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Glycosaminoglycans as Naturally Occurring Combinatorial Libraries: Developing a Mass Spectrometry-Based Strategy for Characterization of Anti-Thrombin Interaction with Low Molecular Weight Heparin and Heparin Oligomers

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Heparin is a densely charged polysaccharide, which is best known for its anticoagulant activity, although it also modulates a plethora of other biological processes. Unlike biopolymers whose synthesis is strictly controlled by a unique genetic template, heparin molecules exhibit a remarkable degree of structural heterogeneity, which poses a serious challenge for studies of heparin−protein interactions. This analytical challenge is often dealt with by reducing the enormous structural repertoire of heparin to a model small molecule. In this paper, we describe a different approach inspired by the experimental methodologies from the arsenal of combinatorial chemistry. Interaction of anti-thrombin III (AT) with heparinoids is studied using a mixture of oligoheparin molecules of fixed degree of polymerization, but varying chemical composition (heparin hexasaccharides obtained by size exclusion chromatography of an enzymatic digest of porcine intestinal heparin with bacterial heparinase), as well as a heparin-derived pharmaceutical preparation Tinzaparin (heparin oligosaccharides up to a 22-mer). AT binders are identified based on the results of ESI MS measurements of complexes formed by protein−oligoheparin association. Additionally, differential depletion of free heparin oligomers in solution in the presence of AT is used to verify the binding preferences. ESI MS characterization of oligoheparin−AT interaction under partially denaturing conditions allowed the conformer specificity of the protein−polyanion binding to be monitored. A model emerging from these studies invokes the notion of a well-defined binding site on AT, to which a flexible partner (heparin) adapts to maximize favorable intermolecular electrostatic interactions. This study demonstrates the enormous potential of ESI MS as an analytical tool to study the interactions of highly heterogeneous glycosaminoglycans with their cognate proteins outside of the commonly accepted reductionist paradigm, which reduces the intrinsic complexity of heparin by using structurally defined homogeneous low molecular weight mimetics.

Heparin is a densely charged polysaccharide, which is best known for its anticoagulant activity, although it also modulates a plethora of other biological processes by interacting with numerous proteins.[1] Heparin belongs to a large class of polysaccharides, collectively known as glycosaminoglycans (GAG), whose other members are heparan sulfate, keratan sulfate, dermatan sulfate, chondroitin sulfate, and hyaluronate. GAG molecules are primary components of the cell surface and the extracellular matrix interface, where they interact with numerous proteins and modulate their activity, playing a major (although not always understood) role in cell growth, morphogenesis, and development.[2] This obviously places a premium on the ability to discern the structure−function correlations in this class of polysaccharides,[2] and so does the need to exploit them for therapeutic purposes.[3]

Although heparin (Hp) chains feature a limited number of building blocks (Scheme 1), the genetic control of their biosynthesis is indirect and involves a set of enzymes rather than a single template. Consequently, heparin molecules exhibit a remarkable degree of structural heterogeneity, which poses a serious challenge for structural studies, thus greatly complicating attempts to elucidate structure−function relations. This analytical challenge is often dealt with by reducing the enormous structural repertoire of heparin to a single model small molecule, whose structure is precisely defined. This "reductionist" approach essentially ignores the intrinsic heterogeneity of heparin and its self-evident biofunctionality. The inhomogeneity of GAGs in general can be visualized by recognizing for example that heparin fractions prepared by either size exclusion, ion exchange, or affinity chromatography can productively be fractionated by either of the other two techniques. Such heterogeneity along multiple dimensions—known as a "complexity distribution" for synthetic polymers—stimulates the development of nonclassical bioanalytical methods capable of dealing with such extreme analyte complexity.[4]

Mass spectrometry (MS) is rapidly gaining popularity in this field due to its unrivaled ability to provide a wealth of structural information on the components of highly heterogeneous systems. While electrospray ionization (ESI) MS alone is capable of

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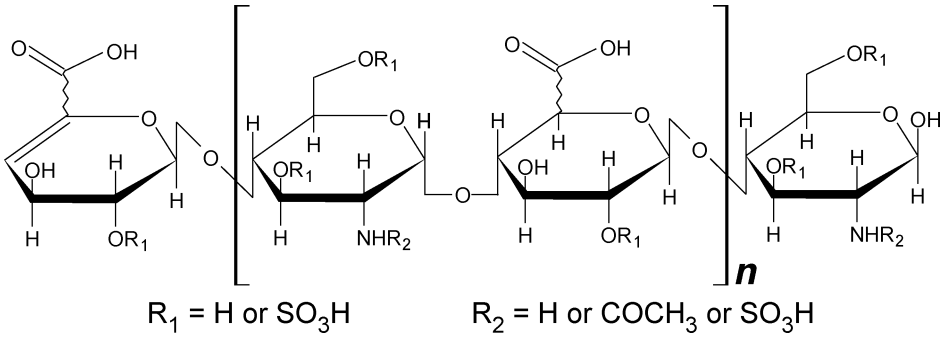
Scheme 1



providing meaningful information on short-chain heparin oligomers up to a decasaccharide level,[5] its combination with either size exclusion chromatography (SEC)[6−8] or ion-pairing reversed-phase HPLC[9,10] results in a dramatic expansion of both size and diversity of heparin oligomers (and, more generally, GAGs) amenable to characterization. Furthermore, methods of tandem mass spectrometry provide a means to obtain sequence information on individual heparin chains.[11−13] While collision-activated dissociation has been shown to be extremely useful for obtaining sulfate group distribution patterns within the GAG chains, as it typically favors glycosidic bond cleavages, other methods of ion activation may provide valuable complementary information. For example, Amster et al. demonstrated that electron detachment dissociation gives rise to abundant cross-ring cleavages, which allow GlcA and IdoA be distinguished in short-chain heparan sulfate oligosaccharides.[14] Finally, a recent report by Barran and co-workers suggests that the ability to make a clear distinction among various isomeric building blocks of heparin (or GAG) chains is likely to be further enhanced by using ion mobility in the gas phase as an orthogonal dimension to the MS analysis.[15]

