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Utility of non-covalent complexes in the matrix-assisted laser desorption ionization mass spectrometry of heparin-derived oligosaccharides

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Abstract

Molecular weights of heparin-derived oligosaccharides ranging from disaccharides to hexadecasaccharides have been determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. While these compounds ionize poorly or not at all when used as such, a strong signal can be obtained of their ionic complexes formed with a basic peptide or protein. The molecular weight of the sulfated oligosaccharide is determined by subtracting the mass of the basic component from that of the complex. Optimization of the experimental conditions resulted in sub-picomole sensitivity, in the elimination of sulfate loss and of the interference from attachment of inorganic cations. Synthetic peptides $(Arg–Gly)_{10}$ and $(Arg–Gly)_{15}$ were specifically designed as complexing agents for synthetic and natural heparin fragments up to decasaccharides. Accurate molecular weight determination on chemically homogeneous oligosaccharides ($\pm 0.05\%$) unambiguously identified the number of saccharide units, and the number of *O,N*-sulfate and *N*-acetyl groups. For oligosaccharides larger than decasaccharides, a small basic protein, angiogenin ($M_r = 14{,}120$), was used to form the complex (an inhomogeneous hexadecasaccharide fraction was the largest available for this study). For inhomogeneous samples larger than decasaccharides, the mass accuracy is lower (± 0.2–0.3%) but still suffices to determine the number of saccharide units present and to estimate the number of sulfate groups, except it is no longer possible to differentiate one sulfate from two *N*-acetyl groups ($\Delta = 4$ Da). However, taking into account known regularities of sulfation and acetylation, the specificity of heparin lyases and chemical degradation steps, the method promises to contribute significantly to the determination of the primary structure of heparin and other sulfated glycosaminoglycans.

Keywords: Heparin; Oligosaccharides; Sulfated glycosaminoglycans

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1. Introduction

In contrast to some classes of biopolymers, such as proteins, nucleic acids or neutral oligosaccharides, the biological significance of peptidoglycans and glycosaminoglycans (GAG) has not been recognized until quite recently. Despite their abundance in living organisms as constituents of the extracellular matrix or certain cell walls and their extensive use in medicine (most importantly, heparin), even the primary structures of some of these highly polar and polydisperse compounds are not well-understood [1–3]. An additional complication is the varying degree of sulfation that is characteristic of several glycosaminoglycans, particularly heparin and heparan sulfate. The primary sequence of GAG heparin or heparan sulfate is not known, in contrast to the high level of detail by which DNA and protein sequences can be determined. To date, only certain, abundant or biologically specific subsequences of GAG degradation products have been characterized.

Perhaps the main difficulty in the studies of the biochemistry of highly sulfated GAGs is the lack of analytical techniques that are efficient, specific, and yet sufficiently general. Affinity and sizing chromatography have been widely used [4–9]. Strong anion-exchange chromatography is well suited to the separation of highly acidic biopolymers, but the isolation and purification of chemically homogeneous heparin fragments larger than disaccharides from a crude mixture are at present extremely difficult or impossible. Identification of the components relies chiefly on external standards [10]. Affinity chromatography can efficiently be used in the study of specific heparin–protein interactions, but the technique is not applicable to the separation of other segments from a GAG chain. Capillary electrophoresis has emerged recently as a powerful separation technique for these highly charged polysaccharides, but the identification of the peaks may be very difficult if the charge-to-size ratio of the components is similar [11]. Sizing of heparin fractions can be carried out by gel-permeation chromatography [12] or gradient polyacrylamide gel electrophoresis [13,14]. Data obtained by these methods are frequently mutually inconsistent, and the use of external standards can also be an additional source of substantial error [15]. Chemically homogeneous fragments of GAG heparin can be structurally characterized by the combination of chromatographic and spectroscopic methods. ^{13}C- and ^{1}H-NMR spectrometry can efficiently be used for resolving structural and stereoisomers [5,16,17], but require a milligram or more of material.

Mass spectrometry is a particularly useful and general analytical method in cases where structural regularities of the material investigated allows one to deduce structural details from molecular weight information. This is certainly the case with GAG heparin and heparan sulfate, where accurate mass measurement ($\pm 0.1\%$ or better) would unambiguously identify oligosaccharides except structural and stereoisomers. Some of these remaining ambiguities could be resolved by specific enzymatic and/or chemical reactions [18,19]. Few mass spectrometric studies of highly sulfated GAGs have been reported probably due to the difficulties of ionizing these compounds in the mass spectrometer. Plasma-desorption mass spectrometric (PDMS) studies were carried out [20], where molecular weight data and extent of sulfation were determined for heparin-derived oligosaccharides up to hexasaccharides from 25–50 μg samples (20–30 nmol).

Ten nmol sensitivity by fast-atom bombardment (FAB) ionization in the negative ion mode was reported for chondroitin sulfate oligosaccharides and synthetic heparin oligosaccharides up to pentamers [21,22]. At the same level of sensitivity, Dell et al. measured the spectra of a disulfated tetrasaccharide and of a trisulfated hexasaccharide derived from heparan sulfate [23]. Additional structural details were deduced upon permethylation and peracetylation, procedures commonly used for mass spectrometric carbohydrate analysis.

Somewhat improved sensitivity was obtained by Mallis et al. who was able to detect heparin-derived oligosaccharides up to the hexamers [24], and later extended to octamers [25] using triethanolamine as the FAB matrix rather than thioglycerol employed earlier [21]. More recently, electrospray studies were conducted on sulfated disaccharides with further improved sensitivity (100 pmol level) [26]. All these efforts are characterized by low sensitivity (in comparison with peptides and proteins), by abundant multiple adducts of alkali cations and partial loss of the sulfate groups. These features interfere with the unambiguous identification of individual components or with the analysis of mixtures at high sensitivity. The poor mass spectrometric response is due to the extremely polar character of the sulfated GAG fragments. Polar and ionic groups of the analyte lead to strong binding to the polar groups of the matrix in which the sample is embedded or dissolved. Thus, a large amount of energy is needed to volatilize and ionize the analyte in the ion source of the mass spectrometer, eventually leading to excessive fragmentation.

