymerization. Diffusion-ordered spectroscopy (DOSY) provides a noninvasive separation of the different mixture components on the basis of differences in their translational diffusion coefficients (97–100). DOSY NMR was used for the routine screening of LMWH and unfractionated heparin by Sitkowski et al. (101) and Bednarek et al. (102). Because the ^{1}H chemical shifts of the *N*-acetyl resonances of heparin and its potential impurities DS and OSCS are well resolved spectrally, DOSY plots can resolve OSCS and DS from both unfractionated heparin and LMWH. However, care must be taken in the design of NMR diffusion experiments because viscosity effects and macromolecular crowding in concentrated solutions of heparin and other GAGs can dominate their translational diffusion behavior.

Limtiaco et al. (103) demonstrated the use of DOSY NMR spectra in conjunction with ^{1}H NMR and UV absorption measurements to monitor the enzymatic depolymerization of heparin with heparinase I. DOSY spectra were acquired throughout the course of the enzymatic depolymerization. **Figure 6** shows a portion of the DOSY spectra for the digestion solution at 1 h and 66 h. In the 1-h spectrum (**Figure 6*a***), the two major anomeric resonances observed for the intact heparin polymer at 5.230 and 5.415 ppm give rise to similar diffusion coefficients. At the conclusion of the enzymatic digestion, the DOSY spectrum (**Figure 6*b***) shows a much greater variability in composition as well as in the observed diffusion coefficients. The DOSY spectra of the reaction solution yielded insight into the extent of the depolymerization and provided information about the size distribution of the heparin oligosaccharides produced. In addition to allowing the investigator to track and optimize the enzymatic digestion, this experiment demonstrated that DOSY NMR can be used to study mixtures of digested GAGs without employing a physical separation.

5.3. Enhancing Nuclear Magnetic Resonance Sensitivity

Although NMR experiments provide unique molecular-level information required for structural characterization, such measurements are often limited by the experiments' low relative sensitivity. This disadvantage is most commonly encountered when the target of the measurement is available only in limited quantities, as is the case for heparin and HS oligosaccharides isolated from biological samples through lengthy and often tedious procedures. To address this limitation, several approaches can be used to improve NMR sensitivity. These methods include increasing the strength of the static magnetic field (which has the added advantage of improving resonance dispersion) and using cryogenically cooled probes (to decrease noise), hyperpolarization techniques, and smaller radio-frequency (rf) coils (microcoils). Although the benefits of increased magnetic field strength are well recognized, investigators are generally limited by the locally available instrumentation. Cryogenically cooled NMR probes and receivers can provide significant improvements in signal-to-noise ratio (S/N), generally by a factor of two to four, depending on the solvent and ionic

