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## Controlled photochemical depolymerization of K5 heparosan, a bioengineered heparin precursor

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### ARTICLE INFO

#### Article history:

Received 4 May 2011

Received in revised form 30 May 2011

Accepted 15 June 2011

Available online 23 June 2011

#### Keywords:

Heparosan

Heparin

Photochemical

Depolymerization

Titanium dioxide

### ABSTRACT

Heparosan is a polysaccharide, which serves as the critical precursor in heparin biosynthesis and chemoenzymatic synthesis of bioengineered heparin. Because the molecular weight of microbial heparosan is considerably larger than heparin, the controlled depolymerization of microbial heparosan is necessary prior to its conversion to bioengineered heparin. We have previously reported that other acidic polysaccharides could be partially depolymerized with maintenance of their internal structure using a titanium dioxide-catalyzed photochemical reaction. This photolytic process is characterized by the generation of reactive oxygen species that oxidize individual saccharide residues within the polysaccharide chain. Using a similar approach, a microbial heparosan from *Escherichia coli* K5 of molecular weight >15,000 was depolymerized to a heparosan of molecular weight 8000. The <sup>1</sup>H NMR spectra obtained showed that the photolyzed heparosan maintained the same structure as the starting heparosan. The polysaccharide chains of the photochemically depolymerized heparosan were also characterized by electrospray ionization-Fourier-transform mass spectrometry. While the chain of K5 heparosan starting material contained primarily an even number of saccharide residues, as a result of coliphage K5 lyase processing, both odd and even chain numbers were detected in the photochemically depolymerized heparosan. These results suggest that the photochemical depolymerization of heparosan was a random process that can take place at either the glucuronic acid or the *N*-acetylglucosamine residue within the heparosan polysaccharide.

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### 1. Introduction

Heparin is a heterogeneous polydisperse mixture of sulfated polysaccharides ranging in molecular weight from 5000 to 30,000 with an average molecular weight of 15,000, and is widely used as an anticoagulant drug (Capila & Linhardt, 2002; Johnson & Mulloy, 1976; Linhardt & Claude, 2003; Munoz & Linhardt, 2004; Raman & Sasisekharan, 2005). In 2008, lots of porcine intestinal heparin were contaminated with oversulfated chondroitin sulfate, which caused anaphylactoid reactions and over 100 deaths in patients receiving the contaminated drug (Guerrini et al., 2008, 2009; Kishimoto et al., 2008). While effective assays are being developed for the detection of known and unknown contaminants and adulterants in heparin (Li et al., 2009; Rudd et al., 2009; Liu, Zhang, & Linhardt, 2009),

new highly regulated processes must be implemented in heparin production. Recently, our laboratory has chemoenzymatically synthesized heparin in small quantities by following the heparin and heparan sulfate biosynthetic pathways using cloned and expressed enzymes (Zhang et al., 2008). Starting from *E. coli* K5 heparosan, two chemical steps and four enzymatic steps led our group to prepare a bioengineered heparin with anticoagulant properties comparable to porcine intestinal heparin. The structure of the capsular polysaccharide from *E. coli* O10:K5:H4 heparosan is similar to that of desulfated heparin (Vann, Schmidt, Jann, & Jann, 1981). Since heparin chain size plays a critical role in mediating protein binding for blood anticoagulant activity, control of the polysaccharide chain length of the bacterial precursor from *E. coli* K5 is necessary in the development of bioengineered heparin, and other heparin-related drugs (Wang et al., 2010).

Previously, it has been determined that the molecular weight of microbial heparosan is greater than that of heparin, and that controlled depolymerization of heparosan is necessary prior to its conversion into bioengineered heparin (Ly et al., 2011).

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Although controlled depolymerization of heparosan by heparin lyase III treatment can be easily performed, the resulting product contains modified saccharide units within the polysaccharide backbone (Linhardt, 2001). In previous studies we reported the depolymerization of simple, nonsulfated polysaccharides, sodium alginate and pectin, using a photochemical reaction relying on titanium dioxide catalyst (Burana-osot et al., 2009; Burana-osot, Soonthornchareonnon, Hosoyama, Linhardt, & Toida, 2010). Furthermore, we found that titanium dioxide can catalyze depolymerization of unfractionated heparin to prepare photodegraded low molecular weight heparin (pLMWH) without structural modifications (Higashi et al., unpublished data). This photochemical reaction has been investigated using liquid chromatography/mass spectrometry (LC/MS). The polysaccharide chains of reduced length have both an odd and even number of saccharide residues.

