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Microscale preparation of even- and odd-numbered *N*-acetylheparosan oligosaccharides

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Abstract—In order to prepare a series of *N*-acetylheparosan (NAH)-related oligosaccharides, bacterial NAH produced in *Escherichia coli* strain K5 was partially depolymerized with heparitinase I into a mixture of even-numbered NAH oligosaccharides, having an unsaturated uronic acid (ΔUA) at the non-reducing end. A mixture of odd-numbered oligosaccharides was derived by removing this ΔUA in the aforementioned mixture by a 'trimming' reaction using mercury(II) acetate. Each oligosaccharide mixture was subjected to gel-filtration chromatography to generate a series of size-uniform NAH oligosaccharides of satisfactory purity (assessed by analytical anion-exchange HPLC), and their structures were identified by MALDITOF-MS, ESIMS, and ^{1}H NMR analysis. As a result, a microscale preparation of a series of both even- and odd-numbered NAH oligosaccharides was achieved for the first time. The developed procedure is simple and systematic, and thus, should be valuable for providing not only research tools for heparin/heparan sulfate-specific enzymes and their binding proteins, but also precursor substrates with medical applications.

Keywords: *N*-Acetylheparosan; Heparitinase; K5 polysaccharide; Mass spectrometry; Oligosaccharide

1. Introduction

N-Acetylheparosan (NAH) is a glycosaminoglycan (GAG) known as a biosynthetic precursor of mammalian heparin and heparan sulfate (Hep/HS). Its structure is composed of alternating α-(1→4)-linked *N*-acetylglucosamine (GlcNAc) and β-(1→4)-linked glucuronic acid (GlcA), and is not sulfated at all, unlike Hep/HS. It is also known that *Escherichia coli* strain K5 produces an antigenic capsular polysaccharide having a closely similar structure, called 'K5 polysaccharide',[1] which is referred to as 'bacterial NAH' in this article.

In regard to the biosynthesis of Hep/HS, which starts from NAH, the post-glycosylation modification features have been previously elucidated.[2,3] A series of modification enzymes, that is, *N*-deacetylase/*N*-sulfotransferase, some *O*-sulfotransferases and C-5 epimerase, are involved in the process, and as a result of multistep reactions, highly heterogeneous Hep/HS structures are generated. It is widely accepted that such heterogeneous Hep/HS structures contain biologically more significant oligosaccharide domains, which interact with various Hep/HS-binding proteins[2,3] either to potentiate or attenuate their physiological functions. On the other hand, it is also known that a considerable part of the original NAH sequences remain intact in HS.[4,5]

To replace Hep or to define minimal structural requirements for the biological activities of Hep/HS, a variety of artificial Hep/HS fragments have been synthesized by using bacterial NAH. For this purpose, various modification techniques based on either chemical,[6–8] enzymatic[9] or chemoenzymatic[10–12] methodologies have been developed. By using such chemically modified as well as unmodified bacterial NAH, in fact, detailed

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specificities of eliminative enzymes have been investigated.[13] Although such modified polysaccharides may provide clear-cut answers, they are not always homogeneous due to difficulties in quantitative modification of the polymers. More critically, minimum size requirements for enzyme activity cannot be defined rigorously with such polymer samples. In this respect, size-uniform standard NAH oligosaccharides as well as their related structures are essential for gaining a basic understanding of the structure–function relationships of post-glycosylation enzymes and depolymerizing enzymes.

Recently, a partial digest of bacterial NAH was separated by capillary-type, high-performance liquid chromatography (HPLC), followed by electrospray ionization mass spectrometry (ESIMS) analysis.[14] In that work, even-numbered NAH oligosaccharides up to a 40-mer were successfully detected. However, the authors did not isolate these oligosaccharides. In this article, we describe for the first time the isolation of a series of both even- and odd-numbered NAH oligosaccharides on a microscale. These should be valuable, not only as research tools for Hep/HS-specific enzymes and binding proteins, but also as modification substrates for syntheses of a wide variety of derivatives for diverse research fields.

2. Results and discussion

In order to prepare sets of even- and odd-numbered NAH oligosaccharides, a bacterial NAH isolated from *E. coli* K5 strain was first depolymerized into a mixture of even-numbered oligosaccharides by the action of heparitinase I. The resultant oligosaccharides were expected to bear an unsaturated uronic acid at their nonreducing ends. Corresponding odd-numbered oligosaccharides were subsequently obtained by 'trimming' the putative unsaturated uronic acids from the above mixture. As a result, a mixture of odd-numbered NAH oligosaccharides was prepared. The overall scheme is shown in Figure 1.

