Design and Synthesis of Neutralizable Fondaparinux

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ABSTRACT: Fondaparinux, a clinically approved anticoagulant pentasaccharide for the treatment of thrombotic diseases, displays better efficacy and biosafety than other heparin-based anticoagulant drugs. However, there is no suitable antidote available for fondaparinux to efficiently manage its potential bleeding risks, thereby precluding its widespread use. Herein, we describe a convergent and stereocontrolled approach to efficiently synthesize an aminopentyl-functionalized pentasaccharide, which is further used to prepare fondaparinux-based biotin conjugates and clusters. Biological activity evaluation demonstrates that the anticoagulant activity of the fondaparinux-based biotin conjugate and trimer is, respectively, neutralized by avidin and protamine as effective antidotes. This work suggests that our synthetic biotin conjugate and trimer have potential for the development of neutralizable and safe anticoagulant drugs.

KEYWORDS: anticoagulant agent, heparin, oligosaccharide, glycosylation, synthesis design

INTRODUCTION

Heparin, a highly sulfated linear polysaccharide constituted by alternant α-1,4-d-glucosamine (GlcN) and β-1,4-d-glucuronic acid (GlcA) or α-1,4-L-iduronic acid (IdoA) units, has served as an anticoagulant agent to prevent or treat venous and arterial thrombosis for more than 80 years.1,2 Currently, the approved heparin-based anticoagulant drugs for clinical use mainly contain three forms: unfractionated heparin (UFH, average molecular weight ~16,000 Da), low-molecular weight heparin (LMWH, average molecular weight 3500–6000 Da), and ultralow-molecular weight heparin (UMWH, molecular weight 1500–3000 Da) such as synthetic pentasaccharide fondaparinux (molecular weight 1728 Da).3 UFH, isolated from animal sources such as the porcine intestine or bovine lung, exhibits a rapid anticoagulant effect to treat acute thrombotic events, and the effect can be reversed by protamine.4 However, the usage of UFH may result in the occurrences of heparin-induced thrombocytopenia and osteoporosis.5–6 To improve the pharmacokinetic properties and decrease the risks accompanied by the usage of UFH, LMWH, prepared by chemical or enzymatic degradation of UFH, has been increasingly applied in treatment of thrombotic disorders.7 Compared with UFH, LMWH such as enoxaparin shows a longer half-life and can be administered subcutaneously, which contributes to its clinical use. The anticoagulant effect of enoxaparin is only incompletely neutralized by protamine, which may result in the bleeding risks.8–9 Although enoxaparin has a significantly larger market share, it lacks an effective neutralizable agent. More importantly, the quality control and safety of heterogeneous animal-sourced UFH and LMWH are still concerns due to batch-to-batch differences and potential contaminations. The clinical use of contaminated heparins with over-sulfated chondroitin sulfate resulted in hundreds of patient deaths and other adverse events in 2008.9 As a result, a structurally homogeneous heparin pentasaccharide fondaparinux (Arixtra) as an alternative is extensively used to treat thrombotic events.10,11

Unlike heterogeneous animal-sourced heparins, fondaparinux is a pure synthetic pentasaccharide exhibiting antithrombin III-mediated exclusive factor Xa (FXa) inhibition activity.12 Since the pentasaccharide is obtained by chemical synthesis,13–15 the quality in terms of purity and reproducibility can be readily controlled. Additionally, fondaparinux exhibits a longer half-life and better antithrombotic efficacy and biosafety than the aforementioned two forms.8,16 However, clinical use of fondaparinux is still restricted because the anticoagulant effect cannot be immediately neutralized in the bleeding events.17 Although many elegant methodologies and strategies20–36 have been developed to synthesize heparin oligosaccharides for drug discovery and great endeavors have been dedicated to the development of reversible heparin-based anticoagulant agents such as biotinylated idraparinux derivatives that can be neutralized by non-toxic avidin protein
the design and efficient synthesis of neutralizable fondaparinux still remain challenging. Given the drug/antidote pair by binding-assisted clearance from circulation such as biotin/avidin and large-size homogeneous heparin oligosaccharide/protamine, we describe here the chemical preparation of fondaparinux-based biotin conjugate and clusters relying on a synthetic aminopentyl-functionalized pentasaccharide (Figure 1). Additionally, all these fondaparinux-based molecules were evaluated to explore the anti-FXa activity and neutralizable anticoagulant activity.