In this article, crude and pure heparosan are depolymerized by titanium dioxide with visible light of >370 nm. Structures of photodegraded heparosan were same as a starting material, suggesting that photochemical depolymerization of heparosan is useful for preparation of a bioengineered heparin. Finally, the odd and even chain lengths of photodegraded heparosan were characterized by electrospray ionization Fourier-transform mass spectrometry (ESI-FT-MS).

## 2. Materials and methods

### 2.1. Chemicals and equipment

Titanium dioxide (anatase type, particle size average, 50  $\mu\text{m}$ ) was purchased from Wako Pure Chemical Co. (Osaka, Japan). Electrophoresis-grade acrylamide, *N,N'*-methylene-bis-acrylamide, sucrose, glycine, ammonium persulfate (APS), and *N,N,N',N'*-tetramethylethylenediamine (TEMED), were from Bio-Rad (Hercules, CA, USA). Boric acid, disodium salt of ethylenediaminetetraacetic acid (EDTA), phenol red, and Alcian blue were from Fisher (Pittsburgh, PA, USA).  $\text{D}_2\text{O}$  (99.96 atom%) was from Sigma-Aldrich (St. Louis, MO, USA).

The photochemical reaction device (Sen Lights Corporation, Osaka, Japan) consisted of a VG1500 reaction tank with 5 inlets, a UV light source (high pressure mercury lamp HL100 CH-4, 100 W), a power source (HB100P-1), and lamp jacket-Pyrex glass JW-1G. The apparatus was connected with a circulating water system to cool the lamp.

### 2.2. Growth and harvest of *E. coli* K5

The *E. coli* strain Bi 8337/41 (O10:K5:H4) was from ATCC (Manassas, VA, USA). The *E. coli* K5 was grown in a 7 L fermentor (Wang et al., 2010). Briefly, the fermentation consists of a batch growth stage and a fed batch growth stage. The composition of the medium for the batch growth in the fermentation was: 20 g/L glucose, 10 or 300 mg/L thiamine, 13.5 g  $\text{KH}_2\text{PO}_4$ ; 4.0 g  $(\text{NH}_4)_2\text{HPO}_4$ , 1.4 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.7 g citric acid, and 10.0 mL trace metal solution. Trace metal solution consisted of (per L of 5 M HCl) 10.0 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0 g  $\text{CaCl}_2$ , 2.2 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 1.0 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.1 g  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ , and 0.02 g  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ . The feeding solution used in the fed batch stage consisted of (per L): 250–1000 g glucose, 20 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.15 or 0.25 g thiamine (Johnson & Mulloy, 1976). The pH was kept at a value of  $\sim 7.0$  throughout the fermentation.

The batch growth stage began with the inoculation of seed culture (300 mL of 5.6 g/L DCW) obtained from a shake flask in late exponential growth. The temperature was maintained at  $\sim 37^\circ\text{C}$ , and the pH was maintained at approximately 7 (by adding 29%

ammonia solution). Air was sparged into the fermentor to supply oxygen, and the stirrer speed was set to 520 rpm.

The second stage of the fermentation began after glucose in the batch growth medium had been depleted and the dissolved oxygen showed a sharp increase. The feeding solution was then fed exponentially as described previously (Wang et al., 2010). Fermentation supernatant was harvested after no further increase in cell density was observed at 32 h.

