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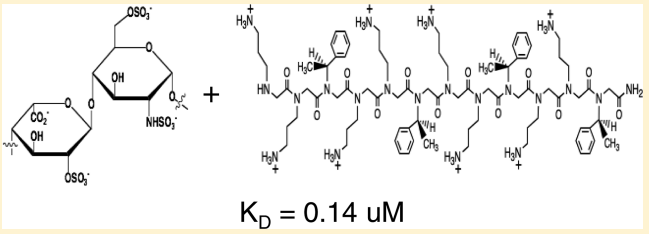
Design, Synthesis, and Characterization of Heparin-Binding Peptoids

Bruce Kevin Ford, Mark Hamza, and Dallas L. Rabenstein*

Department of Chemistry, University of California Riverside, California 92521, United States

Supporting Information

ABSTRACT: A series of N-substituted glycine oligomers (peptoids) of varying length and side chains was synthesized with the aim of producing peptidomimetics that would bind with high affinity to heparin and thereby neutralize its anticoagulant activity. To this end, a library of 29 peptoids was synthesized using solid phase synthesis methodologies. The general design of the peptoids was the repeating trimer sequence *N*(cationic side chain)-*N*(alkyl or benzyl side chain)-*N*(α-chiral side chain), where the monomers are *N*-substituted glycine residues bearing the indicated side chains. The peptoids were designed to have a helical structure with positively charged ammonium or guanidinium groups on side chains that would interact electrostatically with negatively charged sites on heparin. Binding of the peptoids by heparin was characterized by isothermal titration calorimetry (ITC) and heparin affinity chromatography (HAC). The secondary structure of the peptoids was characterized by circular dichroism (CD) spectroscopy. The peptoid design was systematically modified to produce peptoids with high affinity binding to heparin as measured by the above methods, resulting in the synthesis of peptoids with micromolar and sub-micromolar heparin-binding affinity. The efficacy of selected peptoids as agents for neutralization of the anticoagulant activity of heparin was assayed by the Coatest method, which measures restoration of the activity of the serine protease factor Xa (FXa). The results indicate that peptoids show promise as potential therapeutic agents for neutralization of the anticoagulant activity of heparin.



Heparin, a highly sulfated glycosaminoglycan produced in mast cells and basophils, is used as a blood anticoagulant for medical conditions such as atrial fibrillation, deep-vein thrombosis, and pulmonary embolism, and during medical procedures such as cardiac surgery and renal dialysis.[1−3] The heparin used in medicine is obtained from porcine intestine and bovine lung.[1,4,5]

Structurally heparin is a linear polymer consisting of repeating uronic acid-(1→4)-D-glucosamine disaccharide subunits.[1,2] The uronic acid is predominantly 2-O-sulfated L-iduronic acid and the D-glucosamine 6-O-sulfated and N-sulfated, with a small fraction N-acetylated (Figure 1). Under physiological conditions, the carboxylic acid, O-sulfate and N-sulfate groups are deprotonated, with on average 3.8 and 3.5 anionic sites per repeating disaccharide of bovine lung and porcine intestinal mucosal heparin, respectively.[1,6] Heparin binds proteins through electrostatic interactions between protein cationic sites and heparin anionic sites.[2,7] Binding of heparin by the protease inhibitor antithrombin III (AT) is the basis of its anticoagulant activity; binding causes a conformational change that enhances inhibition of the coagulation cascade enzymes thrombin and factor Xa (FXa) by AT.[1,3]

In some clinical situations, it is necessary to neutralize the anticoagulant activity of heparin.[8] For example, the use of heparin in cardiovascular surgeries often leads to a high incidence of bleeding complications. To neutralize its anticoagulant activity, protamine, an arginine-rich protein of average molecular weight 4.5 kDa, is used. Protamine neutralizes the anticoagulant activity of heparin by competitive binding of heparin through electrostatic interactions between its positively charged guanidinium groups and the negatively charged O-sulfate and N-sulfate groups of heparin. Protamine, however, can cause adverse reactions such as bradycardia, hypotension, pulmonary artery hypertension, and other anaphylactoid-type reactions. Thus, there is intense interest in developing synthetic replacements for protamine.[9]

Research on alternative neutralization agents has focused on heparin-binding peptides, including synthetic peptides that have the sequences of heparin-binding domains of heparin-binding proteins.[10−13] For example, it was shown by Carson and coworkers that a peptide with the amino acid sequence of the heparin-binding domain of heparin-interacting protein (HIP) binds to and neutralizes the anti-FXa activity of heparin in blood plasma assays.[10,14] The amino acid sequence of the peptide, CRPKAKAKAKAKDQTK, contains one arginine and six lysine residues; under physiological conditions, the peptide has a net positive charge that accounts for its binding to heparin. Two synthetic peptide analogues of the HIP heparin-binding domain have also been shown to have significant heparin-binding affinity and were found to be effective in neutralizing the anti-FXa activity of heparin.[13]

In the research reported here, we have extended the search for alternatives to protamine with a study of the binding of peptoids by heparin.[15] Peptoids are *N*-substituted glycine

