HHS Public Access

Author manuscript

Carbohydr Polym. Author manuscript; available in PMC 2016 May 20.

Published in final edited form as:

Carbohydr Polym. 2015 May 20; 122: 399–407. doi:10.1016/j.carbpol.2014.10.054.

Combinatorial one-pot chemoenzymatic synthesis of heparin

Ujjwal Bhaskar[a,f], **Guoyun Li**[b,f], **Li Fu**[b,f], **Akihiro Onishi**[c,f], **Matt Suflita**[c,f], **Jonathan S. Dordick**[a,c,d,e,f], and **Robert J. Linhardt**[a,b,c,d,e,*]

[a]Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY, USA

[b]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, NY, USA

[c]Department of Biology, Rensselaer Polytechnic Institute, Troy, NY, USA

[d]Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY, USA

[e]Department of Materials Science and Engineering, Rensselaer Polytechnic Institute, Troy, NY, USA

[f]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA

Abstract

Contamination in heparin batches during early 2008 has resulted in a significant effort to develop a safer bioengineered heparin using bacterial capsular polysaccharide heparosan and recombinant enzymes derived from the heparin/heparan sulfate biosynthetic pathway. This requires controlled chemical *N*-deacetylation/ *N*-sulfonation of heparosan followed by epimerization of most of its glucuronic acid residues to iduronic acid and *O*-sulfation of the C2 position of iduronic acid and the C3 and C6 positions of the glucosamine residues. A combinatorial study of multi-enzyme, one-pot, *in vitro* biocatalytic synthesis, carried out in tandem with sensitive analytical techniques, reveals controlled structural changes leading to heparin products similar to animal- derived heparin active pharmaceutical ingredients. Liquid chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy analysis confirms an abundance of heparin's characteristic trisulfated disaccharide, as well as 3-*O*-sulfo containing residues critical for heparin binding to antithrombin III and its anticoagulant activity. The bioengineered heparins prepared using this simplified one-pot chemoenzymatic synthesis also show *in vitro* anticoagulant activity.

* To whom correspondence should be addressed, Phone: +1-518-2763404, Fax: +1- 518-2763405, linhar@rpi.edu, 4005C Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 8th Street, Troy NY US: 12180.

Keywords

Bioengineered heparin; one-pot synthesis; liquid chromatography-mass spectrometry; United States Pharmacopeia; nuclear magnetic resonance spectroscopy

1. Introduction

Heparin, the first biopolymeric drug, possesses a wide range of structural heterogeneity owing to its biosynthesis (Bhaskar et al., 2012). Heparin's diverse fine structure is further complicated by an animal-sourced, tissue-based recovery, leading to considerable structural differences within commercial heparin active pharmaceutical ingredients (APIs) (Linhardt & Gunay, 1999). Serious concerns about control of livestock, the primary source of heparin, have been raised since the 1990s following a series of incidents involving bovine spongiform encephalopathy, viral infections and prion contamination (Wilesmith, Wells, Cranwell, & Ryan, 1988). Lack of quality control during initial recovery stages led to adulteration of the pharmaceutical heparin supply with oversulfated chondroitin sulfate (OSCS), resulting in an international crisis in 2008 associated with over 100 deaths reported in US alone (Guerrini et al., 2008; Liu et al., 2009).

The inherent problems with animal tissue-based heparin production have motivated us to develop a commercially feasible chemoenzymatic heparin preparation process (Zhang et al., 2008). This is based on bacterial fermentation of *Escherichia coli* K5 to generate a capsular polysaccharide heparin precursor, which is then chemically *N*-deacetylated and *N*-sulfonated (Hickey, Bhaskar, Linhardt, & Dordick, 2013; Wang et al., 2011). The iterative application of selected recombinant enzymes, derived from the heparin biosynthetic pathway and expressed in *E. coli*, epimerize uronic acid residues and sulfates the C2, C3 and C6 positions (Hickey, Bhaskar, Linhardt, & Dordick, 2013; Wang et al., 2011). In summary, C5-epimerase epimerizes uronic acid residues, followed by sulfation at the C2 position by 2-*O*-sulfotransferase (2-OST) in the presence of an aryl sulfotransferase IV (AST IV) based cofactor regeneration system (Bethea, Xu, Liu, & Pedersen, 2008; Burkart, Izumi, Chapman, Lin, & Wong, 2000; Sheng, Xu, Dulaney, Huang, & Liu, 2012; Zhang et al., 2008). This is followed by sulfation at the C6 position by two isoforms of 6-*O*-sulfotransferase (6-OST-1 & −3) in the presence of the cofactor regeneration system leading to generation of non-anticoagulant heparin structure (Chen et al., 2005; Restaino et al., 2013; Zhang et al., 2001; Zhang et al., 2008). 3-*O*-sulfotransferase-1 (3-OST-1) then sulfates the C3 position, also in the presence of the cofactor regeneration system to generate anticoagulant heparin (Myette et al., 2002; Zhang et al., 2008). A similar sequential approach led to another version of bioengineered heparin derived from partially *N*-deacteylated/*N*-sulfonated heparosan as substrate (Wang et al., 2011). This chemoenzymatic approach has also been employed to generate an analogue of ultra low molecular weight heparin (ULMWH), Arixtra (Xu et al., 2011).

The total synthesis of full length heparin polysaccharides is considered infeasible owing to large number of modest yield steps and side product formation (Bhaskar et al., 2012; DeAngelis, Liu, & Linhardt, 2013; Driguez, Potier, & Trouilleux, 2014; Petitou et al., 1987).

One-pot chemical synthesis in organic chemistry is frequently employed for simplified synthesis of glycoconjugates and sugar building blocks (Koeller & Wong, 2000). One-pot chemical synthesis of heparin oligosaccharides from sugar building blocks with low to moderate overall yield has been previously described (Polat & Wong, 2007; Wang et al., 2010). This one step synthesis enables high speed processing of analogues with increased overall process yield. Combinatorial one-pot synthesis can potentially be used towards preparation of heparin mimetic microarrays for deciphering the effect of structural heterogeneity on structure activity relationship (SAR) and heparin-protein interactions (Capila & Linhardt, 2002; Feizi, 2003; Noti, de Paz, Polito, & Seeberger, 2006; Wang et al., 2010). As an alternative to the sequential process design (Figure 1), we aimed at development of a one-pot chemoenzymatic synthesis of heparin from *N*-sulfo heparosan leading to generation of biologically active bioengineered heparin products.

2. Methods

2.1. Recombinant enzymes and N-sulfo heparosan preparation

Recombinant *E. coli* strains expressing fusion proteins of C5-epi, 2-OST, 6-OST- 1, 6-OST-3, 3-OST-1 and AST IV were grown in LB medium (MP Biomedicals) at 37 °C using rotary air shaker (New Brunswick Scientific Innova 44R) (Burkart et al., 2000; Chen et al., 2005; Chen, Jones, & Liu, 2007; Zhang et al., 2008). Recovered cell pellets were stored at −80 °C until purified. Recombinant enzymes were purified from clarified cell lysates using either MBP- or His- affinity chromatography. Briefly, cell pellets were re-suspended in respective extraction buffers, lysed and centrifuged to obtain a clear cell lysate. The clarified cell lysate was then loaded onto respective affinity column connected to a GE Äkta purifier system. Elution was carried out using either high maltose (for MBP tagged proteins) or high imidazole (for His tagged proteins) containing buffers. The eluted protein was stored at −80 °C with 10-15 % glycerol, until further use.

E. coli K5 capsular polysaccharide, heparosan, was purified from the supernatant of fed batch fermentation using ammonium sulfate precipitation (Wang et al., 2011). *N*-sulfo heparosan (NSH) was prepared by partial chemical *N*-deacetylation and *N*-sulfonation of heparosan as described earlier (Wang et al., 2011). Titanium dioxide based depolymerization was employed to reduce the molecular weight, if required (Higashi et al., 2011). Analysis of the NSH product obtained indicated following characteristics: Number average molecular weight (M_n) = 11,100 ± 200 Da; Weight average molecular weight (M_w) = 18,800 ± 200 Da; Polydispersity index (PDI) = 1.69 ± 0.01; % *N*-sulfo groups = 81.4 ± 0.9%.