### 2.3. Preparation of extracellular polysaccharide of *E. coli* K5

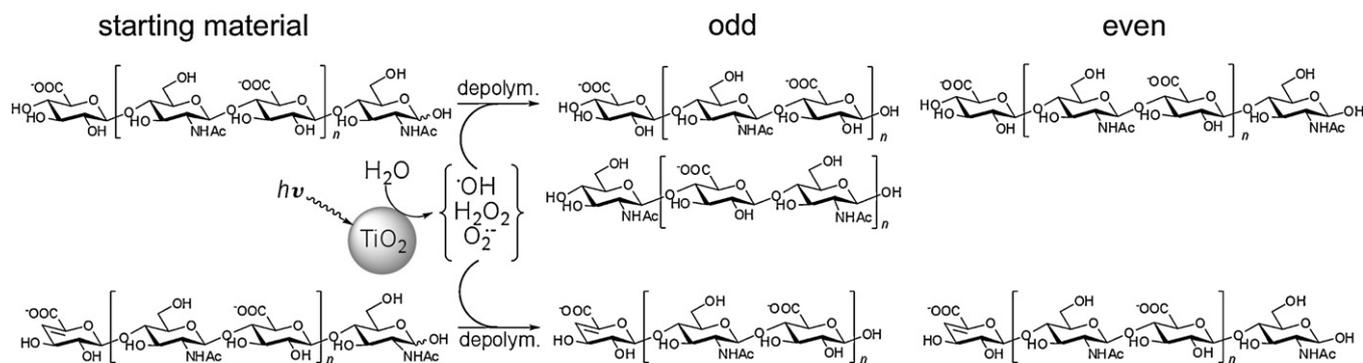
Crude heparosan was prepared from fermentation supernatant (100 mL) by adding absolute ethanol (Fisher Scientific, PA) in a 4:1 ratio. A precipitate of heparosan was allowed to form at  $4^\circ\text{C}$  for 24 h in an explosion-proof refrigerator. Crude heparosan was recovered in a pellet form using Sorvall Evolution RC superspeed centrifuge at  $2400 \times g$  for 1 h. The recovered pellet was resolubilized in water and dialyzed against water using 3500 Da molecular weight cut-off (MWCO) Spectra/por<sup>®</sup> Dialysis membranes (Spectrum Laboratories Inc.) for 3 days. The retentate was concentrated using Buchi rotavapor R-200 and then lyophilized. Pure heparosan was prepared by an alternative route (Capila & Linhardt, 2002). Fermentation supernatant (100 mL) was diluted two folds using buffer A (50 mM NaCl and 20 mM NaOAc, pH 4.0) and the pH was readjusted to 4.0. This diluted fermentation broth was then purified using DEAE Sepharose fast flow resin packed weak anion exchange column: heparosan (20 mg) was bound to 1 mL of DEAE resin (GE Healthcare Bio-Sciences Corp., NJ), which was equilibrated at pH 4.0 using 3 column volumes of buffer A. Diluted fermentation broth (200 mL containing 1 g of heparosan) was then loaded onto a 150 mL column under gravity flow conditions. The bound heparosan was eluted using 2 column volumes of buffer B (1 M NaCl and 20 mM NaOAc, pH 4.0). Pure heparosan was recovered from the eluent by ethanol precipitation, dialysis, rotary evaporation and lyophilization as previously described for the recovery of crude heparosan.

### 2.4. Depolymerization of K5 heparosan by photochemical reaction

K5 heparosans (10 mg) were each dissolved in 1 mL water with 1 mg of titanium dioxide ( $\text{TiO}_2$ ) particles and closed by a screw cap. The sample tube (borosilicate glass) was then placed in the photochemical reaction tank and exposed to light at room temperature. A mechanical stirrer was used in addition to a magnetic stirrer to ensure the dissolution of air into the solution. After the reaction, the sample was centrifuged at  $1500 \times g$  for 5 min at  $20^\circ\text{C}$  and the supernatant was filtered through 0.45  $\mu\text{m}$  membrane filter (Dismic-13HP; Advantec, Tokyo, Japan) to eliminate all of the  $\text{TiO}_2$ , and the product solution was dialyzed and lyophilized. The change in molecular weights of degraded heparosan samples were monitored by gel permeation chromatography (GPC) high performance liquid chromatography (HPLC).

