Protein Interactions

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The Design and Evaluation of Heparin-Binding Foldamers**

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Foldamers, nonbiological oligomers with well-defined secondary or tertiary structures,[1,2] provide novel templates for the design of biologically active molecules that compete for a

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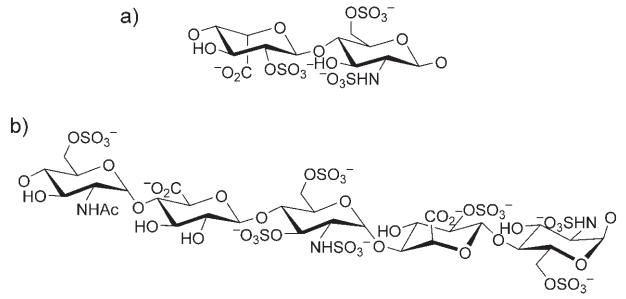
variety of protein–protein[3] and protein–membrane interactions.[4] Their semirigid structures and their adjustable lengths mean that they provide excellent starting points for the elaboration of protein mimics that might be difficult to design based on small-molecule scaffolds. Here we describe the design of aryl amide oligomers that compete for heparin–protein interactions. Heparin, a linear and highly sulfated polysaccharide, is a crucial component in a variety of biological processes mediated by specific heparin–protein interactions including blood coagulation, viral infection, and cell growth. Heparin has become a commonly used clinical anticoagulant to prevent and treat thrombotic diseases.[5,6] However, bleeding complications including hemorrhage and heparin-induced thrombocytopenia (HIT), which is a common immune-mediated disorder, are major adverse effects associated with heparin therapy.[7] Therefore, frequent coagulation monitoring may be necessary to minimize the risk of life-threatening hemorrhages resulting from an overdose, while at the same time maximizing the anticoagulant efficacy.

Low-molecular-weight (LMW) heparins, like unfractionated (UF) heparin, consist of repeating units of L-iduronic acid and D-glucosamine (Scheme 1a). LMW heparins have



Scheme 1. a) The major repeating unit and b) the antithrombin III binding pentasaccharide of heparin.

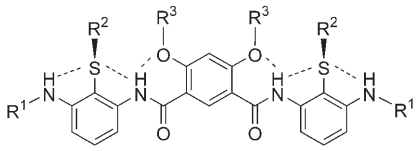
resolved some of the problems associated with UF-heparin use, in that they have a more predictable dose response, an improved bioavailability, and a longer half-life.[8] Protamine is used extensively as a clinical heparin antidote to neutralize the anticoagulation function of heparin following cardiovascular surgery[9] but it is not prescribed for use with LMW-heparin therapy. Protamine treatment can also cause several side effects mediated by nonimmunologic and immunoglobulin-mediated pathways.[10] Thus, much safer and more effective agents for neutralizing the anticoagulant function of heparin are currently of great interest.

The interaction between heparin and antithrombin, a plasma serine proteinase inhibitor that is the major inhibitor of the coagulation cascade, facilitates a conformational change in antithrombin. This conformational change accelerates inhibition of coagulation factors such as thrombin and factor Xa.[11,12] Through X-ray crystallographic analysis of the antithrombin–pentasaccharide complex[13,14] and several studies of site-directed mutagenesis of antithrombin,[15,16] it has been shown that the negatively charged pentasaccharide binds to the basic amino acids (lysine and arginine) of antithrombin.

Furthermore, by comparing various heparin-binding proteins, Cardin and Weintraub classified two consensus sequences responsible for binding, XBBXBX and XBBBXXBX, where B is a basic residue and X is any other amino acid. It was suggested that these sequence motifs fold along one face of an α helix or β sheet.[17] Most heparin-binding sites have a bipolar structure, in which the basic amino acids are facing one side; neutral or lipophilic amino acids generally face the opposite side, regardless of the secondary structure.[18]

A few medium-sized peptides such as protamine analogues,[10,19] antithrombin-derived peptides,[20] and polyarginine peptides,[21] have been reported as potent heparin antidotes. Small-molecule inhibitors of heparin may alleviate complications associated with peptide-based heparin antidotes, such as proteolytic stability, distribution, and difficulty of scale-up. We have previously reported the synthesis of amphiphilic arylamide oligomers that mimic the biological properties of antimicrobial peptides and proteins.[4] Herein, we expand this strategy towards the design of small molecules that strongly interact with highly sulfated heparin.

