Heavy Heparin: A Stable Isotope-Enriched, Chemoenzymatically-Synthesized, Poly-Component Drug

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Abstract: Heparin is a highly sulfated, complex polysaccharide and widely used anticoagulant pharmaceutical. In this work, we chemoenzymatically synthesized perdeuteroheparin from biosynthetically enriched heparosan precursor obtained from microbial culture in deuterated medium. Chemical de-N-acetylation, chemical N-sulfation, enzymatic epimerization, and enzymatic sulfation with recombinant heparin biosynthetic enzymes afforded perdeuteroheparin comparable to pharmaceutical heparin. A series of applications for heavy heparin and its heavy biosynthetic intermediates are demonstrated, including generation of stable isotope labeled disaccharide standards, development of a non-radioactive NMR assay for glucuronosyl-CS-epimerase, and background-free quantification of in vivo half-life following administration to rabbits. We anticipate that this approach can be extended to produce other isotope-enriched glycosaminoglycans.

Stable isotope labeled (SIL) proteins, nucleic acids, and other biomacromolecules have played essential roles in nuclear magnetic resonance (NMR) spectroscopy and small angle neutron scattering structural studies for decades, while labeled small molecules are now central to an array of targeted analytical assays and untargeted metabolomics and proteomics.[10] Recently, the first FDA-approved SIL pharmaceutical with a strategically positioned $^2$H label entered the market exhibiting improved pharmacological properties.[2] Innovative imaging methodologies have also recently capitalized on SILs as imaging probes.[3] As new methods expand with SIL analogs at their core, synthetic methods to build SIL compounds must co-evolve to keep pace. Our recently demonstrated ability to synthesize bioengineered heparin in multi-mg quantities presents one such opportunity.[4]

Heparin and structurally related heparan sulfate (HS) glycosaminoglycans are complex, linear, anionic, heterogeneous, polydisperse, polysaccharides comprised of O-sulfated, 1→4 linked repeating units of uronic acid (ε-iduronic acid (IdoA)) or δ-glucuronic acid (GlcA)) and N-hexosamine (N-acetylg glucosamine (GlcNAc) or N-sulfoglucosamine (GlcNS)).[5] Heparin/HS biosynthesis, a complex, non-template driven process, involves modification of the heparosan backbone →4GlcA(1→4)GlcNAc(1→ through de-N-acetylation, N-sulfation, epimerization of GlcA to IdoA, and 2-, 6-, and 3-O-sulfation catalyzed by a suite of biosynthetic enzymes (Supporting Information, Figure S1 a). Pharmaceutical or unfraccionated heparin (UFH), produced by extraction from pig intestine,[6] is at risk of contamination and can contain prion and viral impurities.[7] While the chemical synthesis of anticoagulant heparin oligosaccharides, such as ArixtraTM,[7] is possible, chemical synthesis of UFH is not. We have established a platform for the scalable chemoenzymatic synthesis (Figure 1) of bioengineered heparin for a safe and sustainable supply of synthetic animal-free heparin, that is virtually indistinguishable from UFH.[8]

The ability to synthesize UFH at scale enables creative control over precise chemical modifications facilitating the development of new heparin products and novel biochemical and pharmacological assays. Selective partial isotopic labeling of UFH was previously reported.[9–11] We chemoenzymatically synthesized small quantities of uniform $^{13}$C$^{15}$N-labeled heparin for NMR studies from the heparosan precursor made by E. coli grown on $^{13}$C-glucose and $^{15}$NH$_4$.[12] Here we demonstrate the first preparation of stable isotope enriched perdeuteroheparin (“heavy” heparin), from microbially produced perdeuteroheparosan. Small deuterated drug molecules have gained increased interest as $^2$H-labeled molecules can display unique pharmacokinetics (PK)/pharmacodynamics (PD) properties.[13–15] Moreover, $^2$H-labeling permits the highest labeling density for a single stable isotope. In heparin up to 14 non-exchangeable $^2$H atoms can be incorporated per disaccharide. This work expands the repertoire of perdeuterated
natural products and exemplifies a new paradigm in SIL analog synthesis involving the combination of biosynthetic scaffold preparation followed by significant chemoenzymatic modification. Heavy heparin represents the most complex perdeuterated product prepared by synthetic means to date.

*E. coli* was used to biosynthesize the perdeuteroheparosan precursor. Preparation of perdeuterated biomolecules by microbes grown in $^2$H$_2$O containing a perdeuterated carbon source has been reported. Perdeuteroheparosan was prepared from *E. coli* strain ATCC 23506, a microbe that naturally produces and sheds this capsular polysaccharide in minimal medium prepared in D$_2$O with heavy glycerol as the sole carbon source. $^2$H enrichment was significantly enhanced in medium prepared with $^2$H$_2$O relative to H$_2$O (Figure S2). Uniform $^2$H-labeling precludes analysis by $^1$H NMR, so $^{13}$C NMR was used to confirm its structure. Peak assignments were comparable to heparosan standard.

