Experimental evidence of chemical exchange over the β(1→3) glycosidic linkage and hydrogen bonding involving hydroxy protons in hyaluronan oligosaccharides by NMR spectroscopy†

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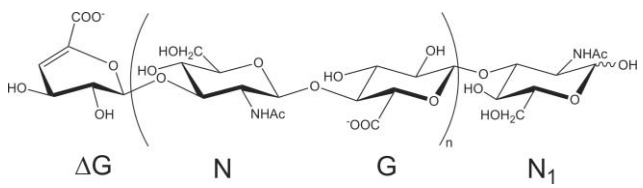
The hydroxy protons of unsaturated di-, tetra-, hexa- and octa-saccharides of hyaluronan (ΔHA_2, ΔHA_4, ΔHA_6 and ΔHA_8) in 85% H_2O/15% acetone-d_6 have been studied by NMR spectroscopy. The chemical shifts (δ), chemical shift differences (Δδ), temperature coefficients (dδ/dT) and nuclear or rotating-frame Overhauser effects (NOEs or ROEs) of hydroxy protons were measured to gain insight into hydration, hydrogen bonds and flexibility of the HA structure. The NMR data give the first experimental evidence that weak hydrogen bonds exist between O(4)H of *N*-acetyl-D-glucosamine (GlcNAc) and O(5) of D-glucuronic acid (GlcA) across the β(1→3) glycosidic linkage and between O(3)H of GlcA and O(5) of GlcNAc across the β(1→4)-linkage. A chemical exchange was observed between O(4)H of GlcNAc and O(2)H of GlcA over the β(1→3)-linkage. The interaction could be mediated through water bridge(s) and thus contribute to the water-retaining ability of hyaluronan. In this study it was also demonstrated how the chemical shifts of exchangeable hydroxy or amide proton signals can be used to describe small structural and conformational perturbations within large oligosaccharides.

Introduction

Hyaluronan (HA), with biomedical applications, is one of the most studied polysaccharides. It is the only glycosaminoglycan that lacks sulfation and it is a linear chain of repeating disaccharide units, consisting of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA), which are linked together with β(1→3) and β(1→4) glycosidic linkages (Scheme 1). Despite a simple primary structure, the conformation of HA has been studied for more than 50 years without obtaining an unambiguous explanation of its remarkably high viscosity and water-retaining properties.[1]



Scheme 1 Chemical structure of ΔHA oligosaccharides ΔHA_2 (n = 0), ΔHA_4 (n = 1), ΔHA_6 (n = 2) and ΔHA_8 (n = 3).

Many techniques such as X-ray diffraction, viscometry, electron microscopy, circular dichroism and nuclear magnetic resonance (NMR) spectroscopy[1-3] have been used to explore the secondary and tertiary structure of HA. Aliphatic protons,[4-10] carbons,[11-15] amide protons[16-19] and hydroxy protons[20-23] of HA have been analyzed by NMR to get information on hydrogen bonds, conformational constraints, and flexibility of the structure. Chemical shifts of ^1H and ^{13}C signals are equivalent from polymer to hexasaccharide, except for the terminal residues of the oligomers, which have perturbed chemical shifts because of end effects.[5,13] End effects arise from the higher mobility of the terminal residues, as shown by ^{13}C NMR T_1 relaxation time experiments.[12] Nuclear and rotating-frame Overhauser effects (NOEs and ROEs)[6-8] and rotational correlation times,[6] together with molecular modeling simulations on oligomers,[6-8,15,24-34] have revealed information on the conformation of the glycosidic linkages.

On the macroscopic level, the structure of HA is described as a stiffened random coil.[1] To explain the stiffness of the polymer, Scott *et al.* proposed, based on ^1H NMR of HA oligosaccharides in DMSO-d_6 solution,[21] that the secondary structure includes a network of inter-residue hydrogen bonds. A hydrogen bonding network correlated with other observed effects, for example a reversible decrease of the viscosity of alkaline HA solutions.[35] Four hydrogen bonds per disaccharide unit were predicted, one of them between the amide proton of GlcNAc and the neighboring carboxylate group of GlcA.[21] Two hydrogen bonds were predicted between ring oxygens and hydroxyl groups across the β(1→3) and β(1→4) glycosidic linkages. The fourth hydrogen bond was proposed to occur between a hydroxyl group of GlcA and the carbonyl oxygen of the N-acetyl group of the adjacent GlcNAc.

Molecular dynamics (MD) simulations of HA oligosaccharides in water,[6,8,24,27-30,34] have shown a pattern of hydrogen bonds similar to that proposed by Scott *et al.*[21] but with short lifetimes and in rapid exchange with the water molecules. However, a hydrogen bond between the amide proton of GlcNAc and the neighboring carboxylate group of GlcA was not found by ^1H NMR of HA oligomers in aqueous solution.[17,18] Hydrogen bonds, observed by

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^{1}H NMR in DMSO-d_6, are usually more difficult to observe in aqueous solution because of the high solvation by water molecules. The amide protons of HA in aqueous solution have been thoroughly investigated[16-19] by NMR, but studies on hydroxy protons have been limited due to their rapid exchange with water. Therefore predictions from MD simulations on the existence of hydrogen bonds involving hydroxy protons in HA have yet not been confirmed experimentally.

