Ultrasensitive NMR Spectroscopy

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Characterization of Picomole Amounts of Oligosaccharides from Glycoproteins by ^{1}H NMR Spectroscopy**

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NMR spectroscopy is a very valuable tool in carbohydrate analysis. However, its use is limited if only small quantities of the sample are available. Literature data suggest that commercial probes are suitable for NMR spectroscopic analysis of samples in the range of a few nanomoles,[1,2] which, for example, is equivalent to many micrograms of a decasaccharide. NMR spectroscopic characterization of oligosaccharides utilizing significantly less material is highly desirable for the analysis of glycan chains of biological origin, for example, glycoproteins. This would complement the analysis of oligosaccharides by mass spectrometry (MS) which is inherently more sensitive but provides less information. Here, we demonstrate that modern equipment can be used to record spectra of minute amounts of sugars, for example, sucrose or a complex N-type decasaccharide, down to 15 picomole. Special sample preparation techniques and instrument setup are required to record spectra at such low quantities. Importantly, water suppression by a factor of 500 000 has been achieved by utilizing a modified water suppression by "excitation sculpting". This also makes it possible to observe signals only 50 Hz away from the solvent signal. Also, sample tube selection and preparation were optimized. Data were recorded with a 700 MHz NMR spectrometer equipped with a commercially available triple-resonance cryoprobe.

Elucidation of the carbohydrate components of glycoproteins is a very important step in understanding the biological function of oligosaccharides. Although more than 60 % of the human proteome is thought to be glycosylated, the role of many glycan structures is not yet clear. Oligosaccharide chains attached to proteins can contribute to, for example, cell recognition, protein folding, and signal transduction.[3,4] Furthermore, it is known that dysglycosylation can cause severe diseases like the congenital disorders of glycosylation.[5] Also, most cancer cells have an altered glycosylation pattern. This is currently the target for the development of vaccines and new diagnostic assays.[6]

Today mass spectrometry and NMR spectroscopy are the major techniques used to identify the structures of glycans. NMR spectroscopy is limited because its sensitivity is relatively low in comparison to that of mass spectrometry (MS).[7] However, in the study of oligosaccharides, NMR spectroscopy is superior to MS as it offers information that cannot be obtained from MS, such as the determination of 1) the configuration of sugar residues that have the same molecular weight, 2) the anomeric configuration (α or β), 3) the target position of glycosidic linkages, 4) the position of substituents linked to the OH groups, like phosphates or sulfates, and 5) the position of functional groups other than OH. Currently it is believed that NMR characterization of molecules with commercial probes requires a few nanomoles of material.[1,2] However, the development of cryoprobes improved sensitivity by a factor of 4, resulting in a reduction of acquisition time by a factor of 16.[8,9]

Here we show that a high-resolution 700 MHz NMR spectrometer equipped with a cryogenic probe can be used to record spectra of molecules down to a level of a few picomoles. This is of special importance as most compounds from biological sources are available in very limited quantities. Sucrose and a complex N-type decasaccharide were used as examples. The decasaccharide was characterized previously by Vliegenthart et al. using multidimensional NMR spectroscopy. It can be assigned easily from 1D NMR spectra using the structural reporter group concept[10–12] (Figure 1).

The lower limit of detection for a signal is defined as when its height is three times the root-mean-square of the noise.[1,13] Following information from Bruker,[18] in many cryoprobes the signal to noise ratio (S/N) measured in 3 mm tubes is about same as that in 5 mm tubes with the same concentration

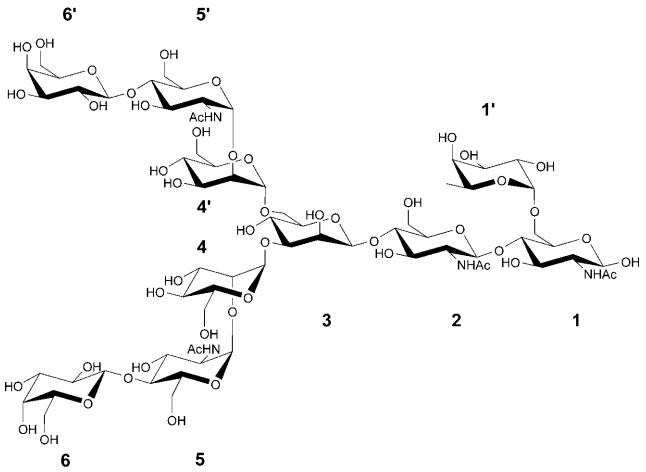

Figure 1. Structure of an N-type decasaccharide from a glycoprotein.

