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Diversity-oriented chemical modification of heparin: Identification of charge-reduced *N*-acyl heparin derivatives having increased selectivity for heparin-binding proteins

Liusheng Huang and Robert J. Kerns*

Division of Medicinal and Natural Products Chemistry, University of Iowa, Iowa City, IA 52242, USA

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Abstract—The diversity-oriented chemical modification of heparin is shown to afford charge-reduced heparin derivatives that possess increased selectivity for binding heparin-binding proteins. Variable *N*-desulfonation of heparin was employed to afford heparin fractions possessing varied levels of free amine. These *N*-desulfonated heparin fractions were selectively *N*-acylated with structurally diverse carboxylic acids using a parallel synthesis protocol to generate a library of 133 heparin-derived structures. Screening library members to compare affinity for heparin-binding proteins revealed unique heparin-derived structures possessing increased affinity and selectivity for individual heparin-binding proteins. Moreover, *N*-sulfo groups in heparin previously shown to be required for heparin to bind specific proteins have been replaced with structurally diverse non-anionic moieties to afford identification of charge-reduced heparin derivatives that bind these proteins with equivalent or increased affinity compared to unmodified heparin. The methods described here outline a process that we feel will be applicable to the systematic chemical modification of natural polyanionic polysaccharides and the preparation of synthetic oligosaccharides to identify charge-reduced high affinity ligands for heparin-binding proteins.

1. Introduction

Heparin and heparan sulfate (HS) are structurally similar members of a class of unbranched, polydisperse, polyanionic polysaccharides called glycosaminoglycans[†] (GAGs).[1,2] Heparin and HS are composed of repeating disaccharide sequences consisting of β-D-glucopyranosiduronic acid or α-L-idopyranosiduronic acid (1 → 4) linked to *N*-acetyl or *N*-sulfo D-glucosamine (Fig. 1). The uronic acid and glucosamine residues are variably substituted with anionic *O*-sulfo (sulfate) and *N*-sulfo (sulfoamino) groups. This variation in anionic group substitution affords diverse saccharide sequences that vary between heparin and HS, that vary among heparin and HS isolated from different tissues or cell types, and that vary along each polysaccharide chain (Fig. 1).[2,3] Sequences composed primarily of β-D-glucuronic acid (1 → 4) linked to *N*-acetyl-D-glucosamine comprise a relatively rigid domain with low charge density (NA domain, Fig. 1). Sequences composed of α-L-idopyranosiduronic acid (1 → 4) linked to *N*-sulfo-D-glucosamine are substituted with sulfate groups, affording sequences with high charge density (NS domain, Fig. 1). The more highly charged sequences in HS are typically short, three to five repeating disaccharide units, and are predominant sites for protein binding.[4] Approximately 70% of heparin structure is comprised of a tetra-anionic disaccharide found in the NS or NS/NA domain of HS (Fig. 1).

Proteins that bind heparin and HS are commonly termed heparin-binding proteins. Heparin is primarily known for its clinical use as an anticoagulant,[5] but has been ascribed an increasing number of biological activities as a result of binding to hundreds of biologically important proteins.[2–4] HS is found on the surface of virtually all mammalian cells and in the extracellular matrix, where it plays a profound role in cellular interactions and physiologic processes.[2,4] Numerous therapeutic applications have been proposed for non-anticoagulant heparin-like or heparin-mimicking polyanions (heparinoids) to block or modulate specific HS–protein interactions.[6] Because of this enormous

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* Corresponding author. Tel.: +1 319 335 8800; fax: +1 319 335 8766; e-mail: robert-kerns@uiowa.edu
† Heparin and HS are biosynthesized as proteoglycans. In this paper, heparin and HS refer to the polysaccharide (glycosaminoglycan) component of these proteoglycans.

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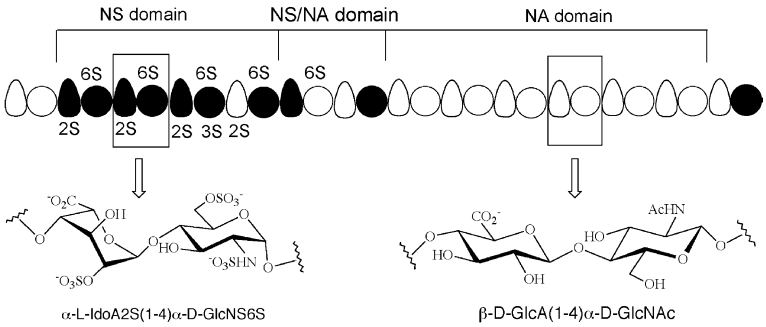


Figure 1. Pictorial representation of high-charge and low-charge domains in heparin and HS.

therapeutic potential, considerable effort has been directed toward the discovery of heparinoids to bind HS-binding proteins and block their interactions with cell surface HS.[7] It is important to distinguish between bind-and-block 'antagonists' of protein–HS interactions, which is the focus of the work here, versus heparin-like 'agonists' that bind heparin-binding proteins and allosterically or otherwise activate protein or promote protein–protein interactions.[6] The latter of which typically requires specific saccharide sequences and/or a unique spatial display of critical charged groups and is exemplified by antithrombin activation upon binding a unique heparin pentasaccharide.

The design and synthesis of saccharide-based antagonists that block HS–protein interactions has primarily focused on optimizing the degree of substitution and/or spatial display of anionic substituents on oligosaccharide-based and non-carbohydrate-based scaffolds. The spatial orientation of arginine and lysine residues in the polyanion-binding site of heparin-binding proteins affords a surface of positive electrostatic potential. This has made it relatively easy to find polyanions having high affinity for any heparin-binding protein, but makes it difficult to identify ligands that selectively bind individual heparin-binding proteins. Regardless of eloquent design and placement of multiple ionic groups on a core structure, these molecules are ultimately highly charged polyanions. Over 50% of all proteins in the human body have an isoelectric point over 7.0 and will non-specifically bind many polyanions. Heparin and other polyanions also bind clusters of basic amino acids in anionic proteins. Protein-binding selectivity is a significant problem thwarting therapeutic application of heparin-mimicking polyanions as antagonists of HS–protein interactions.[8]

Modification of heparin and HS structure toward non-anticoagulant purposes has primarily focused on chemical or biosynthetic alteration of sulfate substitution patterns and length of saccharide sequences.[9] A limited number of reports describe partial *O*-acylation or *N*-acylation of heparin using hydrophobic groups to investigate hydrophobization of heparin toward specific applications.[10,11] Chemical synthesis has been employed to prepare heparin or heparin-like oligosaccharides.[12] Substitution of non-carbohydrate scaffolds has been reported for preparing molecules to block HS-growth factor interactions.[13] Non-carbohydrate-based polyanionic polymers have been studied as ligands for certain heparin-binding proteins.[14] To date, a primary problem with developing polysaccharide-based or oligosaccharide-based antagonists that bind and block the HS-binding site of specific proteins has been the inability to minimize charge and optimize spatially more stringent non-ionic binding contacts with protein.[‡]

In this paper, we demonstrate a strategy for employing the diversity-oriented chemical modification of heparin to prepare charge-reduced heparin derivatives that possess increased affinity and selectivity for heparin-binding proteins. Parallel synthesis has been employed to introduce structurally diverse non-anionic moieties into heparin in place of anionic *N*-sulfo groups, where the presence of *N*-sulfo groups has been shown to be required for high affinity binding of heparin to fibroblast growth factor 1 (FGF1), FGF2, vascular endothelial growth factor (VEGF), and α-thrombin. Studies to evaluate the binding of these structurally diverse *N*-acyl heparin derivatives to these proteins revealed unique heparin-derived structures having increased affinity and selectivity for FGF1, VEGF, and α-thrombin.

