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Heparin-mimetic sulfated peptides with modulated affinities for heparin-binding peptides and growth factors

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Abstract

Heterogeneity in the composition and in the polydispersity of heparin has motivated the development of homogeneous heparin mimics, and peptides of appropriate sequence and chemical function have therefore recently emerged as potential replacements for heparin in selected applications. Here, we report the assessment of the binding affinities of multiple sulfated peptides (SPs) for a set of heparin-binding peptides (HBPs) and for vascular endothelial growth factor isoform 165 (VEGF165); these binding partners have application in the selective immobilization of proteins and in hydrogel formation through non-covalent interactions. Sulfated peptides were produced via solid-phase methods, and their affinity for the HBPs and VEGF165 was assessed via affinity liquid chromatography (ALC), surface plasmon resonance (SPR), and in selected cases, isothermal titration calorimetry (ITC). The shortest peptide, SP_a, showed the highest affinity binding of HBPs and VEGF165 in both ALC and SPR measurements, with slight exceptions. Of the investigated HBPs, a peptide based on the heparin-binding domain of human platelet factor 4 showed greatest binding affinities toward all of the SPs, consistent with its stronger binding to heparin. The affinity between SP_a and PF4_{ZIP} was indicated via SPR ($K_D = 5.27 \mu\text{M}$) and confirmed via ITC ($K_D = 8.09 \mu\text{M}$). The binding by SP_a of both VEGF and HBPs suggests its use as a binding partner to multiple species, and the use of these interactions in assembly of materials. Given that the peptide sequences can be varied to control binding affinity and selectivity, opportunities are also suggested for the production of a wider array of matrices with selective binding and release properties useful for biomaterials applications.

Keywords

Sulfated peptide; Heparin mimic; Heparin-binding peptides; Affinity chromatography; Growth factor delivery; Hydrogel

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2007.08.010.

1. Introduction

Growth factor delivery has been a topic of increased recent research interest owing to both increasing interests in controlled and targeted drug delivery as well as the expansion of recombinant DNA techniques that have facilitated growth factor production [9,45,59]. Heparin, a highly sulfated glycosaminoglycan, has been used to mediate the local delivery of growth factors from various matrices such as microcapsules [14], fibers [33,34], grafts [29,64], and hydrogels [50,53,54,66–69], since it is known to bind and stabilize many growth factors and potentiate their activity. In particular, heparin has been shown to protect growth factors from inactivation [22,45], increase their affinity to receptors [49], improve the loading efficiency of growth factor into delivery vehicles [34], and sustain the release of growth factors over extended periods of time [33,34]. The chemical diversity, versatility, and heterogeneity of heparin (H), and the related heparan sulfate (HS) that is found in tissue, permits the binding of over 50 growth factors [34], as well as mediation by H/HS of many important biological processes including thrombosis, cell adhesion, lipid metabolism, enzyme regulation, and cytokine action [1,3,27,35,51].

We have reported previously the non-covalent assembly of hydrogels via interaction between heparin and heparin-binding peptides or growth factors, as well as the delivery of growth factors from these matrices via passive or receptor-mediated matrix erosion (Scheme 1) [66–69]. Four-arm star poly(ethylene glycol) (PEG) modified with heparin has been shown to form hydrogels when mixed with star PEGs modified with heparin-binding peptides, as illustrated in the left path in Scheme 1; the peptides used are derived from heparin-interacting protein, antithrombin III, or platelet factor 4 (HIP [36–39], ATIII [61], and PF4_{ZIP} [5,69], respectively). Such modified polymers form hydrogels through non-covalent (primarily electrostatic) interactions, and the hydrogels can be loaded with therapeutic growth factors. The mechanical strength of the hydrogels can be controlled by choosing different heparin-binding peptides and polymer compositions [66,69], and the distribution of the charged heparin throughout the matrix may also facilitate homogeneous incorporation of growth factors into the delivery vehicle [34]. The lack of toxic cross-linking agents in the gels [53], coupled with the potential for their non-invasive administration (*e.g.*, via injection) should permit their use in multiple applications.

In addition, dimeric growth factors, such as vascular endothelial growth factor (VEGF), can be directly employed as cross-links in the assembly of these hydrogel networks, since they provide two cross-linking points per molecule (right path in Scheme 1) [68]. Upon administration at a target site, the growth factor may be removed from the gel via binding with its receptors, and subsequently through receptor-mediated endocytosis [43], which would cause erosion due to the loss of physical cross-linking points and would theoretically permit elimination of the polymer matrix should PEG molecules of appropriate molecular weight be used [10,30,46,47,55]. Such a delivery system could be flexibly applied for numerous targets, depending on the desired growth factor employed in the matrix; in theory, multiple growth factors could also be used in a single matrix to permit delivery and erosion on multiple timescales that depend on the affinity of the growth factor for heparin. In addition, the delivery of multiple proteins would be expanded if additional affinity ‘tags’ could be identified with various affinities for heparin-binding proteins of relevance in biomedical applications; such identification would be facilitated by the use of homogeneous molecules with compositions that could be readily and controllably varied.

The inherent heterogeneity of heparin makes its controlled use in the above applications challenging [67]. The number of saccharides in heparin, the sulfation pattern, and the exact site of conjugation of polymers, can vary substantially from sample to sample, which has motivated the identification of heparin surrogates that are homogeneous and straightforward

to synthesize. Among the many kinds of heparin mimics previously reported, including anionic polymers and small molecules [13,20,21,25,28,63], a sulfated tyrosine containing tetrapeptide reported by Maynard and Hubbell [42], which binds specifically to VEGF165, attracted our attention owing to its potential use in growth factor delivery and VEGF-mediated hydrogel assembly [68]. The use of sulfated tyrosine as the sulfated species in these molecules may offer advantages with respect to biological activity, as such post-translational modifications occur naturally [31]; indeed, it has been suggested that the electrostatic interaction mediated by sulfated tyrosine is one of the important factors in the antithrombotic action of heparin cofactor II (HCII) [24], the hirudin–thrombin interaction [2,41,57], and interaction between chemokine receptor CCR5 and HIV (human immunodeficiency virus) gp120 [17]. In addition, the ability to tailor sequences via solid-phase methods will facilitate identification of additional peptides of desired homogeneity for binding with specific heparin-binding partners. Taken together with our studies summarized above, these reports suggest that sulfated peptides of select and defined sequences could be useful in the production of materials with targeted mechanical properties and protein delivery profiles.

