Generation of "Neoheparin" from *E. coli* K5 Capsular Polysaccharide

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Abstract: Heparin remains a major drug in prevention of thromboembolic disease. Concerns related to its animal source have prompted search for heparin analogues. The anticoagulant activity of heparin depends on a specific pentasaccharide sequence that binds antithrombin. We report the generation of a product with antithrombin-binding, anticoagulant, and antithrombotic properties similar to those of heparin, through combined chemical and enzymatic modification of a bacterial (*E. coli* K5) polysaccharide. The process is readily applicable to large-scale production.

Heparin is extensively used to prevent and treat thromboembolic disease.[1] It is a sulfated linear polysaccharide composed of alternating D-glucosamine (GlcN) and hexuronic acid [D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)] units.[2] The requisite blood anticoagulant activity of heparin depends largely on a specific pentasaccharide sequence that mediates binding to antithrombin (AT).[2] The AT-binding sequence (Figure 1) also expresses structural features typical of other parts of the heparin molecule. However, it contains in addition a rare *N*-,3-*O*-sulfated internal GlcN residue (marked by an asterisk in Figure 1 inset) that is essentially lacking in other parts of the heparin chain. Binding to AT shows exquisite specificity in that heparin species merely lacking the GlcN 3-*O*-sulfate group of the AT-binding sequence interact weakly and have low anticoagulant activity.[2,3] Such low-affinity species constitute about two-thirds of commercial heparin preparations. Other sulfate groups (underlined in Figure 1 inset) are equally important to AT binding but are common elsewhere in the molecule, hence also in chains with low affinity for AT.

The commercial production of heparin, currently exceeding 20 tons/year, utilizes mainly porcine slaughter byproducts (intestinal mucosa) as starting material. Drawbacks of the process include unstable supply of raw materials, environmental and ecological hazards, and potential risk of prion and other contaminants (hence, the general trend to avoid animal-based drugs). Attempts to bypass these problems have been aimed at chemical synthesis of heparin analogues containing an AT-binding pentasaccharide domain[4] and modifications of various naturally occurring polysaccharides (see review[5]). Most of these modified polysaccharides deviate appreciably from heparin in structure and show generally weak interaction with AT and low anticoagulant activity.

Recent approaches toward "neoheparin" generation aim for polysaccharides with a carbohydrate backbone similar to that of heparin. The capsular polysaccharide produced by the *E. coli* K5 strain has the same [GlcAβ-(1−4)GlcNAcα(1−4)]$_n$ structure as the precursor polysaccharide generated in heparin biosynthesis.[6] In this process, the polymer is modified through a series of *N*-deacetylation/*N*-sulfation, *O*-sulfation, and epimerization reactions outlined in Figure 1.[2] Most of the reactions are incomplete, thus explaining the structural heterogeneity of native heparin (and in particular of the related heparan sulfate). We previously showed that chemically *N*-deacetylated and *N*-sulfated K5 polysaccharide (K5-PS) will serve as a substrate for GlcA C5-epimerase and *O*-sulfotransferases obtained from a heparin-producing mouse mastocytoma.[6] Under the appropriate incubation conditions, including adequate concentration of the sulfate donor (PAPS), 1−2% of the substrate attained high affinity for AT, suggesting generation of the specific AT-binding pentasaccharide sequence. Using well-defined oligosaccharides as acceptors in *O*-sulfotransferase reactions, incorporation of the critical 3-*O*- and 6-*O*-sulfate groups (Figure 1) was demonstrated. Moreover, only oligosaccharides containing an internal −GlcA−GlcNS−IdoA− sequence served as targets for the 3-*O*-sulfotransferase concluding formation of the AT-binding region.[7,8] These findings were recently confirmed in more refined experimental systems using K5 oligo/polysaccharide as substrate for recombinant enzymes.[9,10] An obvious benefit of an all-enzyme-based *O*-sulfation product would be the lack of sulfate groups at sites (such as C3 of GlcA or IdoA) not substituted in natural heparin (Figure 1). On the other hand, products of enzymatic *O*-sulfation were obtained on a microgram scale only,[9,10] pointing to serious yield problems in relation to commercial-scale heparin production. Here, we describe the generation of a product with AT-binding, anticoagulant, and antithrombotic properties similar to those of heparin, through combined chemical and enzymatic modification of the K5 polysaccharide. The process is readily applicable to large-scale production.

