

# N-Sulfotestosteronan, A Novel Substrate for Heparan Sulfate 6-O-Sulfotransferases and its Analysis by Oxidative Degradation

Guoyun Li,<sup>1,2,3,4,5,6\*</sup> Sayaka Masuko,<sup>2,3,4,5,6\*</sup> Dixy E. Green,<sup>7</sup> Yongmei Xu,<sup>8</sup> Lingyun Li,<sup>2,3,4,5,6</sup> Fuming Zhang,<sup>2,3,4,5,6</sup> Changhu Xue,<sup>1</sup> Jian Liu,<sup>8</sup> Paul L. DeAngelis,<sup>7</sup> Robert J. Linhardt<sup>2,3,4,5,6</sup>

<sup>1</sup> College of Food Science and Technology, Ocean University of China, Qingdao, People's Republic of China 266003

<sup>2</sup> Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY 12180

<sup>3</sup> Department of Biology, Rensselaer Polytechnic Institute, Troy, NY 12180

<sup>4</sup> Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180

<sup>5</sup> Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180

<sup>6</sup> Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180

<sup>7</sup> Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma Center for Medical Glycobiology, Oklahoma City, OK 73126

<sup>8</sup> Department of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599

Received 9 February 2013; revised 13 March 2013; accepted 12 April 2013

Published online 20 April 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bip.22263

## ABSTRACT:

Testosteronan, an unusual glycosaminoglycan (GAG) first isolated from the microbe *Comamonas testosteroni*, was enzymatically synthesized *in vitro* by transferring uridine diphosphate sugars on  $\beta$ -*p*-nitrophenyl glucuronide acceptor. After chemically converting testosteronan to N-sulfotestosteronan it was tested as a substrate for sulfotransferases involved in the biosynthesis of the GAG, heparan sulfate. Studies using <sup>35</sup>S-labeled 3'-phosphoadenosine-5'-phosphosulfate (PAPS) showed that only 6-O-sulfotransferases acted on N-sulfotestosteronan. An oxidative depolymerization reaction was explored to generate oligosaccharides from <sup>34</sup>S-labeled 6-O-sulfo-N-sulfotestosteronan using <sup>34</sup>S-labeled PAPS because testosteronan was resistant to all of the tested GAG-degrading enzymes. Liquid chromatography-mass spectrometric analysis of the oxidatively depolymerized polysaccharides

confirmed the incorporation of <sup>34</sup>S into ~14% of the glucosamine residues. Nuclear magnetic resonance spectroscopy also showed that the sulfo groups were transferred to ~20% of the 6-hydroxyl groups in the glucosamine residue of N-sulfotestosteronan. The bioactivity of 6-O-sulfo-N-sulfotestosteronan was examined by performing protein-binding studies with fibroblast growth factors and antithrombin (AT) III using a surface plasmon resonance competition assay. The introduction of 6-O-sulfo groups enhanced N-sulfotestosteronan binding to the fibroblast growth factors, but not to AT III. © 2013 Wiley Periodicals, Inc. *Biopolymers* 99: 675–685, 2013.

**Keywords:** bacterial polysaccharide; sulfotransferases; protein-binding; sulfonation; heparan sulfate

\*Guoyun Li and Sayaka Masuko contributed equally to this work.

Correspondence to: Robert J. Linhardt; e-mail: linhar@rpi.edu

Contract grant sponsor: NIH

Contract grant number: HL62244, GM38060

© 2013 Wiley Periodicals, Inc.

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the *Biopolymers* editorial office at [biopolymers@wiley.com](mailto:biopolymers@wiley.com)

## INTRODUCTION

Glycosaminoglycans (GAGs) are linear polysaccharides consisting of a repeating hexosamine-containing disaccharide unit. These polymers are usually found in the extracellular matrix of animal cells and have a wide variety of biological functions. In certain pathogenic bacteria, GAGs are synthesized as an extracellular coating or polysaccharide capsule, which acts as a molecular camouflage during the entry into a vertebrate host.<sup>1</sup> This polysaccharide capsule is biosynthesized either by complexes of monofunctional glycosyltransferases or by bifunctional GAG synthases that assemble the GAG from uridine diphosphate (UDP)-sugars.<sup>2</sup> For example, the *Pasteurella multocida* GAG synthases PmHS1 and PmHS2 employ UDP-*N*-acetyl glucosamine (GlcNAc) and UDP-glucuronic acid (GlcA) to construct heparosan, which has the repeating backbone  $[-4\text{-D-GlcA-}\beta\text{1,4-D-GlcNAc-}\alpha\text{1-}]_m$ , and is a biosynthetic precursor to heparan sulfate (HS).<sup>3</sup>

Heparin and HS are sulfated GAGs that are known to regulate important biological processes.<sup>4</sup> In their biosynthesis, their basic backbone heparosan undergoes O- and N-sulfonation and epimerization by modification enzymes: O-sulfotransferases (OSTs), *N*-deacetylase sulfotransferase (NDST), and C5-epimerase (C5-epi). These enzymes have been cloned and expressed and used to modify heparosan-like oligosaccharides in vitro to prepare heparin oligosaccharides with anticoagulant activity.<sup>5,6</sup>

Recently, we reported the cloning of a novel GAG synthase, CtTS from *Comamonas testosteroni*,<sup>7</sup> a Gram-negative aerobic bacteria and human pathogen.<sup>8</sup> The GAG produced by *C. testosteroni* has an unusual backbone structure  $[-4\text{-D-GlcA-}\alpha\text{1,4-D-GlcNAc-}\alpha\text{1-}]_m$ , which contains the same sugar composition as heparosan but a different glycosidic linkage stereochemistry at the GlcA residue. In heparosan, the linkage between GlcA and GlcNAc is in  $\beta$ -form, while in the GAG from *C. testosteroni*, the linkage is in  $\alpha$ -form. As a result of its distinctive glycosidic linkage, this novel GAG, testosterone, is insensitive to digestion by GAG lyases.<sup>7</sup> This suggested that sulfated testosterone could resemble heparin and HS to bind to a variety of proteins, but afford catabolically resistant analogs having therapeutic potential.

In this manuscript, we describe the in vitro enzymatic synthesis of testosterone on a  $\beta$ -*p*-nitrophenyl glucuronide acceptor and the chemical modification of the resulting testosterone polysaccharide to obtain *N*-sulfotestosterone. *N*-Sulfotestosterone is structurally similar to *N*-sulfoheparosan, a natural substrate for HS biosynthetic enzymes.<sup>9</sup> Next, the further modification of *N*-sulfotestosterone by OSTs involved in HS biosynthesis was examined. A non-enzymatic method was required to prepare oligosaccharide fragments to monitor the

enzymatic modification of *N*-sulfotestosterone polysaccharide. The O-sulfonation of *N*-sulfotestosterone oligosaccharides was established using liquid chromatography (LC)-mass spectrometry (MS). Two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy was used to establish the position of the O-sulfo group and surface plasmon resonance (SPR) was used to profile the structure and protein-binding activity of this new polysaccharide.