2.2. Combinatorial one-pot chemoenzymatic synthesis of heparin

Initial combinatorial chemoenzymatic heparin synthesis experiments were carried out using 1 mg of NSH as substrate in 50 mM MES, pH 7 buffer. The reaction mixture consisted of 0.1 mg/mL of NSH substrate and 300 μM of sulfo group donor 3′- phosphoadenosine-5′-phosphosulfate (PAPS), an essential cofactor for sulfotransferases. A 5 mM concentration of *p*-nitrophenyl sulfate (PNPS, Sigma) was used in the reaction mixture as the sulfo group donor to establish an AST IV based co-factor recycling system for PAPS regeneration

(Burkart et al., 2000). The control reaction contained 0.1 mg/mL each of C5-epi, 2OST, 6-OST-1, 6-OST-3 and AST IV. The 3-OST-1 was not included in the initial combinatorial synthesis of heparin to reduce product complexity in order to simplify analysis (3-O-sulfo group containing sequences are resistant to heparin lyases and thus do not afford disaccharide products). The concentration of three groups of enzymes was varied combinatorially by either 2-fold (0.2 mg/mL) or 10-fold (1 mg/mL): 1. C5-Epi & 2-OST individually as well as together; 2. 6-OST-1 & 3 individually as well as together; and 3. AST IV alone. All enzymes not varied were maintained constant at 0.1 mg/mL, as was the reaction control. The reaction mixtures were incubated overnight at 37 °C. The resulting products were analyzed using disaccharide analysis and optimal conditions were identified.

In the second set of experiments, 2 mg of the product formed using the two best conditions, identified through the combinatorial experiments, were treated with 3-OST-1 at a final concentration of 0.1 mg/mL, after the end of overnight incubation and the reaction mixture was incubated for an additional day. The product was purified using anion exchange chromatography and further evaluated using chemical and biological assays.

2.3. Strong anion exchange (SAX) purification of bioengineered heparin

Reaction product, obtained after the second enzymatic one-pot preparation, was boiled and centrifuged, and the supernatant was filtered using a 0.22 μm filter. The clarified permeate was then dialyzed using centrifugal ultrafiltration units (Amicon centrifugal filter units, Millipore) and DI water. Dialyzed polysaccharide solution was then loaded onto a 20 mL Q-Sepharose fast flow (GE life sciences) strong anion exchange (SAX) glass column connected to a GE *Äkta* purifier FPLC system. Prior to loading the sample, Q-Sepharose column was washed with 4 column volumes of DI water, 4 column volumes of 20 % v/v ethanol and 4 column volumes of DI water. After loading the sample, column was washed using 4 column volumes of buffer A (DI water) and 4 column volumes of 0.4 M NaCl by mixing buffer A and buffer B (2 M NaCl in DI water). This was followed by step elution at 2 M NaCl by buffer B. Fractions eluted with 2 M salt were collected, dialyzed and lyophilized. These samples were used for further analysis.

2.4. Enzymatic digestion for disaccharide analysis and tetrasaccharide mapping

For disaccharides analysis, heparin lyases 1, 2, and 3 (10 mU each) in 5 μL of 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer (pH 7.4) were added to 10 μg heparin sample in 100 μL of distilled water and incubated at 35 °C for 10 h to degrade heparin sample completely (Yang, Chang, Weyers, Sterner, & Linhardt, 2012). The products were recovered by centrifugal filtration using a YM-10 micro-concentrator (Millipore), and the heparin disaccharides were recovered in the flow-through and freeze-dried. The digested heparin disaccharides were dissolved in water to concentration of 50-100 ng/2 μL for liquid chromatography (LC)-mass spectrometric (MS) analysis.

For tetrasaccharide analysis, 40 mU of heparin lyase 2 in 20 μL of 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer (pH 7.4) was added to 50-100 μg heparin sample in 100 μL of distilled water and incubated at 35 °C for 10 h. The resulting product was freeze-dried for further LC-MS analysis (Li et al., 2014).

2.5. Disaccharide analysis and tetrasaccharide mapping using liquid chromatography-mass spectrometry

LC-MS analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE) equipped with a 6300 ion trap and a binary pump followed by a UV detector equipped with a high-pressure cell. The column used was a Poroshell 120 C18 column (2.1 × 100 mm, 2.7 μm, Agilent, USA). Eluent A was water/acetonitrile (85:15) *v/v*, and eluent B was water/acetonitrile (35:65) *v/v*. Both eluents contained 12 mM tributylamine (TrBA) and 38 mM ammonium acetate with pH adjusted to 6.5 with acetic acid. For disaccharide analysis, a gradient of solution A for 5 min followed by a linear gradient from 5 to 15 min (0-40% solution B) was used at flow rate of 150 μL/min.

For tetrasaccharide analysis, a gradient of solution A for 2 min followed by a linear gradient from 2 to 40 min (0-30 % solution B) was used at flow rate of 150 μL/min. The column effluent entered the source of the electrospray ionization (ESI)-MS for continuous detection by MS. The electrospray interface was set in negative ionization mode with a skimmer potential of -40.0 V, a capillary exit of -40.0 V, and a source temperature of 350 °C, to obtain the maximum abundance of the ions in a full-scan spectrum (200-1500 Da). Nitrogen (8 L/min, 40 psi) was used as a drying and nebulizing gas.

Quantification analysis of di- or tetra-saccharides was performed using calibration curves established by separation of increasing amounts of unsaturated di- or tetrasaccharide standards (0.1, 0.5, 1, 5, 10, 20, 50, 100 ng/each). Linearity was assessed based on the amount of di- or tetra-saccharide and peak intensity in extracted ion chromatogram (EIC). Disaccharide and tetrasaccharide analyses were performed in duplicates and singlet respectively.

2.6. NMR spectroscopy

Heparin products were analyzed by ^{1}H-nuclear magnetic resonance (NMR) and two-dimensional NMR spectroscopy heteronuclear single quantum coherence (HSQC) to fully characterize its structure (Fu et al., 2013). All NMR experiments were performed on a Bruker Advance II 600 MHz spectrometer (Bruker BioSpin, Billerica, MA) with Topsin 2.1.6 software (Bruker). Briefly, samples were each dissolved in 0.5 mL D_2O (99.996%, Sigma) and freeze-dried repeatedly to remove the exchangeable protons. The samples were re-dissolved in 0.4 mL D_2O and transferred to NMR micro tubes (OD 5 mm, Norell,tubes). The conditions for one-dimensional ^{1}H-NMR spectra were as follows: wobble sweep width of 12.3 kHz, acquisition time of 2.66 S, and relaxation delay of 8 S at 298 K. The conditions for two-dimensional HSQC spectrum were as follows: 32 scans, sweep width of 6.15 kHz, acquisition time of 0.33 S, and relaxation delay of 0.90 S.

2.7. Molecular weight determination

Molecular weight and polydispersity of prepared heparin products was determined using size exclusion chromatography as described earlier (Zhang et al., 2011). TSK-GEL G3000PWxl size exclusion column (Tosoh Bioscience), maintained at 40 °C with an Eppendorf column heater, was connected to a HPLC system consisting of a Shimadzu

LC-10Ai pump, a Shimadzu CBM-20A controller and a Shimadzu RID-10A refractive index detector. The mobile phase consisted of 0.1 M $NaNO_3$. A sample injection volume of 20 μL and a flow rate of 0.6 mL/min were used. The SEC chromatograms were recorded with the LCsolution Version 1.25 software and molecular weight properties determined using the "GPC Postrun" function. Heparin sodium oligosaccharides (Iduron, UK) of different molecular weights (2687 Da, 4300 Da, 5375 Da, 6449 Da and 8060 Da) were used as calibrants for the standard curve. The molecular weight measurements were carried out in duplicates.

2.8. In vitro anticoagulant activity measurement

The anti-factor Xa and anti-factor IIa activities of heparin products was determined by two stage Biophen heparin anti-Xa and anti-IIa colorimetric kits (Aniara, US) using manufacturer's supplied instructions (Fu et al., 2014). USP heparin (Celsus Laboratories) with anti-IIa activity of 200 IU/mg and anti-Xa/anti-IIa activity ratio of 1 was used as a standard for the colorimetric assays. The *in vitro* activity measurements were carried out in duplicates.

