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Oligo(tyrosine sulfate)s as heparin pentasaccharide mimic: Evaluation by surface noncovalent affinity mass spectrometry

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Abstract—Since the discovery of anti-HIV activity in oligo(tyrosine sulfate)s in our laboratory, we have been interested in their potential as heparin pentasaccharide mimics. In this study, we investigated their interactions with synthetic heparin-binding peptides, derived from human antithrombin III (hAT III) and heparin-interacting protein (HIP), using surface noncovalent affinity mass spectrometry. We compared binding affinities to those heparin-binding peptides between oligo(tyrosine sulfate)s and several known sulfated compounds and found that oligo(tyrosine sulfate)s bind to hAT III (123–139) more strongly than a heparin-derived hexasaccharide dp6. Moreover, we found longer oligo(tyrosine sulfate) has higher binding affinity to hAT III (123–139).

1. Introduction

Members of the heparin/heparan sulfate-like glycosaminoglycan (HLGAG) family play important roles in the regulation of many biological processes including blood coagulation, cell growth and differentiation, viral invasion, and cancer metastasis.[1] Because HLGAGs contain many sulfate and carboxyl groups, their interactions with basic amino acids in various binding proteins must be electrostatic. However, these interactions are more complex and are dependent on the sequence and position of sulfate groups.[2] A recent study using well-defined chondroitin sulfate oligosaccharides demonstrated that specific sulfation motifs function as molecular recognition elements (a 'sulfation code'[3]) for growth factors and that these modulate neuronal growth.[4] To understand the sulfation specificity in greater detail, it is necessary to synthesize many analogs with sulfate groups at precise positions along the carbohydrate backbone. Methods have been developed recently to synthesize acidic oligosaccharides, including the antithrombin-binding pentasaccharide, arixtra, and its analogs with longer-lasting activity, such as idraparinux, for clinical use.[5] However, these methods require many steps to prepare a single oligosaccharide.[6] In contrast to oligosaccharide synthesis, methods for peptide synthesis are much simpler and are well established. This study presents an alternative approach, in which homooligomers of tyrosine instead of carbohydrates are used as the backbone.[7]

We recently reported on strong heparin-like anti-human immunodeficiency virus (HIV) activity in fully N- and side-chain O-sulfated homooligomers of tyrosine, which we obtained by simultaneous sulfation and oligomerization of tyrosine.[8,9] Multiply sulfated peptides, which bind to vascular endothelial growth factor (VEGF)[10] and acidic fibroblast growth factor 1 (FGF-1),[11] were also found in combinatorial chemical studies. Therefore, we are interested in the potential use of oligo(tyrosine sulfate)s (**1**, Fig. 1) as an antithrombin-binding heparin

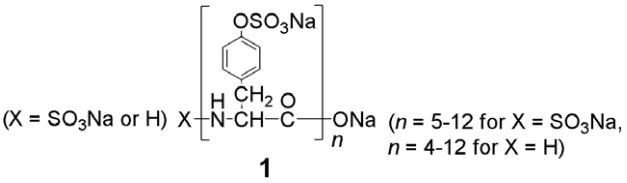

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Figure 1. Structures of oligo(tyrosine sulfate)s.

Keywords: Sulfated peptides; Heparin-binding peptides; Heparin mimics; MALDI–TOF–MS.
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pentasaccharide mimic. We investigated the possible binding of oligo(tyrosine sulfate)s to heparin-binding peptides using surface noncovalent affinity mass spectrometry (SNA–MS), developed by Kaiser et al.[12] to isolate specific HLGAG oligosaccharide binders.

2. Results and discussion

2.1. Affinity chromatography

Heparin binds with high affinity to the plasma protein antithrombin (AT III), and this binding accelerates the inhibitory activity of AT III toward factor Xa. Of the three human antithrombin III (hAT III)-derived peptides [hAT III (39–54), hAT III (123–139), and hAT III (286–301)], only hAT III (123–139) inhibits hAT III–heparin binding.[13] A putative heparin-binding motif of heparin interacting protein (HIP), HIP peptide, competes with AT III for binding to heparin in blood plasma assays.[14] These four peptides can be retained on a commercially available heparin-immobilized column (Table 1).

We used oligo(tyrosine sulfate)-immobilized affinity chromatography to evaluate the relative binding ability of oligo(tyrosine sulfate)s toward these heparin-binding peptides. N-sulfated and N-nonsulfated nonamers, NaO_3S-[Tyr(SO_3Na)]$_9$-OH and H-[Tyr(SO_3Na)]$_9$-OH, were selected as models and immobilized to Affi-Gel 10. Each heparin-binding peptide was then injected onto the columns and eluted in the presence of increasing NaCl concentrations.

As a result, hAT III (123–139) and the HIP peptide were bound to both of the oligo(tyrosine sulfate) nonamer columns (Table 1 and Fig. 2). In contrast, no hAT III (39–54) or hAT III (286–301) was retained in the columns. From these results, we decided to use hAT III (123–139) and the HIP peptide in further investigations.

2.2. Surface noncovalent affinity mass spectrometry

Affinity mass spectrometry is a technique that combines affinity purification and mass-spectrometric analyses such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS)[15] and surface-enhanced laser desorption/ionization mass spectrometry (SELDI–MS).[16] In this approach, a binding molecule, such as an antibody, lectin or receptor, is covalently attached to the surface of a MALDI probe. This permits the analyte of interest to be selectively captured and concentrated on the probe surface before MS analysis. This technique is also useful for investigating the relative affinities in protein–protein interactions.

Table 1. Retention time of various heparin-binding peptides on oligo(tyrosine sulfate)- or heparin-immobilized affinity columns[a]

Column	Retention time (min)			
	hAT III (39–54)	hAT III (123–139)	hAT III (286–301)	HIP peptide
HiTrap Heparin HP	29.3	54.7	29.9	44.0
NaO_3S-[Tyr(SO_3Na)]$_9$-O-AffiGel 10	1.8	37.5	4.0	39.2
H-[Tyr(SO_3Na)]$_9$-O-AffiGel 10	1.8	34.7	1.2	34.7

[a] Elution: after sample loading, columns were washed with 20 mM Tris–HCl buffer (pH 7.6) for 30 min and peptides were eluted with a linear gradient from 0 to 2 M NaCl in a 20 mM Tris–HCl buffer (pH 7.6) over 30 min; flow rate 0.5 mL/min. All the experiments were performed at ambient temperature.

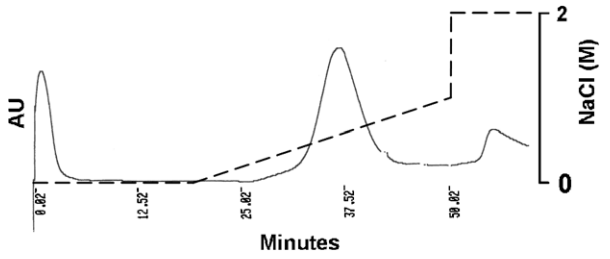

Figure 2. NaO_3S-[Tyr(SO_3Na)]$_9$-immobilized affinity chromatography of hAT III (123–139). Suspension of hAT III (123–139) in 20 mM Tris buffer (pH 7.6) was loaded onto the column equilibrated in 20 mM Tris buffer (pH 7.6). The peptide was eluted with a gradient of 0–2 M NaCl/20 mM Tris buffer, pH 7.6 (dashed line), at a flow rate of 0.5 mL/min, detection at 220 nm.

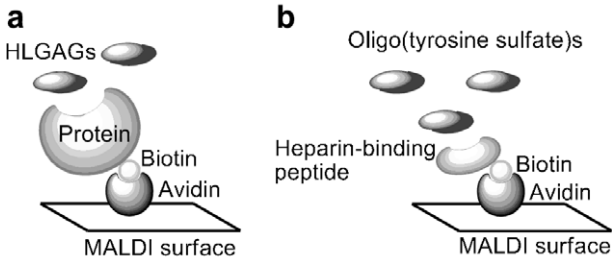

Figure 3. Schematic of surface noncovalent affinity mass spectrometry. (a) The method by Kaiser et al. (b) The method used in this study.