While the extreme heterogeneity of intact heparin chains inevitably complicates MS analysis of their interactions with biological partners, several groups have been successful in using various MS-based strategies to probe protein-binding properties of short GAG chains. Robinson et al. used ESI MS to evaluate the stoichiometry of a complex formed by fibroblast growth factor, its receptor, and heparin fragments with defined length.[16,17] Orlando and co-workers used Fourier transform ion cyclotron resonance (FT ICR) mass spectrometry to monitor hyaluronan interactions with a tumor necrosis factor-related protein and detected protein binding to an octa- (but not tetra-) saccharide chain.[18] Leary et al. employed FT ICR MS to monitor oligomerization of small polypeptides (chemokines) coupled to GAG binding.[19]

The enormous structural repertoire of native heparin (and other GAG) chains inevitably invokes the notion of a "natural" library, akin to synthetic combinatorial libraries.[20] While the natural diversity of GAG molecules can inspire generation of synthetic libraries using the elaborate tools of organic chemistry,[21] a much easier (and more physiologically relevant) approach to generating "organic" GAG libraries utilizes enzymatic methods. For example, Leary and co-workers used mass spectrometry to screen a small heparin oligosaccharide library with limited structural variation (prepared by heparinase digestion of intact heparin chains followed by isolation of a decamer fraction by SEC) for high-affinity octameric species by observing their binding to small polypeptides from the chemokine family.[22]

Heparin and heparin-derived therapeutics play a major role in treatment and management of thrombotic and cardiovascular disorders[23] by interacting with a variety of target proteins including anti-thrombin (AT), renin, myosin ATPase, and many others. The understanding of the corresponding structure−function relationships has been heavily influenced by numerous studies of the interaction of AT with model heparinoids. The adoption of this paradigm has emerged in large part because the complexity and diversity of native heparin chains makes their structural analysis appear to be an unrealistic goal.[4]

In this work, we adapted an experimental approach inspired by combinatorial chemistry to characterize AT interaction with short-chain heparin oligomers (heparin hexamer oligosaccharides obtained by SEC fractionation of an enzymatic digest of porcine intestinal heparin with bacterial heparinase), as well as a low

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molecular weight heparin-derived therapeutic, Tinzaparin.[24] We demonstrate that AT binding-competent heparin oligomers can be identified in both cases, providing valuable information on the distribution of negative charges along the heparin chain required for efficient protein−GAG interaction. Furthermore, ESI MS provides a means to monitor the binding processes in a conformation-specific fashion under conditions when multiple protein folding states are populated under equilibrium. While the surviving natively folded AT molecules retain their heparin-binding properties, the unfolded polypeptide chains are binding-incompetent. Therefore, nonspecific electrostatic interaction between highly flexible polyanionic (heparin) and polycationic (unfolded AT) chains is negligible and a highly organized heparin-binding site on the surface of the natively folded protein is required for strong binding. A model emerging from these studies invokes the notion of a rigid template (AT) to which a flexible partner (heparin) adapts to maximize the electrostatic attraction.

EXPERIMENTAL SECTION

Materials. Hexameric heparin fragments dp6 (produced by treatment of porcine liver heparin with bacterial heparinase followed by fractionation with size exclusion chromatography) was purchased from Medipol (Geneva, Switzerland). Therapeutic-grade low molecular weight heparin (commercial anticoagulant Tinzaparin) was generously provided by Dr. Jens Henriksen of LEO Pharma A/S (Ballerup, Denmark). All heparin samples were used as supplied without any further purification. Human anti-thrombin III was a gift of Prof. Frank Church, University of North Carolina (Chapel Hill, NC). The protein sample was desalted and reconstituted in 10 mM ammonium acetate by ultrafiltration with a 5-kDa MW cutoff (Millipore, Billerica, MA). All solvents and salts were of analytical grade or higher.

Methods. Electrospray ionization mass spectra of dp6 and Tinzaparin, AT, and its mixtures with the heparinoids were carried out using a QSTAR-XL (Applied Biosystems/MDS-Sciex, Toronto, Canada) hybrid quadrupole/time-of-flight mass spectrometer at a nominal resolution of 13 000. Heparin ion dissociation in the interface region was avoided by using very mild ion desolvation parameters (DP = 25−30 V, Q0 = 15 V, RO2 = 9.8 V, DP2 = 10 V). Mass distribution of protein-bound ligands was obtained from the ESI MS data using a linear deconvolution routine with a user-defined basis function (MS profile of a ligand-free protein ion) in Origin 6.0 (OriginLab Corp., Northampton, MA).

Exact mass measurements of various species in the dp6 sample were carried out with an APEX-III (Bruker Daltonics, Billerica, MA) Fourier transform ion cyclotron resonance mass spectrometer equipped with a 4.7-T magnet using internal calibration at a 80 000 mass resolution. Heparin ion dissociation was avoided by using the following set of parameters in the ion source: capillary exit, 50 V; skimmer 1, 100 V; skimmer 2, 60 V; offset potential, 10 V. Renin substrate (monoisotopic mass 1757.925 33 u), bradykinin fragment 2−9 (903.459 73 u), and melittin (2844.730 36 u) were used as internal calibrants.