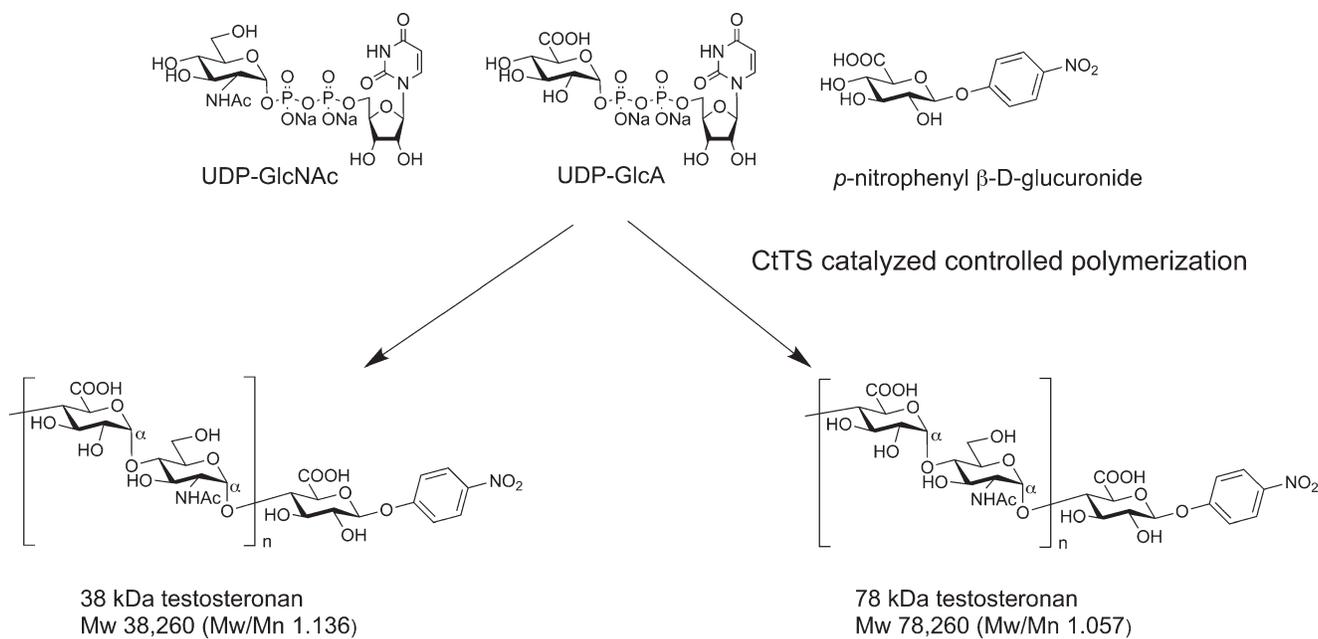
## RESULTS

### In Vitro Synthesis of Testosterone

Testosterone was synthesized in vitro using *p*-nitrophenyl  $\beta$ -glucuronide as the acceptor, UDP-sugars as the donors, and recombinant testosterone synthase, CtTS, as the catalyst (Figure 1). The acceptor was not efficiently incorporated (thus was present at high concentration in the reaction), but its presence narrowed the size distribution of testosterone significantly over “no acceptor” reactions. Two sizes of the polysaccharide were prepared from the reaction mixture by a strong anion exchange chromatographic separation. The size of the resulting testosterone polysaccharides was determined using polyacrylamide gel electrophoresis (PAGE) (Figure 2) and Multi-Angle Laser Light Scattering (MALLS). By MALLS, the average molecular weight/polydispersity values for the 0.8M and the 1.0M ammonium formate-eluted fractions were 38.3 kDa/1.136 and 78.3 kDa/1.057, respectively.

### Chemical Synthesis of *N*-Sulfotestosterone

The chemical conversion of testosterone to *N*-sulfotestosterone was based on our previously described conversion of heparosan to *N*-sulfoheparosan (Figure 3).<sup>9,10</sup> The *N*-acetyl group of testosterone was removed by chemical de-*N*-acetylation by treatment with 2M NaOH at 60°C overnight followed by chemical *N*-sulfonation by the addition of NMe<sub>3</sub>·SO<sub>3</sub> (Figure 3). After purification of the polysaccharide, the structure of *N*-sulfotestosterone was confirmed using one-dimensional <sup>1</sup>H-NMR (Figure 4). The successful removal of the *N*-acetyl group from testosterone was established by the nearly complete disappearance of the signal at 2.0 ppm, corresponding to the methyl group of the *N*-acetyl group in GlcNAc. Integration of the residual *N*-acetyl peak demonstrates a conversion of more than 95% on the GlcNAc residues to GlcNS residues. The conversion of GlcNAc to GlcNS could also be confirmed by the downfield chemical shift of the anomeric proton in GlcNAc (5.3 ppm) with the formation of GlcNS at 5.6 ppm. In the heteronuclear multiple-quantum correlation (HMQC) spectrum, the <sup>13</sup>C signal for C2 of GlcNS residue was observed



**FIGURE 1** In vitro CtTS-catalyzed synthesis of testosterone on *p*-nitrophenyl glucuronide acceptor.

at 58.0 ppm as compared to 51.9 ppm in GlcNAc residue (Figure 5A), corresponding to a significant downfield shift. These chemical shift changes are comparable to the chemical shift changes observed in the de-*N*-acetylation and *N*-sulfonation of heparosan in the preparation of *N*-sulfoheparosan.<sup>9</sup> Based on these spectral data, it was concluded that testosterone had been successfully converted to *N*-sulfotestosteronan,  $[-4\text{-D-GlcA-}\alpha\text{1,4-D-GlcNS-}\alpha\text{1-}]_n$ .

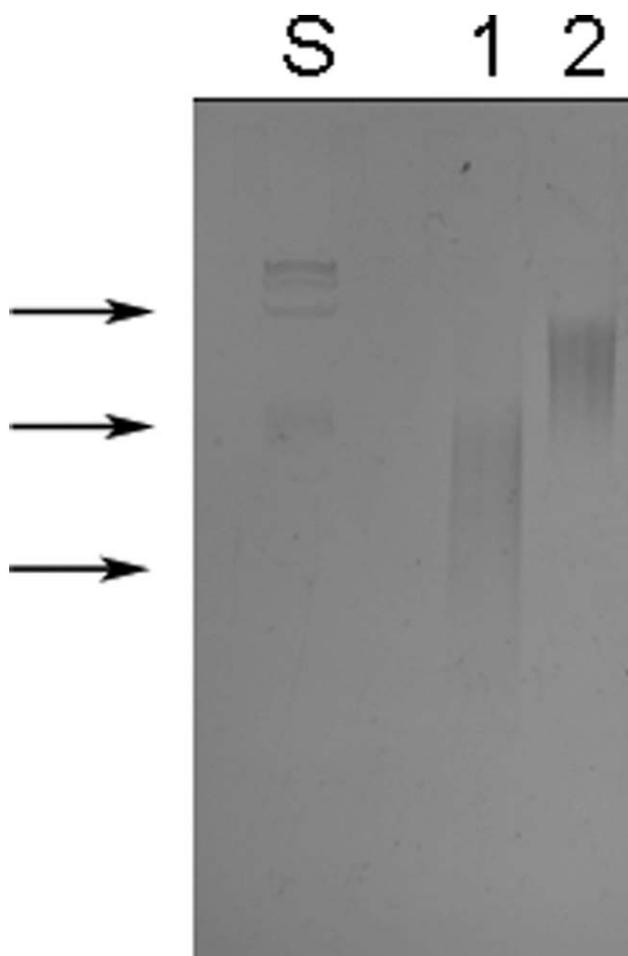
### Survey of the Reactivity of *N*-Sulfotestosteronan with *O*-Sulfotransferases

