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Antithrombin III-Binding Site Analysis of Low-Molecular-Weight Heparin Fractions

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ABSTRACT

Low-molecular-weight heparins (LMWHs) are widely used as clinical anticoagulant drugs. LMWHs are heterogeneous and highly negatively charged glycans prepared by chemical or enzymatic depolymerization of unfractionated heparin. The detailed structural analysis of a LMWH is essential for the drug quality control. In this study, an LMWH, enoxaparin sodium (a generic version of Lovenox) was separated into different molecular weight fractions by a Superdex peptide column. The disaccharide compositions, 3-*O*-sulfo group–containing tetrasaccharides composition, and antithrombin III-binding affinity of the fractions from this LMWH were analyzed. The results showed that all the fractions had very similar disaccharide and 3-*O*-sulfo group–containing tetrasaccharide compositions, but the fraction containing larger-sized chains had higher antithrombin III-binding affinity.

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Introduction

Heparin and low-molecular-weight heparin (LMWH) derivatives are complex mixtures of sulfated glycosaminoglycan chains, which are widely used as anticoagulants.[1] Heparin has an average molecular weight of ~16,000 Da, whereas LMWH has an average molecular weight of ~4000 to ~8000 Da.[2] Both heparin and LMWH have a major repeating trisulfated disaccharide unit (TriS) of the structure [→4) α-L-IdoA2S (1→4) α-D-GlcNS6S (1→]n, where IdoA is iduronic acid, GlcN is glucosamine, and S is sulfo.[1] They also contain pentasaccharide sequences that represent their antithrombin III (AT)-binding site responsible for most of their anticoagulation activity.[1] Currently, LMWHs are the most widely used anticoagulant drugs (e.g., LMWHs hold ~70%, whereas unfractionated heparin [UFH] holds ~30% of the heparin market share in the United States).[3] This preference is principally due to LMWHs having better bioavailability, longer half-lives, and reduced adverse effects, such as heparin-induced thrombocytopenia.[4]

Although heparin was discovered in 1916 and has been studied for over 100 years, additional biological activities of heparin are being discovered each year.[5,6] All the structures within heparin are still unknown and, thus, the structure-activity relationship of heparin chains are also not fully understood. Because heparin and LMWHs are polydisperse mixtures of poly/oligosaccharide chains with sequence heterogeneity, a multifaceted approach is required for the analysis of their chemical structure. The methodology, developed in our laboratory and by others, has converged on 4 major analytical aspects: (1) disaccharide compositional analysis, (2) oligosaccharide mapping of domain structures, (3) nuclear magnetic resonance for fine structural analysis and domain mapping, and (4) analysis of protein-binding sites corresponding to heparin activity. Recently, we developed top-down and bottom-up approaches for the structural characterization of LMWH products using liquid chromatography-mass spectrometry (LC-MS) and capillary electrophoresis-MS.[7-13] The goal of the present study is to understand how the disaccharide composition, the composition of 3-*O*-sulfo group–containing tetrasaccharides, and the AT-binding affinity of LMWH vary as a function LMWH chain size.

Experimental Section

Materials

The LMWH selected for the study was 4 different lots of enoxaparin sodium injections (a generic version of Lovenox™) from

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Teva Parenteral Medicines (Irvine, CA). Unsaturated disaccharide standards of HS (ΔUA-GlcNAc [0S]; ΔUA-GlcNS [NS]; ΔUA-GlcNAc6S [6S]; ΔUA2S-GlcNAc [2S]; ΔUA2S-GlcNS [2SNS]; ΔUA-GlcNS6S [NS6S]; ΔUA2S-GlcNAc6S [2S6S]; and ΔUA2S-GlcNS6S [TriS]), where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid, S is sulfo, and Ac is acetyl, were from Iduron (Manchester, UK). Recombinant *Flavobacterium heparinum* heparinase I, II, and III were expressed in our laboratory using *Escherichia coli* strains that were gifts of Dr. Jian Liu (University of North Carolina). Pharmaceutical heparin samples were obtained from the United States Pharmacopeia (USP). Tetrasaccharide standards (ΔUA-GlcNAc6S-GlcA-GlcNS3S; ΔUA-GlcNAc6S-GlcA-GlcNS3S6S; ΔUA-GlcNS6S-GlcA-GlcNS3S6S; ΔUA2S-GlcNAc6S-GlcA-GlcNS3S6S; ΔUA2S-GlcNS6S-GlcA-GlcNS3S6S) were prepared in our laboratory as previously described.[14]