Our design is based on a 1,3-substituted arylamide oligomer with additional hydrogen-bonding substituents included to increase solubility and conformational rigidity and to provide appropriately spaced cationic groups to interact with heparin. The thioether and ether groups of the generic oligomer **1** provide internal hydrogen-bond acceptors, while simultaneously providing points of attachment for additional functional groups.



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Initially, a molecular dynamics study on binding of one of the arylamide analogues (shown in Figure 1, compound **8c**) to heparin in aqueous solution was undertaken. Analysis of the obtained trajectory suggests that these compounds might interact strongly with the pentasaccharide sequence of heparin (PDB code: 1E03). Owing to the extensive charge complementarity between the inhibitor and the heparin analogue, the binding is dominated by electrostatic interactions. At early stages in the trajectory, the two guanidium cations of the terminal side chains (R^4, Table 1) anchor compound **8c** to the pentasaccharide in a nearly parallel mode, by closely interacting with CH_2–OSO_3^- groups of the two terminal pentasaccharide sugars (Figure 1a). The other positively (NH_3^+) and negatively (COO^-, OSO_3^-) charged groups of compound **8c** and the pentasaccharide, respectively, are then aligned. The limited flexibility of the pentasaccharide (Figure 1a) allows these groups to assume optimal positions, thereby maximizing the attractive interactions (Figure 1b) and hydrogen bonding without incurring an overly large loss in conformational entropy, which might occur with more flexible small peptides. All the side chains of compound **8c** are involved in the binding, with the terminal ones playing an

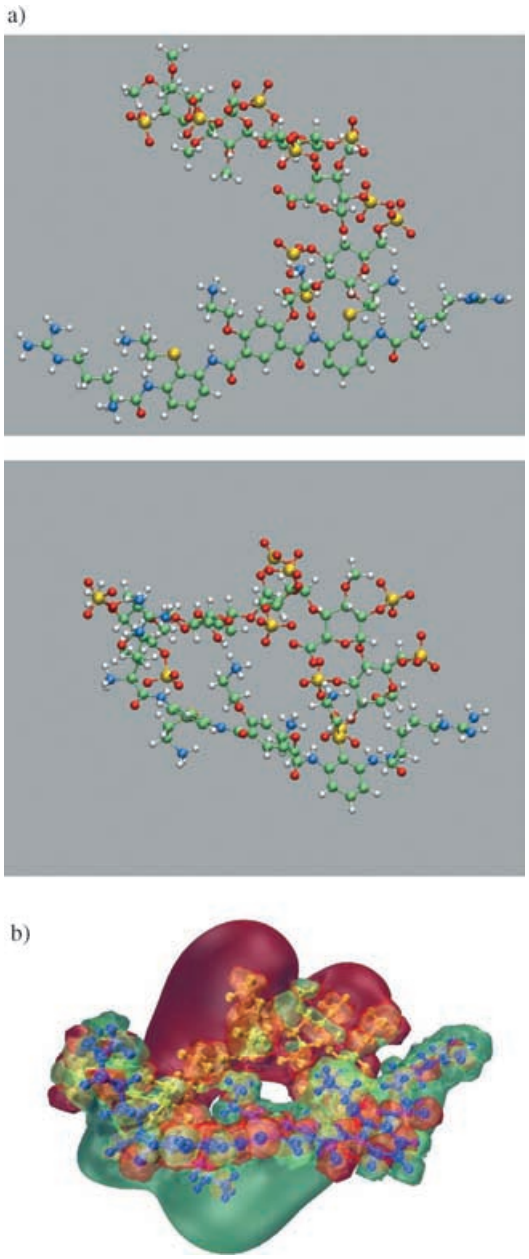
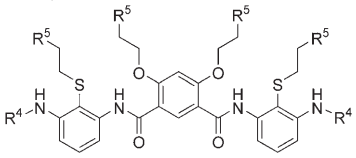
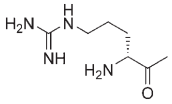
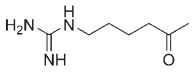


