Glycoprotein Analysis: Using Nuclear Magnetic Resonance

Bas R. Leeflang and J.F.G. Vliegenthart

in
Encyclopedia of Analytical Chemistry
R.A. Meyers (Ed.)
pp. 821–834

Glycoprotein Analysis: Using Nuclear Magnetic Resonance

Bas R. Leeflang and J.F.G. Vliegenthart
Utrecht University, Utrecht, The Netherlands

The majority of proteins are decorated with one or more covalently linked carbohydrate chains, and are thus glycoproteins. These carbohydrate chains may consist of mono-, oligo- or polysaccharides, which can affect the physiological behavior and biological functioning of the glycoprotein. Insight into the structural aspects and characteristics of glycoprotein glycosylation is an important step in unraveling the function of carbohydrate chains in a glycoprotein. In most cases the glycan and protein moieties are cleaved and analyzed separately. Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool in this respect. A plethora of NMR techniques are available to obtain NMR parameters such as chemical shift, scalar coupling constants and relaxation and cross-relaxation properties. From these, both primary structural, three-dimensional structural and dynamical features can be determined for these molecules. In this article, several aspects of glycoprotein structural studies will be discussed, and a selection of NMR experiments, including experimental conditions, will be presented.

1 INTRODUCTION

Proteins are among the most important biomolecules. More than 50% of all proteins are decorated with one or more covalently linked carbohydrate chains, and are thus glycoproteins. These carbohydrate chains may consist of mono-, oligo- or polysaccharides, which can affect the physiological behavior and biological functioning of the glycoprotein. Insight into the structural aspects and characteristics of glycoproteins glycosylation is an important step in unraveling the function of carbohydrate chains in a glycoprotein.[1-3]

Until recently, the NMR analysis of intact glycoproteins was too elaborate and/or too difficult to do. Only in recent years have some glycoprotein NMR studies been presented. In most cases, the structural analysis is subdivided into smaller problems. Initially, the glycan and the protein moieties are cleaved, and analyzed separately. In some cases this is followed by a study of the intact glycoprotein. The cleavage can be achieved enzymatically or chemically. The peptide sequence has to be analyzed also, in cases where it is not yet known. The monosaccharide composition and branching information can be established through chemical means. NMR spectroscopy has proven to be a suitable tool to determine the primary sequence of the glycan in combination with the monosaccharide data. In many studies the analysis was considered complete when the primary glycan structure had been determined. The combination of modern equipment and the desire to gain a fundamental understanding of the structure–function

relation resulted in more advanced studies. These studies include conformational analysis and spatial structure determination of both the glycan and the protein, as well as of the intact glycoprotein. Insight into the molecular mobility and flexibility is important in order to understand complex functions and interactions, such as molecular recognition processes. Again, NMR spectroscopy is a suitable tool to obtain this information. In this article, several aspects of glycoprotein structural studies will be discussed.

2 SAMPLE PREPARATION

In order to obtain the most information from NMR spectra, the preparation of the samples is of vital importance. Obviously much attention has to be given to the choice of solvent, to solvent conditions and to sample purity. In particular, contamination by protonated molecules should be avoided as much as possible. Depending on the type of NMR equipment, various sample holders are available, ranging from conventional NMR tubes to dedicated low-concentration, low-volume sample holders.

2.1 Solvent

For ^{1}H-NMR experiments on carbohydrates, the samples are normally dissolved in deuterium oxide (D_2O or ^2H_2O), a solvent that closely resembles the natural, aqueous solution, however, without the characteristic huge amount of protons. In this way, the skeleton carbohydrate protons can readily be observed. In D_2O solution all exchangeable protons (−OH and −NH) are nonobservable. Often the exchange of these protons with deuterium atoms is done in advance in one or more D_2O-dissolution/lyophilization cycles. However, in some cases one is particularly interested in these labile protons, for instance when glycopeptides or glycoproteins are studied. The amide signals are needed to obtain a complete sequential assignment of the peptide moiety. Furthermore, the observation of carbohydrate hydroxyl signals or amine and amide resonances has definite applications in the field of conformational analysis. In these cases the sample is generally dissolved in an H_2O/D_2O mixture with a D_2O content between 5% and 10%, needed for the deuterium lock system. It should be noted here that for the latter type of experiments the pH plays a crucial role. The chemical exchange of these labile protons is readily catalyzed at a pH above 7. In many cases the pH is adjusted between pH 5.5 and pH 6.5 to ensure a slow exchange. The exchange rate of hydroxyl protons is larger than that of NH-protons. The presence of metal ions (or salts in general) will have a catalytic effect on the labile proton exchange. The occurrence of paramagnetic impurities is harmful to most NMR experiments. Metal ions are conveniently removed from the sample by passing it through a small column of Calix (Bio-Rad™).

2.2 Purification and Desalting

Next to the choice of solvent, a second condition for obtaining good NMR spectra is careful sample preparation. Next to a functional fractionation of the samples using several chromatographic techniques, all proton-containing contaminating substances, such as buffer compounds, must be removed from the samples. Anionic carbohydrates and large oligosaccharides are easily desalted on small (1 cm × 20 cm) gel filtration columns (e.g. Bio-Gel™ P-2, Bio-Rad™) eluted with water. The sample is recovered in the void volume and is ready for D_2O exchange after lyophilization. If the sample contains high amounts of acetate, as can be the case after high-pH anion exchange chromatography, the use of 5 mM NH_4HCO_3 as eluent instead of pure water facilitates the removal of the acetate. Small neutral oligosaccharides can be desalted on a mixed-bed of cation (e.g. AG 50 W, Bio-Rad™) and anion (e.g. AG 1, Bio-Rad™) exchange resins. A recent advance in sample desalting techniques is the introduction of the Hi-trap column (Pharmacia).

2.3 Sample Amounts and Sensitivity

The amount of information that can be obtained by NMR spectroscopy is immense. The technique, however, is rather insensitive compared with other spectroscopic or spectrometric techniques. The first solution is to put a large amount of sample in the NMR instrument. This can be achieved by using high sample concentrations and/or using NMR tubes with a large diameter. Therefore, sensitivity is not a problem in cases where ample amounts of material are available and are soluble. However, in the analysis of released glycoprotein glycans, the total available amounts are often small (nanomolar and micromolar ranges). Using the standard NMR hardware, the best choice is to use special NMR tubes with a thick glass bottom and a glass insert (plunger) on top of the sample. These kind of NMR tubes are often referred to as "Shigemi-tubes", named after the main manufacturer. The glass has a magnetic susceptibility close to that of water. Consequently, the magnetic field lines are not refracted at the sample–glass interface. The result is that the effective sample volume can be reduced from 500 to 250–300 μL. Therefore, the NMR spectra can be recorded at increased sample concentrations. To reduce the sensitivity problem further, the manufacturers of NMR instruments offer "micro-probes" or "nano-probes". In comparison with conventional NMR measurements, the

sample volumes are drastically reduced from typically 500 to 150–50 μL, respectively. In this kind of NMR probe the sensitivity gain is achieved by placing the receiver coils much closer to the (smaller) sample. Especially with the nanoprobe technology, NMR spectra of released glycoprotein glycans can be recorded with sample amounts as small as 10 nmol.

