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Preparation of heparin/heparan sulfate oligosaccharides with internal N-unsubstituted glucosamine residues for functional studies

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Abstract The rare N-unsubstituted glucosamine ($GlcNH_3^+$) residues in heparan sulfate (HS) have important biological and pathophysiological roles. However, it is difficult to prepare naturally-occuring, $GlcNH_3^+$-containing oligosaccharides from HS because of their low abundance, as well as the inherent problems in both excising and identifying them. Therefore, the ability to chemically generate a series of structurally-defined oligosaccharides containing $GlcNH_3^+$ residues would greatly contribute to investigating their natural role in HS. In this study, a series of heparin/HS oligosaccharides, from dp6 up to dp16 in length that possess internal $GlcNH_3^+$ residues were prepared by a combination of chemical modification and heparinase I digestion. Purification and structural analysis of the major species derived from the octa- to dodeca-saccharide size fractions indicated the introduction of between 1 and 3 internal $GlcNH_3^+$ residues per oligosaccharide. In addition, a $GlcNH_3^+$ residue was selectively introduced into an internal position in a tetrasaccharide species by direct chemical modification. This selectivity has potential as an alternative procedure for preparing internally-modified oligosaccharides of various lengths. The utility of such oligosaccharides was demonstrated by a comparison of the binding of three different tetrasaccharide species containing 0, 1 and 2 free amino groups to the NK1 truncated variant of hepatocyte growth factor/scatter factor.

Keywords Heparin · Heparan sulphate · N-unsubstituted disaccharide · Free amino group · Heparinase

Abbreviations

AMAC	2-amino-acridone
CyPB	Cyclophilin-B
DMSO	Dimethyl sulfoxide
dp	Degree of polymerization (*i.e.* number of monosaccharide units *e.g.* dp2 is disaccharide)
GAG	Glycosaminoglycan
GlcA	β-D-glucuronic acid
GlcNAc	β-D-*N*-acetylglucosamine
$GlcNH_3^+$	β-D-*N*-unsubstituted glucosamine
GlcNS	β-D-*N*-sulfoglucosamine
GMSA	Gel mobility shift assay
ΔHexA	Δ^{4-5}-unsaturated hexuronic acid
HS	Heparan sulfate
HSV	Herpes simplex virus
HS 3-OST	Heparan sulfate 3-*O*-sulfotransferase
IdoA	α-L-iduronic acid
NDST	N-deacetylase/N-sulfotransferase
NK1	Truncated variant of hepatocyte growth factor/scatter factor containing only the N-terminal and the first Kringle domain
S	Sulfate
SAX-HPLC	Strong anion-exchange HPLC

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Introduction

Heparin and heparan sulfate (HS) are complex, sulfated glycosaminoglycans (GAGs) synthesised on specific proteins, forming proteoglycans, that have important biological activities in developmental processes [1, 2], angiogenesis [3], blood coagulation [4], cell adhesion [5], and tumor metastasis [6]. These involve interactions with a wide variety of proteins, *i.e.* enzymes, cytokines, growth factors, and extracellular matrix proteins [7–12], primarily mediated by the HS/heparin chains.

HS and heparin share a common biosynthetic pathway, being initially synthesized as repeating disaccharides of alternating β1→4 glucuronic acid (GlcA) and α1→4 *N*-acetylglucosamine (GlcNAc) [8, 10, 13, 14]. This non-sulfated precursor is then modified by co-ordinated N-deacetylation/N-sulfation of GlcNAc residues by N-deacetylase/N-sulfotransferase enzymes (NDST), forming *N*-sulfoglucosamine (GlcNS), *via* an N-unsubstituted glucosamine ($GlcNH_3^+$) intermediate. Subsequently, further modifications occur, *i.e.* C5-epimerization of GlcA to α1→4 iduronic acid (IdoA), O-sulfation at C2 of hexuronic acid (primarily IdoA), O-sulfation at C6 of GlcNS or GlcNAc, and lastly the rare, but functionally important, 3-*O*-sulfation of GlcNS by 3-*O*-sulfotransferases (HS 3-OST).

In the case of HS, the limited extent, but co-ordinated nature, of these enzymic modifications leads to a structurally complex, block co-polymer, in which highly modified sequences are interspersed with relatively unmodified sequences, with short transition sequences bridging the two [15]. HS structure can vary between cell and tissue types. By contrast, a more complete level of modification gives the more homogeneous, more sulfated heparin that is restricted to only a few cell types.

Rare structural components may contribute to selective protein binding. It has become clear that the $GlcNH_3^+$ unit also exists in mature HS [16, 17], where it is implicated in important cell-biological and pathophysiological phenomena. Its presence correlates with the ability of bovine and human endothelial HS to bind L-selectin [18]. $GlcNH_3^+$ residues have also been identified as additional targets for 3-*O*-sulfation. The specific HS 3-OST-3A isoform generates a sequence that is utilized as a binding site by the herpes simplex virus (HSV) glycoprotein D, thus making cells susceptible to HSV-1 entry [19–21]. Similarly, 3-O-sulfation by the HS 3-OST-3B isoform provides specific binding sites for cyclophilin B (CyPB) on responsive cells. The $GlcNH_3^+$ residue is specifically positioned two saccharides from the non-reducing end of the CyPB-binding octasaccharide [22].

Two monoclonal antibodies have been reported to recognise $GlcNH_3^+$; these highlighted distinctive localisations of their respective epitopes in tissues. One bound to extracellular components in rat kidney [23], whereas the other reacted with scrapie lesions in murine brain [24, 25]. In addition, it has been proposed that $GlcNH_3^+$ residues provide cleavage sites in HS for the endogenous NO-derived nitroxyl anion, contributing to a recycling mechanism for glypican-1 [26].

The existence of $GlcNH_3^+$ residues in mature HS may be due to an occasional interruption of the NDST-mediated, catalytic linkage between N-deacetylation and re-N-sulfation, thereby trapping the intermediate $GlcNH_3^+$. This may be exacerbated under conditions of limiting availability of 3′-phosphoadenosine 5′-phosphosulfate, which drives the final sulfation [27]. In general, the content of $GlcNH_3^+$ in HS species is low but variable, mostly ranging from 0.2% to 4% of disaccharide units [16, 17, 28–30], but reaching a particularly high 12% in bovine kidney HS [29].

$GlcNH_3^+$ residues may subsequently become 6-*O*- or 3-*O*-sulfated. Their location within the chain also varies, though they occur mostly within the N-acetylated domains, or in the short transition sequences between N-acetylated and N-sulfated domains [17]. However, structures both up- and down-stream of $GlcNH_3^+$ residues are highly diverse. A nonsulfated tetrasacharide sequence containing $GlcNH_3^+$ is associated with prion infectivity and disease progression [24]. In contrast, a highly sulfated hexasaccharide with an internal 3-O-sulfated $GlcNH_3^+$ (*i.e.* GlcA±2S-GlcNS - IdoA2S-$GlcNH_3^+$3S±6S - GlcA±2S-GlcNS) is the binding site for the HSV gD protein [21].

