Homogeneous low-molecular-weight heparins with reversible anticoagulant activity

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Low-molecular-weight heparins (LMWHs) are carbohydrate-based anticoagulants clinically used to treat thrombotic disorders, but impurities, structural heterogeneity or functional irreversibility can limit treatment options. We report a series of synthetic LMWHs prepared by cost-effective chemoenzymatic methods. The high activity of one defined synthetic LMWH against human factor Xa (FXa) was reversible in vitro and in vivo using protamine, demonstrating that synthetically accessible constructs can have a critical role in the next generation of LMWHs.

Heparin is a widely used anticoagulant to prevent and treat arterial and venous thrombosis. There are three US Food and Drug Administration (FDA)-approved forms of heparin: unfractionated heparin (UFH, average molecular weight (MW avg) ~14,000 Da), LMWH (MW avg 3,500–6,000 Da) and fondaparinux (MW 1,508 Da). UFH is rapid-onset, safe for renal-impaired patients and its effects can be reversed using the cationic-peptide drug protamine; however, it shows a 1–6% incidence of heparin-induced thrombocytopenia (HIT), a life-threatening complication. Introduced in the 1990s, LMWHs are subcutaneously administered and have a longer half-life than UFH, permitting their outpatient use and self-administration. Due to these advantages, LMWH is the most widely prescribed heparin in the US. However, LMWH can be used in renal-impaired patients only at reduced doses and is incompletely neutralized with protamine, thereby increasing the risks of bleeding. Fondaparinux, a synthetic pentasaccharide, is subcutaneously bioavailable and has reduced risks of HIT and osteoporosis. However, it is primarily excreted through the kidney and thus is not suitable for renal-impaired patients, and it lacks an antidote. The US FDA recently approved generic forms of LMWH and fondaparinux, underscoring the rapid growth in heparin-based drugs.

Heparin consists of a disaccharide repeating unit of either iduronic acid (IdoA) or glucuronic acid (GlcA) and glucosamine (GlcN) residues, each capable of carrying sulfate groups. The locations of sulfate groups on IdoA and GlcA dictate the anticoagulant activity of heparin1. In vivo, heparin is synthesized by a series of heparan sulfate (HS) biosynthetic enzymes (Supplementary Results, Supplementary Fig. 1). Recombinant HS biosynthetic enzymes, expressed in Escherichia coli, show comparable substrate specificities to their mammalian counterparts2. These recombinant enzymes offer a strategy to synthesize heparin oligosaccharides using a chemoenzymatic approach2-12.

LMWH is a depolymerized derivative of heparin, isolated from porcine intestine. A worldwide contamination of heparin in 2007 affected the purity and safety of LMWHs14 and was associated with over 200 deaths in the US14. This crisis revealed the fragility of the LMWH supply chain. The cost-effective preparation of a synthetic LMWH could improve drug safety and efficacy15. Currently available LMWHs are complex mixtures, having average molecular masses of 3,500–6,000 Da, corresponding to 12–20 saccharide units. Until now, the preparation of a homogeneous LMWH has not been possible owing to difficulties in the chemical synthesis. Thus, we sought to design and synthesize a potential lead compound for a new generation of LMWHs, particularly to explore reversibility of treatment.

Five synthetic LMWHs (1-5, Fig. 1a and Supplementary Table 1), ranging from hexasaccharide to dodecasaccharide, were synthesized (Supplementary Note). The structures of 1-4 contain a different number of IdoA2S-GlcN5S6S (where S is sulfate) repeating units near their reducing ends. Synthetic LMWH 5 differs from 4, as it has two 3-O-sulfate groups (Fig. 1). The synthesis of 1 was initiated from a commercially available monosaccharide, 1-O-(para-nitrophenyl)-glucuronic acid (GlcA-pNP) (Supplementary Fig. 2). Elongation of GlcA-pNP to a hexasaccharide used two bacterial glycosyltransferases (Supplementary Table 2), an intermediate for subsequent syntheses. The syntheses of 2, 3 and 4 were initiated from hexasaccharide 6 through intermediates 7, 8 and 9, respectively (Fig. 1b and Supplementary Table 1). These intermediates contain multiple IdoA2S-GlcN5S6S repeating units, posing a synthetic challenge owing to the substrate specificity of C7-epimerase (C7-epi). A carefully designed sequence of enzymatic steps was used for high purity and yields. The conversion of GlcA to IdoA2S involves two steps: C7-epi catalyzes epimerization of a GlcA to an IdoA, and 2-O-sulfotransferase (2-OST) transfers a sulfate group to IdoA. C7-epi catalyzes both the forward and reverse reactions, leading to the incomplete conversion of GlcA to IdoA2S and a complex mixture of products17. The placement of a pentasaccharide domain, GlcN-trifluoroacetyl(TFA)-GlcA-GlcNS-GlcA-GlcNS, into the substrate directs C7-epi to irreversibly react with only the GlcA residue flanked with two GlcNS residues and avoids incomplete conversion.

