Addressing endotoxin issues in bioengineered heparin

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Abstract.

Heparin is a widely used clinical anticoagulant that is prepared from pig intestine. A contamination of heparin in 2008 has led to a reexamination of animal-derived pharmaceuticals. A bioengineered heparin prepared by bacterial fermentation and chemical and enzymatic processing is currently under development. This study examines the challenges of reducing or removing endotoxins associated with this process that are necessary to proceed with preclinical *in vivo* evaluation of bioengineered heparin. The current process is assessed for endotoxin levels, and strategies are examined for endotoxin removal from polysaccharides and enzymes involved in this process.

1. Introduction

Heparin is a polysaccharide in widespread clinical use as an intravenous anticoagulant with more than 100 tons produced annually worldwide [1]. Nearly a century after its discovery, heparin is still derived from animal sources such as porcine intestine [2]. Heparin has a complex and diverse fine structure and is a polydisperse mixture of varying polysaccharide chain lengths. The heparin polysaccharide is composed primarily of a trisulfated disaccharide repeating unit, 2-*O*-sulfo-α-L-iduronic acid 1–4 linked to 6-*O*-sulfo-*N*-sulfo-α-D-glucosamine (Fig. 1a). Heparin is polydisperse with an average molecular weight of 12,000 Da and a molecular weight range of 5,000–40,000 Da [3]. In addition to the trisulfated disaccharide, heparin also contains disaccharides with lesser degrees of sulfation leading to its structural heterogeneity. Anticoagulant activity associated with heparin is primarily attributed to a highly specific interaction with an antithrombin III (AT)-binding pentasaccharide sequence containing a central 3,6 di-*O*-sulfo, 2-*N*-sulfo-glucosamine residue [4]. When this pentasaccharide sequence is bound to AT, AT undergoes a conformational change, thereby accelerating its inhibition of factor IIa (thrombin) and factor Xa in the blood coagulation cascade. AT and thrombin both need to bind to the same heparin chain in a ternary bridging complex to inhibit thrombin and prevent conversion of fibrinogen into an insoluble fibrin clot. Low-molecular-weight heparins (LMWHs) of average molecular weight 6,000–8,000 Da are prepared from heparin through its controlled chemical or enzymatic depolymerization. The development of LMWH was initially aimed at controlling heparin's hemorrhagic effect by exploiting heparin chain size-dependent differences in binding to antithrombin and factor Xa and factor IIa [5] but ultimately LMWHs gained popularity as a result of their subcutaneous bioavailability and improved pharmacodynamics [6].

An international health crisis, associated with contamination of several heparin batches, began in early 2008, reportedly resulting in nearly 100 deaths in the United States alone. These adverse side effects resulted in the withdrawal of a number of heparin batches from US markets in March 2008, followed by an investigation for the presence of contaminants in these
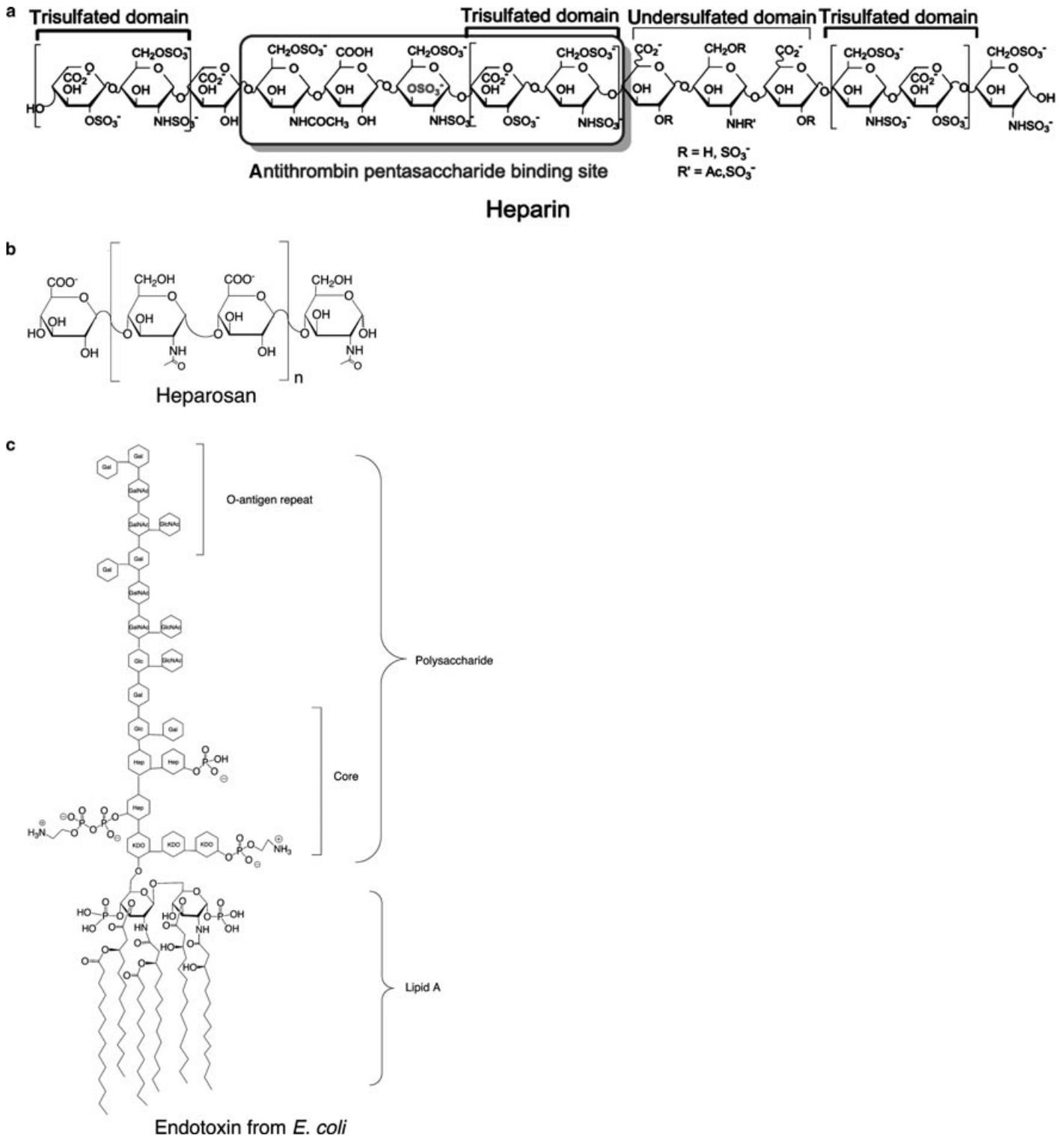
Fig. 1. Chemical structures. (a) Structure of heparin. (b) Structure of heparosan. (c) Structure of LPS. Depicted is the structure of the endotoxin from the *E. coli* O111:B$_4$ [7]. The specific sugars for K5 have not been determined. KDO, 2-keto-3-deoxyoctanonic acid; Hep, L-glycero-D-manno-heptulose; Gal, galactose; GalNAc, *N*-acetyl-galactosamine; GlcNAc, *N*-acetyl-glucosamine, Glc, glucose.