Recently, we have reported [27] that highly acidic (anionic) biomolecules can be efficiently mass analyzed by the matrix-assisted laser desorption ionization technique [28–30], if the charged groups are shielded by a properly selected basic polypeptide. Intact complex ions can then be generated and detected in both ion polarities and with greatly improved sensitivity. Moreover, strong interaction between the basic and acidic components completely displaces alkali cations from the anionic groups of the acidic components. This feature considerably improves the reliability of molecular weight measurement.

We now report a more systematic study of this approach for the molecular weight measurement of heparin-derived oligosaccharides at the low picomole, or sub-picomole level. Heparin fractions up to hexadecasaccharides were detected in the form of ionic complexes with basic peptides or proteins. The design of synthetic arginine-rich peptides in conjunction with the use of new matrix materials eliminated the previously observed [27] detrimental elimination of multiple SO_3 moieties in the ionization process. This feature now makes it possible to analyze mixtures of heparin-related sulfated oligosaccharides.

2. Materials and methods

Matrix-assisted laser desorption ionization mass spectrometry.—Matrix-assisted laser desorption ionization (MALDI) utilizes short-pulse irradiation of the sample from ultraviolet (UV) or infrared (IR) lasers [30,31]. MALDI time-of-flight (TOF) mass

spectra have been recorded on a VT2000 (Vestec Corp., Houston, TX) linear instrument described in detail in an earlier publication [32]. Ultraviolet MALDI experiments were carried out using a N_2 laser (Laser Science, Newton, MA; 337 nm wavelength, 3 ns pulse width). Infrared MALDI mass spectra were generated by means of an Er:YAG laser (2.94 μm wavelength, 120–150 ns pulse width, Schwartz Electro-Optics, Orlando, FL). The ions are accelerated to 30 keV energy and, following a two-meter drift, are detected by a hybrid detector consisting of a microchannel plate and a discrete dynode secondary electron multiplier. The detector signal was preamplified and digitized by a digitizing oscilloscope (LeCroy 9450A, Chestnut Ridge, NY) at a sampling rate of 400 or 200 MHz. In order to improve the signal-to-noise ratio, a number of laser shots were averaged. In UV-MALDI experiments, 30–50 single-shot mass spectra were automatically averaged at a laser irradiance close to the ion generation threshold. In IR-MALDI, due to substantial shot-to-shot variation of the ion signal, only those mass spectra were averaged where visual inspection on the oscilloscope screen indicated satisfactory quality. Averaged data were transferred to a local area VAXcluster for processing using software developed in our laboratory.

Materials.—For IR-MALDI, a 5-(trifluoromethyl)uracil (TFMU) matrix was used, dissolved in 1:1 water–acetonitrile (MeCN) mixture at 10–12 g/L concentration. For UV-MALDI, sinapinic acid and caffeic acid were used in 10 g/L concentration, the former in 2:1, the latter in 1:1 water–MeCN mixture as the solvent, and 3-hydroxypicolinic acid was used at a concentration of 25 g/L in 1:1 water–MeCN. Approximately 10 w/w% α-D-fucose was always added to sinapinic acid and 3-hydroxypicolinic acid, respectively, because this slightly improved spectrum quality. Fresh matrix solutions were prepared every week, but sinapinic acid solutions had to be prepared daily because of the photosensitivity of this compound. All the matrix materials were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI).

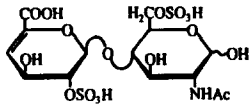
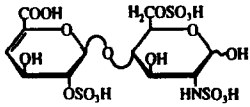
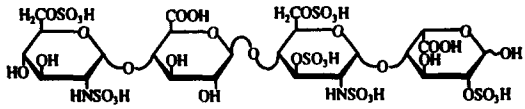
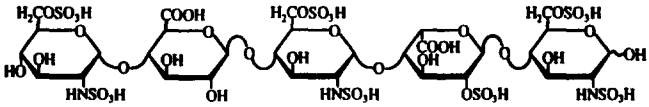
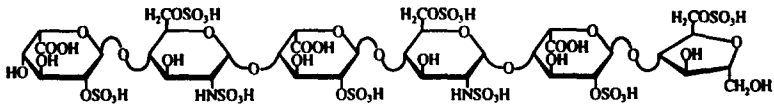
Heparin-derived oligosaccharides and basic peptides/proteins were mixed in the presence of the matrix in equimolar proportions. When the components were mixed in advance as aqueous solutions and the matrix added later, less efficient complex formation was observed for reasons still unknown. The concentration of sample components was 0.5–10 pmol/μL (although an order of magnitude less could still be used). Of this sample-matrix solution, 0.5–1.0 μL was placed on the probe surface and dried under a stream of air.

Peptides SP1, SP2, and SP3 (see Table 1) were synthesized by the Biopolymer Laboratory at MIT. MALDI mass spectra with caffeic acid matrix were found to be affected by the presence of inorganic anions in the preparation of peptides SP2 and SP3.

Table 1
Notation and structure of basic peptides used for ionic complexes with heparin oligosaccharides

Basic peptide	Sequence	Molecular weight
SP1	RRRRRRPYIL	1441.76
SP2	$(RG)_{10}$	2150.41
SP3	$(RG)_{15}$	3216.61

Table 2
Notation and structure of oligosaccharides investigated

Symbol/ MW	Structure
D1 539.4	
D2 577.4	
T1 1173.0	
P1 1414.2	
H1 1655.4	

It was useful to exchange the anions to hydroxide anions by means of AG 1-X2 resin (BioRad, Hercules, CA). As free bases, these peptides are quite unstable in aqueous solution which, therefore, has to be freshly prepared daily.

Chemically homogeneous, well-characterized heparin-derived oligosaccharides used in this study are listed in Table 2. Disaccharides D1 and D2, the end products of enzymatic depolymerization of GAG heparin, were purchased from Sigma Chemical Co. (St. Louis, MO) and used as sodium salts. A great advantage of the complex formation method is that salts can be analyzed as efficiently as the free acids without the interference of cation adducts [27]. Tetrasaccharide T1 and Pentasaccharide P1 were a gift from S.A. Carr (SmithKline Beecham Pharm., King of Prussia, PA). Both compounds had been synthesized previously [33], and characterized by FAB mass spectrometry [22]. These compounds were also used as sodium salts. The ammonium salt of hexasaccharide H1 was isolated, purified, and characterized by D.J. Tyrrell and coworkers (Glycomed Inc., Alameda, CA) [17,34].