The conversion of 6 to 7 to construct the pentasaccharide domain recognized by C7-epi was completed in four steps. The GlcNTFA residue (residue A) of hexasaccharide 6 was first converted to a GlcNS residue (step a, Fig. 1b). The resultant hexasaccharide was then elongated
to an octasaccharide in two enzymatic steps (steps b and c, Fig. 1b), to obtain the desired pentasaccharide domain (dashed box, Fig. 1b). The conversion of GlcA (residue E) to IdooA2S in 7 was achieved by C5-epi and 2-OST (step d, Fig. 1b). The formation of the IdooA2S residue removes its reactivity toward further C5-epi modification. Repeating these steps (steps a–d, Fig. 1b) once or twice produced 8 and 9, respectively. The syntheses of 7, 8 and 9 were completed (Supplementary Table 2), and structural characterization and purity are shown in Supplementary Figures 22–33. The octasaccharide 7 and decasaccharide 8 were converted to 2 and 3, respectively, after N-sulfation, 6-O-sulfation and 3-O-sulfation; decasaccharide 9 was converted to 4 after 6-O-sulfation and 3-O-sulfation. The conversion of 4 to 5 was achieved by 3-O-sulfotransferase isoform 5 (3-OST-5) modification (Fig. 1b). Final-product purity was confirmed by HPLC. Structures were determined by MS and NMR analyses (Supplementary Figs. 4–19) and MS-assisted sequence analysis18 (Supplementary Figs. 20 and 21).

A clinical benefit of UFH and some LMWH chains is their ability to be cleared from the circulation through the liver, allowing these agents to be used in patients with renal impairment. We examined whether the synthetic LMWHs showed similar clearance profiles. UFH and larger chains in LMWH are known to bind to Stabilin-2, a scavenger receptor present on liver sinusoidal endothelial cells that mediates their clearance19. Like UFH and the LMWH enoxaparin, 3, 4 and 5 showed significant (P < 0.0001) endocytosis in cells stably transfected with Stabilin-2, whereas 1 and 2 showed very low internalization (Supplementary Fig. 34). Using a mouse model, we compared the retention of synthetic LMWHs in the liver with that of UFH and enoxaparin (Fig. 2a). Larger constructs (3, 4 and 5) were retained in the liver, whereas smaller-size constructs (1 and 2) showed a very low level of liver retention (Fig. 2a).

We determined the anticoagulant activities of the synthetic LMWHs. All compounds (1–5) showed strong antithrombin (AT)-binding affinity (Kd 5–30 nm) (Supplementary Table 1). Results of anti-FXa activity assays showed lower half-maximal inhibitory concentration (IC50) values for 1–5 than UFH and enoxaparin, confirming their potent anti-FXa activity (Supplementary Fig. 35). Unlike UFH and enoxaparin, synthetic LMWHs have no detectable anti-factor IIa (FIIa) activity, and, thus, these compounds are FXA-specific inhibitors. Our data also suggest that compounds larger than decasaccharides are required for anti-FIIa activity. Bemiparin, a ‘second-generation’ LMWH, was recently approved by the European Medicines Agency (EMA). It has considerably lower anti-FIIa activity than other LMWH drugs but similar clinical utility, suggesting that anti-FIIa activity is not critical20.

We next determined the protamine reversibility of the anticoagulant activity of five synthetic LMWHs (Fig. 2b). In contrast to the activities of 1–3, the activity of 4 was partially reversed by protamine and the activity of 5 was more reversible than enoxaparin and showed the same protamine reversibility as UFH activity (Fig. 2b). Using an ex vivo mouse model, we confirmed that 5 has similar sensitivity to protamine neutralization as UFH. As expected, enoxaparin was only partially neutralized by protamine (Fig. 2c). Finally, using a mouse-tail-clip bleeding model, we demonstrated that protamine shortened the prolongation of bleeding time induced by 5 (Fig. 2d), confirming the sensitivity of 5 to protamine neutralization in vivo. The US FDA has recently approved three new anticoagulants—dabigatran etexilate, rivaroxaban and apixaban—however, none of these drugs has an antidote. Although other strategies to inhibit anticoagulant drugs using an engineered FXA-like protein21 or antithrombin mutant22 have been reported, these methods remain to be approved by a regulatory agency. Synthetic LMWH 5 can be neutralized by protamine, an FDA-approved antidote for UFH.

