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Tailored Glycopolymers as Anticoagulant Heparin Mimetics**

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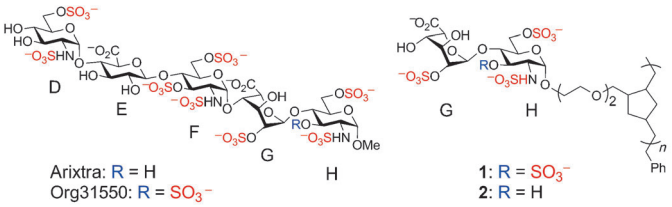
Heparin, a heterogeneously sulfated glycosaminoglycan, has been used as an anticoagulant drug for over 80 years. Although it is a highly effective and inexpensive agent, heparin in its unfractionated form has several limitations as a therapeutic. First, it is extracted from porcine or bovine mucosa and tissue, and carries the potential risk of contamination. The global distribution of contaminated heparin in 2007, for example, resulted in over 100 deaths and hundreds of additional cases reporting adverse clinical effects.[1] Second, heparin displays a variable dose–response relationship among patients, in part owing to its structural heterogeneity, and thus often requires active monitoring to fine-tune the dosages.[2] Third, approximately 3% of patients undergoing prolonged heparin therapy experience heparin-induced thrombocytopenia (HIT),[3] a severe autoimmune response triggered by formation of a complex between heparin and platelet factor 4 (PF4). Altogether, these factors underscore the critical need to develop safer alternatives to animal-derived heparin that have more predictable bioactivity and reduced side effects.

In recent decades, two forms of heparin-based anticoagulants have emerged as alternatives to unfractionated heparin. Low-molecular-weight heparin (LMWH) is produced by the chemical or enzymatic degradation of heparin, yielding fragments with an average molecular weight of 6000 Da.[4] Shorter ultralow-molecular-weight heparin (ULMWH; ca.1500 Da) is prepared through the synthesis[5] of a specific heparin pentasaccharide that represents the minimal epitope required for antithrombin III (ATIII) activation.[6] The commercial drug Arixtra (GlaxoSmithKline; Scheme 1) is a methylated derivative of this pentasaccharide. The addition of another 3-*O*-sulfate group has been shown to confer even greater specificity for ATIII activation (Org31550; Scheme 1).[7] For the prophylaxis and treatment of thromboembolic diseases, LMW and ULMW heparins are preferred over unfractionated heparin because of their more predictable pharmacokinetics, longer half-lives, and reduced risk of side effects.[3] However, animal-derived LMWH still has considerable structural heterogeneity and is susceptible to external contamination.[1b] ULMWH is notoriously difficult and expensive to synthesize (ca. 50 chemical steps, ca. 0.1% overall yield in the case of Arixtra)[5a,b] and exhibits limited inhibitory activity toward thrombin (also known as factor IIa (FIIa)) owing to its shorter length.[5a] Thus, despite the notable advantages of LMW and ULMW heparins, new methods are greatly needed for the efficient preparation of defined heparin-like molecules with broad anticoagulation profiles and improved safety.



Scheme 1. Chemical structures of Arixtra, Org31550, and synthetic glycopolymers **1** and **2**.

Synthetic glycopolymers[8] bearing short, well-defined heparin epitopes may offer an attractive alternative to natural heparins and Arixtra. Here, we utilize ring-opening metathesis polymerization (ROMP)[9] chemistry to generate a series of heparin-based glycopolymers and demonstrate that their structures can be tailored to recapitulate the potent activity of anticoagulant drugs. Previously, we showed that glycopolymers containing defined chondroitin sulfate (CS)[10] and heparan sulfate (HS)[11] disaccharide epitopes could mimic the activity of natural glycosaminoglycans in neurite outgrowth[10] and immune cell migration[11] assays, respectively. However, the application of ROMP to heparin, the most highly charged and synthetically challenging glycosaminoglycan, has not been investigated. Moreover, synthetic glycopolymers with medicinally significant anticoagulant activity have not been extensively explored as potential heparin mimetics. Our approach overcomes the challenges of heterogeneous sulfation and complex oligosaccharide synthesis and shows promise for the development of a new class of medicinal agents.