RESULTS AND DISCUSSION

Identification of Principal Components in the "Natural Library" of Short Heparin Oligomers. ESI MS of the oligoheparin sample dp6 obtained both in the positive (Figure 1) and negative (Figure 2) ion modes highlight its structural heterogeneity by showing a distribution of several species with varying degrees of sulfation. Regardless of the polarity and the number of charges carried by the oligomer ions in the gas phase, the hexameric species carrying eight sulfate groups and no acetyl groups (i.e., (6, 8, 0) in the Roepstorff−Henriksen nomenclature[7]) give rise to the most abundant ionic signal. Assignment of individual peaks in the negative ion mode is very straightforward based on the nominal masses of detected ions. Since the detected oligomers have significant levels of sulfation, the isotopic patterns of each anionic species can be used to provide additional confirmation of its identity due to high natural abundance of ^{33}S and ^{34}S isotopes (see the inset in Figure 2).

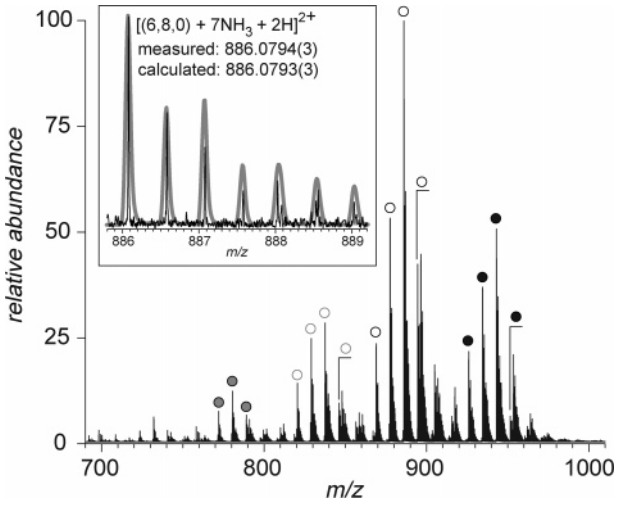

Figure 1. ESI mass spectrum (positive ion mode) of dp6 (0.015 mg/mL in 10 mM ammonium acetate) showing the region of doubly charges ions. Peaks marked with circles represent NH_3 adducts of (6, *N*, 0) species, where *N* ranges from 6 (gray-shaded circles) to 9 (black). Inset shows a high-resolution MS data used to obtain empirical formulas of the principle species by measuring their exact masses (gray trace shows medium-resolution MS data used to obtain nominal masses).

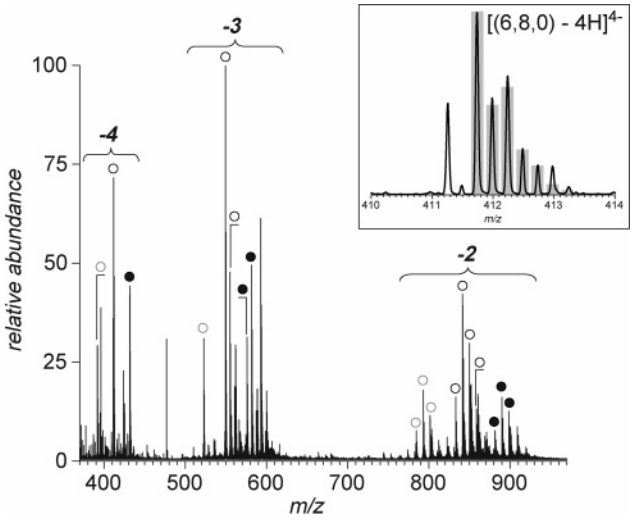

Figure 2. ESI mass spectrum (negative ion mode) of dp6 (0.015 mg/mL in 10 mM ammonium acetate). Inset shows measured (black trace) and calculated (gray bars) isotopic distribution for a tetraanionic species (6, 8, 0).

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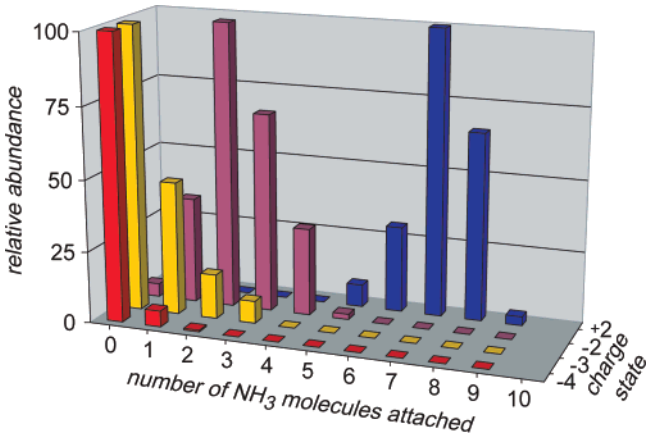

Figure 3. Extent of NH_3 adduct formation for ions representing the (6, 8, 0) species in the negative and positive ion modes.

As the number of negative charges is reduced, the appearance of the ionic signal becomes more complicated due to the emergence of ammonium adducts (see Figure 3). The extent of adduct formation becomes particularly significant in the positive ion mode, where the nominal mass of the (6, 8, 0) dications reveals the presence of at least five NH_3 molecules. While the extent of adduct formation in ESI MS can often be reduced by using mild collisional activation in the interface region, the labile nature of covalent bonds attaching sulfate groups to the polysaccharide backbone[7] may cause their fragmentation in the gas phase as a result of the declustering procedure. Because of this concern, characterization of free heparin oligomers was carried out under the mild conditions in the ESI interface to eliminate the possibility of ion dissociation in the gas phase.