The location of $GlcNH_3^+$ residues and the nature of their surrounding sequences might contribute to their enzyme susceptibility, antibody recognition and protein interactions. Due to their relative scarcity and sequence variability in native HS, and the consequent difficulties in isolating, identifying and analysing them from HS, it would be useful to be able to synthesise such sequences *in vitro*. It is preferable that such sequences contain $GlcNH_3^+$ residues that are disposed internally, rather than terminally, in order to maximise the possibility of correct binding sequence generation and resulting ligand affinity. Thus, the availability of a series of such structurally-defined oligosaccharides would contribute to the investigation of the function of $GlcNH_3^+$ residues in HS. Previously, we demonstrated that the various heparinases have differential specificities toward $GlcNH_3^+$ residues [29], and can thus be useful preparative tools. In this study, we investigated the generation of heparin/HS oligosaccharides containing $GlcNH_3^+$ residues specifically at internal positions, for potential use in protein interaction and functional studies, using a combination of limited chemical modification and partial, specific enzyme digestion.

Materials and methods

Materials Heparin (bovine lung) was purchased from Sigma (Poole, Dorset, UK). A partial, enzymatically-

depolymerised, low molecular weight heparin (Innohep) was obtained from Leo Laboratories Limited (Princes Risborough, Bucks, UK). Heparinase I (*Flavobacterium heparinum*; heparin lyase EC 4.2.2.7), heparinase II (*F. heparinum*; no EC number assigned) and heparinase III (*F. heparinum*; heparitin-sulfate lyase EC 4.2.2.8) were all purchased from Grampian Enzymes (Orkney, UK). Bio-Gel P-10 (fine grade) was from Bio-Rad Laboratories (Hemel Hempstead, Herts., UK). ProPac PA-1 analytical HPLC columns were obtained from Dionex (Camberley, Surrey, UK). All HPLC solutions were prepared using MilliQ (Millipore; Watford, Herts., UK) ultra pure water. Amberlite IR-120 (H^+ form) and all reagents used for de-N-sulfation were from Sigma. Prepacked PD-10 desalting columns were purchased from Amersham Biosciences (Chalfont St. Giles, Bucks., UK).

Partial de-N-sulfation of heparin Partially de-N-sulfated heparin was prepared as previously described [29]. Briefly, the pyridinium salt of intact heparin (30 mg) was obtained by passage through an Amberlite IR-120 (H^+ form) column and titration of the resulting protonated form with pyridine. The pyridinium salt was then treated with 3 ml of 95% DMSO, 5% water at 20°C for 60 min followed by extensive dialysis and freeze-drying. The degree of de-N-sulfation was calculated by disaccharide analysis (see below).

Preparation of oligosaccharides from partially de-N-sulfated heparin Partially de-N-sulfated heparin dissolved in 6 ml of 0.1 M sodium acetate buffer pH 7.0, containing 0.1 mM calcium acetate and 100 μg/ml of bovine serum albumin, was passed through a 0.2 μm filter. It was then digested at 37°C with 66 mIU of heparinase I, added in three equal aliquots every 24 h. Digestion was monitored by absorption at 232 nm and was complete after 72 h, when it was stopped by heating at 100°C for 2 min. After centrifugation at 13,000 g for 15 min, the supernatant was concentrated to 2 ml and was then applied to two Bio-Gel P-10 columns (2.5×115 cm each) linked in series and eluted with 0.2 M NH_4HCO_3 at a flow rate of 10 ml/h with collection of 2.5 ml fractions. Elution of oligosaccharides was monitored by absorbance at 232 nm. NH_4HCO_3 was removed from pooled fractions by heating in an open container at 55°C for 24 h, followed by three cycles of freeze-drying.

Preparation of heparin tetrasaccharide (dp4) and its partial de-N-sulfation Dp4 was recovered from a partial heparinase I digestion of heparin (*i.e.* Innohep) by Bio-Gel P-10 chromatography, as described above. The fully sulfated dp4, comprised of two tri-sulfated disaccharides, was isolated from the dp4 mix by strong anion-exchange HPLC (SAX-HPLC) on a preparative ProPac PA1 column (9×250 mm). Samples were applied in water adjusted to pH 3.5 with HCl, washed with water for 2 min, and then eluted using a biphasic, linear gradient of NaCl, pH 3.5 from 0 to 0.6 M (2.1–7.1 min) followed by 0.6 to 1.3 M (7.1–47.1 min), at a flow rate of 4 ml/min. Elution was monitored by on-line absorbance at 232 nm.

Partial de-N-sulfation of dp4 was slightly modified from that used for intact heparin (above), in that the pyridinium salt of the dp4 was treated with 95% DMSO, 5% water at 50°C for 30 min. Instead of using dialysis, the reaction chemicals were removed by chromatography on a Sephadex G25 gel filtration column (2.5×20 cm) run in distilled water and monitored at 232 nm. The recovered, partially de-N-sulfated dp4 mix was fractionated by SAX-HPLC using the conditions described above.

Separation of oligosaccharides with N-unsubstituted glucosamine residues Further charge separation of each size fraction of partially de-N-sulfated heparin oligosaccharides was carried out by SAX-HPLC on a ProPac PA-1 column (4.6×250 mm). All samples were applied in water adjusted to pH 3.5 with HCl, washed for 2 min with water, pH 3.5, and then eluted using a biphasic, linear gradient of NaCl, pH 3.5 at a flow rate of 1 ml/min. The NaCl gradients for each oligosaccharide size fraction were: dp6 from 0 to 0.5 M (2.1–7.1 min) and then 0.5 to 1.3 M (7.1–57.1 min); dp8 from 0 to 0.6 M (2.1–7.1 min) and then 0.6 to 1.4 M (7.1–57.1 min); dp10 from 0 to 0.6 M (2.1–7.1 min) and then 0.6 to 1.5 M (7.1–57.1 min); dp12 from 0 to 0.6 M (2.1–7.1 min) and then 0.6 to 1.6 M (7.1–57.1 min). Eluates were monitored by on-line absorbance at 232 nm. Oligosaccharide fractions were concentrated and then desalted on prepacked PD-10 desalting columns run in water.

Disaccharide analysis Oligosaccharides were completely digested to disaccharides using a mixture of heparinases I (5 mIU), II (10 mIU), and III (5 mIU) in 0.1 ml of 0.1 M sodium acetate buffer pH7.0 containing 0.1 mM calcium acetate and 100 μg/ml of bovine serum albumin at 37°C overnight. The reaction was terminated by heating at 100°C for 2 min.

SAX-HPLC separation of disaccharides was performed on a Hewlett Packard-1100 HPLC system with an analytical ProPac PA-1 column (4.6×250 mm). Disaccharides were applied in 1 ml of water pH 3.5 at a flow rate of 1 ml/min. After a 2 ml wash with pH 3.5 H_2O, a biphasic linear gradient of NaCl was applied from 0 to 0.5 M (2.1–35.1 min) and then 0.5 to 1.1 M (35.1–57.1 min). The elution profile was monitored by on-line absorbance at 232 nm and compared with that of standard disaccharides.

