

A Purification Process for Heparin and Precursor Polysaccharides Using the pH Responsive Behavior of Chitosan

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DOI 10.1002/btpr.2144

Published online July 16, 2015 in Wiley Online Library (wileyonlinelibrary.com)

*The contamination crisis of 2008 has brought to light several risks associated with use of animal tissue derived heparin. Because the total chemical synthesis of heparin is not feasible, a bioengineered approach has been proposed, relying on recombinant enzymes derived from the heparin/HS biosynthetic pathway and Escherichia coli K5 capsular polysaccharide. Intensive process engineering efforts are required to achieve a cost-competitive process for bioengineered heparin compared to commercially available porcine heparins. Towards this goal, we have used 96-well plate based screening for development of a chitosan-based purification process for heparin and precursor polysaccharides. The unique pH responsive behavior of chitosan enables simplified capture of target heparin or related polysaccharides, under low pH and complex solution conditions, followed by elution under mildly basic conditions. The use of mild, basic recovery conditions are compatible with the chemical N-deacetylation/N-sulfonation step used in the bioengineered heparin process. Selective precipitation of glycosaminoglycans (GAGs) leads to significant removal of process related impurities such as proteins, DNA and endotoxins. Use of highly sensitive liquid chromatography-mass spectrometry and nuclear magnetic resonance analytical techniques reveal a minimum impact of chitosan-based purification on heparin product composition. © 2015 American Institute of Chemical Engineers *Biotechnol. Prog.*, 31:1348–1359, 2015*

Keywords: bioengineered heparin, polyelectrolyte based purification, liquid chromatography-mass spectrometry (LC-MS), United States Pharmacopeia (USP), nuclear magnetic resonance (NMR)

Introduction

Heparin, a widely used parenteral anticoagulant, has been shown to possess important biological activities that vary based on its fine structure.¹ Variability within the structure of heparin, and the related glycosaminoglycan (GAG) heparan sulfate (HS) occurs owing to its biosynthesis and animal

Additional Supporting Information may be found in the online version of this article.

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tissue-based recovery, and adds another dimension to its complex polymeric structure. Structural variations in chain length and sulfation patterns of heparin mediate its interaction with many heparin-binding proteins, thereby, eliciting complex biological responses. The advent of novel chemical and enzymatic approaches for polysaccharide synthesis coupled with high throughput combinatorial approaches for drug discovery have facilitated an increased effort to understand heparin's structure-activity relationships.²⁻⁴ An improved understanding would offer potential for new therapeutic development through engineering of GAG polysaccharides.

Contamination of several commercial heparin batches, with over-sulfated chondroitin sulfate (OSCS), led to nearly 100 deaths world-wide between late 2007 and early 2008.^{3,5,6} In response to this 2008 contamination crisis, our research group proposed a bioengineered version of heparin possessing biological and chemical equivalence to United States Pharmacopeia (USP) and European Pharmacopeia (EP) heparins as an alternative to porcine mucosa derived heparins.^{2,5-9} A detailed review of the bioengineered heparin process was published earlier by our research group.¹ In summary, bioengineered heparin is prepared through the modification of *E. coli* K5 capsular polysaccharide, heparosan, which resembles a non-sulfated heparin backbone.^{10,11} In the first step of preparing bioengineered heparin, chemical modification of heparosan yields a partially *N*-deacetylated/*N*-sulfonated heparosan (NSH), which is further modified using a series of recombinant enzymes derived from the heparin/HS biosynthetic pathway. This approach modifies the polysaccharide chains at specific positions based on enzyme specificity, minimizing the risk of generating unnatural modifications. Orthogonal purification techniques will be employed, post chemoenzymatic steps, to achieve desired levels of product- and process- related impurities leading to generation of clinically safe bioengineered heparin product. The design of the current bioengineered heparin process is heavily reliant upon traditional anion exchange chromatography followed by an ultrafiltration/diafiltration (UFD) step for separation of bioengineered heparin intermediates from co-factors, immobilized/free enzymes and salt. This coupled approach is required to ensure low conductivity levels and high substrate concentrations (~ 10 mg mL⁻¹) that are ideally suited for these *in vitro* biocatalytic systems. Our experience suggests that up to 10 enzymatic modification cycles may be required to achieve bioengineered heparin structures similar to commercial heparins. This repeated use of coupled ion exchange and UFD is not ideal for commercial manufacturing. Development of a one-step purification technique for heparin and its undersulfated variants is highly desirable for simplification of our bioengineered heparin process, in its current form.

Precipitation of biological molecules for recovery and purification has generated significant interest among process engineers as a scalable alternative to chromatographic separations. A wide array of polyelectrolytes, stimulus responsive polymers, and small molecules have been investigated in capture or polishing steps, in immunoglobulin processing.¹²⁻¹⁷ The advent of such non-chromatographic techniques promise simplified process design with ease of scale-up and low operational cost without sacrificing selectivity.¹⁷⁻¹⁹ Chitosan is a commercially available polysaccharide, prepared through the controlled chemical de-*N*-acetylation of chitin, which is comprised of randomly distributed D-glucosamine

and *N*-acetyl-D-glucosamine residues that introduces a pH-dependent positive charge. Thus, chitosan is water soluble under acidic conditions behaving as a cationic polyelectrolyte, but is water insoluble under basic conditions where chitosan is deprotonated.²⁰ Chitosan is biocompatible, non-inflammatory, non-toxic and biodegradable, and these properties have led to its extensive application in tissue engineering, gene therapy and drug delivery.²¹⁻²⁶ Chitosan is also widely used for recovery of metal ions and waste water treatment owing to its ubiquitous availability and low operational cost.^{27,28} The use of chitosan for flocculation of impurities leads to enhanced clarification of mammalian cell culture.^{29,30} Heparin and its undersulfated/non-sulfated precursors possess a net negative charge owing to glucuronic acid ($pK_a = 3$)/iduronic acid ($pK_a = 3$)/sulfated ($pK_a < 1$) sugar residues. Under acidic conditions, the cationic chitosan interacts spontaneously to form a polyelectrolyte complex with anionic heparin or its undersulfated/non-sulfated precursor. The release of counter ions provides the entropy gain which drives this polyelectrolyte formation^{31,32} and has been reported for other polyelectrolyte complexes. In the current study we utilize chitosan's unique pH responsive behavior to capture heparin and its unsulfated or less sulfated precursors and characterize various process parameters that influence their recovery. A brief outline of our study is presented in Figure 1. This approach can simplify heparosan capture from high titer *E. coli* K5 fermentation broth (~ 10 – 20 g L⁻¹) and intermediate chemoenzymatic steps and provide an alternative to anion exchange chromatography. Moreover, pH-responsive chitosan precipitation can be scaled-up for metric ton production of heparin, required for meeting the world-wide market demand.