Extensive adduct formation components in the positive ion mode makes identification of the principal components not very straightforward when based solely on the nominal mass. Therefore, peak assignment was carried out using exact mass measurements, which were shown in the past to be a powerful tool for the quality control analyses of small synthetic molecule combinatorial libraries.[25] The high resolving power and the superior mass accuracy afforded by FT ICR MS easily allow the ionic masses to be measured with nearly 0.1 ppm accuracy (see inset in Figure 1). However, even this accuracy is not sufficient to establish a unique empirical formula for a molecule whose mass approaches 2 kDa, especially when Na and K are included as potential cationizing agents contributing to the total mass of the ion. To circumvent this difficulty, we restricted the search to a subset of candidates whose empirical formulas do not conflict with the hexamer template structure shown in Scheme 1. The empirical formula of this template is $C_{36}H_{45}O_{30}N_3$, and the allowed subset of hexamers included those that could be built on this template using a combination of up to 12 H atoms, up to 12 SO_3H groups, and up to 3 $COCH_3$ groups to fill the vacancies indicated with R_1 and R_2 in Scheme 1. Also allowed was an unlimited number of Na and K atoms and NH_3 molecules. Finally, a provision was made for a possible hydroxylation of the hexamer at the reducing end, as proposed earlier by Roepstorff and Henriksen.[7] Once these limitations were imposed, 5 empirical formulas out of the initial list of 1094 gave masses falling within 1.0 ppm of the exact mass for the peak at m/z 886 (inset in Figure 1), and only 2 allowed formulas agreed with the measured exact mass to within 0.2 ppm. One of these structures corresponded to a (6, 8, 0) species carrying two protons (H^+) and seven noncovalently attached NH_3 molecules (measured m/z ratio 886.0794(3) versus calculated 886.0793(3) for a dication). Similar analysis was carried out for all major peaks in the positive ion spectrum, allowing in many cases their confident identification.

Binding of Heparinoid Oligomers to Anti-Thrombin under Near-Native Conditions. One of our ultimate goals is to develop MS-based assays to probe interaction of heparin, its derivatives, and analogues with its biological partners, which are represented here by AT. Although binding to AT is not heparin's sole raison d'etre, obvious clinical importance of this particular interaction made it a paradigm for heparin−protein binding. AT−Hp has been extensively studied in the past two decades using a variety of techniques, although a complete understanding is still lacking. One of the issues still being debated in the research community is the existence of structurally precisely defined AT-binding domain(s) within the heparin chain, akin to well-defined binding interface regions in proteins. The search for such a domain was catalyzed by the obvious interest from the pharmaceutical community and resulted in synthesis of a pentasaccharide motif (shown in black in Scheme 2), which is often referred to as the "active domain" of heparin.[26] While some structural variations of the original pentasaccharide structure do not have an adverse effect on its interaction with AT and may in fact increase the binding affinity, the notion of the heparin active domain had become widely accepted and indeed culminated in a recent approval of a synthetic antithrombotic drug Arixtra in both the E.U. and United States.[26]

One of our immediate objectives was to search for AT binders in the pool of short heparin fragments and see if their chemical compositions are consistent with the inclusion of the pentasaccharide fragment shown in Scheme 2, which is often considered to be the AT-activating domain of heparin and was used as a blueprint for designing Arixtra.[26] To achieve this objective, we employed an experimental strategy inspired by MS-based experimental tools from the arsenal of combinatorial chemistry.[27] In addition to becoming a potent tool for characterization of small combinatorial libraries (vide supra), ESI MS can also provide a means to screen them with respect to affinity toward specific targets.[28,29] Binding preferences are usually determined by measuring mass shifts of target protein ions and relating these changes to masses of specific library members. While this approach works well for small homogeneous proteins, additional complications presented by AT are its relatively large size (nearly 60 kDa) and significant extent of glycosylation, which is almost always synonymous with structural heterogeneity.

Glycosylation patterns of AT derived from human plasma were studied extensively by Rizzi et al., who determined that carbohy-

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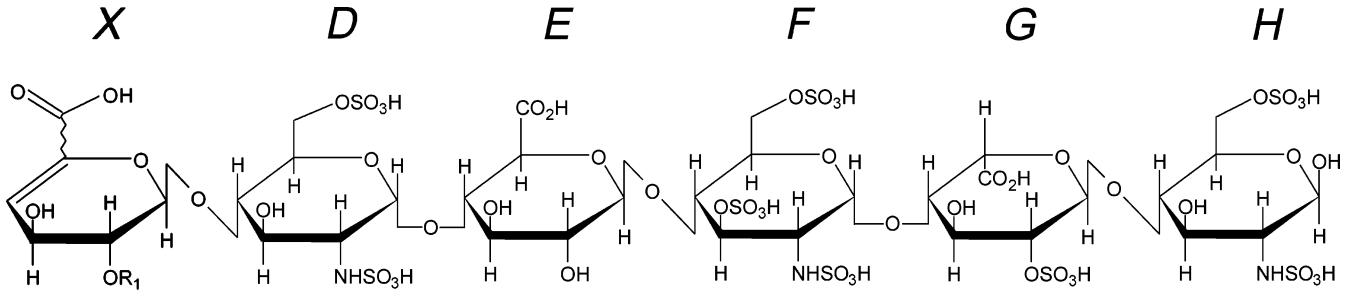
Scheme 2



drates contribute ~15% to the protein total mass (58 kDa) and do introduce inevitable heterogeneity.[30−32] The ESI mass spectra of human AT obtained in this work under near-native conditions (Figure 4) are consistent with these conclusions and also provide evidence of AT dimerization even at modest concentration (10 μM). The latter observation is not surprising, given the high dimerization propensity of AT.[33] Addition of dp6 to the AT solution gives rise to the appearance of peaks corresponding to ions whose mass exceeds that of AT (black trace in Figure 4). Another notable change is the disappearance of the protein dimer peaks from the spectrum. Although polyanions such as GAGs are frequently found associated with the amyloid deposits and have in fact been implicated in catalyzing aggregation processes linked to Alzheimer-type pathologies,[34,35] the heparin hexamer is too short to be an affective template for aggregation. It seems likely that the hexamer binding reverses (or at least neutralizes) the positive charge of the heparin-binding domain of AT. Since association of proteins at low ionic strength near pI is often driven by their positive domains,[36] such charge neutralization would obviously have a negative effect on the ability of AT to form homodimers. A similar effect has been observed in the ability of heparin or dextran sulfate to suppress the aggregation of fibroblast growth,[37] keratinocyte growth factor,[38] and heat-denatured RNAse.[39]