We explored various routes toward AT-binding anticoagulants by chemoenzymatic modification of K5-PS.

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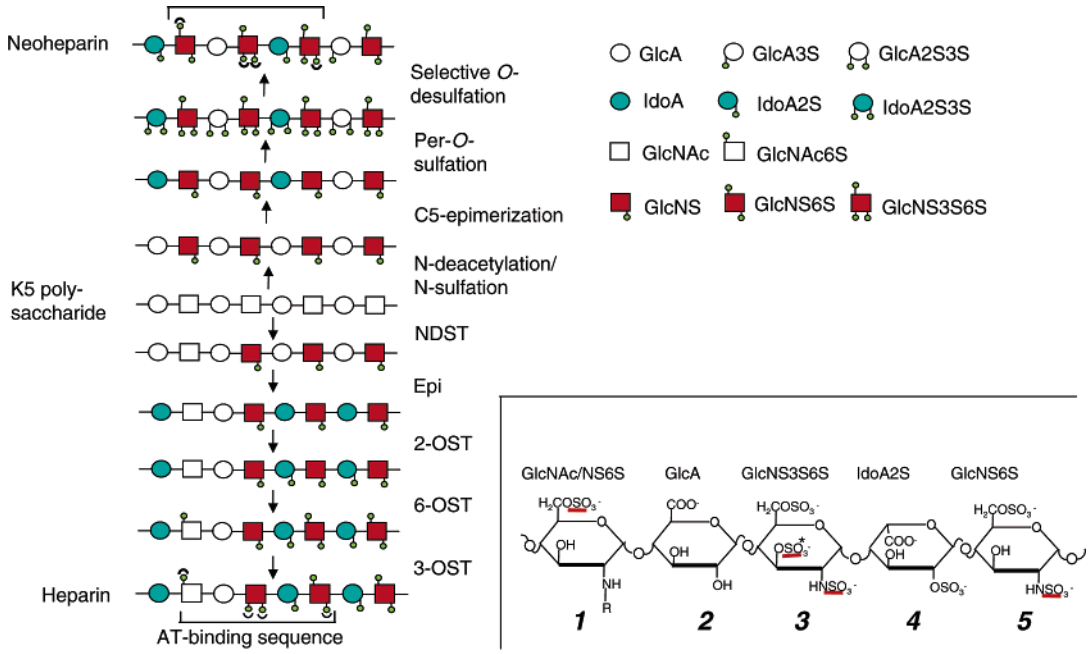

Figure 1. Generation of the AT-binding sequence through biosynthetic or chemoenzymatic modification of precursor polysaccharide. The course of heparin biosynthesis is indicated by arrows pointing downward, whereas the reactions utilized in chemoenzymatic conversion of the *E. coli* K5-PS (identical to the precursor polymer in heparin biosynthesis) into neoheparin are indicated by arrows pointing upward. The enzymes catalyzing the biosynthetic reactions are GlcNAc *N*-deacetylase/*N*-sulfotransferase (NDST), GlcA C5-epimerase (Epi), IdoA 2-*O*-sulfotransferase (2-OST), GlcN 6-*O*-sulfotransferase (6-OST), and GlcN 3-*O*-sulfotransferase (3-OST). Only the epimerization reaction is enzymatic in the conversion of the bacterial polysaccharide; all other steps involve chemical modification. It is noted that the selective *O*-desulfation process is concluded by a 6-*O*-resulfation step (see Supporting Information). Sulfate groups essential for interaction with AT are semicircled. The inset shows the AT-binding pentasaccharide sequence. R is $-COCH_3$ or $-SO^-_3$; sulfate groups essential for interaction with AT are underlined in red.