Chemical conversion of GlcNac residues to GlcNS residues was marked by C2 shift to a lower field (Figure 2 and Table S1). $^2$H NMR further supported $^2$H incorporation through a characteristic peak at 2 ppm, with CD$_2$ of GlcNac (Figure S3 and Table S1). LC-MS analysis of enzymatically depolymerized heavy heparosan and 2-amino- acridine-labeling, yielded a disaccharide corresponding to the unlabeled heavy heparosan disaccharide (0S, m/z = 585, 98.6 atm. %) (Figure 3a, b and Figure S2).

Preparation of heparin from microbial heparosan involves two sequential chemical and enzymatic modifications (Figure S1). First, perdeuteroheparosan was chemically de-N-acetylated using aqueous NaOH and N-sulfated with (CH$_3$)$_2$N-SO$_3$ to generate heavy N-sulfoheparosan (NSH). LC-MS analysis of heavy NSH disaccharide composition indicated 90% NAc to NS conversion, supported by near complete loss of $^2$H signal from GlcNac C$^2$H of the N-acetyl group between the perdeuteroheparosan and heavy NSH $^2$H spectra. As these reactions were performed in H$_2$O, tautomeration took place at the residual NAc moieties resulting in the transformation of NAc C$^2$H$_2$ to CH$_3$ (treatment of heavy NSH with heparinases generate 0S as m/z = 582). Perdeuteroheparin contains only a very low quantity of NAc-containing disaccharides, thus, this $^2$H→$^2$H exchange was deemed acceptable, but could be avoided by performing de-N-acetylation in $^2$H$_2$O and NaOH.

Simultaneous epimerization (in $^2$H$_2$O to prevent replacement of C5 $^2$H with $^2$H) and 2-0-sulfation of this chemically derived intermediate, with *E. coli*-expressed recombinant glucuronosyl-C5-epimerase (*Homo sapiens*, GlcC) and 2OST-1 (*Cricetus griseus*, Hs2st1), yielded perdeutero-2-0-sulfo-NSH. Analysis of heavy C5-epimerized, 2-0-sulfo-NSH by $^{13}$C NMR (Table S1) focused on a decrease in the signal for (C2, GlcNS) and (C1, GlcA) and an increase in the signal for (C1, IdoA). The $^{13}$C-spectrum for heavy 2-0-sulfo-NSH reveals the nearly complete disappearance of C1 signal from GlcA indicating high levels of epimerization. This intermediate was sulfated through the action of recombinant 6OST-1 (*Mus musculus*, Hs6st1) and 6OST-3 (*M. musculus*, Hs6st3) to generate non-anticoagulant heparin with similar disaccharide content as UFH (Figure 3c, d and Table S2). The final heavy heparin product was prepared by treatment of non-anticoagulant perdeuteroheparin with recombinant 3OST-1 (*M. musculus*, Hs3st1), to generate the antithrombin pentasaccharide-binding site providing anticoagulant activity comparable to heparin. NMR analysis of non-anticoagulant perdeuterated heparin confirmed the generation of a product similar to UFH (Figure 2). In all enzymatic reactions $p$-nitrophenylsulfate was a sacrificial sulfate donor to regenerate $5'$-phosphoadenosine-$5'$-phosphosulfate using recombinant arylsulfotransferase-IV (*Rattus norvegicus*, SULT1A1).

LC-MS quantification of analytes in biological samples is often complicated by variability introduced during sample extraction and preparation of technical and biological replicates. Spiking a SIL-internal standard into a drug prior to administration for PK studies, or even into a biological sample preceding or following analyte extraction, can compensate for this variability. Thus, assays utilizing an appropriate SIL-internal standard are often preferred as their use benefits
accuracy, precision, and reproducibility. \[\text{21}\] We prepared perdeuterated heparin disaccharides, as SIL-interned standard for quantification of heparin/HS in biological samples (Figure 4). 2-aminoacridone labeled perdeuterated heparin disaccharides were confirmed by LC-MS with matched retention times and an appropriate mass increase compared to 2-aminoacridone labeled UFH disaccharide internal standard (Figure S5). Although deuteration has been shown in some cases to alter retention, the 2-aminoacridone-labeled heparin and heavy heparin disaccharides in this study exhibited no significant mobility shifts.

During reversible glucuronosyl-C5-epimerization of the heparin backbone precursor, hydrogen is abstracted from hexuronic acid C5 and replaced by a hydrogen from the solvent \(\text{H}_2\text{O}\). \[\text{22–24}\] Quantifying the activity of epimerase presents unique challenges as substrate and product possess the same mass. Moreover, LC-MS assay of disaccharide composition involves heparin lyase-catalyzed depolymerization, generating an unsaturated uronic acid, resulting in a loss of information on C5-epimer identity. Although a direct colorimetric assay for this enzyme does not exist, an indirect colorimetric assay involves a coupled reaction with 2OST. \[\text{25}\]