Received: February 11, 2013
Revised: May 1, 2013
Published: May 3, 2013

   dx.doi.org/10.1021/bi4001722 | *Biochemistry* 2013, 52, 3773−3780

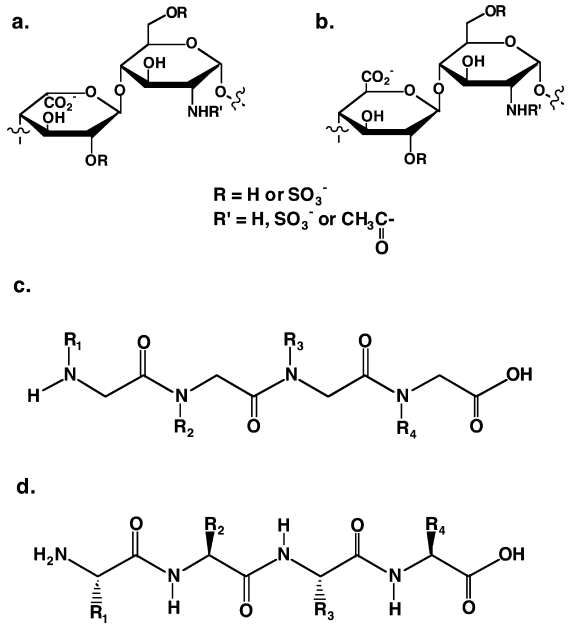


Figure 1. Structural formulas of the repeating L-iduronic acid-(1→4)-D-glucosamine (a) and D-glucuronic acid-(1→4)-D-glucosamine (b) repeating disaccharides of heparin. The major repeating disaccharide of heparin is (a) with the L-iduronic acid 2-O-sulfated and the D-glucosamine 6-O- and N-sulfated. Generic tetrapeptoid (c) and tetrapeptide (d). R_1−R_4 represent side chains. Note that the peptoid backbone is achiral and lacks backbone NH hydrogens.

oligomers, with the side chains shifted from the alpha carbons of peptides to the backbone nitrogens. The general structure of peptoids in comparison to peptides is shown in Figure 1, where R_1−R_4 represent side chains.

The shift of the side chains to the backbone nitrogens has profound consequences for the biological properties of peptoids. As a result, peptoids offer several advantages as drug candidates: peptoids are protease resistant,[16,17] they pass through biological membranes with greater efficiency than peptides,[18] and they are nonimmunogenic.[19−21] Because the *N*-substituted glycine monomers are constructed using primary amines, they lend themselves to synthesis of oligomers with a wide range of side chains.[22]

The structural properties of peptoids also differ from those of peptides. The peptoid backbone is achiral, there are no backbone amide protons that stabilize α-helical secondary structures in peptides and proteins, and the population of cis isomers of the backbone tertiary amide bonds of peptoids can be much higher than that of the backbone secondary amide bonds of peptides.[23] The result is that peptoids can be a mixture of 2^{n-1} configurational isomers, where n is the number of monomer units.[23] Of particular relevance to this research: the presence of bulky α-chiral side chains starting with the side chain on the *N*-substituted glycine monomer at the C-terminus has been reported to induce a polyproline type 1 helical structure stabilized by steric interactions, with a periodicity of three residues per turn.[24−26]

The peptoids studied in this research were constructed using a general scaffold consisting of the repeating trimer sequence: H-[*N*(cationic side chain)-*N*(alkyl or benzyl side chain)-*N*(α-chiral side chain)]$_n$-NH_2, where *N*(cationic side chain) represents a *N*-substituted glycine residue with a cation-bearing side chain on the nitrogen, etc. Binding constants and thermodynamic parameters for the binding interaction were determined by isothermal titration calorimetry (ITC). Relative binding affinities were determined by heparin affinity chromatography (HAC), and circular dichroism (CD) spectra were measured to gain insight into the peptoid secondary structure. The efficacy of selected peptoids for neutralizing the anti-FXa activity of heparin was measured using the Coatest heparin assay.

MATERIALS AND METHODS

Chemicals. Triisopropylsilane (99%), piperidine (99.5%), (*R*)-1-phenylethylamine (98%), (*S*)-1-phenylethylamine (98%), *n*-butylamine, *n*-propylamine, ethylamine, diisopropylethylamine, methylamine (40% aqueous solution), dimethylformamide (DMF), methanol, and heparin sodium salt from porcine intestinal mucosa (avg. m.w. 12 kDa, 180 USP units/mg) were purchased from Sigma-Aldrich. *N*,*N*′-Diisopropylcarbodiimide (DIC), bromoacetic acid (97%), and (*S*)-(+)-*sec*-butylamine were obtained from TCI America. *N*-(*tert*-Butoxycarbonyl)-1,3-diaminopropane, *N*-(*tert*-butoxycarbonyl)-1,4-diaminobutane, *N*-(*tert*-butoxycarbonyl)-1,5-diaminopentane, and trifluoroacetic acid (TFA) were purchased from Chem-Impex International Inc. Rink amide 100−200 mesh (MBHA) resin (0.56 mmol/g substitution) was obtained from Novabiochem. *N*-Methyl-2-pyrrolidone (NMP) was purchased from Alfa Aesar Inc. 1-*H*-Pyrazole carboxamidine HCl was purchased from AK Scientific Inc. The Coatest heparin assay kit was obtained from DiaPharma.

Solid Phase Peptoid Synthesis. Peptoids were synthesized on Rink amide MBHA resin either manually using 20 mL Teflon syringes with fritted disks as reaction vessels or on an Applied Biosystems 433A peptide synthesizer running Applied Biosystems SynthAssist software version 2.0 modified for the two-step submonomer synthesis methodology.[22] In the first step, bromoacetic acid is coupled to a resin-bound amine. In the second step, a primary amine is added by an S_N2 nucleophilic displacement reaction to form the C-terminal *N*-substituted glycine residue.[22] The two-step process is then repeated to construct the peptoid, using specific amines to create *N*-substituted glycine residues with the desired side chains. Solutions of the amines in DMF were used at a concentration of 1 M to facilitate a high level of monomer formation. Up to 20% DMSO was added to the DMF solutions of four and five carbon chain-bearing amines to aid in solubility and to avoid clogging of the needle assembly on the synthesizer. In the automated synthesis, the amine solutions were placed in synthesizer cartridges and sealed with septum caps. 200 mg of Rink amide resin were used per run. Reaction times of 2 h and 1 h were used for the acylation reaction to add bromoacetic acid and the S_N2 reaction to add amines, respectively.

