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Glycosamino-glycans

Methods and Protocols

Second Edition

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Glycosaminoglycans

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Second Edition

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Cover Caption: Drosophila ovary, one of the best model systems for the study of the stem cell niche where glycosaminoglycans play major roles. Green color shows expression of Dally, a Drosophila glypican.

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Chapter 1

One-Pot Enzymatic Synthesis of Heparin from *N*-Sulfoheparosan

Li Fu and Robert J. Linhardt

Abstract

Heparin, a glycosaminoglycan-based anticoagulant drug, is prepared as an extract of animal tissues. Heparosan, an *Escherichia coli* (*E. coli*) K5 capsular polysaccharide with the structure →4)-β-D-glucuronic acid (1 → 4)-β-D-*N*-acetylglucosamine (1→, corresponds to the precursor backbone in the Golgi-based biosynthesis of heparin. Anticoagulant heparin is prepared in a one-pot synthesis using a chemically prepared derivative of heparosan called *N*-sulfoheparosan (NSH), recombinant Golgi enzymes expressed in *E. coli*, and the 3-phosphoadenosine-5-phosphosulfate (PAPS) cofactor.

Key words Heparin, Heparan sulfate, Heparosan, Enzymes, 3-Phosphoadenosine-5-phosphosulfate, Biosynthesis

1 Introduction

Heparin (Fig. 1), a glycosaminoglycan-based anticoagulant drug, is prepared as an extract of animal tissues, including beef lung and pig, beef, and sheep intestine [1]. Heparin, discovered over 100 years ago [2], is a widely used essential drug for the practice of modern medicine [3]. Since heparin is prepared from the tissues of food animals, its production involves both the food and drug chains and thus is difficult to control and regulate [4]. In 2007–2008, as a result of a regulatory failure, pharmaceutical heparin products were contaminated with an inexpensive adulterant, oversulfated chondroitin sulfate (OSCS), leading to a number of patient deaths [5]. While the control and regulation of heparin production from animal tissues have been improved by upgraded pharmacopoeial monographs, regular inspections of manufacturing facilities, and improved record keeping [6], there remain concerns over the safety and availability of these critically important drugs [4].

The biosynthesis of heparin in the Golgi of animal cells has been extensively studied over the past 50 years [7]. A number of laboratories have cloned and expressed many of the Golgi enzymes

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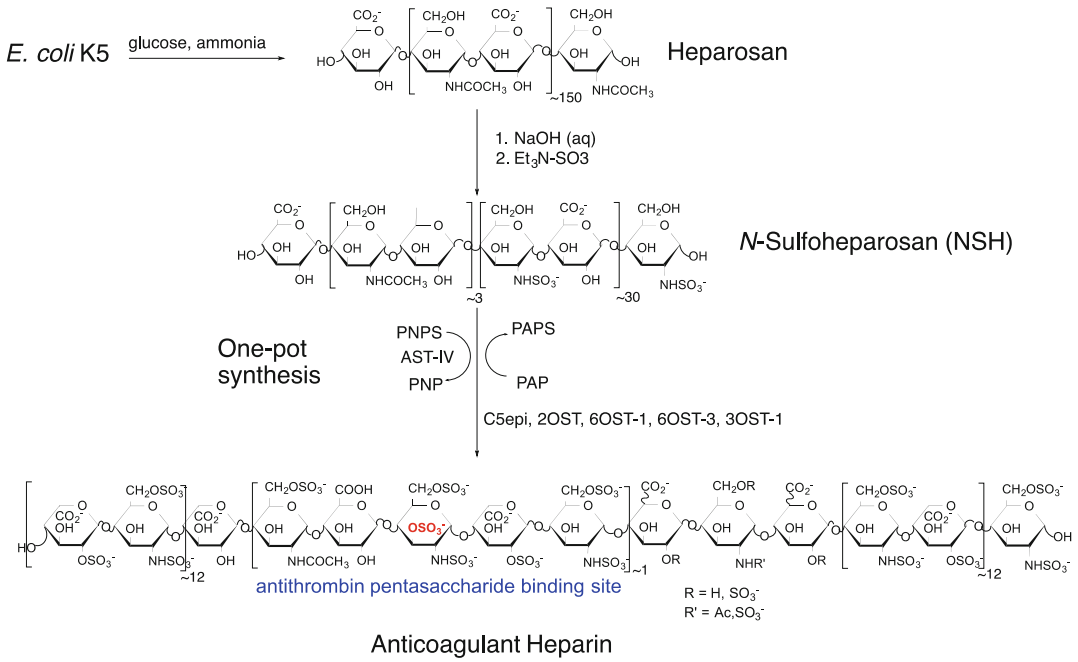