batches. Oversulfated chondroitin sulfate, a semisynthetic polysaccharide of average molecular weight 18,000 Da having a charge density slightly greater than heparin, was dis-covered to be the contaminating agent [8]. This contaminant also carried through the production process of certain LMWH products [9]. The rapid and acute response elicited by

this oversulfated chondroitin sulfate was associated with an anaphylactoid response generated because of the activation of the kinin–kallikrein pathway in human plasma, leading to the formation of vasoactive mediator bradykinin, which caused vasodilation and a sometimes lethal drop in blood pressure [10].

The health crisis in 2008 brought to light flaws within animal tissue-based heparin production, which begins at slaughterhouses. On the basis of the initial small-scale studies in our laboratory [11], we proposed to replace animal-sourced heparin with a clinically safer, nonanimal bioengineered heparin. Bioengineered heparin begins with the fermentative production of the polysaccharide precursor of heparin, heparosan (Fig. 1b), from *Escherichia coli* K5 [12]. Heparosan is then converted to *N*-sulfoheparosan through base treatment and chemical *N*-sulfonation [13]. The resulting *N*-acetyl, *N*-sulfoheparosan is enzymatically converted to bioengineered heparin by treatment with recombinant mammalian enzymes expressed in *E. coli*, including 2-*O*-sulfotransferase (2-OST), C-5 epimerase, 6-OST, and 3-OST [14]. The resulting bioengineered heparin closely resembles porcine intestinal heparin in its chemical properties and *in vitro* anticoagulant activity. However, before it can be extensively studied *in vivo*, it must be free of endotoxins associated with the Gram-negative *E. coli* used in its production.

Endotoxins are lipopolysaccharides (LPS) present in integral parts of outer cellular membranes of most Gram-negative bacteria, and consist of a hydrophilic heteropolysaccharide domain covalently bound to hydrophobic lipid A tail (Fig. 1c). The molecular structure of lipid A is constituted by a diphosphorylated D-glucosamine disaccharide backbone acylated by up to seven asymmetric fatty acid chains. The heteropolysaccharide region is made by a sequence of oligosaccharide unit (*O*-antigen) and several rare sugars. The *O*-antigen structure provides serotype specificity and polar properties to the overall structure, whereas lipid A is responsible for most of the pathogenesis of septic shock.