Fractionation of LMWH With Different Molecular Weights

Fractions of LMWH having different average molecular weights were prepared by separation on a Superdex peptide column (31 × 1 cm) followed by HPLC-gel permeation chromatography (GPC) eluted with 0.2 M ammonium carbonate at a flow rate of 0.4 mL/min. Refractive index detection was used, and fraction samples were collected by the flowing out time. Buffer salt was removed by lyophilizing 3 times.

Top-Down Analysis of LMWH Fractions

Top-down LC-MS analysis method was based on our previously developed approach.[10] Briefly, a hydrophilic interaction liquid chromatography (HILIC) column (2.0 × 150 mm^2, 200 Å; Phenomenex, Torrance, CA) was used to separate the LMWH fractions into their oligosaccharide and polysaccharide components. This separation used 2 mobile phases. Mobile phase A was 5 mM ammonium acetate, and mobile phase B was 5 mM ammonium acetate in 98% acetonitrile. After injection of an LMWH fraction (8.0 μL, 1 mg/mL), a linear gradient of mobile phase A ($t_{0\text{-}40}$ min 10%-35%) was applied with a flow rate of 150 μL/min. The effluent from the HILIC column was electrosprayed into an LTQ-Orbitrap XL FT-MS (Thermo Fisher Scientific, San Jose, CA). The optimized parameters were spray voltage of 4.2 kV, capillary voltage of −40 V, tube lens voltage of −50 V, capillary temperature of 275°C, and sheath flow rate and an auxiliary gas flow rate of 30 and 6 L/min, respectively. A mass accuracy of better than 3 ppm was afforded through external calibration.

MS Data Processing

DeconTools (web source from PNNL at OMICS. PNL.GOV) was used for charge deconvolution. Web-based GlycReSoft 1.0 (http://code.google.com/p/glycresoft/downloads/list) was used to assign oligosaccharide structures using parameters: minimum abundance of 1.0; minimum number of scans of 1; molecular weight lower boundary of 500 Da; molecular weight upper boundary of 6000 Da; mass shift of ammonium; match error of 5.0 ppm; grouping error of 80 ppm; and adduct tolerance of 5.0 ppm. Identification of LMWH components relied on a theoretical database generated by GlycReSoft 1.0 using the following parameters: A, ΔHexA = 0 or 1; B, HexA = 0 − 12; C, HexNAc = A + B − 1 to A + B + 1; D, Ac = 0 − 5; E, SO_3 = B to A + B + (C * 2) + 1 − D; modification, adduct = ammonium from 0 to 14. Anhydro-component identification was performed by adding one extra water loss to A to generate the theoretical andydro-database keeping the other parameters the same.

Heparinase Digestion of LMWH Fractions

LMWH fraction samples (200 μg) in 100 μL of 50 mM sodium phosphate buffer (pH 7.4) were digested by heparin lyase II (20 mU) at 37°C for 4 h. The digestion solutions were heated in a 100°C water bath for 15 min to denature the enzyme and terminate the digestion. The disaccharides and tetrasaccharides were obtained by centrifugation for 15 min (8000 × *g*).