3. Results and Discussion

3.1. Effect of C5-epi and 2-OST on non-anticoagulant heparin composition in one-pot synthesis

On treatment with a mixture of heparin lyase 1, 2, and 3, heparin is degraded into unsaturated disaccharides along with a small quantity of lyase resistant 3-*O*-sulfo group-containing tetrasaccharide, associated with the AT binding site. The trisulfated (TriS) disaccharide comprising of IdoA2S and GlcNS6S is the major disaccharide sequence (66-85%) in porcine heparins (Zhang et al., 2011). The microheterogeneity of the saccharides in bioengineered heparin is critical for chemical and biological equivalence to porcine heparin and the TriS disaccharide content equivalence is an important prerequisite for the regulatory approval of any generic product. Our chemoenzymatic process begins with partial chemical *N*-deacetylation/*N*-sulfonation of heparosan, as an alternative to enzymatic modification by the *N*-deacetylase/*N*- sulfotransferase (NDST) family of enzymes *in vivo*. C5-epi catalyzes the isomerization of GlcA residue to an IdoA residue in NSH flanked by GlcNS or GlcN. The reversible biphasic catalytic mechanism of C5-epi adds further complexity (Sheng et al., 2012). Addition of a sulfo group derived from PAPS at the C2 position of IdoA by 2-OST renders the isomerization irreversible and drives the overall equilibrium towards an IdoA rich 2-*O*-sulfo group-containing product. The wide variation in potential substrates with regards to sulfation levels (0-3 sulfo groups/disaccharide) and polymer chain lengths, coupled with lack of reliable real time assays, further complicate our understanding of this C5-epimerization/2-*O*-sulfotransferase step. Often, additional enzymatic modifications are required to achieve a high level of 2-*O*-sulfo groups and a high content of IdoA, comparable to that observed in porcine heparin (Xiong et al., 2013).

Based on these considerations, we focused on combinatorial modulation of C5- epi/2OST activity while maintaining the other required biosynthetic enzymes (except 3-OST-1) at control levels. The control reaction (**1**) was designed with a 1:1 enzyme to substrate (E:S)

ratio by mass, similar to previous work (Wang et al., 2011; Zhang et al., 2008). The C5-epi and 2-OST E:S ratio was increased by either two-fold or ten-fold, individually and together, with respect to the control. The disaccharide composition (mass percentages) of the digested products, as determined by LC-MS quantification, is presented in Table 1. Reaction **1** contained a moderate level of TriS disaccharide (~ 35.1%) with significant formation of NS2S (~ 24.6%) and NS6S (~ 17.5%) disaccharides. Variation of C5-epi alone (Reactions **2** and **3**) had relatively little impact on the overall structure and resulted in similar composition of TriS, NS2S and NS6S. However, a similar variation in 2-OST had a profound effect on the resulting disaccharide composition. Use of a ten-fold higher E:S ratio for 2-OST alone (Reaction **5**) led to the formation of a product with high TriS disaccharide content (~ 58.9%). Use of ten-fold higher E:S ratio for C5-epi and 2-OST (Reaction **7**) led to further improved TriS content (~ 67.9%) with significant NS2S (~ 15.7%) and NS6S (~ 7.8%) disaccharide formation. The low NS content (~ 1.6%) of Reaction **7** signifies near complete conversion of available substrate sites into NS2S, NS6S and TriS disaccharides in one single step. Use of excess 2-OST, in Reactions **5** and **7**, led to products with similar core structure as porcine heparins. This is indicative of the action of 2-OST being the rate-limiting step and makes 2-OST an ideal target for protein engineering to improve its activity. Although better results were achieved with ten-fold higher C5-epi and 2-OST compared to the ten-fold higher 2-OST alone, NMR spectroscopy is clearly required to accurately assess the role of C5-epi on the chemical composition of the heparin product (see Section 3.6). The formation of minor disaccharides particularly 6S, 2S and 2S6S in all combinatorial one-pot syntheses suggests the complexity introduced by substrate sites tied to the acetylated regions of the polysaccharide chains towards enzymatic action. It should be noted that the molar enzyme to substrate ratio is still highly favorable for these one-pot synthesis owing to large number of available substrate sites (~ 20 per chain) and high molar mass of enzymes. Ten-fold higher E:S ratio of 2-OST alone or with C5-epi were chosen for incorporation of 3-*O*-sulfo groups to obtain heparin with anticoagulant activity.

3.2. Effect of 6-OST-1 & 6-OST-3 on non-anticoagulant heparin composition in one-pot synthesis

*T*he 6-*O*-sulfotransfrase enzyme family *in vivo* contains three different isoforms, and sulfates the C6 position on GlcNS or GlcNAc, particularly those in GlcNS-IdoA2S domains. The three different isoforms possess similar substrate specificity and can introduce 6-*O*-sulfo groups into polysaccharide chains with various levels of *N*-sulfo and 2-*O*-sulfo group substitution, however, the 6-OST-1 prefers the absence of 2-*O*-sulfo group (Bhaskar et al., 2012; Habuchi et al., 2003; Sterner et al., 2014).

The iterative bioengineered heparin chemoenzymatic process developed in our laboratory (Wang et al., 2011) utilizes 6-OST-1 & 3 isoforms. Thus, in the combinatorial experiments, the E:S mass ratio for 6-OST-1 & −3 was varied two-fold and ten-fold, alone or in combination, with respect to the control. The resulting product disaccharide compositions are presented in Table 2. A two-fold increase in E:S ratio for 6-OST-1 (Reaction **8**) or 6-OST-3 (Reaction **10**) resulted in an increase in the formation of TriS disaccharide content. In contrast, a ten-fold increase of 6-OST-1 (Reaction **9**) or 6-OST-3 (Reaction **11**) E:S ratio led to a decrease in TriS content. The impact of altered 6- OST-1 E:S ratio was more evident on

the minor disaccharide composition and can be explained by the relative rates of *O*-sulfation and substrate specificity. The total 6-OST, sum of 6-OST-1 & −-3 mass, in reaction mixtures **8** and **10** is 1.5 times higher than Reaction **1**, without any change in the amount of 2-OST. With the C5-epi/2OST composition unchanged and comparable to 6OSTs the rate of 2-*O*-sulfonation is similar to 6-*O*-sulfation and results in products qualitatively similar to Reaction **1**. The percentage of 2-*O*-sulfation, the sum of NS2S and TriS, remains the same in Reactions **8** and **10**, while a small increase in TriS and NS6S content indicates greater extent of 6- *O*-sulfation with respect to Reaction **1**. Both Reactions **9** and **11** have 5.5-times higher amounts of 6-OSTs, compared to Reaction **1**, and display remarkably different disaccharide compositions. The NS6S disaccharide was the most abundant disaccharide (> 64%) while TriS disaccharide was lower than Reaction **1**.

Enhanced 6-*O*-sulfation rapidly catalyzes the formation NS6S in place of the desired TriS, as the formation of latter is limited by NS2S generation from its relatively slower 2-*O*-sulfation. The NS6S disaccharide, thus formed, may be an unsuitable substrate for C5-epi/2OST, blocking the generation of TriS disaccharide. The disaccharide composition follows similar trend for Reactions **12** and **13** wherein both 6-OST-1 & −3 are simultaneously varied by either two-fold or ten-fold. In Reaction **12**, with two-fold higher 6-OST-1 & −3, a composition similar to Reactions **8** and **10** is observed, while use of ten-fold higher 6-OST-1 & −3 leads to NS6S as the most abundant disaccharide in Reaction **13**. The blocking of potential epimerization/2-*O*-sulfonation sites due to enhanced 6-*O*-sulfonation rate is evident, as negligible proportion (~ 7.1 %) of 2-*O*-sulfo groups is present in Reaction **13**. The use of higher E:S ratio of 6-OSTs failed to achieve products structurally similar to porcine heparins and resulted in a low TriS content. These results support the presumed order of sulfation in the heparin/HS biosynthesis as followed in our iterative process and suggest epimerization/2-*O*-sulfation takes place prior to 6-*O*-sulfation. Microarray-based high throughput screening can be further employed to investigate potential anti-inflammatory properties of these highly 6-*O*-sulfated products (Parish, 2006; Wang, Brown, Varki, & Esko, 2002).