The IdoA unit 4 (Figure 1) is important to AT binding, since it tethers the adjacent GlcNS unit 5 into appropriate apposition to the protein.[2,11] IdoA must be enzymatically generated through C5-epimerization of GlcA. The epimerase substrate was provided by chemical N-deacetylation followed by *N*-sulfation of the K5-PS (see Supporting Information), yielding a >90% *N*-sulfated sample. The GlcA C5-epimerase has been molecularly cloned and expressed in insect cells with a specific catalytic activity similar to that of native enzyme.[12] Application of such enzyme to *N*-sulfated K5-PS typically yields products in which about one-third of the GlcA residues have been converted to IdoA units.[13] We recently found that up to 60% conversion of GlcA to IdoA may be achieved in the presence of divalent cations,[14] and this finding has been exploited in the present study (see Supporting Information).

On the basis of pilot experiments,[15] regioselective *O*-sulfation of IdoA-containing K5 derivatives was undertaken with the aim of approaching the distribution of sulfate residues in heparin, i.e., primarily at C2 of IdoA and C6 of GlcN units, with some sulfation also at C3 of GlcN as required for AT binding (Figure 1). We predicted that a process designed to exceed certain minimal yields of *O*-sulfate residues in key positions, though stochastic, would generate significant amounts of AT-binding domain. Importantly, sulfation at C3 of IdoA should be avoided, since such modification of heparin chains was shown to impede the anticoagulant (antifactor Xa) activity.[16] However, model experiments involving direct chemical *O*-sulfation of *O*-desulfated heparin consistently yielded products with IdoA units preferentially sulfated at C3[17] and not at C2 as claimed;[18] hence, there is modest anticoagulant activity.

An alternative strategy was therefore explored involving per-*O*-sulfation of all available hydroxyl groups followed by graded solvolytic desulfation (Figure 1). Model experiments using per-*O*-sulfated heparin showed that the IdoA 3-*O*-sulfate groups were removed more rapidly than the 2-*O*-sulfate groups over a wide range of experimental temperatures and reaction times. Moreover, whereas the 6-*O*-sulfate groups of GlcN residues were extensively removed, the 3-*O*-sulfate groups of these residues were more resistant to solvolysis.[17] In the present work, conditions for graded *O*-desulfation were applied to an *N*-sulfated, ~45% C5-epimerized, and subsequently per-*O*-sulfated K5-PS (see Supporting Information). *N*- and 6-*O*-resulfation of the product, followed by controlled cleavage with nitrous acid, yielded a heparin-like material of low molecular weight (neoheparin, $M_r \approx 8000$; purity >95%, see Supporting Information) in which ~60% of the IdoA was 2-*O*-sulfated and ≤10% was 3-*O*-sulfated (Table 1). Almost half of the GlcN units remained 3-*O*-sulfated (more than in heparin), a significant fraction of these residues occurring adjacent to nonsulfated GlcA (Table 1; Figure 2A), as in the AT-binding sequence.[2] Retention of the original degree of epimerization was confirmed by ^{1}H NMR analysis of desulfated neoheparin (see Supporting Information). The structural difference between neoheparin and native heparin is largely quantitative in nature. However, the order of 2-*O*- and 3-*O*-desulfation of GlcA residues was found reversed compared to that of IdoA units, leading to a high proportion of 3-*O*-