In this study, a series of sulfated peptides, including the previously reported sulfated tetrapeptide [42], was synthesized. The binding affinities between the sulfated peptides and heparin-binding peptides or VEGF165 were measured via affinity liquid chromatography (ALC) and surface plasmon resonance (SPR) techniques. The results were compared with the binding affinities of heparin. The importance of the amino acid sequence of the sulfated peptides in the binding event was indicated by the differences in affinities among the various binding pairs, despite the similarity of sequence and charge of the sulfated peptides. Isothermal titration calorimetry (ITC) experiments confirmed the dissociation constant of the SP-HBP pair of highest binding affinity. These results suggest the potential use of the SPs for the immobilization of multiple proteins with different affinities in biomaterials and delivery systems where heterogeneous heparin has been conventionally used.

2. Experimental

2.1. Materials

Fmoc-Tyr(OH)-OH, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 2-chlorotriyl chloride resin, Rink-amide MBHA resin and all other peptide synthesis reagents were purchased from Novabiochem® (Merck Biosciences AG, Switzerland), except for piperidine (Aldrich, Allentown, PA), 4-methylmorpholine (Aldrich, Milwaukee, WI), and *N,N*-dimethylformamide (DMF, Fisher Scientific Inc., Newark, DE). The sulfur trioxide-pyridine complex and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich (Milwaukee, WI). Triisopropylsilane (TIS) and 1,1,1,3,3,3-hexafluoro-isopropanol (HFIP) were purchased from Aldrich (Allentown, PA). The tetrabutylammonium hydrogen sulfate was purchased from Sigma (St. Louis, MO). The 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Pierce (Rockford, IL). Other chemicals were purchased from Sigma–Aldrich unless otherwise noted. All chemicals were used as received. A pRSET-VEGF165 plasmid was a generous gift from Dr. Andreas H. Zisch (Department of Obstetrics and Gynecology, University Hospital Zurich, Switzerland). The recombinant VEGF165 protein was expressed and isolated from *Escherichia coli* according to the reported protocol [68,70,71], and purified via heparin–agarose affinity chromatography. All HPLC experiments were conducted on a Waters Delta 600 HPLC system (Waters Co., Milford, MA), with various choices of columns and eluent conditions. All nuclear magnetic resonance (NMR) spectra were acquired on a DRX400 (Bruker BioSpin Co., Billerica, MA) under standard quantitative conditions.

2.2. Synthesis and characterization of the sulfated peptides

Fmoc-Tyr(SO₃-NnBu₄)-OH was synthesized as previously described [62]. Fmoc-Tyr(OH)-OH was sulfated via treatment with sulfur trioxide-pyridine complex in DMF. The counterion of the sulfate was exchanged to tetrabutylammonium in order to improve the acid-stability [6,65] and the solubility of the amino acid. The degree of sulfation of the amino acid was determined via ¹H NMR (400 MHz, in MeOD containing an internal reference TMS, 512 scans): Fmoc-Tyr(OH)-OH, ¹H NMR (400 MHz, MeOD, 512 scans): δ = 6.69 (2H, tyrosyl meta, d), 7.04 (2H, tyrosyl ortho, d), 7.29 (2H, Fmoc, m), 7.39 (2H, Fmoc, t), 7.59 (2H, Fmoc, d), and 7.78 ppm (2H, Fmoc, d); Fmoc-Tyr(OSO₃)-OH, ¹H NMR (400 MHz, MeOD, 512 scans): δ = 7.20 (4H, tyrosyl aromatic, m), 7.31 (2H, Fmoc, m), 7.39 (2H, Fmoc, t), 7.59 (2H, Fmoc, d), and 7.78 ppm (2H, Fmoc, d). The purity of the sulfated tyrosine was confirmed via reverse phase HPLC on a Waters DeltaPak® C18 column. The eluent was subjected to a linear gradient from 95:5 to 30:70 of 0.1 M ammonium acetate aqueous/acetonitrile over 35 min at 5 ml/min, and the absorbance was observed at 214 nm.

The sulfated tyrosine containing peptides were synthesized via standard solid-phase peptide synthesis methods on the PS3™ automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ), using 2-chlorotrityl chloride resin as a polymer support and HBTU as a coupling reagent. The synthesized peptides were cleaved from the resin by treating the resin with HFIP/DCM (1:4, v/v) at room temperature for 1 h [4,62]. The solution was precipitated in cold ether to obtain a white solid. The side-chain protecting groups of the peptides were removed in pre-cooled TFA/TIS/water mixture (95:2.5:2.5, v/v/v) in an ice bath for 1 h. The solution was precipitated into cold ether to give a white solid. The sulfated peptides were purified via anion-exchange chromatography on an ÄKTA™ explorer system equipped with a HiTrap DEAE FF 5 ml column (GE Healthcare Bio-Sciences Corp.: formerly Amersham Biosciences, Piscataway, NJ). The peptides were eluted with a linear gradient of sodium chloride concentration from 0 to 2 M in 5 mM sodium phosphate (pH 7.4), over 20 min at 5 ml/min, and the absorbance was observed at 215 nm. Fractions with the highest affinity (requiring the highest concentration of salt for elution) were collected and lyophilized. The collected peptides were then purified in a final step via RP-HPLC with elution via a linear gradient from 100:0 to 30:70 of water/acetonitrile over 35 min; this was conducted to confirm the purity of the sulfated peptide and to remove the salts from the previous purification step. Obtained peptides were characterized via amino acid analysis (Molecular Analysis Facility, University of Iowa), and showed purities of greater than 95%. The degree of sulfation was confirmed via NMR; ¹H NMR (400 MHz, D₂O, 512 scans): δ = 7.20 ppm (8H, sulfated tyrosyl aromatic, m); the peptides showed essentially 100% sulfation after purification. Reliable mass spectral analysis of intact, sulfated peptides was impossible owing to the lability of the sulfate groups under the required experimental conditions.