Fig. 1 One-pot synthesis of heparin. (**a**) Conversion of heparosan to NSH. (**b**) One-pot conversion of NSH to anticoagulant heparin with cofactor recycling

responsible for heparin biosynthesis [8–10]. These enzymes, which are commonly expressed in *Escherichia coli* (*E.* coli) as truncated forms and/or fusion proteins to improve their solubility, stability, and catalytic properties [11], have been used as useful tools in understanding Golgi function and more recently in heparin and heparin oligosaccharide synthesis [12]. There are multiple approaches at different stages of development for using recombinant heparin biosynthetic enzymes to prepare heparins and heparin oligosaccharides. Two such approaches, metabolic engineering, involving the cell-based synthesis of heparin [13], and iterative chemoenzymatic synthesis [14], involving the stepwise assembly of heparin oligosaccharides using glycosyltransferases and activated sugar donors along with polysaccharide modifying enzymes (i.e., sulfotransferases and epimerases), are not described in this methods chapter.

This method chapter describes in detail a simple one-pot approach for the enzymatic conversion of a polysaccharide substrate, *N*-sulfoheparosan (NSH), to anticoagulant heparin (Fig. 1) [15]. NSH is prepared from heparosan (with the structure →4)-β-D-glucuronic acid (1 → 4)-β-D-*N*-acetylglucosamine (1→, which has an average molecular weight of 50–150 kDa) and the capsular polysaccharide of *E. coli* K5 obtained from the supernatant of its fermentation on either glucose or glycerol (Fig. 1) [16]. Treatment of heparosan with sodium hydroxide and

Table 1
Enzymes used in the one-pot synthesis of heparin

Enzyme	Source species	Truncation	Fusion protein or tag	Reference
C5Epi	Human	– Transmembrane domain	Mannose binding protein	[18]
2OST	Chinese hamster	– Transmembrane domain	Mannose binding protein	[18]
6OST-1	Mouse	– Transmembrane domain	Mannose binding protein	[19]
6OST-3	Mouse	– Transmembrane domain	Mannose binding protein	[20]
3-OST-1	Mouse	– Transmembrane domain	His-tag	[21]
AST-IV	Rat	– None	His-tag	Unpublished

N-sulfonation with triethylamine-sulfur trioxide complex affords NSH with the structure →4)-β-D-glucuronic acid (1 → 4)-β-D-*N*-sulfoglucosamine (1→, which contains some residual *N*-acetylglucosamine residues (2–15%) and an average molecular weight of 10–20 kDa (Fig. 1) [17].

In this one-pot synthesis, NSH is treated with recombinant Golgi enzymes, 2-*O*-sulfotransferase (2OST), C5-epimerase (C5epi), 6-*O*-sulfotransferase-1 and -3 (6OST-1 and 6OST-3), and 3-*O*-sulfotransferase (3-OST-1), which are all expressed in *E. coli* (Table 1), as well as a sub-stoichiometric amount of 3-phosphoadenosine-5-phosphosulfate (PAPS) together with a cofactor regenerating system consisting of p-nitrophenylsulfate (PNPS) and arylsulfotransferase-IV (AST-IV) (Fig. 1) [15]. The resulting heparin product has a specific activity of >100 units/mg based on either anticoagulation assay or amidolytic assays.