Materials and Methods

Material

Heparin (USP) and HS (Supporting Information Table S1) sodium salts, both derived from porcine intestine, were purchased from Celsus laboratories, Cincinnati, OH. Low molecular weight heparin (LMWH), Lovenox (Enoxaparin), was purchased from Sanofi-Aventis, Bridgewater, NJ. V-shaped 96-well plates were purchased from Corning (Corning, NY) and sealing mats were purchased from Thermo Fischer Scientific (Waltham, MA). Low-, medium-, and high-molecular weight chitosan were purchased from Sigma-Aldrich (Saint Louis, MO). Medium molecular weight chitosan is referred to as chitosan unless otherwise mentioned. For screening experiments, 20 mM sodium acetate buffer was used for maintaining pH 4 while 20 mM sodium phosphate buffer was used for maintaining pH 6 and at 8. Additional salt was added as required and pH adjustments were carried out using aqueous solutions of hydrochloric acid and sodium hydroxide. All buffer components were purchased from Sigma-Aldrich.

E. coli K5 fermentation and heparosan purification

E. coli K5 fed batch fermentation was carried out at 100-L scale using a modified M9 medium supplemented with glucose feeding, as described earlier.¹¹ Heparosan from the *E. coli* K5 capsular polysaccharide was shed into the fermentation broth reaching a final titer of ~ 20 g L⁻¹. Ammonium sulfate precipitation, of a 1-L portion of the sample, followed by dialysis using a 6 kDa MWCO membrane

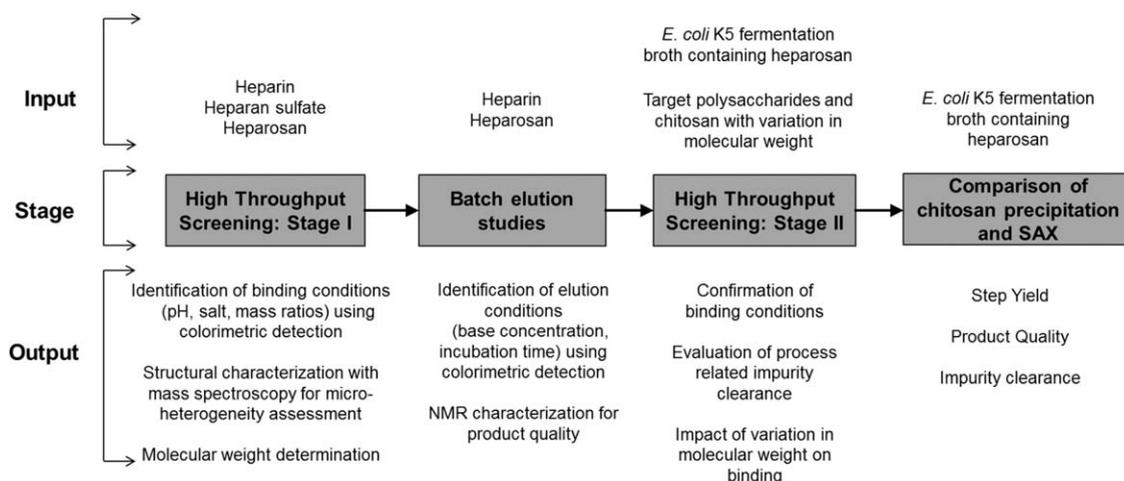


Figure 1. Overview of high throughput screening approach followed for development of chitosan precipitation based purification process for heparin and related polysaccharides.

(Spectrum, Rancho Dominguez, CA) was carried out to recover heparosan from the fermentation broth. Recovery of the entire product afforded in the 100-L fermentation was not undertaken, as this would require specialized ultrafiltration/diafiltration equipment not available in our laboratory. This ammonium sulfate purified heparosan is referred to as heparosan throughout the remainder of this article.

Screening chitosan-GAG interaction using 96-well plate assay

GAG stock solutions (10 mg mL^{-1}) were prepared by dissolving lyophilized GAG powder and were diluted to 1 mg mL^{-1} using appropriate buffer for screening experiments. Fermentation broth experiments were carried out using 1 mg mL^{-1} solutions obtained by serial dilution of clarified *E. coli* K5 broth, obtained by centrifugation at $3500g$, in appropriate buffer. In this work, crude heparosan refers to the heparosan product present in the diluted fermentation broth. Chitosan solution (1%, 10 mg mL^{-1}) was prepared in 1% aqueous acetic acid. The 96-well plate screening was carried out using V-shaped polypropylene plates to identify suitable pH and salt concentrations. GAG solution ($150 \mu\text{L}$) was added to each well in triplicate and varying amounts of chitosan were added at room temperature and followed by 1 h incubation. Sealing mats were used to prevent loss of liquid due to evaporation. Plates were centrifuged at $3,500g$ for 30 min and supernatant was assayed for presence of GAG using micro-carbazole assay. Elution, if required, was carried out using 1 M NaOH at 4°C , unless otherwise specified, to prevent any polysaccharide degradation. Chitosan remained precipitated under basic elution condition. Supernatant containing the target polysaccharide was recovered post centrifugation at $3500g$ and was used for analysis by liquid chromatography (LC)—mass spectrometry (MS).

Micro-carbazole assay

A modified 96-well assay was used to determine the GAG concentration in the supernatant recovered in the screening experiments.³³ Reagent A was prepared by dissolving 0.95 g of sodium tetraborate decahydrate in 2 mL of hot water, which was then added to 98 mL of concentrated sulfuric acid, stored at $2\text{--}8^\circ\text{C}$, carefully with stirring. Reagent B was

prepared by dissolving 125 mg of carbazole (recrystallized from ethanol) in 100 mL of absolute ethanol. Aliquots ($50 \mu\text{L}$) of GAG samples/standards ($0\text{--}1 \text{ mg mL}^{-1}$) were added to wells of a flat bottom transparent 96-well plate (Corning, USA). Ice-cold reagent A ($200 \mu\text{L}$) was then added to each well using a multi-channel pipette. The 96-well plate was heated at 100°C for 10 min in an oven followed by cooling at room temperature. Reagent B ($50 \mu\text{L}$) was added to each well and mixed using a multi-channel pipette, followed by reheating at 100°C for 10 min in an oven. The 96-well plate was cooled at room temperature. Absorbance at 550 nm was measured and concentration of unknown samples was determined using a calibration curve made with known concentration of GAG standard samples in $0\text{--}1 \text{ mg mL}^{-1}$ range.