2.3. Synthesis of heparin-binding peptides

Two of the three heparin binding peptides (HBP) investigated, i.e. ATIII (NH₂-KAFKLAARLYRKA-CONH₂) [61,66] and HIP (NH₂-RPKAKAKAKAKDQTK-CONH₂) [36–39,66,67], were synthesized via a standard Fmoc solid-phase peptide techniques as previously described [8,66,67]. Each peptide was purified via preparative HPLC on a Waters Symmetry® C18 column, under a linear gradient from 95:5 to 5:95 of water/acetonitrile (containing 0.1% of trifluoroacetic acid) at 5 ml/min. The molecular weights of each peptide were verified via matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOFMS, OnmiFlex-NT, Bruker Daltonics Inc., Billerica, MA), m/z = 1608.33 [(M + H)⁺, calcd 1607.00] for the ATIII peptide, and m/z = 1667.44 [(M + H)⁺, calcd 1668.02] for the HIP peptide. The purities of peptides were re-confirmed via HPLC. The ATIII was 99% pure as determined via separation on Waters Analytical Symmetry® C18 column under a linear gradient of 95:5 to 5:95 of water/acetonitrile

(containing 0.1% trifluoroacetic acid) over 50 min. HIP was 99% pure as determined via separation on a Waters Symmetry® C18 column under a linear gradient from 100:0 to 0:100 of water/acetonitrile (containing 0.1% trifluoroacetic acid) over 50 min. The PF4_{ZIP} peptide (NH₂-RMKQLEDKVKLLKKNYHLENEVARLKKLVG-CONH₂) [5,69] was a generous gift from Le Zhang and was of similar purity.

2.4. Affinity liquid chromatography

A heparin-mimetic column was produced via immobilization of the sulfated peptides on a solid support. The sulfated peptides were immobilized on an NHS-preactivated cross-linked agarose column (GE Healthcare Bio-Sciences Corp.: formerly Amersham Biosciences, Piscataway, NJ) via amine coupling of the single amine at the N-terminus of the peptide. The sulfated peptides were dissolved in the binding buffer at a concentration *ca.* 15 mg/1 ml (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3), and then mixed with the NHS-activated resin. The reaction was conducted for 40 min at room temperature with constant exposure to peptide solution. Any excess active groups were deactivated and any non-specifically bound ligands were removed by washing the columns with 0.5 M ethanolamine/0.5 M NaCl (pH 10) followed by 0.1 M sodium acetate/0.5 M NaCl (pH 6.5), with a total of five wash cycles. The modified columns were used in binding assays on the ÄKTA™ Explorer system (GE Healthcare Bio-Sciences Corp.: formerly Amersham Bio-sciences, Piscataway, NJ).

The heparin-binding peptides or VEGF165 were injected onto each column and unbound molecules were removed by washing the column with 5 mM sodium phosphate buffer (pH 7.4) over five column volumes. The elution of peptides was detected via UV, at an absorbance of 215 nm. The bound peptides were eluted with a linear gradient of sodium chloride at concentrations ranging from 0 to 2 M in 5 mM sodium phosphate solution (pH 7.4). The relative binding affinities between each sulfated peptide and heparin-binding peptide/protein are estimated from the concentration of NaCl (M) required to elute the heparin-binding peptide from the column. The experiments were done in at least triplicate and the average NaCl concentrations reported. The binding affinity between heparin and VEGF165 was measured on a heparin sepharose column (HiTrap Heparin HP, 1 ml). The VEGF165 was injected and eluted under a linear gradient of 0–1 M NaCl in 5 ml sodium phosphate (pH 7.4) at 1 ml/min for 20 min, with UV detection at 215 nm.

Control experiments to probe the role of non-specific electrostatic interactions in the binding of the HBPs to the modified columns were conducted on non-peptide functionalized columns equipped with carboxylate groups. The NHS-preactivated agarose column was hydrolyzed to give free carboxyl end-groups by treating with 20 column-volumes of water, 20 column-volumes of 5 mM sodium phosphate solution (pH 7.4), and then 20 column-volumes of 2 M sodium chloride solution (in 5 mM sodium phosphate, pH 7.4). The HBPs and VEGF165 were loaded and eluted from this column via the same protocols as described above.

2.5. Surface plasmon resonance

The association and dissociation between sulfated peptides and HBPs were monitored via SPR, in which the refractive index change and resulting surface plasmon angle change, due to binding at a modified gold surface, are measured. Corresponding dissociation constants (K_D) were obtained via non-linear regression fitting to a Langmuir binding model, as described in a previous report [66,69]. All experiments were conducted on the Biacore® 3000 with running buffer HBS-EP (Biacore Inc., Piscataway, NJ). The sulfated peptides were immobilized on a carboxymethylated dextran CM5 sensor chip (Biacore Inc., Piscataway, NJ) through an amine coupling reaction. The surface of the sensor chip was activated by injecting a mixture of 0.15 M NHS and 0.6 M EDC for 2 min at 5 µl/min. The

prepared sulfated peptide solution (*ca.* 12 mg/0.1 ml) in HBS-EP running buffer (100 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4 containing 0.005% (v/v) P-20 surfactant) was then injected for 10 min at 5 μ l/min. The unreacted NHS groups were inactivated via injection of 1 M ethanolamine. Permanent increases in the resonance units of the chips confirmed immobilization (122.7 RU for SP_a, 58.4 RU for SP_b, and 45.4 RU for SP_c). A reference flow cell was treated with ethanolamine as a ligand instead of sulfated peptide. For binding studies, a series of concentrations of each heparin-binding peptide (0, 5, 10, 25, 50, 100, and 250 μ M in HBS-EP buffer) or of VEGF (0, 0.85, 1.7, 3.4, 6.79, 13.6, and 27.2 μ M in HBS-EP buffer) were injected over the sulfated peptide-modified chips. After a 15 min dissociation phase, the sensor chip surface was regenerated for the next peptide sample injection, via treatment with two 1 min pulse injections of regenerating buffer (2 M NaCl in HBS-EP, pH 7.4) at 100 μ l/min. The resonance units of the baseline returned to the initial value after the regeneration step, confirming the removal of all bound analytes. The SPR measurement of the binding affinities of HBPs to a heparin-modified sensor chip has been described in a previous report [66,69].

