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Chemoenzymatic Synthesis of Homogeneous Ultralow Molecular Weight Heparins

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Abstract

Ultralow molecular weight (ULMW) heparins are sulfated glycans that are clinically used to treat thrombotic disorders. ULMW heparins range from 1500 to 3000 daltons, corresponding from 5 to 10 saccharide units. The commercial drug Arixtra (fondaparinux sodium) is a structurally homogeneous ULMW heparin pentasaccharide that is synthesized through a lengthy chemical process. Here, we report 10- and 12-step chemoenzymatic syntheses of two structurally homogeneous ULMW heparins (MW = 1778.5 and 1816.5) in 45 and 37% overall yield, respectively, starting from a simple disaccharide. These ULMW heparins display excellent in vitro anticoagulant activity and comparable pharmacokinetic properties to Arixtra, as demonstrated in a rabbit model. The chemoenzymatic approach is scalable and shows promise for a more efficient route to synthesize this important class of medicinal agent.

Heparin has been used as an anticoagulant drug for more than 50 years (1). It is currently marketed in three forms: un-fractionated (UF) heparin [average molecular weight (MW_{avg}) ~14000]; low molecular weight (LMW) heparin (MW_{avg} ~6000); and the synthetic ultralow molecular weight (ULMW) heparin pentasaccharide Arixtra (GlaxoSmithKline) (MW 1508.3). UF heparin is used in surgery and kidney dialysis due to its relatively short half-life and its safety for renal-impaired patients (2). LMW heparins and Arixtra, introduced over a decade ago, have played an increasingly important role in preventing venous thrombosis among high-risk patients (3, 4) because of their more predictable anticoagulant doses, long half-lives, and reduced risks of osteoporosis (5). Recent research on LMW heparin has resulted in the European approval of Bemiparin (6), a second-generation LMW heparin, and the U.S. approval of a generic LMW heparin, M-Enoxaparin.

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Supporting Online Material
www.sciencemag.org/cgi/content/full/334/6055/498/DC1
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UF heparin is isolated from porcine intestine or bovine lung, and LMW heparins are prepared through the chemical or enzymatic degradation of this animal-sourced UF heparin. Worldwide distribution of contaminated heparin in 2007 has raised concerns over the reliability and safety of animal-sourced heparins and LMW heparins (7, 8). As a result, a cost-effective method for preparing new synthetic heparins is highly desirable (9). Heparin is a polysaccharide that consists of a disaccharide-repeating unit of either iduronic acid (IdoA) or glucuronic acid (GlcA) and glucosamine (GlcN) residues, each capable of carrying sulfo groups. The locations of the sulfo groups and IdoA residues are crucial for heparin's anticoagulant activity. The chemical synthesis of Arixtra, the most successful example to date for preparing a synthetic heparin (10), entails ~50 steps with an overall yield of ~0.1% (11); as such, Arixtra is the most expensive drug among heparins (12). Efforts to improve the synthesis of Arixtra with a purely chemical approach have achieved only limited success (13).

A chemoenzymatic approach, relying on a series of heparan sulfate (HS) biosynthetic enzymes, mimics the biosynthesis of heparin and HS (fig. S1) (14). Heparin and HS have similar disaccharide repeating units; however, heparin carries more sulfo groups and a higher level of IdoA residues and possesses the strongest anticoagulant activity among this class of polysaccharide isolated from natural sources (15). HS polymerases synthesize the backbone with a disaccharide repeating unit of GlcA and *N*-acetylated glucosamine (GlcNAc). Subsequent modification relies on sulfotransferases and an epimerase, including *N*-deacetylase/*N*-sulfotransferase [containing separate *N*-deacetylase and *N*-sulfotransferase (NST) domains], C_5-epimerase (C_5-epi), 2-*O*-sulfotransferase (2-OST), 6-*O*-sulfotransferase (6-OST), and 3-*O*-sulfotransferase (3-OST). Using these enzymes, we and others have succeeded in the preparation of heparin polysaccharides (16–19) as well as structurally well-defined HS oligosaccharides (20, 21). In particular, we developed a protocol to control the size of the oligosaccharides, positions of the *N*-sulfo glucosamine, 2-*O*-sulfo IdoA or 6-*O*-,*N*-disulfo glucosamine residues (20). However, the utility of this approach for the targeted synthesis of medicinally significant heparin oligo-saccharides requiring all four controlled sulfation steps, *N*-sulfation, 6-*O*-, 2-*O*- and 3-*O*-sulfation, has not been demonstrated. Furthermore, until now, low recovery yields in each purification step have cast doubt on the scalability of such chemoenzymatic synthesis.