3.3. Effect of enhanced cofactor recycling on non-anticoagulant heparin composition in one-pot synthesis

The consumption of PAPS, a critical sulfo group donor, by sulfotransferases leads to generation of 3′-adenosine 5′-phosphate (PAP). AST IV reversibly catalyzes the transfer of a sulfo group from PNPS to PAP and converts it into PAPS, thereby establishing an efficient *in vitro* cofactor recycling system (Burkart et al., 2000). This cofactor recycling system is essential to overcome the prohibitively high cost of commercially available PAPS for improved process economics and allows for use of catalytic amount of PAPS. The results for a two level combinatorial variation of AST IV E:S mass ratio are presented in Table 3. Use of ten-fold higher AST IV (Reaction **15**) showed an elevated TriS disaccharide content compared to two-fold higher AST IV (Reaction **14**) and Reaction **1**. These results are in agreement with the observed rapid kinetics of AST IV and suggest that cofactor recycling is not the rate limiting step in sulfotransferase coupled *in vitro* biocatalytic systems (Sterner et al., 2014).

3.4. Disaccharide analysis of anticoagulant bioengineered heparins containing 3-O-sulfo groups

There are seven different isoforms of 3-OST that act either on GlcNS or GlcNS6S. The iterative bioengineered heparin process utilizes 3-OST-1 as the last enzymatic step for completion of the pentasaccharide binding sequence responsible for heparin's anticoagulant activity. The number of 3-*O*-sulfo groups required to impart anticoagulant activity is very low (occupying < 5% of the total C3-positions of GlcN residues). This makes the 3-OST step a relatively simpler enzymatic transformation than the 2-OST and 6-OST steps, which are required to act at 80-90% of the 2-*O*- and 6-*O*- positions within the polysaccharide chains. Combinatorial one-pot preparation of heparins, Reactions **5** and **7**, was repeated at 2 mg scale with incorporation of 3-*O*-sulfo groups using 3-OST-1. After treatment with heparin lyases, the samples were analyzed using LC-MS. Disaccharide composition (on a weight basis) of Reactions **16** (ten-fold level of 2-OST) and **17** (ten-fold level of C5-epi/2-OST) are presented along with USP porcine heparins in Table 4. The TriS disaccharide, representative sequence of heparin, is present in a range from 66 to 84% by mass in heparin APIs suggesting that the manufacturing processes and/or source material have an impact on disaccharide composition. The one-pot bioengineered heparins, Reactions **16** and **17**, were very similar to Reactions **5** and **7** with TriS disaccharide as their major component. Reaction **17** had a higher mass percentage (~ 71%) of TriS disaccharide, in comparison to Reaction **16**, within observed range for porcine heparins (Fu et al., 2013). Six out of eight disaccharides reported for porcine heparins were detected in Reactions **16** and **17**, with significant abundance of NS2S and NS6S. The small percentage of NS disaccharide is indicative of near complete sulfation of available substrate sites on the polysaccharide chains. In Reaction **17**, 5 out of 8 disaccharides (excluding only NS2S, 6S and 2S6S) were within the reported range observed for porcine heparin. The higher standard deviations (> 20%) observed in analytical measurements for minor disaccharides (NS2S, 6S and 2S6S with mass percentages <5%) makes accurate determination of their abundance difficult. The impact of these individual minor disaccharides on biological activity is also not well understood. The similarity of disaccharide compositions obtained for Reactions **16** and **17** to the combinatorial study (Reactions **5** and **7**) suggests a high degree of reproducibility, essential for robust process design.

3.5. HPLC-MS tetrasaccharide mapping of bioengineered anticoagulant heparins

Heparin binds to antithrombin III (AT) through a pentasaccharide sequence. The bound AT undergoes a conformational change that enhances its ability to inactivate thrombin, responsible for conversion of soluble fibrinogen into an insoluble fibrin clot, leading to observed anticoagulant activity of heparins. The representative pentasaccharide binding sequence of heparin is a collection of sequences and can vary depending upon the source (Li et al., 2014). When heparin is exhaustively treated with heparin lyase 2, in addition to the disaccharides formed, some lyase resistant tetrasaccharides are observed due to the presence of 3-*O*-sulfo containing glucosamine residues (Li et al., 2014). Their molecular ratio provides a fingerprint of the heparin from which they were derived as well as an insight into the structural diversity of the AT- binding pentasaccharide sequence within heparin. A positive correlation between the 3- *O*-sulfo containing glucosamine content to *in vitro*

biological activity of USP porcine heparins has been observed (Li et al., 2014). We analyzed seven different USP heparins obtained from commercial sources, as described previously (Zhang et al., 2011). Five lyase resistant tetrasaccharides (T1, T2, T3, T4 and T5) were observed in both porcine and bovine heparins. Mass spectroscopy properties and mass percentages of each individual tetrasaccharides are presented in Figure 2. In porcine heparin and samples **16** and **17**, T1 (0.4-1.0%) and T4 (0.4-3.0%) were identified as the major components with T2 and T5 present as minor components. Two additional lyase resistant tetrasaccharides (T1' and T5') were identified in Reactions **16** and **17** (Figure 2). The masses of T1' and T5' were same to T1 and T5 respectively, but T1' and T5' displayed different retention times from T1 and T5, indicating that they have different structures. Unfortunately, their detailed sequences could not be determined owing to the small amounts of these T1' and T5' formed. Presence of 3-*O*-sulfo group-containing tetrasaccharides suggests potential biological activity in Reactions **16** and **17** (Section 3.8). The total tetrasaccharide content of Reaction **17**, though higher than Reaction **16**, was closer to lowest tetrasaccharide content observed in porcine heparins.

3.6. NMR Spectroscopy of bioengineered heparins

The one-pot bioengineered heparins, Reactions **16** (2.5 mg dry mass) and **17** (2.7 mg dry mass), were purified using SAX chromatography. Anticoagulant heparin consists of IdoA2S (I_{2S}) and GlcNS3S6S (A_{NS3S6S}). Both ^{1}H NMR and HSQC evaluation of Reactions **16** and **17** gave clear views of the 3S and I_{2S} peaks in anomeric regions. All the anomeric peaks of I_{2S}, A_{3S} and A_{NS} were visible in ^{1}H-NMR and HSQC spectra of Reactions **16** and **17** (spectra not shown for Reaction **16**) (Figure 3). None of the product impurities coming from pharmaceutical heparins produced from animal tissues, such as xylose (Xyl), galactosamine (Gal) and the GlcA-Gal repeating units were observed in the HSQC spectra. A high level of IdoA and sulfation was detected in both Reactions **16** and **17**. All the other peaks in sugar region were identified in the ^{1}H-NMR and HSQC studies. Additionally, peaks of critical features in the IdoA residues and GlcN residues, including *N*-sulfo, *N*-acetyl, 2-*O*-sulfo and 3-*O*-sulfo could be fully assigned by ^{1}H-NMR and HSQC spectra (Figure 3). The percent substitution (on a molar basis), calculated using peak integration, of glucosamine (A), iduronic acid (I) and glucuronic acid (G) in Reaction **16** (A_{NS}= 76.2 %, A_{NAC}= 18.0 %, A_{3S}= 5.8 %, I_{2S}= 58.5 %, I= 6.2 % and G= 35.3 %) and Reaction **17** (A_{NS}= 73.3 %, A_{NAC}= 19.2 %, A_{3S}= 7.5 %, I_{2S}= 53.5 %, I= 13.9 % and G= 32.6 %) was very similar to USP porcine heparins (A_{NS}= 80 ± 0.9 %, A_{NAC}= 13 ± 0.5 %, A_{3S}= 6.7 ± 0.7 %, I_{2S}= 61 ± 2.6 %, I= 10.5 ± 0.7 % and G= 28.4 ± 2.0 %).