Using SNA–MS, Kaiser et al. succeeded in isolating specific HLGAG oligosaccharide binders using AT III that was biotinylated and immobilized to the MALDI surface by avidin[12] (Fig. 3a). In this method, a transparency film, to which avidin was adsorbed and immobilized noncovalently, was used as the MALDI surface.

To apply SNA–MS to sulfated peptides, which are easily desulfated during MALDI–MS measurements, an additional technique is necessary. We recently developed a highly selective MALDI–MS method to detect sulfated peptides using various kinds of onium salts as the co-matrix.[17,18] As with oligo(tyrosine sulfate)s, quaternary phosphonium (Q^P) salts were the most convenient for giving the strongest molecular ions in the form of $[M(Q^P_n) + Q^P]^+$, where n corresponds to the number of sulfate groups, and typical ladder fragmentation patterns correspond to the loss of $SO_3{\cdot}Q^P$.[17] Accordingly, we applied SNA–MS to sulfated peptides.

2.3. Sample preparation

Although the method of Kaiser et al. used transparency film to immobilize avidin, we found that avidin could be

Table 2. A list of oligo(tyrosine sulfate)s and sulfated compounds used in this study

Compound	Molecular formula	Molecular weight	t_R[a] (min)	Number of sulfate group (n)	Molecular mass in the form of $[M(TBP_n)+TBP]^+$
N-Sulfated 12-mer	NaO_3S-[Tyr(SO_3Na)]$_{12}$-ONa	3324.68	26.8	13	6635.92
N-Sulfated 11-mer	NaO_3S-[Tyr(SO_3Na)]$_{11}$-ONa	3059.46	25.5	12	6134.26
N-Sulfated 10-mer	NaO_3S-[Tyr(SO_3Na)]$_{10}$-ONa	2794.54	24.7	11	5632.59
N-Sulfated 9-mer	NaO_3S-[Tyr(SO_3Na)]$_9$-ONa	2529.02	23.2	10	5130.93
N-Sulfated 8-mer	NaO_3S-[Tyr(SO_3Na)]$_8$-ONa	2263.80	21.2	9	4629.26
N-Sulfated 7-mer	NaO_3S-[Tyr(SO_3Na)]$_7$-ONa	1998.58	17.8	8	4127.60
N-Sulfated 6-mer	NaO_3S-[Tyr(SO_3Na)]$_6$-ONa	1733.36	16.5	7	3625.93
N-Sulfated 5-mer	NaO_3S-[Tyr(SO_3Na)]$_5$-ONa	1468.14	12.6	6	3124.26
N-Nonsulfated 12-mer	H-[Tyr(SO_3Na)]$_{12}$-ONa	3222.64	25.4	12	6297.43
N-Nonsulfated 11-mer	H-[Tyr(SO_3Na)]$_{11}$-ONa	2957.42	22.8	11	5795.77
N-Nonsulfated 10-mer	H-[Tyr(SO_3Na)]$_{10}$-ONa	2692.20	20.1	10	5294.10
N-Nonsulfated 9-mer	H-[Tyr(SO_3Na)]$_9$-ONa	2426.98	19.1	9	4792.44
N-Nonsulfated 8-mer	H-[Tyr(SO_3Na)]$_8$-ONa	2161.76	16.4	8	4290.77
N-Nonsulfated 7-mer	H-[Tyr(SO_3Na)]$_7$-ONa	1896.54	12.0	7	3789.11
N-Nonsulfated 6-mer	H-[Tyr(SO_3Na)]$_6$-ONa	1631.32	8.8	6	3287.44
N-Nonsulfated 5-mer	H-[Tyr(SO_3Na)]$_5$-ONa	1366.10	5.5	5	2785.78
N-Nonsulfated 4-mer	H-[Tyr(SO_3Na)]$_4$-ONa	1100.88	16.9[b]	4	2284.12
$VEGF_{165}$-binding peptide	Ser-Tyr(SO_3H)-Asp-Tyr(SO_3H)-Gly	763.71		2	1540.00 1798.42[c] 2056.85[c]
Hexasaccharide dp6	$\Delta U_{2S}H_{NS,6S}I_{2S}H_{NS,6S}I_{2S}H_{NS,6S}$	1996.19		9	4317.68

[a] Retention time in high-performance liquid chromatography (conditions: column, 5 μm Symmetry C18 (3.9 × 150 mm); linear gradient elution, CH_3CN/0.05 M aq $^nBu_4NHSO_4$ 55:45 to 85:15 over 30 min; flow rate 1.0 mL/min).

[b] Conditions: column, 5 μm Symmetry C18 (3.9 × 150 mm); eluent, CH_3CN/0.05 M aq $^nBu_4NHSO_4$ 35:65 for 5 min, linear gradient elution, 35:65 to 60:40 over 25 min; flow rate 1.0 mL/min).

[c] Molecular mass that carboxyl groups of C-terminus and/or aspartic acid side-chain also formed TBP salts.

immobilized directly onto the metal surface of the Opti-TOF plate (Applied Biosystems, Foster City, CA, USA). Next, we immobilized N-terminal biotinylated heparin-binding peptides on the MALDI surface through avidin (Fig. 3b). We added a solution of analytes and removed nonspecific binders with a low-salt (0.1 M Na_2SO_4) wash. MALDI samples were prepared by addition of 2,4,6-trihydroxyacetophenone (THAP) as the matrix and tetrabutylphosphonium bromide (TBP·Br) as the co-matrix.[17]

At first, we tested the binding of each oligo(tyrosine sulfate) to hAT III (123–139) using N-sulfated oligomers NaO_3S-[Tyr(SO_3Na)]$_n$-ONa (n = 5–12) and N-nonsulfated oligomers H-[Tyr(SO_3Na)]$_n$-ONa (n = 4–12). Their molecular weights and the masses of their molecular ions in the form of their TBP salts are listed in Table 2. When samples were prepared without the 0.1 M Na_2SO_4 wash and analyzed in the positive mode, all oligo(tyrosine sulfate)s could be detected as their TBP salts. The signal of biotinylated hAT III (123–139) (m/z = 2252) was also observed, but no signal of the oligo(tyrosine sulfate)–hAT III (123–139) complex was observed (data not shown). This suggests that addition of THAP and TBP·Br causes dissociation of the complexes. In samples treated with the 0.1 M Na_2SO_4 wash, N-nonsulfated oligomers shorter than the hexamer (n = 6) were not retained. These results show that the specific binding affinity of oligo(tyrosine sulfate)s to hAT III (123–139) can be observed and that nonspecific binders can be removed with a low-salt wash. This also shows the effectiveness of hAT III (123–139) immobilized to the surface through an N-terminal biotin.

2.4. Comparison of the relative binding affinity to hAT III (123–139) between oligo(tyrosine sulfate)s and various kinds of sulfated compounds

To compare the binding affinities of oligo(tyrosine sulfate)s with those of known sulfated compounds, we performed competitive binding experiments. A commercially available heparin-derived hexasaccharide dp6 ($\Delta U_{2S}H_{NS,6S}I_{2S}H_{NS,6S}I_{2S}H_{NS,6S}$) was selected for this purpose. Our previous study showed that sulfated oligosaccharides can be detected as their onium salts when onium salts are used as the co-matrix in MALDI–MS.[19] Equimolar mixtures of each oligo(tyrosine sulfate) and the hexasaccharide were analyzed using the same method described above with or without the 0.1 M Na_2SO_4 wash. In this analysis, large excess amounts of each oligo(tyrosine sulfate) and the hexasaccharide were applied to the immobilized hAT III (123–139). When a mixture of N-nonsulfated tetramer (n = 4) and the hexasaccharide was analyzed without the 0.1 M Na_2SO_4 wash, only the hexasaccharide was retained (Fig. 4a). As with oligomers from pentamer to dodecamer, the oligomers were retained and detected selectively (Fig. 4b). With the 0.1 M Na_2SO_4 wash, no signal of either compound was observed in the analysis of mixtures of N-nonsulfated short oligomers (n = 4 or 5) and the hexasaccharide (Fig. 4c). In the cases of oligomers from hexamer to dodecamer, the oligomers were detected selectively (Fig. 4d). These results suggest that the binding affinity to hAT III (123–139) is stronger for oligo(tyrosine sulfate)s than for the hexasaccharide and is dependent on chain length.