We targeted two ULMW heparins, constructs **1** and **2,** because of their apparent compatibility with our chemoenzymatic approach (Fig. 1). These new constructs contain the antithrombin (AT)–binding domains of porcine and bovine heparin, respectively, which constitute the pharmacophores of anticoagulant heparin (22). Construct **1** also resembles the AT-binding site of human heparin (23). Construct **2** has the same structure as Arixtra except for replacement of a methyl aglycone with disaccharide **3**. This structural similarity facilitates comparison of the synthetic efficiency and the in vitro and in vivo biological activities of these two homogeneous ULMW heparins to Arixtra.

Our synthesis of ULMW heparins includes backbone elongation and saccharide modification (Fig. 1). Disaccharide **3** was chosen as the starting material because it can be elongated by glycosyl transferases and can be prepared in multigram quantities from heparosan, readily obtained by fermentation (24). Elongation of disaccharide **3** to tetrasaccharide **4** was completed using two bacterial glycosyl transferases, *N*-acetyl glucosaminyl transferase of *Escherichia coli* K5 (KfiA) (25) and heparosan synthase-2 (pmHS2) from *Pasteurella multocida* (26). Tetrasaccharide **4** was designed with an unnatural monosaccharide, GlcNTFA (*N*-trifluoroacetyl glucosamine), because the *N*-TFA group can be readily converted to an *N*-sulfo group (20) in a later step. In preparation of ULMW heparin construct **1**, tetrasaccharide **4** was elongated to heptasaccharide **5** in three steps, with an overall yield of 80% (Fig. 1, steps a, b, and c). Heptasaccharide **5** was converted to the

final product by a series of chemoenzymatic reactions, including conversion of the GlcNTFA residue to GlcNS (Fig. 1, left column, steps d and e), epimerization and 2-*O*-sulfation (Fig. 1, left column, step f; here, epimerization of GlcA to IdoA is accompanied by 2-*O*-sulfation using 2-OST to form an IdoA2S at residue D), 6-*O*-sulfation (Fig. 1, left column, step g), and 3-*O*-sulfation (Fig. 1, left column, step h; the 3-*O*-sulfation occurred at residue C). After these 10 steps, we obtained 3.5 mg of construct **1,** corresponding to 45% overall yield as assessed by nuclear magnetic resonance (NMR) spectral integration (fig. S7). Selective epimerization/2-*O*-sulfation of residue D but not residue B (step f) takes advantage of known enzyme specificity, as residue D is flanked by two GlcNS residues (27). Similarly, in the 3-*O*-sulfation step (step h), 3-OST-1 selectively adds a 3-*O*-sulfo group to residue C but not to residue E, because residue C is flanked by a GlcA residue at its nonreducing end (28). In the conversion of heptasaccharide **5** to ULMW heparin construct **1**, it was critical to ensure that each modification was completed. For this purpose, small-scale reactions were carried out in parallel using [^{35}S]3′-phosphoadenosine 5′-phosphosulfate ([^{35}S]PAPS) to form the ^{35}S-labeled intermediates; monitoring by diethylami-noethyl high-performance liquid chromatography (DEAE-HPLC) enabled optimization of the reagent concentrations and reaction times required in the synthesis (fig. S2).