Disaccharide and Tetrasaccharide Composition

Disaccharide and tetrasaccharide composition were analyzed by reversed-phase ion-pairing−HPLC-MS on an Agilent 1200 LC/MSD system (Agilent Technologies, Wilmington, DE) equipped with a Poroshell 120 C18 column (2.1 × 100 mm, 2.1 μm). This separation used 2 mobile phases. Mobile phase A was water/acetonitrile (85:15, v/v), 12 mM tributylamine, 38 mM ammonium acetate, pH 6.5, and mobile phase B was water/acetonitrile (35:65, v/v), 12 mM tributylamine, 38 mM ammonium acetate, pH 6.5. The gradient used was based on our previous studies.[8,15] Briefly, LC conditions for disaccharide composition analysis used a gradient of eluent A for 8 min, followed by a linear gradient of 0% to 80% eluent B from 8 to 15 min and maintained at 80% eluent B from 15 to 25 min. LC conditions for tetrasaccharide compositional analysis relied on a gradient of eluent A for 2 min, followed by a linear gradient of 0% to 30% eluent B from 2 to 40 min and 30% to 60% eluent B from 40 to 50 min, was used at a flow rate of 100 μL/min. The column effluent entered the source of the electrospray ionization−MS for continuous detection by MS. Electrospray was set in negative ionization mode at a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source temperature of 350°C, and maximum abundance of the ions were obtained in a full-scan spectrum (200−1500 Da). Nitrogen was used as a drying (8 L/min) and nebulizing (40 psi) gas. The 3-*O*-sulfo group−containing tetrasaccharides were quantified using calibration curves, prepared with tetrasaccharide standards. The linear response curve of MS peak intensity as a function of concentration (in triplicate) was obtained.

Surface Plasmon Resonance Analysis for AT-Binding Affinity

Competition surface plasmon resonance (SPR) (Biacore 3000; GE Healthcare, Uppsala, Sweden) was used to test the AT-binding affinity of the different LMWH fractions.[16] USP heparin was biotinylated and immobilized on the SA chip (GE Healthcare). AT (50 nM) was premixed with different LMWH fractions (5 μg/mL) in HBS-EP buffer (0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid, and 0.005% surfactant P20, pH 7.4). These mixtures were injected over the heparin chip each at a flow rate of 30 μL/min at 25°C. After each run, the sensor surface was regenerated by injecting with 30 μL of 2 M NaCl and 30 μL of running buffer. AT solution (50 nM without LMWH) was flowed across the chip's surface as a control experiment.

Results and Discussion

The Preparation of LMWH Fractions With Different Molecular Weights

LMWH was fractioned by Superdex peptide column, which is well suited for fractionating linear polysaccharides of the chain size range found in heparin and LMWH. In the GPC chromatogram (Fig. 1), different molecular weight LMWH did not show a symmetrical distribution as was observed in USP (unfractionated) heparin. The peaks were pooled into 4 fractions based on retention time and labeled as fraction 1, fraction 2, fraction 3, and fraction 4. The

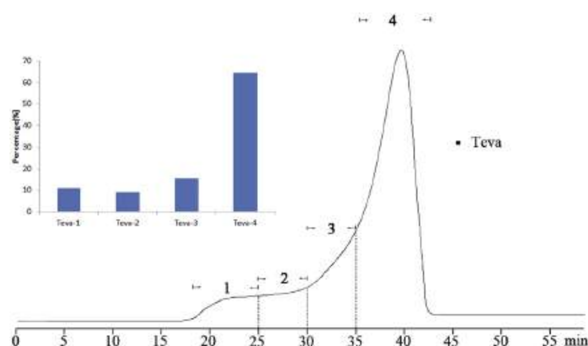

Figure 1. GPC chromatogram of Teva LMWH on Superdex peptide column and the content of the 4 fractions, which were separated based on the retention time.

contents of LMWHs of the 4 fractions were determined by carbazole assay.[17] The mass ratio of fraction 1, fraction 2, fraction 3, and fraction 4 was approximately 11%, 9%, 16%, and 64%, respectively.