2.6. Isothermal titration calorimetry

All ITC experiments were conducted on a MicroCal VP-ITC calorimeter (MicroCal, LLC, Northampton, MA). A solution of the PF₄ZIP peptide (0.2 mM in 5 mM sodium phosphate buffer, pH 7.4, 1.34 ml) was placed in the sample cell. After baseline stabilization, a total of 125 aliquots of 10 μ l SP_a solution (2.146 mM in 5 mM sodium phosphate buffer, pH 7.4) were injected, with a time interval of 600 s and stirring at 260 rpm. The reference cell power and temperature were set at 10 μ cal/s and 25 °C, respectively. Control experiments were conducted to measure the heats of dilution, via titration of buffer solution into buffer solution, buffer solution into PF₄ZIP solution, and SP_a solution into buffer. The data from these control experiments were subtracted from the appropriate sets of data prior to the analysis. The raw data was fit to various binding models using Origin® 7.0 software; the best fit was assessed on the basis of a minimized χ^2 value. The thermodynamic parameters were obtained from the standard Gibbs free energy equation, $\Delta G = \Delta H - T\Delta S = -RT \ln K_a$ as described elsewhere [11,12], to determine K_D values and the number of binding sites.

3. Results

3.1. Sequences and synthesis of sulfated peptides

Despite the utility of heparinized polymers in the production of hydrogels in our previous reports [66–69], variation in chemical modification and hydrogel mechanical properties resulted from the intrinsic heterogeneity of the heparin employed. The goal of this study was to investigate the potential feasibility, through assessments of binding affinities, of employing heparin mimetic sulfated peptides in the non-covalent assembly of hydrogels via interaction with heparin-binding peptides. The sequences of studied sulfated peptides are shown in Table 1, with the regions that mediate binding indicated in bold. The SP_a peptide was chosen as a first target given its previous identification, via a combinatorial library method, as a binding partner for VEGF165 [42]. The terminal glycine residues were added to improve accessibility to the peptide in solid-phase binding assays. In these sequences, serine, aspartic acid, and sulfated tyrosine are chosen as mimics of the hydroxyl, carboxyl, and sulfate functional groups in heparin, as reported by Maynard and Hubbell [42]; these functional groups are purported to be the most important in the binding activities of the glycosaminoglycan.

For comparison, SP_b was synthesized to investigate the effect of the size of the peptide containing the same pattern of functional groups as SP_a. It has been reported that the minimum required size of heparin for binding to ATIII and HIP is a pentasaccharide, while

four tetrasaccharides are required for high affinity binding to PF4. The heparin disaccharide has on average one to two *O*-sulfates, which suggests an average minimum of two to four *O*-sulfates to mediate binding to the peptides. Therefore, sulfated peptides of different sizes (and therefore different numbers of *O*-sulfates) might show modulated binding affinities to the peptides. The sequence of SP_c was chosen to probe the effect of variation in the positioning and number of the sulfate groups in binding to these different targets. The fact that SP_c might have some binding affinity for heparin-binding proteins and peptides was suggested from the screening of a combinatorial library (Maynard, personal communication). Studies were therefore conducted to determine if this sequence had any increased affinity for the heparin-binding peptides of interest in hydrogel studies.

The synthesis of sulfated Tyr-containing peptides has not been widely employed due to the intrinsic acid-lability of *O*-sulfates during synthesis. In these studies, sulfated, Fmoc-protected Tyr was first synthesized via previously reported protocols [62] and employed in standard Fmoc-based solid-phase peptide synthesis strategies employing the highly acid-labile, 2-chlorotrityl chloride resin, which permitted cleavage of the peptide from the resin with mild acid treatment and minimal desulfation. The cleavage was conducted under mild acidic conditions, and the side chains were deprotected in cold TFA solution (0 °C) in order to minimize desulfation. A two-step purification via anionic-exchange chromatography followed by RP-HPLC resulted in a highly pure and homogeneous peptide. The final degree of sulfation of the peptides was confirmed via ¹H NMR. Fig. 1 shows the comparison of ¹H NMR spectra of non-sulfated Fmoc-Tyr-OH, sulfated Fmoc-Tyr(SO₃)-OH and the sulfated SP_a peptide. The aromatic ortho and meta proton shifts of the non-sulfated Fmoc-Tyr-OH were distinctly separated at 7.04 and 6.69 ppm, respectively (Fig. 1a). After sulfation, the ortho and meta protons shift and overlap as a multiplet near 7.20 ppm (Fig. 1b). The degree of sulfation of the peptide can therefore be estimated from the integration of the aromatic protons of the sulfated portion and comparison to the total number of aromatic protons. As shown in the figure, the spectrum of SP_a (Fig. 1c) shows a high degree of sulfation with a negligible extent of desulfation. High degrees of sulfation of SP_b and SP_c were confirmed in the same manner. The data for all three peptides reproducibly indicated essentially 100% sulfation for the peptides produced and purified via these protocols (supporting information).

3.2. Binding studies via affinity liquid chromatography

Binding affinities between sulfated peptides and select heparin binding peptides, as well as VEGF165, were measured via ALC. In our studies designed to probe the binding of these HBPs to the sulfated peptides, the sulfated peptides were immobilized on an NHS-preactivated column. The unbound sulfated peptide molecules were removed and the non-reacted NHS groups were deactivated by washing the column with ethanolamine solution and sodium acetate buffers alternately, as described in Section 2. Heparin-binding peptides (or VEGF165) were then applied and allowed to bind to the sulfated column. The bound HBPs (or VEGF165) were eluted under a linear concentration gradient of NaCl in a phosphate buffer.