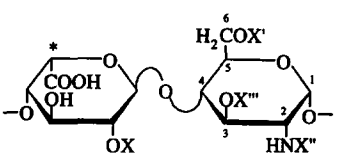
In addition to these well-characterized, homogeneous heparin-related oligosaccharides, inhomogeneous heparin fractions produced by nitrous acid depolymerization of GAG heparin as well as recombinant human angiogenin expressed in *E. coli* [35] were provided by F. Soncin (Harvard Medical School, Boston, MA). Heparin degradation products had been separated by gel filtration into fractions with increasing numbers of disaccharide units.

3. Results and discussion

As had been reported previously [27], strongly acidic compounds, such as heparin-derived oligosaccharides, form tightly bound ionic complexes with arginine-rich peptides or proteins. MALDI mass spectra of such mixtures contain complex ions of various composition, and sometimes also ions due to one or both of the individual protonated (or deprotonated) components. However, it is not unusual that only the protonated (or deprotonated) complex ions are present. For heparin-derived oligosaccharides, usually the 1:1 complex ion is observed in the MALDI mass spectrum in either ion polarity (protonated in the positive, deprotonated in the negative ion mode). Protonated molecules, $(M + H)^+$, can often be observed for the basic peptide in the positive ion mode. These can then be used as internal standards, which is desirable for better mass accuracy; therefore, the positive ion mode is preferred.

The molecular mass of the unknown acidic component is then determined by subtracting the protonated molecular mass of the basic peptide from the mass of the protonated complex. In most cases, calibration was carried out by means of an external standard (such bovine insulin or ubiquitin), and a mass accuracy of 0.1% could usually be attained. If the basic peptide exhibited more than one peak in the mass spectrum (for instance, the singly and doubly protonated peptide molecules), these peaks could be used as internal references for calibrating the mass scale, reducing the error of mass measurements by a factor of 2–3.

Disaccharides.—Glycosaminoglycan heparin is built up from disaccharide units: a hexuronic acid (D-glucuronic acid or L-iduronic acid) 1-4-linked to D-glucosamine [1]. There are four possible sulfation sites in this "repeating unit" (as indicated in Scheme 1): position 2 on the hexuronic acid, and positions 2, 3, and 6 on the glucosamine residue. To date, heparin disaccharides containing up to three sulfate groups have been



Scheme 1. Repeating unit of GAG heparin. X, X', and X''' –H or SO_3H, X'' –SO_3H or $COCH_3$. Both epimers can occur at * (L-iduronic or D-glucuronic acid).

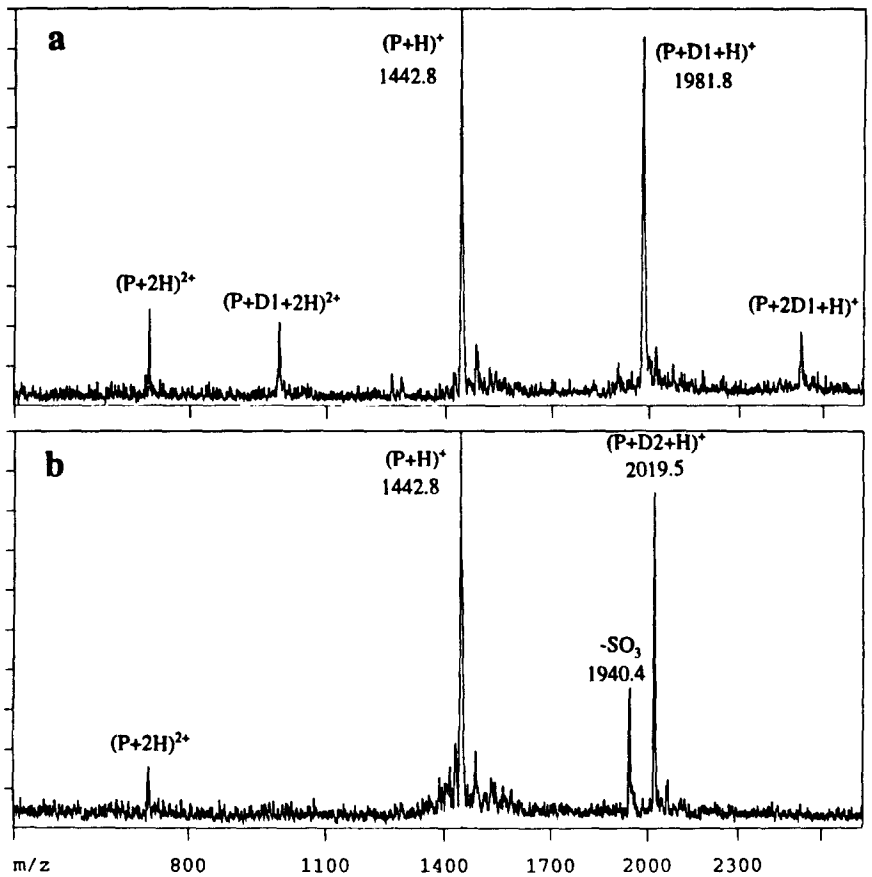


Fig. 1. IR-MALDI mass spectra of heparin disaccharides D1 and D2 mixed with the synthetic peptide SP1 (M_r = 1441.8). Matrix: 5-(trifluoromethyl)uracil. (a) Disaccharide D1 (M_r = 539.4), 7 laser shots averaged. (b) Disaccharide D2 (M_r = 577.4), 19 laser shots averaged. The lability of *N*-sulfate group(s) is obvious from spectrum (b). In these and all other spectra, the notation "P" stands for the peptide or protein identified in the legend.

observed [36]. The amino group of the glucosamine moiety is always acetylated, if not sulfated (see D1 in Table 2).

It turns out that ionic complexes of disaccharides are less easily detected than those with larger oligosaccharides, probably because the small number of sulfate groups is not sufficient to provide strong binding to the basic peptides. In UV-MALDI, with the exception of the trisulfated disaccharide D2, only very weak complex ion signals could be obtained. The best wavelength/matrix combination was determined to be 2.94 μm and TFMU matrix. The IR-MALDI mass spectra of the disaccharides D1 and D2 with synthetic peptide SP1 are shown in Fig. 1. The loss of the SO_3 group in the spectrum of D2 (Fig. 1b) must be from the *N*-linked sulfate since it is not observed if only *O*-linked sulfate groups are present, as is the case for disaccharide D1 (Fig. 1a). This finding is corroborated by data (not shown) for other disulfated disaccharides also containing

N-sulfate groups. Although IR-MALDI is claimed to be less sensitive than UV-MALDI [31], as little as 150 fmol of the disaccharide D2 could still be detected by the complexing technique with this basic peptide/matrix/wavelength combination. In these and all other spectra shown, the notation "P" stands for the peptide or protein identified in the figure legend.