### 2.5. Estimation of the average molecular weight

GPC-HPLC was performed using a TSK-GEL G4000PWxl size exclusion column with a sample injection volume of 20  $\mu\text{L}$  (100  $\mu\text{g}$ ) and a flow rate of 0.6 mL/min on an apparatus composed 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  $\text{NaNO}_3$ . The column was maintained at  $40^\circ\text{C}$  with an Eppendorf column heater during the chromatography. The GPC chromatograms were recorded with the LC solution Version 1.25 software and analyzed with its "GPC Postrun" function. Hyaluronan molecular weight markers of 30.6 kDa, 54 kDa, 128 kDa and



**Fig. 1.** Controlled photolytic depolymerization of heparosan. When titanium dioxide particles absorb irradiated light (>370 nm), a photogenerated electron ( $e^-$ ) and hole ( $h^+$ ) react with the oxygen or water on the surface of the activated titanium dioxide particles, generating reactive oxygen species ( $H_2O_2$ ,  $O_2^{\cdot-}$ ,  $\cdot OH$ ) (Harbour, Tromp, & Hair, 1985; Jaeger & Bard, 1979; Noda, Oikawa, & Kamada, 1992).

262 kDa purchased from Hyalose L.L.C. (Oklahoma City, OK) were used as calibrants for the standard curve.

### 2.6. NMR analysis

All  $^1H$  NMR were conducted on a Bruker 600 MHz NMR spectrometer. Heparosan samples (2 mg) were dissolved in 0.5 mL of  $D_2O$  (99.96%, Sigma–Aldrich Co.) and exchanged 3-times by lyophilization. The samples were prepared in 5 mm standard NMR tubes. Acquisition of the spectra was carried out using TOPSPIN 2.0 software. All the spectra were acquired at the temperature of 298 K. A recycle delay time of 8 s was used. The acquired  $^1H$  NMR spectra were processed with MestReC NMR software for phase and baseline correction.

### 2.7. Continuous-elution preparative polyacrylamide gel electrophoresis PAGE

Photolytically depolymerized heparosan (pure) was fractionated by continuous elution preparative polyacrylamide gel electrophoresis (PAGE) to decrease chain size heterogeneity as described elsewhere (Ly et al., 2011; Laremore, Ly, Solakyildirim, Zagorevski, & Linhardt, 2010). Lower molecular weight fractions of heparosan were separated by a 10% total acrylamide (10% T) resolving gel containing 9.66% (w/v) acrylamide, 0.33% (w/v) *N,N'*-methylene-bis-acrylamide, and 5% sucrose. Monomer T solution (2 mL) was prepared in resolving buffer (0.1 M boric acid, 0.1 M Tris, 0.01 M disodium EDTA, pH 8.3) and allowed to polymerize as a 5 cm  $\times$  7 mm diameter resolving gel column using 2  $\mu$ L TEMED and 12  $\mu$ L of 10% APS. A stacking gel of 0.5 mL was used with 0.5  $\mu$ L TEMED and 30  $\mu$ L of 10% APS.

The heparosan (1.5 mg) was loaded as 25% (w/v) sucrose above the stacking gel. Electrode running buffer was 1 M glycine and 0.2 M Tris at pH 9, and the lower chamber and elution buffer chamber were filled with resolving buffer. A peristaltic pump was set to 80  $\mu$ L/min and subjected to electrophoresis at a constant power of 1 W for 4 h. Collected fractions (numbering 80) were analyzed by analytical PAGE on 0.75-mm  $\times$  6.8-cm  $\times$  8.6-cm minigels cast in-house using 12% T resolving gel monomer solution and 5% T stacking gel monomer solution. Upper chamber and lower chamber were filled with buffer A (0.2 M Tris (pH 9 without adjustment) and 1 M glycine) and buffer B (0.1 M Tris–HCl (pH 8.3), 0.1 M boric acid 10 mM EDTA), respectively. The mini-gels were subjected to electrophoresis at constant 200 V, stained in 0.5% (w/v) alcian blue containing 2% (v/v) aqueous acetic acid solution for 30 min, and destained in water. Heparosan of various molecular weights (fraction 10, 13, 16, 20, 23, 25) were chosen and mixed on a vortex mixer with 240  $\mu$ L of 4 M salt and desalted using 10,000 MWCO

Amicon Ultra-0.5 centrifugal filter devices (Millipore, Billerica, MA, USA) prior to electrospray ionization (ESI) Fourier transform (FT) mass spectrometric (MS) analysis (Ly et al., 2011) for intact chain molecular weights of heparosan.