Following synthesis, the resin was rinsed with methanol. A 95:2.5:2.5 TFA/H_2O/TIPS cleavage solution was added, and the resin suspension was stirred for 2.5 h at room temperature to deprotect amine side chains and cleave the peptoid from the resin. After separation from the resin by vacuum filtration, the peptoid-containing cleavage solution was collected, the resin was rinsed with additional methanol, and the methanol rinse was combined with the cleavage solution. The solvents were then removed under high vacuum to obtain a semiviscous yellow oil which was reconstituted with 5 mL of deionized water. The peptoid was isolated by preparatory-scale reverse-phase HPLC. The HPLC peak corresponding to the target

dx.doi.org/10.1021/bi4001722 | *Biochemistry* 2013, 52, 3773−3780

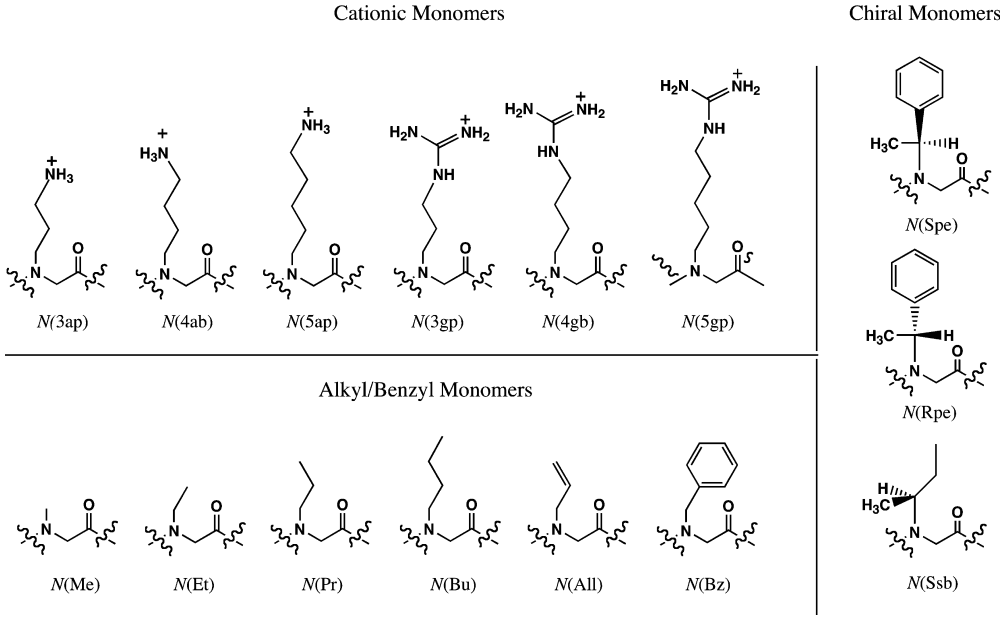


Figure 2. Designations and structures of *N*-substituted glycine monomers used to construct the peptoids synthesized and studied in this research.

peptoid was identified by MALDI-TOF-MS. HPLC solvents were removed under high vacuum. One milliliter of deionized water was then added, and the solution was frozen with dry ice and lyophilized to obtain typically 30−60 mg of solid compound.

Peptoids with guanidinium side chains were obtained by postsynthesis conversion of peptoid amine groups to guanidinium groups by reaction with 1-H-pyrazole carboxamidine HCl and diisopropylethylamine.[27] The reaction was quenched with a 10:90 TFA/H_2O solution until neutral pH was reached. The guanidinylated peptoids were isolated by preparatory-scale reverse-phase HPLC.

Binding Constants and Thermodynamic Parameters of Binding Reactions. Isothermal titration calorimetry was used to determine directly the binding enthalpy, ΔH, the binding constant, K_B, and the number of peptoid molecules bound per heparin oligomer, N. The entropy change, ΔS, was calculated using K_B and ΔH. ITC experiments were performed at 25 °C using a Microcal VP-ITC microcalorimeter. Heparin and peptoid solutions were prepared in 50 mM sodium phosphate buffer (pH 7.4). Peptoid concentrations ranged from 0.1 to 2 mM, and heparin titrant concentrations were from 0.125−1.5 mM.

In a typical ITC experiment, peptoid in buffer solution was placed in the sample cell, and an equivalent quantity of the same buffer solution was placed in the reference cell. Heparin in buffer solution was placed in the titration syringe. Heparin titrant was added to the sample cell in 4−10 μL aliquots with 210 or 360 s between aliquots. The ITC instrument measures the amount of heat absorbed or released from interaction of peptoid with heparin following the addition of each aliquot of titrant. The effect of dilution of heparin titrant in the titration cell was removed by subtracting calorimetric data for a blank titration of heparin into buffer solution. The ITC titration data, corrected for dilution, was integrated and analyzed using Origin 5.0 nonlinear least-squares software supplied with the ITC instrument. Data were fit to a model that considers heparin to be a macromolecule with N equivalent and independent binding sites, the one-set-of-sites model.

Heparin Affinity Chromatography. The relative affinities of the peptoids for heparin were determined by heparin affinity chromatography (HAC). HAC was performed on a Dionex HPLC system using HiTrap heparin affinity columns (0.7 × 2.5 cm, 1.0 mL) purchased from GE Healthcare Biosciences. Twenty microliters of peptoid solution, typically 0.2 mM, was injected onto the column. As heparin is a polyelectrolyte, sodium ions in the mobile phase compete with peptoid for the heparin stationary phase. Peptoids were eluted by gradient elution; mobile phases A and B consisted of 50 mM sodium phosphate buffer (pH 7.2), without and with 1 M added NaCl, respectively. A flow rate of 0.65 mL/min was used. The gradient began with mobile phase A and ended at 60 min with 100% mobile phase B; in most cases, elution of the peptoid was complete within 30 min or less.

Circular Dichroism. Information about the secondary structures of the peptoids was obtained by circular dichroism (CD) spectroscopy. CD spectra were measured on a Jasco J-815 CD spectrometer. Sample solutions were contained in 1 mm quartz crystal QX cuvettes purchased from Fisher Scientific Inc. The instrument was purged with nitrogen gas for 5 min prior to and during measurement of a spectrum to protect the optics and mirrors of the spectrometer. In a typical experiment, the spectrum was obtained using 300 μL of a 200 μM solution of peptoid in deionized water.