Estimation of process contaminants

Micro-BCA assay was performed on undiluted supernatant obtained from 96-well screening to estimate unbound protein contaminants using manufacturer-supplied instructions (Thermo Fischer Scientific, Rockford, IL). DNA measurements were carried out using a Nanodrop detector (Thermo Fischer Scientific, Wilmington, DE). Endotoxin measurements were carried out using Pyrotell gel-clot assay (Associates of Cape Cod, USA) as per manufacturer's instructions. Trinitrobenzene sulfonic acid (TNBS) assay was used to determine free amine content of GAG solution using L-alanine as standard, as described previously.³⁴

Enzymatic digestion for disaccharide analysis

Recombinant heparin lyase 1, 2, and 3 were expressed in *E. coli* and purified using affinity chromatography as described earlier.^{35–37} For disaccharides analysis, heparin lyases 1, 2, and 3 (10 mU each) in $5 \mu\text{L}$ of 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer (pH 7.4) were added to $10 \mu\text{g}$ heparin sample in $100 \mu\text{L}$ of distilled water and incubated at 35°C for 10 h to degrade heparin sample completely. The products were recovered by centrifugal filtration using an YM-10 micro-concentrator (Millipore), and the heparin disaccharides were recovered in the flow-through and freeze-dried. The recovered heparin disaccharides were dissolved in water to concentration of $50\text{--}100 \text{ ng}/2 \mu\text{L}$ for LC-MS analysis.

Disaccharide analysis using liquid chromatography-mass spectrometry

LC-MS analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, 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 mm × 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⁻¹. 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 (200–1,500 Da). Nitrogen (8 L min⁻¹, 40 psi) was used as a drying and nebulizing gas. Quantification analysis of disaccharides was performed using linear calibration curves established by separation of increasing amount of disaccharide standards (0.1–100 ng/each) and their respective peak intensities in an extracted ion chromatogram (EIC). Disaccharide analyses were performed in triplicates.

Molecular weight determination

Molecular weight and polydispersity of prepared heparin products was determined using size exclusion chromatography (SEC) as described earlier.^{38,39} TSK-GEL G3000PWxl size exclusion column (Tosoh Bioscience, Minato, Japan), 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 NaNO₃. A sample injection volume of 20 μL and a flow rate of 0.6 mL min⁻¹ 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 (2,687, 4,300, 5,375, 6,449, and 8,060 Da) (Iduron, Manchester, UK) were used as calibrants for heparin, HS and Lovenox. Hyaluronic acid (HA) (81, 130, and 44.3 kDa) was used as calibrant for heparosan. The molecular weight measurements for processed GAGs/starting materials was carried out in triplicates/duplicates.

NMR spectroscopy

Heparosan and heparin products were analyzed by ¹H nuclear magnetic resonance (NMR). All NMR experiments were performed on a Bruker Advance II 600 MHz spectrometer (Bruker BioSpin, Billerica, MA) with Topspin 2.1.6 software (Bruker) as previously reported.^{40,41} Briefly, samples were each dissolved in 0.5 mL D₂O (99.96%, Sigma) and freeze-dried twice to remove the exchangeable protons. The samples were re-dissolved in 0.4 mL D₂O and transferred to NMR micro tubes (OD 5 mm, Norell, tubes). ¹H NMR experiments were performed with 64 scans and an acquisition time of 1.5 sec at 298 K.

Strong anion exchange (SAX) chromatography

Fermentation broth was diluted using 20 mM sodium acetate pH 4 (buffer A) to a final heparosan concentration of 1 mg mL⁻¹. Diluted fermentation broth (10 mL) was 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, the Q-Sepharose column was sanitized using 0.5 M NaOH and equilibrated with buffer A. After loading the sample, column was washed using four column volumes of buffer A. A linear gradient for 20 column volumes was carried out by mixing buffer A and buffer B (2 M NaCl in 20 mM sodium acetate pH 4). This was followed by a step elution of 100% buffer B for another five column volumes. Collected samples were analyzed using micro-carbazole assay. The fractions that tested positive for the presence of heparosan were pooled and dialyzed using 3 kDa MWCO Amicon centrifugal ultrafiltration devices.

Results and Discussion

Screening of solution conditions for chitosan-GAG binding

Industrial scale fermentations are carried out at or near neutral pH conditions with essential media components leading to a significant salt load that can impact downstream processing of biological molecules. The complex nature of animal derived, raw mucosal feedstock for heparin presents another processing challenge at manufacturing scales. High processing volumes encountered in the initial steps of a typical heparin manufacturing can be addressed by incorporation of a simpler capture step that is unaffected by presence of salts thereby leading to overall process simplification.¹ Toward this goal, screening of chitosan binding to heparin and related polysaccharides was carried out with variation in initial solution pH and salt concentration. Heparin, with nearly 2.7 sulfo groups and 1 carboxyl group per disaccharide, possesses significantly higher charge density compared to its undersulfated counterpart HS, which may have 0–2 sulfo groups and 1 carboxyl group per disaccharide. Heparosan, derived from *E. coli* K5 capsular polysaccharide, is a non-sulfated, completely acetylated precursor of heparin with 1 carboxyl group/disaccharide.

Initial screening was performed to identify suitable pH, between 4 and 8, for precipitation of heparin, HS and heparosan through generation of solubility curves (Figures 2A,C, and E). The ratio, expressed as percentage of polysaccharide concentration remaining in solution (C) with varying chitosan concentrations and initial polysaccharide concentration (C₀), was used to determine the respective solubility curves. The upper pH limit was based on the pK_a of the glucosamine residue (~6.3) and the typical initial fermentation broth pH (~7.0).^{11,20} The lower limit of pH was chosen based on a pK_a of ~3.1 for glucuronic acid residue in heparin so that negative charge on these anionic polysaccharides, particularly the unsulfated heparosan, is retained while most contaminating proteins may exhibit net positive charge.⁴² The observed solubility curves show stark difference across these different polysaccharides in line with their extent of sulfation. However, there is negligible variation observed with pH for each individual polysaccharide resulting in overlapping solubility curves. The observed chitosan/GAG ratio at solubility curve minima varied from 1.6–2.0 for heparin,