2 Materials

2.1 Combinational One-Pot Enzymatic Reaction

1. 500 mM MES buffer: 500 mM MES, pH 7.0. Weigh 0.39 g 2-(*N*-morpholino)ethanesulfonic acid (MES hydrate) and transfer into a 4 mL centrifuge tube. Add 3 mL DI water to dissolve completely. Adjust pH to 7.0 using 2% NaOH and bring the volume up to 4 mL.
2. 50 mM MES buffer: 50 mM MES, pH 7.0. Mix 1 mL of 500 mM MES buffer into 9 mL DI water. Check pH and adjust pH to 7.0 if necessary, using HCl or NaOH. Store at 4 °C.
3. Solution S: Weigh 2 mg of purified NSH (*see* **Note 1**) and dissolve into 2 mL of 50 mM of MES buffer in a 50 mL Eppendorf centrifuge tube.
4. 3 mM PAPS: 3 mM PAPS. Weigh 1.5 mg of PAPS and dissolve into 1 mL DI water completely. Store at 4 °C.

5. 50 mM PNPS: 50 mM PNPS. Weigh 51.5 mg of PNPS and dissolve it into 4 mL DI water completely. Store at 4 °C.
6. Purified C5 epimerase (C5-epi), aryl sulfotransferase (AST) IV, and sulfotransferases (2-OST, 6-OST-1, 6-OST-3, and 3-OST-1): All enzymes (Table 1) are concentrated to at least 5 mg/mL before use. All enzymes are at 5 mg/mL unless otherwise specified (*see* **Note 2**).

2.2 Strong Anion Exchange (SAX) Purification of Bioengineered Heparin

1. 3 kDa molecular weight cut-off (MWCO) centrifugal unit (Amicon centrifugal filter units, Millipore).
2. Strong ion exchange spin column: 20 mL Vivapure Ion Exchange Q-Column (Sartorius).
3. SAX calibration buffer: 50 mM NaCl. Dilute 2 mL of SAX washing buffer in 18 mL of DI water.
4. SAX washing buffer: 500 mM NaCl. Dilute 10 mL of SAX elution buffer in 30 mL of DI water.
5. SAX elution buffer: 2 M NaCl. Weigh 11.69 g NaCl and dissolve into 100 mL of DI water.

2.3 Enzymatic Digestion

1. Ammonium acetate buffer: 50 mM ammonium acetate, pH 7.4.
2. Heparin lyases I, II, and III [22]: 2 mU/μL separately prepared in ammonium acetate buffer (*see* **Note 3**).
3. YM-10 micro-concentrator (Millipore).

2.4 LC-MS

1. 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.
2. Poroshell 120 C18 column (2.1 × 100 mm, 2.7 m, Agilent, USA).
3. LC-MS Eluent A: 12 mM tributylamine (TBA), 38 mM ammonium acetate (NH_4Ac) in water/acetonitrile (85:15, v/v), pH 8.5. Dissolve 2.22 g TBA and 2.93 g NH_4Ac completely in 800 mL DI water. Adjust pH to 8.5 using acetic acid. Bring up volume to 850 mL. Add 150 mL acetonitrile. Adjust pH back to 8.5 if necessary. Store at 4 °C.
4. Disaccharide and tetrasaccharide standards for LC-MS system are prepared to concentrations of 0.1, 0.5, 1, 5, 10, 20, 50, and 100 ng/μL.
5. LC-MS Eluent B: 12 mM TBA, 38 mM NH_4Ac in water/acetonitrile (35:65, v/v), pH 8.5. Dissolve 2.22 g TBA and 2.93 NH_4Ac completely in 300 mL DI water. Adjust pH to 8.5 using acetic acid. Bring up volume to 350 mL. Add 650 mL acetonitrile. Adjust pH back to 8.5 if necessary. Store at 4 °C.