**RESULTS AND DISCUSSION**

**Synthesis Plan of Aminopentyl-Functionalized Pentasaccharide 1**

The chemical synthesis of 1 is challenging because it requires regioselective introduction of sulfate groups, installment of glycosidic bonds in a stereoselective fashion, and differentiation of amino functionalities for N-sulfation in a saccharide moiety and late-stage conjugation in the artificial spacer. It is envisaged that protected pentasaccharide 12 could be synthesized via a convergent and stereocontrolled [3 + 2]...
Also, the 6-iodosuccinimide (TMSOTf) gave exclusively benzyl (Bn) ethers and TEMPO = 2,2,6,6-tetramethyl-1-piperidinylxyloxy, BAIB = (diacetoxyiodo)benzene, and CF$_3$C(NPh)Cl = N-phenyltrifluoroacetimidate.

**Scheme 2. Synthesis of Trisaccharide Donor 10**

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Reagents and conditions: (a) NIS, TMSOTf, CH$_2$Cl$_2$, 0 °C, 85%; (b) (i) hydrazine acetate, DCM/pyridine; (ii) NaH, BnBr, DMF, 88% over two steps; (c) (i) DDQ, CH$_2$Cl$_2$/PBS (100 mM, pH 7.4); (ii) Ac$_2$O, DMAP, pyridine, 80% over two steps; (d) (i) p-TsOH$_2$H$_2$O, CH$_2$Cl$_2$/MeOH; (ii) TEMPO, BAIB, CH$_2$Cl$_2$/H$_2$O; (iii) CH$_3$I, KHCO$_3$, DMF, 80% over three steps; (e) TMSOTf, CH$_2$Cl$_2$, −20 °C, 66%; (f) (i) HF/pyridine, THF; (ii) CF$_3$C(NPh)Cl, Cs$_2$CO$_3$, CH$_2$Cl$_2$, 85% over two steps. Nap = 2-naphthylmethyl, TDS = thexyldimethylsilyl, NIS = N-iodosuccinimide, TMSOTf = trimethylsilyl trifluoromethanesulfonate, DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, p-TsOH$_2$H$_2$O = p-toluenesulfonic acid monohydrate, TEMPO = 2,2,6,6-tetramethyl-1-piperidinylxylo, BAIB = (diacetoxyiodo)benzene, and CF$_3$C(NPh)Cl = N-phenyltrifluoroacetimidoyl chloride.

**Scheme 3. Synthesis of Disaccharide Acceptor 11**

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Reagents and conditions: (a) NIS, TMSOTf, DCM, 0 °C, 84%; (b) (i) p-TsOH$_2$H$_2$O, CH$_2$Cl$_2$/MeOH; (ii) TEMPO, BAIB, CH$_2$Cl$_2$/H$_2$O; (iii) CH$_3$I, KHCO$_3$, DMF, 80% over three steps; (d) (i) HF/pyridine, THF; (ii) CF$_3$C(NPh)Cl, Cs$_2$CO$_3$, CH$_2$Cl$_2$, 85% over two steps. Nap = 2-naphthylmethyl, TDS = thexyldimethylsilyl, NIS = N-iodosuccinimide, TMSOTf = trimethylsilyl trifluoromethanesulfonate, DDQ = 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, p-TsOH$_2$H$_2$O = p-toluenesulfonic acid monohydrate, TEMPO = 2,2,6,6-tetramethyl-1-piperidinylxylo, BAIB = (diacetoxyiodo)benzene, and CF$_3$C(NPh)Cl = N-phenyltrifluoroacetimidoyl chloride.

Method from appropriately protected monosaccharide building blocks 5–9 (Scheme 1). A well-organized protecting group strategy made it possible to achieve O-sulfation, N-sulfation, and highly stereoselective glycosylations. Acetyl (Ac) and benzyloxycarbonyl (Cbz) as permanent protecting groups of the 1,2-trans-glycosidic linkages by neighboring-group participation. Acido groups at C-2 positions of 5, 7, and 9 were employed as amino precursors for selective N-sulfation. Moreover, these azido groups did not perform neighboring-group participation, thus contributing to installing 1,2-α or β-glycosidic linkages by the anomic effect. Also, the 6-O-acetyl or Bz group of 5-glucosazide donors facilitated α- or β-glycosidic linkage due to the remote participation effect. Additionally, benzyl (Bn) ethers and benzyloxycarbonyl (Cbz) as permanent protecting groups could be easily removed for unmasking the hydroxy groups and amino group to afford fondaparinux-based pentasaccharide 1 with an anomic aminopentyl linker.