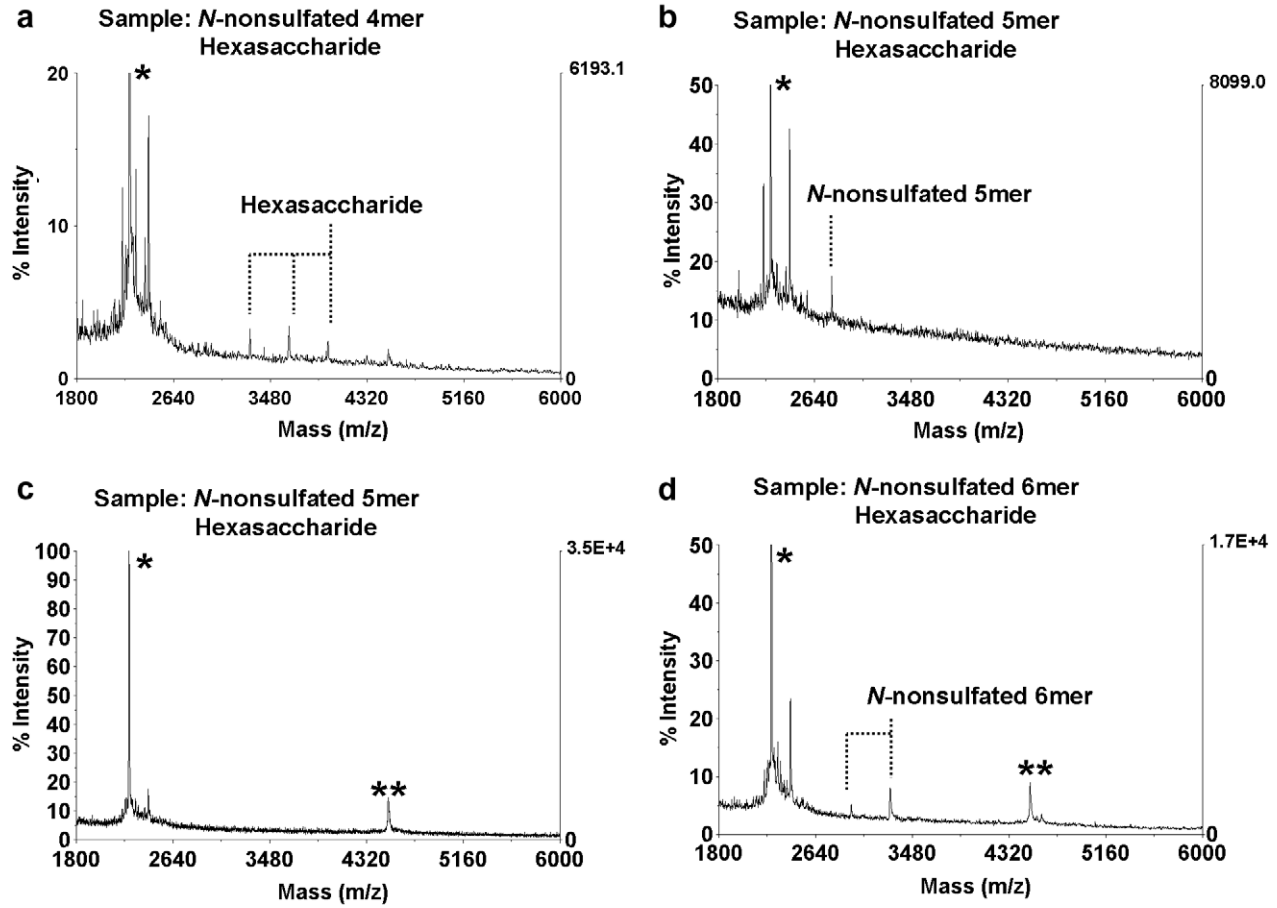

Figure 4. SNA–MS of mixtures of each oligo(tyrosine sulfate) and the hexasaccharide using hAT III (123–139). (a) H-[Tyr(SO_3Na)]$_4$-ONa and the hexasaccharide without a 0.1 M Na_2SO_4 wash. (b) H-[Tyr(SO_3Na)]$_5$-ONa and the hexasaccharide without a 0.1 M Na_2SO_4 wash. (c) H-[Tyr(SO_3Na)]$_5$-ONa and the hexasaccharide with a 0.1 M Na_2SO_4 wash. (d) H-[Tyr(SO_3Na)]$_6$-ONa and the hexasaccharide with a 0.1 M Na_2SO_4 wash. * and ** indicate a monomer and a dimer of molecular ions of hAT III (123–139).

The next target was a disulfated pentapeptide, SY-(SO_3)DY(SO_3)G, where Y(SO_3) denotes tyrosine *O*-sulfate. This short peptide was identified as the strongest binder to $VEGF_{165}$ among the library of tetrapeptides with an additional C-terminal glycine.[10] Its binding affinity to $VEGF_{165}$ as determined by surface plasmon resonance spectroscopy was 100 times stronger than to suramin, which is known to exert an antiangiogenic action that is mediated by inhibition of VEGF function.[20] Equimolar mixtures of each oligo(tyrosine sulfate) and the disulfated pentapeptide were analyzed with or without the 0.1 M Na_2SO_4 wash. Without the low-salt wash, all oligo(tyrosine sulfate)s were detected selectively, as exemplified in the N-nonsulfated tetramer (Fig. 5a). However, neither the N-nonsulfated short oligomers ($n = 4$ or 5) nor the pentapeptide was retained after the low-salt wash (Fig. 5b). In the cases of the N-sulfated oligomers, the N-nonsulfated hexamer ($n = 6$), and the longer oligomers, mixed with the pentapeptide, the oligo(tyrosine sulfate)s were retained selectively and no signal of the pentapeptide was observed (Fig. 5c). Thus, the binding affinity of oligo(tyrosine sulfate)s to hAT III (123–139) was much higher than that exhibited by the $VEGF_{165}$-binding pentapeptide.

2.5. Comparison of the relative binding affinity to hAT III (123–139) between oligo(tyrosine sulfate)s

N-Sulfate group plays an important role in the antithrombin binding by the pentasaccharide of heparin.[21] Recently methods to synthesize carbohydrates have been developed,[6] however, introduction of *N*-sulfate groups is still troublesome. Therefore, *N*-sulfate group has been substituted by *O*-sulfate and other groups for ease in synthesizing heparin mimics, as in the syntheses of chondroitin sulfates[4] and idraparinux[5] mentioned above. Chemokine–glycosaminoglycan interactions[22] suggest the importance of N-sulfation and chain length. We next investigated the dependence of binding affinity on chain length and *N*-sulfate group in the oligo(tyrosine sulfate)–hAT III (123–139) interaction.