To reduce the rate of exchange with water, hydroxy protons are often studied at sub-zero temperatures, which requires for example a solvent mixture of H_2O and acetone-d_6.[36-38] These conditions are used in association with a pulse sequence that efficiently suppresses the water signal without affecting the resonances of the exchangeable protons. It has been shown that the addition of acetone-d_6 does not alter the conformation of carbohydrates.[39,40] Full solvation of dicarboxylic acids even occurred in a 90% acetone-d_6/10% H_2O solution.[41]

Siciñska *et al.* have studied the hydroxy protons of the methyl glycoside of a HA disaccharide dissolved in a 1 : 1 mixture of H_2O and acetone-d_6 as a model for HA.[22] However, the finding of end effects caused by terminal residues[5] has increased the interest in studying longer HA oligomers. Oligomers of HA (HA_6 or longer) have been proposed to mimic the true secondary structure of HA satisfactorily,[8] but effects induced by the tertiary structure of the polymer are probably not present in shorter oligosaccharides. HA polysaccharides of varying lengths have, on the other hand, wide-ranging and often opposing biological functions[42,43] and conformational studies of HA of different size are therefore important.

In this work, we have studied the hydroxy protons of unsaturated di-, tetra-, hexa- and octa-saccharides of HA (referred to as ΔHA_2, ΔHA_4, ΔHA_6 and ΔHA_8) in aqueous solution. Chemical shifts (δ), chemical shift differences (Δδ), temperature coefficients (dδ/dT) and NOEs/ROEs of hydroxy protons were measured to gain insight into hydration, hydrogen bonds and flexibility of the HA structure.

Experimental

Preparation of oligosaccharides from hyaluronan

Bacterial hyaluronan (280 mg) dissolved in water (16 ml) was treated with hyaluronidase (100 U from *Streptomyces hyaluronlyticus*, Calbiochem) at 37 °C for 4 days and then the solution was freeze-dried. The mixture of oligosaccharides formed was separated on a Superdex 30 prep grade HiLoad 16/60 column (GE Healthcare, Uppsala, Sweden) using 0.1 M ammonium acetate in water as solvent (2 ml/min) and the eluate was monitored by UV detection at 230 nm. The separation was performed on an ÄKTA system consisting of a P-900 pump, a UV-900 detector and Frac-900 fraction collector (Amersham Pharmacia Biotech, Uppsala, Sweden). The fractions were analysed by MALDI-TOF MS and ^{1}H NMR spectroscopy.

Sample preparation

Oligosaccharides were treated with a Dowex 50 WX-8 (H^+) cation exchange resin prior to NMR experiments to remove ammonium ions (Fig. 1). After lyophilization, the samples were dissolved in 85% H_2O/15% acetone-d_6 at a sample concentration of

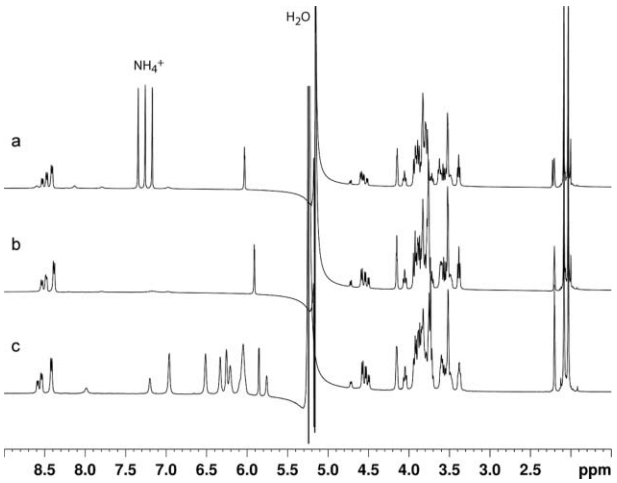

Fig. 1 ^{1}H NMR spectra of ΔHA_4 in 85% H_2O/15% acetone-d_6: **a**) before cation exchange (–10 °C), **b**) after cation exchange, pH 4.5 (–10 °C) and **c**) after adjustment of pH to 6.6 (–8 °C).

13–100 mM (HA_2 100 mM; HA_4 15 mM; HA_6 13 mM; HA_8 13 mM). To minimize absorption of impurities from glassware, the NMR tubes were soaked for at least 2 h in 100 mM sodium phosphate buffer, pH 7 and rinsed with Milli-Q® purified water.[37] The pH of the samples was adjusted to 6.6–6.8 with HCl or NaOH solutions.

NMR Spectroscopy

Spectra of mono- and disaccharides were recorded on a Bruker 400 MHz spectrometer using a 5 mm ^{1}H/^{13}C/^{15}N/^{31}P inverse detection probe, whereas spectra of tetra-, hexa- and octa-saccharides were recorded on a Bruker 600 MHz spectrometer using a 2.5 mm ^{1}H/^{13}C inverse detection probe. Both probes were equipped with z-gradient. For experiments in D_2O, the residual HOD signal was presaturated during the recycle delay. For experiments in 85% H_2O/15% acetone-d_6, water suppression was achieved using the Watergate pulse sequence.[44] The chemical shifts for ^{1}H NMR signals were referenced by setting the residual acetone-d_5 signal to δ_H 2.204 ppm. Homonuclear 2D NMR spectra were recorded in the phase-sensitive mode using the States-TPPI method.[45] TOCSY spectra were recorded with mixing times 20–150 ms and at –10, –5, 0 and 5 °C to obtain the temperature coefficients of hydroxy protons. The ^{1}H-^{1}H homonuclear DQF-COSY, TOCSY, NOESY and ROESY spectra and the ^{1}H-^{13}C heteronuclear HMBC and HSQC spectra were recorded with 2K or 4K data points in t_2 and 256 increments in t_1, using a minimum of 16 scans per increment and a relaxation delay of 1.5–2.0 s. NOESY and ROESY spectra were run with several mixing times (τ_m) ranging from 50 to 500 ms. The data were zero-filled to 2K × 2K before applying a π/4 shifted sine-squared bell function in both dimensions.