Synthesis of β-1,4-linked GlcA-GlcN$_3$ glycosidic linkage in 16 is challenging due to the inherently low reactivity of the glucuronic acid donor and the lack of neighboring-group participation in the presence of 2-O-benzyl (Bn) protection. Although our previous work showed that a Ag$_2$CO$_3$-mediated coupling of glycosyl bromide with the glucosazide acceptor gave the desired disaccharide as a mixture of anomers ($\beta$/$\alpha$ = 7:1), the separation of both stereoisomers was extremely tedious and time-consuming. Furthermore, we tried different glucuronic acid donors for glycosylation, which did not give satisfactory results. To circumvent this problem, the integration of flexible orthogonal protecting group manipulations with a late-stage oxidation strategy made it possible to efficiently synthesize disaccharide acceptor 16. First, the coupling of thioglycoside donor 6 with 7 using N-iodosuccinimide (NIS) and catalytic trimethylsilyl trifluoromethanesulfonate (TMSOTf) gave exclusively β-linked disaccharide 13 ($\beta$-1,4-2 = 8.0 Hz) in 85% yield owing to the neighboring-group participation of 2-O-Lev. Then, the Lev ester was readily converted into the corresponding benzyl ether for permanent protection of the C-2 hydroxyl to give 14 via a two-step procedure involving the removal of the Lev group by hydrazine acetate to afford a free hydroxyl group, followed by benzylation of the resulting hydroxyl group using BnBr and NaH. Subsequently, Nap ethers of 14 were oxidatively cleaved
using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) without affecting any other protecting groups to provide a diol intermediate, which was acetylated using Ac₂O and 4-dimethylaminopyridine (DMAP) to give 15 in 80% yield for two steps. The cleavage of the 4,6-\text{O}-benzylidene group using \(p\)-TsOH·H₂O in CH₂Cl₂/MeOH afforded the corresponding 4,6-diols, which were reacted with TEMPO/BAIB for selective oxidation of the primary alcohol to carboxylic acid. The resulting carboxylic acid was esterified using MeI/KHCO₃ to give the desired disaccharide acceptor 11.

**Synthesis of L-IdoA-α-(1→4)-D-GlcN₁ Disaccharide Acceptor 11**

As illustrated in Scheme 3, the disaccharide acceptor 11 was efficiently prepared by the same late-stage oxidation procedure as that used for the synthesis of 16. An NIS/TMSOTf-promoted coupling of suitably functionalized idosyl donor 8 with acceptor 9 provided only \(\alpha\)-anomeric product 18 in a high yield of 84% owing to neighboring-group participation of Bz at the C-2 position. The benzylidene acetal of 18 was cleaved by treating with \(p\)-TsOH/H₂O in CH₂Cl₂/MeOH to afford the corresponding 4,6-diols, which were reacted with TEMPO/BAIB for selective oxidation of the primary alcohol to carboxylic acid. The resulting carboxylic acid was esterified using MeI/KHCO₃ to give the desired disaccharide acceptor 11.
Synthesis of Fondaparinux-Based Pentasaccharide 1 and Biotin Conjugate 2

With trisaccharide donor 10 and disaccharide acceptor 11 in hand, attention was focused on preparing aminopentyl-functionalized pentasaccharide 1 for late-stage conjugation (Scheme 4). First, a TMSOTf-mediated glycosylation of PTFAI donor 10 and acceptor 11 proceeded in highly stereoselective fashion to afford the fully protected pentasaccharide 12 with a higher yield of 89% than that in our previous result (68%).13 Probably, the use of the PTFAI glycosyl donor improved the coupling yield compared with that of the glycosyl trichloroacetimidate donor by eliminating the potential rearrangement reaction of the donor in glycosylation. The highly stereoselective glycosylation is attributed to the dual effects involving the anomeric effect of the azido group at the C-2 position and the remote participation effect of the acetyl (Ac) ester at the C-6 position in the D-glucosazide moiety of donor 10. The anomeric configuration of the newly generated α(1,4)-glycosidic linkage was verified by detailed NMR analysis (\(J_{H1-H2} = 3.8\) Hz). Next, differential manipulations of protecting groups for regioselective installment of sulfate groups were carried out. The saponification of esters to remove acetyl, Bz, and methyl groups provided partially deprotected pentasaccharide 19 with five free hydroxyl groups via a two-step procedure including treatment of 12 with \(\text{H}_2\text{O}_2\) and LiOH, followed by addition of NaOH. The resulting hydroxy groups in 19 were completely sulfated by the sulfur trioxide trimethylamine complex (SO\(_3\)·NMe\(_3\)) to afford pentasulfate compound 20. Subsequently, three azide groups were reduced by trimethyl phosphine (PMe\(_3\)) to give an amine intermediate, followed by N-sulfation with SO\(_3\)·NMe\(_3\) in the presence of Et\(_3\)N/pyridine. The resulting N-sulfated crude was purified using a reverse-phase C-18 silica gel column, followed by the SCR732 Na\(^+\)-form column to provide 21. Finally, the removal of Bn and Cbz by catalytic hydrogenolysis using Pd(OH)\(_2\)/C in \(\text{t-BuOH}\) and \(\text{H}_2\text{O}\) afforded fondaparinux-based pentasaccharide 1. The stereochemistry of five glycosidic linkages in compound 1 was confirmed by detailed NMR characterization (Supporting Information Page S20). In order to prepare biotinylated pentasaccharide 2, compound 1 was conjugated to biotin by an efficient reaction of the free amine of the artificial spacer with the activated biotin succinimide ester (biotin-OSu). The resulting crude was readily purified by size exclusion chromatography using Bio-Gel P4 to afford fondaparinux-based biotin conjugate 2 in a yield of 79%.