The superb sensitivity toward protamine neutralization shown by 5 suggests that both sulfation pattern and size contribute to its sensitivity to neutralization. Currently marketed LMWHs, for example, enoxaparin, are a mixture of oligosaccharides with a broad size distribution of chains, having different affinities toward protamine. Our data suggest that protamine only neutralizes chains in LMWHs that are larger than decasaccharides. Smaller chains, pentasaccharide to decasaccharide, can have anticoagulant activity23, even though they are not protamine reversible. Consequently, only
understanding of C₃-eptide specificity resulted in the design of a modification sequence leading to the synthesis of the critical IdoA2S-GlcNS repeating domain. The next challenge will be to determine whether a large-scale synthesis of homogeneous LMWH can be accomplished to meet the needs of the anticoagulant drug market. The synthesis of 5 requires 22 synthetic steps, but it is much shorter than the synthesis of fondaparinux, which requires 50 steps. Fondaparinux is now synthesized in kilogram scale and has been a profitable drug for ten years, suggesting that further development of the chemoenzymatic approach will result in cost-effective products, accelerating the modernization of LMWH therapeutics.

Figure 2 | Determination of the clearance, anti-FXa activity and sensitivity to protamine neutralization of synthetic LMWHs. (a) Percentage of ³⁵S-labeled synthetic LMWHs that was retained in the liver in a mouse model. In comparison to synthetic 1, all tested compounds, with the exception of synthetic 2 and exonaparin, showed significantly higher retention in liver (***P < 0.0001). (b) FXa activity of synthetic LMWHs in the presence of different concentrations of protamine under in vitro conditions. (c) Ex vivo reversibility of anti-FXa activity by protamine. The inhibition of FXa activity by the test compounds was significantly affected in the presence of protamine (**P < 0.05 and ****P < 0.0001). Data presented in a-c are the average of three to five determinations ± s.d. (d) Effect of 5 and protamine on tail-bleeding time after tail transection. Protamine significantly shortened the primary bleeding time (**P < 0.01) that was induced by 5. Each data point represents the measured value from an individual mouse in the test group. Data are presented as mean ± s.d. The blood loss and total bleeding time were also measured (Supplementary Fig. 36).

partial neutralization for commercial LMWH can be achieved with protamine. Synthetic LMWH 5 has a uniformly high affinity to protamine because it is a structurally homogeneous compound. In addition, our results demonstrate that an extra 3-O-sulfate group increases the sensitivity for protamine neutralization, suggesting that the sulfation pattern has a critical role in protamine binding.

Additional efforts are necessary to further develop 5 into an LMWH drug candidate. First, replacement of pNP aglycone should eliminate concerns that it might be converted to p-nitrophenol in vivo, leading to a potentially harmful metabolite (http://www.epa.gov/ttnatw/tplt/nitrophe.htm). Second, a comprehensive structure-activity relationship study will be required to understand the contribution of sulfation pattern to the pharmacological effects.

LMWHs are critical for the practice of modern medicine, yet their production still depends on a long supply chain that is vulnerable to contamination and adulteration. After the contamination crisis, the US FDA and EMA implemented a series of new approaches to monitor the purity of heparin drugs. Although these efforts have stopped the influx of contaminated heparin into the market, a long-term solution should be to manufacture synthetic heparin under highly regulated processes, eliminating the need for animal-sourced heparin. Chemoenzymatic synthesis offers a promising approach toward this goal. A homogeneous product reduces the complexity for the quality control during the manufacturing process and is compatible with standard approval processes by regulatory agencies. Although the structures produced through chemoenzymatic synthesis are limited by the substrate specificities of the enzymes, their synthetic capability can be expanded through better understanding of enzyme properties. As demonstrated in the present study, a firm
ONLINE METHODS