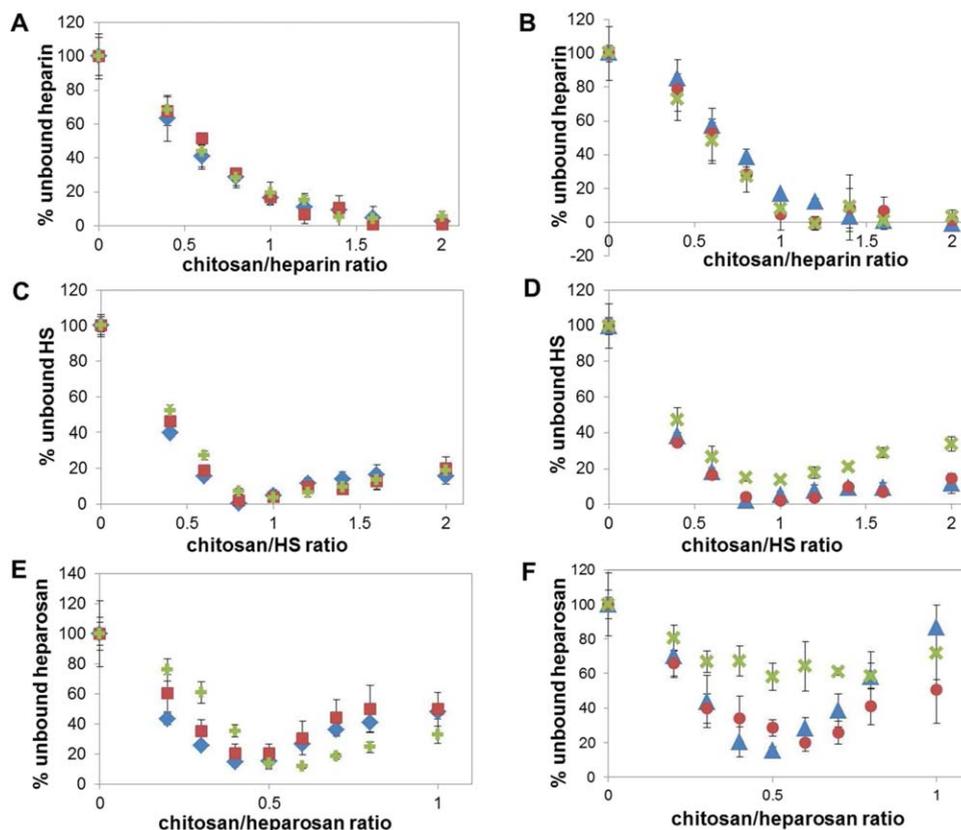


Figure 2. Impact of pH and salt concentration on precipitation of heparin (A, B), HS (C, D) and heparosan (E, F) by chitosan. ◆: pH 4, 0 NaCl; ■: pH 6, 0 NaCl; +: pH 8, 0 NaCl; ▲: pH 4, 20 mM NaCl; ●: pH 4, 100 mM NaCl; x: pH 4, 500 mM NaCl. Chitosan to GAG ratio represented on the x-axis is by weight basis.

0.8–1.2 for HS, and 0.4–0.6 for heparosan. This results from the near complete charge neutralization of these polysaccharides by chitosan and the decrease in net charge per unit disaccharide, which decreases from heparin to heparosan (the amount of chitosan required follows the same trend). A small fraction of heparosan, at minima, appears to remain in solution based on colorimetric assessment while heparin and HS demonstrate near complete capture. The non-precipitating fraction in heparosan may represent interfering components, predominantly media components known to interfere with the carbazole assay, which have been co-purified using the single step ammonium sulfate precipitation step used to recover heparosan from the fermentation broth. It should be noted that chitosan solution prepared using 1% acetic acid can potentially lead to pH reduction, especially at higher chitosan/GAG ratio, depending on buffer strength. In line with these observations, pH 4 was used as solution pH for future studies, as under acidic conditions a larger fraction of proteinaceous impurities will possess a net positive charge thereby minimizing any interaction with positively charged chitosan. The observed re-solubilization of heparosan, in particular, along with HS beyond solubility curve minima with increasing chitosan/GAG ratio is in line with predictions made using Monte-Carlo simulations for protein-polyelectrolyte interactions.^{13,43} Although this behavior was not observed for heparin we anticipate a similar behavior at higher chitosan/heparin ratio beyond the investigated range.

A separate 96-well plate screening was performed to investigate the effect of salt on precipitation of these GAGs (Figures 2B,D, and F). For this purpose, salt concentration was varied from 20 to 500 mM ($\sim 3\text{--}50\text{ mS cm}^{-1}$) in a simi-

lar setup for heparin, HS and heparosan. Earlier work using polyelectrolytes for monoclonal antibody purification has shown very strong effect of salt/conductivity with 5 mS cm^{-1} conductivity levels capable of tuning out any significant complex formation at pH 6 or higher.¹³ In case of heparin, we do not observe any impact of solution conductivity on polysaccharide capture up to 500 mM salt. There is a minor impact of highest salt concentration used in case of HS but its overall capture remains unaffected by salt. The only significant impact of salt is observed for heparosan, wherein high salt concentrations were able to screen out ionic interactions to a greater extent with $\sim 40\%$ capture observed under suitable chitosan/heparosan ratio. These results promise a much easier capture condition for heparosan from the fermentation broth where $40\text{--}50\text{ mS cm}^{-1}$ conductivity levels are commonly observed. Use of chitosan under such conditions would require minimal conditioning/dilution for optimized performance. The ability of chitosan-GAG complex to withstand high salt concentrations will also enable simplified capture of undersulfated and highly sulfated bioengineered heparin intermediates, resembling HS and heparin respectively, from enzymatic co-factors, salts, free or immobilized enzymes.

Evaluation of *E. coli* K5 broth

E. coli K5 fermentation can reach very high cell densities ($>100\text{ g}_{\text{cdw}}\text{ L}^{-1}$) using a modified defined M9 media. The short doubling time of this wild type strain requires efficient control over feeding and solution conditions. Even in well-controlled fermentations cell lysis cannot be completely