Synthesis of Fondaparinux-Based Dimer 3 and Trimer 4

Initially, the coupling of excess aminopentyl-functionalized pentasaccharide 1 (10 equiv) with di-N-hydroxysuccinimidyl suberate in phosphate buffered saline (PBS) buffer (pH 7.4) did not give any dimer product, and in this case, the starting material and the hydrolysis product of the activated monoester were mainly observed by electrospray ionization-mass spectrometry (ESI-MS) analysis (Figure S1). To circumvent this issue, the preparation of fondaparinux-based dimer 3 and trimer 4 was achieved by using thiol−maleimide coupling chemistry with a two-step procedure (Scheme 5). First, the primary amine from the linker of 1 was treated with an excess of dithiobispropanoic acid bis(N-hydroxysuccinimide ester) and trimethylamine (Et\(_3\)N) in dimethyl sulfoxide (DMSO), followed by the treatment with dithiothreitol (DTT) to reduce the disulfide bond to furnish thiol-derivated compound 22. Next, the resulting thiol was reacted with the dimaleimide- and trimaleimide-activated spacer in PBS buffer/dioxane/acetonitrile to afford the desired dimer 3 and trimer 4, which were purified using Bio-Gel P4 and P6, respectively.
Bioactivity Tests

Since the FDA-approved UMWH fondaparinux and LMWH enoxaparin have exhibited excellent anticoagulant activity and the latter is incompletely neutralized by protamine, we wonder whether our synthetic fondaparinux-based biotin conjugate as UMWH and as LMWH can display effective anti-factor Xa (FXa) activity and be neutralized by avidin and protamine, respectively. The anticoagulant activities of compounds 2–4 and corresponding neutralization efficacy were evaluated in vitro with comparison to those of fondaparinux and enoxaparin. Biotinylated pentasaccharide 2 as UMWH exhibited similar anti-FXa activity with fondaparinux (median inhibitory concentration IC\textsubscript{50}: 6.3 nM for 2 vs 4.4 nM for fondaparinux, Figure 2A), and the anti-FXa activity of 2 was efficiently neutralized by the addition of avidin (Figure 2B). Next, we tested the anti-FXa activities of fondaparinux-based clusters 3–4 as LMWH and showed similar anti-FXa activity with enoxaparin (Figure 2C). As compared to enoxaparin, which was only partially neutralized by protamine, dimer 3 showed inferior protamine-mediated reversibility than enoxaparin, while trimer 4 was nearly completely reversed by treatment with protamine (Figure 2D). These results suggested that synthetic fondaparinux-based biotin conjugate 2 and trimer 4 are encouraging targets with great potential for the development of neutralizable and safe anticoagulant drugs.