In modern biotechnology, Gram-negative bacteria are often used to produce recombinant proteins that are generally contaminated with endotoxins [15]. Endotoxins are generally shed in large amounts upon cell death as well as during cell growth and division [16]. They are highly heat stable and are not destroyed under regular sterilizing conditions. Endotoxins have profound biological effects at low concentrations, and thus they require removal before the use of such preparations in biological cell-based assays [17]. Although acid or alkaline treatment can be used to destroy endotoxins, these methods can also damage biological molecules, such as recombinant proteins [18]. Thus, a variety of milder methods are often used for the removal of endotoxins from recombinant proteins, such as anion-exchange chromatography [19], hydrophobic interaction chromatography (HIC) [20],[21], polymyxin B affinity chromatography [22]. The current study examines the endotoxin levels present in the process used in the preparation of bioengineered heparin and assesses various methods for endotoxin reduction/removal.

2. Materials and methods

2.1. Reagents
The equilibration buffer used was purchased from Genscript, Piscataway, NJ, USA. A 1% sodium deoxycholate solution was prepared by adding 50 mL of endotoxin-free water to 50 mg of sodium deoxycholate (Thermo Scientific, Rockford, IL, USA). Deionized (DI) water was obtained by passing distilled water through a BioPak Ultrafilter (Millipore, Billerica, MA, USA) and collected in cleaned glassware.

2.2. Glassware cleaning
The glassware was soaked overnight in a 1% solution of alkaline detergent. It was then rinsed 8–10 times with warm tap water. Next, it was rinsed with distilled water five times and finally once with endotoxin-free water. The glassware was placed in a hot air oven for 1 H at 100°C, removed when completely dried, and finally autoclaved for 1 H.

2.3. Protein determination
Protein concentration, specifically arylsulfotransferase (AST)-IV, was quantified using the Bradford assay [23] using a 5-μL sample volume in a 96-well microtiter plate containing 250 μL of Coomassie Plus Protein Assay Reagent (Thermo Scientific). Absorbance was read at 595 nm. Standards of 2.0–0.025 mg/mL were prepared from bovine serum albumin (Thermo Scientific), with water as the control.

2.4. Uronic acid determination
A carbazole assay [24] was used to determine uronic acid content based on a heparin standard curve. Standards were prepared to include concentrations of heparin of 2.5, 2.0, 1.5, 1.0, and 0.5 μg/mL with water as control, and read at 525 nm.

2.5. Preparation of *E. coli* K5 heparosan
E. coli K5 strain (American Type Culture Collection #23506) was cultured at a 100-L scale using the same media and conditions as previously described for a 15-L fermentation [12],[13]. The K5 capsular polysaccharide, heparosan, shed into the culture supernatant was treated overnight at room temperature with 1.2% (w/v) sodium hypochlorite. After this beaching step, the heparosan was recovered by ammonium sulfate precipitation at 60% saturation (∼361 g/L). The recovered heparosan pellet (∼900 g) was dissolved in endotoxin-free water and dialyzed against endotoxin-free water using a 6,000–8,000 Da molecular weight cutoff (MWCO) cellulose membrane. The resulting heparosan was >95% pure as determined by nuclear magnetic resonance (NMR) spectroscopy and disaccharide analysis [13],[14]. The average molecular weight determined by size-exclusion chromatography (SEC) was ∼40,000 Da [25]. The purified heparosan was freeze-dried and stored desiccated at −20°C.

2.6. Preparation of recombinant AST-IV

All recombinant proteins were expressed in *E. coli* as either His-tagged or maltose-binding fusion proteins and purified either on Ni-nitrilotriacetic acid (NTA) resin or amylose column as previously described [26]. As a typical example, a detailed protocol is provided for AST-IV. The seed cultures were removed from a $-80°C$ freezer and thawed on ice to prepare for small-scale tube culture. Luria broth (3 mL) along with 3 μL of seed bacteria and 50 μg/mL kanamycin antibiotic were placed on a shaker at $37°C$ and incubated overnight. The cultures were then transferred to 1 L-scale flasks along with the antibiotic. Optical density (OD) at 600 nm was measured every hour and the flasks were transferred to $22°C$ shakers once they were in the range of OD 0.55–0.75. After 30 Min, 0.2 mM of isopropyl β-D-1-thiogalactopyranoside was added and the culture shaken overnight at $22°C$. The cultures were poured into centrifuge bottles and centrifuged at 3,000g, $4°C$ for ~30 Min, and the collected cell pellet was resuspended in 20 mL of 25 mM Tris pH 7.4 buffer containing 50 mM NaCl. Sonication for ~3 Min at power level 7 and 50% cycle was used to disrupt the cells. Cell debris was removed by centrifuging at $4°C$ for 1 H in 50 mL tubes at 9,400g. Purification was performed by first passing the supernatant through a 0.45 μm filter and onto a 20 mL Ni-NTA agarose (His-tagged) affinity column, which had been first cleaned with one column volume of 20% ethanol and then two column volumes of DI water. The Ni-NTA agarose column was washed with 5 mL of 25 mM Tris, pH 7.4, containing 500 mM NaCl, and eluted with 30 mM imidazole buffer. Enzyme was then added and washed with 5 mL of 25 mM NaCl and 30 mM Tris, pH 7.4, 500 mM NaCl, and 30 mM imidazole. Elution occurred with 10 mL of 25 mM Tris, pH 7.4, 500 mM NaCl, and 300 mM imidazole. The eluted enzyme was added to a 30,000 MWCO spin column (Millipore) and centrifuged at 4,000 rpm for 10 Min. The bottom portion was discarded and 10 mL of phosphate-buffered saline (PBS), pH 7.0, was added and the process repeated 2–3 times. The bicinchoninic acid (BCA) assay was performed to quantify protein concentration and it was read at $\lambda = 562$ nm. Copper(II) sulfate solution (0.2 mL) was added to 10 mL of BCA and vortexed for reagent preparation.