Results

Importance of sample preparation

The importance of sample preparation for the observation of hydroxy protons in the NMR spectra of ΔHA oligosaccharides is illustrated in Fig. 1. The large signal from ammonium counter

ion interfered with the signals of the hydroxy protons (Fig. 1a). After cation exchange, the hydroxy proton signals did not appear (Fig. 1b) unless the pH was adjusted to 6–7 (Fig. 1c).

Assignments and nomenclature of ΔHA oligosaccharides. ^{1}H NMR resonances of ΔHA oligosaccharides in D_2O were assigned from COSY, TOCSY, HSQC and HMBC spectra obtained at 30 °C. Chemical shifts of aliphatic proton signals in 85% H_2O/15% acetone-d_6 at −5 °C were obtained from TOCSY experiments, with the help of the assignments in D_2O. Exchangeable protons were assigned from cross peaks with ring protons in TOCSY experiments with 20 ms mixing time. Protons are abbreviated as sugar residue (N or G)$_{\text{number in the chain}}$_atom next to the proton (C, O or N)(numbering)H. The torsion angles at the glycosidic linkages are defined as $\varphi_{H1\text{-}C1\text{-}O1\text{-}C3}$ and $\psi_{C1\text{-}O1\text{-}C3\text{-}H3}$ at the β(1→3)-linkage and as $\varphi_{H1\text{-}C1\text{-}O1\text{-}C4}$ and $\psi_{C1\text{-}O1\text{-}C4\text{-}H4}$ at the β(1→4)-linkage.

Aliphatic protons. Chemical shifts of signals from the aliphatic protons of ΔHA_4 (see Electronic supplementary information†) were consistent with those reported by Blundell *et al.*[5] A complete list of the chemical shifts of ΔHA_6 and ΔHA_8 has not been reported before, but the differences compared to ΔHA_4 were small. Chemical shifts of some of the ring proton signals were found to be sensitive to temperature change, especially those for protons at the glycosidic linkages and G_C(5)H, which was in accordance with previous studies.[9]

Chemical shifts of exchangeable protons. Chemical shifts of amide and hydroxy proton signals were compared within each oligosaccharide and between different oligomers (Fig. 2, Table 1). The existence of end effects was apparent from the difference in chemical shifts between terminal and interior residues. We sought for regions with negligible or no end effects to mimic the hyaluronan polysaccharide. ΔHA_2 was ruled out since any observed chemical shift changes could arise from the effect of the unsaturation of the glucuronic acid. For the longer ΔHA oligosaccharides, the terminal unsaturated GlcA residue (G_n),

Table 1 Chemical shifts (ppm) and dδ/dT (ppb/°C) of exchangeable protons in GlcNAc, GlcA, ΔHA_2, ΔHA_4, ΔHA_6 and ΔHA_8. Chemical shifts were measured at −5 °C

			GlcNAc/GlcA	ΔHA_2	ΔHA_4	ΔHA_6	ΔHA_8
$N_{1\alpha}$	O(1)H	δ	7.22	7.22	7.18	7.19	7.19
		dδ/dT	−6.7	−6.3	−6.2	−6.6	−7.4
	NH	δ	8.35	8.51	8.51	8.52	8.52
		dδ/dT	−9.8	−8.6	−10.4	−10.5	−10.8
	O(4)H	δ	6.43	6.32	5.72	5.74	5.74
		dδ/dT	−11.0	−9.4	−8.4	−9.2	−10.1
	O(6)H	δ	5.91	5.92	5.98	6.00	6.00
		dδ/dT	−12.5	−11.7	−13.7	−14.0	−14.8
$N_{1\beta}$	O(1)H	δ	7.85	7.92	7.95	7.97	7.97
		dδ/dT	−10.2	−10.2	−11.5	−9.7	−12.5
	NH	δ	8.43	8.66	8.56	8.56	8.56
		dδ/dT	−7.8	−7.4	−8.2	−8.1	−8.4
	O(4)H	δ	6.46	6.27	5.72	5.72	5.72
		dδ/dT	−11.4	−8.6	−8.4	−9.7	−10.2
	O(6)H	δ	5.99	5.99	6.05	6.07	6.07
		dδ/dT	−12.7	−11.7	−12.6	−12.7	−14.8
G	O(2)H	δ			6.22	6.23	6.22
		dδ/dT			−12.8	−13.9	−14.5
	O(3)H	δ			6.01	6.00	6.01
		dδ/dT			−10.6	−10.1	−11.0
N	NH	δ				8.32	8.31
		dδ/dT				−7.8	−8.2
	O(4)H	δ				5.72	5.72
		dδ/dT				−9.7	−10.2
	O(6)H	δ				6.19	6.19
		dδ/dT				−13.7	−14.1
G_{n-2}	O(2)H	δ				6.23	6.22
		dδ/dT				−13.9	−14.5
	O(3)H	δ				6.03	6.03
		dδ/dT				−11.2	−12.1
N_{n-1}	NH	δ			8.39	8.39	8.38
		dδ/dT			−7.1	−7.5	−7.6
	O(4)H	δ			6.30	6.30	6.30
		dδ/dT			−9.7	−10.3	−10.6
	O(6)H	δ			6.17	6.19	6.19
		dδ/dT			−12.8	−13.7	−14.1
$G_{n\alpha}$	O(2)H	δ	6.12	6.40			
		dδ/dT	−11.3	−10.6			
	O(3)H	δ	6.29	5.96			
		dδ/dT	−11.6	−13.3			
$G_{n\beta}$	O(2)H	δ	6.51	6.43	6.47	6.50	6.50
		dδ/dT	−11.9	−11.3	−12.4	−12.7	−13.3
	O(3)H	δ	6.43	5.96	6.01	6.04	6.03
		dδ/dT	−12.0	−13.3	−13.7	−14.9	−15.6