Next, to discover whether *N*-sulfotestosteronan was a substrate for HS biosynthetic enzymes, we treated *N*-sulfotestosteronan with various sulfotransferases and <sup>35</sup>S 3'-phosphoadenosine-5'-phosphosulfate (PAPS) *N*-sulfoheparosan as a positive control (Figure 3). Initial studies examining a mixture of 6OST-1, 6OST-3, and 2OST effectively sulfated the substrate, while 2OST alone was unable to incorporate [<sup>35</sup>S] sulfate into the polysaccharide, suggesting that the 6OSTs catalyzed most of the radiolabel [<sup>35</sup>S] sulfo group incorporation (Table I). Next, 3OST-1 was examined and showed a low level of radiolabel incorporation (Table II), suggesting that 3OST-1 cannot modify *N*-sulfotestosteronan. Finally, 6OST-1 and 6OST-3 were compared and both isoforms showed similar levels of radiolabel incorporation (Table III). The [<sup>35</sup>S] PAPS used varied in its specific activity over the timeframe during which the data presented in Table I were collected. To normalize the data in these three sets of

experiments, a negative control (containing no added enzyme) was set to 0% incorporation and a positive control (using *N*-sulfoheparosan treated with both 6OST-1 and 6OST-3) was set to 100% incorporation. While the data obtained, in each set of experiments, on the incorporation of <sup>35</sup>S into *N*-sulfotestosteronan treated with both 6OST-1 and 6OST-3 varied somewhat (114–169% of the positive control), the data demonstrated that the combination of 6OST-1 and 6OST-3 generally gave the highest level of incorporation. On the basis of these studies we decided to examine the incorporation of *O*-sulfo groups into *N*-sulfotestosteronan using a mixture of 6OST-1 and 6OST-3. Based on the [<sup>35</sup>S] sulfo group incorporation studies we anticipated a 20% incorporation of 6-*O* sulfo group.

### Synthesis of <sup>34</sup>S-Labeled 6-*O*-Sulfo-*N*-sulfotestosteronan

Using a mixture of 6OST-1 and 6OST-3, *N*-sulfotestosteronan was sulfonated using <sup>34</sup>S-labeled PAPS to incorporate a non-radioactive isotope, which would assist in measuring incorporation of *O*-sulfo groups by MS analysis. *N*-sulfotestosteronan was incubated for overnight at 37°C with 6OST-1, 6OST-3, and <sup>34</sup>S-labeled PAPS and the product was purified by a diethylaminoethyl (DEAE) column for the structural analysis. It was essential to use <sup>34</sup>S-labeled PAPS (Figure 5A) as exhaustive chemical *N*-sulfonation can introduce very small amounts of *O*-sulfo groups through a mechanism that is still unclear (possibly direct *O*-sulfonation followed by *N*- to *O*-sulfo group



**FIGURE 2** Analysis of testosterone by PAGE. A 6% PAGE gel was subjected to electrophoresis at 250 V for 15 min and stained with 0.05% Alcian Blue. Lanes: S, HA LoLadder; 1, 38 kDa testosterone (3  $\mu$ g); 2, 78 kDa testosterone (3  $\mu$ g). The positions of the 110 and 27 kDa bands of the ladder, and a 12 kDa HA standard (not shown on this portion of the gel) are marked with arrows (top to bottom, respectively).

migration) as previously observed by LC-MS analysis (results not shown).

### Oxidative Cleavage of 6-O-Sulfo-*N*-sulfotestosteronan and LC-MS Analysis

To utilize LC-MS to analyze the incorporation of *O*-sulfo groups into *N*-sulfotestosteronan, the polysaccharide must first be broken down to oligosaccharide fragments. Hydroxyl free radical-mediated, controlled depolymerization of 6-*O*-sulfo-*N*-sulfotestosteronan using copper (II) and hydrogen peroxide<sup>11</sup> at pH 7.0 afforded oligosaccharides that were then analyzed by hydrophilic liquid interaction chromatography (HILIC) LC- Fourier transform mass spectrometer (FTMS). The total ion chromatogram showed oligosaccharides ranging from trisaccharide,

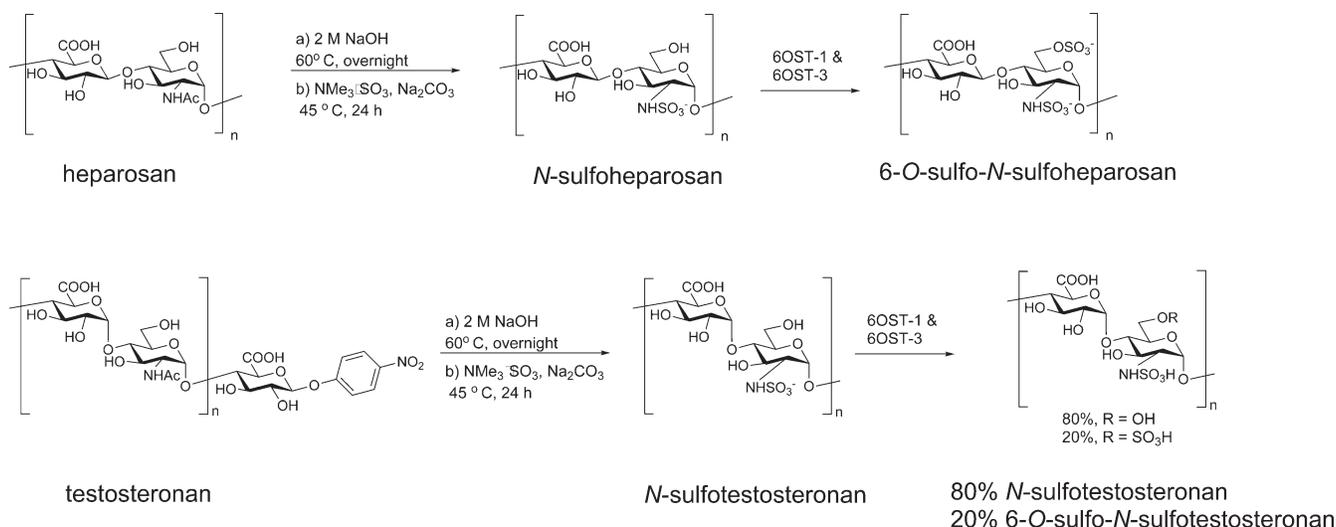
degree of polymerization (dp)3, to dp>22 (Figure 5B). <sup>34</sup>S-labeled oligomers can be identified from dp3 to dp12, and both even and odd chains of oligomers were detected in the depolymerized samples. Every odd number chain has two corresponding peaks, the first peak corresponding to a chain with an additional GlcNS residue, and the second (labeled with an asterisk \*) corresponding to an additional GlcA residue. From the mass spectra of the oligosaccharides, <sup>34</sup>S-labeled-sulfated oligosaccharides were detected and confirmed by FTMS elemental component analysis (dp4 and dp8 are shown in Figure 5C as examples). Based on these analyses, peaks corresponding to oligosaccharides containing <sup>34</sup>S-*O*-sulfo groups were integrated and peak areas of dp4, -6, -8, -10 were compared to peaks corresponding to oligosaccharides without *O*-sulfo groups. The ratio of peak areas indicated that the enzymatic 6-*O*-sulfonation of *N*-sulfotestosteronan was  $14.13 \pm 0.32\%$  efficient.

### Structural Analyses of 6-*O*-Sulfo-*N*-sulfotestosteronan Using NMR Spectroscopy

Two-dimensional <sup>1</sup>H-<sup>13</sup>C-HMQC NMR spectroscopy was used to evaluate the structure of the 6OST-treated *N*-sulfotestosteronan. HMQC NMR spectroscopy clearly demonstrated that *N*-sulfotestosteronan (Figure 6) had been converted to mixture containing *N*-sulfotestosteronan and 6-*O*-sulfo, *N*-sulfotestosteronan (~20% as determined by integration of the one-dimensional <sup>1</sup>H-NMR spectrum, not shown). The spectrum of the 6-*O*-sulfo, *N*-sulfotestosteronan clearly shows a new peak that corresponds to the C6 position of the newly formed GlcNS6S (circled). The <sup>13</sup>C chemical shift of this signal is 69.6 ppm, is in excellent agreement with the 6-*O*-sulfo group substituted carbon of the related 6-*O*-sulfo, *N*-sulfoheparosan (Figure 3).<sup>9</sup> Based on these spectral data, the treatment of *N*-sulfotestosteronan with 6OST-1 and 6OST-3 and <sup>34</sup>S-labeled PAPS confirmed the formation of 6-*O*-sulfo, *N*-sulfotestosteronan in a moderate yield.