In an equimolar mixture of two oligo(tyrosine sulfate)s, the oligo(tyrosine sulfate) with higher affinity could be detected selectively. For example, in a mixture of two N-nonsulfated oligomers with different chain lengths, the longer oligomer was bound and detected selectively (Fig. 6a). The same chain-length dependence was observed in the analysis of mixtures of two N-sulfated oligomers (Fig. 6b), but in some cases both oligomers were

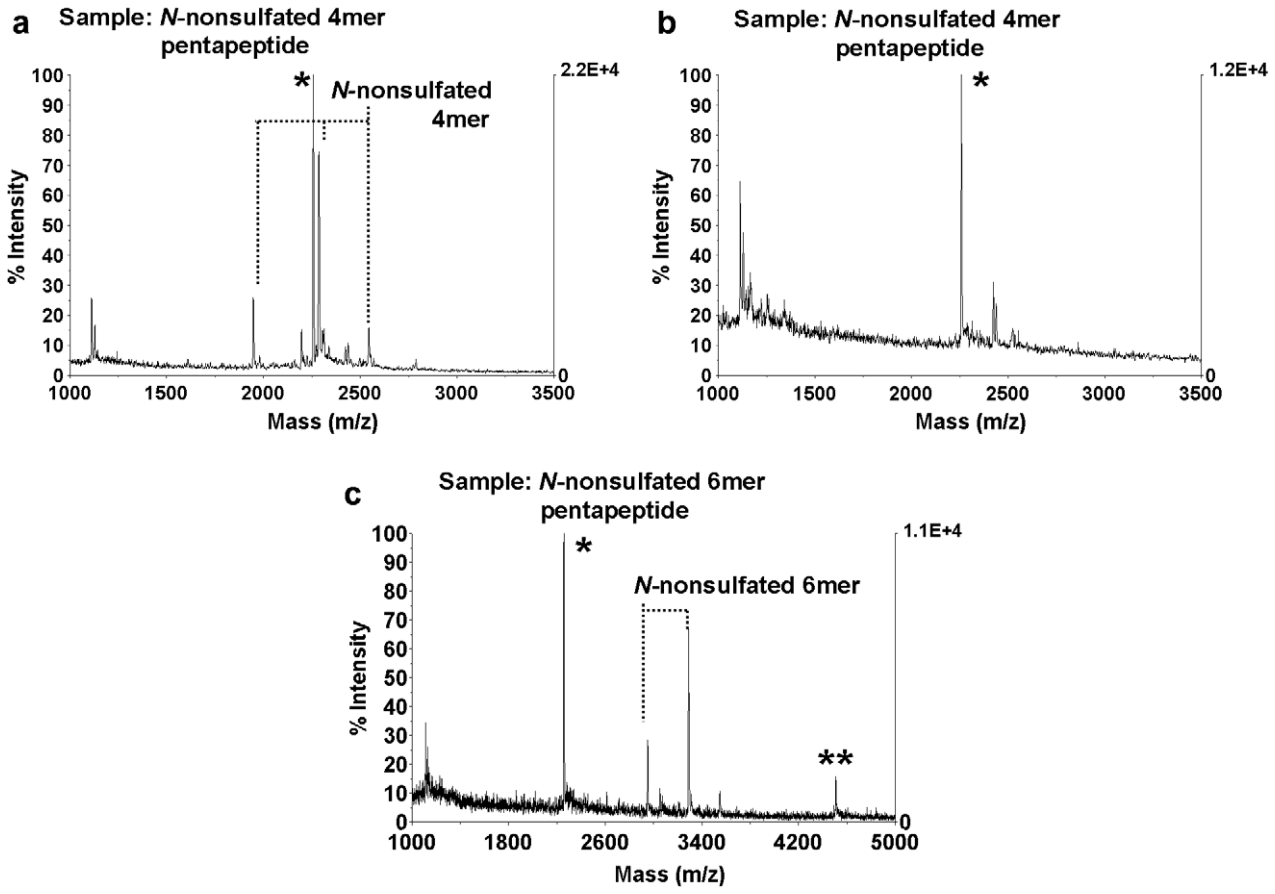

Figure 5. SNA–MS of mixtures of each oligo(tyrosine sulfate)s and the pentapeptide [SY(SO_3)DY(SO_3)G] using hAT III (123–139). (a) H-[Tyr(SO_3Na)]$_4$-ONa and the pentapeptide without a 0.1 M Na_2SO_4 wash. (b) H-[Tyr(SO_3Na)]$_4$-ONa and the pentapeptide with a 0.1 M Na_2SO_4 wash. (c) H-[Tyr(SO_3Na)]$_6$-ONa and the pentapeptide with a 0.1 M Na_2SO_4 wash. * and ** indicate a monomer and a dimer of molecular ions of hAT III (123–139).

detected. These results suggest that the difference in binding affinity between the two N-sulfated oligomers is relatively small. Thus, we succeeded in observing chain-length dependence in the noncovalent complex formation of oligo(tyrosine sulfate)s with hAT III (123–139).

To evaluate the effects of N-sulfation and the number of sulfate groups on the binding affinity to hAT III (123–139), we compared N-sulfated oligo(tyrosine sulfate)s with N-nonsulfated oligomers containing the same number of sulfate groups or one more. For example, in the analysis of a mixture of N-sulfated oligomer with tyrosine residue number n, NaO_3S-[Tyr(SO_3Na)]$_n$-ONa (n = 8 or 9), and N-nonsulfated decamer, H-[Tyr(SO_3Na)]$_{10}$-ONa, signals of both oligomers were observed (Fig. 6c and d). These results suggest that N-sulfation is important in enhancing the binding affinity of oligo(tyrosine sulfate)s to hAT III (123–139).

2.6. Comparison of the relative binding affinity to the HIP peptide between oligo(tyrosine sulfate)s and various kinds of sulfated compounds

To further investigate the heparin-like affinity of oligo(tyrosine sulfate)s, the SNA–MS method established with hAT III (123–139) was applied to the HIP peptide. Analytical samples were prepared by the same method described above using N-terminal biotinylated HIP peptide. Equimolar mixtures of each oligo(tyrosine sulfate) and the hexasaccharide were analyzed with the 0.1 M Na_2SO_4 wash and produced selective signals of oligo(tyrosine sulfate)s from hexamer to dodecamer (Fig. 7). In mixtures of N-nonsulfated short oligomers (n = 4 or 5) and the hexasaccharide, neither was detected after the 0.1 M Na_2SO_4 wash (data not shown). Without the 0.1 M Na_2SO_4 wash, each oligomer was detected (data not shown). These results suggest that the binding affinity of oligo(tyrosine sulfate)s to HIP peptide was stronger than that of the hexasaccharide and as strong as that to hAT III (123–139).

Subsequently, equimolar mixtures of each oligo(tyrosine sulfate) and the $VEGF_{165}$-binding sulfated pentapeptide, SY(SO_3)DY(SO_3)G, were analyzed with or without the 0.1 M Na_2SO_4 wash. In the N-nonsulfated short tetramer (n = 4), neither was retained after the 0.1 M Na_2SO_4 wash (Fig. 8a). However, without the low-salt wash, signals of both compounds were observed (Fig. 8b). This suggests that the affinity of the pentapeptide for the HIP peptide is as weak as that of the N-nonsulfated tetramer. In the cases of N-sulfated oligomers,