### 2.8. Electrospray ionization Fourier-transform mass spectrometry

ESI-FT mass spectra were acquired at a resolution of 60,000 on an LTQ-Orbitrap–XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) in the negative ionization mode as previously (Ly et al., 2011). Parameters used include spray voltage 3.5 kV, capillary temperature 270  $^\circ$ C, capillary voltage  $-15^\circ$ C, tube lens  $-100$  V; the sheath and auxiliary gas flow rates were set to 10 and 0, respectively. Mobile phase consisting of 1:1  $H_2O$ : methanol with 0.2% formic acid was delivered by an Agilent 1200 nano-LC pump at a flow rate of 50  $\mu$ L/min, and 1  $\mu$ L of each samples was infused through an Agilent 1200 autosampler.

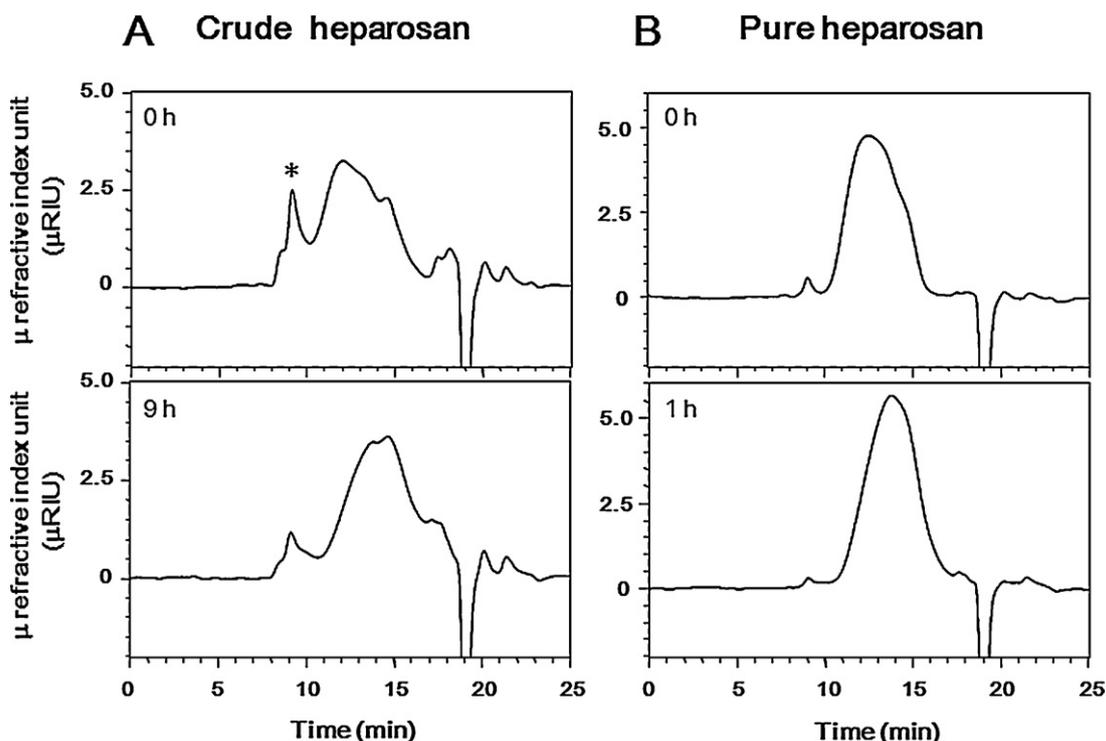
## 3. Results and discussion

Heparosan was prepared by fermentation of *E. coli* K5. Crude heparosan was recovered from the fermentation supernatant by ethanol precipitation and dialysis. Pure heparosan was recovered and purified from the fermentation supernatant by weak anion exchange chromatography, ethanol precipitation and dialysis. Heparosan isolated from *E. coli* K5 was photolyzed using  $TiO_2$ -catalyzed photochemical depolymerization conditions developed for other acidic polysaccharides (Burana-osot et al., 2009, 2010). The structure of heparosan and the putative reaction products are shown in Fig. 1.