2. Rationale for replacing anionic groups in heparin with non-anionic moieties

Three distinct types of charged moieties exist within the heterogeneous structure of heparin; *O*-sulfo (sulfate), *N*-sulfo (sulfoamino), and carboxylate (Fig. 1). At the outset of this study we anticipated non-anionic moieties incorporated into heparin in place of anionic groups required for protein binding would contribute to increased binding selectivity and possibly increased affinity for individual heparin-binding-proteins. First, it was antici-

[‡] The diversity-oriented modification of heparin reported here employs 'replacing anionic groups' on heparin with non-anionic moieties to obtain heparin-based antagonists of HS–protein interactions. In contrast, *O*-methylation of hydroxyl groups in heparin with or without additional modifications to the core saccharide 'while maintaining requisite anionic groups' has led to synthesis of heparinoid-based agonists having improved activity and physical characteristics. In particular, the *O*-methylated heparin pentasaccharide *Idraparinux* binds and activates antithrombin with improved affinity and diminished non-specific interactions in vivo. Hydropathic interaction analysis has also been used to identify non-sugar activators of antithrombin.[15]

pated that aryl moieties substituted in place of anionic groups on heparin could properly align with lysine or arginine residues in the heparin-binding site of select heparin-binding proteins to afford cation–π interactions (Fig. 2), directly replacing a charge–charge binding contact.[16,17] Aryl groups also afford the potential to form π-stacking interactions with aromatic amino acids in heparin binding sites (Fig. 2). Second, heteroaromatic groups and other moieties that possess multiple hydrogen bond donor/acceptor motifs are capable of forming spatially stringent hydrogen bond networks with basic amino acids.[18] Such interactions have the potential to replace a charge–charge interaction (e.g., arginine-sulfate), albeit with diminished affinity, or provide new binding contacts with protein (Fig. 2). Third, simple alkyl groups afford the potential for forming hydrophobic interactions within heparin-binding sites, although the introduction of alkanoate chains into heparin without altering anionic content has previously met with limited success.[10,11] Replacing anionic moieties in heparin with groups capable of forming cation–π, π-stacking or hydrogen bond contacts with protein can theoretically afford binding interactions through native heparin–protein binding orientations or through reorientation of the heparin–protein interaction.

Heparin–protein binding is complex, involving charge–charge interactions in addition to other protein–saccharide binding contacts. Most HS-binding proteins display some level of affinity for all sequences within heparin (NS and NS/NA domains, Fig. 1); the proteins simply bind with highest affinity to specific highly charged sequences or sequences with optimal display of anionic groups. Binding contacts between a non-anionic moiety substituted into heparin in place of a charged group required for protein binding and protein/amino acid structures have much greater steric, spatial, and directional requirements than charge–charge interactions. These more stringent binding requirements dictate increased binding site selectivity, even when affinity for a given protein is simply maintained. Incorporation of non-ionic groups into heparin in place of anionic groups will sterically prohibit binding to many heparin-binding proteins, thus inherently affording heparin-derivatives that only bind proteins which can accommodate such a substitution on the heparin chain. Modified sequences derived from regions of heparin normally possessing high affinity for a given protein may bind the protein in a conformation similar to that of the parent saccharide sequence or in an altered conformation of the saccharide. Modification of heparin sequences in regions of heparin that normally possess low affinity for a given heparin-binding protein may yield new, high affinity sequences for the target protein through a reorientation of binding contacts between the modified heparin and the protein. Guided by these theories for replacing charged groups on heparin, we embarked on the diversity-oriented chemical modification of heparin reported here. Our primary goal was to demonstrate proof for the concept that charged groups on heparin required for high affinity binding of heparin to many proteins can be replaced with structurally diverse non-anionic moieties to yield charge-reduced heparin derivatives that selectively bind individual, or a limited number of, heparin-binding proteins without loss of affinity.

3. Results and discussion

Here, the diversity-oriented synthesis of charge-reduced heparin derivatives was envisioned to arise from selective *N*-desulfonation of heparin followed by selective *N*-acylation of the resulting amine groups. Complete removal of *N*-sulfo groups from heparin reduces net charge by approximately 25%. The *N*-sulfo groups on heparin are required for heparin to bind many different heparin-binding proteins. Selective *N*-desulfonation followed by *N*-acetylation is commonly employed to demonstrate the requirement of *N*-sulfo groups for heparin to bind a protein. To this end the introduction of non-anionic moieties into heparin in place of *N*-sulfo groups would be anticipated to diminish or destroy binding affinity for most heparin-binding proteins. However, guided by the conceptual basis of this work outlined

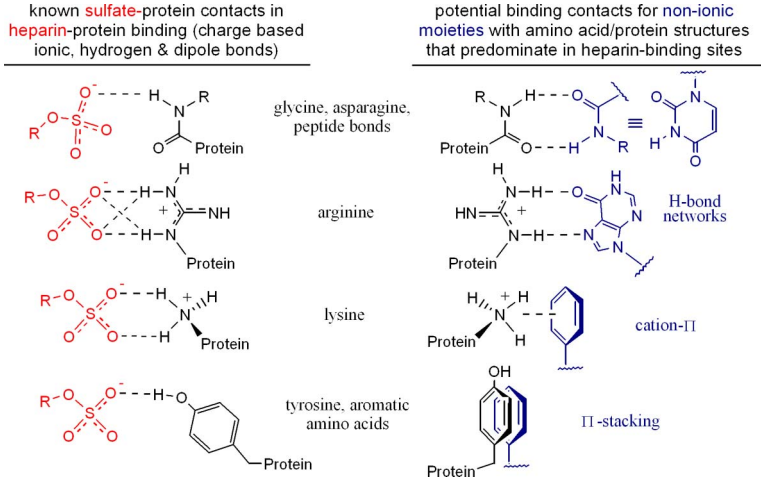


Figure 2. Representations of known binding contacts between sulfate (anion) groups on heparin and amino acids/peptide backbone of heparin-binding proteins; in comparison to well-established binding contacts that non-anionic moieties (blue) are known to form with these protein structures.

above, we anticipated that replacing *N*-sulfo groups along the heparin chain with structurally diverse non-anionic moieties would afford unique heparin derivatives possessing equivalent or greater affinity than parent heparin for specific heparin-binding proteins or a limited number of heparin-binding proteins.

3.1. Preparation of amine-containing heparin-derived scaffolds

Heparin fractions containing varied percentages of free amine were prepared by solvolytic *N*-desulfonation of heparin.[19] Multi-gram quantities of the pyridinium salt of heparin underwent *N*-desulfonation in DMSO/H_2O (19:1) at 20 and 50 °C. One-gram aliquots were removed at time intervals and processed to afford heparin fractions having varied percentages of free amine. Amine content of samples from all fractions was determined using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay[20] and plotted versus time of *N*-desulfonation (Fig. 3). Amine content of fractions was also determined by analysis of *N*-sulfo groups remaining after *N*-desulfonation.[21] ^{1}H NMR was employed to confirm complete removal of *N*-sulfo groups from the 100% *N*-desulfonated fraction, where complete disappearance of the H-2 signal for 2-*N*-sulfo-D-glucosamine residues was observed.[22] Seven *N*-desulfonated heparin fractions containing 12%, 21%, 28%, 33%, 47%, 70%, and 100% free amine were chosen for *N*-acylation (Fig. 3).[23]

3.2. Chemical rehearsal for selective *N*-acylation

Heparin is stable to mild acylation conditions employing NHS activated acids or anhydrides in aqueous sodium bicarbonate solutions.[24] Loss of sulfate and *N*-sulfo groups is known to occur under certain acylation conditions.[25] With this in mind, we set out to identify structurally diverse activated carboxylic acids that would be amenable to selective *N*-acylation of glucosamine residues under the requisite mild, aqueous reaction conditions. Diverse Functional Moieties (DFMs) obtained as carboxylic acid anhydrides or NHS esters were selected for their potential to provide different types of protein-binding contacts as previously discussed (Fig. 2). Selective *N*-acylation of β-D-glucosamine-1-thiophenol

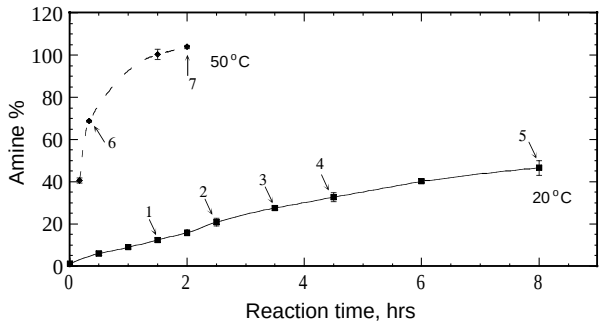


Figure 3. Amine content of heparin fractions after solvolytic *N*-desulfonation of pyridinium heparin at 20 and 50 °C. Percent amine was determined using the TNBS assay, where absorbance values for parent heparin and completely *N*-desulfonated heparin were set at 0% and 100% amine, respectively.

by these structurally diverse anhydrides and pre-activated carboxylic acids was initially studied to identify acylating agents that provided complete *N*-acylation under requisite reaction conditions for *N*-acylation of heparin. Comparative rates of selective *N*-acylation for DFMs found to provide complete *N*-acylation in two or fewer coupling steps are shown (Table 1).