^{1}H-NMR analysis of heparin dp4 species Heparin dp4 samples (0.5 mg) were dissolved in 550 μl of 100% D_2O

with traces of deuterated EDTA, and the pH was adjusted to 6.0 (without any correction for the presence of deuterated solvent). All NMR spectra were collected at 25°C on an 800 MHz Bruker Avance spectrometer equipped with a TCI cryoprobe. 1D TOCSY spectra were acquired using 60–120 ms DIPSI-2 spin-lock [31] and 256 scans. Selective excitation was achieved by 20 or 40 ms Gaussian pulses arranged in a DPFGSE [32]. 2D ROESY spectra were acquired using t_1 and t_2 acquisition times of 80 and 160 ms, respectively. Twenty-four scans were accumulated in each of 512 complex t_1 increments. The relaxation time was 1.5 s, and a 250 ms CW spin lock was applied at $\gamma B_1/2\pi$= 2,500 Hz. The total acquisition time was 14 h.

Gel mobility shift assay (GMSA) Human NK1 was purified from the culture medium of a *Pichia pastoris* recombinant expression system kindly provided by Dr Ermanno Gherardi (MRC Centre, Cambridge, UK), as previously described [33].

Dp4 species (quantified by UV absorbance at 232 nm) were tagged at their reducing ends with 2-amino-acridone (AMAC), as described by Lyon *et al*. [34]. Individual AMAC-tagged dp4 species were pre-incubated with NK1 protein (20 μg) in a 1:1 molar ratio for 30 min in a total volume of 10 μl of PBS. In the selection mode, a fixed amount of a mixture of equimolar AMAC-tagged dp4 species was pre-incubated with a varying amount of NK1. Both sets of samples were then electrophoresed on a non-denaturing, 6% (*w/v*) polyacrylamide (2.6% crosslinker) gel at a constant 100 V for approx. 10 min, and then directly imaged under UV light [34].

Results and discussion

Preparation of partially de-N-sulfated heparin In this study, $GlcNH_3^+$ residues were introduced, by chemical modification, into the commonly-available heparin, which is structurally related to the protein-binding, sulfated regions of HS.

Partially de-N-sulfated heparin was prepared by controlling the time course of a standard desulfation protocol. We demonstrated previously that heparinases II and III (but not heparinase I) are able to cleave at $GlcNH_3^+$ residues [29], therefore the partially de-N-sulfated heparin was exhaustively digested with a mixture of heparinases I, II, III, to efficiently cleave both chemically modified and unmodified segments of the chain, before SAX-HPLC analysis of the released disaccharides. The disaccharide compositions are summarized in Table 1. Three $GlcNH_3^+$-containing, sulfated disaccharides, *i.e.* ΔHexA-$GlcNH_3^+$(6S), ΔHexA(2S)-$GlcNH_3^+$ and ΔHexA(2S)-$GlcNH_3^+$(6S), were detected at levels of 3.02%, 4.12% and 59.67% of total disaccharides, respectively, indicating an overall 66.8% de-N-sulfation (*NB.* no $GlcNH_3^+$ residues were detected in the parent heparin). Interestingly, the levels of de-N-sulfation of the three parent disaccharides, *i.e.* ΔHexA-GlcNS(6S), ΔHexA(2S)-GlcNS and ΔHexA(2S)-GlcNS(6S), were 51.7%, 67.1% and 71.2% respectively. Though the latter two values are similar, the first is noticeably lower, suggesting that not all disaccharide structures in a chain are equally susceptible to de-N-sulfation under limiting conditions; it may be that the presence or absence of 2-*O*-sulfation on the adjacent non-reducing hexuronate (and possibly on the reducing side too) may have some influence on chemical susceptibility.

Preparation of N-unsubstituted oligosaccharides It has been demonstrated that the sulfated disaccharide IdoA(2S)-$GlcNH_3^+$(±6S) is a target for the HS 3-OST-3 enzyme, which generates binding sites for the gD glycoprotein of HSV-1 [20] and for CyPB [22]; this disaccharide would be

Table 1 Disaccharide compositions of heparin, partially de-N-sulfated heparin, and subsequent oligosaccharide size fractions derived from these by partial enzyme cleavage and gel filtration chromatography (Fig. 1)

Disaccharide Structures	Frequency (%)							
	Heparin	De-NS-heparin	dp16	dp14	dp12	dp10	dp8	dp6
Δ HexA-GlcNH3$^+$(6S)	n.d.	3.02	2.7	2.38	3.21	2.44	2.57	3.79
Δ HexA(2S)-$GlcNH_3^+$	n.d.	4.12	2.59	2.34	2.3	1.97	2.05	2.34
Δ HexA-GlcNS	1	1.78	2.31	1.43	2.87	2.59	3.73	4.02
Δ HexA-GlcNAc(6S)	1.14	1.98	2.64	2.16	3.46	4.11	3.33	2.98
Δ HexA(2S)-GlcNAc	0.42	0.48	0.68	0.4	0.89	0.75	0.53	0.32
Δ HexA(2S)-$GlcNH_3^+$(6S)	n.d.	59.67	58.83	58.84	52.17	45.47	38.39	27.55
Δ HexA-GlcNS(6S)	5.62	2.82	2.33	2.38	2.58	3.03	3.13	3.18
Δ HexA(2S)-GlcNS	5.47	2.02	1.89	1.9	1.89	2.03	1.99	1.92
Δ HexA(2S)-GlcNS(6S)	86.34	24.11	26.04	28.17	30.64	37.62	44.3	53.9
NH_3^+-Disaccharides	0	66.8	64.11	63.56	57.68	49.87	42.99	33.68

primarily located within the highly sulfated domains of HS. Structural analyses of such protein-binding oligosaccharides indicated that the $GlcNH_3^+$ residues were present in internal locations rather than terminally. Therefore, oligosaccharides containing highly sulfated $GlcNH_3^+$ residues preferentially at internal positions would be particularly valuable substrates for testing protein binding activities and also enzyme susceptibilities.

In order to excise oligosaccharides with internal, as opposed to terminal, $GlcNH_3^+$ residues from partially de-N-sulfated heparin we specifically used heparinase I. This heparinase does not cleave at $GlcNH_3^+$-containing disaccharides [29], but does efficiently cleave at unmodified, highly sulfated disaccharides in heparin. After exhaustive digestion with heparinase I, the digest was fractionated by gel filtration into fractions corresponding to dp6-dp16, by reference to the elution positions of known oligosaccharide standards (Fig. 1.). The recoveries of dp6-dp16 were 1.16 mg, 0.95 mg, 0.52 mg, 0.48 mg, 0.32 mg and 0.05 mg, respectively, from an initial 30 mg of fractionated heparin. No clear peak corresponding to dp4 could be identified, possibly because of the selective specificity of heparinase I.