### SPR Competition Assay with Heparin-Binding Proteins

The SPR competition assay was applied to determine the binding of 6-*O*-sulfo, *N*-sulfo testosterone to various heparin-binding proteins (acidic fibroblast growth factor (FGF1), basic fibroblast growth factor (FGF2), and antithrombin (AT)). Binding to these proteins requires sulfo group substitutions so unsulfated GAGs (e.g., testosterone, heparosan, hyaluronan) do not bind and serve as negative controls. Different sulfated GAGs were used as positive controls. SPR competition sensorgrams and bar graphs of 6-*O*-sulfo-*N*-sulfotestosteronan GAG competition levels are displayed in Figure 7. The 6-*O*-sulfo-*N*-sulfotestosteronan showed good binding ability to FGF1; its

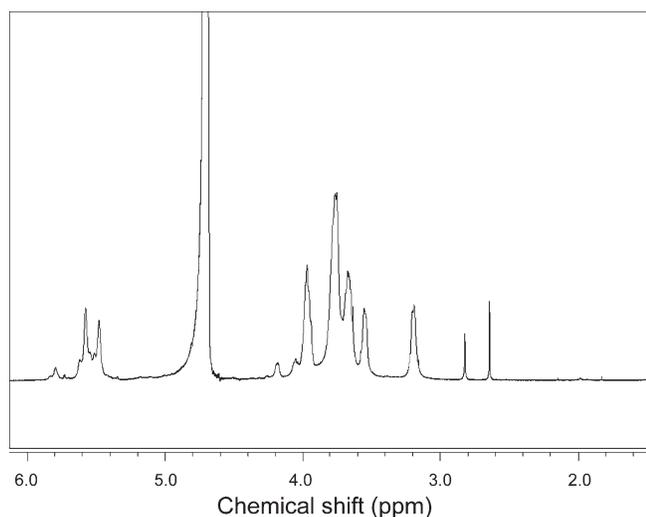


**FIGURE 3** Chemoenzymatic synthesis of 6-*O*-sulfo-*N*-sulfo heparosan and 6-*O*-sulfo-*N*-sulfotestosteronan.

binding affinity was comparable to chondroitin sulfate E (CSE). In comparison with *N*-sulfotestosteronan, the data also showed that 6-*O*-sulfonation greatly improved the FGF1 binding affinity (Figures 7A and 7B). 6-*O*-Sulfo-*N*-sulfotestosteronan showed weak binding ability to FGF2 and ATIII and 6-*O*-sulfonation slightly improved the FGF2 and AT III binding affinity (Figures 7C–7F).

## DISCUSSION

GAGs regulate a wide variety of biological processes and, thus, are attractive therapeutic targets. Heparin, for example, is a

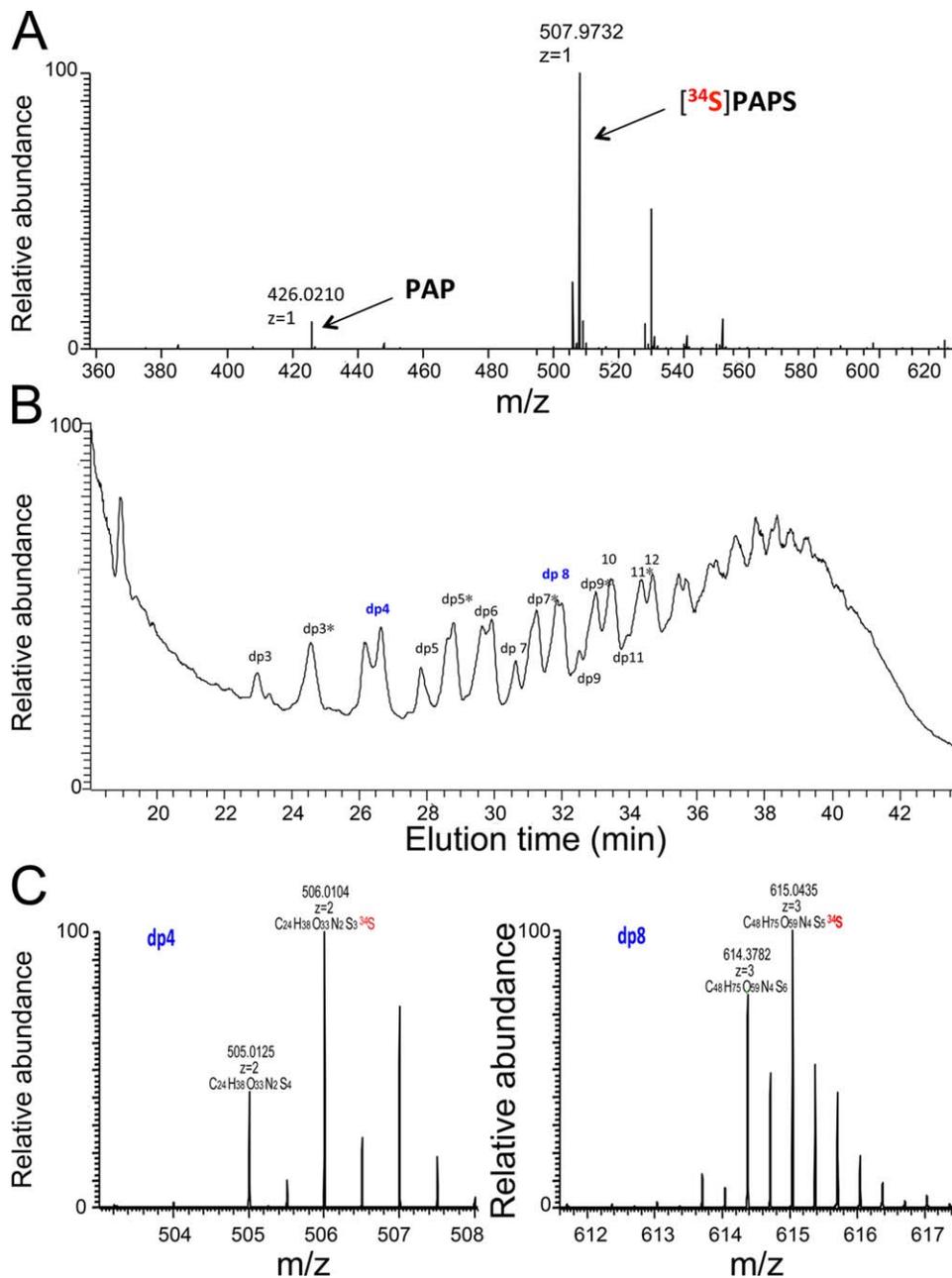


**FIGURE 4** One-dimensional  $^1\text{H-NMR}$  spectrum of *N*-sulfotestosteronan.

widely used clinical anticoagulant.<sup>12</sup> Previously, we identified a GAG synthase from *C. testosteroni*, which produced a novel GAG, testosteronan. Its backbone structure was determined as  $[-4\text{-D-GlcA-}\alpha\text{1,4-D-GlcNac-}\alpha\text{1-}]_n$ , similar to heparosan but with an  $\alpha$ -configuration in the GlcA residue. Due to the different stereochemistry of the GlcA glycosidic linkage, testosteronan is not susceptible to GAG-digesting enzymes. We also speculate that it would not be lysosomally degraded if administered to an animal. The similarity to heparosan led us to investigate whether we could synthesize a novel GAG with interesting biological activities that might potentially have long in vivo half-lives because their unusual glycosidic linkage should either prevent or slow their catabolic breakdown. It is necessary to point out that semi-synthetic GAGs, such as over sulfated chondroitin sulfate (OSCS) have shown unexpected toxicity<sup>13</sup> and substantial in vivo studies in animals would be necessary before sulfonated testosteronan derivatives could be considered for human use.