The concentration of NaCl required to elute bound HBPs or VEGF165 was measured; the results are shown in Fig. 2. The SP_a-modified column required 0.290 ± 0.002 , 0.240 ± 0.002 , 0.498 ± 0.002 , and 0.509 ± 0.007 M NaCl for the elution of ATIII, HIP, PF4_{ZIP}, and VEGF165, respectively. The SP_b-modified column required 0.090 ± 0.011 , 0.25 ± 0 , 0.444 ± 0.008 , and 0.283 ± 0.024 M for the elution of ATIII, HIP, PF4_{ZIP}, and VEGF165, while the SP_c-modified column required 0.172 ± 0.006 , 0.163 ± 0.007 , 0.468 ± 0.012 , and 0.222 ± 0.006 M of NaCl. The NaCl concentrations required to elute the peptides or VEGF165 from an unmodified column were also measured; the hydrolyzed column carries carboxylic acid moieties that can interact with the positively charged peptides and protein via electrostatic

interactions. The results of these control experiments indicated that only 0.156 ± 0.001 , 0.136 ± 0.003 , 0.082 ± 0.007 , and 0.148 ± 0.011 M NaCl was required for elution of the ATIII, HIP, PF4_{ZIP}, and VEGF, confirming that while some non-specific electrostatic interaction may occur during the ALC measurement, this electrostatic attraction is not the primary source of the measured binding affinity of the HBPs and VEGF for the SP-modified columns.

3.3. Binding studies via surface plasmon resonance

The binding kinetics and affinities between sulfated peptides and heparin binding peptides/VEGF165 were also assessed via surface plasmon resonance (SPR) [23,52]. In our study, each sulfated peptide was directly immobilized on the sensor chip via amine coupling, and changes in the refractive index accompanied by the binding of analytes (*i.e.* HBPs and VEGF165) were recorded. Prior to the concentration measurement, appropriate control experiments were conducted to confirm that there were no mass transfer limitations at the flow rates used in this study and that there was no non-specific binding during injection of analyte. The binding between the surface of the reference cell and analytes, which was insignificant compared to the binding of the analytes to the sulfated peptide-modified surface, was subtracted from the data in order to exclude the contribution of any non-specific electrostatic interactions.

A series of binding sensorgrams was obtained upon injection of various concentrations of HBPs to each sulfated-peptide-modified chip. The equilibrium dissociation constants, K_D , were estimated via a non-linear regression fitting to a Langmuir binding model, as previously described [69]; the results are shown graphically in Fig. 3. The SP_a showed K_D values of 9.09 ± 7.71 , 29.4 ± 19.0 , 5.27 ± 4.72 and 0.907 ± 0.118 μ M for ATIII, HIP, PF4_{ZIP}, and VEGF165, respectively. The SP_b showed K_D values of 69.0 ± 9.4 , 34.4 ± 18.3 , 10.4 ± 8.72 , 6.29 ± 1.32 μ M for ATIII, HIP, PF4_{ZIP}, and VEGF165, while the SP_c showed K_D values of 60.8 ± 2.8 , 64.4 ± 32.7 , 55.3 ± 25.2 , and 6.10 ± 1.10 μ M. The k_{on} and k_{off} values for these measurements are given in supporting information.

3.4. Isothermal titration calorimetry

The SP_a and PF4_{ZIP} interaction was shown to be of high affinity in both ALC and SPR experiments. Therefore, these binding partners would be appropriate first choices in the generation of homogeneous assembling polymers, as described in Scheme 1, and would provide useful comparisons to previously reported hydrogels assembled via interactions of LMWH and PF4_{ZIP}, mainly in assessing the role of homogeneity in hydrogel assembly and properties. Therefore, ITC experiments were carried out in order to characterize the binding of SP_a and PF4_{ZIP} in greater detail, and under the solution conditions in which assembly would be conducted. The ITC experiment provides a direct measurement of the heat associated with binding of two molecules in solution, and provides assessments of both the enthalpic and entropic contributions, as well as the stoichiometry, of the binding event [11,12].

In this study, the PF4_{ZIP} solution was titrated with SP_a solution and the evolved heat was detected. Heats of dilutions of PF4_{ZIP} solution and SP_a solution were subtracted from the data prior to analysis. The reverse titration data, *i.e.* PF4_{ZIP} titration into SP_a solution, suggested that the PF4_{ZIP} peptide exists in different association states at the different concentrations in the ITC experiment, consistent with its expected behavior (data not shown). Therefore, when small aliquots of high concentration of PF4_{ZIP} peptide were titrated into the SP_a solution, the heat of dissociation of the PF4_{ZIP} oligomer dominated the overall enthalpy change. Thus, the experiments were conducted in the order described for determination of the thermodynamic parameters in the binding event.

The molar enthalpy of binding, after subtracting the heats of dilution, is shown in Fig. 4. The fit to the data, obtained with a sequential three-site binding model (chosen on the basis of minimal χ^2 values) is shown in Fig. 4 as a solid line. The $K_{D1} = 8.09 \mu\text{M}$, $\Delta H_1 = -1320 \text{ cal/mol}$, and $\Delta S_1 = 18.9 \text{ cal/mol-K}$; $K_{D2} = 113 \mu\text{M}$, $\Delta H_2 = -1339 \text{ cal/mol}$, and $\Delta S_2 = 13.6 \text{ cal/mol-K}$; $K_{D3} = 5515 \mu\text{M}$, $\Delta H_3 = -20690 \text{ cal/mol}$, and $\Delta S_3 = -59.0 \text{ cal/mol-K}$, indicating that the first and second binding events are tight and the third sequential binding event is of very low affinity.