3 PRIMARY STRUCTURE

To characterize the primary structure of a glycoprotein, several structural parameters have to be determined: amino acid sequence, type of linkage of the carbohydrate chain to the polypeptide backbone, position of the glycosylation site, and the primary structure of the carbohydrate chain. The amino acid sequence can be obtained in several ways. Thus far, several different glycan–protein linkage types have been identified. *N*-glycosyl linkages between *N*-acetylglucosamine and asparagine residues, *O*-glycosylation of serine and threonine by *N*-acetylgalactosamine, *N*-acetylglucosamine, or fucose residues all occur widely. In the linkage region of proteoglycans,[4] *O*-glycosylation of serine by xylose is found. Less well known are *C*-glycosidic linkages that were discovered in recent years.[5,6] In this article the focus will be mainly on the analysis of *N*- and *O*-glycosidic linkages.

3.1 Glycan Characterization

Glycoproteins are rarely single compounds. Even when they are homogeneous in the amino acid sequence, glycoproteins may consist of a family of compounds. This is due to (micro)heterogeneity that may occur in each glycan at each glycosylation site. This feature forms a serious complication in the structure determination of the glycans. Therefore, characterization of a glycoprotein sample often starts with the cleavage of the carbohydrate chains from the intact glycoprotein along chemical or enzymatic routes. *N*-linked glycans can be split off through hydrazinolysis. Although this technique has often been applied, in general the use of enzymes has to be preferred, because fewer artifacts are introduced. Several different types of enzymes can be taken into consideration for this purpose: first the so-called endo-enzymes that cleave within the *N*,*N*-diacetylchitobiose unit. The enzymes belonging to this category have a rather strict specificity, dependent on the glycan structure. The peptide-*N*-(*N*-acetyl-β-glucosaminyl)asparagine amidase F cleaves the linkage between the first *N*-acetylglucosaminyl residue and the amide group of asparagine. Since it is an amidase, it has a very broad specificity. However, PNGase F is almost inactive when fucose is (α 1–3)linked to the first *N*-acetylglucosamine residue. In that case, PNGase A has to be preferred. Following the enzyme digestion, the cleaved oligosaccharides are purified using several chromatographic steps.

For the *O*-linked carbohydrate chains, there are no enzymes available that are widely applicable for cleavage of the whole chain. The most generally applicable cleavage reaction is based on the alkaline borohydride treatment. The resulting oligosaccharide alditols are fractionated through chromatography. Instead of splitting the carbohydrate chains from the glycoprotein, glycopeptides can be prepared through digestion of the glycoprotein with proteolytic enzymes. The structure determination of glycan fragments comprises invariably multi-step processes. A first indication of the character of the glycans with regard to molecular size and charge is obtained from the fractionation by different high-performance liquid chromatography (HPLC) techniques. The qualitative and quantitative monosaccharide composition is established after methanolysis followed by gas chromatography (GC). The absolute configuration of the monosaccharides is determined after solvolysis of the methyl glycosides with 2-butanol by GC.[7,8] The molecular masses of oligosaccharides are derived by mass spectrometric techniques such as fast atom bombardment mass spectrometry or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

NMR spectroscopy is by far the most informative method for the determination of the glycan structures. For the primary structure, it is the only method that provides all details, comprising types of constituting monosaccharides including ring size and anomeric configuration, position of glycosidic linkages as well as type and position of non-carbohydrate substituents. Since NMR spectroscopy is a nondestructive technique, it is the most obvious first approach in the analysis of unknown glycans. Prior to NMR spectroscopic analysis, the dry samples are dissolved in D_2O and lyophilized in order to exchange the hydroxyl and amide protons for deuterium and dissolved finally in 500 μL D_2O with a high isotopic purity.

3.2 Structural Reporter Groups

The one-dimensional ^{1}H-NMR spectra of glycoprotein-derived glycans in ^2H_2O solutions show that two groups of signals can be distinguished: the so-called bulk signal and the structural reporter group signals. The former contain mainly the nonanomeric protons, present in a rather narrow spectral area, between 3.2 and 3.9 ppm. Owing to overlap this group of resonances can hardly be interpreted in terms of individual protons. The structural reporter group signals are found outside the bulk region. It appears that it is not necessary to assign all ^{1}H-NMR signals for a full characterization in terms of a carbohydrate structure.

Table 1 Structural reporter groups for glycoprotein derived glycans

- Anomeric protons
- Alkyl and acyl substituents like methyl, acetyl, glycolyl
- Sialic acid H3 atoms
- GalNAc-ol H2, H3, H4 and H5, Man-H2 and Gal-H4 atoms
- Protons shifted out of the bulk-region due to glycosylation shifts or under the influence of substituents, such as sulfate, phosphate, and acyl groups
- 6-Deoxy sugar H6 atoms

The exact location of a proton resonance in the spectrum is determined by the average magnetic field experienced by this proton. This local magnetic field depends on the primary structure (i.e. electron distribution), the spatial structure, and the mobility of the structure. The relation between the chemical shift and the structural parameters is very complex, and not easily accessible for theoretical approaches. Prominent structural reporter groups are the signals as summarized in Table 1.

The various chemical-shift patterns comprising chemical shifts and couplings are translated into structural information based on a comparison with patterns in a library of relevant reference compounds.[9–11] The comparison of NMR data for many closely related glycans resulted in empirical rules to correlate chemical shift values with carbohydrate structures. Obviously, the application of this approach requires accurate calibration of the experimental conditions like sample temperature, solvent, and pH. It is remarkable that the reporter group signals are such excellent probes of local structural elements. They are rather insensitive for alterations in the structure distant from the corresponding locus. Although the concept has proved its usefulness in the structure elucidation of numerous compounds, it should be applied with some care in the investigation of completely new structures. For definitive conclusions on the identity of novel compounds, additional experimental data from independent techniques should be collected. The application of the ^{1}H-NMR structural reporter group approach to determine the structure of *N*-glycans derived from glycoproteins was very successful.[9] A groundbreaking study was the elucidation of the structure of the glycans of α-acid glycoprotein.[12] For *O*-linked glycans of glycoproteins released as oligosaccharide alditols, we demonstrated that analogous structural reporter group signals can be identified.[9,11] Since the peripheral parts of the *N*- and *O*-linked glycans have several elements in common, the same type of structural reporter signals can be used in both cases.