2.1. Oligosaccharide preparation

So far, several lyase enzymes have been reported to depolymerize NAH.[5,13–17] They cleave *N*-acetylglucosaminyl α-(1→4)-glucuronic acid (i.e., GlcNAc-α-(1→4)-GlcA) linkages in an eliminative manner. As a result, products of various lengths are generated. Of these reported enzymes, heparitinase I is the most readily available and is frequently used for degradation of HS and NAH. This enzyme can convert NAH completely into unsaturated disaccharides, that is, ΔUAβ-(1→4)-GlcNAc by exhaustive digestion, while a series of even-numbered oligosaccharides with polydisperse molecular weights may be produced under appropriate conditions.[14,18]

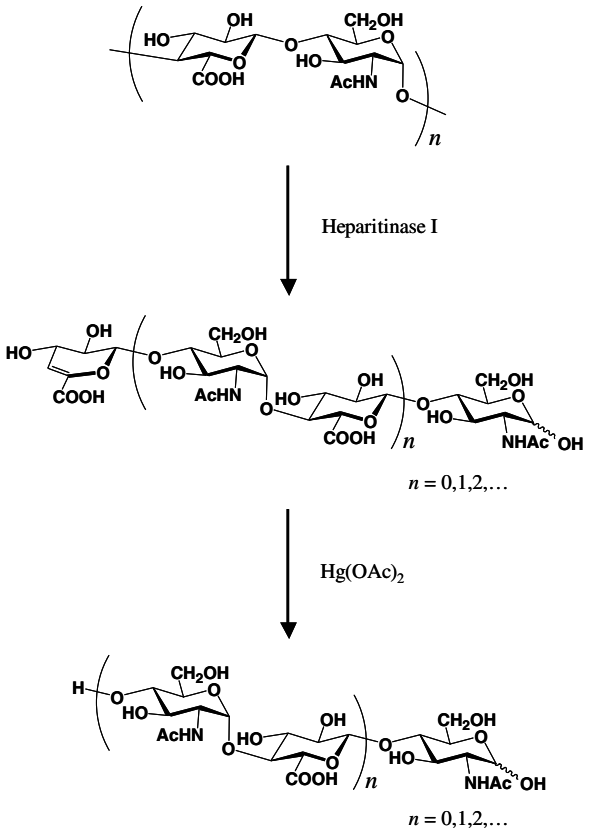

Figure 1. Scheme for preparation of NAH oligosaccharides. All oligosaccharides have *N*-acetylglucosamine at their reducing ends.

Matrix-assisted laser-desorption ionization (MALDI-TOF-MS) analysis of the desalted digestion product, obtained under the depolymerization conditions employed, revealed the expected feature of the products, that is, a series of even-numbered oligosaccharides (Fig. 2A). The MS spectrum clearly reflects the cleavage due to heparitinase I: all of the mass differences between adjacent major signals corresponded to the disaccharide unit, GlcAβ-(1→4)-GlcNAc (i.e., *m*/*z* 379, as a deprotonated ion). These results indicate the structural regularity of NAH.

Subsequently, the above mixture of even-numbered oligosaccharides was reacted with mercury(II) acetate under established conditions to remove the non-reducing terminal unsaturated uronic acid.[19,20] This procedure seems useful for preparing odd-numbered oligosaccharides from unsaturated, even-numbered ones in a mild and rapid manner.[21] The MALDITOF-MS spectrum (Fig. 2B) showed that the 'trimming' reaction had occurred almost completely, the products being the desired, odd-numbered oligosaccharides, with no apparent byproducts. The MS spectrum also confirmed the regularity of the repeating disaccharide unit observed for the even-numbered oligosaccharides.

Both mixtures of even- and odd-numbered oligosaccharides obtained above were subjected to gel-filtration