Determination of the in vitro and ex vivo anti-FXa activity. Assays were based on a previously published method26,27. Briefly, human factor Xa (FXa) (Enzyme Research Laboratories) was diluted to 50 U ml⁻¹ with PBS. The chromogenic substrate S-2765 was from Diapharma and made up at 1 mg ml⁻¹ in water. UFH (from US Pharmacopeia), exonaparin (Lovenox from local pharmacy) and synthetic 1 to 5 were dissolved in PBS at various concentrations (3–600 μg ml⁻¹). The reaction mixture, which consisted of 20 μl of human plasma (Sigma-Aldrich) and 8 μl of the solution containing the sample, was incubated at room temperature for 5 min. Factor Xa (100 μl) was then added. After incubation at room temperature for 4 min, 30 μl of S-2765 substrate was added. The absorbance of the reaction mixture was measured at 405 nm continuously for 5 min. The absorbance values were plotted against the reaction time to measure the reaction rate. The initial reaction rates were used to measure the activity of FXa.

Preparation of ¹³⁵S-labeled LMWHs. UFH (from US Pharmacopeia) and exonaparin (from local pharmacy) were modified by NST. The reaction consisted of MES (2-(N-morpholino)ethanesulfonic acid, Sigma) 50 mM pH 7.0, NST 0.1 mg ml⁻¹ and 0.5 nmol [¹³⁵S]PAPS (specificity activity of [¹³⁵S]PAPS was 2.2 × 10³ cpm pmol⁻¹), 50 μg of UFH or exonaparin in total 500 μl at 37 °C overnight. The products were purified by a diethylaminoethyl (DEAE) column. ¹³⁵S-labeled synthetic 1 to 4 were prepared from the synthetic LMWH constructs intermediates without 3-O-sulfate groups. The reaction consisted of MES 50 mM pH 7.0, 10 mM MnCl₂, 5 mM MgCl₂, 3-OST-1.0 mg ml⁻¹ and 0.5 nmol [¹³⁵S]PAPS and oligosaccharide (5 μg) in total 500 μl at 37 °C overnight. ¹³⁵S-labeled synthetic 5 was prepared from synthetic 4. The reaction consisted of MES 50 mM pH 7.0, 10 mM MnCl₂, 5 mM MgCl₂, 3-OST-5.0 mg ml⁻¹ and 0.5 nmol [¹³⁵S] PAPS and 4 (5 μg) in total 500 μl at 37 °C overnight. The ¹³⁵S-labeled 5 was purified by a DEAE-HPLC column.

Determination of the binding affinity of synthetic LMWHs to antithrombin (AT). The dissociation constant (Kᵣ) of each sample and AT was determined using affinity co-electrophoresis45. Approximately 1,500–2,500 c.p.m. of ¹³⁵S-labeled synthetic 1 to 5 was loaded per lane with zones of AT at concentrations 0, 8, 16, 32, 60, 120, 250, 500, and 1,000 nM. The gel was performed at 300 mA for 2 h, dried and analyzed on a Phospholmage (Amersham Biosciences, Storm 860). The retardation coefficient was calculated at R = (Mₛ/Mₐ)EMU wherein Mₛ is the mobility of the polysaccharide through the zone without AT, and Mₐ is the mobility of the sample through each separation zone. The retardation coefficient was then plotted against the retardation coefficient divided by its respective concentration of AT. The slope of the line represents −1/Kᵣ.

HPLC analysis. Both DEAE-HPLC and polymebrane-based ion exchange (PAMN)-HPLC were used to analyze the purity of the products. The elution conditions for the HPLC analysis were described elsewhere46. Briefly, for DEAE-HPLC method, the DEAE-NPR column (Tosohaas) was eluted with a linear gradient of NaCl in 20 mM sodium acetate buffer (pH 5.0) from 0 to 1 M in 60 min at a flow rate of 0.4 ml min⁻¹. For PAMN-HPLC, the column (Waters) was eluted with a linear gradient of KH₂PO₄, from 0 to 1 M in 40 min at a flow rate of 0.5 ml min⁻¹.

Neutralization of synthetic LMWHs by protamine in vitro. The procedures followed those reported in a previous publication47. The synthetic LMWH constructs and protamine chloride (Sigma-Aldrich) were dissolved in PBS. The concentrations of the synthetic LMWH samples for each construct were different because each sample had different Iₛₐₚ values for the anti-FXa activity. Generally, the final concentration of each test compound was about four times its Iₛₐₚ value. The reaction mixture consisted of 20 μl of human plasma (Sigma-Aldrich), 2 μl of stock solutions of synthetic LMWHs and 8 μl of protamine with various concentrations (0–90 μg ml⁻¹), and was incubated at room temperature for 5 min. The concentrations of 1, 2, 3, 4 and 5 stocks were 4.4 μg ml⁻¹, 5.4 μg ml⁻¹, 4.7 μg ml⁻¹, 5.7 μg ml⁻¹ and 6.6 μg ml⁻¹, respectively. The concentrations of exonaparin and UFH stocks, two control samples, were 126 μg ml⁻¹ and 21 μg ml⁻¹, respectively. The mixture (30 μl) was then subjected to anti-FXa activity measurement as described above.