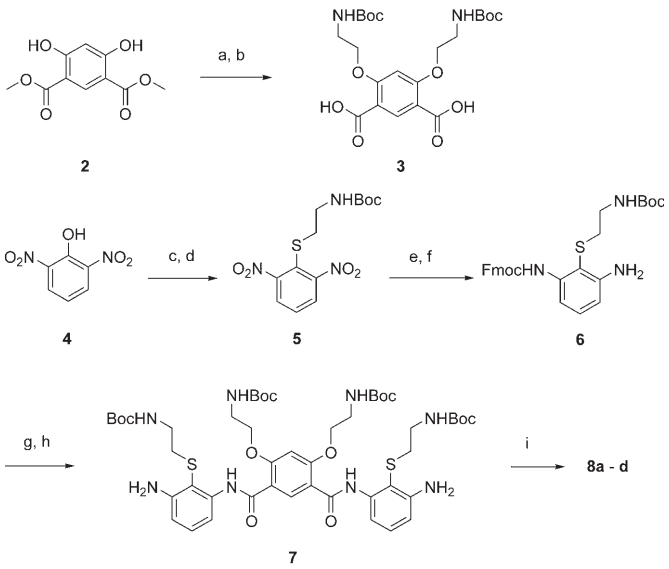
Figure 1. a) The initial (top) and final (after 7 ns, bottom) conformations of the aryl amide oligomer–pentasaccharide heparin system. In the initial stage of the simulation, the aryl amide backbone undergoes an internal twist, which facilitates the anchoring of the terminal aryl amide groups to pentasaccharide. Color code: S: yellow; C: cyan; N: blue; O: red. Water molecules and hydrogen atoms are not shown for clarity. b) The final aryl amide oligomer–pentasaccharide conformation, with electrostatic potential surfaces[29] shown in red (aryl amide oligomer) and green (pentasaccharide).

Table 1: Biological activity of small molecules **8a–d**.



Compound	R^4	R^5	IC_{50} [μM]	K_B [μM][a]	HC_{50} [μM][b]
8a	H	NH_2	256	6.7	>1540
8b	H		77.9	3.2	>1363
8c		NH_2	22.5	1.8	>1087
8d		NH_2	28.1	2.0	927

[a] The dissociation constant (K_B) was measured by Schild plot analysis by using the anti-Factor Xa assay. [b] The HC_{50} value (measurement of hemolytic activity) was obtained by measuring 50% lysis of human erythrocytes.



Scheme 2. a) Boc-NH$(CH_2)_2$OH, DEAD, PPh_3, THF; b) 2 N LiOH, MeOH, THF; c) TsCl, DIEA, CH_2Cl_2; d) Boc-NH$(CH_2)_2$SH, DIEA, CH_2Cl_2; e) $SnCl_2{\cdot}2H_2O$, $NaOAc{\cdot}3H_2O$, EtOH, 78 °C; f) FmocCl, pyridine, THF, 0 °C; g) **2**, $(COCl)_2$, pyridine, DMF (cat.), THF, then **6**, Et_3N, DMAP, CH_2Cl_2; h) 20% PIP in DMF; i) for **8a**: 50% TFA in CH_2Cl_2; for **8b** and **8a**: *N*,*N*′-bis(*tert*-butoxycarbonyl)-1*H*-pyrazole-1-carboxamidine, DIEA, MeCN/H_2O, then 50% TFA in CH_2Cl_2; for **8c** and **8d**: RCO_2H, $POCl_3$, pyridine, −15 °C, then 50% TFA in CH_2Cl_2. Boc = *tert*-butoxycarbonyl, DEAD = diethylazodocarboxylate, DIEA = *N*,*N*-diisopropylethylamine, DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide, Fmoc = 9-fluorenylmethoxycarbonyl, PIP = piperidine, TFA = trifluoroacetic acid, THF = tetrahydrofuran, Ts = toluene-4-sulfonyl.

additional role of initial anchoring and aligning. However, some of the pentasaccharide charged groups are pointing away from the complex, toward the solution.

Based on these observations, we synthesized a series of aryl amide oligomers, as described in Scheme 2.