2.7. *N*-deacetylation/*N*-sulfonation of K5 heparosan

N-deacetylation/*N*-sulfonation of K5 heparosan was typically carried out as described previously [14]. Specifically, purified K5 heparosan (1 mL of 20 mg/mL) was mixed with 1 mL NaOH at varying concentrations. This solution was then incubated at different temperatures for different lengths of time. The pH was adjusted to 7.0 ± 0.5 with 0.1 M hydrochloric acid. Sodium carbonate (60 mg) and trimethylamine–sulfur trioxide complex (60 mg) were both added to each sample in a single step, and the resulting mixtures were incubated at $47°C$ for 2 days. The mixtures were desalted using a 4-mL desalting column (MWCO 3 K; Amicon-Ultra 4, Millipore, Billerica, MA, USA) and freeze-dried to obtain *N*-acetyl, *N*-sulfoheparosan. The purity of the *N*-acetyl, *N*-sulfoheparosan was determined by NMR spectroscopy and disaccharide analysis [13],[14] as >95% with 12%–18% *N*-acetyl and 88%–82% *N*-sulfo content. The average molecular weight determined by SEC was 11,000–12,300 Da [25].

2.8. Quantification of endotoxin

Endotoxin contents in heparosan and enzyme samples were quantified by the *Limulus amebocyte* lysate (LAL) assay gel-clot method according to the manufacturer's instructions (Associates of Cape Cod, East Falmouth, MA, USA). Briefly, with fresh stock endotoxin, dilutions of 0.5, 0.25, 0.125, 0.06 endotoxin units (EU)/mL along with a negative control were prepared in endotoxin-free dilution tubes. Sample dilutions were made and 100 μL of each dilution and of each standard were transferred to their own clotting test tube. Reconstituted Pyrotell (100 μL) was added to each sample and dilution, then vortexed for 3 Sec and placed in a dry block heater at $38°C$ for 60 Min. The assay has a sensitivity of 0.25 EU/mL, corresponding to 0.05 ng of endotoxin/mL. Results were reported in EU with reference to control standard endotoxin (Associates of Cape Cod).

2.9. Diethylaminoethyl removal of endotoxin from heparosan

A 400-μL sample of AST-IV was centrifuged at 2,000g for 5 Min. The diethylaminoethyl (DEAE) resin, Vivapure™ Mini Q (Sartorius Stedim Biotech GmbH, Goettingen, Germany) was equilibrated to pH 7.2 with one wash of water, NaOH (0.5 M), and two washes of water and PBS (50 mM sodium phosphate plus 100 mM NaCl), pH 7.2. The sample was then added and eluted with PBS. The protein concentration was checked with the Bradford Assay.

2.10. DEAE removal of endotoxin from recombinant proteins

AST-IV (pI 5.0) was dissolved in PBS adjusted to pH 4.5. The Vivapure™ mini DEAE spin column (Sartorius Stedim Biotech GmbH) was washed sequentially with endotoxin-free water, 0.5 M NaOH and PBS, pH 4.5, by loading each solution into the device followed by centrifugation at 2,000g for 15 Min. The AST-IV sample was added and centrifuged as described above. The flow through from each column was then stored at $4°C$. Residual protein was recovered by washing with PBS. Samples of the flow through and protein were collected as they eluted from the column and the concentration of protein was measured in all samples using the Bradford assay.

2.11. Polymyxin B removal of endotoxin

Sodium deoxycholate (10 mL of 1% aqueous solution) was passed through a Detoxi-Gel Endotoxin Removing Gel Column (Thermo Scientific) followed by 15 mL of equilibration buffer (Genscript) and then 2 mL of PBS solution. AST-IV was then added and allowed to seep into the matrix, and the void volume of PBS was collected. The sample was allowed to remain on the column for 1 H before eluting with 3 mL of PBS and 2 mL of equilibration buffer. Sodium deoxycholate (20 mL of 1% solution) was then used to rinse the column. The sample was collected as it eluted from the column, and the concentration

of protein was measured using the Bradford assay. The concentrations of heparin and heparosan were measured using the carbazole assay.