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of the sample. With our spectrometer the S/N recorded for a sample in a 3 mm tube is 90% of that with the same concentration in a 5 mm tube. This in turn results in 90% of the S/N for only 32% of the amount of the compound, which implies that the mass sensitivity of the spectrometer is increased by a factor of about 3.

We determined the intrinsic sensitivity for our cryoprobe using the anomeric proton of the test sample (2 mM sucrose in H_2O/D_2O 9:1) in a 5 mm tube to be S/N = 631 for 8 scans. Therefore, the minimum amount of sample that can be measured with 40000 scans corresponds to about 18 pmol of material within the coil's volume. A more convenient experiment would require about 60 pmol of compound and 2000 scans using a 700 MHz NMR spectrometer equipped with a cryoprobe. This quantitative assessment was the basis for the following experiments.

Samples of the compounds at low concentrations were prepared from 2 mM stock solutions in D_2O. These were diluted two or three times (see the Supporting Information). For easy handling 3 mm NMR tubes were then filled with a total volume (V_{tot}) of 200 μL. The sample volume within the RF coil (V_{obs}) corresponds to 80 μL (TXI cryoprobe, see the Supporting Information). Sample amounts in the following text always refer to the amount within the RF coil (V_{obs}).

In our initial attempts to prepare samples containing 60 pmol we encountered three major problems: 1) We observed impurities with intensities significantly higher than those of the saccharides; 2) sample signals close to the strong solvent signal are drastically reduced in their intensity or invisible because of the water suppression; and 3) huge oscillation of the baseline occurred in the vicinity of the water signal as a result of solvent suppression (cf. Figure 3 and Figure S1 in the Supporting Information). These issues are addressed in the following.

First, in experiments with saccharides in the picomole range, impurities of just a few nanograms generate signals stronger than those of the target samples. We observed several broad signals up to 30 times more intense than those of the sample which overlapped with the signals of the saccharide and complicated its characterization (cf. Figure S2 in the Supporting Information).

To obtain pure spectra of the compounds we used glass materials instead of plastics whenever possible. Pipettes with disposable tips, the only plastic material in contact with the sample, did not give rise to contaminations. However, impurities in the range of nanomoles originated from the glass walls of new NMR tubes. These are probably remainders of the production process. Therefore, we purified the glass vials and NMR tubes in a plasma cleaner immediately before usage. This procedure led to spectra with no or just very minor impurities (see the Experimental Section and the Supporting Information).

Second, suppression of the water signal is a common problem in recording NMR spectra of biomolecules. In the present study, the excitation sculpting sequence devised by the group of Shaka proved to be the most robust technique.[14] However, when we used the published parameters, the oligosaccharide signals close to the H_2O/HDO resonance were strongly suppressed.

When a longer selective pulse is applied, satisfactory reduction of the intensity of the solvent signal is still achieved and oligosaccharide signals close to the H_2O signal can be observed. Figure 2 shows the spectra of a 1 mM decasaccharide sample in H_2O/D_2O (9:1) acquired with selective pulse lengths of 2 ms and 8 ms. At a distance of 50–200 Hz from the