The results of the current study demonstrate the efficient chemical conversion of testosteronan to *N*-sulfotestosteronan. Furthermore, our study demonstrates that *N*-sulfotestosteronan could not be significantly modified by native sequence 2OST or 3OST-1 under typical reaction conditions. However, 6OST-1 and 6OST-3 converted up to  $\sim 20\%$  of *N*-sulfotestosteronan substrate into an 6-*O*-sulfo-*N*-sulfotestosteronan.

This conversion was confirmed using a new oxidative degradation method 11 allowing the preparation of oligosaccharides from a polysaccharide not susceptible to GAG digesting enzymes. By carefully controlling the reaction conditions depolymerization can be controlled to proceed to desired levels,



**FIGURE 5** MS analysis of  $^{34}\text{S}$ -labeled PAPS and of oligosaccharides prepared by free radical depolymerized 6-*O*-sulfo $^{34}\text{S}$ -*N*-sulfotestosteronan by HILIC LC-FTMS. **A:** Spectrum of  $^{34}\text{S}$ -labeled PAPS. **B:** Separation and LC-FTMS detection of total ion chromatograms of oxidatively depolymerized 6-*O*-sulfo-*N*-sulfotestosteronan synthesized by chemoenzymatic method. The number above each peak shows its degree of polymerization. Every odd number component shows two peaks, the one eluting first has one additional GlcNS residue, and the eluting second one labeled with an asterisk (\*) has one additional GlcA residue. **C:** As an example, dp4 and dp8 FTMS data were shown to confirm the  $^{34}\text{S}$ -labeled-sulfate has been added onto the *N*-sulfotestosteronan by FT-MS elemental component analysis within 3-ppm mass accuracy.

enabling even the analysis of oligosaccharides as large as dp20. NMR analysis showed that the *O*-sulfo group modification was at the C6-position of the glucosamine residue as expected.

Mass spectral analysis suggests that the 6-*O*-sulfo groups are uniformly distributed throughout the oligosaccharide products examined (ranging from dp4 through dp16), which were

**Table I** Modification of Polysaccharide Substrates with a Mixture of Biosynthetic Enzymes

Substrates	Sulfotransferases in Mixtures	<sup>35</sup> S Incorporation cpm (%)
<i>N</i> -Sulfotestosteronan	2OST, 6OST-1 and 6OST-3	251,220 (127%)
<i>N</i> -Sulfotestosteronan	2OST only	8610 (4%)
<i>N</i> -Sulfotestosteronan	6OST-1 and 6OST-3	256,840 (129%)
<i>N</i> -Sulfoheparosan (positive control)	6OST-1 and 6OST-3	198,650 (100%)
Negative control	No enzymes	510 (0%)

**Table II** Comparison of the Relative Susceptibility of the Mixture of 6OST-1 and 6OST-3 to *N*-Sulfotestosteronan and *N*-Sulfoheparosan

Polysaccharide	Sulfotransferase Enzymes	<sup>35</sup> S Incorporation cpm (%)
<i>N</i> -Sulfotestosteronan	6OST-1 and 6OST-3	114,950 (114%)
<i>N</i> -Sulfotestosteronan	3OST-1	3560 (2%)
<i>N</i> -Sulfoheparosan (positive control)	6OST-1 and 6OST-3	100,690 (100%)
Negative control	No enzyme	1130 (0%)

formed using oxidative cleavage. We conclude that the positioning of these 6-*O*-sulfo groups by 6OST-1 and 6OST-3 are most likely random based on the assumption that radical-based oxidation is a random process. Finally, SPR analysis confirmed that the 6-*O*-sulfo-*N*-sulfotestosteronan showed enhanced binding to FGF-1 and FGF-2.

## MATERIALS AND METHODS

### In Vitro Synthesis of Testosteronan

The polysaccharide was produced by extension of 1.9 mM *p*-nitrophenyl glucuronide primer (Calbiochem) with 5 mM UDP-GlcNAc (Sigma) and 5 mM UDP-GlcUA (Sigma) in 50 mM Tris, pH 7.2, 1 mM MgCl<sub>2</sub> at room temperature using an *E. coli*-derived recombinant CtTS-containing lysate (3.4 mg/mL total protein final; prepared as in Ref. 7) in a 9.6 mL reaction. After overnight polymerization, the protein was heat precipitated at 100°C for 2 min and the reaction mixture was clarified by centrifugation at 20,000g for 5 min. The supernatant was extracted three times using *n*-butanol, followed by a single chloroform extraction (all 1:1 vol/vol). The polymer in the aqueous phase was adjusted to 0.3M NaCl and then precipitated with three volumes of ethanol at 4°C overnight. The pellet with ~6 mg of polymer

(determined using carbazole assay with GlcA standard) was re-suspended in 5.6 mL water and subjected to strong anion exchange chromatography on a 5 mL HiTrap Q HP column (GE Healthcare) equilibrated in 0.2M ammonium formate. After washing with 10-column volumes (CV) of the same buffer, UDP and UDP-sugars were removed by a 4 CV step of 0.5M ammonium formate. To isolate two different polymer sizes, the column was eluted with steps of 0.8 and 1.0M ammonium formate (each 4 CV); the smaller chains are eluted with lower ionic strength than the larger chains. The pooled 0.8M and 1.0M fractions were separately lyophilized three times from water to remove the volatile buffer. The two testosteronan polymers were assessed using 6% PAGE in 1× TBE<sup>7</sup> using hyaluronan (HA) standards (HA LoLadder and other standards kindly provided by Hyalose, LLC, Oklahoma City, OK). The average molecular weights were obtained using SEC-MALLS with the parameters used for hyaluronan, a polysaccharide with the identical monosaccharide composition to testosteronan.<sup>14</sup>

### Synthesis of *N*-Sulfotestosteronan

*N*-Sulfotestosteronan was prepared according to a modified procedure using NMe<sub>3</sub>·SO<sub>3</sub> as the sulfonating agent.<sup>15</sup> Briefly, testosteronan (1.5 mg) was dissolved in 2M NaOH (375 μL) and stirred overnight at 60°C. The reaction mixture was cooled to 0°C and neutralized using 1M HCl (aq). To this reaction mixture, NMe<sub>3</sub>·SO<sub>3</sub> (4.5 mg) and

**Table III** 6-*O*-Sulfation of *N*-Testosteronan by Individual and Mixture of 6-*O*-Sulfotransferases

Polysaccharide	Sulfotransferase Enzymes	<sup>35</sup> S incorporation in cpm (%)
<i>N</i> -Sulfotestosteronan	6OST-1	4861 (144%)
<i>N</i> -Sulfotestosteronan	6OST-3	6796 (202%)
<i>N</i> -Sulfotestosteronan	6OST-1 and 6OST-3	5674 (169%)
<i>N</i> -Sulfoheparosan	6OST-1	3283 (98%)
<i>N</i> -Sulfoheparosan	6OST-3	3740 (111%)
<i>N</i> -Sulfoheparosan (positive control)	6OST-1 and 6OST-3	3359 (100%)
Negative control	No enzyme	101 (0%)