The disaccharide compositions of the dp6-dp16 fractions are shown in Table 1. The three $GlcNH_3^+$-containing, sulfated disaccharides, ΔHexA-$GlcNH_3^+$(6S), ΔHexA(2S)-$GlcNH_3^+$ and ΔHexA(2S)-$GlcNH_3^+$(6S), were detected in

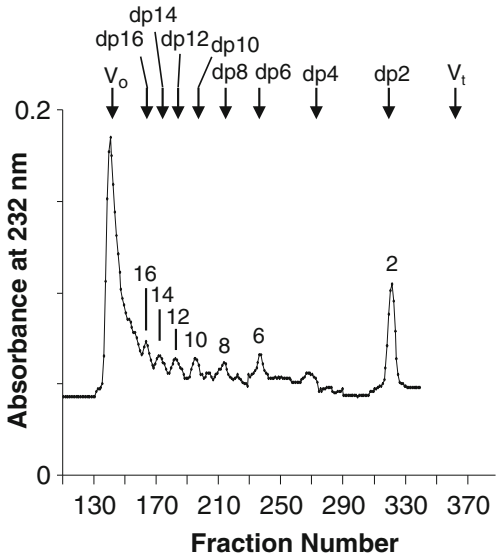

Fig. 1 Fractionation by gel filtration chromatography of partially de-N-sulfated heparin after digestion with heparinase I. Partially de-N-sulfated heparin was exhaustively digested by heparinase I and then fractionated by Bio-Gel P10 chromatography (see Materials and Methods). Elution was monitored by absorbance at 232 nm. *Arrows* indicate the elution positions of known dp2-dp16 size standards. V_o and V_t indicate the void volume and total volume determined using Dextran Blue and sodium dichromate, respectively. Peaks corresponding to dp6-dp16 were separately pooled

all oligosaccharide size fractions. The total $GlcNH_3^+$ content in fractions dp6 to dp16 were 34%, 43%, 50%, 58%, 64%, and 64%, respectively, indicating a gradual increase in occurrence with oligosaccharide length. In parallel, the content of trisulfated ΔHexA(2S)-GlcNS(6S) residues gradually decreased from 54% to 26% through dp6 to dp16. These data are compatible with the $GlcNH_3^+$ residues being internal, with the terminal disaccharides being predominantly trisulfated and unmodified, as expected from the substrate specificity of heparinase I. Interestingly, the contents of the three modified disaccharides were not proportionately constant across the oligosaccharide size range, being a ratio of 1:0.96:21.79 in dp16 changing to 1:0.61:7.27 in dp6, which may reflect sequence heterogeneities in the native heparin and/or differential rates of de-N-sulfation at specific positions within different sequences. In addition to the four disaccharides (three modified and one unmodified) already discussed, five other disaccharides were minor components of all size fractions, suggesting that they are all highly heterogeneous in sequence.

Purification and structure analysis of N-unsubstituted oligosaccharides To address the structural heterogeneity of the various size fractions, the dp6, dp8, dp10 and dp12 fractions were individually subfractionated on the basis of anionic charge density by SAX-HPLC. The compositions of the major separated oligosaccharide species were analysed (summarized in Table 2), and used to calculate the disaccharide ratios in each species, and thus indicate the likely sequences.

The dp6 fraction yielded one major peak (designated dp6a), and a few very minor additional peaks (Fig. 2a). Disaccharide analysis of the recovered dp6a revealed two disaccharides, ΔHexA(2S)-$GlcNH_3^+$(6S) and ΔHexA(2S)-GlcNS(6S), essentially in a 1:2 ratio (Table 2). This, combined with the known specificity of heparinase I, gives a likely structure for dp6a of ΔHexA(2S)-GlcNS(6S) - HexA(2S)-$GlcNH_3^+$(6S) - HexA(2S)-GlcNS(6S), with a central $GlcNH_3^+$.

As shown in Fig. 2b, two major peaks, designated dp8a and dp8b, were found in the dp8 fraction. Disaccharide analysis showed that dp8a contains ΔHexA(2S)-$GlcNH_3^+$(6S) and ΔHexA(2S)-GlcNS(6S) in a disaccharide ratio close to 2:2 (Table 2), indicating that it has two internal $GlcNH_3^+$ groups in a sequence of ΔHexA(2S)-GlcNS(6S) - [HexA(2S)-$GlcNH_3^+$(6S)]$_2$ - HexA(2S)-GlcNS(6S). In contrast, dp8b contains ΔHexA(2S)-$GlcNH_3^+$(6S) and ΔHexA(2S)-GlcNS(6S) in a ratio of approximately 1.3:2.7 (Table 2). This suggests that this particular peak may contain more than one compositional species, though the major species probably contains only one internal $GlcNH_3^+$ disaccharide. Its sequence could thus

Table 2 Disaccharide compositions of the major $GlcNH_3^+$-containing oligosaccharides purified from dp6 to dp12 size fractions by SAX-HPLC (Fig. 2)

Disaccharide structures	Frequency (%) [disaccharide number[a]]						
	dp6a	dp8a	dp8b	dp10a	dp10b	dp12a	dp12b
Δ HexA(2S)-$GlcNH_3^+$(6S)	32.6 [0.98]	46.4 [1.86]	32.2 [1.29]	56.9 [2.85]	39.9 [2.0]	59.6 [3.58]	47.3 [2.84]
Δ HexA(2S)-GlcNS(6S)	67.2 [2.02]	53.6 [2.14]	67.8 [2.71]	43.1 [2.15]	60.1 [3.0]	40.4 [2.42]	52.7 [3.16]

[a] Number of constituent disaccharides in the sequence of the sized oligosaccharide calculated from the disaccharide composition

be either ΔHexA(2S)-GlcNS(6S) - HexA(2S)-$GlcNH_3^+$(6S) - $[HexA(2S)\text{-}GlcNS(6S)]_2$ or ΔHexA(2S)-GlcNS(6S) - HexA(2S)-GlcNS(6S) - HexA(2S)-$GlcNH_3^+$(6S) - HexA(2S)-GlcNS(6S), or possibly a mixture of the two if the SAX-HPLC is unable to resolve these two positional isomers.

The dp10 fraction also gave two major peaks, dp10a and dp10b, upon SAX-HPLC (Fig. 2c). Dp10a was comprised of ΔHexA(2S)-$GlcNH_3^+$(6S) and ΔHexA(2S)-GlcNS(6S), in a ratio close to 3:2 (Table 2), suggesting the presence of three $GlcNH_3^+$, and a potential sequence of ΔHexA(2S)-GlcNS(6S) - $[HexA(2S)\text{-}GlcNH_3^+(6S)]_3$ - HexA(2S)-GlcNS(6S). In contrast, dp10b contained ΔHexA(2S)-$GlcNH_3^+$(6S) and ΔHexA(2S)-GlcNS(6S) in a ratio of 2:3 (Table 2). With two of the three internal disaccharides containing $GlcNH_3^+$ and one being unmodified, there are then three possibilities for the position of the unmodified disaccharide, and thus three potential isomeric sequences for dp10b.

Finally, the dp12 fraction also resolved into two major species by SAX-HPLC (Fig. 2d), namely dp12a and dp12b. Disaccharide analysis showed dp12a to be comprised of ΔHexA(2S)-$GlcNH_3^+$(6S) and ΔHexA(2S)-GlcNS(6S) in a ratio of approximately 3.6:2.4 (Table 2). This suggests a degree of heterogeneity in this peak, but that the major species may contain four $GlcNH_3^+$, and a potential sequence of ΔHexA(2S)-GlcNS(6S) - $[HexA(2S)\text{-}GlcNH_3^+(6S)]_4$ - HexA(2S)-GlcNS(6S). Dp12b, in contrast, contained approximately equimolar proportions (Table 2) of ΔHexA(2S)-$GlcNH_3^+$(6S) and ΔHexA(2S)-GlcNS(6S). This peak is therefore likely to be a mixture of up to four positional isomers in which the core four disaccharides contain three $GlcNH_3^+$ and one unmodified disaccharide, with the latter being potentially present at any one of the four internal positions. The level of separation and purification illustrated here is sufficient to demonstrate the principle of the approach, though clearly further, higher-resolution chromatographic separation and analysis would be required to resolve some compositional (*e.g.* with dp8b and dp12a) and probable isomeric heterogeneities, in order to generate a library of pure, characterised sequences.