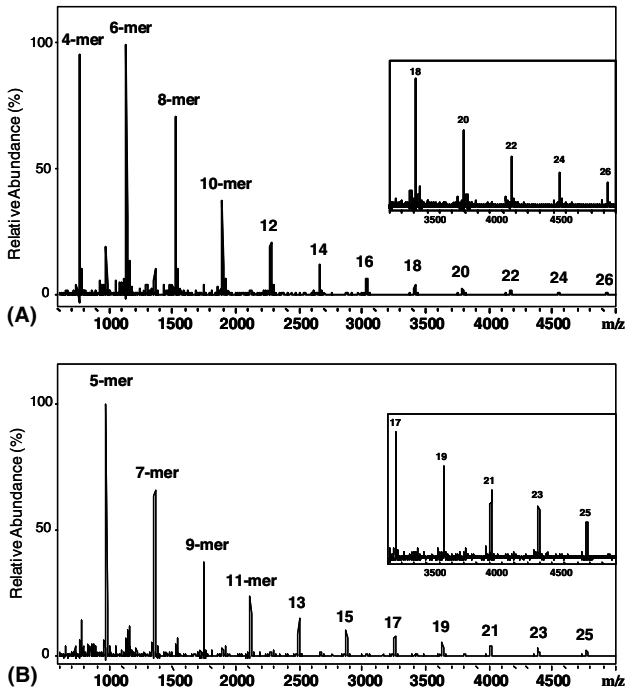

Figure 2. MALDITOF-MS analyses of (A) even- and (B) odd-numbered NAH oligosaccharides. DHB was used as matrix, and signals below *m*/*z* 600 were suppressed by deflection mode.

chromatography (Fig. 3A and B) to isolate each NAH oligosaccharide in a size-uniform manner. Based on the UV absorption at 210 nm (Fig. 3A), it is estimated that approximately a half amount of the starting NAH was converted into appropriate size oligomers (2- to 20-mers). The considerable decrease in the absorption at 232 nm (Fig. 3B) should be noted in the profiles of odd-numbered oligosaccharides. This confirms the loss of unsaturated uronic acids by the 'trimming' reaction. The yields estimated by absorption at 210 nm were 224 μg (2-mer), 160 μg (4-mer), 117 μg (6-mer), 99 μg (8-mer), 97 μg (3-mer), 76 μg (5-mer), and 76 μg (7-mer), respectively. Each oligosaccharide fraction was subjected to the following analyses to confirm its structure: (1) MALDITOF-MS, (2) ESIMS, and (3) analytical anion-exchange HPLC. Further analyses were performed for representative oligosaccharides by ^{1}H NMR spectroscopy.

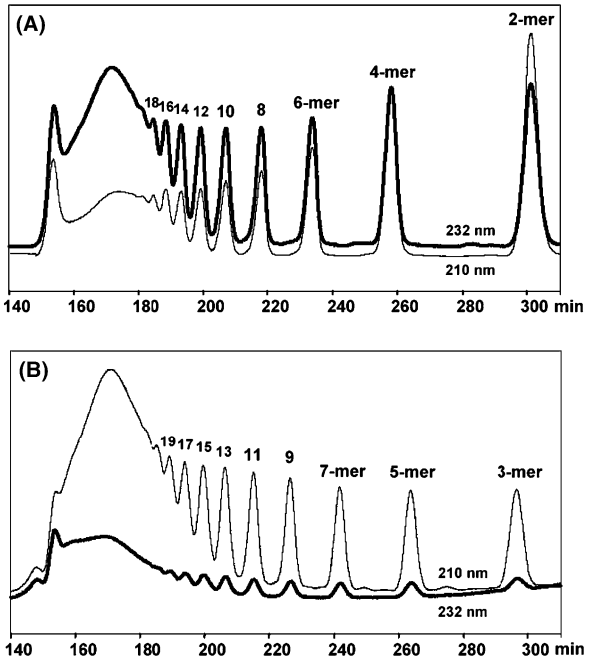

Figure 3. Gel-filtration chromatograms of (A) even- and (B) odd-numbered NAH oligosaccharide mixtures. Highly resolved profiles (up to the 20-mer) are shown.

2.2. MALDITOF-MS analysis

Each isolated NAH oligosaccharide fraction was first analyzed by negative-ion MALDITOF-MS, using 2,5-dihydroxybenzoic acid (DHB) as matrix. Measurements in the negative-ion mode gave superior sensitivity to that in the positive-ion mode. Moreover, DHB gave the best sensitivity and performance of the matrices tested, with undesirable in-source decay (ISD) fragmentation being only rarely observed.