Two extra steps were required to add a GlcNS6S residue to the nonreducing end in the synthesis of ULMW heparin construct **2**. Tetra-saccharide **4** was first converted to hexasaccharide **6**, and the *N*-TFA groups were replaced by *N*-sulfo groups to afford hexasaccharide **7** (Fig. 1, right column). Hexasaccharide **7** was elongated to a heptasaccharide with a nonreducing end GlcNTFA (residue A). This heptasaccharide was treated with C_5-epi and 2-OST enzymes (Fig. 1, right column, step f) to place an IdoA2S at residue D forming heptasaccharide **8**. The introduction of a GlcNTFA residue at the nonreducing end was a critical control point because it prevented the action of C_5-epi and 2-OST on the GlcA (residue B in step f). Heptasaccharide **8** was then converted to construct **2** in a sequential one-pot reaction format (Fig. 1, right column, steps d, e, g, and h). Carrying out a small-scale reaction using [^{35}S]PAPS ensured complete 6-*O*-sulfation (fig. S3). We obtained 7.2 mg of ULMW heparin construct **2** in 12 steps with an overall yield of 37%, as determined by NMR spectral integration (fig. S7). The synthesis of construct **2** was achieved by rearranging the order of the modification and elongation steps without employing additional enzymes or reagents, thus demonstrating that both structural control and target diversification are possible in chemoenzymatic synthesis.

The structure of construct **1** was confirmed by electrospray ionization mass spectrometry (ESI-MS), as well as one-dimensional (1D) and two-dimensional (2D) NMR analysis (Fig. 2). The 3-*O*-[^{35}S]sulfo labeled construct **1** showed a single symmetric peak in a high-resolution DEAE-HPLC trace (Fig. 2A), demonstrating that the purity of the product was above 95% [in the large-scale reaction, purity was confirmed by poly-acrylamide gel electrophoresis (PAGE)] (fig. S4). The ESI-MS analysis revealed construct **1** to have a molecular mass of 1778.5 ± 0.8 daltons, which is identical to the expected calculated molecular mass (1778.5 daltons) (Fig. 2B). High-resolution ESI-MS exhibited a signal at a mass/charge ratio of 887.5313, consistent with $[M\text{-}2H]^{2-}$ (calculated mass/charge ratio, 887.5324). The 2D ^{1}H NMR spectrum clearly demonstrates the presence of six anomeric protons that resonate as doublets (Fig. 2, C and D). The small coupling constants (~3 Hz) of three anomeric protons indicate α linkages between the A–B, C–D, and E–F rings; larger coupling constants (~8 Hz) indicate β linkages between the B–C, D–E and F–G rings. The presence of an internal IdoA2S is clearly indicated by a broad characteristic anomeric signal at δ5.09 parts per million (ppm) that resonates ~0.6 ppm downfield relative to the anomeric proton of GlcA residues. The complete assignment of the spectrum is shown in table S1A. The structure of ULMW heparin construct **2** was confirmed using the same methods (fig. S6 and table S1B). High-resolution ESI-MS of construct **2** afforded a value consistent with

$[M-2H]^{2-}$ of 906.5047 (calculated mass/charge ratio, 906.5056). A ^{1}H-^{13}C heteronuclear multiple-quantum coherence (HMQC) analysis of constructs **1** and **2** further confirmed the assignments discussed above (fig. S5). The structures of all intermediates were confirmed by ESI-MS analysis (table S2).

The in vitro anticoagulant activities of construct **1** and **2** and in vivo pharmacokinetic properties were next assessed and compared with those of Arixtra. We anticipated that these ULMW heparins would exhibit anticoagulant activity by forming a 1:1 complex with AT, which subsequently inactivates factor Xa in the blood coagulation cascade (29). The binding affinities of AT to constructs **1** and **2** were 5.2 ± 0.2 nM and 9.1 ± 0.2 nM, respectively, very similar to the 5.9 ± 1.5 nM value measured for Arixtra. Next, the in vitro anti-Xa activity of each ULMW heparin was determined (16) (Fig. 3A). The median inhibitory concentration (IC_{50}) values of constructs **1** and **2** were 2.8 nM and 3.6 nM, respectively, again very close to the 3.0 nM value measured for Arixtra. Finally, the pharmacokinetic (anti-Xa) property of each ULMW heparin was examined in vivo using a rabbit model (30, 31). A standard curve for each ULMW heparin was prepared in rabbit plasma (fig. S8). After the subcutaneous administration of 120 μg per kg of weight of each ULMW heparin to three anesthetized rabbits, plasma samples were collected and anti-Xa activity was measured over a 24-hour period. The pharmacokinetic profiles of Arixtra, construct **1,** and construct **2** were very similar (Fig. 3B).