2.5 NMR Spectroscopy

1. D_2O: 99.996% D_2O (Sigma).
2. NMR micro tubes, OD 5 mm (Norell).
3. Bruker Advance II 600 MHz spectrometer (Bruker BioSpin).
4. Topsin software (Bruker Topspin).

2.6 Molecular Weight Determination

1. HPLC system consisting of an LC-10Ai pump, a CBM-20A controller, and an RID-10A refractive index detector (Shimadzu).
2. GPC mobile phase: 0.1 M $NaNO_3$. Dissolve 17 g $NaNO_3$ in 2 L of DI water. Store at 4 °C.
3. TSK-GEL G3000PWxl size exclusion column (Tosoh Bioscience).
4. Column heater (Eppendorf).
5. LC solution Version 1.25 software (Shimadzu).
6. USP heparin sodium Mw calibrant and system suitability solution (US Pharmacopeia).

2.7 In Vitro Anticoagulant Activity Measurement

1. BIOPHEN ANTI-IIa and ANTI-Xa (Two-Stage Heparin Assay) kits (Aniara).

3 Methods

3.1 Combinational One-Pot Enzymatic Reaction

1. Add 4 mL of purified 2OST-1, C5-epi, and AST-IV each into solution S.
2. Add 0.4 mL of purified 6OST-1, 6OST-3, and 3OST-1 into solution S.
3. Add 2 mL of 3 mM PAPS and 50 mM PNPS each into solution S.
4. Bring up the final volume to 20 mL, gently mix, transfer the tube into 37 °C immediately, and incubate overnight.

3.2 SAX Purification of Bioengineered Heparin

1. After an overnight one-pot enzymatic reaction, boil the sample for 10 min, cooldown to room temperature, and filter through 0.22 μm membrane.
2. Wash a 20 mL 3 kDa MWCO centrifugal unit with DI water to remove glycerol.
3. Desalt the clarified permeate by loading into washed centrifugal unit and centrifuge at 5000 × *g* for 20 min, bring up the volume back to 20 mL with DI water. Repeat the centrifugation **steps 3–5** times to remove the salt and other low molecular weight substances.

4. Wash a strong ion exchange spin column with SAX calibration buffer by centrifuging at 500 × *g* for 3–5 min (*see* **Note 4**) to remove glycerol and equilibrate the column.
5. Load the desalted polysaccharide solution into the washed column and centrifuge at 500 × *g* for 3–5 min until the solution flows through completely. Repeat the centrifugation step with 10 mL of SAX washing buffer to remove protein or peptide.
6. Elute bioengineered heparin with 5 mL of SAX elution buffer. Collect the elution and desalt using 3 kDa MWCO spin column as **step 2** in Subheading 3.2.
7. Weigh a 2 mL Eppendorf centrifuge tube and determine its tare weight in mg. Collect the purified bioengineered heparin using the weighed tube and freeze-dry.
8. After the sample is completely dried, weigh the tube containing the dried sample and subtract the tare weight to determine the mass of recovered heparin.
9. Add DI water to dissolve heparin to make 10 μg/μL solution as a test sample for further analysis.

3.3 Enzymatic Digestion

3.3.1 Disaccharide Analysis

1. Add 1 μL of the test sample into a 1.5 mL tube, add 5 μL (10 mU) each of heparin lyase I, II, and III into the test sample, bring up the volume to 100 μL using ammonium acetate buffer, and incubate at 35 °C for 10 h to degrade heparin sample completely [23].
2. Load the resulting disaccharide solution into a YM-10 microconcentrator and centrifuge at 10,000 × *g* for 10 min. Collect the permeation, which contains disaccharide, and freeze-dry.
3. Dissolve the digested heparin disaccharides in DI water to a concentration of 25–50 ng/μL for LC-MS analysis.

3.3.2 Tetrasaccharide Mapping

1. Add 5–10 μL of the test sample and 20 μL (40 mU) of heparin lyase II into a 1.5 mL tube, bring up the volume to 100 μL with ammonium acetate buffer, and incubate at 35 °C for 10 h to degrade heparin sample completely [23].
2. Freeze-dry the resulting tetrasaccharide for further LC-MS analysis [24].