Figure 4 displays representative spectra for the even-numbered oligosaccharides (4-, 10-, and 18-mers). Each spectrum clearly shows identity with the expected oligosaccharide in size as well as uniformity. Minor peaks in the lower *m*/*z* range (*m*/*z* 900–2000), which appeared specifically for oligosaccharides longer than a 16-mer, were observed. However, they seemed to have originated from ISD fragmentation, because relatively high laser power, which may decompose oligosaccharide chains, was required to ionize such longer oligosaccharides in the MALDI experiments. Similarly, representative spectra of odd-numbered oligosaccharides (5-, 11-, and 19-mers) are shown in Figure 5. They also showed clear identity and uniformity. However, the degradative

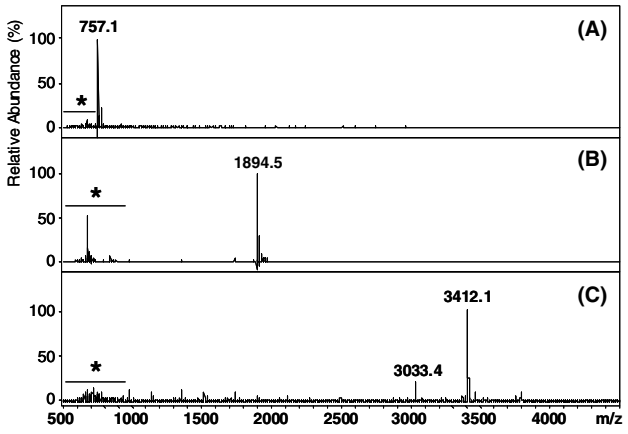

Figure 4. Representative MALDITOF-MS spectra of even-numbered NAH oligosaccharides: (A) 4-mer, (B) 10-mer, and (C) 18-mer.

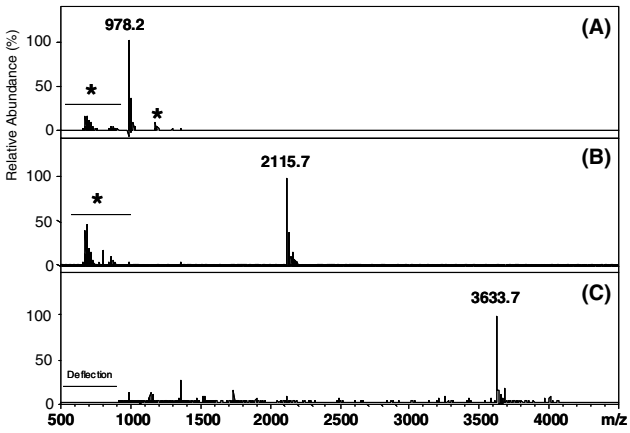

Figure 5. Representative MALDITOF-MS spectra of odd-numbered NAH oligosaccharides: (A) 5-mer, (B) 11-mer, and (C) 19-mer.

effects of ISD were again observed in spectra of oligosaccharides longer than the 15-mer. Thus, for all NAH oligosaccharides prepared, singly charged molecular-relating ions $[M-H]^-$ were observed, which directly reflected their molecular weights, even though some minor ions attributable to cation-salt formation were also seen. The detected *m/z* values for all NAH oligosaccharides are summarized in Table 1.

2.3. ESIMS analysis

Negative-ion ESIMS spectra were measured for both even-numbered (Fig. 6) and odd-numbered (Fig. 7) NAH oligosaccharides. The analysis also clarified the size (length) and purity of the prepared oligosaccharides as did the MALDI measurements above, although generation of multiply charged molecular-relating ions $[M-nH]^{n-}$ was inevitable in the case of ESIMS. As can be seen in Figures 6 and 7 (also see Table 1 for summary), the dominant charge state for each molecular-related ion increased in accordance with oligosaccharide length, although fully charged (i.e., deprotonated) species could no longer be observed for oligosaccharides larger than the 5-mer. Essentially similar results were obtained for hyaluronan oligosaccharides[22–24] as well as for NAH oligosaccharides, although for the latter, ion-pair reversed-phase LC–MS was used.[14] For hyaluronan oligosaccharides, it has been reported that the observed charge states apparently depended on ESI capillary voltage.[22–24]

2.4. NMR analysis

Repeated fractionation of the oligosaccharide mixture using anion-exchange HPLC and subsequent desalting by gel-filtration chromatography yielded milligram quantities of NAH oligosaccharides, which were subjected to ^{1}H NMR analysis. The results of analytical anion-exchange HPLC and MALDITOF-MS (data not shown), on the analyzed oligosaccharides were identical to those obtained by the microscale procedure, and thus, were sufficiently pure.

Figure 8A and B shows the ^{1}H NMR spectra of NAH oligosaccharides, the 4-mer and 8-mer, respectively, both of which are consistent with their assumed structures.