Molecular Weight Profiles of LMWH Fractions

LMWHs produced from heparin are mixtures of polysaccharides and oligosaccharides. The direct characterization of LMWHs by determining their molecular weight variation using GPC provides little information because GPC is relatively insensitive in discriminating compositional differences in LMWHs. In this study, we applied the top-down method, which had been successfully used to profile LMWHs structures,[9] to directly analyze the molecular weight distribution and composition characteristics of the fractions of LMWH having different size. This top-down method, based on our HILIC-FT-electrospray ionization-MS platform, used to characterize LMWH products is simple, reliable, requires no special sample preparation steps, has high ionization efficiency, and can avoid loss of labile sulfo groups.

The top-down analysis showed that the components in the major fraction (fraction 1) from the LMWH mostly were even-numbered chains ranging from dp8 to dp20 containing 2 to 3 sulfate groups/disaccharide (Fig. 2). These components were relatively uniformly distributed. Oligosaccharides (dp12-16) were the major components. As the molecular weight increased, the complexity of the oligosaccharides increased because the oligosaccharides with the same dp would have more sulfate groups and *N*-acetyl groups. The components in fraction 1 contained 0 to 2 *N*-acetyl groups. The sulfo group content decreased as the GlcNAc content increased in the component chains. For example, in dp16 without a GlcNAc, there were 21-24 sulfo groups. The sulfo groups decreased to 17 from 24 when one GlcNAc residue was present and decreased to 15 from 19 when 2 GlcNAc residues were present. The components with 1,6 anhydro reducing end also showed an increased abundance in fraction 1.

Fraction 2 had components similar as that of fraction 1 (Fig. S1). The components below the length of dp14 were most abundant and dp8 was particularly prominent. The polysaccharide chains in the range of dp16-10 were decreased in fraction 2. Fraction 3 had prominent oligosaccharide chains from dp7 to dp16 (Fig. 3). Chains of dp10-14 were particularly prominent. In general, the abundance of components in fraction 3 was higher than in fraction 1 and fraction 2. For the oligosaccharides of the same dp, the ratio of sulfo groups/disaccharide was close to 3. But when a ratio of 3 was reached, the abundance of these chains decreased. Fraction 4 (Fig. S2) was the major LMWH fraction, accounting for more than a half of the total content. The oligosaccharide components ranged from dp4 to dp8. The most prominent component was dp6. Among the 4 fractions, fraction 4 was most abundant, but this fraction showed less compositional variability. Compared with fraction 1, the components with 1,6 anhydro reducing end were dramatically reduced in fraction 4.

Disaccharides Analysis of LMWH Fractions

Disaccharides analysis (Fig. 4) of all 4 LMWH fractions showed comparable compositions. Some minor differences in disaccharide