The large amount of NMR data collected for quite an array of compounds enabled us to develop an NMR database for the computer-assisted identification of carbohydrates.[13] The database, designated SUGABASE, combines 1411 ^{1}H- and 590 ^{13}C-NMR chemical shifts of carbohydrate with 1756 structures and literature references as present in the complex carbohydrate structure database (CCSD).[14] The CCSD currently comprises almost 50 000 records. Starting from an input of chemical shift values, the search program produces a list of partially or completely matching structures. The SUGABASE program is available for UNIX and Microsoft Windows computers, and there is an interface to the database on the Internet at http://www.boc.chem.uu.nl/sugabase/databases.html.

The chemical shifts of these signals, together with their coupling constants, give valuable information on the structure of the glycans.[10] Also, the program is useful for the study of novel compounds, because structural elements can be traced that are occurring in other carbohydrates, already present in the database. The program can be applied for characterizing glycoconjugate-derived glycans and polysaccharides.

3.3 Protein Characterization

In many cases the amino acid sequence of the glycoprotein is known at the start of the characterization thereof. Therefore, and because it goes beyond the scope of an article on the analysis of glycoproteins by using NMR spectroscopy, this aspect of primary structure analysis will not be discussed here. The glycosylation sites, however, are not always known. Although characterization of the glycans is most conveniently done on free oligosaccharides, for the determination of glycosylation sites another approach must be followed. Instead of splitting the carbohydrate chains from the glycoprotein, glycopeptides can be prepared through digestion of the glycoprotein with proteolytic enzymes.

Depending on the specificity of the enzymes, the peptide parts can vary in length and the mixture of peptides and glycopeptides can be rather complex. Often for one glycosylation site more than one glycopeptide is found. Peptides are characterized by using N-terminus analysis in conjunction with comparison to the complete amino acid sequence. Structure analysis of the glycopeptides provides information on the identity of the glycans and on the position of the glycosylation sites in the polypeptide backbone.[15,16] The recent finding of a novel glycosylation type, C-glycosylation, complicates the analysis, because this linkage can thus far not be cleaved without destroying the entire glycoprotein.[5]

NMR spectroscopy is nowadays widely used in protein analysis. The incorporation of ^{13}C, ^{15}N and ^{2}H isotopes in proteins allows very powerful NMR experiments to be performed. Isotope labeling for glycoproteins, however,

is not frequently used as yet. The reason for this is that the common bacterial cell lines used to prepare isotope-labeled proteins do not have the glycosylation machinery that is essential to glycosylate the proteins. This means that the protein may be obtained but without most of all of its glycans, or with a different glycosylation pattern. Furthermore, the native protein folding frequently depends on the presence of glycans. Therefore one cannot be sure whether the labeled protein has adopted the same three-dimensional shape.

3.4 Glycopeptides

Glycopeptides can often be studied as intact molecules. The primary structure characterization of the glycan part is basically identical to that of free glycans. The structural reporter group method can be applied here as well. Although the chemical shifts of the monosaccharide attached to the peptide will be affected, the overall chemical shift profile will be very close to that of the free glycans. Two-dimensional NMR methods, as described in section 4 on spatial structure, can also be applied to determine the final details of the primary structure. Especially in the case of glycopeptides, this approach is very effective, because the structural reporter group approach is only applicable to the glycan moiety and the peptide moiety. Generally, the primary structures of the peptides are known from chemical amino-acid determinations. This means that NMR spectroscopy is merely used here for confirmation, and in the preparation of three-dimensional structure determinations. In most cases, however, glycopeptides are too small to have a defined spatial structure.

4 SPATIAL STRUCTURE

The properties of nuclear spins depend heavily on molecular structure and dynamics. As a consequence, NMR spectra are intrinsically rich in information on these topics. The NMR spectroscopist needs to assign the NMR resonances to individual spins in the molecule. This is a prerequisite for further analysis and extraction of valuable structural information about the molecule under study. The NMR observables used in structure analysis are primarily the nuclear Overhauser effects (NOEs) and three-bond J-coupling constants. Each NOE cross-peak corresponds with a short distance between two protons. Each three-bond coupling provides information on a dihedral angle. When the assignment of many peaks in the NMR spectra has been completed, lists of NOE cross-peaks and/or three-bond coupling constants can be obtained, providing information on interatomic distances and dihedral angles, respectively. This information can be converted into a three-dimensional model, using methods that frequently include force-field calculations.

4.1 Spectrum Assignment

Signal assignment of complex oligosaccharides can partly be done by comparing occurring chemical shifts with data stored in a library of known compounds. This technique uses the structural reporter group method. The comparison can be performed manually or with the aid of computer database programs. Though this method is valuable for characterization purposes, it does not provide sufficient signals assigned for more advanced analysis of the NMR spectra. Furthermore, the application of the structural reporter group method may fail, when a molecule with novel structural properties or with a large complex structure is studied.

Typically, homonuclear correlation type spectra, such as various correlation spectroscopy (COSY) or total correlation spectroscopy (TOCSY) experiments, are needed to assign signals in the bulk area. This kind of correlation spectrum yields spin systems that correspond to monosaccharides or amino acids (or parts thereof). The glycan assignment procedure generally starts from the anomeric proton signal, or any other well-resolved NMR signal. The two-dimensional spectra can be traversed from diagonal peak to diagonal peak via cross-peaks. In this way the resonances in each spin system can be identified.

Homonuclear correlation experiments identify spin systems. They are essential in this respect, but they do not provide sequence information. Mostly nuclear Overhauser effect spectroscopy (NOESY) or rotating frame nuclear Overhauser spectroscopy (ROESY) spectra are used for this purpose. Often the most intense NOESY peak identifies the linkage location. There are, however, several examples known where the most prominent NOESY peak represents a short distance to another proton. Care should be taken here, because NOESY interactions are through-space interactions and the shortest inter-proton distance may be a long-range interaction with respect to the primary structure. An unambiguous method to resolve this problem is the heteronuclear multiple bond coherence spectroscopy (HMBC) experiment, which yields through-bond correlations. This yields cross-peaks between the anomeric carbon and the ring proton of the adjacent sugar residue, and between the anomeric proton and the ring carbon of the adjacent sugar residue. In order to make use of HMBC spectra, the carbon resonances should be assigned as well as the proton resonances. Heteronuclear single quantum coherence spectroscopy (HSQC) or heteronuclear multiple quantum coherence spectroscopy (HMQC) spectra provide one-bond $^{1}H-^{13}C$ correlations. In other words they identify chemical bonds between ^{1}H and ^{13}C, and thus

provide the link between the assigned proton resonances and the carbon resonances. The HMBC experiment is basically a HMQC experiment tuned at small (1–10 Hz) heteronuclear couplings. Often the one-bond correlations are explicitly filtered out of the spectrum. However, in our laboratory this filter is hardly used, because omitting the filter gives many one-bond correlations that are useful in the assignment process.