The first challenge in designing an anticoagulant glycopolymer was to select a minimal unit that encapsulated the key determinants of the ATIII-binding pentasaccharide. Although previous studies had suggested that a pentasaccharide was the minimum active motif,[5,6] we reasoned that a disaccharide epitope might be sufficient when presented on a multivalent polymeric scaffold, which might bring about an enhancement of binding affinity through avidity.[12] Thus, we chose two monosaccharide units (G and H, Scheme 1) from

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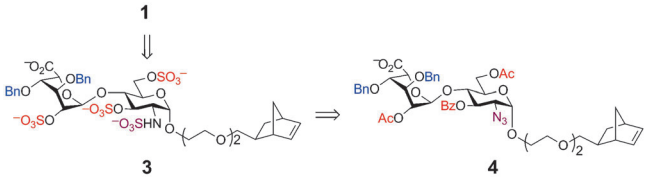
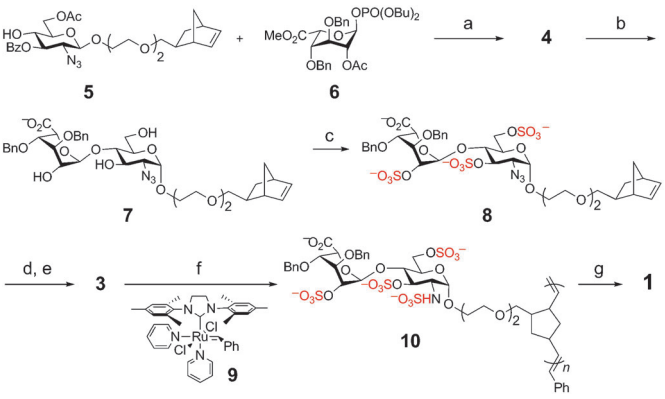
the reducing end of the pentasaccharide, because of their well-characterized interactions with the A-helix (R46, R47) and P-helix (K114, D113) of ATIII.[13] The inclusion of unit G allowed us to exploit the conformational flexibility of L-iduronic acid, which substantially improves the affinity of heparin for ATIII.[14] We chose unit H of Org31550 because it contains a rare 3-*O*-sulfate modification that significantly enhances the interaction of heparin with ATIII.[7]

The tetrasulfated glycopolymer **1**, comprised of units G and H, can be readily derived from disaccharide **3** (Scheme 2). We devised a protecting group strategy for **3** that would allow



Scheme 2. Retrosynthesis of glycopolymer **1** from orthogonally protected disaccharide **3.**



Scheme 3. Synthesis of tetrasulfated heparin glycopolymer **1**. Conditions: a) TMSOTf, CH_2Cl_2, −30 °C, 85 %. b) LiOH (0.7 M), H_2O_2, 25 °C; MeOH, NaOH (4 M), 25 °C, 82 % over two steps. c) SO_3·TMA, DMF, 60 °C, 71 %. d) PMe_3, THF, 25 °C, quant. e) SO_3·pyr, pyr/Et_3N, 60 °C, 54 % over two steps. f) MeOH/$(CH_2Cl)_2$, 55 °C. g) $Pd(OH)_2$/C, H_2 (1 atm), MeOH, phosphate buffer (pH 7.4), 63–92 %. TMSOTf = trimethylsilyl trifluoromethanesulfonate, TMA = trimethylamine, DMF = *N*,*N*-dimethylformamide, pyr = pyridine.

expedient sulfation at four different sites. A benzoyl (Bz) group was used to protect C3, and acetyl (Ac) groups were selected to protect the C2′ and C6 positions. These groups can be removed under the same conditions required for C5′ saponification[15] to simultaneously expose the three sites desired for *O*-sulfation. In addition, the C2′ Ac group was expected to facilitate the formation of the 1,2-*trans* glycosidic linkage through neighboring group participation. Owing to the highly charged nature of the sulfated monomer required for polymerization, we elected to use benzyl (Bn) groups at the nonsulfated positions (C3′ and C4′) to enhance monomer solubility during ROMP.