Neutralization of synthetic LMWHs by protamine in mice. The study was performed on 8-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) (n = 4 per group). The mice experiments were approved by the University of North Carolina Animal Care and Use Committees and complied with US National Institutes of Health guidelines. Under isoflurane anesthesia, mice were subcutaneously administered with PBS, UFH (3 mg kg⁻¹), exonaparin (3 mg kg⁻¹) or 5 (0.6 mg kg⁻¹) 30 min before a protamine administration. Protamine (15 mg kg⁻¹) or PBS was administered intravenously via retro-orbital plexus injection, and 5 min later, blood samples were drawn from the inferior vena cava into syringes preloaded with 3.2% solution of sodium citrate (final volume ratio 9:1). To obtain mouse plasma, blood samples were centrifuged at 4,000g for 15 min at 4 °C. Mouse plasma was then used to determine anti-FXa activity. Ex vivo analysis of anti-FXa activity was done similarly to the in vitro study described above. Briefly, plasma (10 μl) from different groups of mice was incubated with 80 nM human factor Xa (10 μl) at room temperature for 4 min and S-2765 (1 mg ml⁻¹, 30 μl) was then added. The anti-FXa activity in the mouse plasma from the PBS injected mice was defined as 100%. Statistical analysis for multiple comparisons was performed by two-way analysis of variance (ANOVA) with Bonferroni’s post hoc test (GraphPad Prism Software).

Mouse model of tail bleeding. Under isoflurane anesthesia, 8-week-old male C57BL/6J mice (n = 8 per group) were administered with PBS or 5 (0.6 mg kg⁻¹) subcutaneously, and 30 min later PBS or protamine (15 mg kg⁻¹) was administered via retro-orbital intravenous injection. After 5 min the distal part of the tail was transected at the constant diameter (1.5 mm), approximately 3–4 mm from the end, resulting in both arterial and venous bleeding. The tail was immediately placed in a 15-ml Falcon tube containing 13 ml of prewarmed PBS (37 °C), and blood loss was observed for 30 min. The primary bleeding time was defined as the time to the first cessation of bleeding. Subsequently, time for each reinitiated bleeding was also recorded, and used to calculate total bleeding time. One mouse in 5/protamine group received an inaccurate protamine injection and was excluded from the study. The blood collected in PBS was used to calculate total blood volume loss. Formic acid was added to samples (70:30 ratio) and absorbance was measured at 405 nm. A standard curve was generated by mixing 13 ml of PBS with known amounts of blood. Statistical analysis between each group was performed by one-way ANOVA (GraphPad Prism Software) followed by Bonferroni’s multiple-comparison test.

MS analysis. The low-resolution MS analyses were performed on a Thermo LCQ-Deca. A syringe pump (Harvard Apparatus) was used to introduce the sample by direct infusion (35 μl min⁻¹). Experiments were carried out in negative ionization mode. Synthetic nonsulfated oligosaccharides were diluted in 200 μl of H₂O with the electrospray source set to 5 kV and 275 °C. Sulfated LMWHs were diluted in 200 μl of 10 mM ammonium bicarbonate with the electrospray source set to 3 kV and 200 °C. Sulfated oligosaccharide (1 μl) was diluted in a different working solution containing 200 μl of 10 mM ammonium bicarbonate. Experiments for sulfated oligosaccharides were carried out in negative ionization mode with the electrospray source set to 2 kV and 200 °C. The automatic gain control was set to 1 × 10⁶ for full scan MS. The MS data were acquired and processed using Xcalibur 1.3.