Table 1. Summary of the results of MALDITOF-MS and ESIMS measurements of NAH oligosaccharides (*m/z* values for monoisotopic signals)[a]

NAH oligosaccharide			MALDI $[M-H]^-$ (*m/z*)		ESI $[M-nH]^{n-}$ (*m/z*)			Purity (%)
Size	Composition	Monoisotopic mass	Calculated	Found	Charge state (n)	Calcd.	Found	
2-mer	$\Delta UA(GlcNAc)_1$	379.11	—	—	1	378.1	377.7	>99.9
3-mer	$(GlcA)_1(GlcNAc)_2$	600.20	599.2	599.1	1	599.2	599.1	>99.9
4-mer	$\Delta UA(GlcA)_1(GlcNAc)_2$	758.22	757.2	757.1	1	757.2	756.8	>99.9
5-mer	$(GlcA)_2(GlcNAc)_3$	979.31	978.3	978.2	2	488.7	488.5	>99.9
6-mer	$\Delta UA(GlcA)_2(GlcNAc)_3$	1137.33	1136.3	1136.3	2	567.7	567.4	>99.9
7-mer	$(GlcA)_3(GlcNAc)_4$	1358.42	1357.4	1357.3	2	678.2	678.2	>99.9
8-mer	$\Delta UA(GlcA)_3(GlcNAc)_4$	1516.45	1515.4	1515.4	2	757.2	757.0	>99.9
9-mer	$(GlcA)_4(GlcNAc)_5$	1737.54	1736.5	1736.5	2	867.8	867.9	>99.9
10-mer	$\Delta UA(GlcA)_4(GlcNAc)_5$	1895.56	1894.6	1894.5	2	946.8	946.7	>99.9
11-mer	$(GlcA)_5(GlcNAc)_6$	2116.65	2115.6	2115.7	2	1057.3	1057.5	>99.9
					3	704.5	704.6	
12-mer	$\Delta UA(GlcA)_5(GlcNAc)_6$	2274.67	2273.7	2273.6	3	757.2	757.1	>99.9
13-mer	$(GlcA)_6(GlcNAc)_7$	2495.76	2494.8	2494.9	3	830.9	831.2	95.4
14-mer	$\Delta UA(GlcA)_6(GlcNAc)_7$	2653.78	2652.8	2652.8	3	883.6	883.5	97.4
15-mer	$(GlcA)_7(GlcNAc)_8$	2874.87	2873.9	2874.2	3	957.3	957.6	>99.9
16-mer	$\Delta UA(GlcA)_7(GlcNAc)_8$	3032.89	3031.9	3033.4[b]	3	1010.0	1010.0	92.8
17-mer	$(GlcA)_8(GlcNAc)_9$	3253.98	3253.0	3253.1	3	1083.7	1083.9	95.5
18-mer	$\Delta UA(GlcA)_8(GlcNAc)_9$	3412.00	3411.0	3412.1[b]	3	1136.3	1136.5	81.3
19-mer	$(GlcA)_9(GlcNAc)_{10}$	3633.09	3632.1	3633.7[b]	3	1210.0	1210.5	81.7
20-mer	$\Delta UA(GlcA)_9(GlcNAc)_{10}$	3791.11	3790.1	3792.3[b]	3	1262.7	1262.6	70.3
21-mer	$(GlcA)_{10}(GlcNAc)_{11}$	4012.20	4011.2	4013.1[b]	—	—	—	72.7

[a] Oligosaccharide purity as determined by analytical anion-exchange HPLC is also included.
[b] Averaged mass.

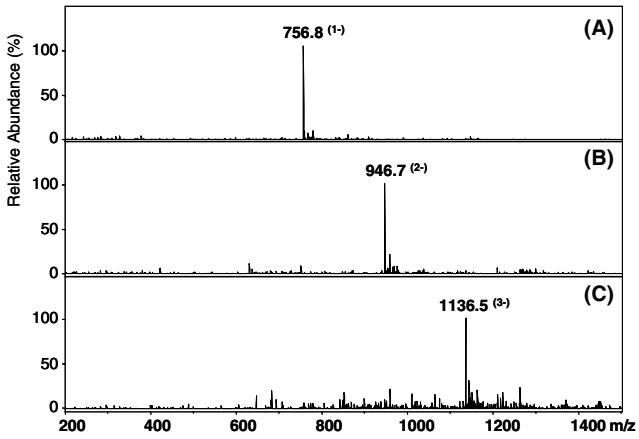

Figure 6. Representative ESIMS spectra of even-numbered NAH oligosaccharides: (A) 4-mer, (B) 10-mer, and (C) 18-mer.