The total IdoA (I_{2S} and I) content of Reaction **16** was identical to Reaction **17** despite use of excess C5-epi for one-pot synthesis. It has been shown that C5-epi complexes with 2-OST, *in vivo*, and such an allosteric effect can explain identical IdoA content and marginally higher TriS levels of Reaction **17** relative to Reaction **16** (Pinhal et al., 2001). The presence of 3-*O*-sulfo glucosamine in Reactions **16** and **17** spectra supports tetrasaccharide analysis and suggests these products should exhibit anticoagulant activity. The requirement of excess C5-epi would be decided based on the measured anticoagulant activity of these bioengineered heparins. Overall, the structural equivalence of bioengineered heparins,

Reactions **16** and **17**, to USP heparins was conclusively demonstrated through ^{1}H and HSQC NMR spectroscopy studies.

3.7. Size exclusion chromatography for molecular weight determination

Post the 2008 contamination crisis, USP has actively engaged in inclusion of enhanced standards of purity and stricter quality control towards minimization of variation within commercial heparin products. For increased clinical safety, the USP has imposed several restrictions on heparin's molecular weight properties as they are known to impact it's biological activity (Holmer, Kurachi, & Soderstrom, 1981). Effective as of May, 2014, the new heparin monograph requires USP heparin APIs to comply with following molecular weight restrictions (Mulloy et al., 2014):

1. Proportion of heparin chains with molecular weight over 24,000 (M_{24000}) is not more than 20 %
2. M_w is between 15,000-19,000
3. The ratio of heparin chains with molecular weight between 8,000-16,000 Da ($M_{8000–16000}$) to heparin chains with molecular weight between 16,000-24,000 ($M_{16000–24000}$) is not less than 1.0

In the current study, the molecular weight properties of USP heparin (M_w = 22,300 ± 200 Da, M_n = 15,000 ± 100 Da and PDI = 1.49 ± 0.01) were determined as an average of seven different commercial heparins previously studied (Zhang et al., 2011). SEC chromatogram of Reactions **16** and **17** showed the presence of a single major peak. M_w values of Reaction **16** (M_w = 26,200 ± 200 Da, M_n = 13,200 ± 100 Da) and Reaction **17** (M_w =29,200 ± 500 Da, M_n = 13,900 ± 200 Da) were higher in comparison to USP heparins while their M_n values were lower than USP heparins (Figure 4A). As a result, the polydispersity index (PDI) of Reaction **16** (PDI = 1.99 ± 0.02) and Reaction **17** (PDI = 2.10 ± 0.04) were significantly higher in comparison to USP porcine heparins (PDI = 1.49 ± 0.01). Higher TriS disaccharide level in Reaction **17** leads to its marginally higher molecular weight (M_w and M_n) than Reaction **16**. The resulting molecular weight of bioengineered heparin products is directly related to the NSH substrate molecular weight. The high PDI of bioengineered heparin products can be attributed to the high PDI of NSH (1.69 ± 0.01). Control over the NSH molecular weight during the chemical *N*-deacteylation/*N*-sulfonation and titanium dioxide depolymerization can effectively generate one-pot heparin products within the new regulatory requirements.

3.8. In vitro anticoagulant activity of bioengineered anticoagulant heparins

USP heparins currently are required to have an anti-factor IIa activity > 180 IU/mg and anti-factor Xa/anti-factor IIa activity ratio between 0.9-1.1 (Zhang et al., 2011). Two-stage colorimetric *in vitro* assays were used to evaluate anti-factor Xa and anti-factor IIa activity of bioengineered heparins Reactions **16** and **17**. The activity value of USP heparin was determined as an average of reported anti-IIa and anti-Xa activity for seven different USP heparins (Zhang et al., 2011). Bioengineered heparin Reaction **17** had an anti-IIa activity of 151 ± 26 IU/mg, which was higher than **16** (73 ± 11 IU/mg) and close to 180 IU/mg required by USP (Figure 4B). Reaction **17** also possessed a higher anti-Xa activity of 159 ±

15 IU/mg in comparison to Reaction **16** (96 ± 4 IU/mg). The anti-Xa to anti-IIa ratios were also determined for Reactions **17** (1.05 ± 0.20) and **16** (1.32 ± 0.21). The one-pot bioengineered heparin products possessed significant anticoagulant activity though they did not meet USP requirements. The higher *in vitro* anticoagulant activity of Reaction **17** can be attributed to its higher tetrasaccharide content (3.4 %). The observed activity of Reactions **16** and **17** was in agreement with their measured tetrasaccharide content and supports the use of tetrasaccharide mapping for understanding heparin's anticoagulant activity at the molecular level.

4. Conclusions

In summary, a successful one-pot chemoenzymatic synthesis of complex full- length heparin/HS polysaccharides has been achieved beginning from NSH. This approach of modulating enzymatic activity through use of an optimized E:S ratio is suited for high throughput screening studies aimed at better understanding of heparin’s structural heterogeneity and its impact on structure activity relationship. Diversity in 3- OST family (7 different isoforms) is primarily responsible for wide array of biological functions attributed to heparin/HS glycosaminoglycans. One-pot chemoenzymatic synthesis of heparin/HS chains provides a way to decipher the substrate specificity of various 3-OST isoforms with easy availability of polysaccharide/oligosaccharide substrates. Rapid oligosaccharide synthesis can also be realized through this one step scheme with inclusion of glycosyltransferases and UDP sugar based chain elongation. Scale up of this process to metric ton scale is relatively infeasible but it is a robust process for laboratory scale preparation of full-length polysaccharides and oligosaccharides.

Acknowledgement

This work was supported by grants from the US National Institutes of Health (grantsHL096972, HL62244, HL094463, GM38060) and the Heparin Consortium.

References

Bethea HN, Xu D, Liu J, Pedersen LC. Redirecting the substrate specificity of heparan sulfate 2-O-sulfotransferase by structurally guided mutagenesis. Proceedings of the National Academy of Sciences USA. 2008; 105(48):18724–9.

Bhaskar U, Sterner E, Hickey AM, Onishi A, Zhang F, Dordick JS, Linhardt RJ. Engineering of routes to heparin and related polysaccharides. Applied Microbiology and Biotechnology. 2012; 93(1):1–16. [PubMed: 22048616]

Burkart MD, Izumi M, Chapman E, Lin C-H, Wong C-H. Regeneration of PAPS for the Enzymatic Synthesis of Sulfated Oligosaccharides. Journal of Organic Chemistry. 2000; 65(18):5565–5574. [PubMed: 10970295]

Capila I, Linhardt RJ. Heparin-protein interactions. Angewandte Chemie International Edition. 2002; 41(3):391–412.

Chen J, Avci FY, Muñoz EM, McDowell LM, Chen M, Pedersen LC, Zhang L, Linhardt RJ, Liu J. Enzymatic redesigning of biologically active heparan sulfate. The Journal of Biological Chemistry. 2005; 280(52):42817–25. [PubMed: 16260789]

Chen J, Jones CL, Liu J. Using an enzymatic combinatorial approach to identify anticoagulant heparan sulfate structures. Chemistry & Biology. 2007; 14(9):986–93. [PubMed: 17884631]

DeAngelis PL, Liu J, Linhardt RJ. Chemoenzymatic synthesis of glycosaminoglycans: re-creating, re-modeling and re-designing nature's longest or most complex carbohydrate chains. Glycobiology. 2013; 23(7):764–77. [PubMed: 23481097]

Driguez P-A, Potier P, Trouilleux P. Synthetic oligosaccharides as active pharmaceutical ingredients: Lessons learned from the full synthesis of one heparin derivative on a large scale. Natural Product Reports. 2014; 31(8):980–9. [PubMed: 24705477]

Feizi T. Carbohydrate microarrays — a new set of technologies at the frontiers of glycomics. Current Opinion in Structural Biology. 2003; 13(5):637–645. [PubMed: 14568620]

Fu L, Li G, Yang B, Onishi A, Li L, Sun P, Zhang F, Linhardt RJ. Structural characterization of pharmaceutical heparins prepared from different animal tissues. Journal of Pharmaceutical Sciences. 2013; 102(5):1447–57. [PubMed: 23526651]

Fu L, Zhang F, Li G, Onishi A, Bhaskar U, Sun P, Linhardt RJ. Structure and activity of a new low-molecular-weight heparin produced by enzymatic ultrafiltration. Journal of Pharmaceutical Sciences. 2014; 103(5):1375–83. [PubMed: 24634007]

Guerrini M, Beccati D, Shriver Z, Naggi A, Viswanathan K, Bisio A, Capila I, Lansing JC, Guglieri S, Fraser B, Al-Hakim A, Gunay NS, Zhang Z, Robinson L, Buhse L, Nasr M, Woodcock J, Langer R, Venkataraman G, Linhardt RJ, Casu B, Torri G, Sasisekharan R. Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. Nature Biotechnology. 2008; 26(6):669–75.