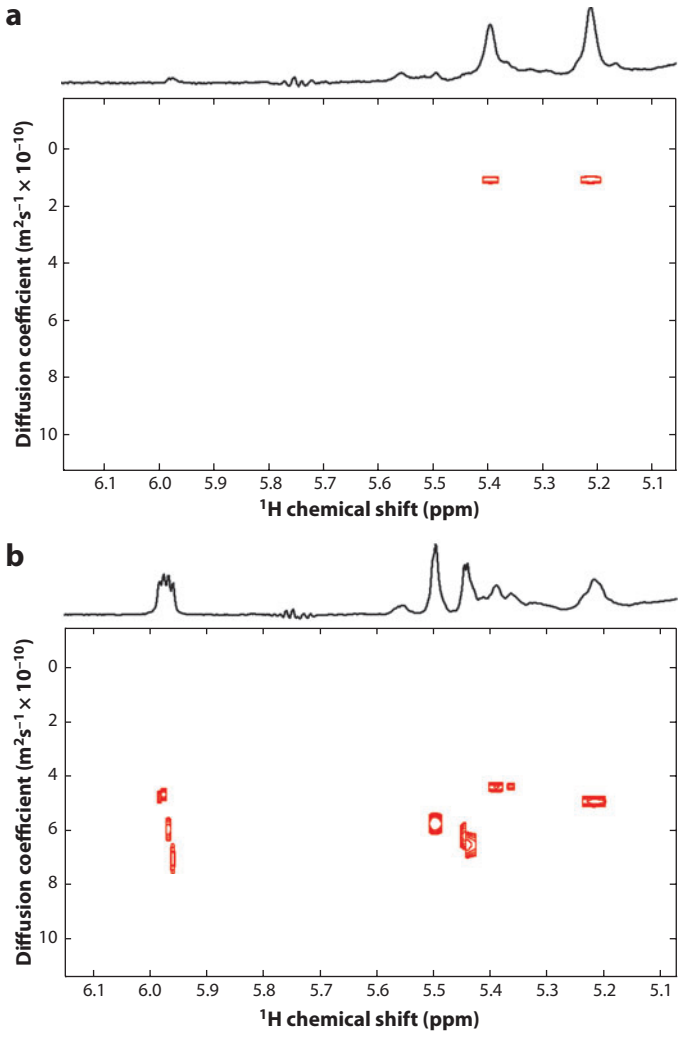


Figure 6

Diffusion-ordered spectroscopy spectra showing the anomeric (5.1–5.6 ppm) and H4 (5.9–6.0 ppm) protons of the ΔUA residues of the heparin digest solution at (*a*) 1 h and (*b*) 66 h. Figure reprinted from Reference 103.

strength (104, 105). A fourfold increase in sensitivity may not seem significant, but for mass-limited samples it can make a significant difference in spectral quality, especially in two-dimensional NMR spectra. Although signal averaging can also be used to improve S/N, it rapidly becomes impractical in two-dimensional experiments, given that a fourfold gain in S/N requires a 16-fold increase in experiment time.

Despite the advantages of cryogenically cooled NMR probes, not every NMR laboratory can accommodate such instrumentation, either because of its initial cost and ongoing maintenance expenses or because the diversity of NMR experiments requires frequent probe changes. For compounds with high solubility, such as heparin oligosaccharides, microcoil NMR probes can provide sensitivity enhancements similar to those offered by cryogenically cooled probes

cITP: capillary isotachophoresis

at a fraction of the cost. For a review of microcoil NMR technology and its application to mass-limited samples, see Reference 106. The smaller active volume of microcoil NMR probes can enable two-dimensional NMR analysis using much smaller sample amounts than required by conventional probes. For example, the three two-dimensional NMR spectra (COSY, TOCSY, and ROESY) shown in **Figure 5** were acquired in only 12 h through the use of a microcoil probe made by MRM/Protasis, Inc. (Savoy, Illinois), with a 600-MHz NMR spectrometer and 74 μg of isolated material in the probe active volume.

Microcoil NMR also allows easy coupling to separation methods such as HPLC, CE, and capillary isotachophoresis (cITP) (107–112). The coupling of cITP to NMR detection holds great promise for analysis of acidic oligosaccharides such as heparin and HS (107, 113). cITP is an electrophoretic technique that can simultaneously focus charged analytes by up to two to three orders of magnitude and separate components on the basis of their electrophoretic mobilities. Sample stacking by cITP allows microliter volumes of a dilute sample to be focused into a concentrated band that closely matches the volume of the rf microcoil (e.g., 25 nl) (114, 115). Sample stacking by cITP both compensates for the poor concentration sensitivity typically associated with rf microcoils and separates analytes according to differences in electrophoretic mobility. For example, cITP-NMR has been used to focus, separate, and detect trace-level acetaminophen-degradation products (116).

Research by our group has demonstrated the use of cITP-NMR for the measurement of ^{1}H NMR spectra for only 1–2 μg of heparin-derived di- and tetrasaccharides (107, 113). Although the resonances are broadened by the interfering magnetic field produced when current is applied to the separation capillary, the unique chemical shift fingerprints of the monosaccharide residues can be used to identify sample components and evaluate sample purity. **Figure 7** compares a ^{1}H NMR spectra acquired for 2.5 μg of a heparin tetrasaccharide by use of cITP-NMR (**Figure 7*a***) with an NMR spectrum measured with 30 μg in the flow cell of a commercial microcoil probe (**Figure 7*b***). Currently, cITP-NMR spectra are obtained on a transient basis as the focused band passes through the detection volume of the rf coil. This process makes it difficult to signal-average or measure longer two-dimensional NMR spectra for the focused samples. To solve this problem, efficient stop-flow methods are needed to park the concentrated band inside the detection volume of the rf microcoil; development of these methods is an area of active investigation.