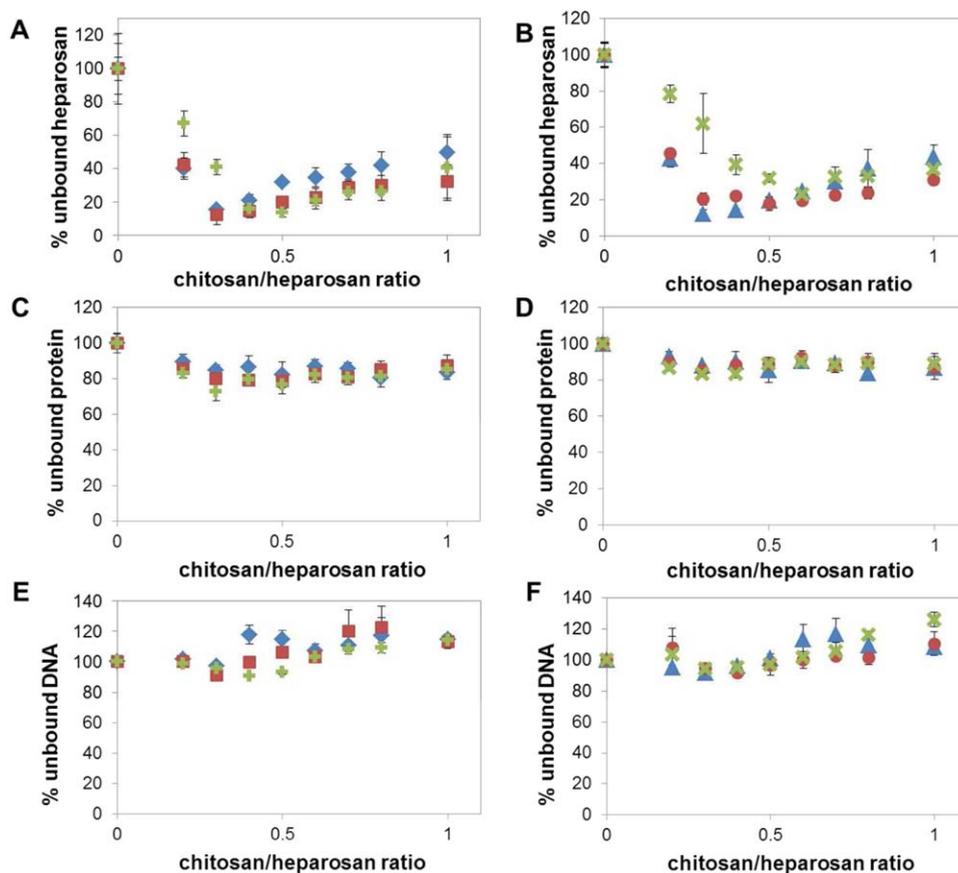


Figure 3. Impact of pH and salt concentration on residual heparosan (A, B), protein contaminants (C, D) and DNA (E, F) present in a fed-batch fermentation broth, containing crude heparosan, with varying chitosan ratio. ◆: pH 4, 0 NaCl; ■: pH 6, 0 NaCl; +: pH 8, 0 NaCl; ▲: pH 4, 20 mM NaCl; ∙: pH 4, 100 mM NaCl; x: pH 4, 500 mM NaCl. Chitosan to GAG ratio represented on the x-axis is by weight basis.

prevented. This can lead to high levels of protein impurities, endotoxins, and DNA, which coupled with a high salt load, increases the overall burden on anion exchange chromatography as a process step. We have been successful in isolating heparosan from fermentation broth using ammonium sulfate precipitation obtaining modest purity and yields. Under these circumstances, use of a selective precipitation method as an initial capture step would provide a significant boost to the overall process economics. As observed above, chitosan is able to precipitate out GAGs with varying levels of sulfonation when using relatively pure starting materials. The results obtained for precipitation of crude heparosan from fermentation broth are in line with what was observed earlier for ammonium sulfate purified heparosan (Figure 3).

Our experimental results suggest that use of chitosan/heparosan ratio of 0.3–0.5 may be most ideally suited for precipitation of crude heparosan. Additionally, solution pH does not seem to have an impact on recovery. Variation in salt showcases a similar screening of ionic interactions at high salt concentrations, as was reported above. An apparent shift of chitosan/heparosan minima and relatively better clearance at higher salt concentrations with crude heparosan can be attributed to potential assay interference. A very large fraction of proteinaceous impurities was found to remain unbound. Based on these estimates, close to a log reduction in protein content can be obtained without inclusion of intermediate washing. Similarly, nearly all DNA present in starting solution remained unbound based on absorbance measurements.

Kinetics of GAG release from polyelectrolyte complex

Release of GAG could be easily accomplished by mild base, which dissociates the polyelectrolyte complex due to deprotonation of chitosan. The GAG product is recovered in the supernatant as chitosan remains insoluble under basic conditions. Different base strengths were tested, in duplicates at 1 mL (1 mg total mass) scale, for release of heparin and heparosan with a chitosan/GAG ratio of 2.0 and 0.5, respectively (Figure 4). Rapid recovery, independent of base strength, is observed for both heparin and heparosan. This reduces the process hold-up time and allows the use of lower base strengths simplifying the purification of process intermediates having low sulfation without significant buffer manipulation. These elution conditions are compatible with chemical *N*-deacetylation/*N*-sulfonation, which represents the first step of the bioengineered heparin process. Heparosan release can be carried out at elevated temperatures and high base strengths for heparosan *N*-deacetylation, either directly or post chitosan removal, followed by immediate chemical *N*-sulfonation (data not shown). Design of experiment studies (DOE) would be required to characterize this process for generation of an *N*-sulfonated product within specifications.^{8,44}

Nuclear magnetic resonance spectroscopy studies

NMR spectroscopy was employed extensively for identification of oversulfated chondroitin sulfate (OSCS) contaminants in adulterated heparin batches.^{3,5} USP also requires use

of ^1H NMR spectroscopy for purity determination and identification of potential contaminants in heparin products. Thus, we carried out NMR studies on heparin and heparosan purified using chitosan with chitosan/GAG ratio of 2.0 and 0.5, respectively, to assess product quality. The spectra reveal that use of chitosan as a purification method did not result in

unknown peaks with all major peaks identified as present in respective controls (Figure 5). This suggests the feasibility of using chitosan for initial capture of heparosan from broth and bioengineered heparin process intermediates. Additionally, no *N*-deacetylation is observed in case of chitosan purified heparosan suggesting that use of higher base strengths are also possible. Further assessment of structural microheterogeneity and molecular weight properties of purified material using sensitive analytical techniques is required to further investigate potential impact of this process on product quality.

Structural characterization of purified heparin and HS using mass spectroscopy

Heparin and HS can exhibit wide variation in sulfation levels and molecular weight properties. These structural variations play an important role in facilitating heparin-protein interactions *in vivo* and determine its biological activity.⁴⁵ LC-MS based disaccharide analysis is a sensitive analytical technique frequently used to determine composition of disaccharides obtained post digestion with heparin lyase 1, 2, and 3. These eight different disaccharides provide a structural fingerprint for heparin related GAGs and can be used to ascertain chemical equivalence.⁴³

The impact of a chitosan-based purification process on the resulting product disaccharide composition was next investigated. Heparin and HS products obtained from 96-well plate screening experiments were analyzed using LC-MS to determine mass percentage of each disaccharide (Supporting Information Table S2). Respective mole percentages, calculated using known molecular weights, were used to determine average sulfo group/disaccharide. The TriS disaccharide, the representative sequence for heparin, is the most abundant disaccharide (66–85%) by mass, observed in all heparin products.³⁹ For a chitosan, or similar polycationic electrolyte, based capture strategy to be incorporated into bioengineered heparin process scheme it is essential that the overall disaccharide composition remains relatively