Habuchi H, Miyake G, Nogami K, Kuroiwa A, Matsuda Y, Kusche-Gullberg M, Habuchi O, Tanaka M, Kimata K. Biosynthesis of heparan sulphate with diverse structures and functions: two alternatively spliced forms of human heparan sulphate 6-O-sulphotransferase-2 having different expression patterns and properties. Biochemical Journal. 2003; 371:131–42. [PubMed: 12492399]

Hickey AM, Bhaskar U, Linhardt RJ, Dordick JS. Effect of eliminase gene (elmA) deletion on heparosan production and shedding in *Escherichia coli* K5. Journal of Biotechnology. 2013; 165(3):175–7. [PubMed: 23583654]

Higashi K, Ly M, Wang Z, Masuko S, Bhaskar U, Sterner E, Zhang F, Toida T, Dordick JS, Linhardt RJ. Controlled Photochemical Depolymerization of K5 Heparosan, a Bioengineered Heparin Precursor. Carbohydrate Polymers. 2011; 86(3):1365–1370. [PubMed: 21841848]

Holmer E, Kurachi K, Soderstrom G. The molecular-weight dependence of the rate-enhancing effect of heparin on the inhibition of thrombin, factor Xa, factor IXa, factor XIa, factor XIIa and kallikrein by antithrombin. Biochemical Journal. 1981; 193:395–400. [PubMed: 6914196]

Koeller KM, Wong C-H. Synthesis of Complex Carbohydrates and Glycoconjugates: Enzyme-Based and Programmable One-Pot Strategies. Chemical Reviews. 2000; 100(12):4465–4494. [PubMed: 11749355]

Li G, Yang B, Li L, Zhang F, Xue C, Linhardt RJ. Analysis of 3-O-sulfo group-containing heparin tetrasaccharides in heparin by liquid chromatography- mass spectrometry. Analytical Biochemistry. 2014; 455:3–9. [PubMed: 24680753]

Linhardt RJ, Gunay NS. Production and chemical processing of low molecular weight heparins. Seminars in Thrombosis and Hemostasis. 1999; 25:5–16. [PubMed: 10549711]

Liu H, Zhang Z, Linhardt RJ. Lessons learned from the contamination of heparin. Natural Product Reports. 2009; 26:313–321. [PubMed: 19240943]

Mulloy B, Heath A, Shriver Z, Jameison F, Al Hakim A, Morris TS, Szajek AY. USP compendial methods for analysis of heparin: chromatographic determination of molecular weight distributions for heparin sodium. Analytical and Bioanalytical Chemistry. 2014; 406(20):4815–23. [PubMed: 24958344]

Myette JR, Shriver Z, Liu J, Venkataraman G, Rosenberg R, Sasisekharan R. Expression in *Escherichia coli*, purification and kinetic characterization of human heparan sulfate 3-O-sulfotransferase-1. Biochemical and Biophysical Research Communications. 2002; 290(4):1206–13. [PubMed: 11811991]

Noti C, de Paz JL, Polito L, Seeberger PH. Preparation and use of microarrays containing synthetic heparin oligosaccharides for the rapid analysis of heparin-protein interactions. Chemistry-A European Journal. 2006; 12(34):8664–86.

Parish CR. The role of heparan sulphate in inflammation. Nature Reviews. Immunology. 2006; 6(9): 633–643.

Petitou M, Duchaussoy P, Lederman I, Choay J, Jacquinet J-C, Sinaÿ P, Torri G. Synthesis of heparin fragments: A methyl α-pentaoside with high affinity for antithrombin III. Carbohydrate Research. 1987; 167:67–75. [PubMed: 3690577]

Pinhal MA, Smith B, Olson S, Aikawa J, Kimata K, Esko JD. Enzyme interactions in heparan sulfate biosynthesis: uronosyl 5-epimerase and 2-O- sulfotransferase interact in vivo. Proceedings of the National Academy of Sciences USA. 2001; 98(23):12984–9.

Polat T, Wong C-H. Anomeric reactivity-based one-pot synthesis of heparin-like oligosaccharides. Journal of the American Chemical Society. 2007; 129(42):12795–800. [PubMed: 17914818]

Restaino OF, Bhaskar U, Paul P, Li L, De Rosa M, Dordick JS, Linhardt RJ. High cell density cultivation of a recombinant *E. coli* strain expressing a key enzyme in bioengineered heparin production. Applied Microbiology and Biotechnology. 2013; 97(9):3893–900. [PubMed: 23318839]

Sheng J, Xu Y, Dulaney SB, Huang X, Liu J. Uncovering biphasic catalytic mode of C5-epimerase in heparan sulfate biosynthesis. The Journal of Biological Chemistry. 2012; 287(25):20996–1002. [PubMed: 22528493]

Sterner E, Li L, Paul P, Beaudet JM, Liu J, Linhardt RJ, Dordick JS. Assays for determining heparan sulfate and heparin O-sulfotransferase activity and specificity. Analytical and Bioanalytical Chemistry. 2014; 406(2):525–36. [PubMed: 24271188]

Wang L, Brown JR, Varki A, Esko JD. Heparin's anti-inflammatory effects require glucosamine 6-O-sulfation and are mediated by blockade of L- and P-selectins. Journal of Clinical Investigation. 2002; 110(1):127–36. [PubMed: 12093896]

Wang Z, Xu Y, Yang B, Tiruchinapally G, Sun B, Liu R, Dulaney S, Liu J, Huang X. Preactivation-based, one-pot combinatorial synthesis of heparin-like hexasaccharides for the analysis of heparin-protein interactions. Chemistry-A European Journal. 2010; 16(28):8365–75.

Wang Z, Yang B, Zhang Z, Ly M, Takieddin M, Mousa S, Liu J, Dordic JS, Linhardt RJ. Control of the heparosan N-deacetylation leads to an improved bioengineered heparin. Applied Microbiology and Biotechnology. 2011; 91(1):91–9. [PubMed: 21484210]

Wilesmith JW, Wells GA, Cranwell MP, Ryan JB. Bovine spongiform encephalopathy: epidemiological studies. The Veterinary Record. 1988; 123(25):638–44. [PubMed: 3218047]

Xiong J, Bhaskar U, Li G, Fu L, Li L, Zhang F, Dordick JS, Linhardt RJ. Immobilized enzymes to convert *N*-sulfo, *N*-acetyl heparosan to a critical intermediate in the production of bioengineered heparin. Journal of Biotechnology. 2013; 167(3):241–7. [PubMed: 23835156]

Xu Y, Masuko S, Takieddin M, Xu H, Liu R, Jing J, Mousa SA, Linhardt RJ, Liu J. Chemoenzymatic synthesis of homogeneous ultralow molecular weight heparins. Science. 2011; 334(6055):498–501. [PubMed: 22034431]

Yang B, Chang Y, Weyers AM, Sterner E, Linhardt RJ. Disaccharide analysis of glycosaminoglycan mixtures by ultra-high-performance liquid chromatography-mass spectrometry. Journal of Chromatography A. 2012; 1225:91–8. [PubMed: 22236563]

Zhang F, Yang B, Ly M, Solakyildirim K, Xiao Z, Wang Z, Beaudet JM, Torelli AY, Dordick JS, Linhardt RJ. Structural characterization of heparins from different commercial sources. Analytical and Bioanalytical Chemistry. 2011; 401(9):2793–803. [PubMed: 21931955]

Zhang L, Beeler DL, Lawrence R, Lech M, Liu J, Davis JC, Shriver Z, Sasisekharan R, Rosenberg RD. 6-O-sulfotransferase-1 represents a critical enzyme in the anticoagulant heparan sulfate biosynthetic pathway. The Journal of Biological Chemistry. 2001; 276(45):42311–21. [PubMed: 11551899]