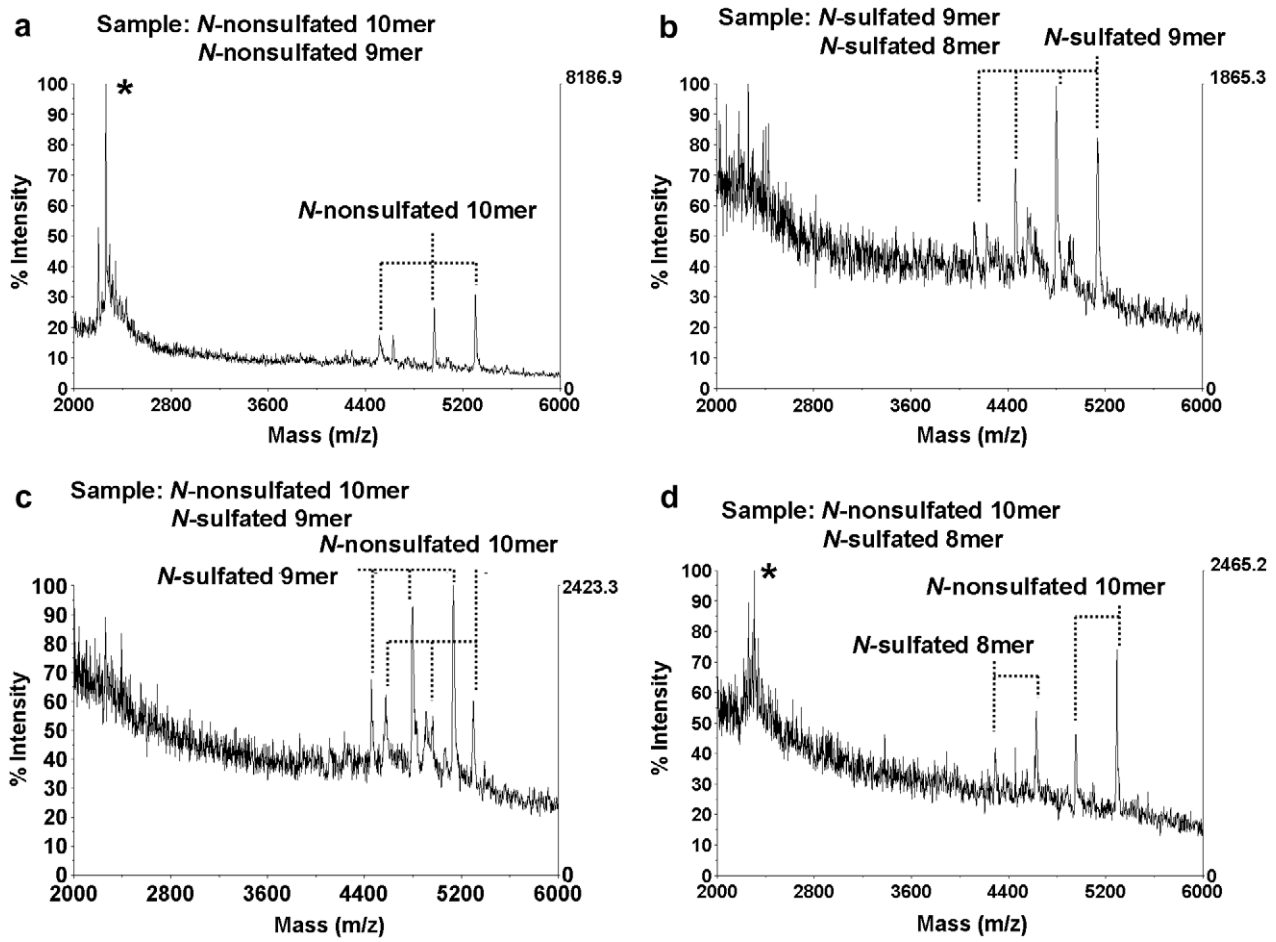

Figure 6. SNA–MS of mixtures of two oligo(tyrosine sulfate)s using hAT III (123–139) with a 0.1 M Na_2SO_4 wash. Samples: (a) H-[Tyr(SO_3Na)]$_{10}$-ONa and H-[Tyr(SO_3Na)]$_9$-ONa. (b) NaO_3S-[Tyr(SO_3Na)]$_9$-ONa and NaO_3S-[Tyr(SO_3Na)]$_8$-ONa. (c) H-[Tyr(SO_3Na)]$_{10}$-ONa and NaO_3S-[Tyr(SO_3Na)]$_9$-ONa. (d) H-[Tyr(SO_3Na)]$_{10}$-ONa and NaO_3S-[Tyr(SO_3Na)]$_8$-ONa. * indicates a molecular ion of hAT III (123–139).

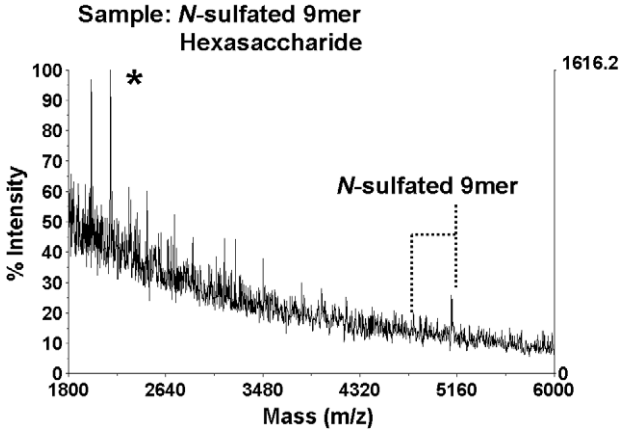

Figure 7. SNA–MS of a mixture of NaO_3S-[Tyr(SO_3Na)]$_9$-ONa and the hexasaccharide using the HIP peptide with a 0.1 M Na_2SO_4 wash. * indicates a molecular ion of the HIP peptide.

N-nonsulfated pentamer ($n = 5$), and the longer oligomers, mixed with the pentapeptide, only signals of the oligo(tyrosine sulfate)s were observed (data not shown). These results show that the binding affinity to the HIP peptide was much higher for oligo(tyrosine sulfate)s than for the pentapeptide.

2.7. Comparison of the relative binding affinity to the HIP peptide between oligo(tyrosine sulfate)s

To examine the dependence of binding affinity on chain length and *N*-sulfate group in the oligo(tyrosine sulfate)–HIP peptide interaction, we investigated the binding of two oligo(tyrosine sulfate)s to the HIP peptide.

A mixture of two N-nonsulfated oligomers or two N-sulfated oligomers with different chain lengths with the 0.1 M Na_2SO_4 wash produced selective signals of the longer oligomer (Fig. 9a and b). However, in mixtures of oligomer with tyrosine residue number n and $n-1$ analyzed without the 0.1 M Na_2SO_4 wash, both oligomers were detected (data not shown). These results suggest that the difference in binding affinity between these two oligomers is relatively small. Thus, we also succeeded in observing chain-length dependence in the interaction between oligo(tyrosine sulfate)s and the HIP peptide.

To evaluate the effect of an *N*-sulfate group on the binding affinity to the HIP peptide, the affinity of N-sulfated oligo(tyrosine sulfate)s was compared with that of N-nonsulfated oligomers containing the same number of

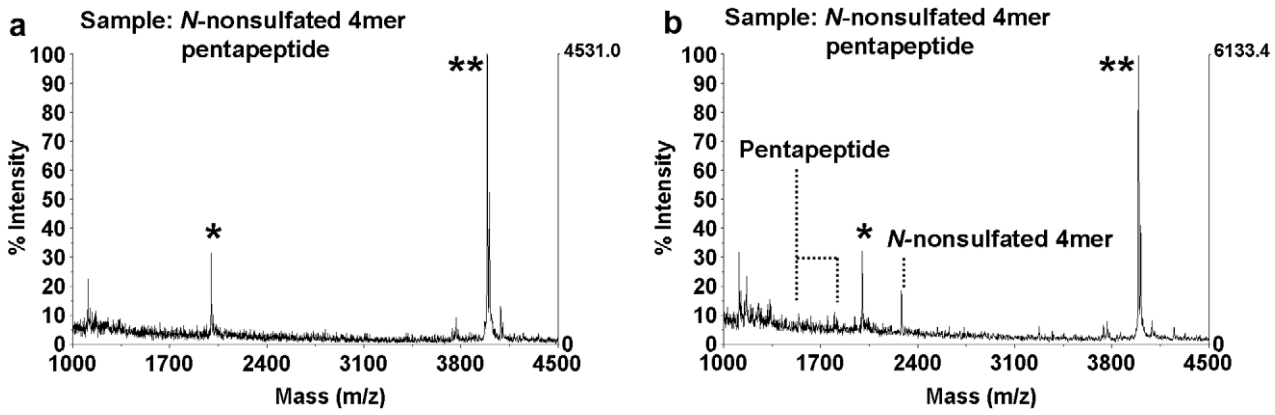

Figure 8. SNA–MS of mixtures of each oligo(tyrosine sulfate) and the pentapeptide [SY(SO_3)DY(SO_3)G] using the HIP peptide. (a) H-[Tyr(SO_3Na)]$_4$-ONa and the pentapeptide with a 0.1 M Na_2SO_4 wash. (b) H-[Tyr(SO_3Na)]$_4$-ONa and the pentapeptide without a 0.1 M Na_2SO_4 wash. * and ** indicate a monomer and a dimer of molecular ions of the HIP peptide.