Since the apo-AT ion peaks have a convoluted shape due to the heterogeneity caused by glycosylation (vide supra), measuring

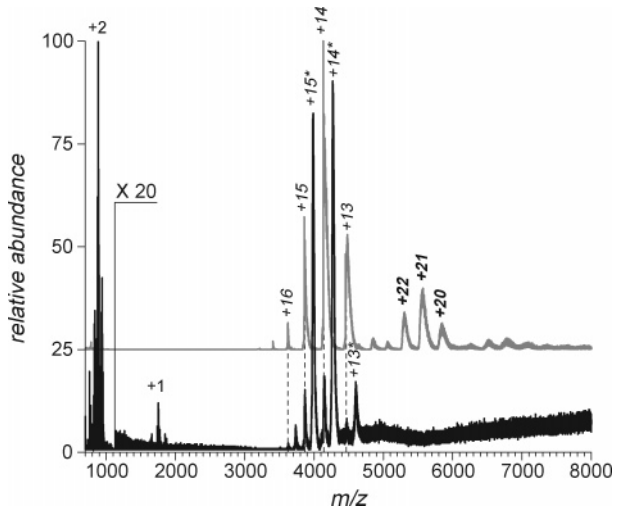


Figure 4. ESI mass spectra (positive ion mode) of AT (10 μM in 10 mM ammonium acetate) in the absence of dp6 (gray trace) and in the presence of 0.05 mg/mL dp6 in solution. Charge states for various ionic species are labeled in italics (apo-AT), italics with asterisks (holo-AT), bold italics (AT dimer), and normal type (unbound heparin oligomers).

m/z shifts alone upon mixing AT and dp6 may not be sufficient to determine the masses of heparin oligomers bound to the protein. Mass distribution of AT binders was obtained by deconvoluting the peak shape of the holoprotein ions (marked with asterisks in Figure 4) using the peak shape of the apo-AT ions. The protein−heparin fragment mixture was prepared in such a way that a fraction of AT remained ligand-free. The shape of the corresponding peak (m/z region 4130−4150 in Figure 5) represents the protein mass distribution function $\mathbf{X}(\xi)$ (signal intensity $\mathbf{X}$ plotted vs m/z values ξ). The peak shape of the ligand-bound protein can be thought of as a shifted and smeared image of $\mathbf{X}(\xi)$:

$$\tilde{\mathrm{X}}(\xi) = \sum_N L_\mathrm{N} \mathrm{X}\left(\xi - \frac{M_\mathrm{N}}{Z}\right) \qquad (1)$$

where M_N represent the set of masses of ligands bound to the protein, L_N are intensities of such complexes (relative to the apoprotein ion), and Z is the charge state of the ion used for this analysis (Z = 14 in Figure 5). The smearing is caused by summation of a set of shifted peaks, where the magnitudes of shifts are determined by the M_N-to-Z ratios.

A set of optimal pairs (M_N, L_N) can be found by running a standard optimization routine to fit the experimentally measured profile of the holoprotein ion, as illustrated in the inset in Figure 5. In the case of heparin hexamers, only two masses are required to fit the experimental data using eq 1, 1651 ± 2 Da and 1731 ± 2 Da, which correspond to heparin fragments (6, 8, 0) and (6, 9, 0). Another abundant hexamer, (6, 7, 0) does not appear to bind to the protein under the conditions tested. Interestingly, inclusion

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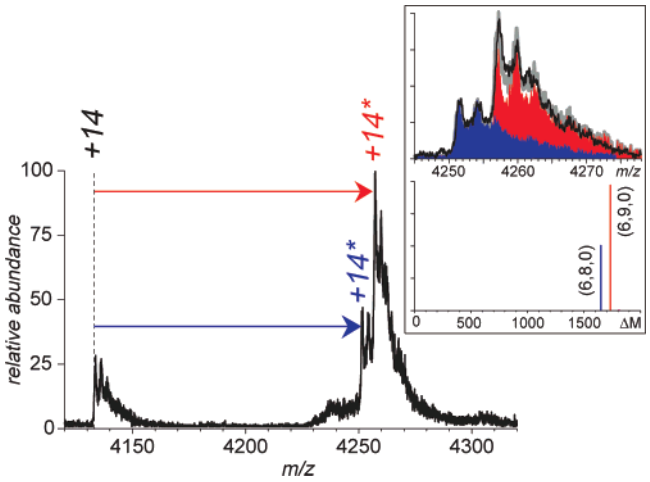

Figure 5. Extraction of masses of binding-competent heparin hexamers from ESI mass spectrum of AT/dp6 mixture. Main panel shows the detailed view of the +14 ion peaks representing both apo- and holoforms of the protein. The top graph in the inset illustrates the deconvolution procedure (see main text for more information), and the bottom diagram presents the calculated mass distribution of AT binders.