Anticoagulation Assays. The efficacy of selected peptoids as agents for neutralization of the anticoagulant activity of heparin was assayed using the Coatest method for determination of heparin in blood plasma. The Coatest assay was performed as described previously.[12]

RESULTS

Design Considerations for Heparin-Binding Peptoids. Binding of biological molecules by heparin is mediated by electrostatic interactions. Thus, the first consideration was to design peptoids with cationic side chains. Side chains bearing ammonium and guanidinium groups were used to create peptoid analogues of lysine-containing and arginine-containing peptides; side chains bearing the imidazole group as an

dx.doi.org/10.1021/bi4001722 | *Biochemistry* 2013, 52, 3773−3780

Table 1. Thermodynamic Parameters for the Binding of Peptoids by Porcine Heparin and Heparin Affinity Chromatography Retention Times

	peptide	K_D[a] (μM)	N	ΔH[b] (cal mol^{-1})	ΔS[c] (cal mol^{-1} °C^{-1})	retention time[d] (min)
1	H-[*N*(3ap)-*N*(Bu)-*N*(Spe)]-NH_2	160 ± 30	25 ± 5	400 ± 200	18.7	3.0 ± 0.6
2	H-[*N*(3ap)-*N*(Bu)-*N*(Spe)]$_2$-NH_2	44 ± 5	18 ± 2	570 ± 60	21.9	9.6 ± 1.1
3	H-[*N*(3ap)-*N*(Bu)-*N*(Spe)]$_3$-NH_2	18.6 ± 3.2	18 ± 3	810 ± 90	24.4	14.1 ± 1.2
4	H-[*N*(3ap)-*N*(Bu)-*N*(Spe)]$_4$-NH_2	4.1 ± 0.6	20 ± 3	1730 ± 50	30.5	21.6 ± 0.6
5	H-[*N*(3ap)-*N*(Bu)-*N*(Rpe)]$_4$-NH_2	7.6 ± 4.2	16 ± 0.9	1300 ± 400	27.8	18.5 ± 0.6
6	H-[*N*(3gp)-*N*(Bu)-*N*(Spe)]-NH_2	140 ± 10	25 ± 2	−633 ± 9	15.5	3.8 ± 0.3
7	H-[*N*(3gp)-*N*(Bu)-*N*(Spe)]$_2$-NH_2	20.6 ± 0.8	17 ± 0.7	−2900 ± 200	11.7	13.5 ± 2.9
8	H-[*N*(3gp)-*N*(Bu)-*N*(Spe)]$_3$-NH_2	4.76 ± 2.3	20 ± 4	−1200 ± 70	20.4	20.8 ± 0.8
9	H-[*N*(3gp)-*N*(Bu)-*N*(Spe)]$_4$-NH_2	3.4 ± 0.4	20 ± 6	−170 ± 40	24.5	29.2 ± 1.0
10	H-[*N*(3ap)-*N*(Pr)-*N*(Spe)]$_3$-NH_2	11.2 ± 0.7	17 ± 0.9	3350 ± 60	33.9	16.5 ± 0.7
11	H-[*N*(3ap)-*N*(Et)-*N*(Spe)]$_3$-NH_2	10.9 ± 0.7	17 ± 0.9	820 ± 30	25.5	16.5 ± 0.5
12	H-[*N*(3ap)-*N*(Me)-*N*(Spe)]$_3$-NH_2	8.3 ± 2.0	15 ± 0.4	1650 ± 10	28.8	18.1 ± 0.5
13	H-[*N*(3gp)-*N*(Pr)-*N*(Spe)]$_3$-NH_2	5.6 ± 1.8	24 ± 0.9	−1630 ± 40	29.6	20.3 ± 0.6
14	H-[*N*(3gp)-*N*(Et)-*N*(Spe)]$_3$-NH_2	4.3 ± 2.2	20 ± 1	−2180 ± 80	31.9	21.5 ± 0.5
15	H-[*N*(5ap)-*N*(Bu)-*N*(Spe)]$_3$-NH_2	11.7 ± 0.1	21.9 ± 0.3	2380 ± 30	30.6	16.3 ± 0.6
16	H-[*N*(4ab)-*N*(Bu)-*N*(Spe)]$_3$-NH_2	16.4 ± 11	16 ± 0.1	3300 ± 60	33.0	14.6 ± 0.2
17	H-[*N*(4ab)-*N*(Bu)-*N*(Spe)]$_4$-NH_2	1.7 ± 0.1	16.1 ± 0.1	2880 ± 20	36.1	20.6
18	H-[*N*(4ab)-*N*(Bu)-*N*(Spe)]$_5$-NH_2	0.53 ± 0.01	20.9 ± 0.4	2460 ± 50	37.0	25.0
19	H-[*N*(4ab)-*N*(Bu)-*N*(Spe)]$_6$-NH_2	0.29 ± 0.02	13.1 ± 0.4	4790 ± 30	46.0	30.2
20	H-[*N*(4gb)-*N*(Bu)-*N*(Spe)]$_3$-NH_2	5.0 ± 0.3	17 ± 3	−600 ± 100	22.3	20.6 ± 0.2
21	H-[*N*(5gp)-*N*(Bu)-*N*(Spe)]$_3$-NH_2	5.5 ± 3.4	20 ± 1	−280 ± 30	23.1	20.1 ± 0.5
22	H-[*N*(3ap)-*N*(All)-*N*(Spe)]$_3$-NH_2	8.3 ± 3.2	19 ± 2	−93 ± 70	20.2	20 ± 2
23	H-[*N*(3ap)-*N*(Bz)-*N*(Spe)]$_3$-NH_2	0.9 ± 0.4	14 ± 0.2	−550 ± 120	25.9	29.3 ± 0.5
24	H-[*N*(4ab)-*N*(Ssb)-*N*(Spe)]$_4$-NH_2	2.6 ± 0.3	15.7 ± 0.1	3440 ± 90	37.0	19.5
25	H-[*N*(4ab)-*N*(Bu)-*N*(Rpe)]-NH_2	222[e]	ND[f]	ND	ND	4.24
26	H-[*N*(4ab)-*N*(Bu)-*N*(Rpe)]$_2$-NH_2	45[e]	ND	ND	ND	10.1
27	H-[*N*(4ab)-*N*(Bu)-*N*(Rpe)]$_3$-NH_2	14.5 ± 0.04	22.6 ± 0.1	1650 ± 9	27.7	16.0
28	H-[*N*(4ab)-*N*(Bu)-*N*(Rpe)]$_4$-NH_2	4.3 ± 1.9	18.92 ± 0.02	2320 ± 50	32.3	20.0
29	H-[*N*(3ap)-*N*(3ap)-*N*(Spe)]$_4$-NH_2	0.14 ± 0.004	8.7 ± 0.9	−5300 ± 100	13.6	38.5 ± 5.0