2.12. Oxidative removal of endotoxin

N-acetyl, *N*-sulfoheparosan (500 μL of 20 mg/mL), or sodium heparin (Celsus Laboratories, Cincinnati, OH, USA; 500 μL of 1 mg/mL), in which 1 unit/mg of endotoxin was deliberately added, was mixed with 1.2% (w/v) sodium hypochlorite. Each mixture was incubated at room temperature for 24 H, and then desalted in a 4-mL desalting column (MWCO 3 K; Amicon-Ultra 4) and freeze-dried.

2.13. HIC for removal of endotoxin

A 500-μL sample of heparin (9.6 mg/mL) was loaded onto a HiScreen Phenyl HP column (GE Healthcare, Life Sciences, Piscataway, NJ, USA) using an AKTA purifier chromatography system. The column was first washed with 0.5 M NaOH for 1 H to remove column impurities. The sample was eluted from the column using 0.7 M $(NH_4)_2SO_4$. For testing of carbohydrate concentrations along with the level of endotoxin, the flow through was collected in 0.5-mL collections and tested using carbazole and the LAL gel-clot assay. The protein was detected by ultraviolet absorbance, and the fractions corresponding to the peak were pooled and the endotoxin level determined.

3. Results

Bioengineered heparin is prepared by a five-step process detailed in Fig. 2. In the first step, heparosan is prepared from *E. coli* K5. Heparosan (∼10 mg/mL) is shed into the culture supernatant and contains 128,000 EU/mL of endotoxin. After bleaching the culture supernatant, the endotoxin level drops to 20,000–64,000 EU/mL. In the second step, heparosan is purified by ammonium sulfate precipitation and dialysis, affording heparosan of >95% purity with an endotoxin level of 10,000–20,000 EU/mL. In the third step, heparosan is converted to *N*-acetyl, *N*-sulfoheparosan by treatment with sodium hydroxide under conditions that reduce its average molecular weight to 10,000 Da and affords an *N*-acetyl to *N*-sulfo ratio consistent with that found in porcine mucosal heparin. The endotoxin level in this intermediate was determined to be 2.5–8.3 EU/mg of *N*-acetyl, *N*-sulfoheparosan. In the fourth step, the *N*-acetyl, *N*-sulfoheparosan is treated with C5-epimerase, 2-OST, 6-OST, and 3-OST in the presence of 3′-phosphoadenosine-5′-phosphosulfate (PAPS). In the *O*-sulfonation process step, PAPS is used catalytically and is recycled using *p*-nitrophenylsulfate and AST-IV [27]. Thus, in this process the *N*-acetyl, *N*-sulfoheparosan is exposed to five *E. coli*-expressed recombinant enzymes each containing ∼300,000 EU/mg endotoxin. Our current process calls for ∼10 wt% of each enzyme based on substrate, resulting in the addition of ∼150,000 EU/mg product.

As a result, we assessed various methods to remove endotoxins from heparosan, recombinant AST-IV, and heparin
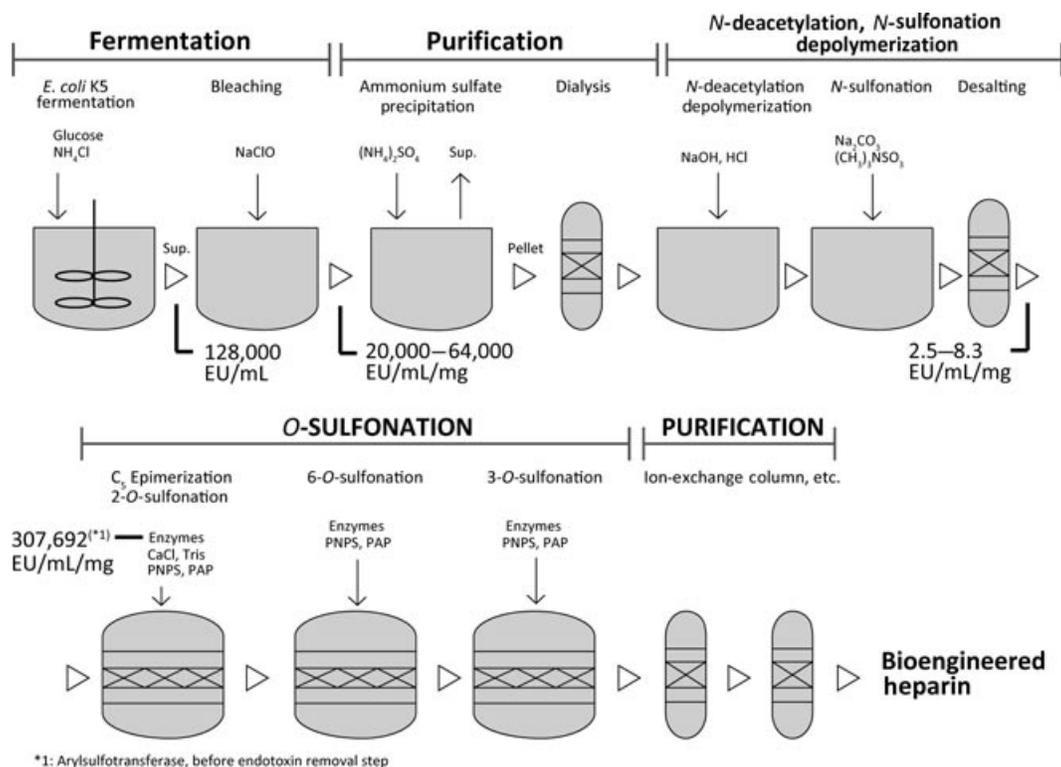