4. Discussion

4.1. Choice of peptide sequences

Delivery of growth factors and other proteins, utilizing the interaction between polysaccharides (*i.e.* heparin) and proteins/polypeptides has recently emerged in the field of drug delivery and tissue engineering [14,29,33,34,50,53,54,64,66–69]. Previously we have reported assembly of non-covalently cross-linked hydrogel systems, via interactions between heparin and heparin-binding peptides, for the delivery of growth factors [66–69]. The intrinsic heterogeneity of heparin, however, resulted in the variations in chemical modification and mechanical properties in such systems. In this study, the feasibility of employment of heparin mimetic sulfated peptides in these types of hydrogels was therefore investigated.

The heparin-binding peptides investigated here were selected because of their successful implementation, via interaction with heparin, in the formation of the previously reported non-covalently assembled networks. Their similar binding to sulfated peptides would permit alternative modes of assembly, and perhaps increased elastic moduli of the resulting materials, owing to increased homogeneity and increased number of binding sites within the network. The ATIII peptide employed in these studies contains the high-affinity heparin-binding domain from the plasma glycoprotein antithrombin III; binding of ATIII with heparin is essential for the anticoagulative properties of ATIII. The HIP peptide is derived from the heparin interacting protein (HIP) [36–39], which has been employed because of HIPs heparin affinity and because HIP and bFGF are reported to have distinct binding sites on heparin; a hydrogel produced via the interactions of HIP and heparin would therefore retain binding sites for bFGF. The PF4_{ZIP} peptide has also been incorporated in growth factor delivery networks in our previous investigations [69], owing to its higher heparin-binding affinity than that of the ATIII and HIP peptides. It is designed to mimic two pairs of lysine residues in the heparin-binding domain of human platelet factor 4, via the use of a GCN4-based coiled-coil motif [5].

The three sulfated peptides studied in this report contain $\text{Y}_{\text{SO}_3}\text{-X-Y}_{\text{SO}_3}$ sequences that had been previously indicated as a common binding motif in the sulfated peptides screened by Maynard and Hubbell [42]; peptides in which the X residue was aspartic acid (D) were indicated to show greatest affinities and this sequence was therefore employed in the three peptides here. The differences in the general trend of affinities observed in our studies, despite the presence of the $\text{Y}_{\text{SO}_3}\text{-D-Y}_{\text{SO}_3}$ in all three peptides, may result from differences in functional group presentation, as well as from conformational entropy considerations upon binding. In the fully extended structures (trans conformation of peptide bonds) expected for these short peptides, the sulfated residues at the n and $n + 2$ positions are presented on one face of the chain, which likely improves their accessibility to the positively charged heparin-binding domains and may also mediate their presentation at distances that approximate and better mimic the presentation of sulfate groups on heparin [44]. These considerations may account for the previously observed frequency of this sequence in sulfated peptides of higher affinity for VEGF165.

4.2. Comparisons of affinities between SPs and HBPs

The interactions between the SPs and HBPs were characterized via ALC and SPR. A high K_D value from SPR experiments corresponds to low binding affinity, which is indicated by the low concentration of salt required for elution in ALC. The results show the same general trends in affinity in both ALC and SPR, although the K_D determined for the binding between ATIII and SP_a appears unusually low. In the binding of SP_b and SP_c, ATIII, HIP, and PF4_{ZIP} showed the same trend in ALC and SPR. In addition, the HBPs generally show similar trends in affinity to the sulfated peptides as the trends in their affinities toward heparin (previously reported [66,69] and included in Figs. 2 and 3). However, the VEGF binding affinity was not consistent between ALC and SPR measurements. The VEGF had the highest affinity among four analytes in SPR while PF4_{ZIP} had the highest in ALC. This discrepancy between SPR and ALC may be due to the fact that the PF4 is known to bind to an oligosaccharide of 16 saccharides while VEGF is thought to bind to a pentasaccharide sequence. Thus, the measured affinity of PF4_{ZIP} may be lower in SPR measurements because of a lower peptide surface density in the SPR experiment versus the ALC experiment. The overall slight differences in binding trends between the SPR and ALC data may also result from the differences in the experimental assessments of binding affinity by the two experiments, as the SPR measures the kinetics of the association and dissociation events versus the bulk assessment of equilibrium binding of the ALC experiment. Slight differences in the sulfated surfaces in the two sets of measurements may also contribute to differences in measured binding.

The kinetics data from the SPR experiment are provided in supporting information. Although there was no single overriding trend, the data suggested that measured changes in affinity resulted mainly from changes in k_{on} , although changes in k_{off} also contributed in select cases. The concentration of immobilized peptides on the surface may affect the k_{on} and k_{off} . In our study, the surface concentrations of peptides were within the range of recommended immobilization values; the permanent increases in RU after immobilization of each SP ligand were 108.0 for SP_a, 58.4 for SP_b, and 45.4 for SP_c, which correspond to 108, 58.4, and 45.4 pg/mm², respectively. The measured k_{off} values for analyte binding to all peptide surfaces were similar, suggesting that variations in surface concentration in this range do not substantially alter dissociation. The k_{off} values for VEGF were only two to five times slower than those of monomeric analytes, further suggesting a minor role, if any, for bivalent binding of the VEGF under these conditions. The k_{on} values for analyte binding to SP_b and SP_c were not higher than those for binding to SP_a, which suggests that excess carboxylates present on the chip surface do not cause significant non-specific binding.

Among the three sulfated peptides, the SP_a peptide showed, in general, the highest affinities toward the HBPs, despite the fact that it is the shortest peptide with the fewest sulfated residues. In comparisons of the three SP peptides, the fewer number of sulfated residues in the SP_a (two) may reduce steric crowding of these important residues versus that experienced by the sulfated residues in the SP_b and SP_c peptides (four and three, respectively). The smaller size of the SP_a may also result in a lower loss in conformational entropy upon binding and contribute to its improved binding relative to the other SPs. The carboxylate and hydroxyl groups in the SPs should also contribute to the binding event, given the known importance of these functional groups in modulating binding by heparin via electrostatic and hydrogen bonding interactions [7,15,18,48,56]; indeed, the reduced affinity of SP_c (particularly as indicated via SPR) toward the HBPs may in part result from its fewer carboxylate and hydroxyl side chains relative to SP_a and SP_b, as well as from the presentation of only three of the four sulfate groups on the peptide.