It is clear that a consistent pattern of modification and resulting oligosaccharide structure does emerge from this study. Dp6 and dp8 species with one internal $GlcNH_3^+$ were generated; these could mimic highly sulfated domains with a rare $GlcNH_3^+$ in native HS. With increasing oligosaccharide length, internal sequences of more than one $GlcNH_3^+$ were also obtained. It is possible that after partial re-acetylation, these could be converted into oligosaccharides that then more closely model mixed N-acetylated/N-sulfated domains of HS.

The oligosaccharides analysed here reflect their derivation from a heparin that has undergone a relatively high level of de-N-sulfation (66.8%). If so wished, it is clearly possible to control and further limit the de-N-sulfation process (data not shown), and also the extent of heparinase I cleavage. This way, longer oligosaccharides with maybe only one internal $GlcNH_3^+$ could be obtained, which might provide even better mimics of native HS sequences.

Preparation of N-unsubstituted dp4 oligosaccharides A dp4 containing a $GlcNH_3^+$ residue cannot be prepared by the above combination of de-N-sulfation and subsequent heparinase I digestion; the $GlcNH_3^+$ would have to be present on either the reducing or non-reducing disaccharide, and such a sequence could not be excised by heparinase I. As an alternative, we attempted to prepare this structure by partial de-N-sulfation of a pre-purified, native heparin dp4.

A dp4 size fraction was prepared from a partial heparinase I digest of heparin by gel filtration on Bio-Gel P-10, as described earlier. The fully sulfated dp4 (*i.e.* ΔHexA(2S)-GlcNS(6S) - IdoA(2S)-GlcNS(6S)) was separated by SAX-HPLC (its retention time of 45.6 min is illustrated with an arrow in Fig. 3), and it was then partially de-N-sulfated using a modified procedure, as described in the Experimental section. Subsequent disaccharide analysis revealed a 65% conversion of GlcNS to $GlcNH_3^+$. The partially de-N-sulfated dp4 fraction was then separated by SAX-HPLC under the same conditions as used for the parent dp4. As shown in Fig. 3, two major peaks (dp4a and dp4b) and one minor peak (dp4c) were detected, with respective elution times of 15, 30.7 and 45.6 min. These were recovered and their disaccharide compositions were analyzed. This confirmed that dp4c corresponded to the unmodified, parent dp4, as expected from its unchanged

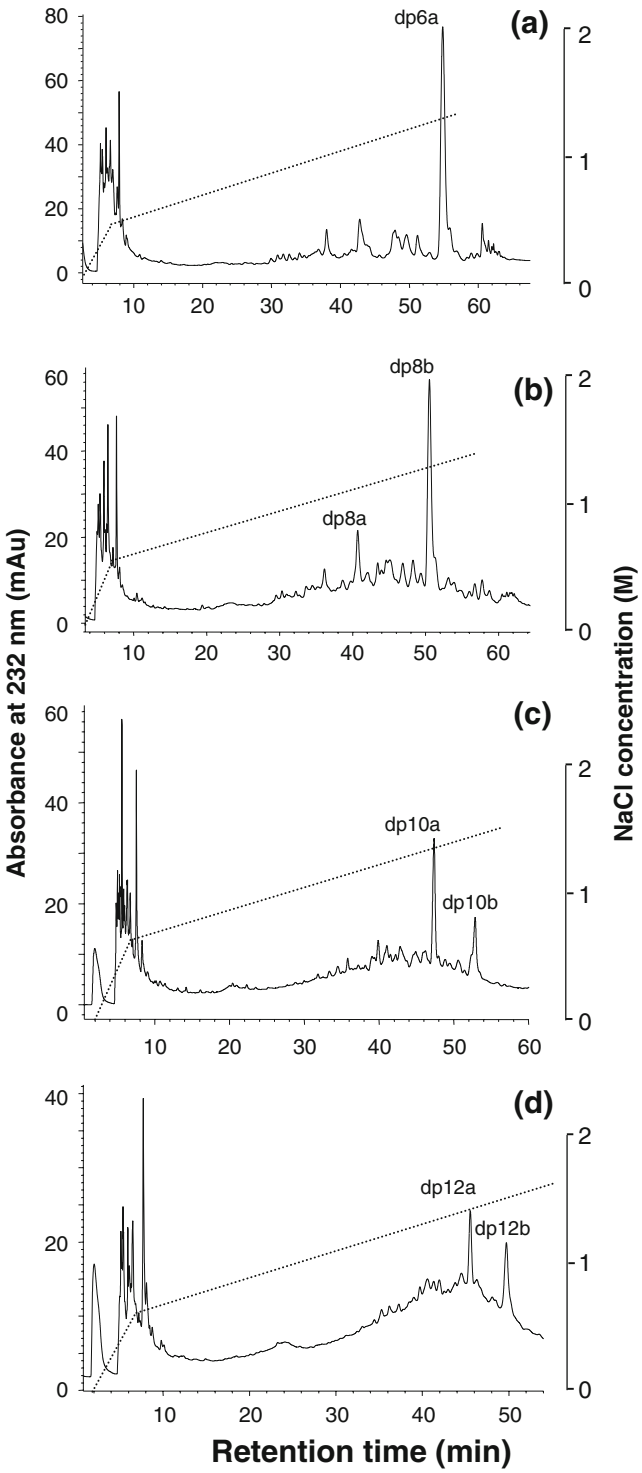

Fig. 2 Subfractionation of partially de-N-sulfated oligosaccharide size fractions by SAX-HPLC. Oligosaccharide size fractions dp6 (**a**), dp8 (**b**), dp10 (**c**) and dp12 (**d**) obtained by gel filtration (Fig. 1) were subfractionated by SAX-HPLC under elution with a biphasic linear gradient of NaCl, and monitored by absorbance at 232 nm (see Materials and Methods)

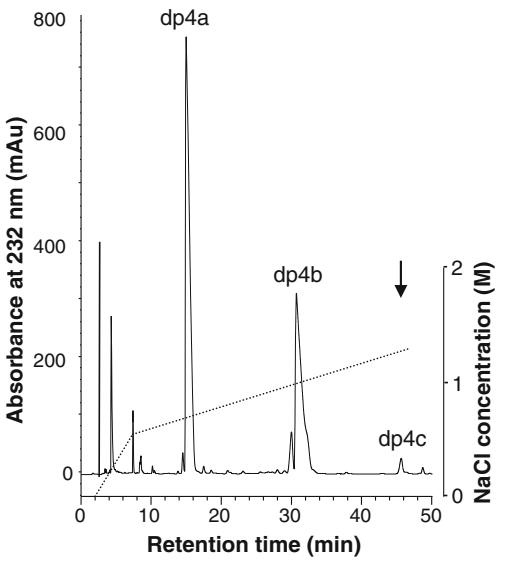

Fig. 3 Separation of partially de-N-sulfated heparin dp4s by SAX-HPLC. The partially de-N-sulfated dp4 fraction was sub-fractionated by SAX-HPLC under elution with a biphasic linear gradient of NaCl, and monitored by absorbance at 232 nm (see Materials and Methods). *Arrow* indicates the elution position of the fully sulphated, parent dp4 (ΔHexA(2S)-GlcNS(6S) - IdoA(2S)-GlcNS(6S))

elution position (compare arrow in Fig. 3). Only ΔHexA (2S)-$GlcNH_3^+$(6S) was detected in dp4a, indicating that it is the fully de-N-sulfated dp4, *i.e.* ΔHexA(2S)-$GlcNH_3^+$(6S) - HexA(2S)-$GlcNH_3^+$(6S). In contrast, dp4b comprised two disaccharides, ΔHexA(2S)-$GlcNH_3^+$(6S) and ΔHexA(2S)-GlcNS(6S), with respective contents of 44% and 56%, *i.e.* close to a 1:1 ratio, showing that it is partially de-N-sulfated. However, this analysis does not yield information on the position of the $GlcNH_3^+$ with respect to the two possible sites; does it occur randomly, and thus equally, at either possible site giving rise to two isomers that are not resolved by SAX-HPLC, or preferentially at one site giving rise to a single, selective structure.To answer this, the sequence of dp4b was investigated by NMR analysis.