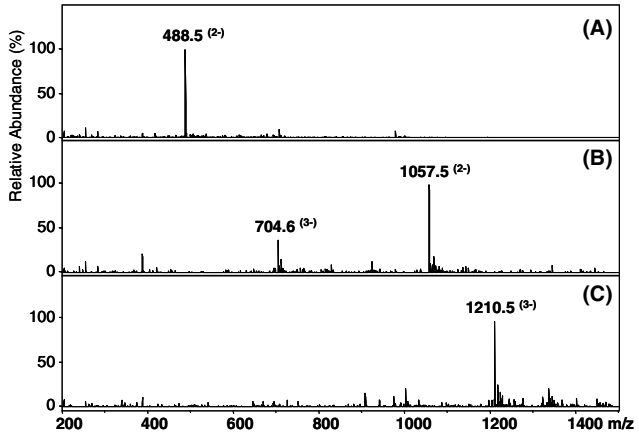

Figure 7. Representative ESIMS spectra of odd-numbered NAH oligosaccharides: (A) 5-mer, (B) 11-mer, and (C) 19-mer.

Characteristic signals observed include (1) the methyl signal attributed to the acetamido group of every GlcNAc (ca. 2.03 ppm), (2) the anomeric signals derived from individual sugar units, and (3) the unsaturated region signal originating from ΔUA at the nonreducing end (ca. 5.78 ppm). The anomeric protons gave chemical shifts each reflecting their differing chemical environments, that is, anomeric configuration (α-anomer for GlcNAc, β-anomer for GlcA, and α/β-equilibrium for reducing GlcNAc; assignments are included in the figures and Table 2). A significant downfield shift was observed for the β-anomeric proton of the non-reducing

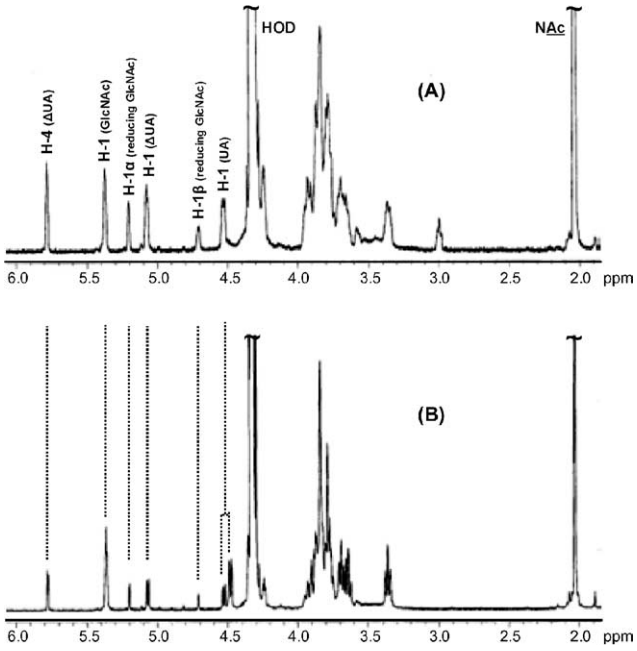

Figure 8. 500-MHz ^{1}H NMR spectra of NAH oligosaccharides: (A) 4-mer and (B) 8-mer.

ΔUA due to the unsaturated bond.[25] Since chemical shift values for these signals are strongly conserved among related oligosaccharides (Table 2), ^{1}H NMR spectroscopy is a powerful analytical tool for determining NAH oligosaccharide structures, provided that sufficient amounts are available.

2.5. Oligosaccharide purity

Isolated NAH oligosaccharides were assessed for their homogeneity by HPLC, and the purity achieved for each oligosaccharide is shown in Table 1. Calculated purities of oligosaccharides up to 12-mers are satisfactorily high (>99 %). On the other hand, much larger oligosaccharides were found to be a mixture, for example, 18-mer fraction (81.3% purity) contained both 16-mer (12.5%) and 20-mer (6.2%) oligosaccharides, whereas 19-mer fraction (81.7%) contained both 17-mer (15.7%) and 21-mer (2.6%) oligosaccharides, respectively. Since the results are consistent with the MALDITOF-MS and ESIMS spectra, our established procedure has proven to yield high purity NAH oligosaccharides in sufficient quantities for structural studies.