Construct **1** was resynthesized at a 15-fold greater scale to demonstrate the scalability and reproducibility of this chemoenzymatic process (table S4). In this larger-scale synthesis, the sequential one-pot format (steps d, e, g, f, and h in Fig. 1) was divided into three steps (fig. S9), permitting the isolation and weighing of the intermediates and their complete structural analysis using NMR and high-resolution MS (fig. S10 and table S4). Construct **1** (49 mg) was obtained at >95% purity based on PAGE and NMR analysis, and in an overall yield of 38% (fig. S10).

Heparin oligosaccharides are generally perceived to be difficult to synthesize by chemical methods. Long synthetic routes—necessitated by the introduction and removal of protecting groups—lead to low overall yield, and there is a lack of efficient methods to separate side products from desired intermediates. The chemo-enzymatic approach demonstrated here shows that targeted, scalable, and high-efficiency synthesis of heparin oligosaccharides is possible. We achieved this goal by carefully selecting the substrate size and optimizing the sequence of sulfo group installation. Careful design of target structures offers a major advance over our previous study (20) by avoiding by-product formation. For example, the order for installing different types of sulfo groups is critically important in synthesizing construct **1**. The optimized sequence is *N*-sulfation followed by epimerization/2-*O*-sulfation, 6-*O*-sulfation, and 3-*O*-sulfation. Reversal of the order in which epimerization/2-*O*-sulfation and 6-*O*-sulfation (or 6-*O*-sulfation and 3-*O*-sulfation) take place results in very low yields of products. Target size selection is also critical, as heptasaccharides and larger oligosaccharides are highly susceptible to sulfotransferase modification and undergo nearly quantitative conversion to the desired intermediate without side products in each modification step. Another crucial innovation was the improvement of purification protocols, raising the 30 to 40% yields from each purification step described in our previous study (20) to ~90% herein by coupling column purification with ESI-MS analysis.

Dabigatran, a direct thrombin inhibitor, was recently approved by the U.S. Food and Drug Administration, a culmination of intensified efforts for new anticoagulant drug development (32). However, none of the currently marketed anticoagulant drugs can replace heparin because of its unique pharmacological properties. A cost-effective approach to prepare heparin is important not only to secure the safety of the heparin supply chain but also to