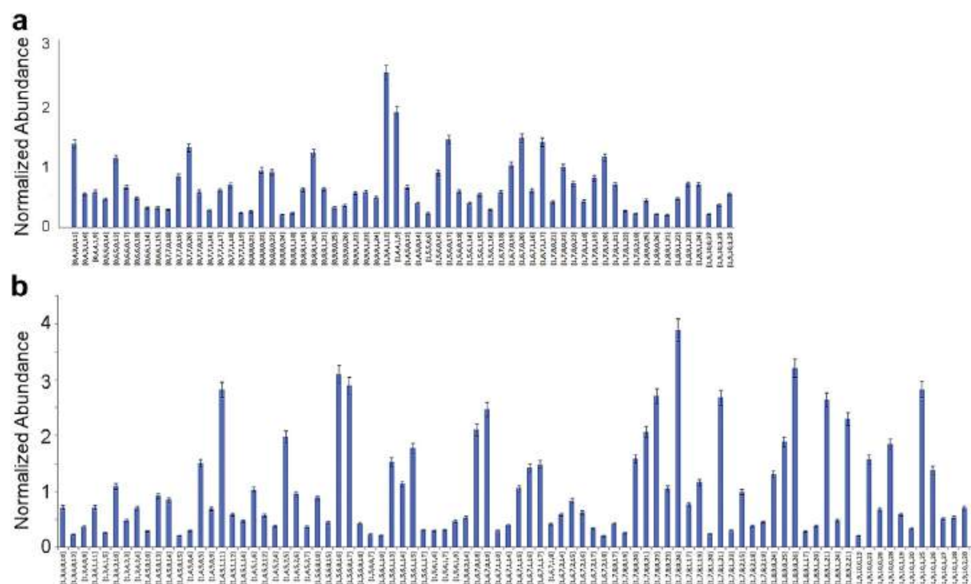

Figure 2. Identified LMWH oligosaccharides from Teva-1; (a) heparin oligosaccharides and (b) anhydro-heparin oligosaccharides. Compositions are given as ΔHexA, HexA, GlcN, Ac, SO_3; error bars are the SDs from triplicate analyses.

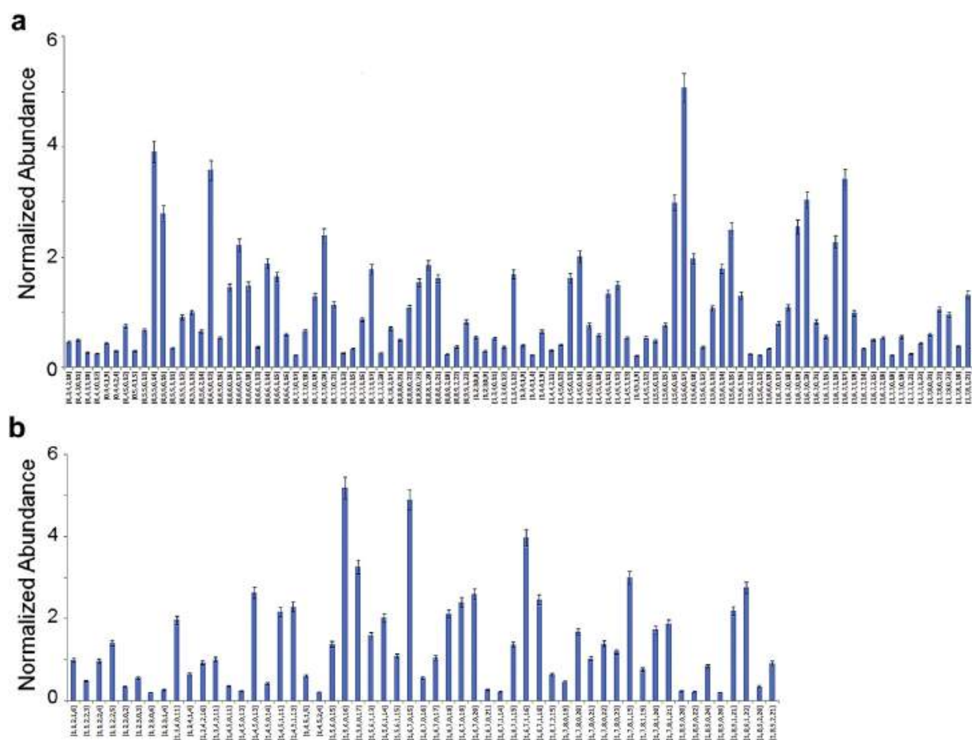

Figure 3. Identified LMWH oligosaccharides from Teva-3; (a) heparin oligosaccharides and (b) anhydro-heparin oligosaccharides; error bars are the SDs from triplicate analyses.

composition were observed as the chain molecular weight decreased. TriS fell slightly from 71.3% to 68.5% and NS2S content increased, from 5.7% to 8.2%, with decreasing molecular weight. The amount of remaining 6 disaccharide components showed no significant differences.