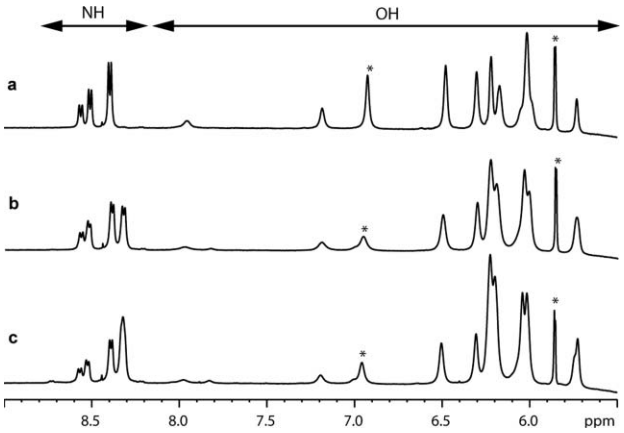

Fig. 2 ^{1}H NMR spectra of **a**) ΔHA_4, **b**) ΔHA_6 and **c**) ΔHA_8 in 85% H_2O/15% acetone-d_6 at −5 °C. The signals from the hydrated form of acetone (6.93–6.96 ppm) and the olefinic proton (5.86 ppm) are marked by asterisks (*). The spectra were processed by applying an exponential function with 1.0 Hz line broadening.

the adjacent GlcNAc residue (N_{n-1}) and the terminal reducing GlcNAc residue (N_1) were affected by end effects (Table 1). Thus, the interior part without end effects was represented by G_{n-2} of ΔHA_4, ΔHA_6 and ΔHA_8, by N_{n-3} and G_{n-4} of ΔHA_6 and ΔHA_8, and by N_{n-5} and G_{n-6} of ΔHA_8. It is important to notice that most of the interior proton signals are averaged over several residues due to spectral overlap. These interior residues will here be abbreviated to N and G respectively, without index number.

Chemical shift differences. To obtain information about the chemical environment of the exchangeable protons, the chemical shifts of signals from the oligosaccharides were compared to those from the corresponding monosaccharide ($\Delta\delta = \delta_{oligosaccharide} - \delta_{monosaccharide}$). This allows detection of the proximity to ring oxygens or to bulky groups, changes in hydration and hydrogen-bonding interactions occurring upon formation of oligosaccharides from the constituent monosaccharides.[38] As can be seen in Fig. 3, the hydroxy protons that are not affected by end effects had large negative $\Delta\delta$ values (>0.25 ppm) except N_O(6)H, which had a positive $\Delta\delta$ (0.20 ppm). The $\Delta\delta$ was largest for N_O(4)H (−0.74 ppm), followed by G_O(3)H (−0.43 ppm) and G_O(2)H (−0.29 ppm).

Temperature coefficients. Temperature coefficients were calculated by measuring chemical shifts at three or four different temperatures between −10 °C and 5 °C, a temperature interval at which hydroxy proton signals were observed. Temperature coefficients of exchangeable protons were negative, but when compared, they are referred to as absolute values. The temperature coefficients of the oligosaccharides and of GlcA were found to be slightly affected by pH, in such a way that $|d\delta/dT|$ increased with increasing pH. Thus, to be able to compare oligosaccharides with