We examined the interaction between compounds **8a–d** and LMW heparin (Lovenox, $M_W = 4500$ Daltons) and UF heparin, respectively, both of which contain an antithrombin-binding domain (pentasaccharide, Scheme 1b). The ability of the compounds to compete with antithrombin for binding to Lovenox at physiological salt concentration (0.15 M NaCl) was assessed by a standard *anti*-factor Xa chromogenic assay (Figure 2).

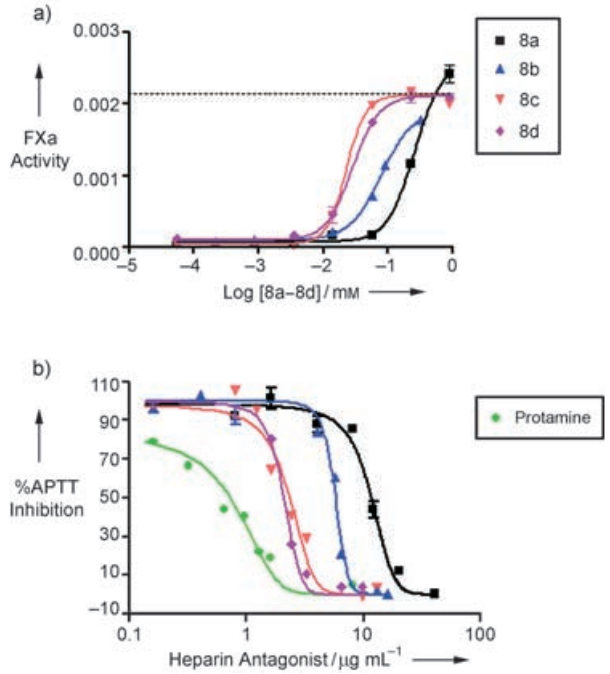


Figure 2. Dose-dependent neutralization of a) LMW heparin and b) UF heparin by compounds **8a–d**. a) Factor Xa assay. b) aPTT clotting assay in the presence of UF heparin (2.42 $\mu g\,mL^{-1}$).

Compound **8a** served as an initial lead compound to inhibit the function of Lovenox. Although it exhibited a low in vitro inhibition ($IC_{50} = 256\ \mu M$, Table 1) against Lovenox, we were inspired to investigate the possible role of the basic side chain of the compound in its interaction with polysulfated heparin. The conversion of the side chains of **8a** to give the polyguanidinylated derivative **8b** resulted in a threefold increase in potency. This result is consistent with observations reported by Linhardt and co-workers. They suggested that arginine binds about 2.5 times more tightly than lysine to the sulfate groups of heparin due to distinct hydrogen bonding.[22] Therefore, we reasoned that the increased activity of compound **8b** arose from differences in hydrogen bonding between ammonium or guanidinium cations and the sulfate groups of LMW heparin.

To obtain more potent heparin inhibitors, further chemical modification was necessary. The introduction of extra positively charged substituents at the terminal amines gave compounds **8c** and **8d**, which exhibited a greater than tenfold increase in potency compared to that of **8a**. Additionally, **8d** was found to have a similar in vitro efficacy to **8c**, in that both compounds provide significant improvement and have similar inhibition constants ($IC_{50} = 28.1$ and $22.5\ \mu M$, respectively, Table 1). The introduction of additional positive charges enhanced the potency of the compounds, as expected from the molecular dynamics study.

To estimate the binding affinities of compounds **8a–d**, Schild plot analysis, which measures agonist dose–response curve shifts by a competitive antagonist, was used to evaluate their capacity to interact with LMW heparin. Table 1 lists the dissociation constants (K_B) of these four compounds. These compounds prevent the binding of heparin to antithrombin in a dose-dependent manner. The Schild plot of inhibition for compounds **8a–d** establishes that they are competitive inhibitors for heparin–antithrombin formation with dissociation constants (K_B) of 6.7, 3.2, 1.8, and 2.0 μM, respectively. These data also show that **8c** was the most potent inhibitor among compounds **8a–d**.