The molecular weight of starting material of crude and pure heparosan were determined using GPC–HPLC (Fig. 2). Number-averaged molecular weight ( $M_N$ ) of starting materials, crude heparosan and pure heparosan were 15.2 kDa and 14.6 kDa, respectively (Table 1). Table 1 also shows weight-average molecular weight ( $M_W$ ), and the polydispersity ( $P$ ) of the crude and pure heparosan before and after photolysis. The molecular weight of starting heparosan determined by GPC–HPLC was consistent with previously reported molecular weight of heparosan (Ly et al., 2011).

**Table 1**  
Molecular weight ( $M_N$ ,  $M_W$  and  $P$ ) of starting heparosan and photochemically depolymerized heparosan.

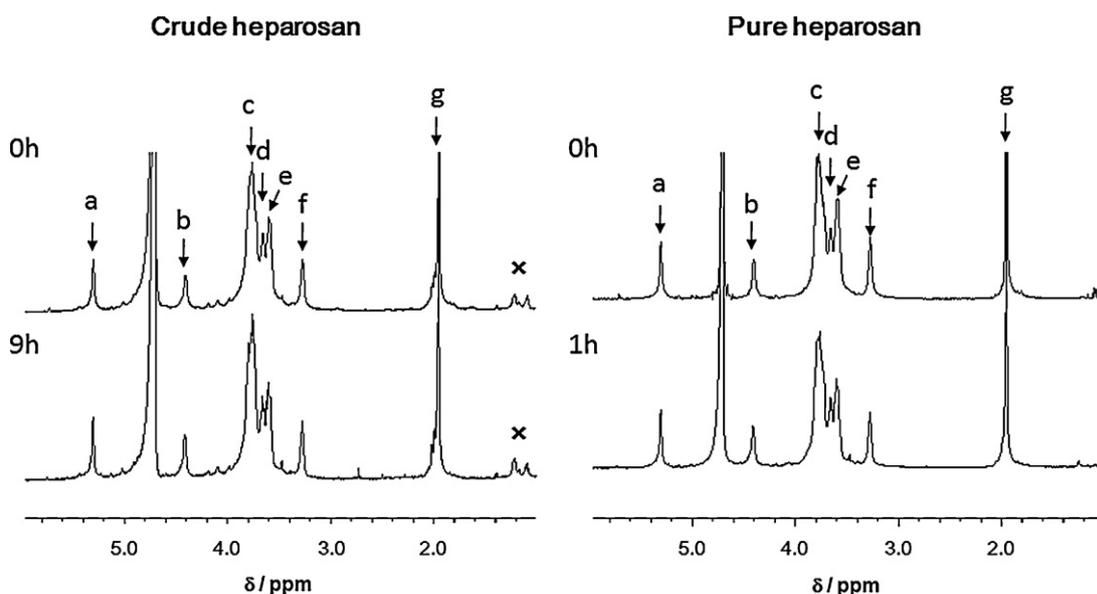
	Photolysis time (h)	$M_N$	$M_W$	$P$
Crude heparosan	0	15,246	77,829	5.10
	9	8158	28,671	3.44
Pure heparosan	0	14,559	29,024	1.99
	1	8501	16,400	1.93