The orthogonally protected disaccharide **4** was prepared from the norbornene-conjugated acceptor **5**[16] and glycosyl phosphate donor **6** (Scheme 3 and Scheme S1 in the Supporting Information). The coupling reaction utilized stoichiometric amounts of TMSOTf at −30 °C, and exclusively produced the α-linked disaccharide **4** in 85 % yield. Late-stage manipulations to the resulting disaccharide were carried out with modifications to reported procedures.[15,17] Briefly, **4** was subjected to the sequential addition of LiOOH and NaOH to saponify the C5′ methyl ester and concomitantly remove the Ac and Bz protecting groups. Global *O*-sulfation of disaccharide **7** using SO_3·NMe_3 in DMF furnished trisulfated intermediate **8** in 71 % yield. Finally, the azide group was reduced under Staudinger conditions, followed by regiospecific *N*-sulfation with SO_3·pyr[18] to give tetrasulfated **3** in 54 % yield over two steps.

With monomer **3** in hand, we evaluated a series of polymerization conditions. Although polar solvents were required for complete solubility of **3**, unfortunately, such conditions compromised the overall efficiency of the ROMP catalyst. Attempts at aqueous ROMP using the water-soluble catalyst [{H_2IMes-poly(ethyleneglycol)}$(Cl)_2$Ru=CH(*o*-*i*PrOC_4H_4)][19] led to incomplete conversion and low-molecular-weight polymers (H_2IMes = 1,3-dimesityl-4,5-dihydroimidazol-2-ylidene). Polymerization reactions in MeOH using the fast initiating catalyst [$(H_2IMes)(Py)_2(Cl)_2$Ru=CHPh] (**9**)[20] produced a similar undesired outcome. Ultimately, we found that the polymerization of **3** was fully compatible with a MeOH/$(CH_2Cl)_2$ cosolvent system at 55 °C,[10] which resulted in high conversions and good glycopolymer polydispersities. Using this cosolvent system, we then explored the relationship between catalyst loading and polymer length. By tuning the amount of catalyst and the MeOH/$(CH_2Cl)_2$ cosolvent ratio, we were able to synthesize a series of glycopolymers with controlled, predictable chain lengths (**10-*n***, where *n* = degree of polymerization (DP); Table 1). Under the tested conditions, polymer lengths were limited to 45 units as a result of premature precipitation of polymer **10** during ROMP. All glycopolymers were characterized at this stage by ^{1}H NMR spectroscopy and size-exclusion chromatography–multiangle light scattering (SEC–MALS). Hydrogenation of the remaining Bn groups using $Pd(OH)_2$/C in a mixture of MeOH and neutral phosphate buffer[21] afforded the desired glycopolymers (**1-*n***) in 63–92 % yield.

We next assessed the anticoagulant activities of the glycopolymers in comparison to heparin, LMW heparin,

Table 1: Heparin glycopolymer properties.

Entry	Polymer	**9** [Mol %]	MeOH/$(CH_2Cl)_2$	*n* (DP)	M_n [g mol^{-1}]	PDI
1	**10-4**[a]	30	1:4	4	4373	2.03
2	**10-6**[a]	17	1:4	6	6167	1.25
3	**10-10**[a]	10.7	1:3	10	11 207	1.29
4	**10-15**[a]	6.5	1:3	15	15 452	1.32
5	**10-30**[a]	5.2	1:2.5	30	32 721	1.41
6	**10-45**[a]	2.0	1:2.5	45	42 970	1.25
7	**2-155**[b,c]	4.0	1:10	155	164 345	1.62

Degree of polymerization (DP), number average molecular weight (M_n), and polydispersity index (PDI) were determined by SEC-MALS in [a] LiBr (0.2 M) in DMF or [b] $NaNO_3$ (100 mM), NaN_3 (200 ppm) in H_2O. [c] See Ref. [11] for the synthesis of **2**.

and Arixtra. The binding of heparin to ATIII induces a conformational change that enables ATIII to inhibit the serine proteases factor Xa (FXa) and FIIa.[13] Protease inhibition was measured using chromogenic substrate assays that monitor the release of *p*-nitroaniline (λ_{max} 405) from specific peptide substrates. In a manner consistent with previous reports,[5,22] heparin, LMWH, and Arixtra attenuated FXa activity in the presence of ATIII, with half maximal inhibitory concentrations (IC_{50}) of (16.5 ± 1.2), (526 ± 71), and (11.0 ± 0.1) nM, respectively (Table 2, Figure S1 in the Supporting Information). To our delight, glycopolymer **1-45** showed greater anti-FXa activity than the clinical anticoagulants; it exhibited an IC_{50} value of (5.76 ± 0.04) nM (Figure 1 A, Table 2). As the polymer length decreased, we observed a significant reduction in anti-FXa activity (($IC_{50} = 684 \pm 60$) and (1470 ± 578) nM for **1-30** and **1-15**, respectively), until no activity was detected (**1-10**, **1-6**, and **1-4**; Table 2 and Figure S2 in the Supporting Information). We also assessed the contribution of the glucosaminyl 3-*O*-sulfate modification on unit H by comparing the activity of the glycopolymers to that of the 3-*O*-desulfated glycopolymer **2-155**.[11] Remarkably, this single alteration in the sulfation pattern abrogated the anti-FXa activity, thereby reaffirming the importance of 3-*O*-sulfation and the specificity of the polymer interaction with ATIII (Figure 1 A, Table 2). Thus, controlling the sulfation sequence and chain length of the glycopolymer allows the fine modulation of ATIII-mediated inhibition at a critical junction in the blood coagulation cascade.