To further address the specificity and affinity of these foldamers in a stringent biological medium, we examined their abilities to inhibit clotting in human plasma by measuring the neutralization of UF heparin by **8a–d** and protamine in an activated partial thromboplastin time (aPTT) clotting assay. The study was conducted with UF heparin because conventional aPTT clotting assays are a relatively insensitive method for measuring the activity of LMW heparin. As shown in Figure 2b, **8a–d** and protamine efficiently reversed heparin-induced aPTT clotting. Also, similar patterns of neutralization of heparin by **8a–d** were observed, which were consistent with those from the factor Xa assay. The most active compounds **8c** and **8d** were only two- to threefold less potent than protamine (average $M_W = 5100$ Daltons), despite the fact that these are small molecules.

Finally, as an initial test of toxicity, we measured hemolytic activities of the compounds against human erythrocytes. None of these small molecules lysed human red cells at concentrations as high as 1000 μM.

In summary, we have described the synthesis of a series of arylamide oligomers, which interact with LMW heparin and UF heparin. This interaction inhibits the ability of LMW heparin to activate antithrombin and, in this way, affects the biological function of LMW heparin. These arylamide oligomers are the first example of low-molecular-weight antagonists that inhibit the anticoagulant function of heparin.

Experimental Section

The synthesis of all compounds is described in the Supporting Information. The factor Xa assay in the presence of Lovenox and inhibitor was measured in a chromogenic assay by using reagents purchased from DiaPharma. In brief, the reagents were reconstituted in a 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.4. Antithrombin was prepared with 0.336 M NaCl for a physiological salt concentration (0.15 M) in the final reaction. Antithrombin ($0.08\ IU\,mL^{-1}$; 50 μL) was mixed with Lovenox ($11.2\ \mu g\,mL^{-1}$; 1 μL). After the incubation for 5 min, different concentrations of inhibitor (1 μL) were added to each row of wells; the wells were then gently shaken and incubated for 20 min. Factor Xa ($2.8\ nkat\,mL^{-1}$; 50 μL) was then added and the reaction mixture was shaken and incubated for an additional 10 min. Substrate (S-2765; 10 μL) was added and the 96-well plate was then shaken and read every 30 s for 7 min at 405 nm on a ThermoLabsystems Multiskan Spectrum. In the assay for Schild plot analysis, we followed the same protocol as for the anti-factor Xa assay except the concentrations of Lovenox and inhibitors were varied.

aPTT activity was resolved by using a BBL Fibrometer (Becton Dickinson, San Jose, CA) and a commercial kit containing Citrex I Control Plasma and Cephalinex-Activated PTT Reagent (Bio/Data Corporation, Horsham, PA). In brief, heparin (5 μL) and antagonist (5 μL) were preincubated (37 °C on a Fibrometer's heating block) for 10 s prior to addition of control plasma (0.1 mL) into the test cuvette. This mixture was allowed to incubate for 2 min at 37 °C. Meanwhile, 25 mM $CaCl_2$ (0.2 mL) was set to preincubate in a separate cuvette. Cephalinex (0.1 mL) was pipetted into the test cuvette containing the

plasma and incubated for exactly 5 min. The preincubated $CaCl_2$ (0.1 mL) was added with simultaneously starting of the Fibrometer's timer. Once a clot formed, the timer stopped and the clotting time was recorded. Biochemicals not provided with the kit were heparin sodium salt from bovine intestinal mucosa (Sigma, H-0777, 150 Units mg^{-1}) and protamine sulfate from salmon (Sigma, p4020).

The details of the interaction between heparin and compound **8c** were investigated by using molecular dynamics simulations. A pentasaccharide heparin sequence (charge −11) was placed in a box ($55.69 \times 53.09 \times 60.04$ Å) with compound **8c** (+8 charges, Table 1) and three Na^+ ions to balance the charge. The system was solvated by TIP3P water,[23] thereby resulting in a size of ≈17500 atoms. Parameters for compound **8c** were derived from the newly developed force field for aryl amide oligomers,[24] to ensure proper description of the backbone conformational dynamics, in combination with semi-empirical (MOPAC) charges.[25] Heparin parameters were developed partly by analogy with the existing Amber parameters,[26] augmented by parameters for the SO_4^- groups,[27] partly from semiempirical calculations.[25] The system was equilibrated for 1 ns and the analysis was performed based on a subsequent 6 ns production run. The simulations were carried out by using the NAMD2 software[28] with full electrostatics by use of the Particle Mesh Ewald method and a time step of 1.5 fs.

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