3.4 Analysis Using LC-MS

3.4.1 Disaccharide Analysis

1. Set the electrospray interface for LC-MS system 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). Use nitrogen (8 L/min, 40 psi) as a drying and nebulizing gas.

2. Calibrate the LC-MS system with Eluent A. For sample testing, set the gradient as solution A for 5 min, followed by 0–40% linear gradient Eluent B from 5–15 min. Set flow rate as 150 μL/min.
3. Inject disaccharide standards first to make quantification calibration curves. Linearity was assessed based on the amount of di- or tetrasaccharide and the peak intensity in extracted ion chromatogram (EIC).
4. Inject the digested test sample (disaccharides) and quantify based on the established calibration curves.

3.4.2 Tetrasaccharide Mapping

1. Use the same LC-MS system as disaccharide analysis (**step 1** in Subheading 3.5) for tetrasaccharide analysis.
2. Calibrate the LC-MS system with Eluent A. For sample testing, the gradient is set as solution A for 2 min, followed by 0–30% linear gradient Eluent B from 2 to 40 min. Set flow rate as 150 μL/min.
3. Inject tetrasaccharide standards first to make quantification calibration curves. Linearity was assessed based on the amount of tetrasaccharide and the peak intensity in extracted ion chromatogram (EIC).
4. Inject the digested test sample (tetrasaccharides) and quantify based on the established calibration curves.

3.5 NMR Spectroscopy

1. Take about 100 μL (~1 mg) of a test sample and freeze-dry.
2. Redissolve the dried test sample in 100–200 μL D_2O.
3. Repeat above deuterium exchange (**steps 1** and **2**) twice to remove most H_2O from the test sample.
4. Dissolve deuterium exchanged test samples into 450 μL of D_2O and transfer the solution to NMR micro tubes for NMR analysis.
5. Tune and shim the Bruker Advance II 600 MHz spectrometer to have optimal conditions.
   (a) 1D 1H-NMR experiment conditions: wobble sweep width of 12.3 kHz, acquisition time of 2.66 s, and relaxation delay of 8 s at 298 K.
   (b) 2D ^{1}H-^{13}C HSQC experiment conditions: 32 scans, sweep width of 6.15 kHz, acquisition time of 0.33 s, and relaxation delay of 0.90 s.
6. Process the NMR data using Topsin software.

3.6 Molecular Weight Determination

1. Set a sample injection volume of 20 μL and a flow rate of 0.6 mL/min in the HPLC system.
2. Maintain TSK-GEL G3000PWxl size exclusion column at 40 °C with a column heater.

3. Record the SEC chromatograms with the LC solution Version 1.25 software and determine molecular weight properties using the "GPC Postrun" function.
4. Inject and analyze USP heparin sodium Mw calibrant and system suitability solution to confirm the system suitability.
5. Inject the test sample and calculate the molecular weight based on the calibration of the USP calibrant.

3.7 In Vitro Anticoagulant Activity Measurement

1. Analyze the anti-IIa and anti-Xa activities using BIOPHEN ANTI-IIa and ANTI-Xa (Two-Stage Heparin Assay) kits following the instruction provided.

4 Notes

1. Purified NSH [17] is freeze-dried before use.
2. The sulfotransferases should be assayed prior to use with a chomogenic assay using NSH and PAPS (coupled with PNPS and AST-IV) [25]. The C5-epimerase should be assayed using chomogenic assay using NSH, 2OST, and PAPS (coupled with PNPS and AST-IV).
3. The heparin lyases should be assayed prior to use with heparin or heparan sulfate as a substrate by measuring a change in absorbance at 232 nm [26].
4. Usually, 10 mL of solution can be all flown through the strong ion exchange spin column. Extend the centrifugation time if there is still a solution on top of the resin.

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