4.2 From Nuclear Overhauser Effect Towards Spatial Structure

NOE cross-peak intensities depend strongly, among other parameters, upon inter-proton distances. Often the NOE cross-peak intensities are assumed to be proportional to r^{-6}, where r is the inter-proton distance of the protons to which the cross-peaks were assigned. Although this is generally incorrect, a slight modification of this approach provides a decent set of intramolecular distances which are only marginally affected by the spin-diffusion process or multiple spin relaxation effects. This method is called the "initial rate approach". For the initial rate approach, several NOESY experiments need to be recorded at various mixing times. The cross-peaks need to be integrated. By fitting a polynomial function to these integrals as a function of the mixing time, τ_m, the slope (initial rate) at $\tau_m = 0$ can be estimated.

The experimental NOE cross-peaks can be either negative or positive. This depends on the magnetic field (i.e. the Larmor frequency) and the molecular tumbling, expressed in the rotational-correlation time τ_c. For molecules having a τ_c near the critical value of $\sqrt{5/(2w)}$ no NOEs can be measured, and NOESY is not applicable. Small oligosaccharides fall in this category. NOE-like information, however, can also be obtained from ROESY data. Quantification of inter-proton distances from rotating-frame nuclear Overhauser effect (ROE) intensities is more complicated than from NOESY data.

For small molecules, knowledge of a few distances may be enough to build an initial molecular model, although in many cases, one could find contradicting short distances. In these cases, the molecule in solution may occur in two or more significantly different conformations (Figure 1). For larger molecules, such as (glyco)proteins, several hundreds of relevant short distances may be obtained, making it virtually impossible to build a molecular model from scratch manually. Just as with smaller molecules, conflicting short distances may occur due to different conformations, making the problem even more difficult. Several computational approaches have been developed to handle this problem, such as distance geometry and simulated annealing methods, or combinations of both. These methods yield many first approximation molecular models that may represent genuinely different conformations. These models are optimized using a short molecular mechanics or molecular dynamics run.

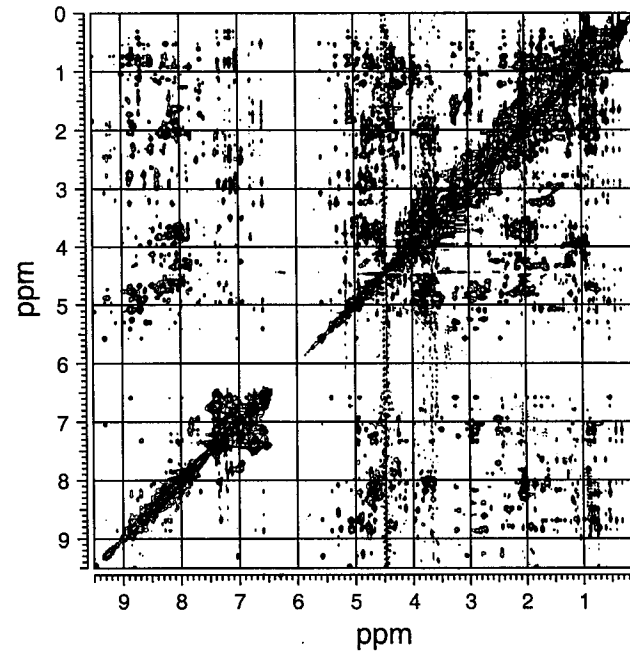

Figure 1 600 MHz NOESY spectrum of fully glycosylated alpha human chorionic gonadotrophin (αhCG) glycoprotein hormone in aqueous solution. The spectrum was recorded on a Bruker AMX-600 spectrometer at an elevated temperature of 333 K, with a NOE mixing time of 125 ms.

5 MOLECULAR MOBILITY

To gain insight into the function of oligosaccharides, glycoproteins, peptides or any other biomolecule, the determination of the spatial structure is a prerequisite. X-ray diffraction, NMR spectroscopy and computational methods are available to obtain molecular structures. Knowledge of the structure alone, however, is often not enough, since molecular flexibility is in most cases essential to their function in biochemical processes. NMR not only provides the tools to determine the spatial structure, but the internal molecular flexibility can also be studied. In NMR spectroscopy, molecular motions are reflected in the relaxation behavior of various NMR observables. The way in which NMR parameters, such as chemical shifts and coupling constants, and dipolar interactions, are affected depends on the relative timescale of the molecular motions. Atomic motion results in reduced NOE cross-peak intensities. The theory behind the NMR relaxation processes is well understood and can be used to determine both the molecular conformation and the molecular flexibility.

5.1 Conformation and Flexibility

Valuable NMR parameters for conformational analysis of biomolecules are the proton–proton NOEs or ROEs. They depend strongly on inter-proton distances and on the mobility of the inter-proton vectors. In the standard approach the mobility of all proton–proton vectors is considered to be constant. This is the so-called isotropic approximation. Now the cross-peak intensities are only a function of all inter-proton distances, and the "overall" tumbling rate (τ_c). This approximation is most useful to determine single conformational molecular models. In practice, however, various distinctly different conformations may occur. How these conformations affect the NMR spectra depends upon the timescale on which the conformations interconvert. In the very slow limit, the NMR spectrum will contain duplicate signals for the same nucleus. In this case, the spectrum is like a spectrum recorded for a mixture of molecules. A typical example of this kind of very slow interconversion is the α/β anomerization of reducing sugars in neutral, aqueous solution. At the other end of the timescale (faster than 1 ps) conformations change so rapidly that from a NMR point of view only the time-averaged structure can be observed. The rotation of a (hydroxy-)methyl group is a characteristic example of a very fast conformational change. It is the timescale region in between the extremes where most relevant conformational changes occur. NOEs and ROEs are especially sensitive for mobility in the microsecond to nanosecond timescale. In this regime the faster conformational changes happen, such as conformational changes in glycosidic linkages and glycan ring conversion. The NOESY spectra are now best analyzed, taking into account local mobility within the molecule that is faster than the overall tumbling rate. This is generally done with dedicated software, such as the CROSREL program.[17] Two application examples are given.[18,19] Conformational changes, however, occur at any timescale. Mostly, the effects can be observed by using T_1, T_2 and $T_{1\rho}$ relaxation times. For exchange rates between 1 μs and 1 ns, NMR spectroscopy does not have suitable parameters to judge the mobility. For the remaining exchange rates various NMR techniques are available.

As an example of the use of relaxation rates we would also like to mention the study on the mobilities of the glycan residues attached to Asn-78 of αhCG.[20–22] (Figure 2).

Figure 2 NMR structure of αhCG glycoprotein hormone obtained from NOESY data using the XPLOR program. Two well-defined protein regions are displayed on an atomic backbone level. The mobile protein region is represented by a ribbon. The glycan moieties are reduced to the first GlcNAc residues at Asn-52 and Asn-78, and are displayed on an all-atom level.