High-resolution ESI-MS analysis was conducted on Thermo LTQ XL Orbitrap under the following conditions. A Luna hydrophilic liquid interaction chromatography (HLIC) column (2.0 × 150 mm², 200 Å, Phenomenex) was used to separate the oligosaccharide mixture. Mobile phase A was 5 mM ammonium acetate prepared with HPLC-grade water. Mobile phase B was 5 mM ammonium acetate prepared in 98% HPLC-grade acetonitrile with 2% HPLC-grade water. After injection of 8.0 μl oligosaccharide mixture (1.0 μg μl⁻¹) through an Agilent 1200 autosampler, an HPLC binary pump was used to deliver the gradient from 10% A to 35% A over 40 min at a flow rate of 150 μl min⁻¹. The LC column was directly connected online to the standard electrospray ionization source of LTQ-Orbitrap XL Fourier transform (FT) mass spectrometer (MS) (Thermo Fisher Scientific). The source parameters for FT-MS detection were optimized using fondaparinux to minimize the ion fragmentation and sulfate loss and maximize the signal-to-noise ratio in the negative-ion mode. The optimized parameters included a spray voltage of 4.2 kV, a capillary voltage of −40 V, a tube-lens voltage of −50 V, a capillary temperature of 275 °C, a sheath flow rate of 30 and an auxiliary gas flow rate of 6. External calibration of mass spectra routinely produced a mass accuracy of better than 3 ppm. All FT mass spectra were acquired at a resolution of 60,000 with 300–2,000 Da mass range.
NMR analysis. Synthetic LMWH constructs and intermediates were analyzed by 1D 1H-NMR and 2D NMR (1H-1H COSY, 1H-13C HMBC). All NMR experiments were performed at 298 K on Bruker Avance II 800 MHz spectrometer with Topspin 2.1 software. Samples (3.0 to 6.0 mg) were each dissolved in 0.5 ml D2O (99.996%, Sigma-Aldrich) and lyophilized three times to remove the exchangeable protons. The samples were re-dissolved in 0.4 ml D2O and transferred to NMR microtubes (OD 5 mm, Norrell). We carried out 1D 1H NMR experiments with 256 scans and an acquisition time of 850 ms. We carried out 2D 1H-1H COSY experiments with 16 scans, 1.5-s relaxation delay and 500-ms acquisition time. We carried out 2D 1H-13C HMBC experiments with 16 scans, 1.5-s relaxation delay and 250-ms acquisition time.

The compounds were also analyzed by 1D 1H-NMR, 1D 13C-NMR and 2D NMR (1H-1H COSY, 1H-13C HSQC) on Varian Inova 500 MHz spectrometer with VNmrfl 2.2D software. Samples (2.0 to 5.0 mg) were dissolved in 0.5 ml D2O (99.994%, Sigma-Aldrich) and lyophilized three times to remove the exchangeable protons. The samples were re-dissolved in 0.5 ml D2O and transferred to NMR microtubes (OD 5 mm, Norrell). We carried out 1D 1H-NMR experiments with 256 scans and an acquisition time of 768 ms; 1D 13C-NMR experiments with 40,000 scans, 1.0-s relaxation delay and an acquisition time of 1,000 ms; 2D 1H-1H COSY experiments with 48 scans, 1.8-s relaxation delay and 204-ms acquisition time; and 2D 1H-13C HSQC experiments with 48 scans, 1.5-s relaxation delay and 256-ms acquisition time.

Determination of the binding of synthetic LMWHs to stabilin-2. The stabilin-2 cell line expressing the 190-HARE19, 19, a truncated form of Stabilin-2, was grown to 90% confluency with DMEM + 8% FBS + 50 μg ml−1 hygromycin B in 24-well plates for at least 2 d before the experiment in a standard tissue-culture incubator. Endocytosis medium (DMEM + 0.05% BSA) containing a known amount of [35S]-labeled synthetic LMWH construct, LMWH, or UFH was injected into the lateral tail vein using a 27G1/2 needle mounted on a 1-ml syringe. The labeled material was allowed to circulate in the blood for 12 min while the mouse lay unconscious. The abdominal cavity was exposed by incision and the liver was collected, washed and weighed. Approximately 100 mg from each of the lobes was homogenized in 0.75 ml 1% NP-40 and then centrifuged at 12,000g for 2 min to pellet insoluble material. The supernatant was then added to 4 ml scintillation fluid, mixed for 30 min by rocking and then radioactivity was assessed by a Beckman Coulter LS6500 scintillation counter. The data is presented as the percentage of CPM in total liver divided by total CPM injected ± s.e.m. of 3–5 mice per ligand. Statistical analysis between each group was performed by one-way ANOVA (SigmaPlot Software, Systat) followed by Bonferroni’s post hoc comparison test.