Table 2: Biological activity of heparin glycopolymers.

Polymer	Anti-FXa IC_{50} [nM]	Anti-FIIa IC_{50} [nM]	APTT[a] [s]	PT[a] [s]
1-4	>2000	>2000	32.5 ± 0.3	13.2 ± 0.1
1-6	>2000	>2000	32.2 ± 0.2	13.2 ± 0.3
1-10	>2000	>2000	59.6 ± 0.3	15.8 ± 0.4
1-15	1470 ± 578	>2000	82.9 ± 0.6	23.4 ± 1.9
1-30	684 ± 60	577 ± 31	100.8 ± 0.6	50.8 ± 6.3
1-45	5.76 ± 10^{-2}	0.114 ± 10^{-4}	119.4 ± 0.5	52.2 ± 7.8
2-155	>2000	>2000	46.1 ± 0.4	12.7 ± 0.1
none	>2000	>2000	31.2 ± 0.3	13.3 ± 0.1
heparin	16.5 ± 1.2	11.0 ± 0.1	>180	84.2 ± 18.
LMWH	526 ± 71	>2000	117 ± 3.0	14.8 ± 0.2
Arixtra	11.0 ± 0.1	>2000	78.3 ± 0.4	15.1 ± 0.3

[a] 150 $\mu g\,mL^{-1}$ compound in citrated human plasma, $n = 3$.

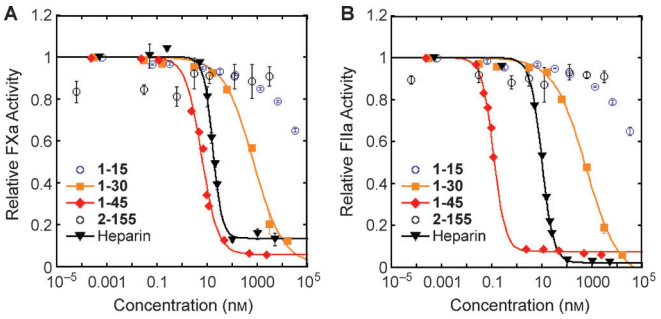


Figure 1. Tetrasulfated glycopolymers (**1-15**, **1-30**, **1-45**) inhibit the ATIII-mediated activity of A) FXa and B) FIIa in a length- and sulfation-dependent manner. Chromogenic substrate assays were used to measure coagulation factor activity in the presence of ATIII and glycopolymer. Data represent the mean ± standard deviation for $n = 3$ experiments. See Table 2 for IC_{50} values.

FIIa is activated downstream of FXa in the coagulation cascade and facilitates blood clotting by converting soluble fibrinogen into insoluble fibrin strands. Unlike FXa, which utilizes a specific pentasaccharide sequence, FIIa requires a longer, more highly sulfated heparin template to form an inhibitory ternary complex with ATIII.[5a,13] In agreement with this molecular mechanism and previous reports,[5,22] heparin displayed strong anti-FIIa activity (Figure 1 B, Table 2; $IC_{50} = (11.0 \pm 0.1)$ nM), whereas LMWH and Arixtra showed no appreciable activity (Table 2, Figure S1 in the Supporting Information). Notably, we found that glycopolymer **1-45** was 100-fold more potent than heparin at inhibiting FIIa ($IC_{50} = (114 \pm 1)$ pM), which is likely due to a greater number of active tetrasulfated motifs and increased polymer length. These results are exciting because they demonstrate that synthetic glycopolymers can be designed to surpass the biological activity of natural glycosaminoglycans. Glycopolymer **1-30** was also active in this assay ($IC_{50} = (577 \pm 31)$ nM), and as expected, the shorter polymers (**1-15**, **1-10**, **1-6**, and **1-4**) and the trisulfated glycopolymer (**2-155**) showed no significant activity (Figure 1 B, Table 2 and Figure S2 in the Supporting Information).