provide the opportunity to design derivatives that eliminate side effects. Although our chemoenzymatic approach, with appropriate optimization, provides a general method for preparing different heparins, including ULMW heparin, and in principle LMW heparin and UF heparin, target selection is restricted by the substrate specificities of the enzymes. Smaller targets, such as the Arixtra pentasaccharide, are more difficult to prepare because pmHS2 inefficiently elongates glucosamine monosaccharide. Despite this drawback, our chemo-enzymatic approach facilitates the scalable synthesis of larger targets through a shorter route. Further optimization will certainly be necessary to make this method amenable to industrial-scale synthesis. We recently developed a method to reduce the cost of the synthesis of the sulfo donor, PAPS, by a factor of more than 5000 (33), facilitating large-scale enzyme-based synthesis. The inclusion of a cofactor recycling system for the regeneration of PAPS (34) could further reduce cost and eliminate potential PAP-involved sulfotransferase inhibition. Continuing efforts should provide a generic and cost-effective approach for the large-scale preparation of antithrombotic therapeutic agents with improved safety and pharmacological effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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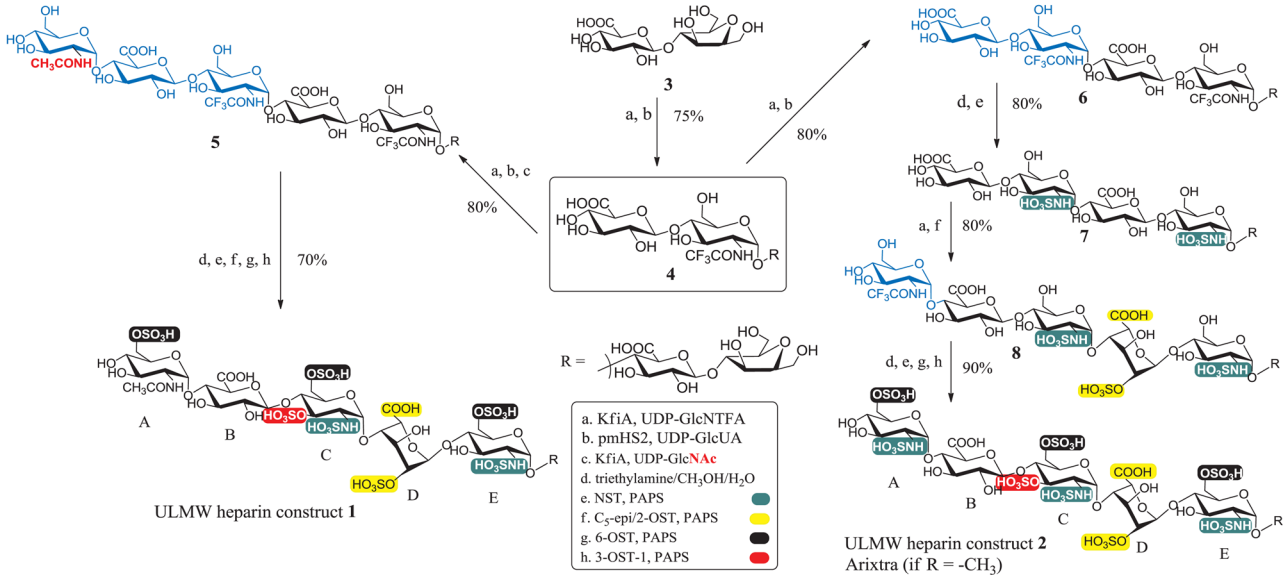

Fig. 1.

Chemoenzymatic synthetic schemes of ULMW heparin construct **1** and **2**. The synthesis started from disaccharide **3**, and it was then elongated to tetrasaccharide **4**. Eight additional steps transformed **4** to construct **1** (**left column**). Steps d through h were combined in sequential one-pot reaction format. Ten additional steps transformed **4** to construct **2** (**right column**). The recovery yield at each purification step was determined by parallel synthesis of the corresponding radioactively labeled oligosaccharide. KfiA, *N*-acetyl glucosaminyl transferase of *E. coli* K5 strain; pmHS2, heparosan synthase-2 of *Pasteurella multocida*; NST, *N*-sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; C_5-epi, C_5-epimerase; 2-OST, 2-*O*-sulfotransferase; 6-OST, 6-*O*-sulfotransferase; 3-OST-1, 3-*O*-sulfotransferase isoform 1. More synthesis details are given in table S3.