[a]Dissociation constants calculated from K_B values determined by ITC. Reported values are the average and standard deviation of results from three or more titrations. [b]Determined by ITC for the binding reaction. [c]Calculated from K_B and ΔH for the binding reaction. [d]HAC retention times for peptoids 1−16, 20−23, and 29 measured with one HAC column; HAC retention times for peptoids 17−19 and 24−28 measured with a second column. [e]Estimated using the linear relationship between log K_D and retention time. [f]Not determined.

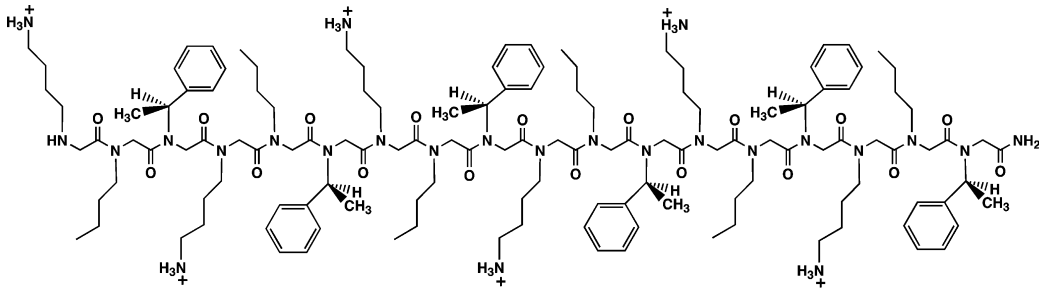
analogue of histidine-containing peptides were not used as the imidazole group is mostly neutral at physiological pH.

The second consideration was location of the cationic side chains along the peptoid oligomer. Peptoids with α-chiral side chains strategically located along a peptoid can induce a helical secondary structure.[24−26] Peptoids were designed with an α-chiral side chain on the C-terminal residue, and then on every third residue thereafter along the peptoid chain. Assuming a helical structure with three residues per turn, location of cationic side chains on the third residue from the C-terminus, and on each third residue thereafter would result in peptoids with cationic side chains aligned along one side of the helix. (*R*)-1-Phenylethylamine and (*S*)-1-phenylethylamine were used to construct the *N*-α-chiral glycine monomers.

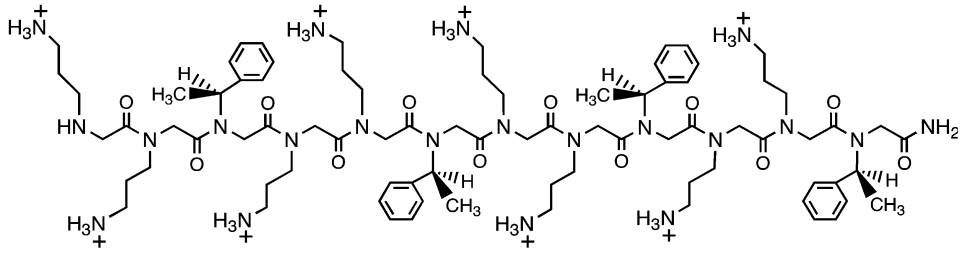
On the basis of the above considerations, the general design of the peptoids was a repeating trimer sequence *N*(cationic side chain)-*N*(alkyl or benzyl side chain)-*N*(α-chiral side chain), where the *N*(alkyl or benzyl) residue is a spacer residue between the *N*(cationic) and *N*(α-chiral) residues, as needed to incorporate the *N*(cationic) and *N*(α-chiral) residues in the desired sequence-specific fashion. The *N*-substituted glycine residues used and their designators are shown in Figure 2. Monomers *N*(3ap) and *N*(4ab) are *N*-substituted glycine analogues of the amino acids ornithine and lysine, and monomer *N*(3gp) is the *N*-substituted glycine analog of arginine. Sequences of the peptoids synthesized based on these design considerations are listed in Table 1. Structures of two peptoids are presented in Figure 3.

Heparin Binding Affinity. Dissociation constants and thermodynamic parameters for binding of the peptoids by heparin were determined by ITC. The results are reported in Table 1. To illustrate, calorimetric data, the titration curve obtained by integration of the calorimetric data, and the fit obtained by fitting the titration curve to the one-set-of-binding sites model for titration of peptoid 27 are shown in Figure 4. Also reported in Table 1 are HAC retention times. The results show that as K_D decreases, the HAC retention time increases. The relationship is logarithmic, as shown by the plot of log K_D vs retention time in Figure 5. A linear least-squares fit of the data yielded the equation log K_D = −0.0864(retention time) + 2.479, R^2 = 0.99989. It is important to note that heparin-binding data for both ammonium-containing and guanidinium-containing peptoids was used to generate the plot in Figure 5, and the data are fit by a single equation. The linear relationship between log K_D and HAC retention time is significant in that it indicates HAC retention times provide a reliable measure of heparin-binding affinity and that, with a "calibration curve" as in Figure 5, K_D values can be estimated from HAC retention times. The data used to create the plot are listed in the figure legend. The remainder of the data in Table 1, which was

dx.doi.org/10.1021/bi4001722 | *Biochemistry* 2013, 52, 3773−3780



Peptoid **19**



Peptoid **29**

Figure 3. Structures of peptoids 19 and 29, the peptoids with the highest binding affinity for heparin.

collected with a different HAC column, is fit by the equation log K_D = −0.1178 (retention time) + 2.846, R^2 = 0.9072. The K_D values listed in Table 1 for peptoids 25 and 26 were calculated using this equation and their retention times.