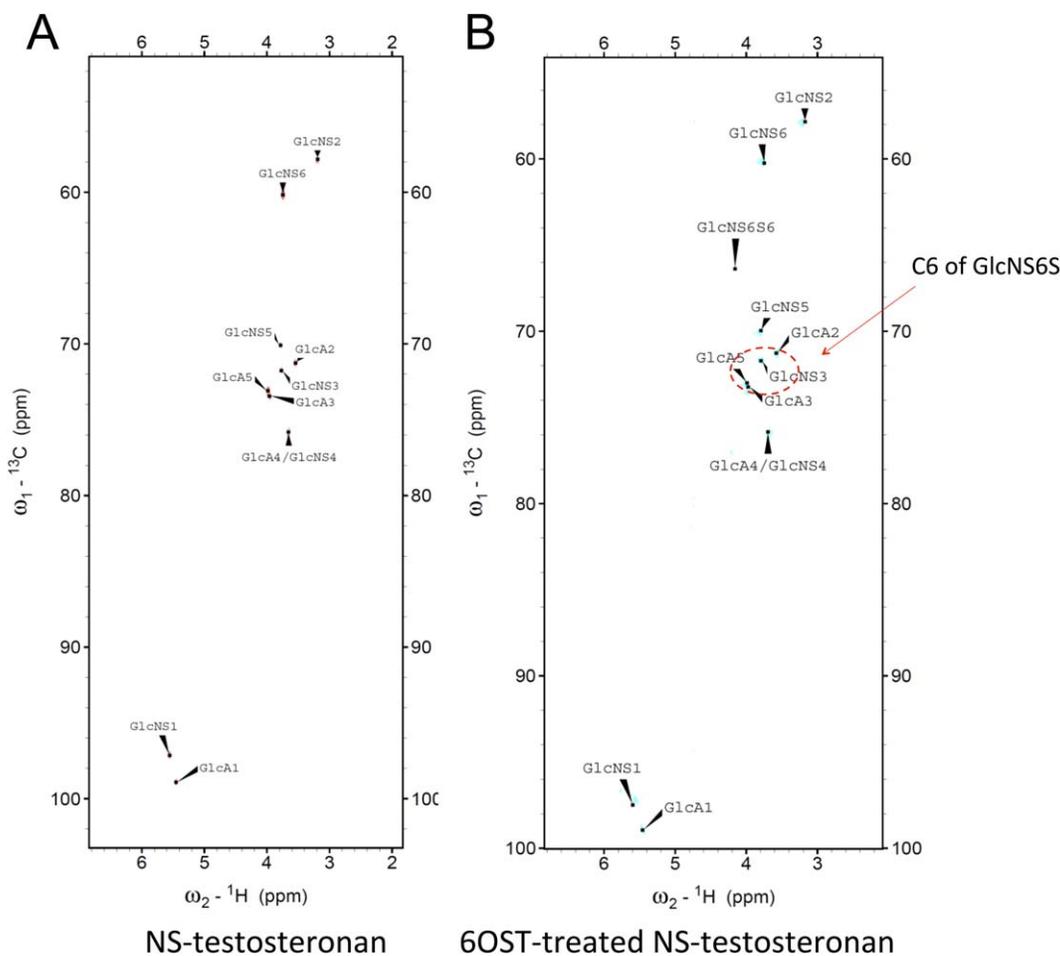


FIGURE 6 2D HMQC (800 MHz) comparison.

$\text{Na}_2\text{CO}_3$  (4.5 mg) were then added under argon atmosphere and stirred for 12 h at 45°C. Another portion of  $\text{NMe}_3\cdot\text{SO}_3$  (4.5 mg) and  $\text{Na}_2\text{CO}_3$  (4.5 mg) was added to the reaction mixture and stirred for 12 h at 45°C. The reaction mixture was cooled to 0°C to quench the reaction. *N*-Sulfotestosteronan was purified by loading onto a 3000 molecular weight cut-off (MWCO) Amicon ultra (Millipore) spin column pre-rinsed with 0.1 M NaOH. The spin column was washed exhaustively with water and the retentate containing *N*-sulfotestosteronan was collected and lyophilized, affording purified *N*-sulfotestosteronan (2.5 mg) for analysis and for further reactions. **Caution:** chemically sulfonated polysaccharides are known to sometimes cause lethal side effects when administered intravenously so caution must be used before in vivo evaluation of *N*-sulfotestosteronan.<sup>16</sup>

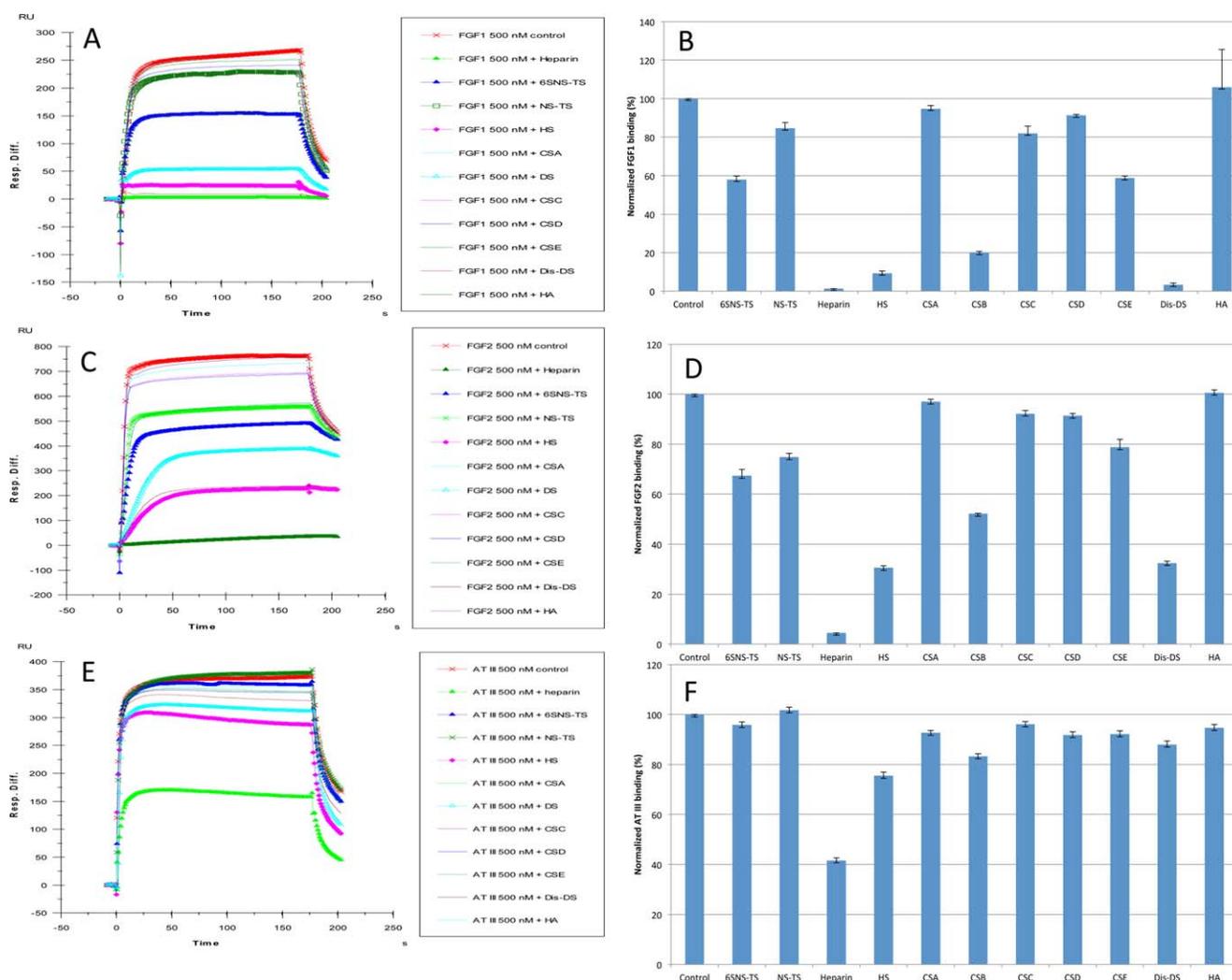
### Synthesis of $^{34}\text{S}$ -Labeled PAPS