NMR characterization of N-unsubstituted dp4 oligosaccharides The fully sulfated parent dp4c, the fully de-N-sulfated dp4a, and the partially-de-N-sulfated dp4b (Fig. 3) were compared by NMR analysis. For descriptive purposes, the individual monosaccharide rings in the dp4 are identified as D-C-B-A, where A is the reducing end hexosamine and D is the non-reducing ΔHexA residue.

The ^{1}H resonances of monosaccharide rings A–D of dp4a and dp4b were identified and assigned by acquiring a series of selective 1D TOCSY spectra using well resolved signals of each ring for selective excitation, and varying mixing times. Enzymatically-derived ΔHexA (ring D) was easily distinguished from the internal IdoA (ring B) by its olefinic proton H4 resonating at ~6.0 ppm. The decision as to which set of resonances of the two GlcN residues belongs to rings A and C was based on analysis of the 2D

ROESY spectra. Here, ROESY cross peaks were observed between the anomeric protons of rings B and D, across the glycosidic linkages, to the protons of rings A and C, respectively. The ^{1}H chemical shifts obtained by these experiments are provided in Table 3.

^{1}H chemical shifts of dp4a and dp4b were compared with those of the fully sulfated heparin dp4 (here referred to as dp4c) obtained previously under identical experimental conditions [35]. The agreement between the chemical shifts of all three tetrasaccharides is very good, with the exception of the H3 resonance of ring C in dp4b (−0.24 ppm) and the H3 protons of rings A and C in dp4a (−0.32 and −0.25 ppm). Smaller chemical shift differences were observed for the H2 protons (−0.10 ppm for ring C in dp4b; −0.07 and −0.13 ppm for rings A and C in dp4a, respectively). It is noteworthy that although the H2 protons are closer to the site of potential N-desulfation than the H3 protons, the largest differences were observed for the latter. Nevertheless, these values are in excellent agreement with the chemical shift differences measured for protons H3 and H2 (−0.26 and −0.05 ppm, respectively) between heparin and N-desulfated heparin (compare compounds 12 and 4 in [36]). Our findings, together with the chromatographic sizing and compositional data from disaccharide analyses, therefore allow us to conclude unambiguously that dp4b is predominantly a specific sequence, corresponding to ΔHexA(2S)-GlcNH$_3^+$(6S) - IdoA(2S)-GlcNS(6S), that shows selective de-N-sulfation at ring C. It also confirmed that dp4a is fully de-N-sulfated at both GlcN positions, as indicated by the disaccharide analysis.

This result was somewhat unexpected. It is clear that de-N-sulfation of dp4 (and presumably any other oligosaccharide size fraction) under sub-optimal conditions does not lead to random, proportionate loss of N-sulfates at both available positions. Instead of generating a mixture of two partially desulfated dp4 isomers, de-N-sulfation has initially and preferentially occurred at an internal position giving rise to a single dp4 species with one GlcNH$_3^+$ residue. It would seem that loss of N-sulfate from the reducing terminal hexosamine occurs much more slowly and/or only after prior loss of the internal N-sulfate (presumably reflecting the markedly different conformations and chemical environments at these two positions). This is evidenced by the presence of a significant amount of fully de-N-sulfated dp4 in the reaction mixture (dp4a in Fig. 3), and by the fact that complete de-N-sulfation is readily achieved by

Table 3 Comparisons of ^{1}H chemical shifts for the ring protons of dp4a, dp4b and dp4c

Ring	Proton	Chemical shift δ (ppm)			Chemical shift difference Δδ (ppm)	
		dp4c	dp4b	dp4a	dp4c-dp4b	dp4c-dp4a
A	H1	5.430	5.417	5.398	0.01	0.03
	H2	3.245	3.229	3.318	0.02	−0.07
	H3	3.682	3.700	3.997	**−0.02**	**−0.32**
	H4	3.732	3.700	3.734	0.03	0.00
	H5	4.117	4.132	4.184	−0.01	−0.07
	H6a	4.290	4.232	4.255	0.06	0.04
	H6b	4.350	4.277	4.293	0.07	0.06
B	H1	5.203	5.220	5.217	−0.02	−0.01
	H2	4.303	4.328	4.332	−0.03	−0.03
	H3	4.198	4.283	4.295	−0.09	−0.10
	H4	4.097	4.136	4.161	−0.04	−0.06
	H5	4.765	4.904	4.841	−0.14	−0.08
C	H1	5.416	5.393	5.413	0.02	0.00
	H2	3.285	3.387	3.410	−0.10	−0.13
	H3	3.631	3.871	3.877	**−0.24**	**−0.25**
	H4	3.823	3.831	3.827	−0.01	0.00
	H5	4.024	3.964	3.974	0.06	0.05
	H6a	4.237	4.30	4.22	−0.06	0.02
	H6b	4.341	4.34	4.34	0.00	0.00
D	H1	5.495	5.460	5.458	0.04	0.04
	H2	4.612	4.606	4.603	0.01	0.01
	H3	4.308	4.281	4.284	0.03	0.02
	H4	5.984	5.963	5.964	0.02	0.02

All analyses were performed at 25°C, pH 6.0, and referenced to the internal HOD signal at 4.766 ppm. Particularly noteworthy shifts are highlighted in bold

extending the reaction conditions. Fortuitously, this is advantageous with respect to generating oligosaccharides with internal $GlcNH_3^+$ residues; such residues if located at the atypical reducing end would not be representative of their environment in a native HS sequence.