3-O-Sulfated Tetrasaccharide Analysis of LMWH Fractions

Heparin's anticoagulant activity depends on its interaction with AT, one of most well-studied carbohydrate-protein interactions.[18] Heparin-AT binding depends on a special pentasaccharide sequence in heparin that contains a 3-*O*-sufo group in its central GlcN residue. LMWHs prepared through the controlled depolymerization of pharmaceutical were depolymerized through exhaustive treatment with the enzyme heparin lyase II. It has been established that this enzyme fails to cleave the glycosidic to the GlcN residue on the nonreducing size of this 3-*O*-sulfo group–containing GlcN residue resulting in resistant tetrasaccharides.[15] The resulting resistant tetrasaccharides containing 3-*O*-sulfo groups originate from AT-binding pentasaccharides and provide structural information on these pentasaccharides. Five common 3-*O*-sulfo group–containing tetrasaccharides were previously isolated and characterized in our laboratory.[14] An HPLC-MS assay was also developed to quantify these 3-*O*-sulfo group–containing tetrasaccharides.[15] Their structures are

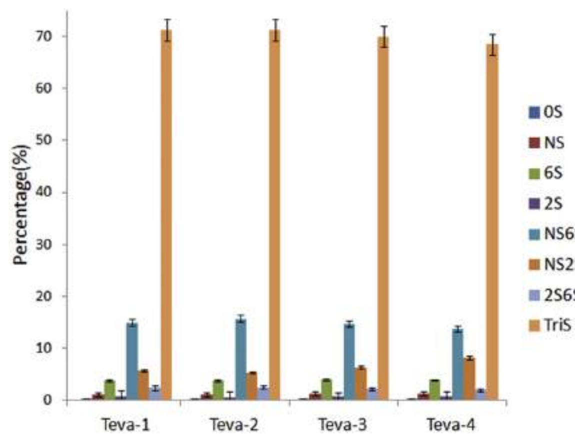

Figure 4. Disaccharide analysis of different fractions from Teva LMWHs; error bars represent the SDs from triplicate analyses.

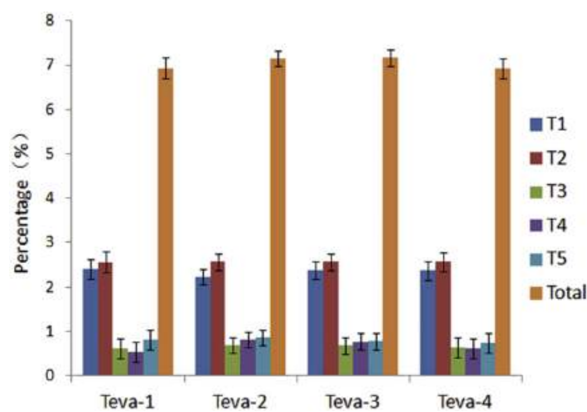

Figure 5. 3-*O*-sulfated tetrasaccharide analysis of different fractions of Teva LMWHs; error bars represent the SDs from triplicate analyses.