Fig. 2. Process flow diagram for bioengineered heparin and endotoxin levels.
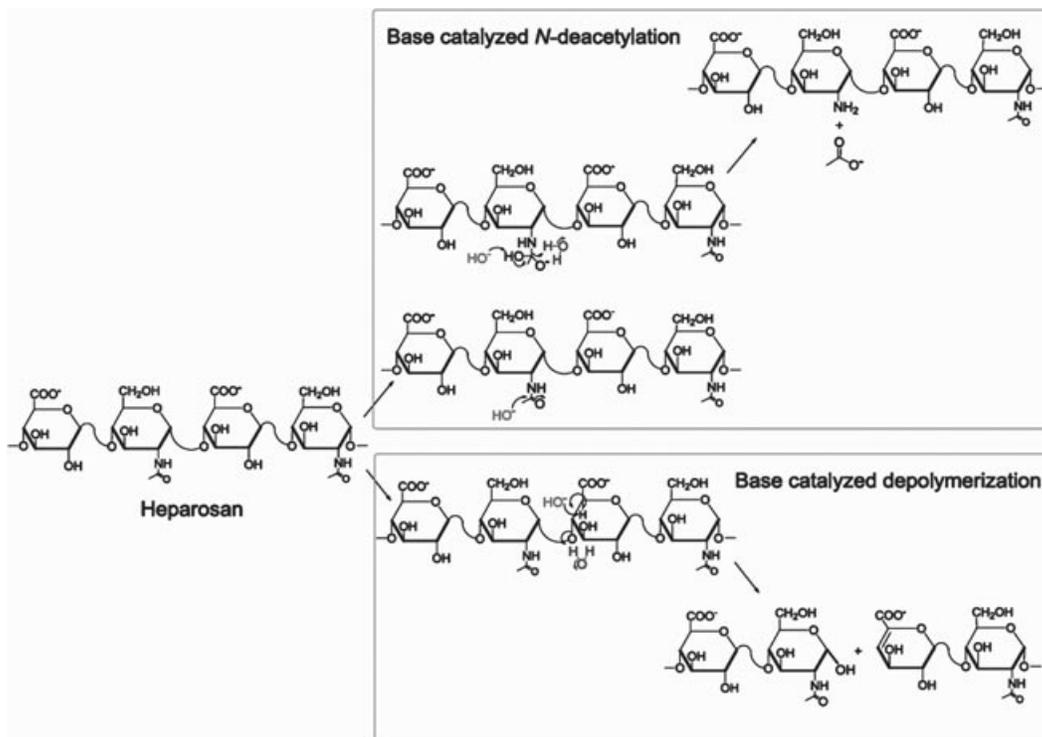
Fig. 3. Proposed mechanism of base catalyzed *N*-deacetylation and depolymerization of heparosan. Depolymerization and *N*-deacetylation of heparosan take place in 0.5–2 M sodium hydroxide at temperatures of 40–60°C in 1–8 H.

Table 1
Efficiency of different methods of endotoxin removal from heparosan, heparin, and recombinant proteins

Interaction	Method	Endotoxin removal (%)		
		Heparosan	AST-IV	Heparin
Oxidation	Bleaching[a]	84.4	ND	99.6
Chemical treatment	Base treatment[b]	99.9	ND	ND
Anion exchange	DEAE	93.6	99.9	ND
Hydrophobic	HIC	96.9	ND	90
Mixed mode	Polymyxin B	71.0	99.9	90.0

[a]Bleaching with 1.2% (w/v) sodium hypochlorite at room temperature for 24 H.

[b]Base treatment with 0.5 M sodium hydroxide at 60°C for 6 H.