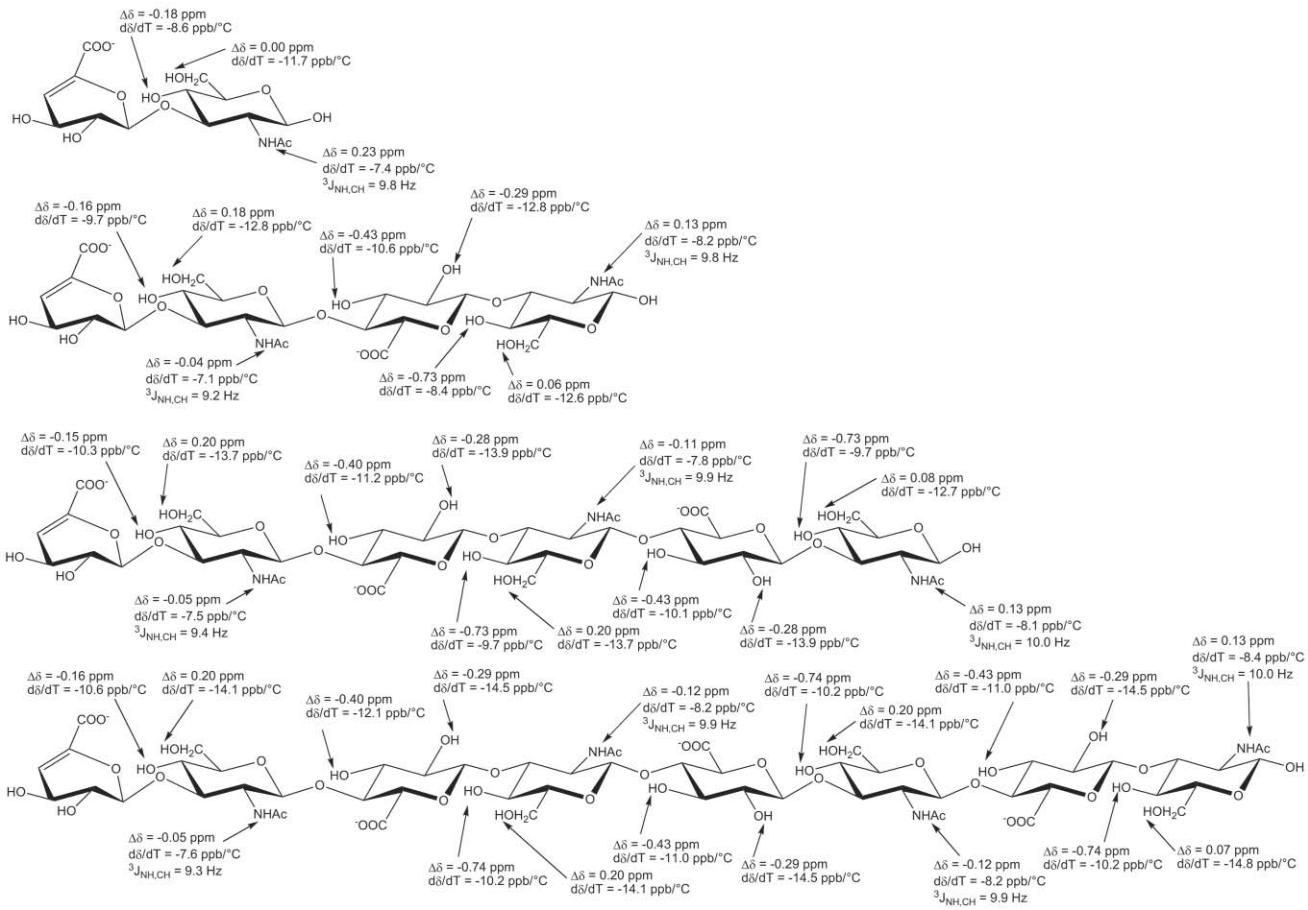

Fig. 3 $\Delta\delta$, $d\delta/dT$ and $^{3}J_{NH,CH}$ of exchangeable protons on ΔHA_2, ΔHA_4, ΔHA_6 and ΔHA_8. Only the β-anomeric forms are displayed.

Table 2 Inter-residue NOEs observed over the β(1→3)-linkage

GlcNAc	GlcA	Oligomers[a]
NH	C(1)H	ΔHA_4, ΔHA_6, ΔHA_8
NH	C(2)H	ΔHA_6, ΔHA_8[b]
NH	O(2)H	ΔHA_6, ΔHA_8[b]
Me	C(1)H	ΔHA_4, ΔHA_6, ΔHA_8
Me	C(3)H	ΔHA_2, ΔHA_8
Me	O(2)H	ΔHA_2, ΔHA_4, ΔHA_6, ΔHA_8
C(2)H	C(1)H	ΔHA_4, ΔHA_6, ΔHA_8
C(3)H	C(1)H	ΔHA_4, ΔHA_6, ΔHA_8[d]
O(4)H	C(2)H	ΔHA_6, ΔHA_8[d]
O(4)H	O(2)H	ΔHA_4, ΔHA_6, ΔHA_8[c,d]

[a] NOESY experiments were run at 50, 100, 200 and 400 ms mixing time, at −5 °C. All cross peaks were visible at 50 ms mixing time. [b] Not observed in ROESY experiments. [c] Chemical exchange. [d] Cross peaks could not be distinguished between interior linkages and reducing-end linkage.

each other and with the monosaccharides, the pH was adjusted to similar values (see experimental). Some differences were observed among the hydroxy protons (Fig. 3, Table 1). In the interior of the oligosaccharides, the hydroxy protons could be divided into protons with large |dδ/dT|, including G_O(2)H (12.8–14.5 ppb/°C) and N_O(6)H (13.7–14.1 ppb/°C) and protons with smaller |dδ/dT|, including G_O(3)H (10.1–11.0 ppb/°C) and N_O(4)H (9.7–10.2 ppb/°C).

NOEs and chemical exchange. Both NOESY and ROESY experiments were run to discriminate between dipolar relaxation and chemical exchange. Through-space interactions can be detected by NOESY experiments, but discrimination between signals from chemical exchange and from dipolar relaxation can only be achieved from ROESY experiments. ROE cross peaks originating from chemical exchange have the same sign as the diagonal peaks, whereas cross peaks originating from dipolar relaxation have the opposite sign. Several mixing times were used to compare the intensities of the NOEs (Table 2). The pattern was similar among the oligomers, even though some cross peaks were not visible in the smaller oligomers (Table 2). Intra-residue NOEs between the N-acetyl amide proton and N_C(1)H, N_C(2)H and N_C(3)H were found in accordance with previous studies.[6,18]

A number of inter-residue NOEs over the β(1→3)-linkage were observed, both between aliphatic protons and between aliphatic protons and exchangeable protons (Table 2). NOEs were observed between the methyl group and both G_C(1)H and G_C(3)H. G_C(1)H showed also NOEs with N_C(2)H, N_C(3)H and the amide proton, which has also been observed by others.[6,7,10,18]

Two NOEs were also observed between hydroxy protons and aliphatic protons; between G_O(2)H and the methyl group, and between N_O(4)H and G_C(2)H. Furthermore, in the NOESY spectra a cross peak was observed between the amide proton and G_O(2)H, and another between N_O(4)H and G_O(2)H (Fig. 4). The ROESY experiments revealed that the interaction between N_O(4)H and G_O(2)H arose from chemical exchange and not from dipolar relaxation. NOE cross peaks for interactions over the β(1→4)-linkage could not be assigned due to severe overlap in the spectra of the oligomers.

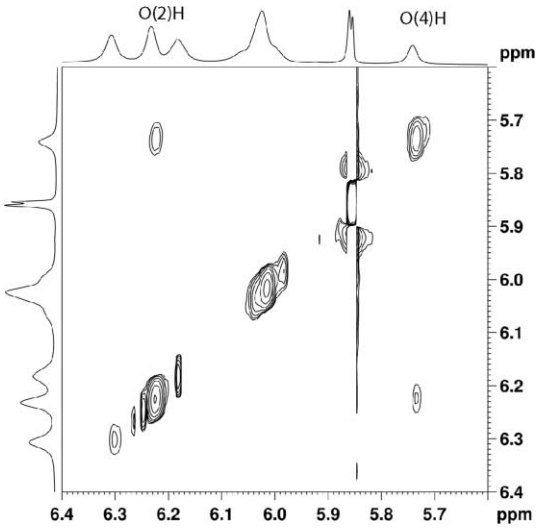

Fig. 4 Part of NOESY spectrum of ΔHA_4 obtained at −5 °C and 200 ms mixing time. Cross peaks between N_O(4)H and G_O(2)H are shown.