3.3. Parallel synthesis of 133-members *N*-acyl heparin library

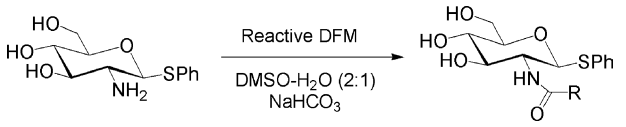
Heparin's high molecular weight, water solubility, and insolubility in organic solvents are normally problematic for chemical manipulation. Here, we took advantage of these physical properties to yield an efficient strategy for the parallel synthesis of heparin derivatives. Structurally diverse acylating agents **D1**–**D19** (Table 1) were coupled to the seven *N*-desulfonated heparin fractions in parallel (Fig. 4). Performing the coupling reactions in the presence of sodium bicarbonate ensured all anionic groups in heparin remained as sodium salts. Acetone–ether precipitation of each reaction mixture followed by centrifugation separated heparin and inorganic salts from organic soluble coupling reagents. Dialysis against water removed remaining low molecular weight salts and any remaining low molecular weight impurities. Although the methods in this parallel coupling strategy require hands-on manipulation, each step is readily amenable to working with large numbers of samples in parallel.

The amine content of each *N*-acylated heparin product was determined using the TNBS assay.[20] Any individual library member showing incomplete *N*-acylation was subjected to a second round of coupling. Final analysis of amine content for all library members demonstrated complete *N*-acylation for each heparin fraction with each DFM (Fig. 5). Structural integrity of the heparin chains was confirmed using HPLC–GPC analyses of select coupling products for each DFM.[26] No decrease in molecular weight was observed. In addition, no residual anhydride, NHS ester or NHS was detected during the HPLC–GPC analyses of *N*-acylated heparin fractions. Analysis of total sulfate for each 100% *N*-desulfonated/*N*-acylated product revealed no detectable loss of *O*-sulfo groups.[27] Analysis of *N*-sulfo content for each 50% *N*-desulfonated/*N*-acylated heparin showed no detectable loss of *N*-sulfo groups during *N*-acylation.[28]

3.4. Competition binding assay for library screening

These studies required a high-throughput binding assay to efficiently determine the relative affinity of each library member for the heparin-binding site of heparin-binding proteins. Filtration-based competition binding assays using both radiolabeled and fluorescent-labeled heparin derivatives have been reported.[30] Here, we adapted these assays to establish a competition-based 96-well filtration assay to screen all heparin-derivatives for binding to virtually any heparin-binding protein. This assay employs high protein binding 96-well filtration plates, where competition between test ligands and fluorescein-labeled heparin (FL-HP) for binding protein affords filtrate levels of

Table 1. Comparative rates of *N*-acylation for DFMs employing reaction conditions required for selective *N*-acylation of heparin



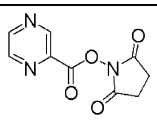
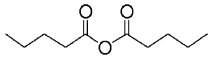
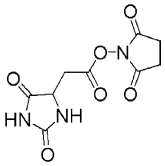
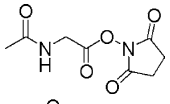
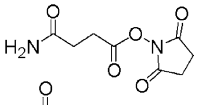
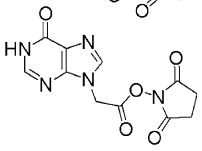
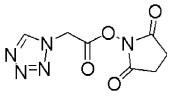
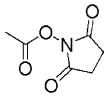
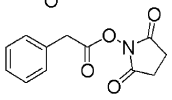
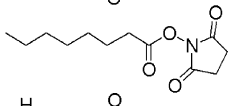
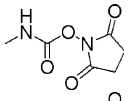
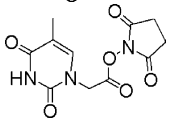
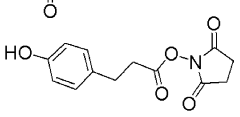
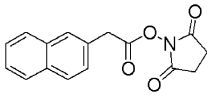
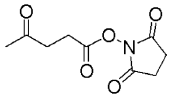
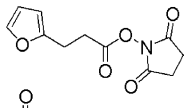
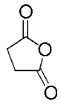
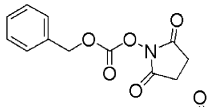
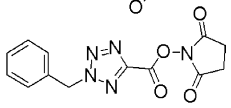
DFM		Reactivity[a]
	D1	++++
	D2	++++
	D3	+++
	D4	+++
	D5	+++
	D6	+
	D7	+
	D8	++
	D9	+++
	D10	++
	D11	+++
	D12	++
	D13	++
	D14	++
	D15	++

Table 1 (*continued*)

DFM		Reactivity[a]
	D16	++
	D17	++++
	D18	++++
	D19	+

[a] Complete within 4 h (++++); complete within 8 h (+++); complete within 16 h (++); complete after second round of coupling (+); DFMs studied but affording incomplete reactions, not shown. The relative rates of acylation here ultimately correlated to efficiency of heparin *N*-acylation.

FL-HP that correspond to affinity of test ligand for protein. To establish requisite assay concentrations of protein and FL-HP for each protein employed in this study, we first identified protein and FL-HP concentrations where saturation of protein by FL-HP was achieved. Subsequent use of these protein and FL-HP concentrations in competition binding provided the maximal signal range between ligands that displace no FL-HP from protein and those that displace all FL-HP from protein. As shown for α-thrombin, employing protein at high concentration resulted in complete retention of FL-HP up to 200 nM (Fig. 6A). At 25 μg/mL α-thrombin FL-HP is observed in the filtrate at approximately 100 nM (Fig. 6B). Complete saturation of protein occurs at approximately 150 nM FL-HP, where increasing FL-HP above 150 nM afforded no further increase in the absolute value of decreased fluorescence signal due to FL-HP binding protein. Based on these data 150 nM FL-HP and 25 μg/mL α-thrombin were employed in screening assays. Identical studies were performed to establish protein and FL-HP concentrations for all other proteins employed here (data not shown).

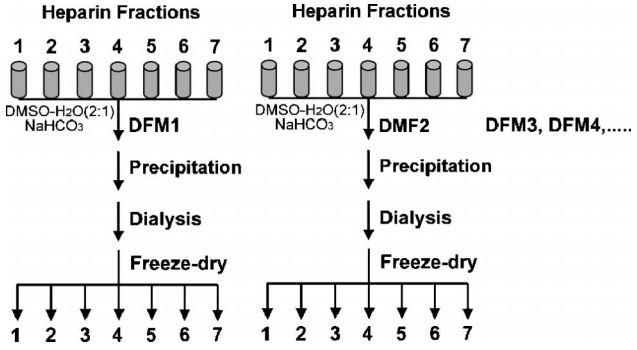


Figure 4. Outline of protocol for the parallel synthesis of variably *N*-acylated heparin derivatives. Heparin fractions 1–7 correspond to the seven *N*-desulfonated heparin fractions containing varied percentages of free amine before coupling and subsequently *N*-acyl groups after coupling.