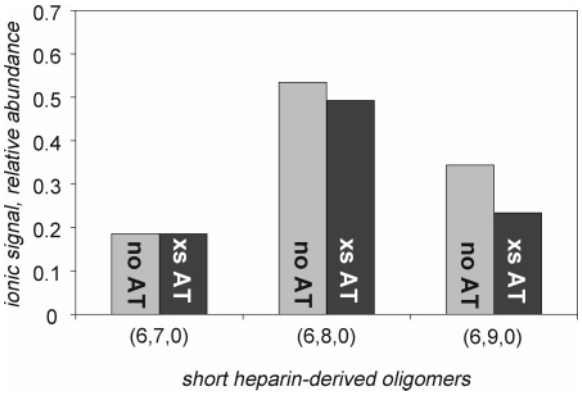

Figure 6. Total ion signal distribution for the three principal species in dp6 in the absence (gray bars) and the presence (black bars) of AT in solution. The latter distribution was normalized with respect to the former using the total ionic intensity of the binding-incompetent (6, 7, 0) species.

of the high AT-affinity pentasaccharide structure within a hexameric heparin fragment places significant restrictions on its levels of sulfation and acetylation (Scheme 1). Specifically, only the (6, 8, 0) and (6, 9, 0) species can contain the high AT-affinity pentasaccharide, both of which bind to AT, as is clearly suggested by deconvolution of the ESI MS data (Figure 5).

Analysis of Free Heparin Oligomer Depletion as a Result of AT Binding. Since the heparin hexamer/AT binding assay was carried out with excess protein (as indicated by the presence of the apoprotein peaks in ESI MS), analysis of the distribution of unbound hexamer species (seen in the m/z range below 2000 in Figure 4) may also provide valuable information vis-à-vis binding preferences. Ionic signal intensity distributions of the three abundant hexamer species are shown in Figure 6. The gray bars represent the total ionic signals for each species in the absence of AT (as seen in Figure 1), and the black bars represent the intensity distribution of the same species in the presence of excess AT. Since the total amount of the bound heparin species cannot be directly determined from the ESI MS data, the latter distribution was normalized with respect to the AT-free distribution using the intensity of the binding-incompetent (6, 7, 0) heparin hexamer. A significant proportion of both (6, 8, 0) and (6, 9, 0) species remain unbound to the protein, most likely representing hexamers with the requisite levels of sulfation, but unfavorable spatial distribution of the sulfate groups along the oligosaccharide backbone.

Although it is clear from both the ligand depletion analysis (Figure 6) and the results of ESI MS deconvolution (Figure 5) that the largest fraction of AT-bound hexamers comprises (6, 9, 0) species, one should not conclude that the extra sulfation at the nonreducing end (unit X in Scheme 2) increases the AT affinity of the hexamer. The AT−heparin hexamer binding experiment carried out in this work is NOT a competition assay, as a fraction of the protein molecules remain ligand-free. Therefore, all binding-competent heparin fragments are expected to associate with AT, regardless of the relative binding affinities. The higher propensity of the (6, 9, 0) species to bind to AT simply points to the fact that distribution of 9 sulfate groups among 12 possible sulfation sites on the hexamer is more likely to produce the binding-competent pattern, compared to distributing 8 sulfate groups within the same template (there are 495 possible ways to distribute 8 sulfate groups among 12 sites in the structure shown in Scheme 1 for $n = 2$, and only 220 possibilities for 9 sulfate groups). Therefore, the distribution of negative charges on the GAG template must play a critical role in the protein−heparin oligomer recognition process.

Characterization of Oligoheparin−AT Binding in a Conformer-Specific Fashion. The results of ESI MS characterization of oligoheparin−AT interaction under near-native conditions provide clear indication that the high extent of sulfation of a heparin hexamer is not synonymous with AT binding competence. Therefore, the distribution of the sulfate groups along the hexasaccharide chain must be an important determinant of the binding strength, as opposed to an association event driven exclusively by nonspecific electrostatic interactions. It is possible to argue, however, that the contribution of such nonspecific electrostatic interaction would increase dramatically if both interacting partners were sufficiently flexible so that the electrostatic attraction could be maximized by conformational adaptation during the association, similar to the zip mechanism of polyanions−polycation interaction.[40] Such adaptations become especially important when polyanions bind to proteins at pH > pI, as here. In such cases, the lowest energy-bound configuration can represent a balance between the attractive interaction between the polyanion and the locally positive electrostatic protein domain, on the one hand, and repulsive interactions between more distal polyanion segments and the globally negative protein on the other.[41]

In order to explore the role of such nonspecific interactions, heparin hexamer/AT binding was monitored under conditions that convert a large fraction of the protein from a folded state to a flexible polycationic chain. Since AT pI is in the 4.8−5.1 range, acidification of protein solution to pH 3.0 was expected to produce a polycation. While acidification of a protein solution generally results in unfolding, several conformations may coexist under

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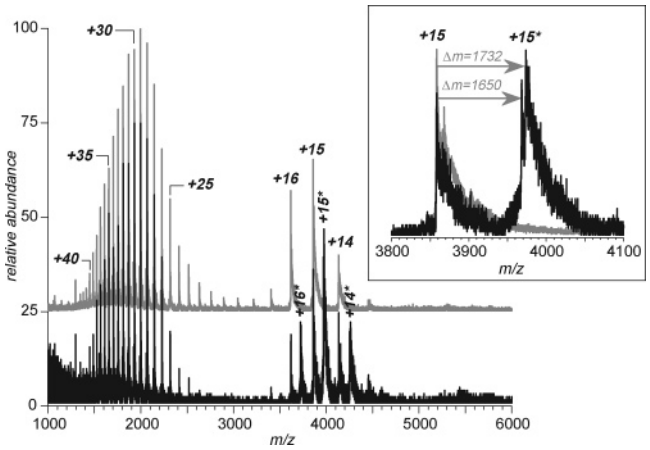

Figure 7. ESI mass spectra (positive ion mode) of 10 μM AT under partially denaturing conditions (pH 3.0) in the absence of dp6 (gray trace) and in the presence of 0.04 mg/mL dp6 in solution. Charge states for various ionic species are labeled in italics (apo-AT) and italics with asterisks (holo-AT).