Interaction of the dp4 species with NK1 In the absence of availability of any protein with a known binding requirement for $GlcNH_3^+$ residues, we used the sequence-determined dp4 species generated above to further extend our knowledge of the binding specificity of NK1, a truncated, splice variant of hepatocyte growth factor/scatter factor that is comprised of the N-terminal and 1st Kringle domains only. NK1 retains the GAG-binding capability of the native growth factor, for which it can thus substitute in *in vitro* experiments, and it conveniently binds to a minimal dp4 sequence [34]. GAG affinity has been shown to be largely dictated by the presence of IdoA combined with a high sulfate density, rather than to a specific oligosaccharide sequence [37, 38]. Although NK1 does not require the presence of $GlcNH_3^+$ residues, it was not known whether the presence of these cationic residues within the dp4 would interfere with the recognition and binding of NK1. To answer this, we employed our previously described GMSA technique [34]. This revealed that both the partially de-N-sulfated dp4b and the fully de-N-sulfated dp4a were able to bind NK1 in a similar manner to the native, fully sulfated dp4c (Fig. 4, left-hand side). Additional analyses, in which a mixture of the three dp4 species was challenged with a varying amount of NK1, allowing it to choose its preferential ligand partners on the basis of their differential affinities, demonstrated that NK1 has a greater binding preference for dp4c over either of the de-N-sulfated species (dp4a and dp4b) (Fig. 4, right-hand side). Further analyses, comparing just the two de-N-sulfated species, revealed that NK1 has a greater preference for the partially de-N-sulfated (dp4b) over the fully de-N-sulfated dp4a (data not shown). Thus, the increasing presence of $GlcNH_3^+$ residues reduces, but does not negate, interaction. It is already known that a serial reduction in sulfate density from six, through five, to four sulfates per tetrasaccharide, in the absence of $GlcNH_3^+$ residues, progressively lowers affinity for NK1 [38]. It may therefore be the case that the reduced affinity seen here in the presence of $GlcNH_3^+$ residues is less a disruptive effect of the acquisition of one or two free amino groups, but primarily a consequence of the coincidental loss of one or two N-sulfates. Nevertheless, it is clear that NK1 can at least accommodate to the presence of free, cationic amino groups in its HS/heparin ligand, which is a novel observation.

Summary Two alternative procedures have been demonstrated that can be used to introduce free amino groups into internal positions in oligosaccharides giving structures that mimic natural $GlcNH_3^+$-containing sequences in HS/heparin. One, involving partial de-N-sulfation of intact heparin/HS, introduces free amino groups randomly; these are subsequently restricted to internal positions within oligosaccharides excised from the polymer by heparinase I scission. By this procedure, modification does not occur on either the reducing terminal hexosamine, or within the non-reducing terminal disaccharide. This has the advantage of generating a library of sizes and sequences, with varying numbers of free amino groups that can also be influenced by controlling the initial extent of de-N-sulfation. The second approach is more targeted, in that it can take a purified oligosaccharide (or maybe an oligosaccharide size

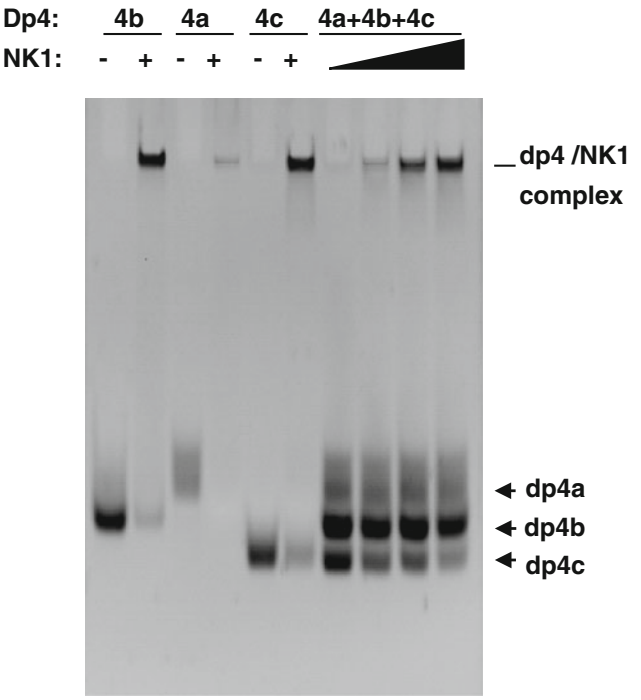

Fig. 4 NK1 interaction with dp4 species assessed by GMSA. The interactions of NK1 with the three AMAC-tagged dp4 species were assessed by GMSA (see Materials and Methods). On the left-hand side of the PAGE gel (lanes 1–6) the three individual dp4 species were tested in 1:1 molar ratios with NK1, On the right-hand side (lanes 7–10) a varying NK1 loading (no NK1 in lane 7) was tested against a fixed, equimolar mixture of the three dp4 species in NK1:dp4 molar ratios increasing from 1:12 through 1:6 to a final 1:3 (*i.e.* in the latter (lane 10) the NK1 ratio to each individual dp4 species was 1:1). Binding of a fluorescent AMAC-dp4 is shown by depletion of the mobile free species (*arrows*), and the reciprocal appearance of a relatively immobile complex with NK1 in, or close to, the well (sometimes this can be partially lost from the well during subsequent gel handling, so the fluorescent intensity of the complex seems less than the corresponding reduction in the free dp4). *NB*: equimolar amounts of the three dp4 species (determined by UV absorbance at 232 nm prior to labeling) were used throughout, though the efficiencies of AMAC-labeling, and thus comparative fluorescent band intensities, can vary considerably between different dp4 samples, as can be seen

fraction) and specifically introduce free amino groups into internal positions. This uses our observation that the conditions of partial de-N-sulfation favour initial modification at internal hexosamines rather than at the reducing terminal hexosamine. Overall, such oligosaccharides would be useful in protein screening and other interaction studies *in vitro*, that could lead to a greater understanding of the biological roles of $GlcNH_3^+$ residues in HS/heparin.