Table 1. Composition of Neoheparin[a]

constituent	reference basis	abundance, %
total GlcNS	total GlcN	92[b]
GlcNS, 6-*O*-sulfated	total GlcN	86[b]
GlcNS, 3-*O*-sulfated	total GlcN	46[b]
total IdoA	total HexA	45[b]
IdoA, nonsulfated	total HexA	7[c]
IdoA, 2-*O*-sulfated	total HexA	>24[c,d]
IdoA, 3-*O*-sulfated	total HexA	<5[c]
GlcA, 3-*O*-sulfated	total HexA	46[b]
disaccharide units[c]		
non-*O*-sulfated	total *N*-sulfated disaccharide	<5
mono-*O*-sulfated	total *N*-sulfated disaccharide	13
di-*O*-sulfated[e]	total *N*-sulfated disaccharide	67
−GlcA−GlcNS3S6S−	total *N*-sulfated disaccharide	5[c]
tri-*O*-sulfated	total *N*-sulfated disaccharide	20

[a] Values are based on NMR data and on disaccharide composition following deaminative cleavage of the sample (see Supporting Information). Abbreviations: NS, *N*-sulfate; 3*S*,3-*O*-sulfate; 6*S*,6-*O*-sulfate. [b] Based on NMR analysis. Values given for 3-*O*- and 6-*O*-sulfated GlcNS may be slightly overestimated because of potential contributions also by *O*-sulfated GlcNAc residues. [c] Based on analysis of disaccharide deamination products. The overall proportions of non-*O*-sulfated, mono-*O*-sulfated, di-*O*-sulfated, and tri-*O*-sulfated disaccharides were estimated by paper electrophoresis. Species of each disaccharide class were identified and quantified by anion-exchange HPLC. [d] The proportion indicated reflects the total disaccharide units identified with a 2-*O*-sulfated IdoA residue and thus is a minimal value. [e] Most of the di-*O*-sulfated disaccharide emerged on anion-exchange HPLC as a distinct component at an elution position different from those of any previously identified species. Because partial acid hydrolysis of this component yielded a mono-6-*O*-sulfated, GlcA-containing disaccharide, we tentatively conclude that the remaining sulfate had been located at C3 of GlcA.

sulfated GlcA residues that have not been reported in heparin. Preliminary structural analysis located the 3-*O*-sulfate groups largely as −GlcA3S−GlcNS,6S− disaccharide units (Table 1).

Affinity chromatography of neoheparin on immobilized AT revealed low- and high-affinity fractions similar to those typical of authentic heparin (Figure 2B). This similarity included not only the proportion of high-affinity components, ~30% of the total polysaccharide, but also their elution position in the salt gradient. We conclude that neoheparin contains saccharide sequences that bind AT as avidly as authentic high-affinity heparin. Moreover, neoheparin showed antifactor Xa and antifactor IIa anticoagulant activities essentially similar to those of standard low molecular weight heparin (Figure 2C, inset). Finally, in vivo tests for antithrombotic activity (see Supporting Information) showed neoheparin to prevent venous thrombus formation with an efficacy related to plasma anti-Xa levels, similar to that of commercial low molecular weight heparin (Figure 2C).

Neoheparin and analogues can be easily prepared and are currently generated at gram scale. The overall recovery of polysaccharide through the six-step modification process was calculated to be ~60%. Potential bottlenecks in the process are limited availability of the K5-PS starting material and of recombinant GlcA C5-epimerase. However, a plasmid has been constructed that increases transcription of the K5 capsule gene cluster. The introduction of this plasmid into a laboratory strain of *E. coli* increased the yield of K5-PS to >1 g/L, a 3- to 5-fold increase from that generated using existing K5 strains (C. Clarke and I. Roberts, unpublished results). Moreover, expression of the epimerase