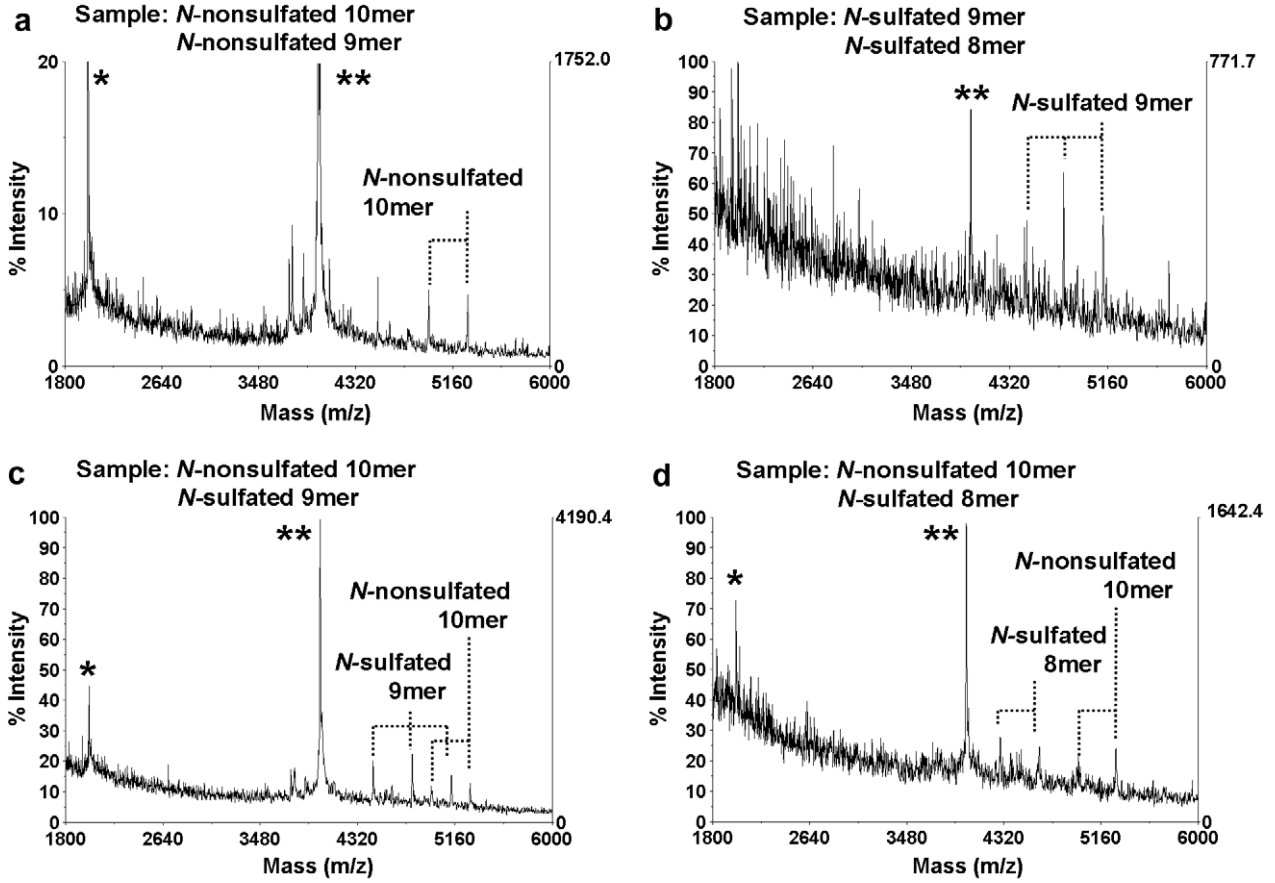

Figure 9. SNA–MS of mixtures of two oligo(tyrosine sulfate)s using the HIP peptide with a 0.1 M Na_2SO_4 wash. Samples: (a) H-[Tyr(SO_3Na)]$_{10}$-ONa and H-[Tyr(SO_3Na)]$_9$-ONa. (b) NaO_3S-[Tyr(SO_3Na)]$_9$-ONa and NaO_3S-[Tyr(SO_3Na)]$_8$-ONa. (c) H-[Tyr(SO_3Na)]$_{10}$-ONa and NaO_3S-[Tyr(SO_3Na)]$_9$-ONa. (d) H-[Tyr(SO_3Na)]$_{10}$-ONa and NaO_3S-[Tyr(SO_3Na)]$_8$-ONa. * and ** indicate a monomer and a dimer of molecular ions of the HIP peptide.

sulfate groups or one more. For example, in a mixture of N-sulfated oligomers with tyrosine residue number n, NaO_3S-[Tyr(SO_3Na)]$_n$-ONa (n = 8 or 9), and N-nonsulfated decamer, H-[Tyr(SO_3Na)]$_{10}$-ONa, signals of both oligomers were observed (Fig. 9c and d). These results suggest that N-sulfation is also important in enhancing the binding affinity of oligo(tyrosine sulfate)s to the HIP peptide.

2.8. Anticoagulant activities of oligo(tyrosine sulfate)s

Finally, we examined the effect of the selected oligomers on the activated partial thromboplastin time (APTT) to determine the in vitro anticoagulant activities of these oligo(tyrosine sulfate)s. Pooled plasma samples obtained from healthy volunteers were incubated with saline or the specific oligomer at final concentrations of

Table 3. Effects of oligo(tyrosine sulfate)s on activated partial thromboplastin time (s)[a]

Compound	Final concentration (mg/mL for oligomers, U/mL for heparin and danaparoid)					
	0	0.01	0.03	0.1	0.3	1
N-Sulfated 6-mer	30.5 ± 0.4	30.8 ± 0.3	31.2 ± 0.3	32.3 ± 0.1	35.7 ± 0.1	64.8 ± 10.8
N-Nonsulfated 7-mer	30.1 ± 0.9	30.4 ± 0.9	30.5 ± 0.8	31.0 ± 0.8	32.4 ± 0.7	40.6 ± 5.7
N-Sulfated 9-mer	30.5 ± 0.6	31.9 ± 0.7	33.1 ± 0.4	36.5 ± 0.6	45.7 ± 1.8	95.8 ± 9.8
N-Nonsulfated 10-mer	30.4 ± 0.5	30.6 ± 0.7	32.1 ± 1.5	41.0 ± 7.9	90.5 ± 8.6	153.7 ± 45.7
N-Sulfated 12-mer	29.8 ± 0.9	32.0 ± 0.2	37.6 ± 1.8	50.3 ± 0.6	75.7 ± 4.8	172.1 ± 28.0
Heparin	29.6 ± 0.4	29.7 ± 0.5	32.4 ± 1.4	35.9 ± 0.6	66.5 ± 7.1	190.3 ± 16.7
Danapaloid	29.6 ± 0.6	30.6 ± 0.9	33.9 ± 1.4	36.4 ± 2.1	59.2 ± 6.9	93.7 ± 7.8

[a] Data are expressed as means ± SD ($n = 3$ per group).