Isotopically  $^{34}\text{S}$ -labeled PAPS was synthesized enzymatically using crude enzymes prepared as previously described.<sup>17</sup> The method was modified from a similar previously described synthesis of  $^{34}\text{S}$ -labeled PAPS substituting  $\text{Na}_2^{34}\text{SO}_4$  (from ISOFLEX USA). The reaction included 90 mM ATP, 100 mM  $\text{MgCl}_2$ , 1M LiCl, 0.8 mg/mL pyrophosphatase, 0.8 mg/mL KAST, 0.8 mg/mL APS Kinase, and 50 mM Tris-HCl at pH 8.0. The reaction was incubated at 30°C for 6 h. The

$^{34}\text{S}$ -labeled PAPS product was analyzed using PAMN-HPLC (polyamine II column, YMC America, Inc.) with a gradient as follows: 100% water for 10 min, followed with a linear gradient of 0–100% of 1M  $\text{KH}_2\text{PO}_4$  for 30 min, followed by 100% 1M  $\text{KH}_2\text{PO}_4$  for 15 min at a flow rate of 1 mL  $\text{min}^{-1}$  with UV 254 nm detection. Purification of PAPS was achieved on a DEAE-Sepharose fast flow column (GE Health; 1.5 cm  $\times$  60 cm). The DEAE column was washed with water, and PAPS was eluted with a gradient of 0–500 mM NaCl at 5.0 mL  $\text{min}^{-1}$  for 200 min. Fractions containing PAPS as determined by PAMN-HPLC were pooled and stored at  $-80^\circ\text{C}$ . The purity of the  $^{34}\text{S}$ -labeled PAPS product was assessed by MS analysis and determined to be 90%+ with less than 10% PAP contamination (Figure 5A).

### Screening of *N*-Sulfotestosteronan as an OST Substrate Using $^{35}\text{S}$ -Labeled PAPS

The susceptibilities of *N*-sulfotestosteronan to the sulfonation by different HS sulfotransferases were examined by reacting with the sulfotransferases in the presence of radioactively  $^{35}\text{S}$ -labeled PAPS. Briefly, *N*-sulfotestosteronan (10  $\mu\text{g}$ ) was incubated with a 6-*O*-sulfotransferase 1 (6-OST-1, 20  $\mu\text{g}$ ) in 200  $\mu\text{L}$  of buffer containing 50 mM MES (pH 7.0) and 60  $\mu\text{M}$  [ $^{35}\text{S}$ ]PAPS (the specific radioactivity of



**FIGURE 7** SPR analysis of protein-binding to GAGs. A: SPR Sensorgrams of solution GAGs/surface heparin competition on FGF1 binding. FGF1 concentration was 500 nM, and concentrations of GAGs in solution were 1000 nM. B: Bar graphs (based on triplicate experiments with standard deviation) of normalized FGF1 binding RU to surface heparin by competing with different GAGs. C: SPR Sensorgrams of solution GAGs/surface heparin competition on FGF2 binding. FGF2 concentration was 500 nM, and concentrations of GAGs in solution were 1000 nM. D: Bar graphs (based on triplicate experiments with standard deviation) of normalized FGF2 binding RU to surface heparin by competing with different GAGs. E: SPR Sensorgrams of solution GAGs/surface heparin competition on ATIII binding. AT concentration was 500 nM, and concentrations of GAGs in solution were 1000 nM. F: Bar graphs (based on triplicate experiments with standard deviation) of normalized AT binding RU to surface heparin by competing with different GAGs.

[<sup>35</sup>S]PAPS was 20,000 cpm/nmole) at 37°C for 2 h. The reaction mixture was then subjected to a DEAE-column (200 µL) washed with 250 mM NaCl. The <sup>35</sup>S-labeled testosterone product was eluted from the DEAE column with 1M NaCl. The amount of radioactivity in the eluent was measured by scintillation counting. To measure the susceptibility of *N*-sulfotestosteronan to 2-*O*-sulfotransferase (2-OST), 6-*O*-sulfotransferase 3 (6-OST-3), and 3-*O*-sulfotransferase 1 (3-OST-1), a very similar procedure was followed by replacing 6-OST-1 with the

appropriate enzyme. The positive control substrates for 6-OST-1, 6-OST-3, and 2-OST modification are *N*-sulfated heparosan.

### Synthesis of <sup>34</sup>S-Labeled 6-*O*-sulfo-*N*-sulfotestosteronan

*N*-Sulfotestosteronan (1 mg) was incubated with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH = 7.0), 6-OST-1 (0.5 mg), 6-

OST-3 (0.5 mg), and 60  $\mu\text{M}$  PAPS in 2 mL. The reaction mixture was incubated at 37°C overnight, and the product was purified by a DEAE column (1 mL). The product was eluted from the DEAE column using 1M NaCl. The product was dialyzed against 25 mM ammonium bicarbonate using 3500 MWCO membrane.

### Copper(II)-Catalyzed Depolymerization of 6-O-Sulfo-N-sulfotestosteronan

Oligosaccharides of 6-O-sulfo-N-sulfotestosteronan were obtained by controlled oxidative depolymerization using hydrogen peroxide and cupric acetate. The 6-O-sulfo-N-sulfotestosteronan polysaccharide (100  $\mu\text{g}$ ) was dissolved in 100  $\mu\text{L}$  0.1M sodium acetate-acetic acid solution containing 0.2 mM copper (II) acetate and adjusted to pH 7.0. Hydrogen peroxide (4  $\mu\text{L}$  of 3% solution) was added with mixing and reacted at 45°C for 3 h. Sodium bisulfite was added to terminate the reaction by removing excess unreacted hydrogen peroxide and the reaction mixture was then lyophilized.

### Mass Spectral Analysis of Free Radical Depolymerized Oligosaccharides

A Luna HILIC column (2.0  $\times$  150 mm<sup>2</sup>, 200 Å, Phenomenex, Torrance, CA) was used to separate the oligosaccharide mixture. Mobile phase A was 5 mM ammonium acetate prepared with high performance liquid chromatography (HPLC) grade water. Mobile B was 5 mM ammonium acetate prepared in 98% HPLC grade acetonitrile with 2% of HPLC grade water. After injection of 8.0  $\mu\text{L}$  oligosaccharide mixture (1.0  $\mu\text{g}/\mu\text{L}$ ) through an Agilent 1200 autosampler, HPLC binary pump was used to deliver the gradient from 10% A to 35% A over 40 min at a flow rate of 150  $\mu\text{L}/\text{min}$ . The LC column was directly connected online to the standard electrospray ionization source of LTQ-Orbitrap XL FTMS (Thermo Fisher Scientific, San-Jose, CA). The source parameters for FT-MS detection were optimized using Arixtra® (purchased at a pharmacy) to minimize the in-source fragmentation and sulfate loss and maximize the signal/ noise in the negative-ion mode. The optimized parameters, used to prevent in-source fragmentation, included a spray voltage of 4.2 kV, a capillary voltage of -40 V, a tube lens voltage of -50 V, a capillary temperature of 275°C, a sheath flow rate of 30, and an auxiliary gas flow rate of 6. External calibration of mass spectra routinely produced a mass accuracy of better than 3 ppm. All FT mass spectra were acquired at a resolution 60,000 with 300–2000 Da mass range.