Peptoid Chirality. To determine the effect of handedness of the peptoid helices, as induced by the chirality of the α-chiral side chain, on heparin binding affinity, the 12mer peptoids 4 and 5 in Table 1 were constructed using the Spe and Rpe α-chiral side chains, respectively. The CD spectrum of peptoid 4 shows a double minimum at 200 and 218 nm, consistent with a right-handed helix. The spectrum of peptoid 5 is a mirror image with a double maximum, consistent with a helix of the opposite handedness (see the Supporting Information).[26,28−30]

The dissociation constant reported in Table 1 for the heparin complex of peptoid 4 is approximately half that for the heparin complex with peptoid 5. Likewise, the HAC retention time for peptide 4 is longer than that for peptoid 5, consistent with a stronger binding affinity of heparin for the Spe-containing peptoid. On the basis of these results, Spe was used as the α-chiral side chain for the majority of the peptoids studied in this research.

Number of Cationic Binding Sites. To investigate the effect of the number of cationic binding sites on binding affinity, three series of peptoids were studied: H-[*N*(3ap)-*N*(Bu)-*N*(Spe)]$_n$-NH_2 and H-[*N*(4ab)-*N*(Bu)-*N*(Rpe)]$_n$-NH_2 where *n* was varied from one to four (peptoids 1−4 and 25−28, respectively) and H-[*N*(4ab)-*N*(Bu)-*N*(Spe)]$_n$-NH_2, where *n* was varied from 3 to 6 (peptoids 16−19). In the first two series, peptoid length varies from three to twelve residues and the number of ammonium side chains from one to four. In the third series, peptoid length varies from 9 to 18 residues and the number of ammonium side chains from three to six. The dissociation constants for binding of heparin by peptoids in each series decrease, and the HAC retention times increase, as the number of ammonium groups increases.

Nature of the Cationic Group. To determine the relative binding affinities of ammonium and guanidinium side chains, the heparin-binding affinity of peptoid analogues of peptoids 1−4 with guanidinium-bearing side chains (peptoids 6−9) was studied. The dissociation constants decrease and HAC retention times increase as the number of guanidinium groups increases. Also, K_D is significantly less and the HAC retention time longer for each guanidinium-containing peptoid than for the analogous ammonium-containing peptoid. Taken together, these results indicate stronger heparin binding of peptoids containing the guanidinium group.

Length of Cation-Bearing Side Chain. The length of the carbon chains bearing cationic groups was varied from 3 to 5 carbons. Both dissociation constants and HAC retention times in Table 1 for the 9mer peptoids 3, 15, and 16 with ammonium-bearing side chains indicate that the heparin-binding affinity increases as the length of the carbon chain increases from three to five carbons. In contrast, both dissociation constants and HAC retention times for the 9mer peptoids 8, 20, and 21 with guanidinylated side chains decrease slightly as the length of the carbon chain is increased from three to five carbons.

The Central N-Substituted Glycine Residue. The effect of the side chain on the nitrogen of the central residue of the repeating trimer sequence was investigated using the *N*(alkyl) and *N*(benzyl) monomers in Figure 2. Dissociation constants and HAC retention times for peptoids 3, 10, 11, and 12 indicate the heparin-binding affinity of the peptoids in this series increases as the size of the alkyl side chain on the central residue decreases. However, peptoid 22, which has the *N*(All) monomer as the central residue, has a heparin-binding affinity similar to that of peptoid 12, which has the much smaller methyl side chain on the central residue. Peptoid 23, which has the even larger benzyl side chain on the central residue, has an even greater heparin-binding affinity, as indicated by its