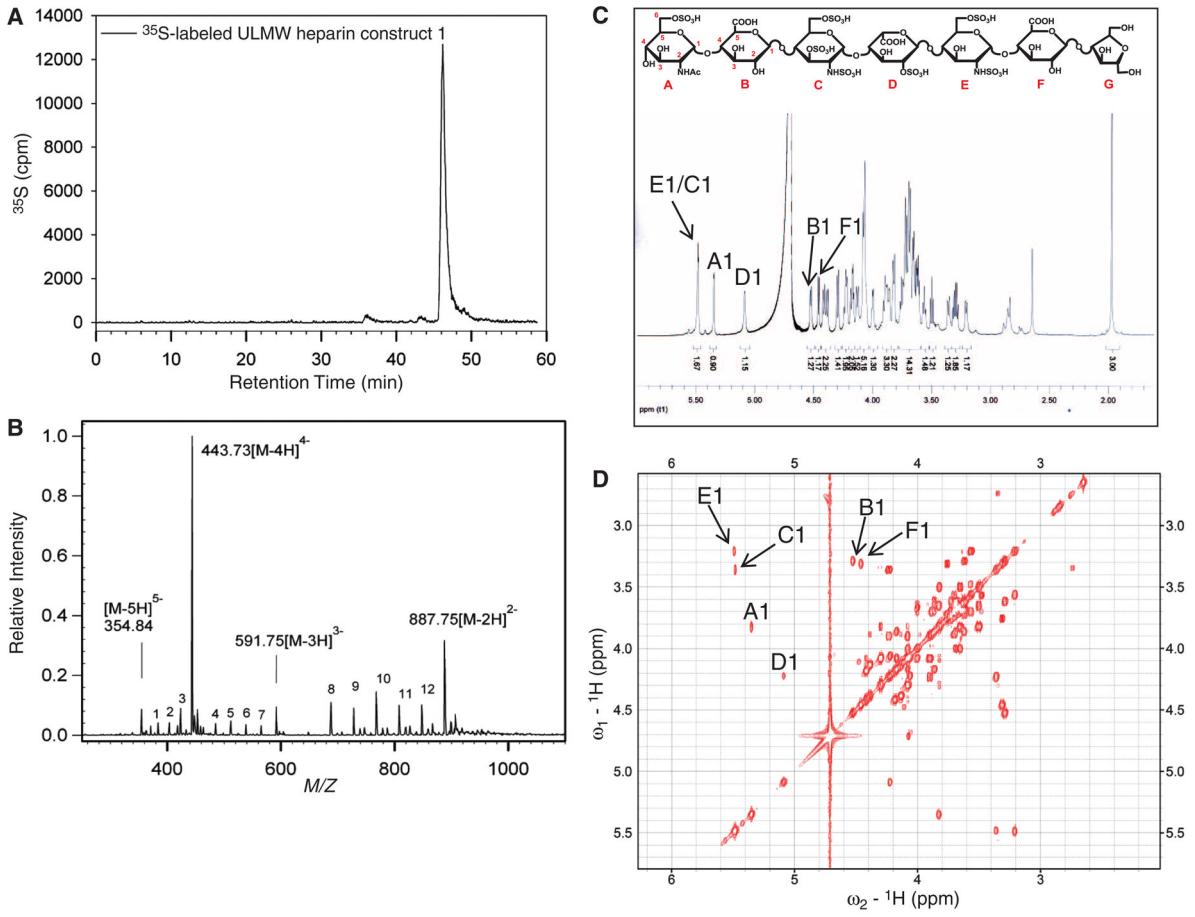

Fig. 2.

Structural characterization of ULMW heparin construct **1**. (**A**) The DEAE-HPLC profile of a ^{35}S-labeled product. (**B**) The ESI-MS spectrum of construct **1**. Peaks 1 to 3 represent the desulfated signals of quadruply charged ions. Peaks 4 to 7 represent the desulfated signals of triply charged ions. Peaks 8 to 12 represent the desulfated signals of doubly charged ions. (**C**) The 1D ^{1}H NMR spectrum of construct **1**. Peaks assigned to the anomeric protons of each hexose ring (A to F) are labeled. (**D**) The 2D correlation spectroscopy spectrum of construct **1** and the corresponding peak assignments of the anomeric protons that resonate as doublets at δ5.48 (d, $J = 3.23$ Hz, 2H), 5.35 (d, $J = 2.94$ Hz, 1H), 5.09 (broad doublet, 1H), 4.53 (d, $J = 8.11$ Hz, 1H), and 4.46 (d, $J = 8.07$ Hz, 1H) ppm. The small coupling constants (~3 Hz) of the anomeric protons indicate an α linkage, and the large coupling constants (~8 Hz) indicate a β linkage.