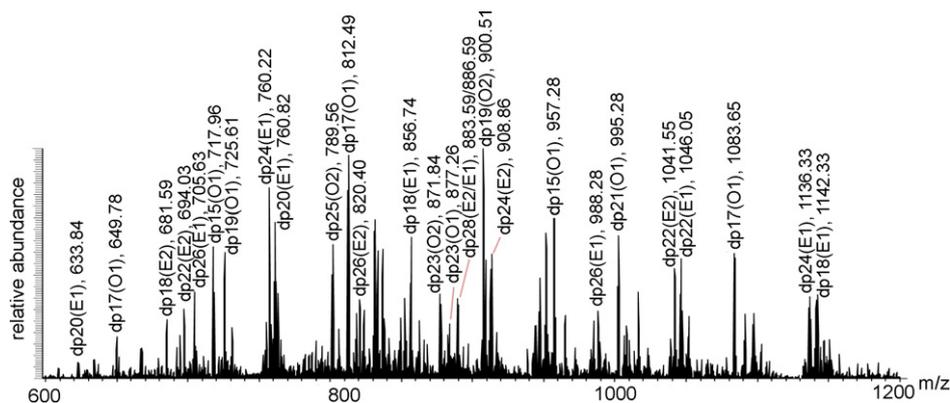
**Fig. 2.** GPC-HPLC analysis of heparosan and photolyzed heparosan. (A) Crude heparosan (upper chromatogram) and crude heparosan photolyzed for 9 h (lower chromatogram). (B) Pure heparosan (upper chromatogram) and pure heparosan photolyzed for 1 h (lower chromatogram). HPLC conditions: column, a TSK-GEL G4000PW<sub>XL</sub> size exclusion column; mobile phase, 0.1 M NaNO<sub>3</sub> at a flow rate of 0.6 mL/min; detection, refractive index.

Heparosan is used in our laboratory as a precursor in the chemoenzymatic preparation of bioengineered heparin (Zhang et al., 2008). Thus, the heparosan precursor must have a molecular weight of ~8 to 9 kDa to afford a bioengineered heparin of molecular weight ~12 to 20 kDa, corresponding to the molecular weight of porcine intestinal heparin used pharmaceutically as a clinical anticoagulant. The goal of the current study was to rely on photolysis to reduce the molecular weight of either crude or pure heparosan obtained from

*E. coli* K5 to the requisite size. Depolymerization of heparosan by time-controlled photochemical reaction was performed (Fig. 1) and resulting MW heparosan were determined by GPC-HPLC (Fig. 2). Heparosan obtained by 9 h photolysis of crude heparosan had a molecular weight of 8.2 kDa (Table 1). In contrast, photolysis of pure heparosan occurred at a much faster rate than crude heparosan and a product of 8.5 kDa was obtained after 1 h of photolysis (Fig. 2 and Table 1).



**Fig. 3.** <sup>1</sup>H NMR spectra of heparosan and photolyzed heparosan. (A) Crude heparosan (upper spectrum) and crude heparosan photolyzed for 9 h. (B) Pure heparosan (upper spectrum) and crude heparosan photolyzed for 1 h (lower spectrum). Peak assignments are: (a) GlcNAc H1, (b) GlcA H1, (c) GlcNAc H2, H3, H5 and H6, (d) GlcA H5, (e) GlcA H3, H4 and GlcNAc H4, (f) GlcA H2, and (g) GlcNAc CH<sub>3</sub>. An impurity is marked with an “x” near 1 ppm.



**Fig. 4.** ESI-FT-MS analysis of heparosan and photolyzed heparosan. FTMS spectra of photolysis reaction of *E. coli* K5 heparosan. Heparosan chains identified from dp17 to dp28 labeled according to different types of fragments formed. Abbreviations for different odd (O1, O2, O3) and even (E1, E2) fragments are: O1; GlcNAc- (GlcA-GlcNAc)<sub>n</sub>, O2; GlcA- (GlcNAc-GlcA)<sub>n</sub>, O3; ΔUA- (GlcNAc-GlcA)<sub>n</sub>, E1; GlcA- (GlcNAc-GlcA)<sub>n</sub>-GlcNAc and GlcNAc- (GlcA-GlcNAc)<sub>n</sub>-GlcA, deltaUA- (GlcNAc-GlcA)<sub>n</sub>-GlcNAc.

Next, the <sup>1</sup>H NMR spectra (600 MHz) of the heparosan samples were examined (Fig. 3). Crude heparosan (~85% pure by NMR) showed additional minor signals associated with protein impurities. Pure heparosan (~95% pure) gave a much cleaner spectrum in which all major signals could be assigned (Vann et al., 1981; Ly et al., 2011). The signals for the photolyzed heparosan sample showed the same chemical shift values as observed for the starting crude or pure heparosan. This suggests that no major modification of the polysaccharide backbone had taken place, consistent with our observations when examining the photolysis of other, structurally related polysaccharides (Burana-osot et al., 2009, 2010).