Discussion

Hydrogen bonding and hydration

Hydroxy protons can be used to monitor hydration and hydrogen-bonding interactions from the measurement of their Δδ, dδ/dT, vicinal coupling constants, rate of exchange with water and from NOEs. The rate of exchange of the hydroxy protons with water was high, resulting in broad hydroxy proton resonances and thus it was not possible to measure the coupling constants between ring protons and hydroxy protons ($^3J_{CH,OH}$). Strong overlapping of hydroxy proton signals in the 2D NOESY spectra of the ΔHA oligosaccharides also prevented accurate determination of exchange rates. Nonetheless, Δδ and dδ/dT are sufficient parameters to obtain information on hydrogen bonding and hydration since they depend on both intramolecular interactions and interactions with water molecules.[38,46,47]

Protons involved in hydrogen bonds are usually deshielded, but to our knowledge the only example of large downfield shift (>1 ppm) of hydroxy proton signals in aqueous solution of carbohydrates is for the intramolecular hydrogen bond between hydroxyl and phosphate groups in *myo*-inositol compounds.[48] We have previously shown that in strongly hydrated systems, such as carbohydrates, the chemical shift of a hydroxy proton signal is a balance between two opposite contributions: a downfield shift due to hydrogen bonding and an upfield shift due to reduced hydration.[38,46] An upfield shift will indicate reduced hydration due to steric hindrance or hydrogen bonding to ring oxygen, whereas a downfield shift will show proximity to another hydroxyl group. The chemical shift of a hydroxy proton involved in a hydrogen bond or with reduced hydration is less affected by temperature changes due to decreased interaction with the solvent. Thus, hydroxy protons with large |dδ/dT| (>11 ppb/°C) are fully hydrated, whereas hydroxy protons with |dδ/dT| <11 ppb/°C are only partially hydrated. For hydroxy protons involved in strong hydrogen bonding, |dδ/dT| of less than 3 ppb/°C have been measured.[36]

The hydroxy proton with largest Δδ (–0.74 ppm) and lowest |dδ/dT| (ΔHA_6 9.7, ΔHA_8 10.2 ppb/°C) was N_O(4)H. This

negative Δδ is due to the proximity of the hydroxy proton to the neighboring ring oxygen of GlcA. In ΔHA_2, N_O(4)H showed a smaller negative Δδ (–0.17 ppm), tentatively attributed to conformational changes caused by the unsaturation of the GlcA residue. Siciǹska *et al.* also measured an upfield shift (Δδ = –0.44 ppm) for N_O(4)H in a methyl glycoside of the HA disaccharide in aqueous solution.[22] We also observed an upfield shift for G_O(3)H (–0.43 ppm) suggesting interaction with the adjacent ring oxygen. These two interactions, N_O(4)H–G_O(5) and G_O(3)H–N_O(5), have been predicted to occur in MD simulations incorporating explicit water molecules[8,24,27-30] and the present study gives the first direct experimental evidence that such interactions occur for HA oligosaccharides in aqueous solution. Whereas MD simulations indicate that the two interactions have similar average distance, average angle and frequency of hydrogen bonds,[8] the larger Δδ and slightly lower |dδ/dT| measured for N_O(4)H suggest however that the hydration of this hydroxy proton is more reduced. In both cases, the relatively high temperature coefficients indicate that these hydrogen bond interactions are weak.

G_O(2)H had a negative Δδ (–0.29 ppm) but a high |dδ/dT| (12.8–14.5 ppb/°C). The upfield shift relative to the shift measured in the monosaccharide is in accordance with spatial proximity to the neighboring N-acetyl group. NOEs between G_O(2)H and both the amide proton and the methyl of the N-acetyl group further confirm such interaction. On the other hand, the large |dδ/dT| suggests that G_O(2)H does not participate in any strong hydrogen-bonding interaction.

N_O(6)H had a positive Δδ (+0.20 ppm) but an unchanged temperature coefficient compared to data from the monosaccharide. According to our previous studies,[46] a positive Δδ indicates spatial proximity to another hydroxyl group. Indeed, a hydrogen bond between N_O(6)H and G_O(3)H over the β(1→4)-linkage has been proposed by Almond *et al.*,[27] but it had short residence time in MD simulations. This is in good agreement with the large |dδ/dT| value as well as with the absence of NOE cross peaks involving N_O(6)H, supporting a relatively freely rotating hydroxymethyl group. Only a small Δδ of 0.05–0.08 ppm was measured for N_O(6)H in the reducing end of the oligomer where such N_O(6)H–G_O(3)H interaction does not occur.