0.01–1 mg/mL for 15 min at room temperature (Table 3). Unfractionated heparin (Sanofi-Aventis) and danaparoid sodium (Orgaran®; Organon)[23] were used as a positive control; these drugs are sulfated glycosaminoglycans that are used widely as injectable anticoagulants to treat a variety of thrombotic disorders.[24,25] As shown in Table 3, all oligomers prolonged blood coagulation time in a concentration-dependent manner. Of note, the anticoagulant activity of the oligomer seemed to be dependent on the length of the oligomer (Table 3). These results are consistent with the results of the AT III binding experiment (see Fig. 5). In contrast, N-sulfation of each oligomer had no obvious effect on enhancement of anticoagulant activity (Table 3).

In conclusion, we observed specific binding of noncovalent complex formation of oligo(tyrosine sulfate)s with heparin-binding peptides using SNA–MS. These results show the potential use of oligo(tyrosine sulfate)s as heparin pentasaccharide mimics. Our next goal is to understand the specifications of the essential sulfate groups.

3. Materials and methods

3.1. Reagents

Sulfur trioxide-pyridine complex, α-cyano-4-hydroxycinnamic acid (CHCA), THAP, and biotin *p*-nitrophenyl ester were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). TBP·Br was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Acetonitrile (LC/MS grade), *N,N*-dimethylformamide (DMF), and trifluoroacetic acid (TFA) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). NeutrAvidin was purchased from Pierce (Rockford, IL, USA). Hexasaccharide dp6 was purchased from Dextra Laboratories (Reading, UK).

3.2. High-performance liquid chromatography

Analytical high-performance liquid chromatography (HPLC) was carried out using a Waters 2695 Alliance system with a Waters 2996 photodiode array detector. Columns and analytical conditions are mentioned in the text. Chromatograms were recorded at 220 nm.

3.3. Synthesis of oligo(tyrosine sulfate)s

N-nonsulfated oligo(tyrosine sulfate)s were synthesized manually by the 9-fluorenylmethyloxycarbonyl (Fmoc) solid phase method as reported previously.[8,26] Their purity was checked by HPLC and the retention times are listed in Table 2. N-sulfated oligo(tyrosine sulfate)s were synthesized by the segment condensation method in solution phase.

3.4. Synthesis of N-sulfated oligo(tyrosine sulfate)s (general procedure)

3.4.1. Fmoc-[Tyr($SO_3{}^{-}{}^{n}NBu_4$)]$_n$-OBzl (n = 2–4). Sulfation of side-chain hydroxyl groups of Fmoc-Tyr$_n$-OBzl (n = 2–4) was performed as reported previously,[8] omitting the pH adjustment before extraction because the C-terminal carboxyl groups of the peptides were protected.

3.4.2. H-[Tyr($SO_3{}^{-}{}^{n}NBu_4$)]$_n$-OBzl (n = 3 or 4). To Fmoc-[Tyr($SO_3{}^{-}{}^{n}NBu_4$)]$_n$-OBzl (n = 3 or 4) cooled in an ice-water bath, 20% diethylamine in DMF (2 mL/mmol) was added and stirred for 20 min at room temperature. The solvent was removed, and the residue was dissolved in methanol, applied to a Sephadex LH-20 column (2 cm × 80 cm), and eluted with methanol at a flow rate of 0.6 mL/min. The eluate was separated into 5 mL fractions, and each fraction was analyzed by HPLC. Fractions containing the desired product were collected, concentrated, and lyophilized from 1,4-dioxane/water (3:1, v/v).

3.4.3. Fmoc-[Tyr($SO_3{}^{-}{}^{n}NBu_4$)]$_n$-OH (n = 2 or 3). Fmoc-[Tyr($SO_3{}^{-}{}^{n}NBu_4$)]$_n$-OBzl (n = 2 or 3) was dissolved in DMF/ethyl acetate (1:5, 10 mL/mmol). To this, 10% palladium on charcoal was added, and hydrogenolysis under atmospheric pressure was carried out for 2 h. The catalyst and the solvent were removed, and the residue was dissolved in methanol, applied to a Sephadex LH-20 column (2×80 cm) and eluted with methanol at a flow rate of 0.6 mL/min. The eluate was separated into 5 mL fractions, and each fraction was analyzed by HPLC. Fractions containing the desired product were collected, concentrated, and lyophilized from 1,4-dioxane/water (3:1, v/v).

3.4.4. Fmoc-[Tyr($SO_3{}^{-}{}^{n}NBu_4$)]$_n$-OBzl (n = 5–12). H-[Tyr($SO_3{}^{-}{}^{n}NBu_4$)]$_n$-OBzl (n = 3 or 4) and Fmoc-[Tyr

$(SO_3{\cdot}^{n}NBu_4)]_n$-OH ($n$ = 2 or 3) were dissolved in DMF (2 mL/mmol). To this, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 1 equiv), 1-hydroxybenzotriazole (HOBt, 1 equiv), and 2,4,6-collidine (3 equiv) were added at −10°C, and the mixture was allowed to react by stirring overnight at room temperature. The mixture was poured into 50 mL of a 5% aqueous Na_2CO_3 solution, and the aqueous solution was extracted with chloroform. The combined extracts were washed three times each with 5% citric acid and water, dried over anhydrous sodium sulfate, and evaporated to dryness in vacuo. The residue was triturated with ether, collected by filtration, and dried. The Fmoc group was removed as described above. The coupling and deprotection steps were repeated until the oligomer of the desired chain length was obtained.

3.4.5. $^{n}Bu_4N{\cdot}O_3S$-[Tyr($SO_3{\cdot}^{n}NBu_4$)]$_n$-OBzl (n = 5–12). The Fmoc group of Fmoc-[Tyr($SO_3{\cdot}^{n}NBu_4$)]$_n$-OBzl (n = 5–12) was removed as described above. Sulfation of the N-terminal amino group was performed as reported previously[8] although the ratio of DMF to pyridine was changed to 1:4 (v/v). After the reaction, the residue was dissolved in methanol/water (3:7, v/v), applied to a Sephadex LH-20 column (2 × 80 cm), and eluted with methanol/water (3:7, v/v) at a flow rate of 0.6 mL/min. The eluate was separated into 5 mL fractions, and each fraction was analyzed by HPLC. Fractions containing the desired product were collected, concentrated, and lyophilized from 1,4-dioxane/water (3:1, v/v).

3.4.6. NaO_3S-[Tyr(SO_3Na)]$_n$-ONa (n = 5–12). Removal of the Bzl group from $^{n}Bu_4N{\cdot}O_3S$-[Tyr($SO_3{\cdot}^{n}NBu_4$)]$_n$-OBzl (n = 5–12) and conversion to the sodium salt were performed as reported previously,[8] and the desired compounds were obtained as a white powder. Their purity was checked by HPLC, and the retention times are listed in Table 2.

3.5. Syntheses of heparin-binding peptides and the $VEGF_{165}$-binding peptide

Heparin-binding peptides and the $VEGF_{165}$-binding peptide were synthesized manually in our laboratory by the Fmoc solid phase method on Wang resin. To introduce a sulfated tyrosine moiety, Fmoc-Tyr($SO_3{\cdot}^{n}NBu_4$)-OH[8] was used as the building block. To introduce the biotinyl group, biotin *p*-nitrophenyl ester was used.