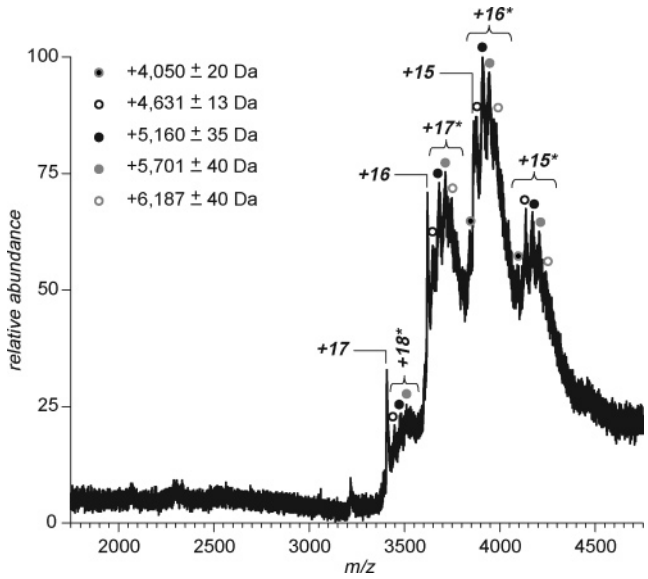

Figure 8. ESI mass spectrum (positive ion mode) of AT (10 μM in 10 mM ammonium acetate) in the presence of 0.06 mg/mL Tinzaparin in solution. Charge states for various ionic species are labeled in italics (apo-AT) and italics with asterisks (holo-AT).

equilibrium. Therefore, a meaningful analysis of AT−heparin interaction under such conditions can be carried out only if the protein is conformationally homogeneous (e.g., completely unfolded) or else if there is a means to monitor the protein−ligand interaction in a conformer-specific fashion.

The unique feature of ESI MS is that it allows distinct protein states to be detected and characterized based on the analysis of protein ion charge-state distributions under equilibrium conditions,[42,43] as well as when they are populated kinetically.[44] In the past, this feature allowed a range of binding processes to be studied in a conformer-specific fashion, ranging from protein interactions with small ligands[45−48] to formation of multiunit protein assemblies.[49−51] In this work, charge-state distribution analysis of AT ions was used in conjunction with AT/heparin hexamer binding monitoring as a means to characterize the protein−polyanion interaction under denaturing conditions in the conformer-specific fashion. ESI MS provides clear evidence of AT partial unfolding as a result of solution acidification, as suggested by a bimodal appearance of the protein ion charge-state distribution (gray trace in Figure 7).

Addition of dp6 to the AT solution under these conditions does not fundamentally change the appearance of the high charge-density part of the protein ion charge-state distribution (charge states +22 through +40), clearly suggesting that the (partially) unfolded AT conformers are incapable of binding short heparin fragments. However, the surviving native conformers of AT (represented by low charge-density ions, charge states +14 through +16) retain their heparin-binding properties. Importantly, peak shape analysis of protein ions in this m/z region indicates that (6, 8, 0) and (6, 9, 0) are the binding species, as was the case under the near-native conditions (Figure 5).

These results suggest that a nonspecific electrostatic interaction between highly flexible polyanionic (heparin) and polycationic (unfolded AT) chains is negligible and a highly organized heparin-binding site on the surface of the natively folded protein is required for strong binding. A model emerging from these studies invokes the notion of a well-defined template (AT) to which a flexible partner (heparin) adapts to maximize the electrostatic attraction.

AT Interaction with Low Molecular Weight Heparin (LMWH): Tinzaparin. While monitoring heparin hexamer fragments binding to AT highlights the unique capabilities of ESI MS that will be essential for characterization of protein−heparin interactions, the structural heterogeneity exhibited by the dp6 sample is very modest compared to the enormous repertoire displayed by the intact heparin chains. In order to evaluate the utility of ESI MS for these analyses, we attempted to characterize AT interaction with unfractionated Tinzaparin. Tinzaparin is a therapeutic LMWH with antithrombotic properties, which inhibits reactions leading to blood clotting. As most other therapeutic LMWH preparations, Tinzaparin activates AT and enables it to bind to and subsequently inactivate both factor Xa and thrombin. It has been shown to be at least as safe and effective as intact heparin and was approved for treatment of acute symptomatic deep-vein thrombosis both in the E.U. and the United States.[24]

The ESI mass spectrum of the AT/Tinzaparin mixture acquired under near-native conditions is presented in Figure 8. As was the case with the AT/dp6 interaction, the relative amounts of the protein and the heparin derivative were selected to ensure a slight excess of the protein (as manifested by the appearance of free protein peaks in the mass spectrum). The mass spectrum provides a clear indication that a range of species with varying masses exist within the Tinzaparin preparation that are capable of binding to AT. Unfortunately, the ion peaks corresponding to the protein−