Table 2. Summary of the chemical shifts of characteristic signals observed by ^{1}H NMR experiments of a representative NAH oligosaccharide

Oligosaccharide	Chemical shift (δ ppm)					
	H-4 of non-reducing UA	H-1 of GlcNAc, other than reducing end	H-1α of reducing GlcNAc	H-1 of non-reducing UA	H-1β of reducing GlcNAc	H-1 of GlcA, other than non-reducing end
4-mer	5.782	5.371 (1H)	5.206 (0.6H)	5.075	4.707 (0.4H)	4.528 (1H)
6-mer	5.778	5.366 (2H)	5.199 (0.6H)	5.067	4.707 (0.4H)	4.527 (1H), 4.487 (1H)
8-mer	5.778	5.366 (3H)	5.199 (0.6H)	5.068	4.707 (0.4H)	4.526 (1H), 4.481 (2H)

3. Concluding remarks

Much attention has been drawn to the biological importance of 'domain structures' found in GAG polymers. Thus, the preparation of structurally defined GAG oligosaccharides is essential for future structure–function studies. Two approaches have been taken so far, that is, organic synthesis and selective GAG digestion followed by isolation. Both approaches have so far had significant practical advantages and disadvantages. Basically, the present procedure employs the latter approach to obtain a series of size-uniform NAH oligosaccharides up to 20-mers. Our method enables at least microscale preparation of NAH oligosaccharides up to the 17-mer with >90% purity simply by gel-filtration purification, without need for anion-exchange chromatography. For further scale-up, a technique recently reported for hyaluronan oligosaccharides[26] can be modified. Thus, the present study enabled us for the first time to achieve systematic isolation of both even- and odd-numbered NAH oligosaccharides. It was also shown that both MALDITOF-MS and ESIMS were effectively used to characterize those oligosaccharides.

Recent developments of MS/MS technologies for detailed and sensitive structural analysis of GAGs are remarkable.[27] For instance, ESIMS/MS studies of chondroitin sulfate oligosaccharides that include positional isomers have been reported.[28,29] Since there are many factors concerning structural isomers in GAGs, for example, position of sulfate, epimerization of uronic acid and diastereomerization of hexosamine, special interest has been given to MS/MS technologies. The MS/MS analysis of NAH oligosaccharides could be extremely valuable in providing fundamental structural data for unmodified precursor compounds, which are related isomerically to hyaluronan oligosaccharides. In this context, MS/MS experiments are in progress and will be described in the near future.

4. Experimental

4.1. Materials

Bacterial NAH (MW: ~44 kDa) was produced in *E. coli* serotype 010:K5(L):H4 in the Central Research Laboratories, Seikagaku Corporation. Detailed conditions for the bacterial culture, and the extraction and purification of this polysaccharide will be described elsewhere.

Heparitinase I (heparan sulfate lyase from *Flavobacterium heparinum*, EC 4.2.2.8) was purchased from Seikagaku Corporation (Tokyo, Japan), mercury(II) acetate from Wako Pure Chemical Industries (Osaka, Japan), recrystallized 2,5-dihydroxybenzoic acid (DHB) from Bruker Daltonik GmbH (Bremen, Germany), MeOH (spectrophotometric grade, 99.9 %) from Aldrich Chemical Co. (Milwaukee, USA), and deuterium oxide (D_2O, 99.98 atom %D) from Acros Organics (Geel, Belgium). Water was purified using a Milli-Q filtration apparatus (Millipore Co., Bedford, MA, USA). All other reagents were of the highest grade commercially available.

4.2. Preparation of NAH oligosaccharide mixtures

Bacterial NAH (100 mg) was dissolved in 50 mM NaOAc buffer (pH 7.0) containing 5 mM $Ca(OAc)_2$ at the concentration of 10 mg/mL. Heparitinase I (0.2 U) was added and digestion was allowed to proceed at 37 °C for 45 min. Reaction was terminated by inactivating the enzyme digest in a boiling water bath for 5 min. After centrifugation, the supernatant solution was lyophilized, and the resultant powder (oligosaccharides) redissolved in water, and desalted by passing through a Sephadex G-10 column (2.2 × 115 cm, Amersham Biosciences, Piscataway, USA). Oligosaccharide fractions were combined and lyophilized (even-numbered NAH oligosaccharides).