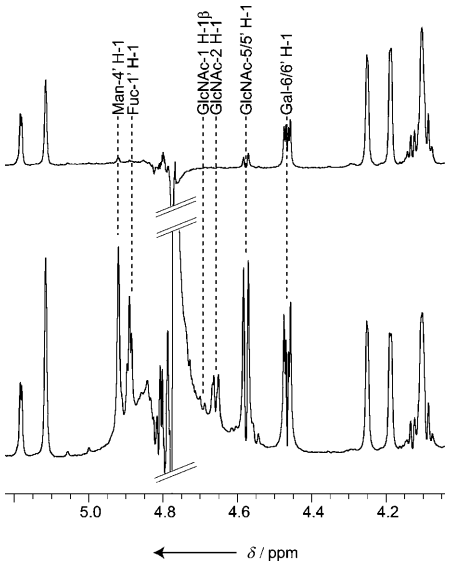

Figure 2. Optimization of solvent suppression for a sample of 1 mM decasaccharide (equivalent to 80 nmol/$V_{obs.}$) in H_2O/D_2O (9:1) with an excitation sculpting pulse sequence. Top: NMR spectrum recorded using the standard conditions described by Hwang et al.[14] with a selective pulse of 2 ms. Signals in the vicinity of the HDO signal have extremely reduced intensities or cannot be identified at all. Bottom: A selective pulse of 8 ms results in an excitation bandwidth of approximately 30 Hz. This is still sufficient to suppress the solvent signal and allows much better observation of signals close to the HDO peak. The H-1 signal of GlcNAc-2, for example, has still one-third of its normal intensity. Even the H-1 signal of the β anomer of GlcNAc-1 can be observed in this spectrum with 7% of its normal intensity at a distance of only 50 Hz from the HDO signal.

H_2O signal, a pulse length of 8 ms results in signals of increased intensity. With the published length of the soft pulse of 2 ms, the anomeric protons of Fuc-1′, GlcNAc-1, and GlcNAc-2 are absent from the spectrum (Figure 2, top) but are clearly visible in a spectrum with 8 ms soft pulses (Figure 2, bottom). At the same time we observe the full intensity of the anomeric signals of Gal-6/6′ and GlcNAc-5/5′ at distances of 210 Hz and 130 Hz, respectively, from the H_2O signal. This is equivalent to an increase of the signals by a factor of 2.5 and 10, respectively. At a distance of 80 Hz we still see signals with an intensity of about 35% (GlcNAc-2, H-1) and at 50 Hz we observe 7% of the original signal intensity (GlcNAc-1, H-1β). Thus, even in H_2O/D_2O (9:1) we can observe signals at a distance of only $\delta = 0.07$ ppm from the H_2O signal.

Third, if many scans must be accumulated, water suppression by excitation sculpting[14] and also WATERGATE[15] result in a strongly distorted baseline (see Figure 3, middle, and Figure S1 in the Supporting Information). We could eliminate this artifact also by using a longer selective soft

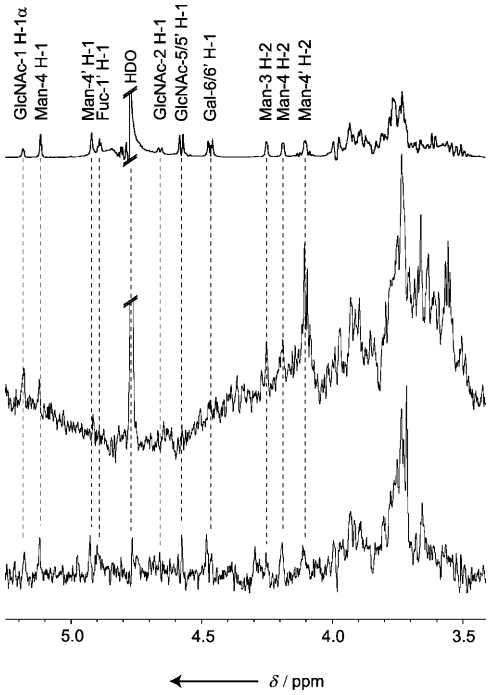

Figure 3. NMR spectra of the N-type decasaccharide. Top: Reference spectrum recorded with 80 nmol of the compound; Middle: Spectrum recorded with a 60 pmol sample, measured before optimization of water suppression and sample preparation. Bottom: Spectrum recorded with a 60 pmol sample under optimized conditions. The two spectra at the bottom were recorded in D_2O with about 1% H_2O with 2048 scans in 2 h each. The middle spectrum has a distorted baseline and some impurities in the region of $\delta = 3.7$–3.5 ppm. The bottom trace presents the nondistorted baseline as a result of optimized solvent suppression. As a consequence, the signals of the anomeric protons of Fuc, GlcNAc-5/5′, and Gal-6/6′ are visible.