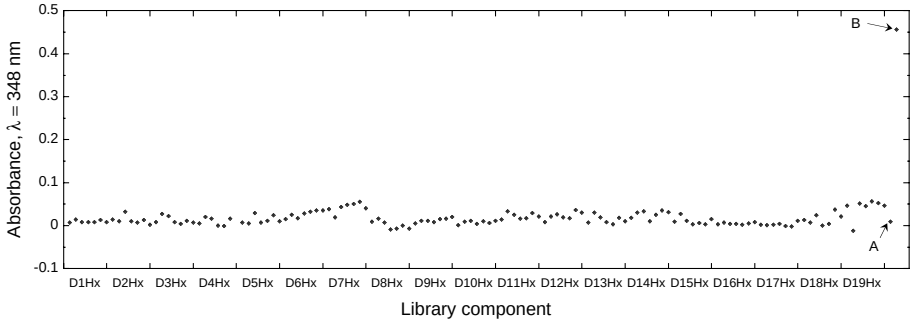


Figure 5. Amine content of all heparin fractions after library coupling as determined using the TNBS assay. Data are reported as the absorbance at $\lambda = 348$ nm, averaged from two separate analyses.[29] Amine content is displayed for coupled products from each DFM (**D1**–**D19**) at each of the seven percentages of *N*-acylation (H*x* = H1–H7). Data point *A* = control readings for unmodified heparin. Data point *B* = control readings for 100% *N*-desulfonated heparin.

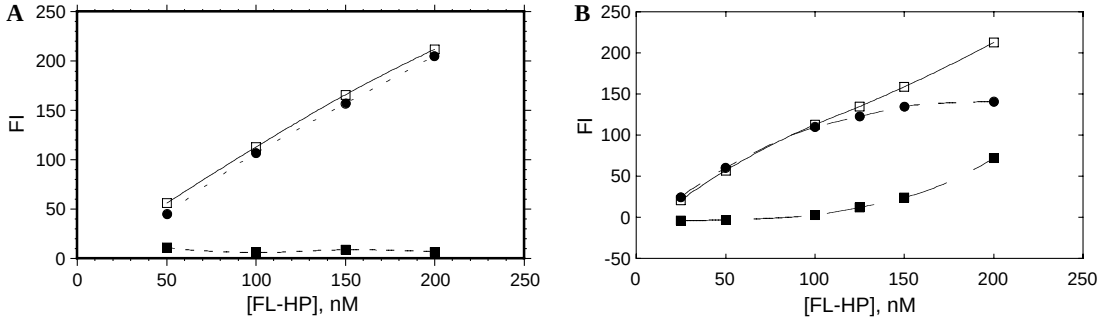


Figure 6. Determination of protein and FL-HP concentrations for screening assays. Representative data for α-thrombin, where α-thrombin at 50 μg/mL (A) and 25 μg/mL (B) was incubated with varied concentrations of FL-HP, 25 nM–200 nM. FI denotes fluorescence intensity of filtrate wells. FI of filtrate for FL-HP with no α-thrombin (□), FI of filtrate for FL-HP incubated with α-thrombin (■), Absolute value of FI decrease due to FL-HP binding α-thrombin (●). (A) shows FL-HP completely protein-bound at all concentrations using 50 μg/mL α-thrombin; (B) (25 μg/mL α-thrombin) shows that FL-HP over 100 nM affords free FL-HP in the filtrate.

Concentration-dependent binding of heparin to each protein was performed in order to establish the single concentration at which all library members were to be evaluated for protein binding. These binding studies provided the concentration at which 50% displacement of FL-HP from each protein was achieved (Fig. 7). Subsequent screening of protein binding for all library members and heparin at or near this single concentration was expected to afford rapid comparison of the affinity each library member possessed for each protein relative to heparin itself.

3.5. Screening library members for binding heparin-binding proteins

Results of screening all library members for binding to FGF2 and VEGF are shown (Fig. 8). It has been established that *N*-sulfo groups on heparin are required for heparin to bind VEGF and FGF2.[31] All library members show diminished affinity for FGF2. This loss of affinity for FGF2 is consistent with removal of the *N*-sulfo groups on heparin required for FGF2 binding. It is notable that competitive binding to the heparin binding site of FGF2 for most *N*-acyl substituents decreased in proportion to increasing percentage of *N*-sulfo groups replaced with non-anionic moieties. Observing this correlation between diminished affinity for FGF2 and increased level of *N*-desulfonation/*N*-acylation demonstrates integrity of our chemical modifications and the integrity of heparin structure to these chemical modifications. Nearly identical results were observed for the binding of most library members to VEFG, again consistent with removing *N*-sulfo groups from heparin that are required for heparin to bind VEGF. However, in contrast to the FGF2 binding results, *N*-acylation of *N*-desulfonated heparin with the aryl DFMs **D13**, **D14**, and **D18** revealed novel heparin derivatives having higher affinity than heparin for VEGF. It is notable that replacing the *N*-sulfo groups on heparin with the phenacyl moiety (**D9**), which is structurally similar to **D13**, **D14**, and **D18**, does not impart improved affinity for VEGF over heparin. A potential explanation for this result is that the distal aryl rings of **D13**, **D14**, and **D18** extend further from the saccharide core than is possible for **D9**. These results demonstrate that *N*-sulfo groups on heparin, which are required for heparin to bind VEGF, have been replaced with structurally diverse non-anionic moieties to afford charge-reduced heparin-derived polysaccharides having increased affinity for VEGF. Moreover, the concomitant decreased affinity of heparin derivatives *N*-acylated with **D13**, **D14**, and **D18** for FGF2 demonstrates improved protein-binding selectivity.

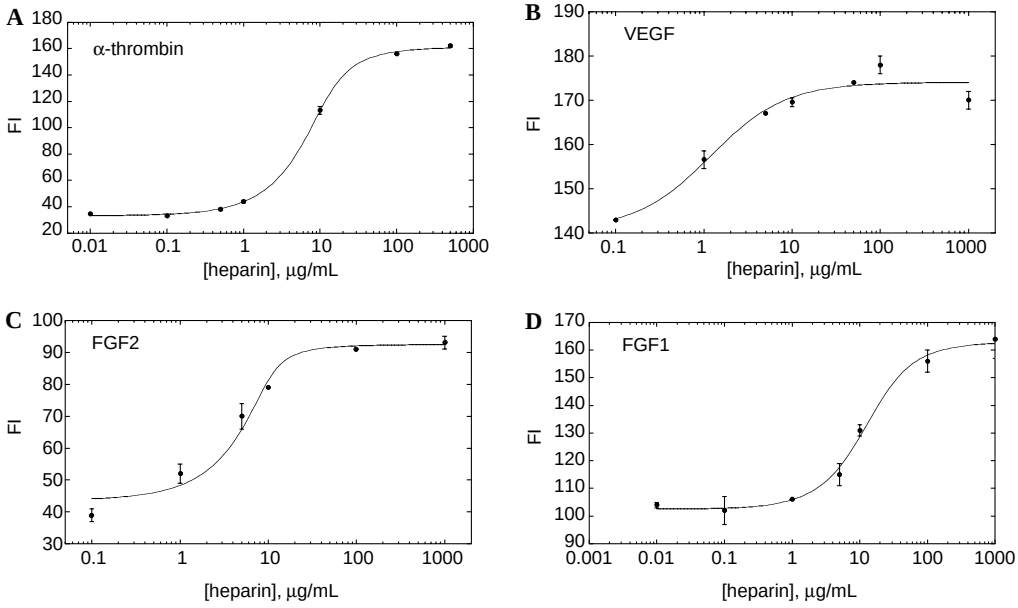


Figure 7. Concentration-dependent binding of heparin to α-thrombin, VEGF, FGF2, and FGF1. All binding curves were obtained by employing competition binding with FL-HP. FI = fluorescence intensity. (A) 150 nM FL-HP, 25 μg/mL α-thrombin. (B) 100 nM FL-HP, 6 μg/mL VEGF. (C) 100 nM FL-HP, 5 μg/mL FGF2. (D) 150 nM FL-HP, 6 μg/mL FGF1.