dx.doi.org/10.1021/bi4001722 | *Biochemistry* 2013, 52, 3773−3780

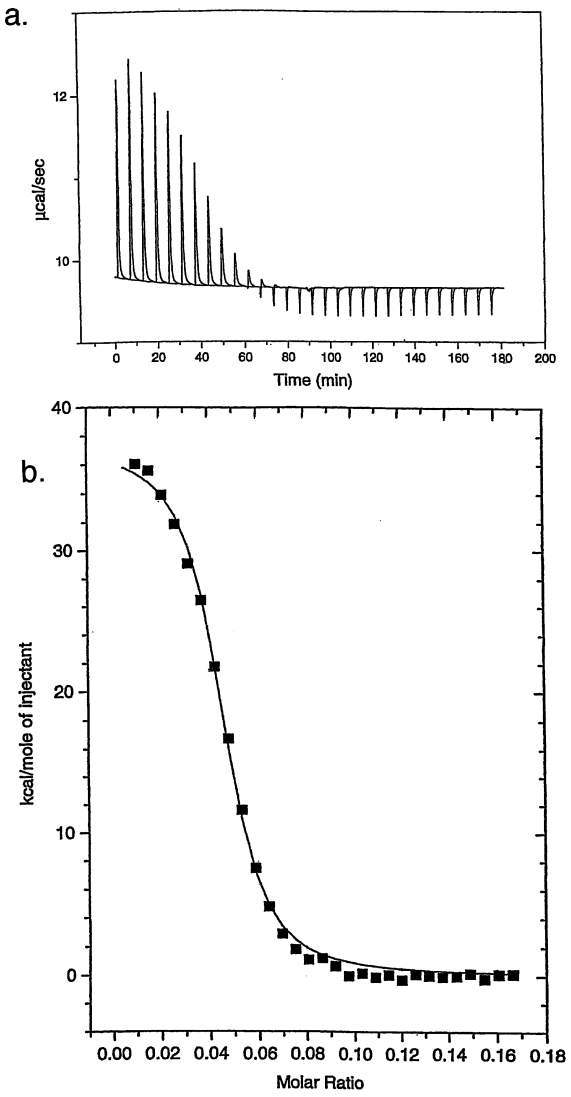


Figure 4. ITC titration data for the titration of 0.4 mM peptoid 27 with 0.5 mM heparin at pH 7.4 and 25 °C. The top panel a presents the calorimetric data obtained during the titration. Upward deflecting peaks show an increase in power to the sample cell indicating an endothermic binding reaction. The bottom panel b presents the titration curve obtained by integration of the calorimetric data after correction for the heat of dilution. The line through the data points is the best fit obtained by fitting the data to the one-set-of-sites binding model. The fit yielded values of K_B = 7.0 (±0.5) × 10^4 M^{-1}, N = 22.5 ± 0.2, ΔH = 1659 ± 16 cal mol^{-1}, and ΔS = 27.7 cal mol^{-1} $°C^{-1}$.

dissociation constant and HAC retention time. In contrast to the results for the ammonium-bearing peptoids 4, 10 and 11, there is essentially no dependence of heparin-binding affinity on the size of the side chain on the central *N*(alkyl) residue for the analogous guanidinium-containing peptoids 9, 13, and 14.

Neutralization of the Anticoagulant Activity of Heparin. The effect of selected peptoids on the anticoagulant activity of heparin was determined using the Coatest heparin assay method. The Coatest method is based on the following reactions:

$$\text{Hep} + \text{AT} \leftrightarrow [\text{Hep-AT}] + \text{AT}_{(\text{excess})} \quad (1)$$

$$[\text{Hep-AT}] + \text{FXa} \leftrightarrow [\text{Hep-AT-FXa}] + \text{FXa}_{(\text{excess})} \quad (2)$$

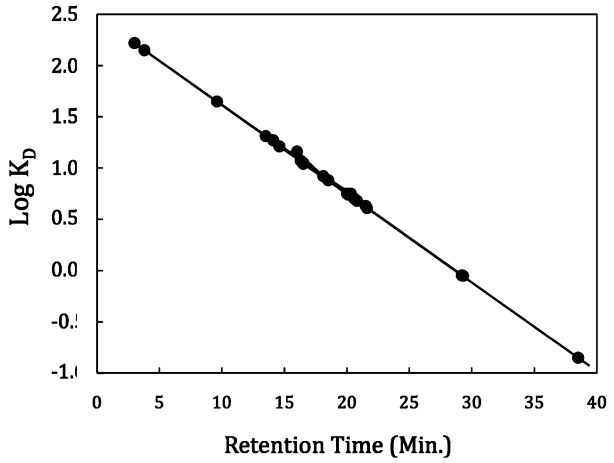


Figure 5. Log K_D vs HAC retention time for peptoids 1−16, 20−23, and 29. The straight line through the data is the linear least-squares best-fit line; see text for equation of the best-fit line. The HAC retention times for the other peptoids in Table 1 were measured with a different HAC column; see text for the best-fit equation for these peptoids.

$$\text{S-2222} \xrightarrow{\text{FXa}_{(\text{free})}} \text{peptide} + \text{pNA (yellow)} \quad (3)$$

where S-2222 represents Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA, a chromogenic substrate cleaved by free FXa to the peptide Bz-Ile-Glu(γ-OR)-Gly-Arg and pNA (*p*-nitroaniline). Heparin binds to AT to form a heparin-AT complex, which in turn binds to and sequesters the activity of FXa. Addition of a peptoid that competes with AT for heparin will increase the concentration of free FXa. Restoration of the activity of FXa, as indicated by the amount of S-2222 converted to peptide and pNA, provides a measure of the ability of a peptoid to compete with AT for heparin and thus neutralize the anticoagulant activity of heparin.[10]

For each peptoid, a range of peptoid concentrations was added to assay solutions to determine its efficacy for neutralization of the anti-FXa activity of heparin. The results in Figure 6 indicate that peptoids restore FXa activity in a

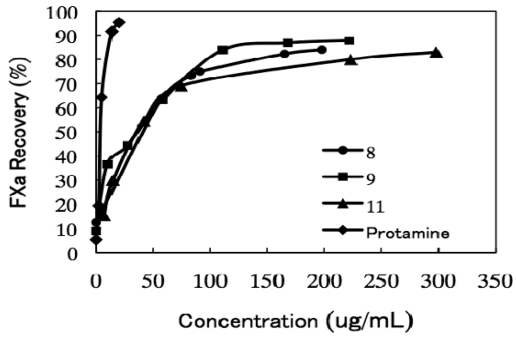


Figure 6. Restoration of the activity of FXa as a function of the concentration of peptoids 8, 9, and 11 and of protamine.

concentration-dependent manner as expected for competitive binding of heparin. (See Supporting Information for additional Coatest results.)

DISCUSSION

The results in Table 1 indicate that peptoids can be designed that bind heparin with micromolar and sub-micromolar affinity,

dx.doi.org/10.1021/bi4001722 | *Biochemistry* 2013, 52, 3773−3780

and they identify important structural features for high affinity heparin-peptoid binding. While protamine is more effective than peptoids 8, 9, and 11 in binding porcine heparin in the Coatest assay (Figure 6), the results nevertheless indicate that peptoids show considerable promise as therapeutic agents for neutralization of the anticoagulant activity of heparin. The key will be to design peptoids with higher binding affinity for heparin. Binding constants of 56.3 μM^{-1}, 68 μM^{-1}, and 122.6 μM^{-1} have been reported for the binding of protamine by porcine heparin,[31,32] which correspond to dissociation constants of 0.018 μM, 0.015 μM, and 0.0082 μM, respectively. All are less than dissociation constants reported in Table 1 for the peptoids, and they are more than 2 orders of magnitude less than K_D values for peptoids 8, 9, and 11. Nevertheless, peptoids 8, 9, and 11, which have total side-chain charges of only +3, +4 and +3, respectively, as compared to a charge of up to +20 on protamine at neutral pH,[31] compete with AT for porcine heparin in the Coatest assay. Considering the large differences between the charges on peptoids 8, 9, and 11 and on protamine, and our ability to design peptoids with significantly higher charge densities, e.g., peptoid 29, the results in Figure 6 and Table 1 clearly indicate that peptoids are good leads for the development of therapeutic agents for neutralization of the anticoagulant activity of heparin.