Higher oligosaccharides.—With the increasing number of saccharide units and sulfate ester and sulfamino groups, binding to the basic peptides becomes stronger and the relative abundances of the protonated complexes increase relative to the $(M + H)^+$ ion of the peptide signal. On the other hand, the tendency to lose sulfate groups also increases, affecting now the *O*-linked SO_3 groups as well [27]. For instance, in IR-MALDI with TFMU matrix, intact (complex) molecular ions of higher oligosaccharides (T1, P1, or H1) could no longer be observed. Unfortunately, loss of one or more SO_3 moieties makes it difficult to determine directly the total number of *O*-linked or *N*-linked sulfate groups and to differentiate compounds of a unique degree of sulfation from mixtures of compounds which vary in this respect. Another conclusion drawn from the earlier results [27] was the unique importance of a high arginine content (such as SP1 in Table 1) for efficient complexing with components that contain a relatively large number of acidic groups. However, it was also observed that peptides with contiguous domains of arginines were less effective than those where the arginine residues were evenly distributed along the polypeptide chain.

Based on these observations and in order to optimize complexing efficiency for highly sulfated heparin fragments, peptides that combine high arginine content and backbone flexibility with the lowest possible molecular weight were most useful. Low molecular weight is desirable to keep that of the complex itself low and thus increases the accuracy of the mass difference. For this purpose, two peptides in which arginine (R) and glycine (G) alternate (SP2 and SP3 in Table 1) were synthesized. Apparently, for the basic components to desorb well with heparin oligosaccharides, the number of arginine residues on the peptide should be no less than the number of sulfate groups on the oligosaccharide (in the positive ion mode).

The choice of the matrix material also had to be optimized. Two known UV matrices, caffeic acid and 3-hydroxypicolinic acid, turned out to be the most efficient if used with basic peptides SP2 and SP3, with respect to the abundance of complex ions and the suppression of sulfate loss. 2,5-Dihydroxybenzoic acid was found to be as effective as caffeic acid. However, very basic peptides induced extensive photoadduct formation with the peptide, leading to several additional peaks spaced 315 Da apart. The identity of these adducts is not known. This feature interferes with the analysis of mixtures, therefore, the use of 2,5-dihydroxybenzoic acid was temporarily abandoned. Thus, 3-hydroxypicolinic acid is the matrix of choice with these peptides.

The efficiency of the complex formation method is illustrated in Figs 2 and 3. The former represents the best mass spectrum of the hexasaccharide H1 in the absence of the basic peptide. To obtain it, 100 pmol of the ammonium salt had to be loaded on the probe and ionized by IR irradiation. Even at this sample level, the signal-to-noise (S/N) ratio is very poor and extensive loss of NH_3 and SO_3 is observed. Obviously, such a mass spectrum cannot be used for accurate molecular weight measurement. Figure 3b and c present the UV-MALDI mass spectra of equimolar mixtures of hexasaccharide H1

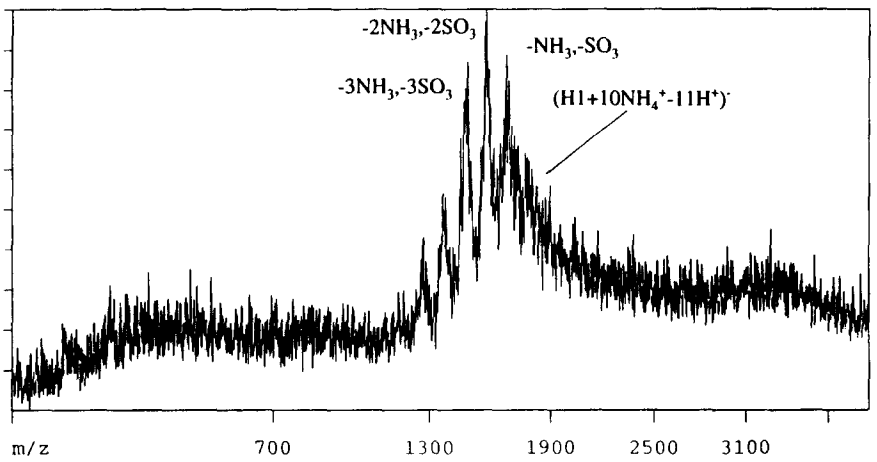
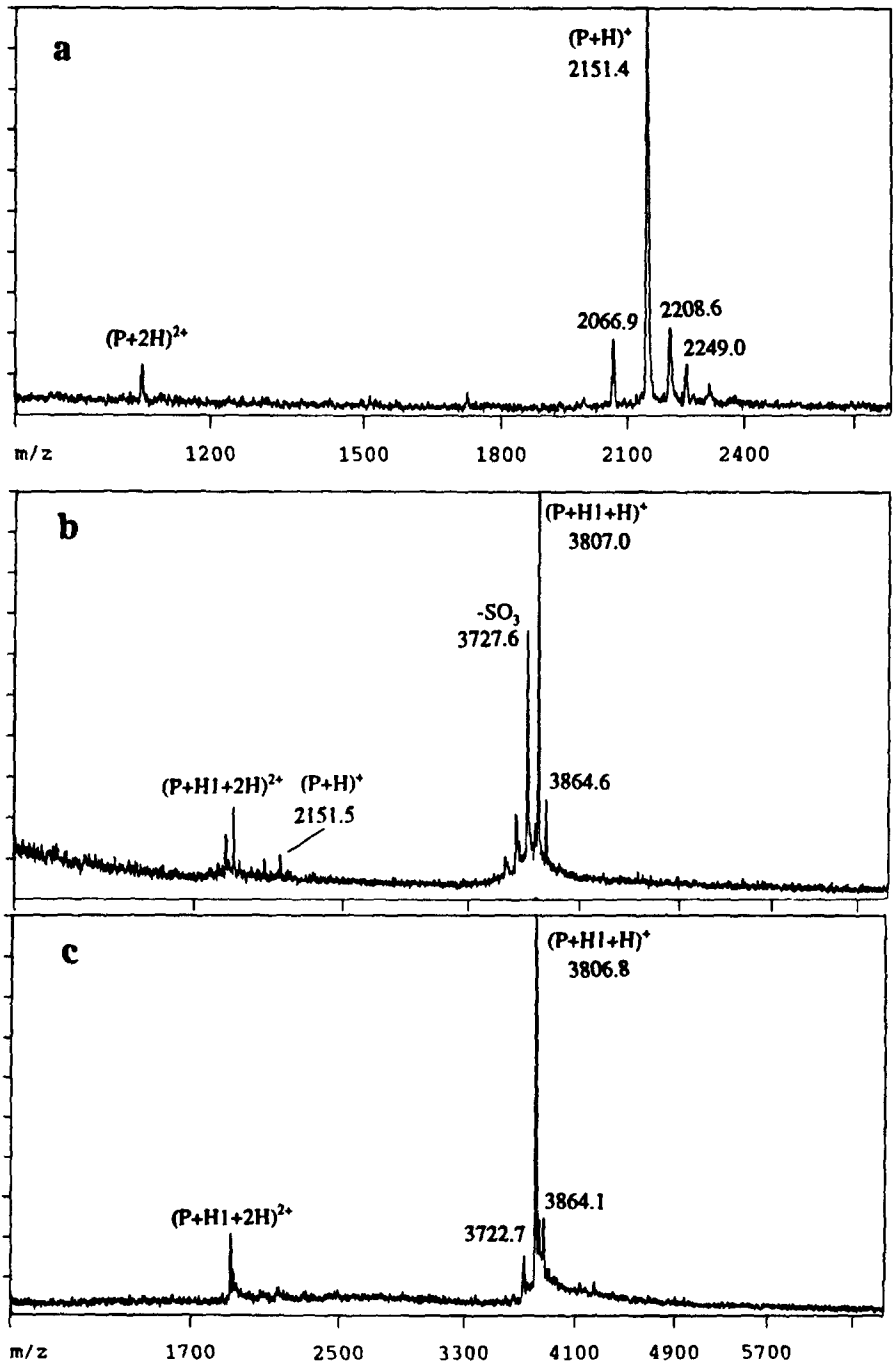