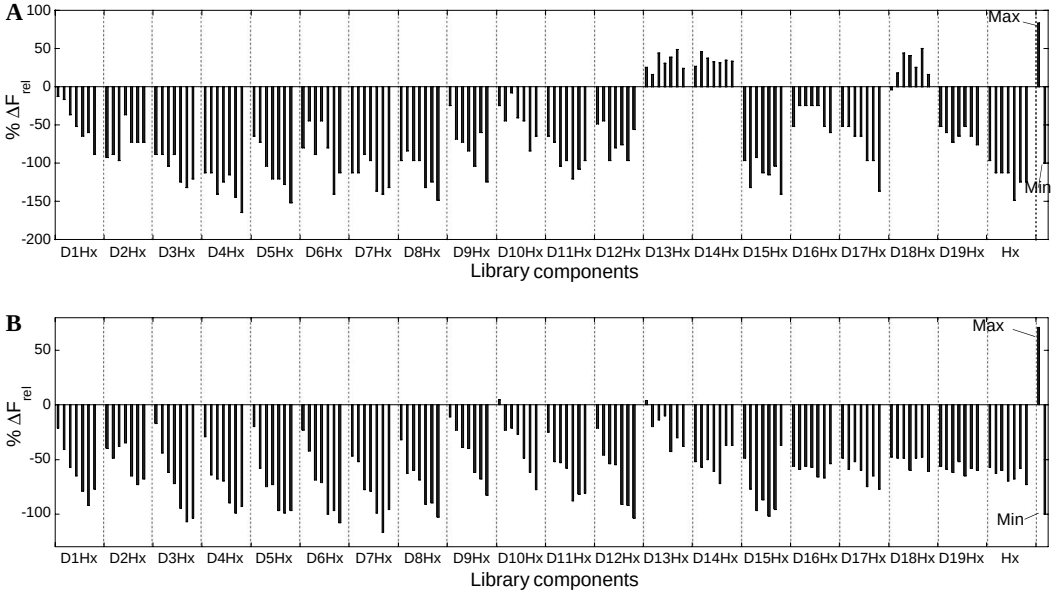


Figure 8. Competitive binding of library members to VEGF (A) and FGF2 (B) relative to heparin. Binding of each library member and heparin for protein was evaluated at 5 μg/mL in the present of 100 nM FL-HP and 6 μg/mL VEGF or 5 μg/mL FGF2. D*n*H*x* defines library members where D*n* (**D1**–**D19**) identifies the acyl group (DFM) coupled to heparin amine groups, and H*x* (H*x* = H1–H7) defines from left to right heparin fractions bearing increasing percentages of *N*-acylation. Data are the average of two separate screening assays. Percent $\Delta F_{rel} = (FI_{DnHx} - FI_{heparin})/(FI_{heparin} - FI_{min}) \times 100$, where FI_{DnHx} is the fluorescence intensity observed for each test compound, $FI_{heparin}$ is fluorescence intensity observed for heparin control, and FI_{min} is the fluorescence intensity observed for FL-HP and protein only. Max is FL-HP reading (control) with no added protein. Library components H*x* are the seven *N*-desulfonated heparin fractions bearing no *N*-acyl group.

It is known that two FGF1 molecules bind the same heparin sequence, at the same time, from opposing sides of the saccharide chain.[30,32] We anticipated this greater promiscuity of FGF1 for heparin sequences and reduced dependence of binding on the *N*-sulfo groups would potentially afford a greater number of highly substituted *N*-acyl heparin derivatives binding FGF1 over VEGF and FGF2. Competition binding of heparin derivatives having 21%, 47%, and 100% of the *N*-sulfo groups replaced with each *N*-acyl group was determined (Fig. 9). Many of the *N*-acyl heparin derivatives having low levels of *N*-substitution display similar or increased affinity for FGF1over heparin. However, substitution of all *N*-sulfo groups on heparin with any of the DFMs diminished affinity for FGF1. These results further demonstrate the improved selectivity of 100% *N*-desulfonated

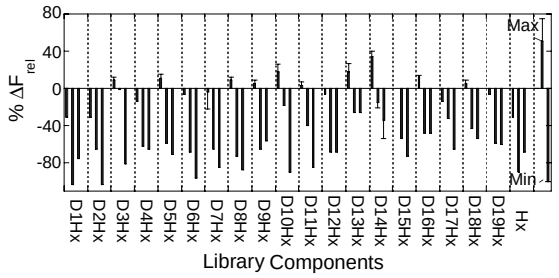


Figure 9. Competitive binding of library members for FGF1 relative to heparin. Each library member and heparin was evaluated at 5 μg/mL in the presence of 150 nM FL-HP and 6 μg/mL FGF1. D*n*H*x* defines library members where D*n* (**D1**–**D19**) identifies the *N*-acyl group coupled to heparin amine groups. H*x* for each D*n* group in the graph corresponds, left to right, to; H*x* = 21%, 47%, 100% *N*-desulfonation/*N*-acylation.[21,23] Data are the average of two separate screening assays. Percent $\Delta F_{rel} = (FI_{DnHx} - FI_{heparin})/(FI_{heparin} - FI_{min}) \times 100$, where FI_{DnHx} is the fluorescence intensity observed for each test compound, $FI_{heparin}$ is the fluorescence intensity observed for heparin control, and FI_{min} is the fluorescence intensity observed for FL-HP and protein only. Max is FL-HP reading (control) with no added protein. Library components noted as H*x* only are the corresponding *N*-desulfonated heparin fractions with no *N*-acyl groups.

heparin derivatives *N*-acylated with **D13**, **D14**, and **D18** for binding the heparin-binding site of VEGF.

Screening results for the three heparin-binding growth factors revealed distinctly different patterns of binding as a consequence of the percent *N*-sulfo groups replaced with *N*-acyl groups. Binding of library members to FGF2 clearly demonstrates that increasing percentages of *N*-sulfo groups replaced by any of the *N*-acyl groups correlates to decreasing affinity. In contrast, replacing *N*-sulfo groups with select *N*-acyl groups afforded unique heparin derivatives having increased affinity for VEGF and FGF1. However, the level of *N*-acyl substitution (*N*-sulfo replacement) differentially affects binding of these heparin derivatives to VEGF versus FGF1. Only low levels of *N*-sulfo substitution with certain *N*-acyl groups afford structures that bind FGF1 with equivalent or greater affinity than heparin. Affinity for FGF1 drops as the percent substitution of *N*-sulfo groups with *N*-acyl groups increases. This binding pattern likely results from the generation of novel heparin sequences that bind FGF1 through both *N*-acyl and *N*-sulfo contacts, which are lost upon increasing levels of *N*-acylation. Alternatively, low levels of *N*-acyl substitution could simply be producing conformational changes within heparin that promote FGF1 interaction with heparin. All *N*-acyl heparin fractions substituted with **D13**, **D14**, and **D18** bind VEGF with increased affinity. This increase in affinity is maintained across each heparin fraction regardless of percent substitution, suggesting that *N*-substitution of heparin with **D13**, **D14**, and **D18** likely affords discrete *N*-acyl sequences within heparin that possess increased affinity for VEGF over heparin in the absence of *N*-sulfo groups. Subsequent removal of additional *N*-sulfo groups does not significantly alter the binding of these discrete sequences with VEGF. Determination of the molecular basis for these binding interactions is certainly necessary to ultimately understand these binding interactions at the molecular level.

Thrombin is an important factor in blood coagulation. Unfractionated heparin catalyzes the inhibition of thrombin through the simultaneous binding of both thrombin and antithrombin, and concomitant activation of antithrombin.[33] The *N*-sulfo groups on heparin are required for high affinity binding of heparin to exosite II of α-thrombin.[34] We screened the library of *N*-acyl modified heparins for binding exosite II of α-thrombin to determine if trends from growth factor binding would be observed (Fig. 10). Similar to VEGF, library members substituted with **D13**, **D14**, and **D18** showed equivalent or increased affinity for exosite II of α-thrombin at all levels of *N*-desulfonation/*N*-acylation. In addition, replacement of heparin *N*-sulfo groups with the phenylacyl group **D9** and caproyl moiety **D10** afforded derivatives that, for the most part, maintain affinity for α-thrombin.