3.6. Affinity chromatography

A HiTrap Heparin HP column (1 mL, Amersham Biosciences, Sweden) was used for heparin-immobilized affinity chromatography. The oligo(tyrosine sulfate)-immobilized affinity gel was prepared as follows. Diamine spacer NH_2–$(CH_2)_6$–NH_2 was introduced to C-terminal carboxyl groups of NaO_3S-[Tyr(SO_3Na)]$_9$-OH and H-[Tyr(SO_3Na)]$_9$-OH. Each oligomer was then immobilized to Affi-Gel 10 (Bio-Rad Laboratories, CA, USA) by the diamine spacer. One milliliter of the affinity matrix was packed into a 1 mL Tricorn empty column (5/50) (Amersham Biosciences, Sweden) and conditioned by repeated washing with 20 mM Tris–HCl buffer (pH 7.6) with a gradient of 0–2 M NaCl. The heparin-binding peptides were dissolved in 20 mM Tris–HCl buffer (pH 7.6) and loaded into the column. The column was washed with 20 mM Tris–HCl buffer (pH 7.6) for 30 min and eluted with a linear gradient from 0 to 2 M NaCl in 20 mM Tris–HCl buffer (pH 7.6) for 30 min at a rate of 0.5 mL/min. All the experiments were performed at ambient temperature. Chromatograms were recorded at 220 nm. Fractions of 0.5 mL were collected, and aliquots were desalted, concentrated using ZipTip C18 (Millipore, USA), and analyzed by MALDI–TOF–MS.

3.7. Surface noncovalent affinity mass spectrometry

3.7.1. Matrix preparation. Matrix solutions were prepared by dissolving 10 mg of the selected matrix in 1 mL of solvent [acetonitrile/0.1% aqueous TFA (1:1, v/v) for CHCA, and acetonitrile/H_2O (1:1, v/v) for THAP].

TBP·Br solution was prepared by dissolving 2.7 mg of TBP·Br in 0.2 mL of acetonitrile/H_2O (1:1, v/v).

3.7.2. Sample preparation. The Opti-TOF plate system (Applied Biosystems, Foster City, CA, USA) was used as the immobilization surface. One microliter of NeutrAvidin solution (4 μg/μL) was spotted on the surface and air-dried. A biotinylated heparin-binding peptide was then immobilized by adding 1 μL of the peptide solution (50 pmol/μL) to the NeutrAvidin spot.

To the immobilized heparin-binding peptide, 1 μL of the analyte solution (containing 500 pmol of each analyte) was added. After 5 min, the spot was washed 10 times with 0.1 M Na_2SO_4 and then washed 10 times with water to remove nonspecific binders. One microliter of TBP·Br solution and 18 μL of THAP solution were mixed, and 1 μL aliquot of the matrix solution was applied to the sample spot and air-dried.

3.8. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Measurements were performed using a Voyager DE time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) equipped with a nitrogen laser (337 nm, 3 ns pulse). The accelerating voltage in the ion source was 20 kV. All samples were measured in the linear positive ion mode. Each mass spectrum was produced by accumulating data from first 50 laser shots from a single spot. All experiments were performed twice. External calibration was performed using the commercial ProteoMass Peptide MALDI–MS Calibration Kit (Sigma Chemical Co., St. Louis, MO, USA).

3.9. Activated partial thromboplastin time measurements

The anticoagulant activity of these oligo(tyrosine sulfate)s was analyzed by APTT. Platelet-poor plasma obtained from at least 10 healthy volunteers was incubated

with saline (control) or with the indicated concentration of oligo(tyrosine sulfate)s, unfractionated heparin, or danaparoid sodium for 15 min at room temperature. APTT was measured by an automated blood coagulation analyzer (CA-500; Sysmex). When blood coagulation was not observed, APTT was defined as 200 s.

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References and notes

1. Capila, I.; Linhardt, R. J. *Angew. Chem. Int. Ed.* **2002**, *41*, 390–412.
2. Raman, R.; Sasisekharan, V.; Sasisekharan, R. *Chem. Biol.* **2005**, *12*, 267–277.
3. Holt, C. E.; Dickson, B. J. *Neuron* **2005**, *46*, 169–172.
4. Gama, C. I.; Tully, S. E.; Sotogaku, N.; Clark, P. M.; Rawat, M.; Vaidehi, N.; Goddard, W. A., III; Nishi, A.; Hsieh-Wilson, L. N. *Nat. Chem. Biol.* **2006**, *2*, 467–473.
5. Petitou, M.; van Boeckel, C. A. A. *Angew. Chem. Int. Ed.* **2004**, *43*, 3118–3133.
6. Karst, N. A.; Linhardt, R. J. *Curr. Med. Chem.* **2003**, *10*, 1993–2031.
7. (a) This work was presented in part: Ueki, M.; Yamaguchi, M. In *Peptide Science 2006 (Proceedings of the International Conference of 43rd Japanese Peptide Symposium and 4th Peptide Engineering Meeting)*; Ishida, H.; Mihara, H., Eds.; The Japanese Peptide Society: Osaka, 2006; pp 188–189; (b) Yamaguchi, M.; Ueki, M. In *Peptides for Youth: 20th American Peptide Society Symposium Proceedings*; Escher, E.; Lubell, W.D.; Valle, S.D., Eds.; The American Peptide Society; in press.
8. Ueki, M.; Watanabe, S.; Ishii, Y.; Okunaka, O.; Uchino, K.; Saitoh, T.; Higashi, K.; Nakashima, H.; Yamamoto, N.; Ogawara, H. *Bioorg. Med. Chem.* **2001**, *9*, 477–486.
9. Ueki, M.; Watanabe, S.; Saitoh, T.; Nakashima, H.; Yamamoto, N.; Ogawara, H. *Bioorg. Med. Chem.* **2001**, *9*, 487–492.
10. Maynard, H. D.; Hubbell, J. A. *Acta Biomaterialia* **2005**, *1*, 451–459.
11. Vazquez-Campos, S.; St. Hilaire, P. M.; Damgaard, D.; Meldal, M. *QSAR Comb. Sci.* **2005**, *24*, 923–942.
12. Kaiser, N.; Venkataraman, G.; Shriver, Z.; Sasisekharan, R. *Nature Med.* **2001**, *7*, 123–128.
13. Onoue, S.; Harada, S.; Nemoto, Y.; Yajima, T.; Kashimoto, K. *Peptides* **2003**, *24*, 821–826.
14. Liu, S.; Zhou, F.; Hook, M.; Carson, D. D. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1739–1744.
15. Brockman, A. H.; Orlando, R. *Anal. Chem.* **1995**, *67*, 4581–4585.
16. Kuwata, H.; Yip, T. T.; Yip, C. L.; Tomita, M.; Hutchens, T. W. *Biochem. Biophys. Res. Commun.* **1998**, *245*, 764–773.
17. Ueki, M.; Yamaguchi, M. In *Peptide Science 2005 (Proceedings of the 42*nd *Japanese Peptide Symposium)*; Wakamiya, T., Ed.; The Japanese Peptide Society: Osaka, 2006; pp 177–180.
18. Ueki, M.; Yamaguchi, M. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1615–1620.
19. Ueki, M.; Yamaguchi, M. *Carbohydrate Res.* **2005**, *340*, 1722–1731.
20. Waltenberger, J.; Mayr, U.; Frank, H.; Hombach, V. *J. Mol. Cell. Cardiol.* **1996**, *28*, 1523–1529.
21. van Boeckel, C. A. A.; Petitou, M. *Angew. Chem. Int. Ed.* **1993**, *32*, 1671–1690.
22. Sweeney, M. D.; Yu, Y.; Leany, J. A. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1114–1119.
23. Meuleman, D. G.; Hobbelen, P. M.; van Dedem, G.; Moelker, H. C. *Thromb. Res.* **1982**, *27*, 353–363.
24. Verstraete, M. *Drugs* **1990**, *40*, 498–530.
25. Ibbotson, T.; Perry, C. M. *Drugs* **2002**, *62*, 2283–2314.
26. Ueki, M.; Takekawa, A.; Yamaguchi, M. In *Peptide Science 2003* (*Proceedings of the 40th Japanese Peptide Symposium*); Ueki, M., Ed.; The Japanese Peptide Society: Osaka, 2004; pp 151–154.