Another observation from these investigations is that the PF4_{ZIP} peptide had the highest binding affinity of the HBPs toward all sulfated peptides. This result is consistent with

results from the heparin affinity chromatography and SPR experiments in our previous reports (included in Figs. 2 and 3) [66,69], and may result from differences in the presentation of positive charges on these HBPs. In general, the ATIII and HIP peptides show similar affinities for the SPs (although this is not perfectly consistent in the data), which may be expected given their similar size and charge. It has been suggested that the heparin-binding domains in ATIII and in HIP adopt a helical conformation that arranges the positive sidechains along the face of the helix for optimized heparin binding [19,39,60,61]. However, the sulfated peptide molecules in our study do not drive such a conformational change (CD data not shown), and therefore the binding of the short HBPs to the SPs may be reduced relative to that of PF4_{ZIP} owing to the suboptimal presentation of the basic residues. In contrast, the PF4_{ZIP} peptide exists as coiled-coil helical dimer under our experimental conditions (concentrations greater than 3.5 μ M) [69]. SPR experiments with PF4_{ZIP} as the analyte were done in the concentration range of 5–200 μ M, as below 5 μ M, the PF4_{ZIP} did not bind measurably to the chip surface, suggesting that the monomeric form of the PF4_{ZIP} does not have significant affinity for the SP peptides. In the dimer, the basic residues are accessible, having been positioned to mimic the presentation of lysines in human platelet factor 4 [5]. The accessibility of the lysines for binding between the SPs and the helical PF4_{ZIP} may therefore minimize any entropic costs of reorganization [40], resulting in a more favorable free energy of binding. Additionally, the HBPs generally have lower affinity for the SPs than they do for heparin, which likely results from a combination of the lack of conformational change for optimized presentation of basic residues (for ATIII and HIP) and from the smaller size of the SPs than the reported binding regions in heparin for ATIII, HIP, and PF4 [26,39,69].

Interestingly, the ITC studies of the binding of PF4_{ZIP} with SP_a suggested the presence of two binding events, which was not indicated in the SPR data. The ITC experiment was done at a PF4_{ZIP} concentration of 0.2 mM; this greater concentration was necessary to generate a reliable binding isotherm. This concentration of PF4_{ZIP} was sufficiently high that the coiled-coil peptide exists as a dimer [69]; four lysine sidechains, which play an important role in heparin binding, are displayed on the two chains [5]. Given that PF4 is reported to bind to an oligosaccharide unit in heparin that contains 16–20 saccharides [26], it is possible that the PF4_{ZIP} heparin-binding domain is sufficiently large to accommodate more than one of the short SP_a, and the two pairs of Lys may therefore provide two binding sites for SP_a peptides on the PF4_{ZIP} peptide. The appropriateness of these determined values is suggested by the fact that the calculated first dissociation constant, K_{D1} , was in the same order of magnitude as the K_D value from the SPR experiment (5.27 μ M), suggesting that the SPR measurement captures the highest affinity binding event. The lack of a measured K_{D2} in the SPR experiments most likely results from the fact that the SPR experiments were conducted with the monovalent SP_a on the chip surface, thus only the first binding event may be detected.

4.3. Comparisons of affinities between SPs and VEGF

The trends in the binding of SPs to VEGF, as determined via SPR, also followed the ALC results and are consistent with the previously reported SPR results for the VEGF–SP_a interaction. The K_D of SP_a and VEGF165 (0.907 μ M) measured here is in good agreement with the K_D value (3.1 μ M) reported by Maynard and Hubbell for binding of the peptide SY_{SO3}DY_{SO3}G with VEGF165 [42], as well as with the estimated K_D for the binding of heparin and VEGF165 (5.5 μ M) based on affinity chromatography [16,32,58]. The slight difference in the values may be caused by differences in the experimental conditions or by the impact of the longer glycine linker in the peptide here. In the previously reported experiments, biotinylated VEGF165 was immobilized on an SPR chip through non-covalent interaction, and the sulfated peptide was injected as an analyte, in contrast to our

experiments in which the VEGF was injected as an analyte across a sulfated peptide-modified surface.

Similar structural considerations as those mentioned above may play a role in the higher affinity of the SP_a for VEGF165, relative to those measured for the other sulfated peptides. The SP_a has a sufficient number of amino acids to span the shortest positively charged region of the heparin-binding domain in VEGF165 [42], and its smaller size may allow unhindered approach to the binding domain in VEGF165, in comparison to the SP_b or SP_c which may be hindered due to unfavorable repulsive interactions near the heparin-binding site in VEGF. For example, Fairbrother et al. have suggested that the heparin-binding domain of VEGF is likely to include Arg13, Arg14, Lys15, Lys30, Arg35, Arg39, and Arg46, with potential contributions from Lys52, Arg54, Arg55 (supporting information). If the SPs also bind at this domain (in the region of highest positive charge density which includes Arg13, Arg14, Arg46 and Arg49), the improvement in binding affinity of the smaller SP_a relative to the longer peptides, SP_b and SP_c, may result from unfavorable enthalpic interactions between the SP_b and SP_c and the neighboring Glu12 (repulsive interaction) and His30 (steric interference). These interactions may not have a large impact on the binding of heparin owing to the greater number of energetically favorable contacts between heparin and the entire heparin-binding domain in VEGF. While detailed experiments to confirm the preceding speculations are outside the scope of this initial report, the data nevertheless indicate that distinct structural differences in the SPs result in measurable differences in binding affinities for both VEGF165 and HBPs.