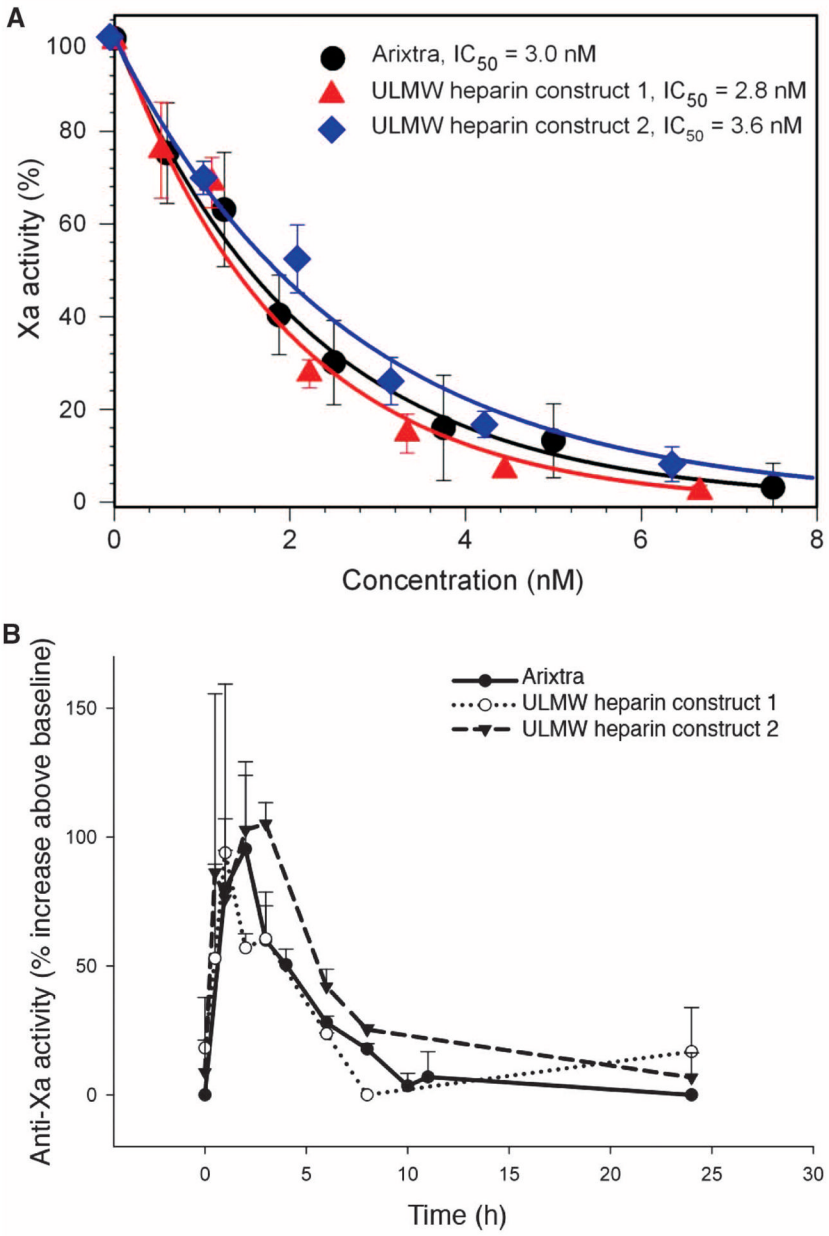

Fig. 3.

Determination of the anticoagulant activities and pharmacokinetic properties of ULMW heparin construct **1** and **2**. (**A**) shows the anti-Xa activity using a chromogenic substrate. Arixtra and constructs **1** and **2** were incubated with AT (240 nM), factor Xa (5.9 nM), and the peptide substrate (289 μM). The activity of Xa was determined by the rate of increase of the absorbance at 405 nm. The activity without drugs was defined as 100%. Each data point represents the average of four determinations ± SD. (**B**) The pharmacokinetic profiles in rabbits. Arixtra and constructs **1** and **2** were each independently administered subcutaneously at 120 μg/kg to three rabbits ($n = 3$) and plasma samples were collected from 0 to 24 hours. The anti-Xa activity of plasma samples was measured against a standard curve (fig. S8). The area under the curve for Arixtra, construct **1**, and construct **2** were 457, 473, and 802, respectively. Error bars, mean ± SD.