Fig. 2. Negative ion mode IR-MALDI mass spectrum of the ammonium salt of the hexasaccharide H1 (M_r = 1842.7 for the ammonium salt). Matrix: hydantoin, the spectrum is an average of 10 laser shots. This is the only wavelength/matrix combination by which signal (although with very poor signal-to-noise ratio) of the intact molecule could be obtained. The total sample load was 100 pmol.

and the basic peptide SP2 with caffeic acid (Fig. 3b) and 3-hydroxypicolinic acid (Fig. 3c) as matrices. Only 2 pmol and 1 pmol, respectively, of the sample were required and the S/N ratio is such that one order of magnitude less sample could still be analyzed. Although some sulfate loss with caffeic acid matrix is observed (Fig. 3b), the most abundant ion is the intact complex. External calibration yielded m/z 3807.5 for the protonated 1:1 complex, thus the measured molecular mass of H1 was 1656.1 Da, in good agreement with the theoretical value (1655.4 Da). The loss of sulfate ($-X-SO_3H \rightarrow -XH$; $\Delta = 80$ Da) is completely eliminated by the use of 3-hydroxypicolinic acid as the matrix. The peak pattern in Fig. 3c is the same as that observed for the peptide itself (Fig. 3a), which is due to the presence of by-products from the synthesis of SP2. The peak at m/z 2208.6 is due to a failure sequence with an additional glycine that adds 57 Da; m/z 2249.0 is an adduct of H_3PO_4 (98 Da) formed in the ionization process due to the presence of phosphate; and another contaminant 84 Da less than the major peak is as yet of unknown identity. The mass spectrum shown in Fig. 3c is an interesting example of how oppositely charged analytes mutually improve mass spectrometric sensitivity: 3-hydroxypicolinic acid matrix yields a poor MALDI spectrum of SP2 alone, no spectrum at all of H1 alone, but the complex ion of the components is generated easily.

Isolation and purification of a single heparin oligosaccharide component are extremely tedious [10], therefore, the capability of analyzing mixtures of sulfated oligosaccharides is important. Three components, tetrasaccharide T1, pentasaccharide P1, and hexasaccharide H1, were mixed and analyzed after adding the peptide SP2. In the mixture, the peptide concentration was 4 pmol/μL and approximately 1 pmol/μL of each oligosaccharide component (for T1 and P1, there is an uncertainty of a factor of two). A 0.5 μL volume of this solution was loaded onto the probe surface corresponding to approximately 0.5 pmol for each oligosaccharide component. In the MALDI mass spectrum with 3-hydroxypicolinic acid matrix (Fig. 4), all three components can easily


a
(P+H)+
2151.4
(P+2H)2+
2066.9
2208.6
2249.0
m/z
1200
1500
1800
2100
2400
b
(P+H1+H)+
3807.0
-SO3
3727.6
(P+H1+2H)2+
(P+H)+
2151.5
3864.6
c
(P+H1+H)+
3806.8
(P+H1+2H)2+
3722.7
3864.1
m/z
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3300
4100
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5700

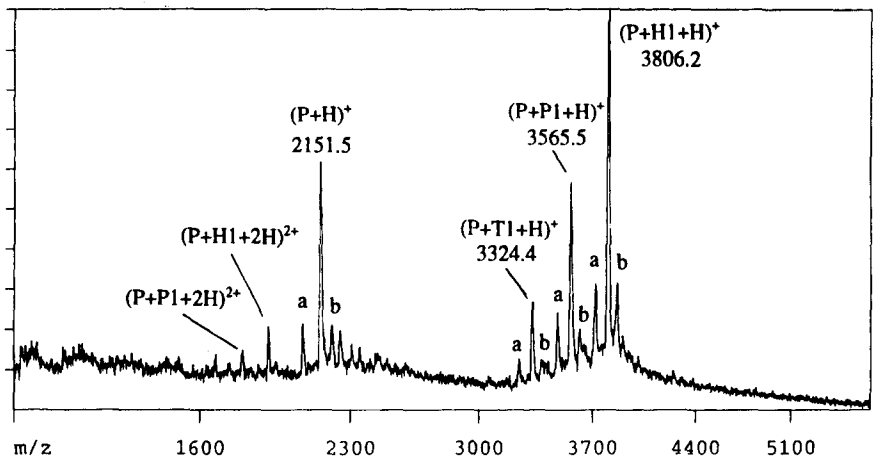

Fig. 4. UV-MALDI mass spectrum of a mixture containing three heparin-derived oligosaccharides: tetrasaccharide T1 ($M_r = 1173.0$), pentasaccharide P1 ($M_r = 1414.2$), and hexasaccharide H1 ($M_r = 1655.4$). The basic peptide was SP2. Total sample load was approximately 500 fmol for each oligosaccharide component and 1.5 pmol for the peptide. Matrix: 3-hydroxypicolinic acid. The satellite peaks labeled a and b are due to the contaminants in the peptide component (see Fig. 3a).