Next, we examined the molecular weight of the photochemically depolymerized pure heparosan by ESI-FT-MS (Ly et al., 2011). Continuous elution preparative PAGE had been previously described by our laboratory as a method to recover chains of a single size (or degree of polymerization, dp) from a complex polydisperse mixture of polysaccharide chains. Using this method, oligosaccharides were prepared and analyzed by ESI-FT-MS. The resulting spectra are shown in Fig. 4. Interpretation of these spectral data allows assignment of exact mass and, hence, chain structure (Supplemental Table 1). As a result of this analysis it is clear that while the starting heparosan (pure) has chains containing primarily an even number of saccharide residues and that these chains are terminated with an unsaturated uronic acid residue (ΔUA) at their non-reducing end. These chains have the general structure ΔUA-(GlcNAc-GlcA)<sub>n</sub>-GlcNAc where *n* = 5–434. In contrast, depolymerized heparosan (pure) had chains with both an odd and even number of saccharide residues some terminated with ΔUA and some terminated with GlcNAc or with GlcA. These chains, which could be detected by ESI-FT-MS, have multiple structures including GlcNAc-(GlcA-GlcNAc)<sub>n</sub> (Type O1), GlcA-(GlcNAc-GlcA)<sub>n</sub> (Type O2), ΔUA-(GlcNAc-GlcA)<sub>n</sub> (Type O3), and GlcA-(GlcNAc-GlcA)<sub>n</sub>-GlcNAc and GlcNAc-(GlcA-GlcNAc)<sub>n</sub>-GlcA (Type E1), ΔUA-(GlcNAc-GlcA)<sub>n</sub>-GlcNAc (Type E2) where *n* = 8–50. Since chains having both an even and odd number of saccharide residues are detected in commercial bovine lung heparin (30% depolymerized by heparinase), using liquid chromatography/mass spectrometry (LC/MS) (Thanawiroon, Rice, Toida, & Linhardt, 2004), photochemically depolymerized heparosan may be directly used as a starting material for bioengineered heparin. Although GlcNAc-(GlcA-GlcNAc)<sub>n</sub> (Type O1), GlcA-(GlcNAc-GlcA)<sub>n</sub> (Type O2), ΔUA-(GlcNAc-GlcA)<sub>n</sub> (Type O3), and GlcA-(GlcNAc-GlcA)<sub>n</sub>-GlcNAc and GlcNAc-(GlcA-GlcNAc)<sub>n</sub>-GlcA (type E1), ΔUA-(GlcNAc-GlcA)<sub>n</sub>-GlcNAc (Type E2) were detected in molecular weight <5000, ΔUA-(GlcNAc-GlcA)<sub>n</sub> (Type O3) was not observed in molecular weight >5000. The photo-degradation mechanism leading to these results is unclear, however, it is possible that reactive oxygen species more easily

attack the [→4]-β-D-GlcA(1→4)-α-D-GlcNAc(1→) than the [→4]-α-D-GlcNAc(1→4)-β-D-GlcA(1→) linkage.

#### 4. Conclusions

In summary, this study shows that titanium dioxide with visible light catalyzes the depolymerization of heparosan. Photochemically depolymerized low molecular weight of heparosan maintained the same structure as the starting heparosan, as confirmed by <sup>1</sup>H NMR. The polysaccharide chains of the photochemically depolymerized heparosan were also characterized by electrospray ionization-Fourier-transform mass spectrometry. The results suggest that the photochemical depolymerization of heparosan is a random process that can take place at either the glucuronic acid or the *N*-acetylglucosamine residue within the heparosan polysaccharide. These results indicate that photochemical depolymerization of heparosan was available for a bioengineered heparin. Future studies will be required to prepare the heparin for clinical use using photochemically depolymerized heparosan as a starting material.

#### Acknowledgements

The authors are grateful for support by a Grant-in-Aid for Scientific Research (20590032), and Special Funds for Education and Research (Development of SPECT Probes for Pharmaceutical Innovation) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (TT), and by the US National Institutes of Health in the form of grants GM38060, HL096972 and HL101721 (RJL).

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.06.042.

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