(Table 1). Oxidative treatment, such as bleaching, can remove LPS through the oxidation of sensitive glycan moieties. Although heparosan and heparin are stable to mild oxidative treatment, proteins such as AST-IV are not. Bleaching successfully removed most of the endotoxin from heparosan and over 99% of added endotoxin from heparin. Strong base treatment decreases heparosan molecular weight and causes 80%–90% *N*-deacetylation effectively as required in the bioengineered heparin process (Figs. 2 and 3) but it also removes 99.9% of the endotoxin from heparosan. Relatively harsh treatment 0.5–2.0 M base at 40–60°C for 2 H is required to remove over 99.9% of endotoxin (Table 2). Such harsh treatment results in loss of heparin's AT-mediated anticoagulant activity [28] and degrades recombinant proteins. At optimal pH, both AST-IV and heparosan fail to interact with the DEAE stationary phase and freely flow through the column eluting as a symmetrical peak for collection and endotoxin analysis. Anion-exchange chromatography on DEAE removes 93% and 99.9% of endotoxin from heparosan and AST-IV, respectively (Table 1). Unfortunately, because heparin contains sulfo groups with pK_a values below 1 [29], heparin will bind to DEAE at all accessible pH values. Thus, DEAE cannot be used for endotoxin removal from heparin. HIC can be effective in removing 96% and 60% of endotoxin from heparosan and heparin, respectively, but HIC resin binds too tightly to proteins to remove endotoxin and allow recovery of active protein. Polymyxin B tightly binds LPS through a mixed hydrophobic–ionic mode, allowing removal of 99.9% of endotoxin from AST-IV but only 71% and 90% endotoxin removal from heparosan and heparin, respectively. AST-IV flows freely through the polymyxin B column, eluting as a symmetrical peak devoid of endotoxin and retaining its sulfotransferase activity (Table 3).

4. Discussion

The most commonly used techniques for endotoxin detection are the rabbit pyrogen test and LAL assay. The simplest form of

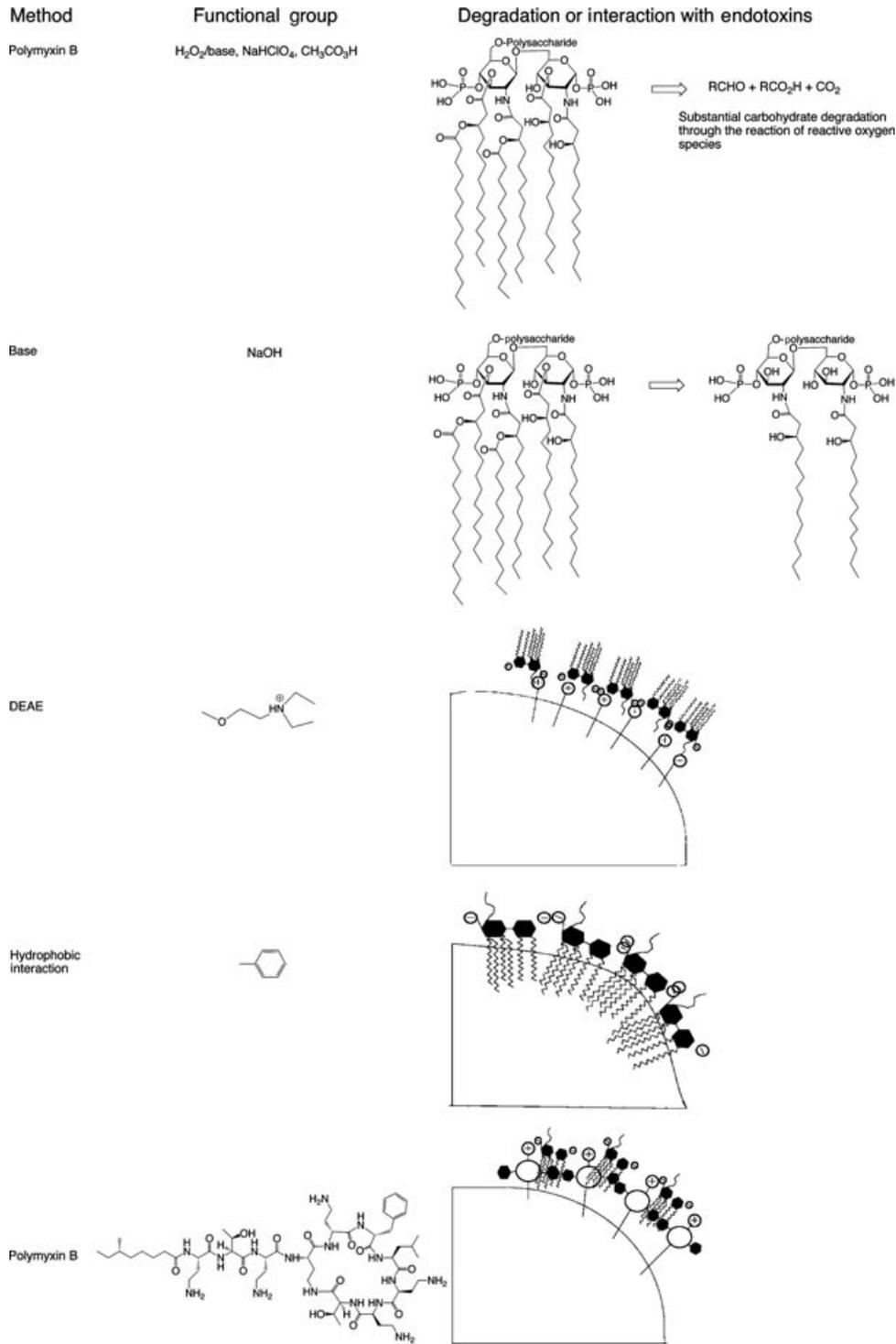| Method	Functional group	Degradation or interaction with endotoxins