To prepare odd-numbered NAH oligosaccharides, 100 mg of the above mixture of even-numbered oligosaccharides was dissolved in water at the concentration of 20 mg/mL, and the pH carefully adjusted to 5.0 with diluted HOAc. An equal volume of 70 mM $Hg(OAc)_2$ solution, also adjusted to pH 5.0, was added and allowed to stand at room temperature for 10 min. The resultant reaction mixture was directly passed through a DIAION PK220 column (bed volume, 30 mL; Mitsubishi Chemical Corporation, Tokyo, Japan) to remove mercury ions. The eluted acidic fractions were combined and neutralized with NaOH solution. After lyophilization, oligosaccharides were redissolved in a minimal volume of water (approximately 300 μL), and applied to Sephadex G-10 column as described above. Oligosaccharide fractions were combined and lyophilized (odd-numbered NAH oligosaccharides).

4.3. Oligosaccharide purification

For purification of either even- or odd-numbered oligosaccharides, 2 mg of oligosaccharide mixture was dissolved in 30 μL of water and applied to tandem connected gel-filtration columns (Superdex Peptide 10/300 GL, column size 1.0 × 30 cm, Amersham Biosciences, Piscataway, USA), previously equilibrated with 5 mM NH_4OAc, pH 6.0. Oligosaccharides were eluted at a flow rate of 0.1 mL/min, and their elution was monitored by UV absorption at 210 and 232 nm. Fractions (~0.2 mL) were collected and each peak was pooled. Each peak was divided into three aliquots for the following analyses: that is, 1 μL (1/200) for MALDITOF-MS, 150 μL (150/200) for ESIMS, and the remainder (49/200) for analytical anion-exchange HPLC.

For ^{1}H NMR measurements, milligram amounts of oligosaccharides were prepared by means of anion-exchange HPLC. Three milligrams of the even-numbered oligosaccharide mixture were applied to a YMC-Pack PA-120-S5 column (4.6 × 250 mm, YMC, Tokyo, Japan), which had been equilibrated with 20 mM Na_2SO_4. The oligosaccharides were eluted by a linear gradient of 20–100 mM Na_2SO_4 (45 min) at an ambient temperature, at a flow rate of 0.5 mL/min. Fractions containing each different-sized oligosaccharides were detected by UV absorbance at 210 nm, pooled appropriately and concentrated. The above separation procedure was repeated 15 times, and the resulting oligosaccharide fractions were desalted on a Sephadex G-25 column. After lyophilization, each sized oligosaccharide was lyophilized.

4.4. MALDITOF-MS

Negative-ion MALDITOF-MS spectra were recorded using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). For sample preparation, 1 μL of oligosaccharide fraction and 4 μL of matrix solution (DHB dissolved in 50% MeOH) were mixed well, and an aliquot (0.5 μL) was spotted on a target plate. After air drying at room temperature, another aliquot (0.5 μL) was superimposed and air dried completely. The instrument was operated using an accelerating potential of 20 kV in the reflection mode. The scanned m/z range was up to 5000, and the matrix suppression (deflection) mode, up to m/z 500–700, was applied according to the sample type.

4.5. ESIMS

ESIMS experiments in the negative-ion mode were performed using a Bruker Daltonik GmbH (Bremen, Germany) Esquire 3000 Plus equipped with an ion-trap analyzer. An aliquot (150 μL) of the isolated oligosaccharide was mixed with an equal volume of MeOH and was infused into the mass spectrometer directly by using a syringe pump at a flow rate of 360 μL/h. The instrument was operated with a capillary voltage of −3.8 kV and an end plate offset of −500 V. Dry nitrogen gas flow was at 4.0 L/min and drying temperature was set at 300 °C. For each experiment, the mass range scanned was m/z 50–2000, with the scan resolution set at 5500 m/z/s.

4.6. Analytical anion-exchange HPLC

The purity of each isolated NAH oligosaccharide (both even- and odd-numbered) was determined by analytical anion-exchange HPLC. Each lyophilized sample was reconstituted with water, and an appropriate aliquot injected into a YMC-Pack Polyamine-II column (4.6 × 250 mm, YMC, Tokyo, Japan), previously equilibrated with 50 mM NaH_2PO_4, pH 5.4. Elution was made with a linear gradient of 50–500 mM NaH_2PO_4 (30 min) at a flow rate of 1.2 mL/min, and at ambient temperature. The purity of each oligosaccharide (%) was calculated from the relative peak area of UV absorbance at 210 nm.

4.7. ^{1}H NMR measurements

500-MHz ^{1}H NMR spectra were recorded using an UNITY INOVA 500 spectrometer (Varian, Palo Alto, USA). Oligosaccharide samples were dissolved in D_2O at a concentration of 2.1–3.6 mg/mL, and sample temperature set at 70 °C. Chemical shifts were expressed in ppm downfield from the reference signal of internal *t*-BuOH (1.230 ppm).

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