4.4. Summary

Despite the slight differences in the exact trends in the ALC and SPR experiments, in general, the PF4_{ZIP} shows the highest affinity toward sulfated peptides, and the SP_a shows the strongest affinity to the HBPs. These data for the sulfated peptides provide additional support for Maynard and Hubbell's suggestion that the Y_{SO3}-X-Y_{SO3} motif, where X is an arbitrary amino acid, is important in the binding to VEGF165. Although the SP_b and SP_c carry the same Y_{SO3}-X-Y_{SO3} motif and double the number of sulfate groups, their lower affinity to all HBPs and VEGF may result from steric interference, unfavorable repulsion, or unfavorable entropic considerations due to their longer lengths. Incorporation of the SP_a as a surrogate for heterogeneous heparin in hydrogels is therefore promising for improving hydrogel homogeneity and protein distribution in the gel. In addition, preliminary data (not shown) indicate that the SP_a does not have any significant anticoagulant effects, while retaining its heparin-like binding to growth factors. Thus the application of SP_a in biomaterials applications would offer the advantages of its small size, good binding to multiple partners, and a low potential for unwanted anticoagulative effects.

The useful binding affinities demonstrated by the SPs toward multiple heparin-binding partners are consistent with the fact that short sequences in heparin are sufficient to bind ATIII and HIP [39,69] and suggest the general utility of SP_a and potentially other similarly short sulfated peptides. Interestingly, the SP_b peptide is suggested to show the best discrimination in the relative binding between the various HBPs and VEGF165, with the same trends as those for the binding of these HBPs to heparin. Although the exact origins of the differences in binding between these molecules are not yet known, these data illustrate that short sulfated peptides may be capable of showing differential binding to multiple binding partners. While it is true that none of the SPs showed binding to HBPs as strong as those between the HBPs and heparin, affinities are sufficiently high to anticipate successful use of these peptides in materials assembly. The affinities observed for SP_b and SP_c, although not higher than that of SP_a, may also have utility in controlled delivery of proteins, and sulfated peptides of higher affinity could be realized via an expanded set of combinatorial libraries and screening to provide information about useful motifs in peptide

design. Specifically, owing to the general utility of solid-phase peptide synthesis and combinatorial library methods, prospects appear promising for the identification of multiple sulfated peptide sequences with high-affinity and selective binding to specific growth factors, which will permit manipulation of individual delivery rates.

5. Conclusions

In the present work we have investigated the binding affinities between three sulfated peptides, including the previously reported VEGF165-binding peptide SP_a, and heparin-binding peptides. The shortest peptide, SP_a (SY_{SO3}DY_{SO3}), had the highest binding affinities to heparin-binding peptides and VEGF165 in spite of its lowest number of sulfate groups per molecule. Of the heparin-binding peptides, the PF4_{ZIP} peptide had highest binding affinities to all sulfated peptides, which was consistent with its highest heparin binding affinity. The SP_a and PF4_{ZIP} are predicted to successfully mediate hydrogel formation between four-arm star PEGs modified with the peptides, as the peptides exhibit a similar binding affinity as that reported for binding partners that we have shown support hydrogel formation (*e.g.*, LWMH with HIP or ATIII). A four-arm star PEG-SP_a should also form hydrogels in presence of dimeric VEGF165 and perhaps other dimeric, heparin-binding growth factors. Owing to the increased homogeneity of the SP_a relative to the structurally and chemically heterogeneous heparin or LMWH, it is expected that hydrogels formed will be more homogeneous with improved mechanical properties that can be more easily attributed to the SP_a and PF4_{ZIP} or VEGF165 interaction. The SPs studied all showed interactions of various affinities to the different HBPs, indicating their potential utility in binding multiple partners. The identification of multiple sulfated peptides with distinct binding affinities for select growth factors is also suggested as a potential and promising route in the design of hydrogels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Non-covalently assembled hydrogels for growth factor delivery. PEG stands for poly(ethylene glycol); LMWH for low molecular weight heparin; HBP for heparin-binding peptide; GF for growth factor.

Fig. 1. The ^1H NMR (400 MHz) spectra of non-sulfated and sulfated tyrosines, and the sulfated tyrosine-containing peptide. (a) Fmoc-Tyr(OH)-OH: $\delta = 6.69$ (2H, tyrosyl *meta*, d), 7.04 (2H, tyrosyl *ortho*, d). (b) Fmoc-Tyr(OSO₃)-OH: $\delta = 7.20$ (4H, sulfated tyrosyl aromatic, m). (c) SP_a peptide: $\delta = 7.20$ ppm (8H, sulfated tyrosyl aromatic, m).

Fig. 2.

The affinity chromatography of sulfated peptides/heparin and heparin-binding peptides/VEGF165. The NaCl concentration required to elute the bound analytes from sulfated peptidyl or heparin columns was recorded as an indicator of binding affinity. The error bars were derived from standard deviations of the average of three or more samples, except for VEGF165. The chromatography data of heparin column and ATIII, HIP, and PF4 were taken from previous reports [66,69].

Fig. 3.

The measured dissociation constants of sulfated peptides/heparin and their binding partners measured via SPR. A series of concentrations of ATIII, HIP, PF4, and VEGF165 analytes were injected to the sulfated peptides/heparin immobilized sensor chip. The binding responses with various concentrations of analytes were fit to a Langmuir model in order to give the binding constants. The insets show the K_D of SP_a and heparin in log-scale. The error bars are derived from the standard deviations of multiple samples. The data of heparin binding with ATIII, HIP, and PF4 were taken from previous reports [66,69].

Fig. 4.

Binding isotherm of SP_a interacting with PF₄ZIP. The total heat of each injection was integrated and plotted (■). The solid line was the fit of the experimental data which was used to calculate K_D , ΔH , and ΔS .

Table 1

Sequences of sulfated peptides

Peptides	Sequences ^a	MW ^b
SP _a ^c	NH ₂ -GGGG- SY _{SO3} DY _{SO3} GGGG-OH	1163.0
SP _b	NH ₂ -GGGG- SY _{SO3} DY _{SO3} SY _{SO3} DY _{SO3} G-OH	1680.3
SP _c	NH ₂ -GGGG- Y _{SO3} Y _{SO3} GGY _{SO3} DY _{SO3} G-OH	1505.4

^aThe sequences in bold font are the purported active binding sites.

^bThe molecular weights are calculated values. Experimental estimates of mass were impossible due to substantial desulfation of the peptides under all mass spectra conditions investigated.

^cThe sequence in bold was previously reported to bind to VEGF165 [42].