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References

1. Perrimon, N., Bernfield, M.: Specificities of heparan sulfate proteoglycans in developmental processes. Nature **404**, 725–728 (2000)
2. Lander, A.D., Selleck, S.B.: The elusive functions of proteoglycans: *in vivo veritas*. J. Cell Biol. **148**, 227–232 (2000)
3. Robinson, C.J., Stringer, S.E.: The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J. Cell Sci. **114**, 853–865 (2001)
4. Marcum, J.A., McKenney, J.B., Galli, S.J., Jackman, R.W., Rosenberg, R.D.: Anticoagulantly active heparin-like molecules from mast cell-deficient mice. Am. J. Physiol. **250**, H879–H888 (1986)
5. Couchman, J.R.: Syndecans: proteoglycan regulators of cell-surface microdomains? Nat. Rev. Mol. Cell Biol. **4**, 926–937 (2003)
6. Liu, D., Shriver, Z., Venkataraman, G., El Shabrawi, Y., Sasisekharan, R.: Tumor cell surface heparan sulfate as cryptic promoters or inhibitors of tumor growth and metastasis. Proc. Natl. Acad. Sci. U.S.A. **99**, 568–573 (2002)
7. Bernfield, M., Cotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., Zako, M.: Functions of cell surface heparan sulfate proteoglycans. Annu. Rev. Biochem. **68**, 729–777 (1999)
8. Casu, B., Lindahl, U.: Structure and biological interactions of heparin and heparan sulfate. Adv. Carbohydr. Chem. Biochem. **57**, 159–206 (2001)
9. Conrad, H.E.: Heparin binding proteins. Academic, San Diego (1998)
10. Esko, J.D., Selleck, S.B.: Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annu. Rev. Biochem. **71**, 435–471 (2002)
11. Liu, J., Thorp, S.C.: Cell surface heparan sulfate and its roles in assisting viral infections. Med. Res. Rev. **22**, 1–25 (2002)
12. Salmivirta, M., Lidholt, K., Lindahl, U.: Heparan sulfate: a piece of information. FASEB J. **10**, 1270–1279 (1996)
13. Lindahl, U., Kusche-Gullberg, M., Kjellén, L.: Regulated diversity of heparan sulfate. J. Biol. Chem. **273**, 24979–24982 (1998)
14. Esko, J.D., Lindahl, U.: Molecular diversity of heparan sulfate. J. Clin. Invest. **108**, 169–173 (2001)
15. Murphy, K.J., Merry, C.L., Lyon, M., Thompson, J.E., Roberts, I. S., Gallagher, J.T.: A new model for the domain structure of heparan sulfate based on the novel specificity of K5 lyase. J. Biol. Chem. **279**, 27239–27245 (2004)
16. Toida, T., Yoshida, H., Toyoda, H., Koshiishi, I., Imanari, T., Hileman, R.E., Fromm, J.R., Linhardt, R.J.: Structural differences and the presence of unsubstituted amino groups in heparan sulphates from different tissues and species. Biochem. J. **322**, 499–506 (1997)
17. Westling, C., Lindahl, U.: Location of N-unsubstituted glucosamine residues in heparan sulfate. J. Biol. Chem. **277**, 49247–49255 (2002)
18. Norgard-Sumnicht, K., Varki, A.: Endothelial heparan sulfate proteoglycans that bind to L-selectin have glucosamine residues with unsubstituted amino groups. J. Biol. Chem. **270**, 12012–12024 (1995)
19. Liu, J., Shriver, Z., Blaiklock, P., Yoshida, K., Sasisekharan, R., Rosenberg, R.D.: Heparan sulfate D-glucosaminyl 3-O-sulfotransferase 3A sulfates N-unsubstituted glucosamine. J. Biol. Chem. **274**, 38155–38162 (1999)
20. Liu, J., Shriver, Z., Pope, M., Thorp, S.C., Duncan, M.B., Copeland, R.J., Sasisekharan, R.: Characterization of a heparan sulfate octasaccharide that binds to herpes simplex viral type 1 glycoprotein D. J. Biol. Chem. **277**, 33456–33467 (2002)
21. Shukla, D., Liu, J., Blaiklock, P., Shworak, N.W., Bai, X., Esko, J. D., Cohen, G.H., Eisenberg, R.J., Rosenberg, R.D., Spear, P.G.: A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell **99**, 13–22 (1999)
22. Vanpouille, C., Deligny, A., Delehedde, M., Denys, A., Melchior, A., Liénard, X., Lyon, M., Mazurier, J., Fernig, D.G., Allain, F.: The heparin/heparan sulfate sequence that interacts with cyclophilin B contains a 3-O-sulfated N-unsubstituted glucosamine residue. J. Biol. Chem. **282**, 24416–24429 (2007)
23. Van den Born, J., Gunnarsson, K., Bakker, M.A., Kjellén, L., Kusche-Gullberg, M., Maccarana, M., Berden, J.H., Lindahl, U.: Presence of N-unsubstituted glucosamine units in native heparan sulfate revealed by a monoclonal antibody. J. Biol. Chem. **270**, 31303–31309 (1995)
24. Leteux, C., Chai, W., Nagai, K., Herbert, C.G., Lawson, A.M., Feizi, T.: 10E4 antigen of Scrapie lesions contains an unusual nonsulfated heparan motif. J. Biol. Chem. **276**, 12539–12545 (2001)
25. McBride, P.A., Wilson, M.I., Eikelenboom, P., Tunstall, A., Bruce, M.E.: Heparan sulfate proteoglycan is associated with amyloid plaques and neuroanatomically targeted PrP pathology throughout the incubation period of scrapie-infected mice. Exp. Neurol. **149**, 447–454 (1998)
26. Cheng, F., Mani, K., van den Born, J., Ding, K., Belting, M., Fransson, L.-A.: Nitric oxide-dependent processing of heparan sulfate in recycling S-nitrosylated glypican-1 takes place in caveolin-1-containing endosomes. J. Biol. Chem. **277**, 44431–44439 (2002)
27. Carlsson, P., Presto, J., Spillmann, D., Lindahl, U., Kjellén, L.: Heparin/heparan sulfate biosynthesis: processive formation of N-sulfated domains. J. Biol. Chem. **283**, 20008–20014 (2008)
28. Rees, M.D., Pattison, D.I., Davies, M.J.: Oxidation of heparan sulphate by hypochlorite: role of N-chloro derivatives and dichloramine-dependent fragmentation. Biochem. J. **391**, 125–134 (2005)
29. Wei, Z., Lyon, M., Gallagher, J.T.: Distinct substrate specificities of bacterial heparinases against N-unsubstituted glucosamine residues in heparan sulfate. J. Biol. Chem. **280**, 15742–15748 (2005)
30. Shi, X., Zaia, J.: Organ-specific heparan sulfate structural phenotypes. J. Biol. Chem. **284**, 11806–11814 (2009)
31. Shaka, A.J., Lee, C.J., Pines, A.: Iterative schemes for bilinear operators—application to spin decoupling. J. Magn. Reson. **77**, 274 (1988)
32. Stott, K., Stonehouse, J., Keeler, J., Hwang, T.L., Shaka, A.J.: Excitation sculpting in high-resolution nuclear magnetic resonance spectroscopy—application to selective NOE experiments. J. Am. Chem. Soc. **117**, 4199 (1995)
33. Chirgadze, D.Y., Hepple, J.P., Zhou, H., Byrd, A., Blundell, T.L., Gherardi, E.: Crystal structure of the NK1 fragment of HGF/SF suggests a novel mode for growth factor dimerization and receptor binding. Nat. Struct. Biol. **6**, 72–79 (1999)

34. Lyon, M., Deakin, J.A., Lietha, D., Gherardi, E., Gallagher, J.T.: The interactions of hepatocyte growth factor/scatter factor and its NK1 and NK2 variants with glycosaminoglycans using a modified gel mobility shift assay. Elucidation of the minimal size of binding and activatory oligosaccharides. J. Biol. Chem. **279**, 43560–43567 (2004)
35. Brisson, J.-R., Sue, S.C., Wu, W.G., McManus, G., Nghia, P.T., Uhrín, D.: NMR of Carbohydrates: 1D Homonuclear Selective Methods. In: Jiménez-Barbero, J., Peters, T. (eds.) NMR of Glycoconjugates, Ch. 4, pp. 59–93. Wiley-VCH, Weinheim (2002)
36. Yates, E.A., Santini, F., Guerrini, M., Naggi, A., Torri, G., Casu, B.: 1H and 13C NMR spectral assignments of the major sequences of twelve systematically modified heparin derivatives. Carbohydr. Res. **294**, 15–27 (1996)
37. Catlow, K.R., Deakin, J.A., Wei, Z., Delehedde, M., Fernig, D.G., Gherardi, E., Gallagher, J.T., Pavão, M.S., Lyon, M.: Interactions of hepatocyte growth factor/scatter factor with various glycosaminoglycans reveal an important interplay between the presence of iduronate and sulfate density. J. Biol. Chem. **283**, 5235–5248 (2008)
38. Deakin, J.A., Blaum, B.S., Gallagher, J.T., Uhrín, D., Lyon, M.: The binding properties of minimal oligosaccharides reveal a common heparan sulfate/dermatan sulfate-binding site in hepatocyte growth factor/scatter factor that can accommodate a wide variety of sulfation patterns. J. Biol. Chem. **284**, 6311–6321 (2009)