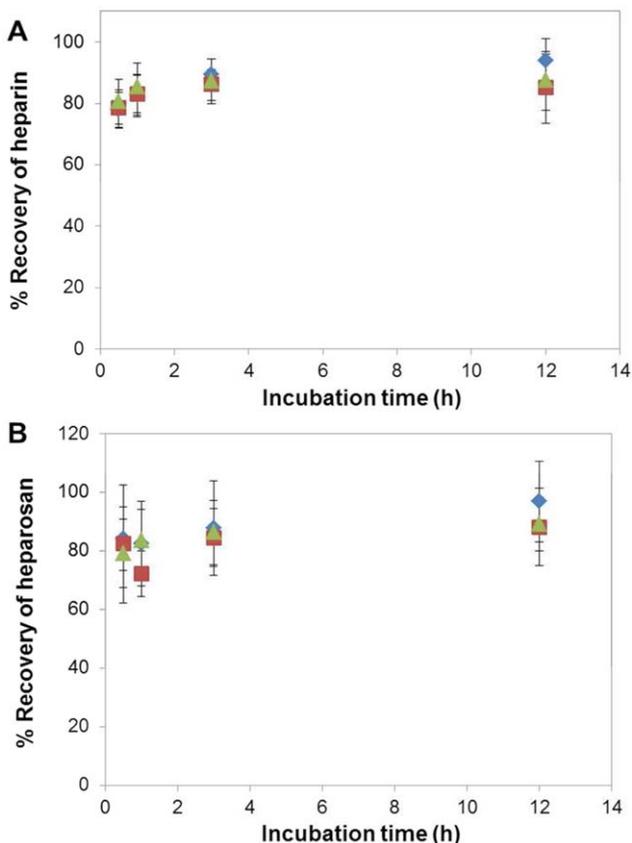


Figure 4. Release kinetics of heparin and heparosan with varying base strength from chitosan-polymer complex. ◆: 2 M NaOH; ■: 0.5 M NaOH; ▲: 0.1 M NaOH.

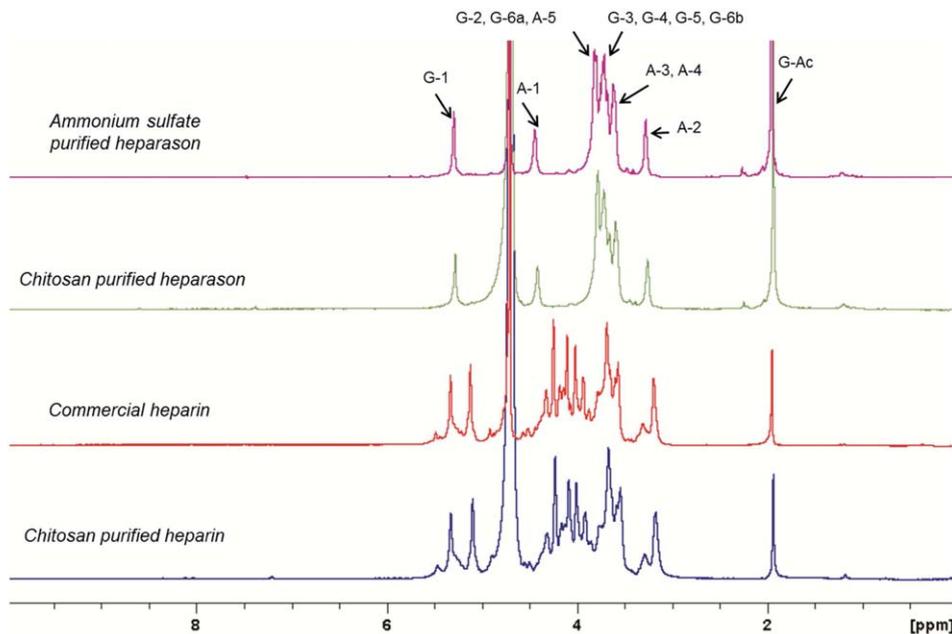


Figure 5. ^1H NMR spectra of heparosan and heparin purified using chitosan (G; GlcNAc, A; GlcA).

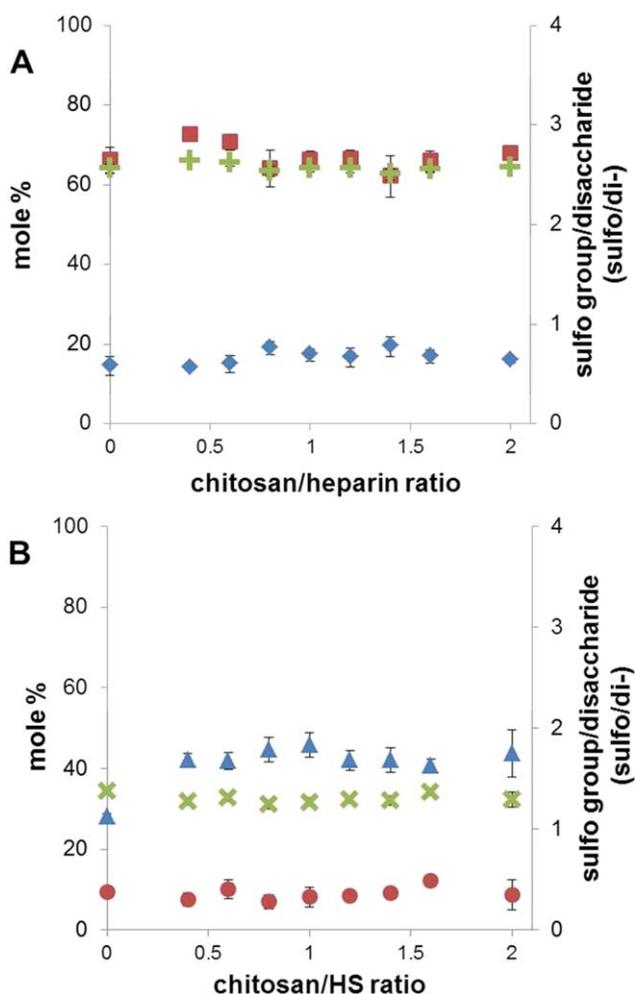


Figure 6. Disaccharide composition of recovered heparin (A) and HS (B) as determined by liquid chromatography-mass spectroscopy based disaccharide analysis. ◆: % NS6S (heparin); ■: % TriS (heparin); +: sulfo group/disaccharide (heparin); ▲: % NS (HS); ●: % TriS (HS); x: sulfo group/disaccharide (HS). Chitosan to GAG ratio represented on the x-axis is by weight basis.

unchanged to maintain chemical equivalence to heparin products purified using traditional process.