CONCLUSIONS

In summary, we have developed a convergent strategy for efficient synthesis of a fondaparinux-based pentasaccharide 1 with an anemic aminopentyl linker. The careful selection of protecting groups makes it possible to construct the target pentasaccharide by stereoregulated chemical glycosylations, selective O-sulfations, and differentiation of amino functionalities of the saccharide moiety and linker for selective N-sulfations. In particular, the integration of flexible orthogonal protecting group manipulations with the late-stage oxidation strategy enables stereoselective and facile synthesis of challenging β-1,4-linked glucuronic acid modular disaccharide 16. Additionally, efficient preparation of fondaparinux-based biotin conjugate 2 and clusters 3–4 was accomplished by coupling pentasaccharide 1 with functionalized biotin and spacers, respectively. More importantly, the anticoagulant effect of biotinylated pentasaccharide 2 can be efficiently neutralized by avidin, and the anticoagulant activity of trimer 4 can be completely reversed by protamine. Therefore, the synthetically neutralizable fondaparinux-based compounds 2 and 4 could serve as safer anticoagulant agents to solve potential medical problems of current clinically used fondaparinux- and animal-sourced low-molecular weight heparin. Taken together, this work will facilitate efficient synthesis of heparin-like oligosaccharides and development of the next generation of safe and effective heparin-based anticoagulant drugs.

METHODS

Materials

Unless otherwise noted, solvents and reagents were purchased and directly used without further purification. Chemical reagents were purchased from J&K Scientific Ltd. and TCI Shanghai (Shanghai, China). Avidin beads and protamine were purchased from Sangon Biotech (Shanghai, China). Bio-Gel P-2, P-4, or P-6 (45–90 μm) was purchased from Bio-Rad Laboratories (Hercules, California, USA). C18 silica gel was purchased from Waters Corporation (Milford,
Synthesis of Fondaparinux-Based Dimer 3

Compound 1 (50 mg, 31.6 μmol) was dissolved in dry DMSO (400 μL) followed by addition of 3,3′-dithiobispropanoic acid bis(N-hydroxysuccinimide ester) (64 mg, 158 μmol) and Et3N (44 μL, 316 μmol). The reaction mixture was stirred at room temperature for 8 h. Subsequently, DTT (48 mg, 311 μmol) was added, and the reaction mixture was stirred for another 3 h. The reaction mixture was lyophilized to give a residue. The resulting residue was purified by Bio-Gel P-2 size-exclusion chromatography (eluent: 0.1 M NH4HCO3) to give 22 (51.5 mg, 98%), which was immediately used for next thiol–maleimide coupling reactions. The resulting compound 22 (40 mg, 24 μmol) was dissolved in a mixture solution of degassed PBS buffer (100 mM, pH 7.4)/dioxane/acetonitrile (v/v/v = 4:1:1; a total volume of 300 μL), followed by the addition of the dopamine-activated spacer (0.5 mg, 1.2 μmol). Then, NaOH (aq) was added to adjust the pH to 7–9, and the reaction mixture was stirred at room temperature for 24 h under Ar atmosphere. Subsequently, DTT (14.8 mg, 96 μmol) was added, and the reaction mixture was stirred for another 3 h. The reaction mixture was loaded on a Bio-Gel P-4 size-exclusion chromatography column (eluent: 0.1 M NH4HCO3) for purification to afford fondaparinux-based dimer 3 (2.9 mg, 65%). 1H NMR (600 MHz, D2O): δ 5.56 (d, J = 3.7 Hz, 2H, 2H-1-GlcN3), 5.52 (d, J = 2.6 Hz, 2H, 2H-1-GlcN2), 5.16–5.11 (m, 4H, 2H-1-GlcN1, 2H-1-IodoA), 4.94–4.89 (m, 2H, 2H-5-IodoA), 4.61 (d, J = 7.8 Hz, 2H, 2H-1-GlcA), 4.45 (d, J = 10.8 Hz, 2H, 2H-6-GlcN), 4.42–4.20 (m, 15H), 4.20–4.10 (m, 7H), 4.09–3.92 (m, 9H), 3.92–3.78 (m, 10H), 3.77–3.48 (m, 3H), 3.48–3.11 (m, 10H, 2H-2-GlcN3, 2H-2-GlcN2, 2H-2-IodoA, 2H-2-GlcA), 3.09–2.74 (m, 7H), 2.73–2.56 (m, 7H), 2.56–2.38 (m, 8H), 1.68–1.58 (m, 4H), 1.56–1.48 (m, 4H), 1.47–1.33 (m, 4H). HRMS (ESI): [M−H]+ m/z calcd for C46H35N4O15S8, 1245.0650; found, 1245.0585.