At the outset of this study, we envisioned a strategy for identifying non-anticoagulant charge-reduced heparin derivatives that would bind heparin binding proteins with equivalent or greater affinity than heparin itself. Results of library screening revealed structurally unique, charge-reduced, heparin derivatives that did maintain affinity for select heparin-binding proteins. Concentration-dependent displacement of FL-HP from α-thrombin was determined for library members having 100% of the *N*-sulfo groups replaced with *N*-acyl moieties **D13**, **D14**, and **D18**. Each derivative achieved 50% displacement of FL-HP from α-thrombin at a modest twofold to fourfold lower concentration than heparin (See, Fig. 11 for representative data).

Anticoagulant activity of these novel heparin derivatives in comparison to parent heparin was evaluated using a standard plasma-based assay to determine activated partial thromboplastin times (aPTT). Heparin binding to exosite II of thrombin alone does not inhibit thrombin. It is the ternary complex of a heparin chain bound to exosite II of thrombin and to antithrombin, with concomitant activation of antithrombin that affords heparin-catalyzed inhibition of thrombin by antithrombin.[33] Removal of the *N*-sulfo groups within heparin here was expected to afford products incapable of activating antithrombin, and thus devoid of anticoagulant activity as a consequence of antithrombin activation. Initial studies demonstrated that none of the library members possess in vitro anticoagulant activity in this assay, where clotting time for each derivative was equivalent to baseline controls containing no heparin or heparin derivative. The aPTT assay does not report on all of the anticoagulant activities observed for heparin-like molecules, and thus heparin derivatives prepared here are being evaluated for activity against individual coagulation factors.

The results of this study demonstrate a novel strategy for preparing charge-reduced heparin derivatives possessing greater affinity and selectivity than heparin for heparin-binding proteins. It is tempting to speculate

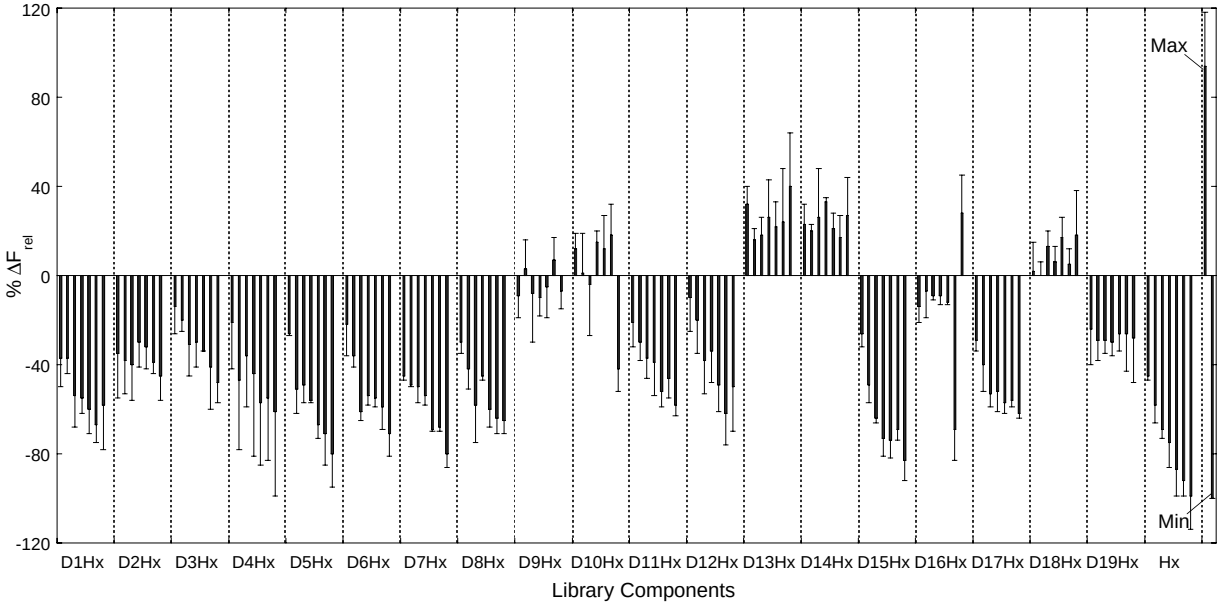


Figure 10. Competitive binding of *N*-acyl heparin derivatives for exosite II of α-thrombin compared to heparin. Competition binding was performed using 150 nM FL-HP, 25 μg/mL α-thrombin, and 5 μg/mL heparin or library member. D*n*H*x* defines library members where D*n* (**D1**–**D19**) identifies the *N*-acyl group attached to heparin and H*x* is used to indicate within each D*n* group levels of increasing *N*-acylation for the seven percentages of *N*-acylation, increasing from left to right. Data are the average of three separate experiments. Percent $\Delta F_{rel} = (FI_{DnHx} - FI_{heparin})/(FI_{heparin} - FI_{min}) \times 100$, where FI_{DnHx} is fluorescence intensity observed for each test compound, $FI_{heparin}$ is the fluorescence intensity observed for heparin control, and FI_{min} is the fluorescence intensity observed for FL-HP and protein only. Max is FL-HP reading (control) with no added protein. Library components noted as H*x* only are the corresponding *N*-desulfonated heparin fractions with no *N*-acyl groups.

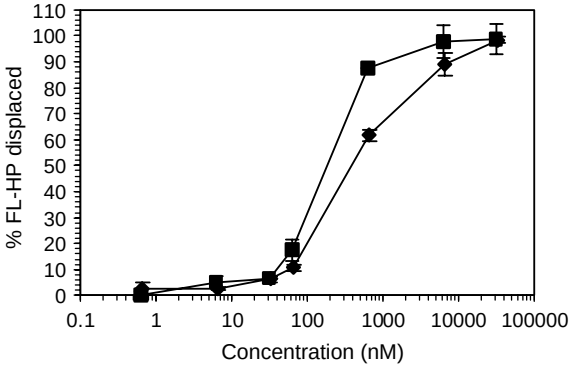


Figure 11. Comparison of concentration-dependent displacement of FL-HP from α-thrombin by heparin (♦) and 100% *N*-desulfonated heparin *N*-acylated with **D13** (■).

that recurrence of aryl groups **D13**, **D14**, and **D18** in binding both VEGF and α-thrombin suggests a cation–π interaction may account for our ability to replace *N*-sulfo groups supposedly required for protein binding and maintain or increase affinity for select proteins. Alternatively, these *N*-acyl groups may make novel binding contacts with protein, with or without conformational changes in the heparin chain, to afford a re-orientation of the heparin–protein binding interaction to maintain a critical level of charge–charge interactions. Indeed, the polyelectrolytic effect is known to provide significant contributions to affinity for many, but not all, heparin–protein interactions under physiological conditions.[35] The heterogeneity of heparin and subsequent heparin-derived structures here complicates identification of the specific molecular interactions that account for these profound results. Studies to fully understand the molecular basis for observed binding here will require the identification and subsequent study of the shortest sequences within these novel heparin derivatives that bind thrombin and VEGF. We are currently preparing second-generation libraries of size-defined and structurally defined heparin to begin answering these questions.

The demonstration here that *N*-sulfo groups on heparin can be replaced with structurally diverse non-anionic moieties to afford unique heparin derivatives having increased affinity for select proteins has implications for modulating heparin–protein binding beyond our goal of identifying selective antagonists of HS–protein interactions. With exception of the few heparin–protein interactions where unique heparin-sequences are required for agonistic activity (e.g., allosteric activation of antithrombin by pentasaccharide sequence), most heparin-binding proteins will bind virtually every sequence within heparin with some level of affinity, although the more highly charged domains typically provide sequences with highest affinity. This is consistent with the concept presented by Lander;[36] that in many cases promiscuous binding of proteins to various sequences of cell surface HS can reduce protein–protein interactions at the cell surface from a three-dimensional diffusion process to a one-dimension diffusion process along the HS chain. Thus, a significant factor governing events from HS–protein binding is likely kinetic, not thermodynamic in nature.[36] That is to say, changes in k_{on} and k_{off} (kinetics) of GAG–protein binding may have important consequences in the absence of a change in k_d (thermodynamics, $k_d = k_{off}/k_{on}$).[36] The possibility

of introducing heparin–protein binding contacts that are not charge-based is expected to afford heparin derivatives with advantageous, but yet unexplored, protein binding kinetics.