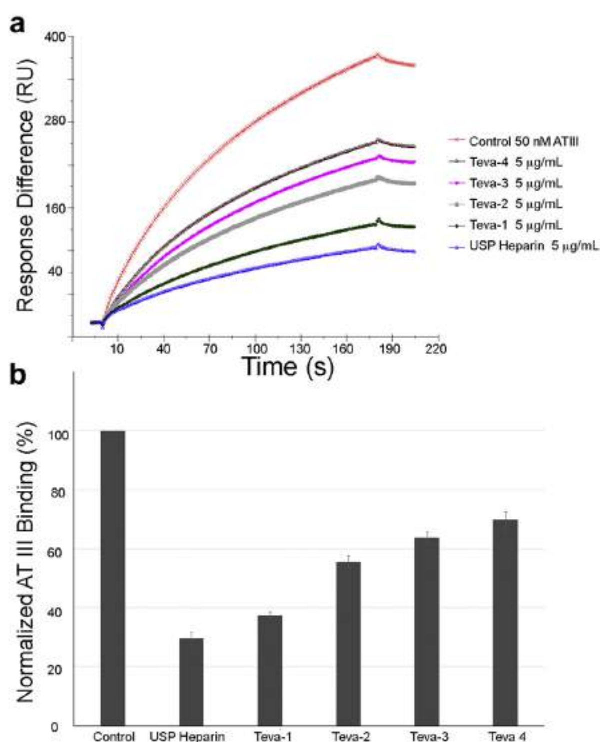

Figure 6. (a) SPR sensorgrams of AT binding to heparin surface competing with different LMWH samples. (b) AT binding to surface heparin as a percentage heparin for different LMWH samples. Error bars are the SDs from triplicate analyses.

ΔUA-GlcNAc6S-GlcUA-GlcNS3S (T1), ΔUA-GlcNAc6S-GlcUA-GlcNS3S6S (T2), ΔUA-GlcNS6S-GlcUA-GlcNS3S (T3), ΔUA2S-GlcNAc6S-GlcUA-GlcNS3S 6S (T4) and ΔUA2S-GlcNS6S-GlcUA-GlcNS3S6S (T5). Thus, we decided to examine the structural variability of the AT-binding site in the LMWH fractions that we had prepared having different molecular weights.

The results (Fig. 5) showed the contents of 3-*O*-sulfo tetrasaccharides of the samples were about 6.5%-7.0%. The major two 3-*O*-sulfo group–containing tetrasaccharides, T1 and T2, were present in the LMWH fractions examined. The contents of 3-*O*-sulfo group–containing tetrasaccharides in LMWH fractions 4 were very similar. This is consistent with our understanding that the controlled depolymerization in which enoxaparin is prepared from UFH is random and the smallest oligosaccharides in fraction 4 were themselves tetrasaccharides. The same 3-*O*-sulfo group–containing tetrasaccharides should, therefore, be present in all fractions.

SPR Analysis for AT-Binding Activity of LMWH Fractions

This SPR method for measuring binding is mainly based on the competition between a sample and standard USP heparin, which was biotinylated and immobilized on the SA chip. Heparin and LMWH samples are then premixed with AT in the solution. The pentasaccharide sequence in heparin or LMWH, containing a 3-*O*-sufo group, will interact and occupy the heparin-binding sites of AT. Once the heparin-binding sites occupied, AT binding to the surface-immobilized heparin will proportionately decrease and the response units of the SPR signal will be reduced. The competitive effect of different LMWH samples were measured though the decrease of the response units resulting from the addition of heparin or LMWH into AT solution and the reduction SPR signal is related to AT-binding affinity. This method correlates well with the AT-mediated anticoagulant activity of a heparin or LMWH sample.[16]

The 4 LMWH fractions prepared from enoxaparin and having different molecular weights were measured by competition SPR and compared USP heparin (UFH) (Fig. 6). The average molecular weight of these fractions showed an obvious change in AT-binding affinity. The slopes of the curves decreased as their average molecular weights increased. Thus, the LMWH fractions having longer chains displayed stronger binding to AT in the solution and, thus, are expected to show higher anticoagulant activity.

The 3-*O*-sulfo group–containing tetrasaccharide contents are similar in the 4 fractions, but their anticoagulant activities were quite different. This suggests that chain size in addition to the presence of 3-*O*-sulfo group contributes to AT bind activity. Alternatively, the 3-*O*-sulfo groups in the smallest chains might be imperfectly positioned (i.e., not in the center of a pentasaccharide domain) leading to nonbinding structures.

In summary, a detailed characterization of LMWHs with different molecular weights fractions was performed in this study. The results showed that all the fractions had very similar disaccharide and 3-*O*-sulfo group–containing tetrasaccharide compositions, but the fraction containing larger-sized chains had higher AT-binding affinity.

Acknowledgments

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