### 1D and 2D NMR Analyses

All samples were dissolved in 400  $\mu\text{L}$  <sup>2</sup>H<sub>2</sub>O (99.9%, Sigma-Aldrich, St. Louis, MO) and lyophilized three-times to remove the exchangeable protons. The samples were dissolved in 400  $\mu\text{L}$  <sup>2</sup>H<sub>2</sub>O and transferred to NMR microtubes. All NMR experiments were performed at 298 K on Bruker Advance II 800 MHz with Topspin 2.1.6 software. One-dimensional <sup>1</sup>H spectra were recorded for 32 scans and two-dimensional HMQC spectra were recorded for 16 scans.

### Protein-Binding Competition Study Using SPR

To test the protein-binding activity of 6-O-sulfo-N-sulfotestosteronan, SPR competition assays were performed with three heparin-binding proteins: FGF1, FGF2, or AT III (Cutter Biological, Berkeley, CA). SPR

measurements were performed on a BIAcore 3000 (GE Healthcare, Uppsala, Sweden) operated using BIAcore 3000 control and BIAevaluation software (version 4.0.1). The GAGs used in this study were porcine intestinal heparin (16 kDa) and porcine intestinal HS (11,700 kDa) (Celsus Laboratories, Cincinnati, OH); chondroitin sulfate A (CS-A) (20 kDa; porcine rib cartilage; Sigma, St. Louis, MO), dermatan sulfate (DS) (also known as chondroitin sulfate B; 30 kDa; porcine intestine; Sigma), chondroitin sulfate C (CS-C) (20 kDa; shark cartilage; Sigma), chondroitin sulfate D (CS-D) (20 kDa; whale cartilage; Seikagaku, Tokyo, Japan), chondroitin sulfate E (CS-E) (20 kDa; squid cartilage; Seikagaku), and hyaluronic acid sodium salt (100 kDa; *Streptococcus zooepidemicus*; Sigma). Dermatan disulfate (Dis-DS) (4,6-di-O-sulfo-DS; 33 kDa), was prepared through the chemical 6-O-sulfonation of dermatan sulfate (Celsus) and is an inhibitor of thrombin and complement activation.<sup>18</sup>

The biotinylated heparin was prepared by reaction of sulfo-N-hydroxysuccinimide long-chain biotin (Pierce, Rockford, IL) with free amino groups of unsubstituted glucosamine residues in the polysaccharide chain following a published procedure.<sup>19</sup> The biotinylated heparin was immobilized to streptavidin (SA) chip (GE Healthcare, Uppsala, Sweden) based on the manufacturer's protocol. In brief, 20- $\mu\text{L}$  solution of the heparin-biotin conjugate (0.1 mg/mL) in HBS-EP running buffer was injected over flow cell 2 (FC2) of the SA chip at a flow rate of 10  $\mu\text{L}/\text{min}$ . The successful immobilization of heparin was confirmed by the observation of a ~150 resonance unit (RU) increase in the sensor chip. The control flow cell (FC1) was prepared by 1 min injection with a saturated biotin solution.

To perform SPR competition assay, protein (500 nM) mixed with 1000 nM of 6-O-sulfo, N-sulfotestosteronan (or other GAGs as positive or negative controls) in HBS-EP buffer were injected over heparin chip at a flow rate of 30  $\mu\text{L}/\text{min}$ , respectively. At the end of the sample injection, the same buffer was flowed over the sensor surface to facilitate dissociation. After a 2 min dissociation time, the sensor surface was regenerated by injecting with 30  $\mu\text{L}$  of 2M NaCl to get fully regenerated surface. The response was monitored as a function of time (sensorgram) at 25°C. For each set of competition experiments on SPR, a control experiment (only protein without any GAGs) was performed to make sure the surface was completely regenerated and that the results obtained between runs were comparable.

Authors thank Bruce Baggenstoss for MALLS analysis and Priscilla Paul for synthesizing the 34S PAPS used in these experiments.

### REFERENCES

- Vann, W. F.; Schmidt, M. A.; Jann, B.; Jann, K. *Eur J Biochem* 1981, 116, 359–364.
- DeAngelis, P. L. *Glycobiology* 2002 12, 9R–16R.
- DeAngelis, P. L.; White, C. L. *J Bacteriol* 2002, 186, 8529–8532.
- Capila, I.; Linhardt, R. J. *Angew Chem Int Ed* 2002, 41, 390–412.
- Liu, R.; Xu, Y.; Chen, M.; Weïwer, M.; Zhou, X.; Bridges, A. S.; DeAngelis, P. L.; Zhang, Q.; Linhardt, R. J.; Liu, J. *J Biol Chem* 2010, 285, 34240–34249.
- Xu, Y.; Masuko, S.; Takieddin, M.; Xu, H.; Liu, R.; Jing, J.; Mousa, S. A.; Linhardt, R. J.; Liu, J. *Science* 2011, 334, 498–501.
- Otto, N. J.; Solakyildirim, K.; Linhardt, R. J.; DeAnglelis, P. L. *Glycobiology* 2011, 21, 1331–1340.

8. Ma, Y.-F.; Zhang, Y.; Zhang, J.-Y.; Chen, D.-W.; Zhu, Y.; Zheng, H.; Wang, S.-Y.; Jiang, C.-Y.; Zhao, G.-P.; Liu, S.-J. *Appl Environ Microbiol* 2009, 75, 6812–6819.
9. Wang, Z.; Yang, B.; Zhang, Z.; Ly, M.; Takiuddin, M.; Mousa, S.; Liu, J.; Dordick, J. S.; Linhardt, R. J. *Appl Microbiol Biotechnol* 2011, 91, 91–99.
10. Wang, Z.; Li, J.; Cheong, S.; Bhaskar, U.; Onishi, A.; Zhang, F.; Dordick, J. S.; Linhardt, R. J. *J Biotechnol* 2011, 156, 188–196.
11. Wu, M.; Xu, S.; Zhao, J.; Kang, H.; Ding, H. *Carbohydr Polym* 2010, 80, 1116–1124.
12. Linhardt R. J. *J Med Chem* 2003, 46, 2551–2554.
13. Liu, H.; Zhang, Z.; Linhardt, R. J. *Nat Prod Rep* 2009, 26, 313–321.
14. Jing, W.; DeAngelis, P. L. *J Biol Chem* 2004, 279, 42345–42349.
15. Maruyama, T.; Toida, T.; Imanari, T.; Yu, G.; Linhardt, R. J. *Carbohydr Res* 1998, 306, 35–43.
16. Li, B.; Suwan, J.; Martin, J. G.; Zhang, F.; Zhang, Z.; Hoppensteadt, D.; Clark, M.; Fareed, J.; Linhardt, R. J. *Biochem Pharmacol* 2009, 78, 292–300.
17. Zhou, X.; Chandarajoti, K.; Pham, T. Q.; Liu, R.; Liu, J. *Glycobiology* 2011, 21, 771–780.
18. Brister, S. J.; Buchanan, M. R.; Griffin, C. C.; Van Gorp, C. L.; Linhardt, R. J. US Patent no. 5,922,690, 1999.
19. Hernaiz, M.; Liu, J.; Rosenberg, R. D.; Linhardt, R. J. *Biochem Biophys Res Commun* 2000, 276, 292–297.

*Reviewing Editor: Eric J. Toone*