5.2 Interaction and Recognition

For the study of the conformation of oligosaccharides in oligosaccharide–protein complexes, transferred NOE (TRNOE) experiments[23,24] can be informative. Weakly bound ligands ($K_d \geq 10^{-6}$) in fast exchange (100–1000 Hz) between free and bound states can transfer NOEs obtained in the bound state to the free state. Since the line width of the ligand in the bound state is generally larger than that in the free state, and since the ligand must be present in excess, the signals from the free state ligand will dominate the spectrum. The NOE obtained in the bound state, however, is much stronger than that obtained in the free state. This causes the observed NOE peaks to be dominated by the conformation in the bound state. The TRNOE technique has been applied in a study of the binding of oligosaccharides to antibodies,[25,26] and to lectins.[26–28]

Laser photochemically induced dynamic nuclear polarization[29,30] is an advanced NMR technique that allows the detection in proteins and glycoproteins of surface exposed side chains of the aromatic amino acid residues tyrosine, tryptophan, and histidine. The

suitability of the method was demonstrated to study carbohydrate–protein interactions intramolecularly within glycoproteins[31] and intermolecularly for carbohydrate–lectin interactions.[32,33]

6 OVERVIEW OF NUCLEAR MAGNETIC RESONANCE EXPERIMENTS

NMR spectroscopy is renowned for its use of acronyms to identify the various available spectroscopic experiments that are mostly called pulse sequences. The large number of available pulse sequences and names obscures the fact that many multidimensional NMR experiments differ only in details. Pulse sequences can be categorized in different ways. Here we will classify them based on the magnetization transfer mechanism, which is linked to the information obtained from the resulting spectra. Two different pathways of magnetization transfer exist. All multidimensional NMR experiments use either one of these pathways, or combinations thereof.

Apart from the huge amount of experimental techniques available, NMR spectroscopy is often treated with caution because of the supposed need of quantum mechanics to understand the behavior of the nuclear spins. Fortunately, most experiments can be simplistically but effectively described in pictorial ways. For more comprehensive reviews of NMR theory, see Harris,[34] Ernst et al.,[35] Derome[36] and van der Ven,[37] and for a detailed practical overview see Braun et al.[38]

NMR experiments can be categorized based on the magnetization used or information transfer pathway. First, magnetization can be transferred through chemical bonds via the so-called scalar or J-coupling. This is the effect that causes the fine-splitting of NMR signals of individual nuclei. NMR spectra based on this route are often used for the assignment of NMR signals, because of the chemical relation between interacting nuclei. Second, magnetization can be transferred through space via cross-relaxation. Cross-relaxation is the effect when relaxation of the spin-state of a nucleus is stimulated by the close presence of one or more other nuclei. NMR spectra based on this route provide information on the spatial structure of the molecule.

6.1 Correlation Spectroscopy[38,39]

The COSY experiment was the first two-dimensional NMR experiment to be proposed and is still used today, although it has several drawbacks. Its strength, however, is that it provides information on proton pairs that are visibly coupled to each other in the one-dimensional NMR spectrum (fine splitting of the signal). With COSY there is no net magnetization transfer, which means that the cross-peaks have a zero integral. Half the multiplet signals are positive, whereas the other half is negative. As long as the J-coupling is larger than the line width this causes no problems. When the J-coupling becomes small compared to the line width, the cross-peak magnitude is reduced by the cancellation of the positive and negative multiplet

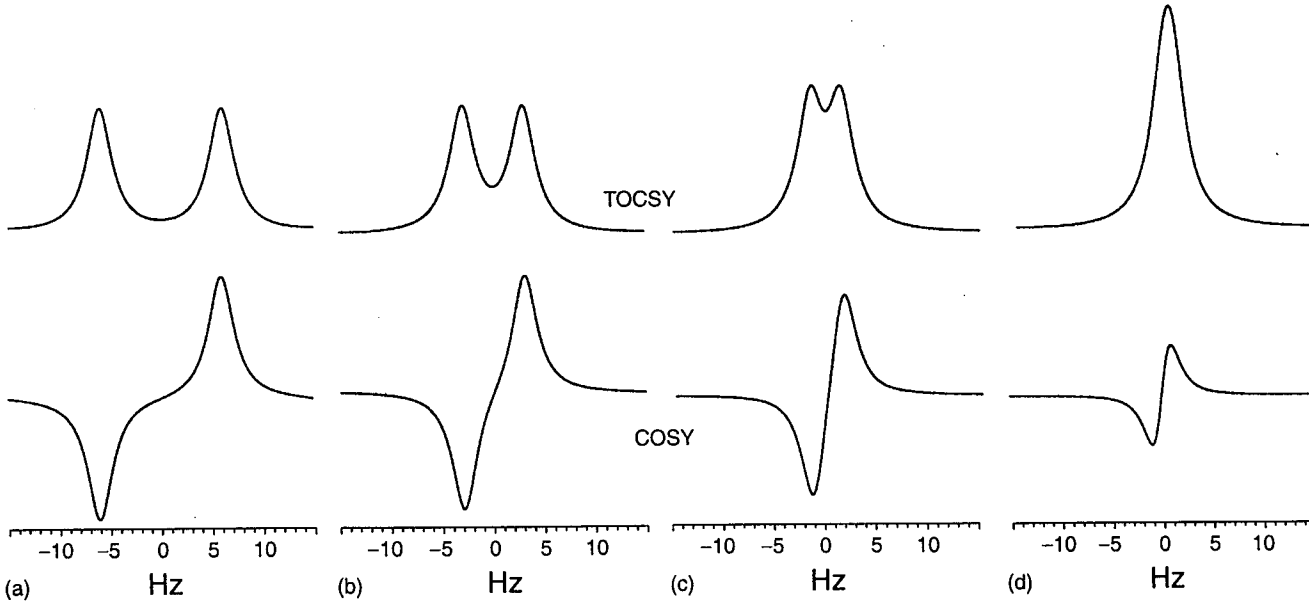

Figure 3 Demonstration of the signal canceling effects in simulated cross-peak cross-sections in COSY type spectra. Canceling occurs in cases where the J-coupling constant is smaller than the line width. A comparison is made between COSY and TOCSY peaks. In cases (a) through (d) the line width was set to 3 Hz, with varying J-coupling values: (a) $J = 12$ Hz; (b) $J = 6$ Hz; (c) $J = 3$ Hz; (d) $J = 1$ Hz.

signals (Figure 3). A second drawback, especially in the field of carbohydrate NMR, is the dispersive character of the diagonal peaks. Because of their long tails, the dispersive signals severely hamper the analysis of cross-peaks close to the diagonal. Preferred alternatives for COSY are double quantum filtered correlation spectroscopy (DQF-COSY) and TOCSY with a short mixing time.

6.1.1 Practical Information

Only hard 90° pulses are needed.

6.2 Double Quantum Filtered Correlation Spectroscopy[38,40]

The dispersive character of the diagonal peaks hampers the analysis of cross-peaks close to the diagonal using conventional COSY. Implementation of a double quantum filter, which allows only peaks involving two different spins, cancels the diagonal signals, including the solvent signal, to a very large extent. Now cross-peaks in the bulk area can also be analyzed. The problem of signal cancelling within the cross-peak multiplet still exists.