The poor sensitivity of NMR spectroscopy arises from the small population differences of nuclear spin states, even in high magnetic fields. A notable way to improve the sensitivity of NMR is to use hyperpolarization techniques (117). In experiments using hyperpolarization, an independent physical means is used to bring the nuclear spin system into a nonequilibrium state before each measurement. One promising technique is dynamic nuclear polarization (DNP), in which a sample aliquot is polarized in its solid state at low temperatures. Following polarization, the sample is dissolved in a stream of hot solvent before being transferred into the NMR spectrometer for acquisition. Compared with spectra acquired at thermal equilibrium in a 9.4-T magnetic field (118), DNP polarization yields signal enhancements of 44,400 for ^{13}C and 23,500 for ^{15}N. A drawback of DNP lies in the short lifetimes associated with the hyperpolarized state, which traditionally restrict the application of DNP to one-dimensional spectra (117). With the introduction of single-scan two-dimensional NMR experiments (119), DNP has allowed the acquisition of fast heteronuclear multiple quantum correlation experiments, thereby permitting the measurement of heteronuclear two-dimensional spectra in a matter of seconds (120). To the best of our knowledge, this technique has not yet been used for the characterization of heparin and HS; however, future work in this area may prove useful for the characterization of mass-limited heparin oligosaccharide samples.

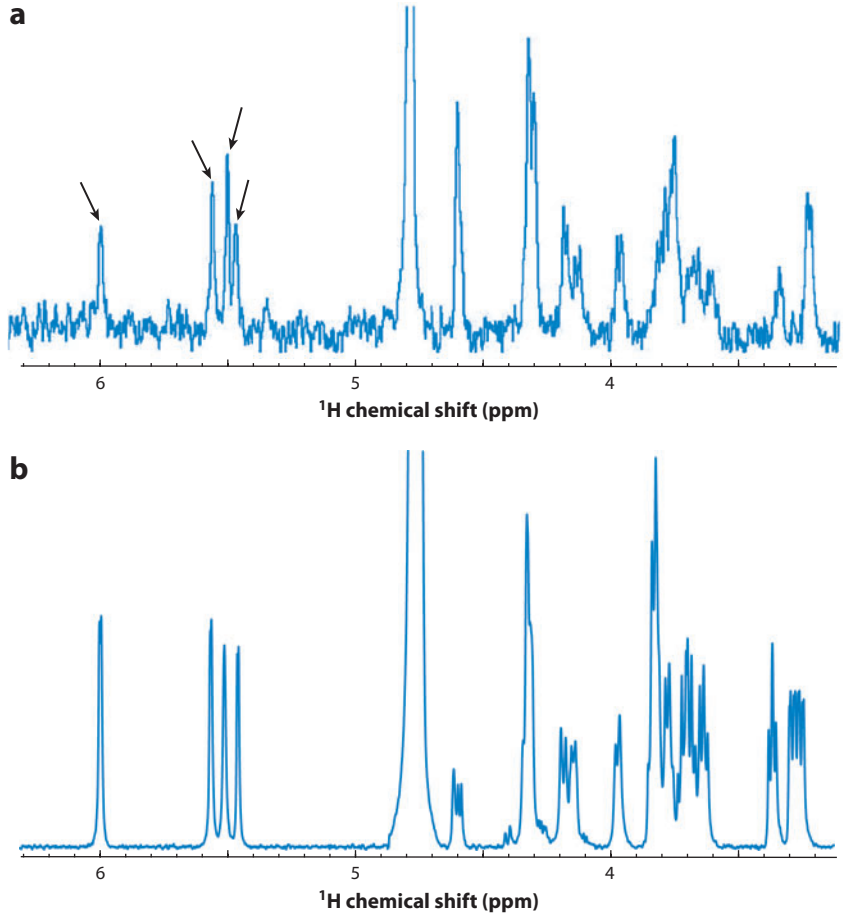


Figure 7

(*a*) ^{1}H NMR (nuclear magnetic resonance) spectrum obtained by postacquisition coaddition of the capillary isotachophoresis (cITP)-NMR spectra for 2.5 μg of a heparin oligosaccharide measured in a home-built probe with a detection volume of 25 nl. The H4 resonance of the ΔUA residue and three anomeric proton resonances (*indicated by arrows*) suggest that the unknown oligosaccharide is a tetrasaccharide containing a GlcA residue. (*b*) ^{1}H NMR spectrum of the heparin tetrasaccharide at a different pH acquired by use of the CapNMR probe with 30 μg of the oligosaccharide in the probe flow cell. Figure reprinted from Reference 107.