4. Conclusions

The diversity-oriented chemical modification of heparin has been employed to prepare charge-reduced heparin derivatives that possess equivalent or increased affinity for select heparin-binding proteins. A parallel synthesis strategy has been exploited for the efficient modification of heparin-based scaffolds. A 96-well plate competition binding assay has been established, which allows simultaneous screening of hundreds of molecules for the heparin-binding site of virtually any heparin-binding protein. The results of this study demonstrate a new approach for replacing certain anionic groups on heparin that are supposedly required for protein binding, where *N*-sulfo groups previously shown to be required for heparin to bind certain proteins have been uniformly replaced with non-anionic *N*-acyl moieties to yield unique heparin derivatives that maintain affinity for those proteins.

The outcome of this work demonstrates a significant first step toward developing new approaches to preparing charge-reduced heparinoids that possess increased selectivity and increased affinity for specific heparin binding proteins. A number of current therapeutic agents, as well as numerous agents in preclinical and clinical development, are based on exploiting polysulfonated saccharides to block HS–protein interactions.[6] Application of this diversity-oriented approach to the chemical modification and/or chemical synthesis of saccharides having defined structure is expected to yield promising new charge-reduced inhibitors of HS–protein interactions. While such molecules may never be reduced to structures that epitomize small, drug-like compounds, this work provides great promise for identifying novel therapeutic agents for the numerous clinical applications where polyanionic heparinoids are already considered viable therapeutic options. Expansion of these strategies is anticipated to yield smaller, lower-charge agents that are certainly more drug-like than current heparinoids.

The ability to employ diversity-oriented chemical modification of heparin to remove charged groups involved in heparin–protein interactions demonstrates a fundamental principle required for the systematic derivation of new therapeutic agents from bioactive polyanionic oligosaccharides and polysaccharides. Our current research efforts are aimed at isolating and characterizing the shortest *N*-desulfonated/*N*-acylated heparin sequences that bind α-thrombin and VEGF. Rigorous characterization of the molecular interactions between these structurally defined oligosaccharides and protein will reveal the molecular basis for affinity in the absence of supposedly required *N*-sulfo moieties. We are also exploiting the methods described here for the diversity-oriented modification of heparin to expand molecular diversity, to target specific heparin-binding proteins related to specific therapeutic applications, and to develop strategies for uniformly replacing additional anionic groups on heparin as well as anionic moieties on other bioactive polysulfonated oligosaccharides.

5. Experimental

5.1. *N*-Desulfonation of heparin and evaluation of amine content

Variable *N*-desulfonation of heparin was performed by modification of established methods.[19] Heparin was converted from the sodium to pyridinium salt form by dissolving in water and passing through a cationic exchange resin (Dowex 50WX4-400, H^+ form) at 4 °C. The resulting acidic fractions of aqueous H^+ heparin were immediately combined and titrated with pyridine to pH 4.72, frozen, and lyophilized. Partial *N*-desulfonation at 20 °C was performed as follows: pyridinium heparin (6.0 g) was dissolved in 320 mL DMSO/water (95:5) at 10 °C, warmed to rt (20 °C), and stirred vigorously. A 40 mL aliquot of the reaction solution was individually removed at 0.5, 1, 1.5, 2, 2.5, 3.5, 4.5, 6, and 8 h. Aliquots were immediately added to 40 mL ice-cooled water to quench the reaction and the pH adjusted to 9.14 with 0.1 N NaOH. The resulting solutions were dialyzed against water (MWCO = 3500) and lyophilized to provide *N*-desulfonated heparin fractions. Partial and complete *N*-desulfonation at 50 °C was performed as follows: pyridinium heparin (3.2 g) was added to 100 mL DMSO/water (95:5) at 50 °C and stirred vigorously. Aliquots (25 mL) were removed at 10, 20, 90, and 120 min after the reaction temperature reached 50 °C. Each aliquot was immediately added to 25 mL cold water and adjusted to pH 9.14 with 0.1 N NaOH. The resulting solutions were dialyzed against water (MWCO = 3500) then lyophilized affording *N*-desulfonated heparin fractions.

Amine content of each heparin fraction was determined using the TNBS assay.[20] A sample of each heparin fraction, unreacted heparin (control), and completely *N*-desulfonated heparin (control) was accurately weighed and dissolved in water to afford 2.0 mg/mL test solutions. Standard curves employing glucosamine derivatives were also employed to assure stability and consistency of the assay. To 380 μL water in separate glass test tubes was added 120 μL of each saccharide (2.0 mg/mL in water) followed by 1.0 mL of 0.5 M phosphate buffer (pH 8.0) and 1.00 mL freshly prepared 0.15% TNBS w/v in water. This solution was incubated at 40 °C in an orbital shaker bath for 1.5 h. The resulting yellow solutions were each treated with 0.5 mL 6 N HCl, mixed well, and UV absorbance at 348 nm was determined immediately. Percent free amine of each fraction was calculated by linear fitting of absorbance at 348 nm relative to 100% *N*-desulfonated heparin (100% free amine) and control heparin (0% free amine). ^{1}H NMR (400 MHz, D_2O) demonstrated complete *N*-desulfonation of the final 50 °C *N*-desulfonation sample: the glucosamine H-2 signal of *N*-sulfo heparin assigned at

δ 3.21 completely disappeared, whereas a downfield-shifted signal at δ 3.34 appeared, which was the expected signal for H-2 bearing a $-NH_2$ substituent. This sample was used as the 100% free amine control.

5.2. Carboxylic acid derivatives and *N*-acylation studies

NHS activated acids **D4**, **D8**, **D10**, **D11**, **D18**, **D13**, and acid anhydrides **D2** and **D17** were purchased from commercial sources. NHS esters **D1**, **D9**,**D14**, and **D15** were prepared from their corresponding commercially available carboxylic acids essentially as reported.[37] Preparation and isolation of NHS esters of heteroaryl-substituted carboxylic acids 5-hydantoin acetic acid (**D3**), 1*H*-tetrazole-1-acetic acid (**D7**), and 3-(2-furyl)propanoic acid (**D16**), as well as succinamic acid (**D5**) have not been reported.[38] To this end, NHS-activated acids **D3**, **D5**, **D7**, and **D16** were prepared via DCC-mediated coupling of NHS with the corresponding commercially available carboxylic acids. Preparation and isolation of the NHS ester of thymine-1-acetic acid (**D12**) has not been reported, although this acid has undergone direct coupling to a variety amines and alcohols including coupling reactions for preparing peptide nucleic acid structures. **D12** was prepared by *N*-alkylation of thymine with bromoacetic acid,[39] followed by conversion to the NHS ester via DCC-mediated coupling with NHS.[40] Previously unreported hypoxanthine-9-acetic acid NHS ester (**D6**) was prepared and isolated from hypoxanthine-9-acetic acid, which was prepared in five steps from adenine using methods previously reported with some modifications.[41] Similarly, 2-Benzyl-tetrazole-5-carboxylic acid was prepared by modifications of reported methods[42] and converted to a previously unreported NHS ester **D19** via DCC-mediated coupling with NHS. Experimental details for the preparation and isolation of all NHS esters employed in this study are provided with Supplementary data. See Supplementary data also for details of the *N*-acylation studies reported in Table 1.