Zhang Z, McCallum SA, Xie J, Nieto L, Corzana F, Jiménez-Barbero J, Chen M, Liu J, Linhardt RJ. Solution structures of chemoenzymatically synthesized heparin and its precursors. Journal of the American Chemical Society. 2008; 130(39):12998–3007. [PubMed: 18767845]

Highlights

- A simple one pot method for heparin synthesis
- Bioengineeered heparin with activity similar to USP heparin
- 2-*O*-sulfation must occur before 6-*O*-sulfation

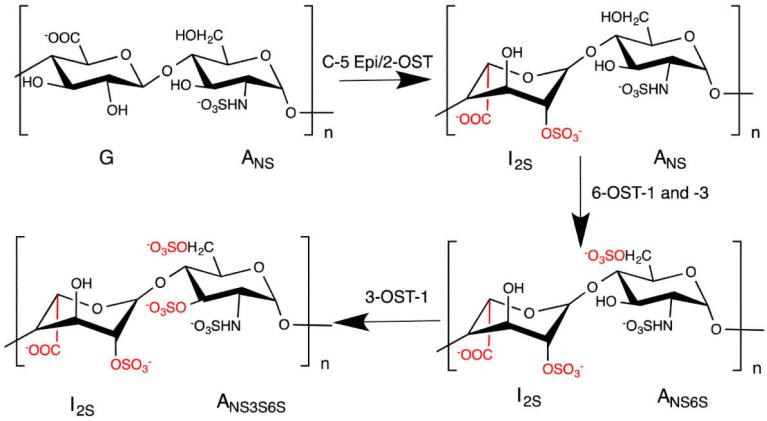

Figure 1.
The chemical structure of typical heparin repeating disaccharides.

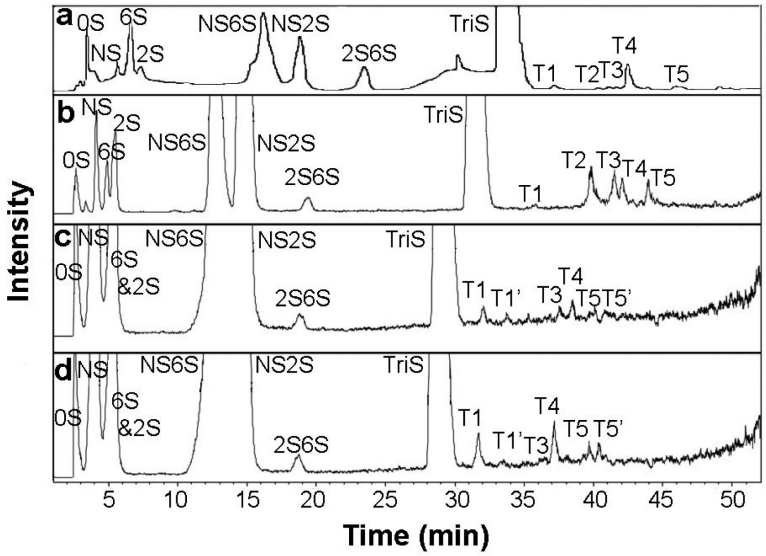

Figure 2.

Extracted ion chromatogram (EIC) of terasaccharide analysis of BRP samples a. Porcine intestinal heparin T1 = 0.8 %; T2 = 0.4 %; T3 = 0.3 %; T4 = 3.0 %; T5 = 0.5 %; Total = 5.0 % (Fu et al., 2013); b. Bovine lung heparin T1 = 0.2 %; T2 = 1.7 %; T3 = 1.2 %; T4 = 0.9 %; T5 = 0.8 %; Total = 4.8 %; c. Bioengineered heparin **16** (T1 = 0.4 %; T1' = 0.2 %; T2 = 0.0 %; T3 = 0.4 %; T4 = 0.4 %; T5 = 0.4 %; T5' = 0.4 %; Total = 2.2 %) d. Bioengineered heparin **17** (T1 = 1.0 %; T1' = 0.2 %; T2 = 0.0 %; T3 = 0.2 %; T4 = 1.0 %; T5 = 0.4 %; T5' = 0.6 %; Total = 3.4 %). The fractions identified are **T1** (m/z = $[477.4]^{2-}$, Calculated molecular mass = 956.8, Theoretical molecular mass = 956.1, Sequence = ΔUA-GlcNAc6S-GlcA-GlcNS3S), **T1'** having the same mass as T1, but of undetermined structure, **T2** (m/z = $[496.6]^{2-}$, Calculated molecular mass = 994.4, Theoretical molecular mass = 994.0, Sequence = ΔUA-GlcNS-GlcA-GlcNS3S6S), **T3** [m/z = $[496.6]^{2-}$, Calculated molecular mass = 994.4, Theoretical molecular mass = 994.0, Sequence = ΔUA-GlcNS6S-GlcA-GlcNS3S], **T4** (m/z = $[517.4]^{2-}$, Calculated molecular mass= 1036.8, Theoretical molecular mass = 1036.0, Sequence = ΔUA- GlcNAc6S-GlcA-GlcNS3S6S), **T5** (m/z = $[536.3]^{2-}$, Calculated molecular mass = 1074.6, Theoretical molecular mass = 1074.0, Sequence = ΔUA-GlcNS6S-GlcA-GlcNS3S6S), **T5'** having the same mass as T5, but of undetermined structure.

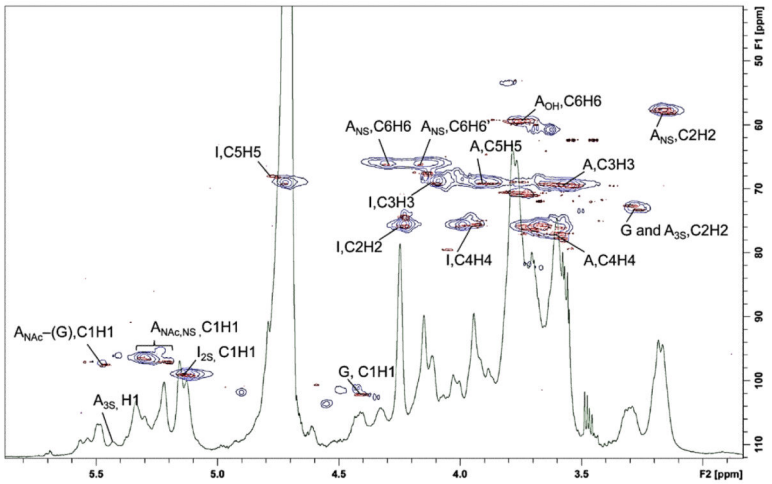

Figure 3.

Two-dimensional HSQC spectra of porcine heparin (blue), **17** (red) and one- dimensional of ^{1}H spectra of **17**. (*A*, glucosamine; *I*, iduronic acid; *G*, glucuronic acid)

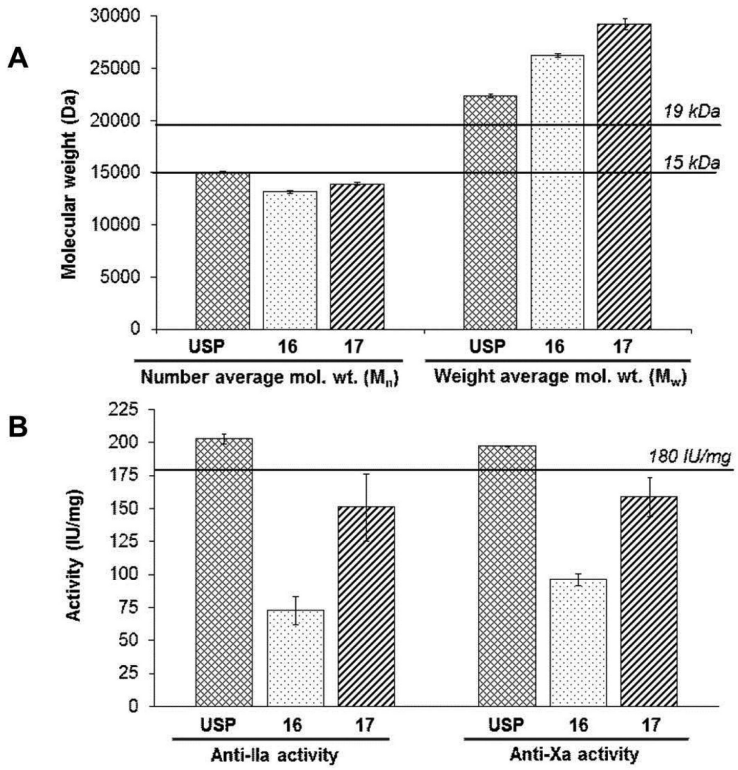

Figure 4.