5.4. Residual Dipolar Couplings

The conformation of the sugar residues of heparin is believed to contribute to its unique ability to bind to a myriad of different proteins. Recent studies have focused on determining the conformations of the internal IdoA and GlcA residues in an effort to understand the unique protein-binding properties of heparin (121, 122). Through measurements of proton-proton (J_{HH}) coupling constants and nuclear Overhauser effect (NOE) cross peaks, Mikhailov and coworkers (123, 124) characterized the internal iduronic acid residue of the fully sulfonated tetrasaccharide UA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S) as being in the skewed-boat conformation, $^2S_0$. Although NOEs and coupling constants are often used to determine secondary structure, for flexible molecules such

as heparin oligosaccharides, the measured values are averaged by rapid interchange between two or more different conformations. As a result, these experimentally determined parameters cannot be used directly in the development of a conformational model. Measurement of residual dipolar couplings (RDCs) is a powerful approach to overcome the problem of conformational averaging (125, 126). RDC measurements are performed in field-oriented media, such as bicelles, to preserve the dipolar coupling information that is generally eliminated as a result of isotropic tumbling in solution. Jin et al. (127) measured ^{1}H–^{1}H and ^{1}H–^{13}C RDCs for a fully sulfonated heparin tetrasaccharide in the presence of a fourfold excess of Ca^{2+}. The torsion angles of the glycosidic bonds determined from the RDC-restrained molecular dynamics structures were consistent with the three-bond coupling constants measured experimentally for this tetrasaccharide. RDC measurements of heparin-derived oligosaccharides show great promise for structural characterization and should ultimately lead to a better understanding of the relationship between structure and dynamics in heparin and other GAGs (124, 128).

6. CONCLUSIONS

More efficient analytical strategies that will allow the determination of unique oligosaccharide structures are needed to improve our understanding of the biological role of heparin and HS. However, subtle variations in structure, such as sulfonation patterns, positional isomers, and hexuronic acid epimers, are almost impossible to identify in intact biopolymers. As a consequence, heparin and HS characterization proceeds through a bottom-up approach via enzymatic or chemical depolymerization, followed by size- and charge-based separations to isolate individual oligosaccharide sequences for MS and NMR characterization. Although advances have been made, given the complexity of heparin and the difficulty in its characterization, there are still many unanswered questions regarding its molecular-level structure. Numerous analytical techniques are being developed to alleviate this deficiency. Improvements to the speed and resolution of separations are increasing the throughput of the analysis, and mass spectra with higher information content, produced by novel methods such as EDD, hold promise for the rapid sequencing of oligosaccharides. In addition to their role in structural characterization, NMR DOSY and RDC measurements have the potential to provide unique information about heparin structure and dynamics; additionally, higher-field magnets, advances in cryogenically cooled and microcoil probes, and DNP experiments are increasing the sensitivity of NMR measurements, thereby removing a bottleneck in the analytical process.

SUMMARY POINTS

1. The highly anionic, polydisperse, and microheterogeneous nature of heparin and HS makes their analysis and characterization challenging.
2. Most techniques for studying heparin involve a three-part process: chemical or enzymatic digestion; separation of digested oligosaccharides; and detection or characterization by techniques such as UV, MS, and NMR.
3. Numerous methods for the separation of heparin- and HS-derived oligosaccharides have been explored, but the most commonly used are SEC, CE, SAX-HPLC, and RPIP-HPLC.
4. MS and NMR are useful methods for the structural characterization of heparin and HS, and they provide complementary information.

5. Hyphenated techniques such as HPLC-MS/MS, HPLC-NMR, and cITP-NMR promise to increase the efficiency of characterization of heparin and HS oligosaccharides in complex mixtures.

FUTURE ISSUES

1. The biological significance and pharmaceutical use of heparin will continue to make the further study of this unique GAG a topic of important and exciting research.
2. As research interests turn toward a deeper understanding of the interplay among molecular structure, dynamics, and the biological roles of heparin and HS, more rapid and sensitive characterization methods will need to be developed to meet the challenges of studying heparin and HS in mass-limited quantities.
3. EDD will probably lead to exciting new applications of MS/MS for the rapid characterization of heparin and HS oligosaccharides.
4. Continued increases in the sensitivity of NMR measurements and the refinement of hyphenated HPLC- and cITP-NMR methods will streamline NMR-based structural studies.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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