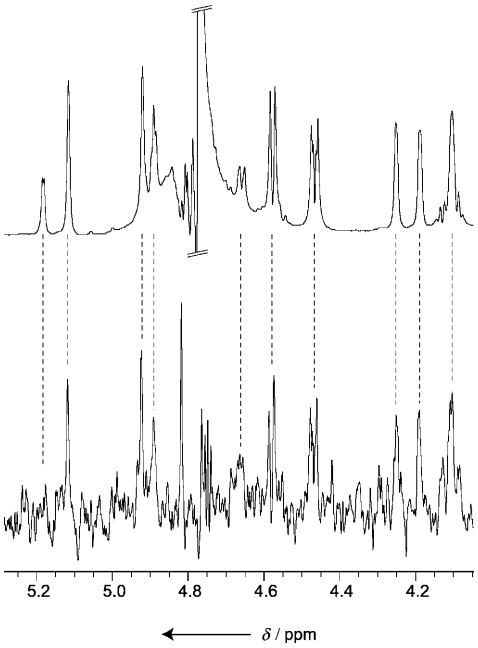

Figure 4. NMR spectrum of 25 pmol decasaccharide (bottom, 32 768 scans in 27 h) and the reference spectrum of 80 nmol decasaccharide (top). The anomeric protons of Fuc, Man-4/4′, GlcNAc-5/5′, and Gal-6/6′ and the H-2 signals of the three core-mannosidic residues can be identified clearly in the expansion of the decasaccharide spectrum recorded from 25 pmol of oligosaccharide.

pulse in the excitation sculpting sequence (see the Experimental Section).

Spectra recorded in D_2O contained roughly 40 μmol residual H_2O (about 1%). Under optimized conditions for the solvent suppression, the residual HDO signal has the same intensity as the oligosaccharide's peaks. Thus, a suppression of the solvent peak by a factor of at least 500 000 was achieved.

As a test case, 42 pmol sucrose in V_{obs} was measured under optimized conditions with 2048 scans and a total acquisition time of 2 h (see the Supporting Information) yielding a S/N of about 3.0. These experiments could be verified by measuring the N-type decasaccharide. The bottom spectrum of Figure 3 was recorded with 60 pmol of decasaccharide and 2048 scans. Because of the optimized solvent suppression nearly all structural reporter groups can be identified with S/N values of 4.2–5.0 (see Table S1 in the Supporting Information).

Even less concentrated samples can be measured, if the number of scans is extended. The spectrum of 25 pmol of the decasaccharide, measured with 32 768 scans in 27 h is shown in Figure 4. At these extremely low concentrations the structural reporter groups can be observed within the spectrum and therefore a definite assignment of the decasaccharide is possible (see Table S1 in the Supporting Information). A spectrum still sufficient for interpretation can even be recorded using only 15 pmol of oligosaccharide (see Figure S5 and Table S1 in the Supporting Information).

As we have shown in earlier studies, neural networks are capable of recognizing oligosaccharides at S/N far below the mentioned limit of detection for interpretation by eye. Approximately 90% of N-type oligosaccharide structures were recognized at S/N of 1.25.[16,17] Thus, spectra with lower S/N than those shown in Figures 3 and 4 can still be recognized by using artificial neural networks.

These techniques further expand the tremendous potential of NMR spectroscopy as an analytical tool, as they not only provide differentiated information but can also be used to analyze carbohydrates in nanogram and glycoproteins in microgram quantities, respectively. In this way it is much easier to obtain structural information for compounds available in only minute quantities.

Experimental Section

All glass material was purified with a plasma cleaner (SPI Plasma-Prep II, SPI Supplies/Structure Probe, Inc., West Chester, USA). The material was exposed to an oxygen plasma for 25 min ($p = 1.0$ mbar, $I = 60$ mA). All samples were handled in a laminar flow bench.

NMR experiments were performed at 300 K on a Bruker Avance 700 MHz NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with an inverse 5 mm triple-resonance cryoprobe. All final spectra were recorded with the excitation sculpting pulse sequence to suppress the HDO or H_2O signal (acquisition time 2.3 s, relaxation delay between 0.5 and 1 s). The selective square pulse length was set to 8 ms. Chemical shifts were referenced to acetone

($\delta_H = 2.225$ ppm). A more detailed procedure can be found in the Supporting Information.

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