be detected in the presence of each other. Using external calibration molecular masses of the three protonated complex ions are 3324.4, 3565.5, and 3806.2 Da (the peaks labeled a and b are due to the contaminants in the peptide, as discussed earlier and shown in Fig. 3a). After subtracting the molecular weight of the protonated peptide, 1173.0, 1414.1, and 1654.8 Da result for the oligosaccharide components T1, P1, and H1, respectively. This is within $\pm 0.05\%$ of the expected values (compare with Table 2). Because of the regularity of the glycosaminoglycan structure, measurement of the molecular weight of oligosaccharides components (generated by well-characterized methods) to this level of accuracy allows one to unambiguously determine the number of saccharide units and the extent of sulfation and *N*-acetylation (approximately up to 4000 Da molecular mass).

In addition to the above mentioned uncertainty of the concentrations of T1 and P1, the relative intensities of the signals for these three complex ions seem to indicate a decreasing relative ionization efficiency of smaller sulfated oligosaccharides complexed with the same peptide. The spectrum shown in Fig. 4 is not meant to demonstrate quantitation of the components. This is generally difficult by MALDI, although quantitative analysis of neuropeptides by this method has been achieved under more controlled conditions [37].

Angiogenin as the basic protein component.—It was of interest to determine whether such complexes can also be observed with heparin-binding proteins. The most widely

Fig. 3. UV-MALDI mass spectra of: (a) synthetic peptide SP2 with caffeic acid as the matrix (for minor peaks, see text); (b) equimolar mixtures of the hexasaccharide H1 ($M_r = 1655.4$ for the free acid) and the synthetic peptide SP2 ($M_r = 2150.4$) with caffeic acid as matrix, total sample load 2 pmol; (c) same mixture, but with 3-hydroxypicolinic acid as matrix, total sample load 1 pmol, minor peaks are due to the impurities in the peptide (compare with panel a).

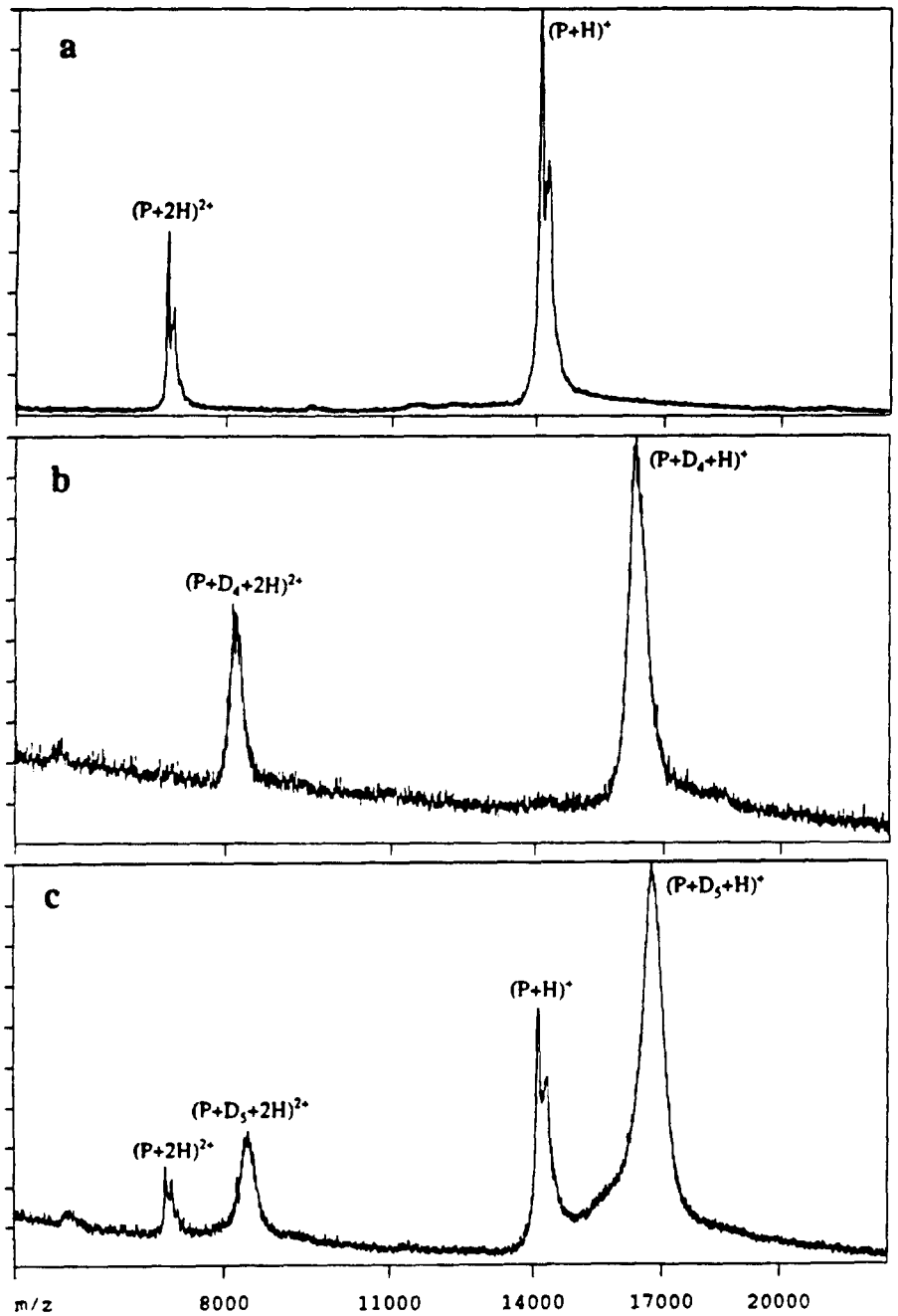

Fig. 5. Complexing of heparin fragments with angiogenin as the basic component (with sinapinic acid matrix at 337 nm irradiation). (a) Neat angiogenin (M_r = 14,120). (b) "Equimolar" mixture of angiogenin and the octasaccharide fraction, D_4. For the average molecular mass of the heparin fraction, 2192 Da was found. (c) "Equimolar" mixture of angiogenin and the decasaccharide fraction, D_5, average molecular mass: 2693 Da.