Results pertaining to TriS (disaccharide with maximal charge), NS6S (second most abundant disaccharide in heparin), NS (most abundant disaccharide in HS) and sulfo group/disaccharide are presented in Figure 6. A minor increase in TriS disaccharide is observed for heparin products purified using low chitosan/heparin ratio (< 0.6) with a corresponding increase in sulfo group/disaccharide. The NS6S disaccharide profile remains unaffected with variation in chitosan/heparin ratio. Overall, the obtained heparin products display similar disaccharide compositions to the starting heparin product. Similar variations were observed for HS, albeit with minor differences in comparison to heparin. The HS employed for these studies had an abundance of NS6S disaccharide (~31% by weight) followed by NS disaccharide (~26% by weight). However, purified products showcased a moderately elevated level of NS disaccharide in comparison to NS6S disaccharide (Supporting Information Table S1). We attribute this variation to potential loss of GAG chains during membrane ultrafiltration (3 kDa MWCO), required for removal of interfering salts, due to lower molecular

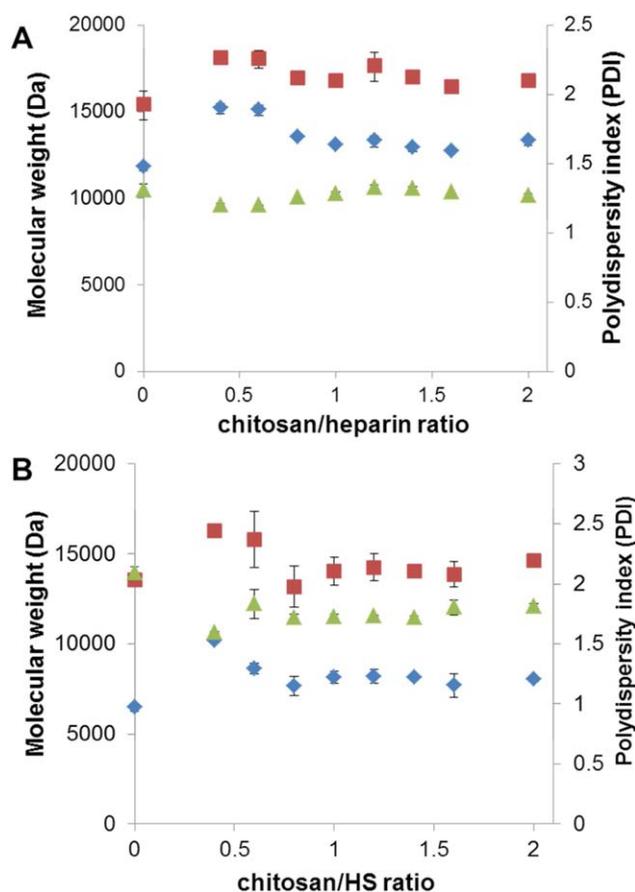


Figure 7. Molecular weight properties determined using size exclusion chromatography of recovered heparin (A) and HS (B). ◆: Number average molecular weight M_N ; ■: Weight average molecular weight M_w ; ▲: Polydispersity index (PDI). Chitosan to GAG ratio represented on the x-axis is by weight basis.

weight of HS (Supporting Information Table S1). The small loss across this intermediate purification process is also observed for heparin but it may be inconsequential towards final product quality due to highly uniform sulfation levels. Overall, NS disaccharide, TriS disaccharide and sulfo group/disaccharide levels remain nearly unchanged with chitosan variation. These observations suggest that chitosan purification of heparin and HS has negligible impact on product quality.

Apart from these disaccharides, a small percentage of five different lyase-resistant tetrasaccharides (<5% by mass) related to the antithrombin III binding pentasaccharide binding region of heparin are also formed due to enzymatic degradation.^{35,46} However, it was not included in this study as the impact of variation in each individual tetrasaccharide on biological activity is not fully understood.

Size exclusion chromatography for molecular weight determination

Post the 2008 contamination crisis, the USP has actively engaged in inclusion of enhanced standards of purity and stricter quality control towards minimization of variation within commercial heparin products. Molecular weight properties of heparin, known to impact its biological activity, were previously not included in the USP monograph.⁴⁷ However, effective as of May 2014, the new heparin monograph

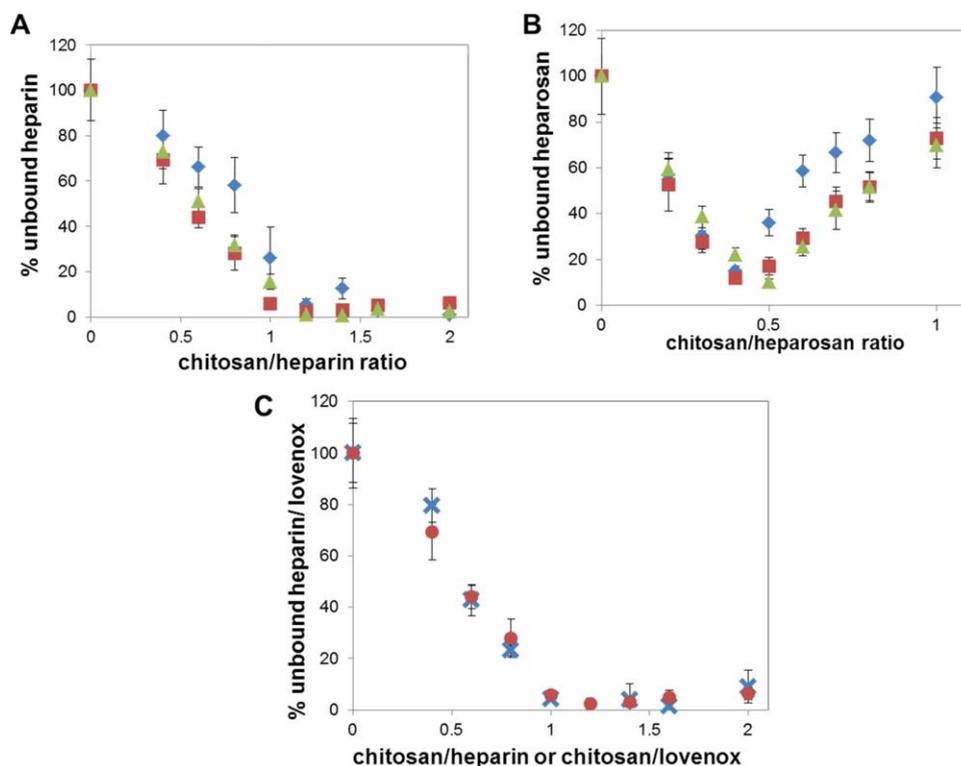


Figure 8. Effect of variation in chitosan molecular weight on precipitation of heparin (A) and heparosan (B). Variation in molecular weight of target polymer using medium molecular weight chitosan is shown in (C). \blacklozenge : Low molecular weight chitosan; \blacksquare : Medium molecular weight chitosan; \blacktriangle : High molecular weight chitosan; \bullet : heparin; \times : Lovenox. Chitosan to GAG ratio represented on the x-axis is by weight basis.

requires USP heparin APIs to comply with the following molecular weight restrictions to ensure minimal variation across commercially available USP heparin products⁴⁸:

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 and 19,000
3. The ratio of heparin chains with molecular weight between 8,000 and 16,000 Da ($M_{8000-16000}$) to heparin chains with molecular weight between 16,000 and 24,000 ($M_{16000-24000}$) is not <1.0

Size exclusion chromatography was used to determine the molecular weight properties of heparin and HS products derived from 96-well screening experiments (Figure 7). The lower chitosan/GAG ratio showed a higher increase in number average molecular weight (M_n) compared to weight average molecular weight (M_w) resulting in a decreased polydispersity. This was observed in case of both heparin and HS and was more pronounced in case of HS, which had higher variation in starting material. It should be noted that in case of both heparin and HS the molecular weight of recovered products with higher chitosan/GAG ratio (corresponding to near complete recovery) is above that for respective starting materials. We attribute this difference to intermediate centrifugal membrane ultrafiltration step, which was employed to remove salts from the recovered step. With restrictions on molecular weight properties being enforced, a chitosan-based clearance of high molecular weight species can provide a simplified solution without significantly affecting the structural composition.