Fig. 4. Mechanism of endotoxin removal using various processing steps. Oxidation—the oxidation of the lipid A portion of the endotoxin occurs close to the KDO sugars, mainly to the chains with the easiest access. Base—sodium cleaves the ester side chains of lipid A portion of endotoxin. Usually only one of the chains of the lipid needs to be cleaved for loss of endotoxin activity. DEAE—the diethylamino group on the stationary phase has a positive charge, which attracts the negatively charged phosphate groups on the endotoxin. HIC—phenyl groups on the stationary phase strongly interact with the lipid A portion of the endotoxin due to a hydrophobic effect. Polymyxin B—mixed mode interaction with lipid portion of endotoxin being attracted to the phenyl group of the polymyxin B on the stationary phase, and the phosphate groups being attracted to the positively charged ammonium groups on the polymyxin B.

Table 2
Base treatment of heparosan; endotoxin levels (in EU) are indicated

Reaction time (H)	NaOH at 40°C			NaOH at 50°C			NaOH at 60°C		
	0.5 M	1 M	2 M	0.5 M	1 M	2 M	0.5 M	1 M	2 M
1	>250	>40	>100	>25	25	>25	>25	25	2.5–25
2	>250	40	2.5–25	>25	2.5–25	2.5–25	25	2.5–25	2.5–25
4	250	20	2.5–25	2.5–25	2.5–25	2.5–25	2.5–25	2.5–25	2.5–25
6	125	5	2.5–25	2.5–25	2.5–25	2.5	2.5	2.5–25	2.5
8	25	5	2.5–25	2.5–25	2.5–25	2.5	2.5	2.5–25	2.5

Table 3
AST-IV recovery and endotoxin level

Sample	Protein (mg/mL)	Recovery (%)	EU (mg/mL)
Load AST-IV	7.0	–	5.7×10^5–1.1×10^6
Flow through	6.1	86.4	660
Wash	0.6	8.5	83

LAL assay is the LAL gel-clot assay. For heparin, the LAL assay is well behaved, with concentration–response curves that are parallel to the standard curve. Indeed, this assay is currently part of the United States Pharmacopeia compendium for heparin. In this study, we show that this assay is also well behaved for assessing endotoxins in heparosan and AST-IV.

Bioengineered heparin is prepared using a complex process and involves different types of intermediates (*e.g.*, polysaccharides such as heparosan) and reagents (*e.g.*, enzymes such as the AST-IV protein) that can introduce endotoxins into the process and ultimately into the bioengineered heparin product. Thus, careful consideration needs to be given to develop a strategy to remove endotoxins from this process. Similar consideration has been given to removal of endotoxins from therapeutic nucleic acids such as plasmid DNA. Alkaline treatment or affinity chromatography has been used to remove endotoxins from nucleic acids [30],[31].

Lipid A carries a 2-keto-3-deoxyoctonic acid (KDO) residue with a phosphate group, affording anionic endotoxin with a $pK_1 = 1.3$ and $pK_2 = 8.2$. Moreover, LPS contains lipid A with five hydrophobic acyl chains. Because endotoxins are amphipathic, having both an anionic and hydrophobic functionality, the removal of endotoxins can rely on anion-exchange (DEAE), hydrophobic interaction (HIC), or multimodal (anion-exchange and hydrophobic) interaction using polymyxin B chromatography (Fig. 4). The objective of these schemes is to tightly bind endotoxin while allowing the endotoxin-free product to freely elute. For anion-exchange chromatography to be effective, the endotoxin contaminated must have a p*I* greater than a phosphate anion $pK_1 = 1.3$. AST IV has p*I* 5.0, resulting in a positive charge at pH <5, and allowing it to flow unimpeded through a DEAE column with bound LPS. Unfortunately, such an approach will not remove endotoxin from heparin, as the p*K* of its sulfate half ester is <1 [29]. In contrast, HIC allows the removal of amphipathic LPS from hydrophilic heparosan and heparin, but not from fragile proteins containing hydrophobic amino acid residues. The multimodal polymyxin B column is highly efficient in removing endotoxin from AST-IV but somewhat less effective in removing endotoxin from heparin and heparosan. Moreover, it is noteworthy that polymyxin B is less effective in removing endotoxin from LMWH and nearly completely ineffective in removing endotoxin from ultra-LMWH.

Process steps such as bleaching hold promise in destroying endotoxin from polysaccharide intermediates, such as heparosan and from heparin but cannot be used to remove endotoxins from process enzymes. Finally, the strong base used in *N*-deacetylating heparosan is extremely effective in destroying endotoxin. Current efforts are focused on sufficiently reducing endotoxin levels in bioengineered heparin to allow for successful preclinical *in vivo* evaluation in animal studies such as those recently carried out on ultra-LMWH prepared using a chemoenzymatic process [32].

Acknowledgements

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