Chemical shifts, coupling constants and temperature coefficients of the amide protons (Table 1) were consistent with those reported by Blundell *et al.* for studies in H_2O/D_2O solutions,[18,19] except that the |dδ/dT| of the amide protons were 0.5–1.8 ppb/°C higher in our study. As mentioned earlier, differences in pH could explain differences in |dδ/dT|. The |dδ/dT| of the amide protons (7.1 to 8.2 ppb/°C) were only slightly lower than that of N_O(4)H. Studies by Blundell *et al.* have shown that the amide protons are not involved in strong hydrogen-bonding interactions.[18,19]

In the disaccharide, the amide proton had a positive Δδ (0.23 ppm, see Fig. 3) attributed to the proximity to G_n_O(2)H. Upon "building up" a tetra-, hexa- or octa-saccharide, this amide proton is close, not only to G_n_O(2)H, but also to the carboxylate group of the other neighboring GlcA residue. This proximity reduces the hydration of the amide proton leading to an upfield shift of its NMR signal. The two effects nearly cancel each other out since in ΔHA_4, ΔHA_6 and ΔHA_8, the amide proton adjacent to the non-reducing unsaturated GlcA terminus had a very small Δδ (–0.05 ppm). The amide proton of the reducing GlcNAc residue in ΔHA_4, ΔHA_6 and ΔHA_8 has no neighboring carboxylate group and the Δδ was positive due to the spatial proximity to G_O(2)H only (0.15–0.17 ppm for the α-anomer and 0.13 ppm for the β-anomer). The amide protons in the interior of the oligosaccharides had a small negative Δδ (–0.12 ppm) from the contribution of two opposite effects: spatial proximity to G_O(2)H leading to a downfield shift (+0.13 ppm) and proximity to the carboxylate group resulting in reduced hydration leading to an upfield shift. The effect of reduced hydration by the carboxylate group on the chemical shift of the amide proton was thus calculated to be –0.25 ppm, by comparing the different amide protons in the HA structures (Table 3).

Table 3 The effects of adjacent groups on Δδ (ppm) of amide protons

	G_{m+1}[a]	G_{m-1}[a]	Δδ	Δ(Δδ)[b]
ΔHA_2	O(2)H		0.23	–0.28
N_{n-1}	O(2)H	COO^-	–0.05[c]	
N_1	O(2)H		0.13[c]	–0.25
N	O(2)H	COO^-	–0.12[c]	

[a] Interfering groups on adjacent residues to the GlcNAc residue (N_m). [b] The difference between Δδ, showing the explicit effect of the carboxylate group on hydration. [c] Δδ values were obtained from ΔHA_8 (see Fig. 3).

The hydroxy protons of compounds similar in structure to the HA disaccharide have previously been studied in aqueous solution. Leeflang *et al.*[49] investigated the hydroxy protons of methyl β-cellobioside in H_2O/CD_3OD (4 : 1, w/w) and concluded, based on the large |dδ/dT| value, that strong hydrogen bonding did not occur. However, the chemical shifts of the hydroxy proton signals were not analyzed. Kindahl *et al.* studied the hydroxy protons of the disaccharide β-D-GlcNAc-(1→4)-β-D-GlcNAc in 85% H_2O/15% acetone-d_6.[50] It resembles the β(1→4)-linkage of HA, with the same substituents around the glycosidic bond except that the carboxylate function in HA is replaced by a hydroxymethyl group on the reducing end sugar. The amide proton signal of the non-reducing residue showed a downfield shift (Δδ = 0.22 ppm) whereas O(3)H, adjacent to the ring oxygen, was shifted upfield (–0.46 ppm). The upfield shift of the O(3)H signal is similar to that measured for G_O(3)H in the ΔHA oligosaccharides (Δδ = –0.43 ppm). The involvement of O(3)H of β-D-GlcNAc-(1→4)-β-D-GlcNAc in hydrogen bonding was also confirmed by a small $^3J_{H,OH}$ coupling constant (2.2 Hz) and a reduced rate of exchange. |dδ/dT| was only slightly smaller than for the other hydroxy protons (9.1 ppb/°C) and it was therefore concluded that O(3)H is incorporated in a weak hydrogen bond to the adjacent ring oxygen. In the same work,[50] the disaccharide β-D-Gal-(1→3)-α-D-GalNAc-OMe was also investigated. An upfield shift was observed for O(2)H (–0.39 ppm) of the non-reducing end sugar. A decreased rate of exchange was observed compared to other hydroxy protons but there was no deviation in coupling constant or temperature coefficient.[50] The lower rate of exchange of O(2)H was interpreted as a limited accessibility of water because of proximity to the N-acetyl group of the neighboring residue, and not to hydrogen bonding.

Conformation of the β(1→3)-linkage

Monte Carlo (MC) and MD simulations[6-8,15,25,26,32-34] have derived the torsion angles at the glycosidic linkages, which have been

verified by NOE measurements.[6-8] For the global minimum energy conformation, the torsion angles of the β(1→3)-linkage are restricted to a rather narrow region (φ = 40–52°, ψ = −20–25°), which is in agreement with the *exo*-anomeric effect. In aqueous solution this *syn* conformation is supported by NOEs between N_C(3)H and G_C(1)H and between the amide proton and G_C(1)H (Table 2). However, a number of NOEs, not reported at room temperature, were observed at −5 °C (Table 2). Some of these NOEs are not consistent with the global minimum energy conformation at the β(1→3)-linkage. A cross peak in NOESY spectra was observed between G_C(2)H and the amide protons in ΔHA_6 and ΔHA_8 at −5 °C. This NOE was not observed by us for ΔHA_8 or by others for an octasaccharide[8] in aqueous solution at room temperature. Furthermore, the NOEs between N_C(2)H and G_C(1)H (also observed by Donati *et al.*[6]), and between N_O(4)H and G_C(2)H, would probably not be observed in the normal *syn* conformation.