The results in Table 1 will provide guidance for the development of peptoids with significantly higher heparin-binding affinity. The dissociation constants and HAC retention times for peptoids 4 and 5 indicate a small dependence on the chirality of the α-chiral side chain, and thus the chirality of the peptoid, with the binding affinity being larger for peptoid 4 with Spe as the α-chiral side chain. The effect of chirality is likely related to the structure of heparin, which, as determined by a low resolution X-ray study of heparin fibers, is an extended, right-handed helix with a 1.65−1.73 nm repeating tetrasaccharide sequence along the molecular chain axis.[33] The solution structure of a heparin dodecasaccharide as determined by NMR and molecular modeling studies is also helical with a repeating tetrasaccharide sequence.[34]

Dissociation constants for the ammonium-containing peptoids 1−4, 17−19, and 25−28 decrease and HAC retention times increase as the number of ammonium groups increases. Likewise, heparin-binding affinity for guanidinium-containing peptoids 6−9 increases as the number of guanidinium groups increases. A comparison of dissociation constants and HAC retention times for ammonium-bearing peptoids 1−4 and the analogous guanidinium-bearing peptoids 6−9, and for the ammonium peptoid/guanidinium peptoid analogue pairs 10 and 13, 11 and 14, 16 and 20, and 15 and 21, indicates that the guanidinium group has a higher affinity for heparin. This is consistent with the relative heparin binding affinities of the guanidinium and ammonium groups of arginine and lysine.[35]

It also is of interest to compare heparin-binding affinities of the peptoids with those of heparin-binding peptides. K_D values for heparin binding of the two analogue peptides of the HIP peptide are 18.9 μM and 22.2 μM.[13] The peptides each have a net +6 charge at neutral pH, larger than the charge on all the peptoids in Table 1 with the exception of peptoids 19 and 29, which have charges of +6 and +8, respectively. However, the K_D values for peptoids 19 and 29 are significantly less than those for the two peptides. Indeed, the K_D values in Table 1 are all less than those of the two peptides, with the exception of those peptoids that have charges of only +1 or +2. As another comparison, the heparin complexes of peptides based on the heparin-binding consensus sequences $(XBBXBX)_n$ and $(XBBBXXBX)_n$, where X and B are hydropathic and lysine or arginine residues, respectively, have K_D's ranging from 40 μM to 0.094 μM (n = 1−6) and 6.2 μM to 0.042 μM (n = 2−5), respectively, with net charges up to +18 and +20, respectively.[36] The maximum charge on the peptoids studied in this research is +8, indicating that, on a per-positive-charge basis, the peptoids studied in this research have a high affinity for heparin as compared to peptides studied previously.

SUMMARY

The goal of this research was to design and characterize peptoids that would bind heparin with high affinity. To this end, a library of 29 peptoids was designed and synthesized, and their binding by heparin was characterized. Structural features have been identified that result in high binding affinity, including the number of cationic sites, *N*(Bz) as the central *N*-substituted glycine residue of the trimer sequence, guanidinium groups as the cationic side chain with three carbons in the carbon chain, and *N*(Spe) as the *N*-substituted glycine residue with the helix-inducing α-chiral side chain. The next phase of this research will build on the results presented above. Taking a cue from protamine, in which some 67% of the amino acids are arginine, this will include peptoids with a higher density of cationic side chains. In preliminary research, we synthesized peptoid 29, in which 67% of the side chains bear ammonium groups. The dissociation constant and HAC retention time for peptoid 29 indicate that it has the greatest heparin binding affinity of all the peptoids studied.

ASSOCIATED CONTENT

Supporting Information

Additional supplemental figures are available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: dallas.rabenstein@ucr.edu. Phone: (951) 827-5649.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the Barron Research Group at Stanford University for software modifications for the ABI 433A for synthesis of peptoids by the two-step sub-monomer method.

ABBREVIATIONS USED

FXa, Factor Xa; AT, antithrombin III; s-2222, chromogenic substrate used in Coatest assay; ITC, isothermal titration calorimetry; HAC, heparin affinity chromatography; HPLC, high performance liquid chromatography; MALDI TOF MS, matrix-assisted laser-desorption-ionization time-of-flight mass spectroscopy; CD, circular dichroism spectroscopy; Spe, (*S*)-1-phenylethylamine; *N*(Spe), *N*-[(*S*)-1-phenylethyl]glycine; Rpe, (*R*)-1-phenylethylamine; *N*(Rpe), *N*-[(*R*)-1-phenylethyl]-glycine; *N*(Ssb), *N*-[(*S*)-sec-butyl]glycine; *N*(3ap), *N*-(3-aminopropyl)glycine; *N*(4ab), *N*-(4-aminobutyl)glycine); *N*(5ap), *N*-(5-aminopentyl)glycine; *N*(3gp), *N*-(3-guanidinopropyl)glycine; *N*(4gb), *N*-(4-guanidinobutyl)-glycine; *N*(5gp), *N*-(5-guanidinopentyl)glycine; *N*(Bz), *N*-benzylglycine; *N*(All), *N*-allylglycine; *N*(Me), *N*-methylglycine;

N(Et), *N*-ethylglycine; *N*(Pr), *N*-propylglycine; *N*(Bu), *N*-butylglycine; TIPS, triisopropylsilane

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