6.2.1 Practical Information

Only hard 90° pulses are needed.

6.3 Triple Quantum Filtered Correlation Spectroscopy[38,40]

In DQF-COSY a filtering technique is used that allows for cross-peaks between coupled protons that share at least a third proton to which they are mutually coupled. Triple quantum filtered correlation spectroscopy (TQF-COSY) is a COSY experiment with a triple quantum filter, which yields cross-peaks only when at least three spins are directly coupled. In practice this technique is often used to obtain COSY spectra containing H5/H6/H6′ cross-peaks in hexapyranosides, or H4/H5e/H5a cross-peaks for xylose, or similar triply coupled signals. This may be needed to overcome signal overlap in DQF-COSY spectra. TQF-COSY is not often used. It may, however, be useful in the case of Gal or GalNAc residues to assign the H5, H6 and H6′ atoms that cannot be found in TOCSY spectra.

6.3.1 Practical Information

Only hard 90° pulses are needed.

6.4 Total Correlation Spectroscopy[38,40,41]

In our laboratory, TOCSY, formerly also called HOHAHA (Homonuclear Hartmann Hahn spectroscopy) is the method of choice to assign as many protons as possible. TOCSY with a short mixing time, i.e. 8–16 ms, yielding spectra with approximately the same information content as DQF-COSY spectra would do, although with increased sensitivity. These experiments mostly have intense cross-peaks between the directly coupled protons. Often low-intensity cross-peaks are found, due to the relay of magnetization to the next coupling partner. This serves as an aid in the assignment. Not only the directly coupled proton is found on one track, but also those that are one or more coupling steps further. TOCSY with longer mixing times, typically up to 120 ms, may yield the complete subspectrum of one monosaccharide. The intensity of a TOCSY cross-peak depends on the magnitude of the J-coupling constants and on the mixing time. For Gal and GalNAc, where the coupling constants between H4 and H5 are small (<1.5 Hz), it is virtually impossible to obtain a full subspectrum. In these cases the magnetization transfer from the well-resolved H1 atom reaches the H4 but not the H5. H5, H6 and H6′ could be identified using TQF-COSY in combination with NOESY or ROESY experiments.

TOCSY does not suffer from the cross-peak cancellation when the line width is greater than the J-coupling (Figure 3). Occasionally the fact that TOCSY relays the magnetization over the whole spin system is a drawback. One is not always sure whether a cross-peak is a genuine 'COSY' type cross-peak or a RELAY-COSY peak. In these cases we still use a DQF-COSY experiment.

The occurrence of cross-peaks due to NOEs in the rotating frame (ROESY type cross-peaks) is a common artifact in TOCSY spectra. They can be distinguished from the intended TOCSY peaks by their invert sign. This effect is a smaller problem for free oligosaccharides than for larger molecules such as proteins or glycoproteins. The reason for this is that free oligosaccharide ROESY type cross-peaks build up slowly. For large molecules clean-TOCSY is often used, where the ROESY type relaxation is cancelled by NOESY type relaxation.

A second potential problem is the sample heating that may be caused by using intensive radiofrequency power, especially in cases when long mixing times are used. Heating can be detected by a changing lock signal level and will result in t_1 noise. In practice, a warming-up period of some 5 min suffices to overcome the effects of sample heating. At the end of this period the most critical shim(s) can be adjusted.

6.4.1 Practical Information

Mixing sequence: MLEV-17. Excitation using a hard 90° pulse and softer pulses (60, 90 and 180° pulses) to obtain a spin-lock field strength of 8–10 kHz. Effective 90° pulse: 25–30 μs. For TOCSY the effective 90° pulse corresponds to the actual 90° pulse. For clean TOCSY the length of the

clean delay should be included in the calculation. In our laboratory the clean delay is set to the same value as the length of the 90° pulse. Thus a 90° pulse of 16.66–20 μs is used. Mixing time: 8–150 ms for small molecules; 8–80 ms for (glyco-)proteins.

6.5 Heteronuclear Single Quantum Coherence Spectroscopy[38,41]

The HSQC experiment exploits the high sensitivity of protons compared to other nuclei such as ^{13}C and ^{15}N. This experiment yields a correlation spectrum for ^{1}H, ^{13}C and ^{1}H, ^{15}N, respectively, by a double insensitive nuclei enhanced by polarization transfer (INEPT) transfer step. For many years it was difficult to acquire heteronuclear correlation spectra for compounds having a natural abundance of ^{13}C isotopes. The emerging magnetic field gradient techniques have reduced these limitations dramatically. For HSQC the proton signal is detected. With a natural abundance of about 1.1% for ^{13}C, 98.9% of the protons are bound to a ^{12}C atom. This means that 98.9% of the detected signal must be eliminated. Without the use of magnetic field gradients, this could only be achieved by phase-cycling. Phase-cycling is a technique that cancels the unwanted signal by subtraction of free induction decays (FIDs) recorded with different phases of the radiofrequency pulses. Each FID, however, contains the unwanted signal for which the receiver has to be put at lower sensitivity. Furthermore, subtraction cancels the unwanted signal but not the associated noise. Using gradient techniques, the unwanted signal can be eliminated before the acquisition, resulting in a much higher sensitivity and preventing dynamical range problems. A further development in recent years is "sensitivity enhancement", mostly together with gradient coherence selection. To obtain narrow cross-peaks, normally the absorption mode signal is recorded and the dispersive signal is cancelled by phase cycling. Sensitivity-enhanced spectra allow both the absorption and dispersive mode signals to appear in the FID. In the processing stage these are separated, processed in such a way that an absorption mode spectrum is obtained with a sensitivity enhancement up to 1.41 ($\sqrt{2}$). The pulse sequence is longer, which allows for some relaxation and consequently loss of intensity. Typical sensitivity enhancements are about 1.3.

6.5.1 Practical Information

Hard 90 and 180° ^{1}H pulses and hard 90 and 180° ^{13}C pulses inversely detected. In order to obtain the shortest ^{13}C pulses the spectrometer is wired such that the output of the amplifier goes directly into the probehead. This wiring scheme prevents radiofrequency power loss in the pre-amplifier circuit, but at the same time prevents detection of the ^{13}C-NMR signals.

Depending on the available hardware, ^{13}C decoupling is possible during acquisition. A composite pulse decoupling scheme such as WALTZ or GARP is mostly used here.

To obtain a higher digital resolution in the indirect dimension, the carrier frequency and the sweep width in the ^{13}C domain may be adjusted, such that some signals are folded back into the spectrum in an empty region.