studied one, antithrombin III, is not very suitable for such experiments because this glycoprotein of 56 kDa has a carbohydrate portion comparable in size with the relevant heparin fragments investigated. Due to the high molecular weight of the glycoprotein and the carbohydrate inhomogeneity, accurate mass measurement based on the mass difference between that of the 1:1 complex and the protein cannot be expected. There are, however, low molecular weight, chemically homogeneous heparin-binding proteins that are better suited for this application.

Angiogenin, isolated from human tumor cells [38], also binds to heparin [39] and seemed to be ideal. As the name implies, this protein of 14.1 kDa molecular weight is an angiogenic factor and is capable of inducing blood vessel formation in chick embryo chorioallantoic membrane and the rabbit cornea. Its sequence has been determined by Edman degradation [40] and DNA sequencing as well [41]. The former indicated a blocked *N*-terminus, identified as pyroglutamic acid, which was confirmed by the MALDI mass spectrum using horse skeletal cytochrome *c* as internal standard. Recombinant angiogenin[35] gave a $(M + H)^+$ peak at m/z 14,122, in good agreement with the molecular mass of the protein assuming the *N*-terminus is pyroglutamic acid (14,120 Da calculated).

Larger heparin segments prepared by cleavage of GAG heparin with nitrous acid and followed by reduction and by two-step gel filtration fractionation were obtained from another laboratory. The individual fractions were believed to differ by additional disaccharide units. To measure their average molecular weights, angiogenin was used as the basic protein in the complex formation experiments. Because of the poorer ionization efficiency of proteins with 3-hydroxypicolinic acid, sinapinic acid was used instead as the matrix to generate the MALDI mass spectra, some of which are shown in Figs 5a–c and 6. Complex ions are abundant in these spectra, but due to the inhomogeneity of the heparin fractions and to the high molecular weight of the complexing protein, the

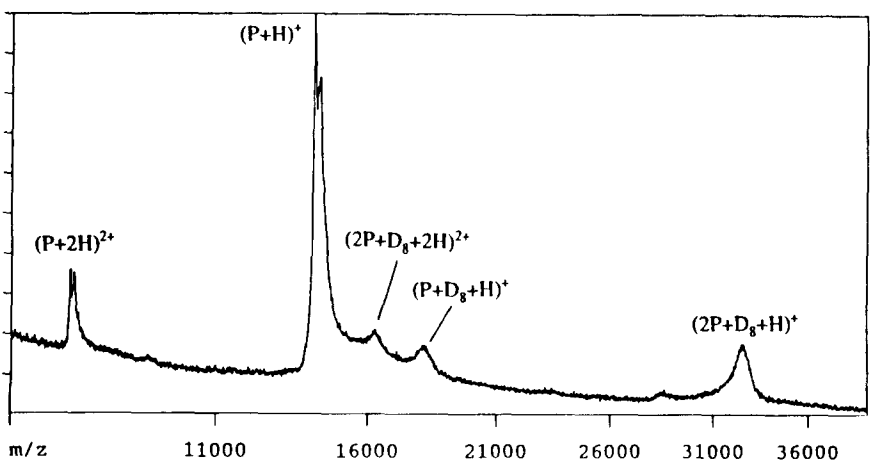

Fig. 6. UV-MALDI mass spectrum of a mixture of angiogenin and the hexadecasaccharide heparin fraction, D_8. Matrix: sinapinic acid. The preferred complex composition is 2:1 protein–oligosaccharide. The average molecular weight of the 2:1 complex distribution is 32,471, and after subtracting the contribution of the protein, 4230 is found for the oligosaccharide distribution.

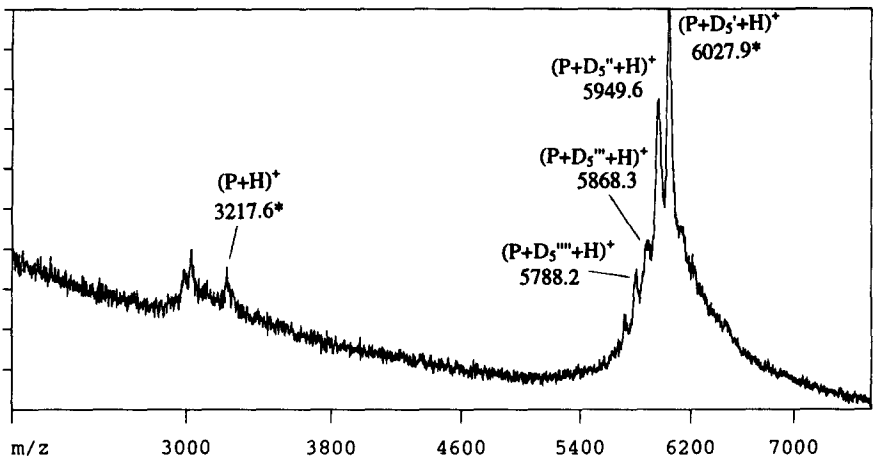

Fig. 7. UV-MALDI mass spectrum of the decasaccharide heparin fraction, D_5, mixed with the synthetic peptide SP3 (M_r = 3216.6). Matrix: 3-hydroxypicolinic acid. In this m/z range, individual heparin components can be resolved. The two most abundant heparin components correspond to decasaccharides with 14 (D_5') and 13 (D_5'') sulfate groups (M_r = 2810.3 and 2730.3, respectively), with all the glucosamine groups *N*-sulfated. For mass assignments, see text.