A. Molecular weight properties of **16** and **17** as determined by size exclusion chromatography. New USP requirements for M_w ($15000 < M_w < 19000$) are depicted by solid lines. B. *In vitro* anti-IIa and anti-Xa activity of 16 and 17. Solid line marks the minimum anti-IIa activity of 180 IU/mg required by USP. Molecular weight and anticoagulant activity values for USP heparins were reported previously (Zhang et al., 2011).

Table 1

Effect of C5-Epi and 2-OST on disaccharide composition of heparin products generated using combinatorial one-pot enzymatic preparation. The numbers depict mass percentage of each disaccharide in the digested product. (OS=ΔUA-GlcNAc, NS= ΔUA-GlcNS, 6S= ΔUA-GlcNAc6S, 2S= ΔUA2S-GlcNAc, NS6S= ΔUA-GlcNS6S, NS2S= ΔUA2S-GICNS, 2S6S= ΔUA2S-GlcNAc6S, Tris= ΔUA2S-GlcNS6S). ΔUA corresponds to 4-deoxy-α-L-threo-hex-4-eno-pyranosyluronic acid.

Sample(ID)	OS (%)	NS (%)	6S (%)	2S (%)	NS6S (%)	NS2S(%)	2S6S (%)	TriS (%)
Control (1)	12.9±1.2	9.7±0.3	0.0±0.0	0.3±0.0	17.5±0.4	24.6±0.5	0.0±0.0	35.1±1.6
2-fold C5 (2)	13.7±0.9	9.5±0.6	0.0±0.0	0.1±0.2	14.0±3.1	31.9±1.8	0.8±1.2	29.9±0.0
10-fold C5 (3)	11.9±3.0	5.7±0.2	0.0±0.0	0.3±0.4	17.3±0.7	24.2±0.4	0.0±0.0	40.5±2.0
2-fold 2OST (4)	10.5±2.0	50.±1.1	0.0±0.0	0.8±0.5	13.7±1.4	29.9±1.8	0.0±0.0	40.9±0.1
10-fold 2OST (5)	5.4±0.8	1.7±0.2	0.0±0.0	0.0±0.0	11.1±2.7	22.1±1.2	0.0±0.0	58.9±3.8
2- fold C5 & 2OST (6)	10.7±1.4	5.7±0.2	0.0±0.0	0.8±0.1	10.1±0.1	35.1±1.1	0.0±0.0	38.4±2.6
10-fold C5 & 2OST (7)	6.1±1.2	1.6±0.6	0.0±0.0	0.0±0.0	7.8±0.7	15.7±0.9	0.0±0.0	67.9±2.1

Table 2

Effect of 6-O-Sulftransferase-1 & 3 on disaccharide composition of heparin products generated using combinatorial one-pot enzymatic preparation. The numbers depict mass percentage of each disaccharide in the digested product. (OS=ΔUA-GlcNAc, NS= ΔUA-GlcNS, 6S= ΔUA-GlcNAc6S, 2S= ΔUA2S-GlcNAc, NS6S= ΔUA-GicNSeS, NS2S= ΔUA2S-GICNS, 2S6S= ΔUA2S-GlcNAc6S, Tris= ΔUA2S-GlcNS6S). ΔUA corresponds to 4-deoxy-α-L-threo-hex-4-eno-pyranosyluronic acid.

Sample (ID)	OS (%)	NS (%)	6S (%)	2S (%)	NS6S (%)	NS2S (%)	2S6S (%)	TriS(%)
Control (1)	12.9±1.2	9.7±0.3	0.0±0.0	0.3±0.0	17.5±0.4	24.6±0.5	0.0±0.0	35.1±1.6
2-fold 6OST1 (8)	9.9±1.3	5.1±09	0.0±0.0	0.0±0.0	24.6±0.8	13.7±0.9	0.0±0.0	46.7±2.1
10-fold 6OST1 (9)	5.3±0.0	2.1±0.7	0.2±0.0	0.0±0.0	64.9±0.7	2.6±0.2	0.0±0.0	24.9±0.2
2-fold 6OST3 (10)	9.5±3.5	5.4±0.2	0.0±0.0	0.0±0.0	27.8±0.9	11.4±0.7	0.0±0.0	46.0±2.7
10-fold 6OST3(11)	3.9±0.5	0.6±0.9	0.2±0.0	0.0±0.0	84.0±0.6	0.5±0.8	0.0±0.0	10.7±1.6
2-fold 6OST1&3(12)	8.5±0.5	3.7±0.8	0.0±0.0	0.0±0.0	38.5±1.0	10.6±0.3	0.0±0.0	38.8±0.5
10-fold 6OST1&3(13)	3.2±1.2	0.0±0.0	0.2±0.0	0.0±0.0	89.6±1.0	0.0±0.0	0.0±0.0	7.1±0.2

Table 3

Disaccharide composition of heparin products generated using enhanced PAPS regeneration. The numbers depict mass percentage of each disaccharide in the digested product. (OS=ΔUA-GlcNAc, NS= ΔAUA-GlcNS, 6S= ΔUA-GlcNAc6S, 2S= ΔUA2S-GlcNAc, NS6S= ΔUA-GlcNS6S, NS2S= ΔUA2S-GlcNS, 2S6S= ΔUA2S-GlcNAc6S, Tris= ΔUA2S-GlcNS6S). ΔUA corresponds to 4-deoxy-\g=a\-L-threo-hex-4-eno-pyranosyluronic acid.

Sample (ID)	OS (%)	NS (%)	6S (%)	2S (%)	NS6S (%)	NS2S (%)	2S6S (%)	TriS(%)
Control (1)	12.9±1.2	9.7±0.3	0.0±0.0	0.3±0.0	17.5±0.4	24.6±0.5	0.0±0.0	35.1±1.6
2-fold AST IV (14)	14.7±1.0	7.2±0.1	0.0±0.0	0.0±0.0	18.7±1.8	23.3±1.3	0.0±0.0	36.1±0.5
10-fold AST IV (15)	14.2±00	4.5±0.0	0.0±0.0	0.0±0.0	26.8±1.7	9.0±0.8	0.0±0.0	45.6±2.5

Table 4

Disaccharide composition of heparin products prepared using one-pot preparation with 3-*O*-sulfonation. The numbers depict mass percentage of each disaccharide in the digested product. (OS=ΔUA-GlcNAc, NS= ΔUA-GlcNS, 6S= ΔUA-GlcNAc6S, 2S= ΔUA2S-GlcNAc, NS6S= ΔUA-GlcNS6S, NS2S= ΔUA2S-GlcNS, 2S6S= ΔUA2S-GlcNAc6S, TriS= ΔUA2S-GlcNS6S). ΔUA corresponds to 4-deoxy-α-L-threo-hex-4-eno-pyranosyluronic acid.

Sample	OS (%)	NS (%)	6S (%)	2S (%)	NS6S (%)	NS2S(%)	2S6S (%)	TriS(%)
USP heparin	0.3-3.2	0.8-2.9	1.2-3.2	0.8-1.6	5.4-10.8	3.7-11.6	0.77-2.6	66.9-83.8
16 (10-fold 2OST)	1.4 ± 0.8	2.5 ± 0.8	0.0 ± 0.0	2.0 ± 0.7	6.5 ± 0.53	28.9 ± 0.6	0.0 ± 0.0	58.8 ± 1.6
17 (10-fold C5-epi/2OST)	2.7 ± 0.7	1.7 ± 0.4	0.0 ± 0.0	1.8 ± 0.3	7.9 ± 0.2	15.5 ± 0.6	0.0 ± 0.0	70.5 ± 0.7