To assess potential interactions with PF4, we evaluated the ability of PF4 to neutralize the anti-FIIa activity of glycopolymers **1-45** and **1-30**. PF4 was added to the glycopolymers or heparin (0.5–500 $\mu g\,mL^{-1}$) in the presence of ATIII and excess FIIa, and FIIa activity was measured using the same chromogenic assay. Both heparin and glycopolymer **1-45** interacted strongly with PF4, a result consistent with reports that longer polysaccharides are more likely to engage PF4.[4,23] However, the anti-FIIa activity of glycopolymer **1-30** was only partially neutralized by PF4 (Figure S3 in the Supporting Information), thus indicating that the PF4 reactivity associated with HIT can be minimized to some extent by modulating the polymer length.

Finally, we examined the ex vivo clotting times of the glycopolymers, with comparison to clinical anticoagulants, using human plasma samples. Since the presence of other proteins in complex serum can interfere with anticoagulant activity, this assay represents a more stringent test of anticoagulant efficacy. We measured the activated partial thromboplastin time (APTT) and prothrombin time (PT) of each compound to determine its ability to inhibit blood clotting through the intrinsic and extrinsic pathways of the coagulation cascade, respectively.[24] Heparin increased both the APTT and PT for clotting compared to the saline control (Table 2), whereas LMWH or Arixtra at the same concentration increased only the APTT. Notably, the APTT of the glycopolymers could be controlled by varying the polymer length, with a minimum of 10 disaccharide epitopes (**1-10**) required to prolong the APTT. A slight increase to 15 units (**1-15**) endowed the polymer with APTT properties similar to Arixtra, whereas the PT was not appreciably altered in either case. Glycopolymers **1-30** and **1-45** modulated both the APTT

and PT, a result consistent with their ability to inhibit FXa and FIIa in vitro. Remarkably, tuning of the polymer structure produced anticoagulants with unique hybrid properties. Whereas the APTTs of **1-30** and **1-45** were comparable to those of LMWH and Arixtra, their PTs more closely resembled that of heparin. Lastly, as expected, the trisulfated glycopolymer **2-155** produced no appreciable change in APTT or PT, thus reinforcing the importance of the 3-*O*-sulfate modification and the potential to direct the selectivity of the glycopolymers toward specific heparin/HS-binding proteins by modulating the sulfation pattern. Overall, the ex vivo profile of the glycopolymers validates not only their efficacy in human plasma, but also their ability to replicate the activity profiles of the clinical anticoagulants. Moreover, we find that fine-tuning of the glycopolymer structure can lead to agents with novel properties not found in naturally occurring oligo- and polysaccharides.

Heparin has been used clinically as an anticoagulant since 1935 for the treatment and prevention of deep vein thrombosis during surgery, blood transfusion, and renal dialysis. Recent contamination issues associated with animal-sourced heparin have highlighted the importance of developing safer, more homogeneous alternatives to unfractionated heparin. While structurally defined oligosaccharides such as Arixtra offer a valuable solution to this problem, they are notoriously challenging and expensive to synthesize. Herein, we have reported the design and expedient synthesis of homogeneously sulfated glycopolymers based on a tetrasulfated disaccharide motif found in heparin. We have demonstrated that these glycopolymers have potent anticoagulant activity, as a result of the inhibition of the serine proteases FXa and FIIa through potentiation of ATIII, and replicate the ex vivo plasma clotting activity of the clinical anticoagulants heparin, LMWH, and Arixtra. Most interestingly, we found that systematic alterations to the glycopolymer length and sulfation pattern enabled the fine modulation of their anticoagulant activity, thus leading to the creation of new agents with unique hybrid activities distinct from those of both natural and synthetic glycosaminoglycans. These findings demonstrate the potential of this neoglycopolymer approach for the control of medicinally significant glycosaminoglycan-mediated events, and raise the prospect of developing a novel class of anticoagulants through systematic exploration of the glycopolymer structure.

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