5.3. Library synthesis and analysis

The following protocol was employed to couple each DFM to each amine-containing heparin fraction. Amine-containing heparin fractions were individually weighed in 4 mL vials (8–10 mg/vial). A small stir bar and 1 mL of a 2:1 solution of DMSO/H_2O saturated with $NaHCO_3$ were added to each vial and vials were placed on stir plates (7 vials/plate). To each vial was added activated DFM (6 equiv, based on 1 equiv DFM corresponding to 1 equiv disaccharide unit, estimated MW = 600) in equal portions at 1–2 h intervals over 6–8 h, and then the reactions were stirred overnight. Each coupling reaction was precipitated by addition to 30 mL acetone/ether (1:1), centrifuged, decanted, and washed with acetone. The remaining residue was dried, dissolved in 1 mL water, filtered through a 20 micron syringe filter into dialysis tubing (MWCO = 3500), and dialyzed against water. Dialyzed products were lyophilized. Amine content of each coupling product was evaluated using the TNBS assay as reported above. Products possessing detectable amine underwent a second, identical, coupling procedure to achieve complete *N*-acylation. Total sulfate, *N*-sulfate, and GPC–HPLC analyses were performed using established methods to confirm integrity of the heparin chains.[26,28]

5.4. Library screening, general

All competition-binding assays were performed in a darkroom using a Multiscreen Filtration System and high protein binding 96-well filtration plates (Millipore, MAHA N4510). FI was detected using a Fluorescence Multi-well Plate Reader (Cytofluor series 4000, Perseptive Biosystems, Inc. Framingham, MA). Fluorescence readings of each well were obtained from the average of three consecutive scans of a plate using λ_{ex} = 485 nm, λ_{em} = 530 nm. All solutions were made in PBS pH 7.4 unless otherwise indicated. 96-well plates were pre-wetted with 100 uL PBS for 10 min and then vacuum applied to completely remove buffer from wells before use. α-thrombin (Human α-thrombin, 13.3 mg/mL in 1:1 glycerol/H_2O, cat. No. HCT-0020) was purchased from Haematologic Technologies Inc. FGF1 (rhFGF-acidic, cat. No.232-FA and 232FA/CF), FGF2 (rhFGF-basic, cat. No. 233-FB), and VEGF (rhvEGF, cat. No. 293-VE) were from R&D Systems, Inc., among which FGF2 and VEGF were generously supplied by the NCI Biological Resources Branch. FL-HP (Heparin, fluorescein conjugate; Molecular Probes, Eugene, OR) was purified before use. To this end, FL-HP (3 mg) in 0.5 mL water was loaded onto a Sephadex G-50 column (1 × 50 cm) and eluted with ultrapure DI water. Fractions containing product were identified using UV detection (λ = 460 nm), combined, and lyophilized. HPLC analysis with fluorescence detection (λ_{ex} = 492, λ_{em} = 515) (GPC column: GSWX 2300, Tosohas, 0.5 mL/min elution with 25 mM Tris, 150 mM NaCl, pH 7.5) showed a single peak for FL-HP at 11 min.

5.5. Protein binding assays

Experimental details for evaluating binding of all *N*-acyl heparin derivatives to α-thrombin are presented here. Growth factor binding studies were performed similarly and experimental details are presented with Supplementary data. The concentrations of α-thrombin and FL-HP employed in library screening were established as follows: α-thrombin was diluted in PBS to afford 50 μg/mL and 100 μg/mL stock solutions. 50 μL of each α-thrombin stock solution and 50 μL PBS (control) were added to individual filtration-plate wells containing 50 μL FL-HP in PBS at concentrations of 50, 100, 200, 300, and 400 nM, affording final concentrations of α-thrombin (0, 25, and 50 μg/mL) and FL-HP (25–200 nM). The plate was incubated on a shaker at rt for 30 min, vacuum filtered into a receiver plate, and the fluorescence intensity of filtrate wells read. Graphs for each α-thrombin concentration and control (no protein) were plotted as fluorescence intensity versus the concentration of FL-HP (Fig. 6).

The concentration at which heparin and all heparin derivatives were to be employed in the library screening assays was determined as follows: α-thrombin (25 μL of

100 μg/mL in PBS) and FL-HP (25 μL of 600 nM FL-HP in PBS) were combined in individual wells of a 96-well filtration plate. To each well was added 50 μL heparin in PBS from stock solutions (0, 0.02, 0.2, 2, 20, 200, and 1000 μg/mL), respectively, affording a final concentration of 0, 0.01, 0.1, 1, 10, 100, and 500 μg/mL heparin. The plate was incubated on a shaker at rt for 30 min, filtered into a receiver plate, and fluorescence intensity of the filtrates read. Data were plotted as the concentration of heparin in logarithmic scale versus fluorescence intensity (Fig. 7). Comparison of concentration-dependent displacement of FL-HP by heparin and *N*-desulfonated/*N*-acylated heparin derivatives was performed in an identical fashion.

Library screening for competitive binding of each library member and heparin (control, zero point reference for all library members) to α-thrombin was performed as follows: α-thrombin (25 μL of 100 μg/mL in PBS) and FL-HP (25 μL of 600 nM FL-HP in PBS) were combined in wells of a 96-well filtration plate, followed by addition of each library component and heparin (50 μL of a 10 μg/mL stock solution PBS) to individual wells. Plates were incubated on a shaker at rt for 30 min, filtered into receiver plates, and fluorescence intensity of the filtrates read. Graphical representation of the data was reported as percent change in observed fluorescence relative to heparin. Data points were calculated for each library member using the equation: $\% \ \Delta F_{rel} = (FI_{DnHx} - FI_{heparin})/(FI_{heparin} - FI_{min}) \times 100$, where FI_{DnHx} is the fluorescence intensity observed for each test compound, $FI_{heparin}$ is the fluorescence intensity observed for heparin control, and FI_{min} is the fluorescence intensity observed for FL-HP and protein only.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.11.013.

References and notes

1. Heparin and HS polysaccharides are polydisperse (comprised of chains of varied lengths) and microheterogeneous (varying sequences within each chain due to varied levels of sulfate substitution and iduronic acid versus glucuronic acid).
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25. The loss of sulfate via acetylative-desulfonation has been studied, see: (a) Hyatt, J. A. *Carbohydr. Res.* **1993**, *239*, 291–296; Loss of *N*-sulfo groups was also observed, but overcome, during previous studies looking to *O*-acylate heparin-derived disaccharides using amine bases in organ-

ic solvent, see: (b) Kerns, R. J.; Vlahov, I. R.; Linhardt, R. J. *Carbohydr. Res.* **1995**, *267*, 143–152.
26. Molecular weight analysis using HPLC-GPC was performed on a random sampling of heparin fractions for each DFM. Photodiode array detection afforded detection of heparin regardless of chromophoric properties of the *N*-acyl groups. Analytical samples of the modified heparins were spiked with the carboxylate form of the DFM, the activated acid (NHS ester or anhydride), and NHS to establish HPLC detection and subsequently demonstrate complete removal of reagents from the heparin products.
27. Total sulfate was determined by acid hydrolysis followed by turbidimetric analysis of inorganic sulfate, see: Dodgson, K. S.; Price, R. G. *Biochem. J.* **1962**, *84*, 106–110, All 100% *N*-desulfonated, *N*-acylated heparin products showed total sulfate content consistent with the parent 100% *N*-desulfonated heparin, demonstrating no loss of sulfate during *N*-acylation.
28. Determination of *N*-sulfate employed nitrous acid treatment followed by turbidimetric analysis of inorganic sulfate, see Ref. 21. Each 50% *N*-desulfonated/*N*-acylated heparin product showed the same level of *N*-sulfo substitution as the parent 50% *N*-desulfonated heparin.
29. Use of the TNBS assay to determine low percentages of amine content for the original *N*-desulfonated heparin fractions was possible because gram quantities of each fraction were available for analysis, which allowed total amine in assays to be well above detection limits. Analysis of coupling products for residual amine after *N*-acylation required the use of smaller sample quantities. With these smaller quantities, the TNBS assay afforded stable, reproducible, detection of amine in heparin fractions having greater than 5% free amine remaining.
30. For examples of filtration binding assay employing column techniques to evaluate heparin-FGF1 and GAGs-PF4 binding, respectively, see: (a) Kreuger, J.; Prydz, K.; Pettersson, R. F.; Lindahl, U.; Salmivitra, M. *Glycobiology* **1999**, *9*, 723–729; (b) Petersen, F.; Brandt, E.; Lindahl, U.; Spillmann, D. *J. Biol. Chem.* **1999**, *274*, 12376–12382; (c) Pejler, G. *Biochem. J.* **1996**, *320*, 897–903.
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