6.6 Heteronuclear Multiple Quantum Coherence Spectroscopy[38,41,42]

The information content of HMQC and HSQC spectra is the same. Both provide a correlation between ^{1}H and an X nucleus, mostly ^{13}C. The differences are hidden in the pulse-sequence. HSQC has more pulses and delays than HMQC and the period between the excitation pulse and the acquisition is longer in the case of HSQC. Therefore, HMQC is the preferred technique in cases where the accuracy of radiofrequency pulse lengths and radiofrequency pulse phases is not considered optimal. This may apply for the older hardware. Also, HMQC may be preferred in cases where T_2 relaxation is fast. A disadvantage of HMQC is that the cross-peak line widths are generally larger than those in the HSQC, because there is no proton decoupling in t_1.

6.6.1 Practical Information

See HSQC.

6.7 Heteronuclear Multiple Bond Coherence Spectroscopy[38,41,42]

The HMBC experiment is very important in unambiguous sequential assignment of carbohydrate residues, through the inter-glycosidic heteronuclear long-range coupling. Experimentally, the HMBC experiment is very similar to the HMQC experiment, where magnetization transfer is tuned to small heteronuclear couplings and the decoupling during acquisition is turned off. In many HMBC experiments the residual one-bond interactions are filtered out. In our laboratory this filter is purposely omitted. The presence of single bond correlations is useful for assignment purposes, because both HMBC and HMQC information are present in the same spectrum.

6.7.1 Practical Information

See HSQC.

6.8 Nuclear Overhauser Enhancement Spectroscopy[38,41,43]

NOESY spectra provide information related to inter-proton distances, and is therefore a valuable tool for structural analysis of carbohydrates, proteins and glycoproteins. The first application is determination of the primary structure of oligosaccharides and/or proteins. The linkage information cannot be found out unambiguously with NOESY and the observed NOE may be long range. This should be evaluated very carefully. HMBC provides this kind of information unambiguously, but at much lower sensitivity. The second application, by further analysis of NOESY spectra, is the determination of the (average) three-dimensional solution state structure of the molecules under study. NOEs can be used in a modeling study, either directly or by converting them into distance constraints. The sign and magnitude of the NOE do not only depend upon the inter-proton distances, but also on the overall (and local) mobility expressed in the rotational correlation time (τ_c). For large molecules (small τ_c) the NOESY cross-peaks have the same sign as the diagonal peaks, whereas for very small molecules the cross-peaks have the opposite sign. Di- and trisaccharides are generally in the area where the sign of the cross-peaks is changing, and can therefore not be analyzed with NOESY. In these cases ROESY experiments are the only alternative.

6.8.1 Practical Information

Only hard 90° proton pulses are needed. Solvent presaturation is normally done, using a low power radiofrequency pulse of 0.75 to 1.5 s. During the NOESY mixing time the solvent can be saturated as well. When the spectrometer is equipped with magnetic field gradients, a purging gradient may be given in the NOE mixing period, otherwise a homo-spoil pulse can be used.

6.9 Rotating Frame Nuclear Overhauser Enhancement Spectroscopy[38,44]

ROESY experiments yield spectra with cross-peaks of closely spaced protons. In this respect it is pretty much comparable to NOESY, although the experimental conditions differ. In contrast to NOESY, this experiment does not have a sign change depending on the size or mobility of the molecule. The cross-peaks are always negative relative to a positive diagonal. Positive cross-peaks do occur in ROESY spectra. Three possible causes for these phenomena exist. First, with chemical exchange, positive cross-peaks are obtained. Second, spin diffusion can be the cause. The first ROESY transfer inverts the sign of the cross-peak. Propagation via a second ROESY transfer within the same mixing period inverts the sign again, yielding a positive spin diffusion peak. Recording both ROESY and NOESY spectra is a method to analyze spin diffusion. Third, with ROESY being a spin-locking technique like TOCSY, it may suffer from TOCSY transfer, causing positive TOCSY peaks. TOCSY transfer may also give spurious peaks due to TOCSY transfer of cross-peak information to a third proton. Minimization of TOCSY transfer in the experimental phase is important for which several techniques are available, such as transverse ROESY (T-ROESY)[45] and off-resonance ROESY.[46] Equally important is coping with TOCSY transfer in the analysis phase. The CROSREL program[17-19] corrects for decreased ROESY cross-peak intensities caused by TOCSY transfer between the protons involved. Relay effects are not considered in this program.

6.9.1 Practical Information

Normally a hard 90° proton pulse is used for excitation. Solvent presaturation is normally done using a low-power radiofrequency pulse of 0.75 to 1.5 s. Spin-locking for conventional (not T-ROESY[45] or off-resonance ROESY[46]) is achieved at a field strength of 2–3 kHz (90° pulse corresponding to 125–90 μs). T-ROESY and off-resonance ROESY can be performed at higher field strengths, comparable to TOCSY experiments (10 kHz). For conventional ROESY the carrier frequency during spin-locking needs to be carefully chosen. This is important to reduce TOCSY artifacts on the one hand, and to obtain meaningful ROESY intensities on the other hand. The tilt angle θ of the most distant signal in the spectrum should be larger than 60°. Equation (1) shows the relation between θ, the spin-lock frequency ν_0 and the frequency of the most distant signal, ν_1:

$$\tan(\theta) = \frac{|\nu_1 - \nu_0|}{\gamma B_1} = \frac{|\nu_1 - \nu_0|}{4P_{90}} \qquad (1)$$

where P_{90} is the length of the 90° pulse at the spin-locking power in microseconds and B_1 is the magnetic field strength of the radiofrequency pulse.

ACKNOWLEDGMENTS

The authors acknowledge Prof. Dr J.P. Kamerling, Dr K. Hård, Dr P. Erbel and Dr J.A. van Kuik for their valuable contributions.

ABBREVIATIONS AND ACRONYMS

CCSD	Complex Carbohydrate Structure Database

COSY	Correlation Spectroscopy
DQF-COSY	Double Quantum Filtered Correlation Spectroscopy
FID	Free Induction Decay
GC	Gas Chromatography
HMBC	Heteronuclear Multiple Bond Coherence Spectroscopy
HMQC	Heteronuclear Multiple Quantum Coherence Spectroscopy
HPLC	High-performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence Spectroscopy
INEPT	Insensitive Nuclei Enhanced by Polarization Transfer
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
ROE	Rotating-frame Nuclear Overhauser Effect
ROESY	Rotating Frame Nuclear Overhauser Spectroscopy
TOCSY	Total Correlation Spectroscopy
TQF-COSY	Triple Quantum Filtered Correlation Spectroscopy
TRNOE	Transferred NOE
T-ROESY	Transverse ROESY
αhCG	Alpha Human Chorionic Gonadotrophin

RELATED ARTICLES

Biomolecules Analysis **(Volume 1)**
Biomolecules Analysis: Introduction • Circular Dichroism in Analysis of Biomolecules • High-performance Liquid Chromatography of Biological Macromolecules • Infrared Spectroscopy of Biological Applications • Nuclear Magnetic Resonance of Biomolecules

Pharmaceuticals and Drugs **(Volume 8)**
Nuclear Magnetic Resonance Spectroscopy in Pharmaceutical Analysis