Dependence on chitosan and target GAG molecular weight

Use of polyelectrolytes requires determination of a suitable molecular weight range for the precipitating polymer to

achieve desired results. This has been demonstrated for use of polyelectrolytes in purification of monoclonal antibodies, which are monodisperse, barring aggregates and fragments.¹³ Polydispersity of heparin and related polysaccharide adds another level of complexity towards use of a polyelectrolyte based approach. We characterized binding of chitosan with varying molecular weights to heparin and heparosan (Figures 8A,B). Heparin and its low molecular weight (LMWH) analogue, Lovenox, were studied separately to determine impact of target polysaccharide molecular weight on capture by chitosan (Figure 8C). There was no difference observed between medium and high molecular weight chitosan. Low molecular weight chitosan displayed a slightly different profile compared to others with slightly lower binding of heparin and even more so of heparosan, yet it was able to achieve the same minima for both heparin and heparosan. Therefore, use of any of these three commercially available products should not significantly impact capture and subsequent recovery of heparin products. Lovenox possesses similar disaccharide composition to heparin but has a M_n of ~ 3.9 kDa, which is lower than heparin ($M_n = 11.8$ kDa) (Supporting Information Table S1). Capture of heparin and lovenox with medium molecular weight chitosan was identical to each other. These studies further demonstrate the overall robustness of this process towards molecular weight variations of chitosan and heparin or other GAGs.

Characterization of heparosan products purified using SAX chromatography and chitosan precipitation

Heparosan was purified from 10 mL of diluted fermentation broth containing 1 mg mL^{-1} crude heparosan using SAX chromatography and chitosan precipitation (chitosan/

Table 1. Comparisons Between Products Purified Using SAX Chromatography and Chitosan

	SAX Purified Heparosan	Chitosan Purified Heparosan
% Recovery (based on carbazole assay)	78.3 ± 3.5	81.7 ± 7.4
Free amine content ($\mu\text{g mg}^{-1}$)	2.3 ± 3.0	3.3 ± 2.6
DNA (ng/mg)	N.D.	N.D.
Proteinaceous contaminants		
Protein/heparosan ($\mu\text{g mg}^{-1}$)	2.3 ± 1.2	4.8 ± 0.6
Fold reduction	~55	~26
Endotoxin		
Endotoxin/heparosan (EU mg^{-1})	45,977	44
Log reduction	–	~2.5
Molecular weight properties		
Number average M.W. (M_n)	37,400 ± 1,800	35,100 ± 2,200
Weight average M.W. (M_w)	59,300 ± 300	61,700 ± 100
Polydispersity index (PDI)	1.6 ± 0.1	1.8 ± 0.1

Error represents error in respective measurements. Fermentation broth contained 24,000 EU mg^{-1} of heparosan, $126.4 \pm 0.6 \mu\text{g}$ protein contaminants/mg of heparosan. Endotoxin measurements were carried out in singlet. N.D.: Not detected

GAG ratio of 0.4). The recovered products (yield ~80%) were dialyzed using DI water and Amicon centrifugal filters and characterized for presence of contaminants and molecular weight properties (Table 1). Proteinaceous impurities detected in the final product were negligible and more than a log reduction was observed in case of SAX and chitosan purified heparosan. Both these samples did not contain measurable quantity of DNA impurities. A significant reduction in endotoxin levels (2.5 log) was recorded for chitosan-purified heparosan while SAX purified heparosan still contained a high endotoxin load. This endotoxin reduction is important, as use of chitosan in the bioengineered heparin process can circumvent the need for a change in enzyme expression systems to mitigate endotoxin risk arising due to use of *E. coli* based expression systems.^{49–52} Further characterization of this process, however, is necessary to ascertain selectivity. The purified products, appearing as a single peak, suggest a higher molecular weight of heparosan compared to ammonium sulfate purified heparosan derived from the same fermentation broth. This is not surprising as the quantification of heparosan molecular weight is complicated by presence of co-purified impurities and interfering peaks that result in a lower molecular weight (Supporting Information Table S1). TNBS assay was carried out separately to determine presence of free amine groups in these purified products as these chitosan products have a high degree of de-acetylation (>70% as determined by the manufacturer). Data suggests absence of free amine containing molecules above and beyond the detected levels of proteinaceous impurities thereby confirming absence of chitosan in purified products as observed in ¹H NMR spectra (Supporting Information Figure S2).

Conclusions

The proposed bioengineered heparin process utilizes six different recombinant enzymes used to catalyze chemically *N*-deacetylated/*N*-sulfonated heparosan thereby generating heparin like structures with characteristic high TriS content and *in vitro* biological activity. Intensive process optimization will be required in the near future to bring manufacturing cost of bioengineered heparin down to the level of

commercially available porcine heparins. Toward this goal, we demonstrate a chitosan based purification of heparin and related polysaccharides as a replacement for anion exchange based chromatography. This robust process can be carried out under acidic conditions and is unperturbed by high conductivity levels. The ability to purify even non-sulfated heparosan polysaccharides from complex fermentation broth makes it readily applicable to all stages of bioengineered heparin process. Use of basic conditions for elution further simplifies the process design and allows for recovery of GAG products at high concentrations leading to minimal buffer manipulation. Normal flow filtration followed by recirculation of mild base for elution of bound GAG products offers a scalable precipitate recovery approach, which will need to be evaluated.⁵² Further optimization may still be required for its use in controlling the molecular weight of commercial heparin products by selectively precipitating out high molecular weight species without causing changes in structural composition. Chitosan appears to be selective towards heparosan polysaccharide leading to many-fold decrease in process related contaminants such as proteinaceous impurities, DNA and endotoxins. These bench scale studies will need to be extended to larger-scale systems to demonstrate feasibility for large-scale purification. Use of this approach will not require use of expensive chromatography skids, slower volumetric throughputs encountered in chromatographic operations, cleaning in place procedures, repeated tangential flow filtrations and use of moderately expensive ion exchange resins. The versatility of chitosan, as demonstrated by its wide spread use in biomedical applications, makes it an ideal polymeric precipitating agent which can be incorporated into the development of a bioengineered manufacturing process for parenterally delivered heparin.

Acknowledgment

This work was supported by grants from the US National Institutes of Health (grants HL096972, HL62244, HL094463, and GM38060) and the Bioengineered Heparin Consortium.

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Manuscript received May 6, 2015, and revision received Jun. 18, 2015.