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Table 1. Long-Chain (Tetradecasaccharide and Above) Heparin Species from Tinzaparin and Their Ability To Bind AT

species	av mass (calcd)	presence in Tinzaparin as identified by Roepstorff et al.[7] by SEC/ESI MS: measd mass	presence in Tinzaparin as identified by Roepstorff et al.[7] by SEC/ESI MS: rel abund, % of most abund peak in the fraction	AT-binding competent species, measd mass (this work)
(14,16,1)	3684	3683	38	
(14,17,1)	3764	3763	76	
(14,18,0)	3802	3801	35	
(14,18,1)	3844	3843	31	
(14,19,0)	3882	3881	86	
(14,19,1)	3924	3923	39	
(14, 20, 0)	3962	3961	100	
(14, 21, 0)	4041	4042	53	4050 ± 20
(16,18,1)	4181	4180	47	
(16,18,2)	4223	4221	41	
(16,19,1)	4262	4261	66	
(16,19,2)	4304	4303	36	
(16,20,1)	4342	4341	79	
(16,21,0)	4380	4379	52	
(16,21,1)	4422	4420	71	
(16,22,0)	4460	4459	100	
(16,22,1)	4502	4499	35	
(16,23,0)	4540	4540	80	
(16,24,0)	4621	4620	41	4631 ± 13
(18,21,1)	4759	4760	53	
(18,22,1)	4839	4837	68	
(18,23,0)	4877	4878	31	
(18,22,2)	4881	4878	31	
(18,23,1)	4919	4917	100	
(18,24,0)	4957	4955	39	
(18,24,1)	4999	4999	54	
(18,25,0)	5037	5037	46	
(18,26,0)	5117	5117	57	
(18,27,0)	5194		not detected	5160 ± 35
(20,29,0)	5695		not analyzed	5701 ± 40
(20,30,0)	5775		not analyzed	
(22,31,0)	6188		not analyzed	6187 ± 40
(22,32,0)	6268		not analyzed	

heparin derivative complexes partially overlap with the peaks of the apoprotein ions, making it impossible to obtain the mass distribution of AT binders using the deconvolution procedure applied earlier to characterize AT−dp6 interaction. Instead, masses of the holoprotein ions were calculated using a standard deconvolution routine for multiply charged ions in ESI MS. Masses of AT-bound Tinzaparin species were then calculated by subtracting a mass of the most abundant AT glycoform (57 858 Da). Overall, five masses of AT binders have been calculated, the highest of which corresponds to a heparin chain containing 22 saccharide units (docosamer). AT binders were identified by checking their masses against the list of various Tinzaparin species obtained recently by Roepstorff et al. using a combination of SEC and ESI MS,[7] and the results are presented in Table 1. The comparison suggests that, within each set of heparin oligomers of a given chain length, the AT binders have the highest level of sulfation (Figure 9). The apparent failure of heparin oligomers with lower levels of sulfation to form complexes with AT despite availability of free protein in solution suggests that the probability of having a requisite sulfation pattern within such species is very low.

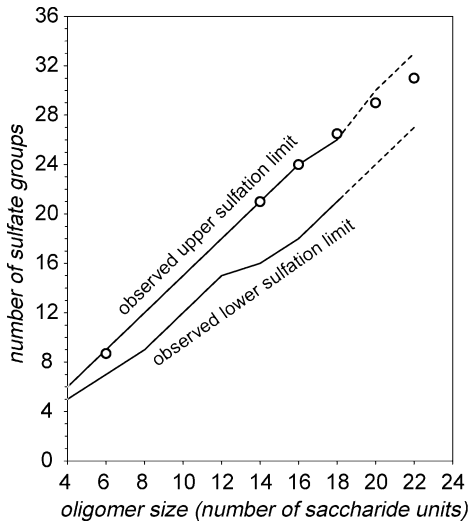

Figure 9. Sulfation level of dp6 and Tinzaparin species whose binding to AT is detected by ESI MS (see Figures 4 and 8) as a function of the heparin oligomer chain length. The region between the two lines corresponds to the heparin oligomer species detected in Tinzaparin by Roepstorff et al.[7]

CONCLUSIONS

The experimental methodology presented in this work allows heparin preparations of low and medium levels of complexity to be screened with respect to their affinity toward large glycosylated proteins. While the ESI mass spectra of oligoheparin preparations highlight their structural heterogeneity, species represented by only few distinct masses (all of them highly sulfated and completely nonacetylated) bind to AT under near-native conditions in solution as determined by ESI MS. While the high extent of sulfation is not synonymous with AT binding competence, the sulfation levels of binding-competent heparin hexamers are consistent with the presence of the high AT-affinity pentasaccharide within such species. Although detailed structural characterization of AT binding-competent heparin oligomers remained outside of the scope of the present work, such studies can certainly be made possible by complementing the experimental approach presented here with tandem MS.

ESI MS also allows oligoheparin−AT interaction to be characterized in the conformer-specific fashion by monitoring the protein−polyanion binding under partially denaturing conditions. While the surviving native conformers of AT retain their heparin-binding properties under acidic conditions, the unfolded polypeptide chains are binding-incompetent. Therefore, nonspecific electrostatic interaction between highly flexible polyanionic (heparin) and polycationic (unfolded AT) chains is negligible and a highly organized heparin-binding site on the surface of the natively folded protein is required for strong binding. A model emerging from these studies invokes the notion of a structurally well-defined binding template (AT) to which a flexible partner (heparin) adapts to maximize the electrostatic attraction.

This work demonstrates the enormous potential of ESI MS as an analytical tool to study the interactions of highly heterogeneous biopolymers with their biological partners outside of the commonly accepted reductionist paradigm, which reduces heparin complexity by using homogeneous mimetics.

Direct ESI MS measurements provide information on AT binding to heparin chains up to 22 saccharide residues long. Although intact (unprocessed) heparin chains were not a subject of this work, it appears almost certain that ESI MS, especially in combination with various separation techniques, will become a powerful tool to study interactions of such highly heterogeneous systems with a variety of their biological targets without reducing their complexity to physiologically irrelevant levels.

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