A chemical exchange cross peak was observed between N_O(4)H and G_O(2)H (Fig. 4). N_1_O(4)H from both α- and β-anomers gave rise to cross peaks with G_2_O(2)H. However, because of spectral overlap it was not possible to distinguish between cross peaks originating from this terminal linkage at the reducing end and from the interior linkages. A direct chemical exchange interaction is not expected in the *syn* conformation[8,25,26] since the two hydroxyl groups are situated on different sides of the glycosidic linkage. The distance between the two protons (5.4 Å between the oxygens N_O(4) and G_O(2) in the middle of HA_8, measured from an average structure calculated by Almond *et al.*[8]) is too long for a direct exchange but could be mediated by water bridge(s) (Fig. 5a). One possible interaction could involve one water molecule bridging N_O(4)H and the glycosidic oxygen, and another water molecule bridging the glycosidic oxygen and G_O(2)H. These water bridges could contribute to the high water-retaining ability of HA. Indeed, the molecular basis of the stiffness of HA has been attributed from MD simulations to water caging around the glycosidic linkages.[24,28] Several water bridges have previously been predicted from MD simulations of a HA tetrasaccharide; *e.g.* between G_O(2)H and carbonyl oxygen (24% of the time; dimer water bridge 23% of the time, direct hydrogen bonding 28% of the time), between N_O(4)H and adjacent ring oxygen (8%; dimer 4%; direct hydrogen bonding 35%) and between N_O(4)H and carboxylate oxygen (19%; dimer 74%; direct hydrogen bonding 0%).[30] A direct or water-mediated interaction between N_O(4)H and G_O(2)H has however not been reported before.

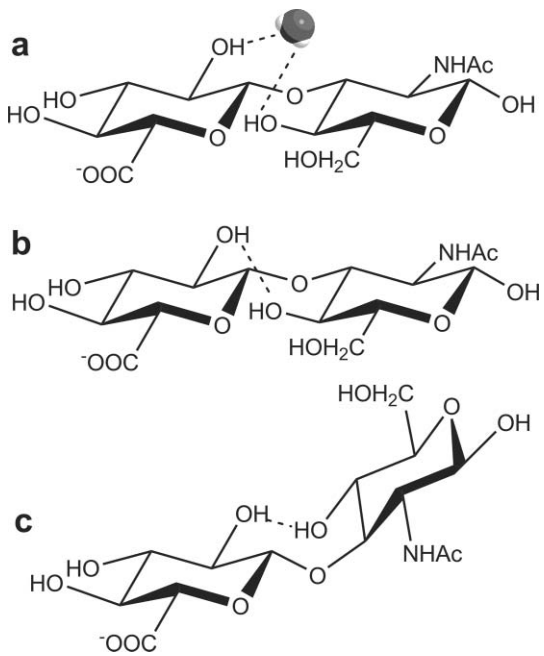

Fig. 5 Schematic representation of three possible conformations explaining the chemical exchange observed between N_O(4)H and G_O(2)H: **a)** water bridge(s), **b)** minor *syn* conformation, in which the two hydroxyl groups are closer to each other, and **c)** *anti* conformation.

An alternative explanation of the chemical exchange observed between N_O(4)H and G_O(2)H could be the occurrence of a minor *syn* conformation in equilibrium with the major conformation and in which the two hydroxyl groups are closer to each other (Fig. 5b). Minor *syn* conformations have been found in MD simulations on HA_2 (φ = −19°, ψ = −26°),[33] and on HA_4, HA_6 and HA_8 (φ = −30°),[8] which also correspond well with the additional NOEs observed by us.

A third alternative is that the chemical exchange is observed for an *anti* conformation at the glycosidic linkage (Fig. 5c). *Anti* conformations have been found in MC and MD simulations of the β(1→3)-linkage (φ = 160–177°, ψ = 4–10°), which are 1.3–5.2 kcal/mol higher in energy compared to the global minimum.[25,26,32,33] However, the calculated local minima are inaccessible at lower temperatures or in the case of longer HA chains and their existence has not been confirmed experimentally.[25,26,32] A *global* minimum energy with an *anti* orientation at the β(1→3)-linkage has also been obtained from low-level *ab initio* calculations (φ = 130°, ψ = −90°) on HA_2[51] and semi-empirical quantum mechanical calculations (φ = 165°, ψ = −122°) on HA_2 and HA_3.[52] However, the *anti* conformation of glycosidic linkages has rarely been found experimentally in oligosaccharides. Nevertheless, Dabrowski *et al.* reported the presence of a hydrogen bond between Glc O(4)H and Gal O(2)H in β-D-Gal-(1→3)-β-D-Glc-OMe proving the existence of an *anti* conformer in equilibrium with the "normal" *syn* conformer.[53] This was verified by ROEs in DMSO-d_6 solution, showing dipolar relaxation between the two hydroxy protons. In aqueous solution, chemical exchange between hydroxy protons has been attributed to close proximity or weak, transient hydrogen bonds, *e.g.* in maltose[39] and sucrose.[54]

Conformation of the β(1→4)-linkage

For the global minimum energy conformation, the angles at the β(1→4)-linkage calculated from MC and MD simulations (φ = 30–50°, ψ = −10–8°) are similar to those of the β(1→3)-linkage.[7,8,25,32,34] No NOEs could be observed over the β(1→4)-linkage because of overlap in the spectra of the oligomers. However, NOEs between N_C(1)H and G_C(4)H, and between the amide proton and G_C(4)H have previously been observed by others.[6]

Individual carbohydrate-water interactions have been proposed to determine the carbohydrate structure and functionality, rather than the amount of overall hydration.[55] Thus, individual water bridges across the glycosidic linkages of HA, together with direct

hydrogen bonds, could play a crucial role in the location of the conformational minima. In the investigations of such effects, ^{1}H NMR of hydroxy protons in aqueous solution is a powerful tool. The measurement of Δδ yields much information about the chemical surrounding of a hydroxy proton in terms of hydration and hydrogen bonds. In particular, in this study we show that Δδ is very sensitive to small structural or conformational perturbations within a molecule and that it can be used as a probe to monitor these small changes. We have also shown how additional inter-residual NOEs involving hydroxy protons can be used, together with the few NOEs from ring protons, to more accurately describe the conformation of the glycosidic linkages.

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