Synthesis of Fondaparinux-Based Biotin Conjugate 2

Fondaparinux-based biotin conjugate 2 was dissolved in a mixture solution of degassed PBS buffer (100 mM, pH 7.4)/dioxane/acetonitrile (v/v/v = 4:1:1; a total volume of 300 μL) followed by the addition of the thiolmaleimide-activated spacer (0.5 mg, 1.2 μmol). Then, NaOH (aq) was added to adjust the pH to 7–9, and the reaction mixture was stirred at room temperature for 24 h under Ar atmosphere. Subsequently, DTT (13.1 mg, 85.2 μmol) was added, and the reaction mixture was stirred for another 3 h. The reaction mixture was loaded on a Bio-Gel P-6 size-exclusion chromatography column (eluent: 0.1 M NH4HCO3) for purification to afford fondaparinux-based trimer 4 (2.3 mg, 57%). 1H NMR (800 MHz, D2O): δ 6.50–5.54 (m, 6H, 3× H-1-GlcN3, 3× H-1-GlcN2), 5.25–5.10 (m, 6H, 3× H-1-GlcN1, 3× H-1-IodoA), 4.98–4.88 (m, 2H, 3H, 3× H-5-IodoA), 4.68–4.59 (m, 3H, 3× H-1-GlcA), 4.47–4.07 (m, 31H), 4.06–3.79 (m, 20H), 3.78–3.50 (m, 29H), 3.49–3.16 (m, 34H, including 3× H-2-GlcN3, 3× H-2-GlcN2, 3× H-2-GlcN1, 3× H-2-IodoA, 2H-2-IodoA, 3× H-2-GlcA), 3.11–2.77 (m, 9H), 2.73–2.66 (m, 3H), 2.64–2.50 (m, 3H), 2.58–2.53 (m, 3H), 2.52–2.47 (m, 3H), 2.46–2.42 (m, 3H), 1.75–1.60 (m, 6H), 1.46–1.36 (s, 6H), 0.85 (s, 3H, −CH3). HRMS (ESI): [M−H]+ m/z calcd for C60H56N14O17S8, 1139.0711; found, 1139.2380.

Evaluation of In Vitro Anti-FXa Activity of Compounds 2–4

The Biophen anti-Xa (two-stage heparin assay) kit was used to evaluate anti-FXa activity of compounds 2–4 according to the procedures provided by the manufacturer. First, antithrombin [anti-Xa reagent 1 (R1)], factor Xa (R2), and factor Xa-specific chromogenic substrates (R3) were dissolved using distilled water (1 mL) for reconstitution. Also, the resulting reagents R1, R2, and R3 were diluted five times by using Tris-ethylenediaminetetraacetic acid (EDTA)-NaCl-poly(ethylene glycol) (PEG) buffer (pH 8.4) for immediate use. Fondaparinux and biotinylated fondaparinux conjugate 2 were dissolved in the above-mentioned buffer at various concentrations (0–500 ng/mL), and enoxaparin and fondaparinux-based dimer 3 and trimere 4 were also dissolved in the same buffer varying from 0 to 5000 ng/mL. 40 μL of each sample solution was introduced into a 96-well plate, followed by the addition of 20% AcOH (80 μL). minced thrombin (100 mU) was added. BioTek Synergy 2 was used to record the absorbance at 405 nm. The absorbance values were calculated to measure the activity of FXa.

Neutralization of Fondaparinux-Based Biotin Conjugate 2 by Avidin In Vitro

The fondaparinux and biotinylated fondaparinux conjugate 2 were dissolved in buffer (Tris-EDTA-NaCl-PEG, pH 8.4) at a concentration of 1000 ng/mL. The avidin beads from Sangon Biotech were suspended at various concentrations (0–100 μg/mL) in the above-mentioned buffer. The avidin beads were incubated with fondaparinux or biotinylated fondaparinux conjugate 2 for 30 min at room temperature (a total volume of 300 μL). Samples were then put on a magnetic rack, and anti-FXa activity was determined in the supernatant following the anti-FXa measurement protocols as described above.
Neutralization of Fondaparinux-Based Dimer 3 and Trimer 4 by Protamine In Vitro

The enoxaparin and fondaparinux-based dimer 3 and trimer 4 were dissolved in buffer (Tris-EDTA-NaCl-PEG, pH 8.4) at a concentration of 5000 ng/mL. Protemine from Sangon Biotech was dissolved in the same buffer and diluted at various concentrations (0–200 μg/mL). Samples (40 μL) and protamine (20 μL) with various concentrations were added into a 96-well plate and incubated for 10 min at 37 °C; then, the mixture (60 μL) was subjected to anti-FXa measurement protocols as described above.

ASSOCIATED CONTENT
Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.2c00537.

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Notes
The authors declare no competing financial interest.

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