Epimerase activity is often quantified using a radioactive assay involving abstraction of \(^3\text{H}\) into the solvent from GlcA C5 \(^3\text{H}\) of NSH that has been labeled with \(^3\text{H}\) at GlcA C5 by reaction with epimerase in \(^3\text{H}_2\text{O}\). \[\text{9}\] Recent reports detail alternative assays utilizing SIL: (1) epimerase treatment of NSH in \(\text{D}_2\text{O}\) to site-specifically deuterate GlcA C5, followed by quantification of deuterated disaccharides released by acid-catalyzed hydrolysis\[\text{26}\] and (2) 2D-\(^1\text{H}-\text{H}_{\text{HSQC}}\)-NMR to quantify C5-epimerization of \(^1\text{H}\)-labeled NSH over time. \[\text{10}\]

Treatment of heavy NSH with epimerase in \(\text{H}_2\text{O}\) catalyzes the replacement of hexuronic acid \(^3\text{H}\) with \(^3\text{H}\), associated with the conversion of GlcA to IdoA (and subsequent IdoA/GlcA interconversion). Incorporation of C5 H5 in heavy NSH by GlcA C5-epimerization in \(\text{H}_2\text{O}\) is easily detected using 2D-\(^1\text{H}-\text{H}_{\text{HSQC}}\), free from interference due to absence of other \(^1\text{H}\) atoms in the substrate (Figure 5).
Figure 5. Heavy NSH serves as a substrate in a non-radioactive 2D $^1$H-$^13$C HSQC NMR epimerase assay. a) Site-specific $^1$H incorporation at hexuronic acid CS positions along the heavy NSH chain is achieved by enzymatic GlcA epimerization and subsequent interconversion of GlcA and IdoA residues, reactions involving CS $^1$H abstraction and replacement by $^1$H from solvent (H$_2$O). b) Integration of GlcA (C5,HS) peak indicates incorporation of $^1$H at GlcA C5 following CS epimerase treatment. c) $^1$H-$^13$C HSQC spectra of heavy NSH before (left) and after (right) incorporation of $^1$H at C5 of hexuronic acid by epimerase treatment in H$_2$O.

Figure 6. Time course of drug clearance from plasma following intravenous heparin administration (mixture of 90% natural abundance and 10% perdeuterated by mass) to rabbits. a) PD: Anti-factor Xa activity of heparin and perdeuteroheparin mixture in plasma quantified over time. b) PK: heparin (red circles) and perdeuteroheparin (blue squares) concentration in plasma over time as quantified by MRM LC-MS/MS. Symbols and error bars are mean and SEM of biological triplicates. Lines show fit of first order decay to mean values, where calculated half-life and area under the curve (AUC) values are reported.

Stable isotope-enriched analogs of biomolecules are easily distinguished from their endogenously biosynthesized counterparts by MS, making these indispensable as exogenously supplied metabolic tracers. However, in quantitative studies, a labeled analog should exhibit similar PK parameters to the natural compound. Given the recent surge of interest in deuterated drugs to the increased hal夫-lives reported for some heavy analogs, we wondered whether intravenously administered heparin and heavy heparin would exhibit comparable PK parameters. We determined the in vivo half-life of perdeuteroheparin and the unlabeled bioengineered heparin. A mixture of natural abundance and heavy heparin (10 wt %) was administered by intravenous bolus to three rabbits, and the two analogs were quantified in collected plasma samples (Figure 6a). LC-MS/MS (monitoring heparin's major disaccharide component, Tris$^{[29]}$) was used to directly detect and quantify isotopolog concentrations in plasma over time (Figure S4). The anticoagulant activity of administered heparin mixture in plasma was quantified by anti-factor Xa activity assay as a measure of PD (Figure 6a).

The 6 min half-lives for heavy heparin and for Figure 6b were identical and comparable to literature values.$^{[30]}$ Notably, oxidatively metabolized drugs often exhibit accelerated in vivo degradation relative to their deuterated counterparts due to large $^2$H kinetic isotope effects.$^{[27]}$ Since heparin is thought to be cleared from the circulation through a non-oxidative mechanism,$^{[31]}$ it was not surprising that perdeuteration did not confer a prolonged plasma half-life. Thus, heavy heparin can be used as a surrogate for heparin in animal studies.

Heparin is among the most widely used pharmacological agents and, along with HS, is an important endogenous regulator of multiple biological pathways.$^{[32]}$ Thus, heavy heparin and its intermediates should provide a better understanding of heparin/HS biological activities. In NMR spectroscopy and small angle neutron scattering, heavy heparin should be a useful binding partner for the structural biology studies of heparin-binding proteins.$^{[33]}$ In addition, stimulated Raman scattering imaging examines a silent region in the Raman spectrum that is only excited when C–$^2$H bonds are stimulated, allowing high-resolution, real-time imaging of $^2$H labeled metabolites in tissue slices and cells.$^{[34,35]}$ Thus, heavy heparin and potentially heavy HS should enhance our understanding of the critical roles of these glycans by facilitating the development of new analytical approaches making use of SIL.

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Conflict of interest

The authors declare no conflict of interest.

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