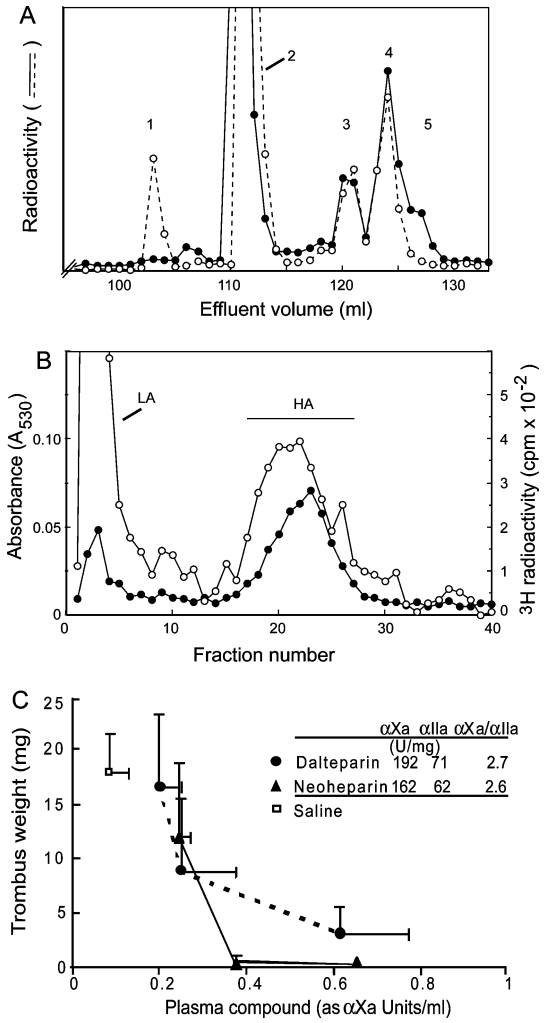

Figure 2. Anticoagulant and antithrombotic properties of neoheparin. (A) Identification and quantification of −GlcA−GlcNS,3,6S− sequence in neoheparin. Polysaccharide was treated with nitrous acid (pH 1.5 procedure), and the resultant disaccharides were reduced with NaB^3H_4. Labeled disaccharides were separated by anion-exchange HPLC (Partisil-10 SAX column) before (closed circles) and after (open circles) digestion with β-D-glucuronidase (devoid of significant α-L-iduronidase activity). The figure shows the part of the chromatogram corresponding to di-*O*-sulfated disaccharides. The numbered peaks indicate elution positions for deamination products ([^{3}H]anhydromannitol derivatives) corresponding to (1) GlcNS,3,6S monosaccharide, (2) sequence tentatively identified (see Table 1, footnote e) as −GlcA3S−GlcNS,6S−, (3) unknown structure, (4)−IdoA2S−GlcNS,6S− sequence, and (5) −GlcA−GlcNS,3,6S− sequence. The ^{3}H label at peak 1 corresponds to ~5% of the total labeled disaccharide. (B) Affinity chromatography of neoheparin prototype on AT-Sepharose. A sample (1.6 mg) of neoheparin was applied to a 3 mL column of AT-Sepharose in 0.05 M NaCl, 0.05 M Tris-HCl, pH 7.4, and eluted using a salt gradient (0.2−3 M NaCl). Effluent fractions were analyzed for hexuronic acid by the carbazole reaction (open circles). The retarded high-affinity (HA) fraction accounted for 30% of the total neoheparin hexuronic acid. LA is the nonretarded low-affinity fraction. A sample of ^{3}H-labeled high-affinity heparin (filled circles) was included as internal standard. (C) Effects of neoheparin and dalteparin on thrombus weight in a rat model of venous thrombosis. The weights of the thrombi are plotted against the plasma levels of neoheparin and dalteparin, expressed as antifactor Xa activity. n = 6/point. Inserted are the specific anticoagulant activities (αXa, antifactor Xa; αIIa, antifactor IIa) recorded in purified assay systems.

in yeast provides ample amounts of enzyme. The enzyme may be immobilized in an insoluble matrix with retained catalytic activity, allowing multiple reuse of the same preparation. Finally, we note that the current neoheparin preparation is a prototype with considerable potential for refinement. We thus predict that optimization of reaction conditions will lead to products with *O*-sulfate patterns more closely similar to those of heparin.

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Supporting Information Available: Materials and methods used to prepare and analyze neoheparin, and purity data. This material is available free of charge via the Internet at http://pubs.acs.org.

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