individual oligosaccharide components cannot be resolved. The average molecular masses of the protonated 1:1 complexes were determined either by external calibration or by internal calibration if the $(M + H)^+$ and $(M + 2H)^{2+}$ ions from angiogenin were also present in the mass spectrum. After averaging the results from 4–10 mass spectra, the protonated 1:1 complexes were found (in reverse order of elution by gel filtration) at m/z 16,313 ± 35, 16,814 ± 14 (Fig. 5b and c), 17,298 ± 35, and 17,731 ± 43 (spectra not shown), but the earliest fraction produced a more abundant 2:1 protein–heparin complex (Fig. 6) at m/z 32,471 ± 48. After subtracting the mass of the protonated protein (14,121 Da), we obtained 2192 ± 35, 2693 ± 14, 3177 ± 35, 3610 ± 43, and 4230 ± 48 Da, respectively (in the last case 28,241 Da was subtracted). It was concluded that this series of average values corresponds to 4, 5, 6, 7, and 8 disaccharide units, and these fractions are therefore denoted as D_4, D_5, D_6, D_7, and D_8. Theoretical molecular masses assuming the most abundant trisulfated disaccharide repeating units (with a terminal residue of 2,5-anhydromannitol-6-sulfate) are 2232.8, 2810.3, 3387.8, 3965.2, and 4542.7 Da. The discrepancy between experimental and theoretical values arises partly from lower degree of sulfation within a given fraction, and possibly from loss of SO_3 groups upon ionization.

In the case of the two smaller fractions, D_4 and D_5, it was possible to resolve the major oligosaccharide components using SP3 as the basic peptide and 3-hydroxypicolinic acid as the matrix. In Fig. 7 at least four components are observed for the fraction D_5. External calibration gave m/z 5800.3, 5880.3, 5962.5, and 6041.2. Subtracting the contribution of the protonated peptide (m/z 3217.6), the molecular masses of the four oligosaccharide components are 2582.7, 2662.7, 2744.9, and 2823.6 Da. The absolute mass differences of these are 80.0, 82.2 and 78.7 Da, respectively, clearly indicating the

presence of four components differing from each other by the number of SO_3 groups. From the molecular weights, one can conclude that these components are decasaccharides with 11, 12, 13, and 14 sulfation sites. However, the calculated molecular weights of these would be 2570.3, 2650.3, 2730.0, and 2810.3, respectively, indicating a systematic error of about +13 Da. Such a relatively large error can be due to the differences in irradiance used for the sample and the external calibration spectrum, or to the quite broad peak shapes, which do not permit resolving adducts of low mass such as H_2O, NH_3, or Na, although these were not observed with the more highly purified samples discussed earlier. If one assumes that the heaviest component is a decasaccharide with 14 sulfation sites (the maximum number possible for a decasaccharide, unless a hitherto not observed higher sulfation pattern for GAGs is present), internal calibration using this peak and the $(P + H)^+$ ion from the peptide results in 2570.6, 2650.7, and 2732.0 for the molecular weights of the other three components. These values are within 0.3 and 2.0 Da of the theoretical values calculated above for the sulfated, unacetylated species.

As the tetra-, penta-, and hexasaccharides with $(RG)_{10}$ [also with $(RG)_{15}$, data not shown] and 3-hydroxypicolinic acid as the matrix (Figs 3c and 4) do not show any sulfate loss, one can safely assume that this will also not occur for the complex of a decasaccharide and $(RG)_{15}$. Thus, Fig. 7 should reflect real inhomogeneity of the sample and not sulfate loss upon ionization. Calculation of the average molecular mass corresponding to the four resolved components in Fig. 7 (that is, weighing the abundances of the components with their molecular weights, summing, and properly normalizing) gives 2745 Da. This value is larger than the 2693 ± 14 Da, determined from the experiment with angiogenin (Fig. 5c). We conclude then, that the angiogenin complex may lose some sulfate upon ionization, and this loss increases with the number of sulfate groups present. This trend may be due in part to the fact that angiogenin has only 13 arginines. On the other hand, two angiogenin molecules contain 26 arginines, thus SO_3 elimination from the 2:1 complex is less pronounced.

Nevertheless, the data indicate that a protein-like angiogenin, which has a low arginine:molecular weight ratio, is not well-suited as the basic component for the analysis of mixtures of sulfated oligosaccharides. Its complexing under MALDI conditions should also not be expected to correlate with biological activity. For all these reasons, we are in the process of designing synthetic peptides that can be used for heparin fragments larger than decasaccharides, which seem to be the limit for $(RG)_{15}$ as the basic component.

4. Conclusions

Matrix-assisted laser desorption ionization mass spectrometry is useful for the molecular weight determination of heparin-derived oligosaccharides relying on ionic complexes formed with basic polypeptides. Highly sulfated oligosaccharides as such give very poor mass spectrometric response almost independent of the ionization technique. The extremely polar (ionic) character of these compounds renders it difficult to produce intact gas phase ions for mass spectrometric analysis. However, when the

anionic groups of the heparin oligosaccharides are shielded by a basic polypeptide, preferably containing a number of arginine residues exceeding the number of sulfate groups on the oligosaccharide, intact protonated complexes could be detected at a sensitivity three orders of magnitude higher. Tightly bound complexes of the heparin oligosaccharides and basic polypeptides also eliminated small cation adducts on the former, resulting in more reliable mass measurement. Well-characterized, chemically homogeneous samples (and mixtures thereof) containing up to six saccharide units and up to eight sulfate groups were studied. Loss of sulfate groups, a common problem in the mass spectrometry of heparin oligosaccharides, could be eliminated by using synthetic peptides of the composition $(RG)_n$, where $n = 10$ or 15, and 3-hydroxypicolinic acid (with 10% α-D-fucose) as the matrix. A mass accuracy of $\pm 0.05\%$ or better was obtained from low picomole or sub-picomole amounts of these chemically homogeneous samples, as well as of inhomogeneous fractions (up to decasaccharides) obtained by gel filtration after partial depolymerization of heparin. Spectra of fractions containing up to sixteen saccharide units were obtained at the same level of sensitivity (picomoles or less) but with somewhat lower mass accuracy.

The mass accuracy of the complex formation technique using the synthetic peptides $(RG)_{10}$ and $(RG)_{15}$ is sufficient to deduce the number of saccharide units and the extent of sulfation, at least if the origin and the method of preparation of the oligosaccharide are known. Therefore, if combined with enzymatic/chemical degradation steps and powerful separation techniques (e.g., capillary electrophoresis), this method may make significant contributions to sequencing of such highly sulfated glycosaminoglycans, as heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, and keratan sulfate.

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