Nuclear Magnetic Resonance and Electron Spin Resonance Spectroscopy **(Volume 13)**
Nuclear Magnetic Resonance and Electron Spin Resonance Spectroscopy: Introduction • Carbon-13 Nuclear Magnetic Resonance Spectroscopy • Chemical Shifts in Nuclear Magnetic Resonance • High-performance Liquid Chromatography Nuclear Magnetic Resonance

Nuclear Magnetic Resonance and Electron Spin Resonance Spectroscopy cont'd **(Volume 14)**
Nuclear Magnetic Resonance Instrumentation • Parameters, Calculation of Nuclear Magnetic Resonance • Relaxation in Nuclear Magnetic Resonance, General • Scalar Couplings in Nuclear Magnetic Resonance, General • Two-dimensional Nuclear Magnetic Resonance of Small Molecules • Two-, Three- and Four-dimensional Nuclear Magnetic Resonance of Biomolecules

REFERENCES

1. J. Montreuil, J.F.G. Vliegenthart, H. Schachter, 'New Comprehensive Glycoproteins I', in *Biochemistry*, Elsevier, Amsterdam, Vol. 29a, 1995.
2. J. Montreuil, J.F.G. Vliegenthart, H. Schachter, 'Glycoproteins and Disease', in *New Comprehensive Biochemistry*, Elsevier, Amsterdam, Vol. 30, 1996.
3. J. Montreuil, J.F.G. Vliegenthart, H. Schachter, 'Glycoproteins II', in *New Comprehensive Biochemistry*, Elsevier, Amsterdam, Vol. 29b, 1997.
4. H. Lis, N. Sharon, 'Protein Glycosylation, Structural and Functional Aspects', *Eur. J. Biochem.*, **218**, 1–27 (1993).
5. J. Hofsteenge, D.R. Müller, T. de Beer, A. Löffler, W.J. Richter, J.F.G. Vliegenthart, 'New Type of Linkage Between a Carbohydrate and a Protein: C-Glycosylation of a Specific Tryptophan Residue in Human RNase Us', *Biochemistry*, **33**, 13 524–13 530 (1994).
6. T. de Beer, J.F.G. Vliegenthart, A. Löffler, J. Hofsteenge, 'The Hexopyranosyl Residue that is C-Glycosidically Linked to the Sidechain of Tryptophan-7 in Human RNase Us is Alpha-mannopyranose', *Biochemistry*, **34**, 11 785–11 789 (1995).
7. G.J. Gerwig, J.P. Kamerling, J.F.G. Vliegenthart, 'Determination of the D and L Configuration of Neutral/Monosaccharides by High-resolution Capillary g.l.c.', *Carbohydr. Res.*, **62**, 349–357 (1978).
8. G.J. Gerwig, J.P. Kamerling, J.F.G. Vliegenthart, 'Determination of the Absolute Configuration of Monosaccharides in Complex Carbohydrates by Capillary g.l.c.', *Carbohydr. Res.*, **77**, 1–7 (1979).
9. J.F.G. Vliegenthart, H. van Halbeek, L. Dorland, 'The Applicability of 500-MHz High-resolution ^{1}H-NMR Spectroscopy for the Structure Determination of Carbohydrates Derived from Glycoproteins', *Pure Appl. Chem.*, **53**, 45–77 (1981).
10. J.F.G. Vliegenthart, L. Dorland, H. van Halbeek, 'High-resolution, ^{1}H-Nuclear Magnetic Resonance Spectroscopy as a Tool in the Structural Analysis of Carbohydrates Related to Glycoproteins', *Adv. Carbohydr. Chem. Biochem.*, **41**, 209–374 (1983).
11. J.P. Kamerling, J.F.G. Vliegenthart, 'High-resolution H-1-Nuclear Magnetic Resonance Spectroscopy

of Oligosaccharide-Aldjtols Released from Mucin-type *O*-glycoproteins', *Biol. Magn. Reson.*, **10**, 1-194 (1992).

12. B. Fournet, J. Montreuil, G. Strecker, L. Dorland, J. Haverkamp, J.F.G. Vliegenthart, J.P. Binette, K. Schmid, 'Determination of the Primary Structures of 16 Asialo-Carbohydrate Units Derived from Human Plasma Alpha 1-acid Glycoprotein by 360-MHz H-1-NMR Spectroscopy and Permethylation Analysis', *Biochemistry*, **17**, 5206-5214 (1978).
13. J.A. van Kuik, J.F.G. Vliegenthart, 'Databases of Complex Carbohydrates', *Trends Food Sci. Technol.*, **4**, 73-77 (1993).
14. S. Doubet, K. Bock, D. Smith, A. Darvill, P. Albersheim, 'The Complex Carbohydrate Structure Database', *Trends Biochem. Sci.*, **14**, 475-477 (1989).
15. J.J.M. van Rooijen, A.F. Voskamp, J.P. Kamerling, J.F.G. Vliegenthart, 'Glycosylation Sites and Site-speicifc Glycosylation in Human Tamm-Horsfall Glycoprotein', *Glycobiology*, **9**, 21-30 (1999).
16. G.J. Gerwig, J.F.G. Vliegenthart, 'Analysis of Glycoproteins-derived Glycopeptides', in *Proteomics in Functional Genomics*, eds. P. Jollès, H. Jörnval, Birkhäuser Publishers, Basel, 2000.
17. B.R. Leeflang, L.M.J. Kroon-Batenburg, 'CROSREL: Full Relaxation Matrix Analysis for NOESY and ROESY NMR Spectroscopy', *J. Biomol. NMR*, **2**, 495-518 (1992).
18. L.M.J. Kroon-Batenburg, J. Kroon, B.R. Leeflang, J.F.G. Vliegenthart, 'Conformational Analysis of Methyl Beta-cellobioside by ROESY NMR spectroscopy and MD Simulations in Combination with the CROSREL Method', *Carbohydr. Res.*, **245**, 3787-3797 (1993).
19. J.P.M. Lommerse, L.M.J. Kroon-Batenburg, J. Kroon, J.P. Kamerling, J.F.G. Vliegenthart, 'Conformations and Internal Mobility of a Glycopeptide Derived from Bromelain Using Molecular Dynamics Simulations and NOESY Analysis', *J. Biomol. NMR*, **6**, 79-94 (1995).
20. T. De Beer, C.W.E.M. van Zuylen, B.R. Leeflang, K. Hård, R. Boelens, R. Kaptein, J.P. Kamerling, J.F.G. Vliegenthart, 'NMR Studies of the Free Alpha-subunit of Human Chorionic Gonadotropin; Structural Influences of *N*-glycosylation and the Beta-subunit on the Conformation of the Alpha-subunit', *Eur. J. Biochem.*, **241**, 229-242 (1996).
21. C.W.E.M. van Zuylen, J.P. Kamerling, J.F.G. Vliegenthart, 'Glycosylation Beyond the Asn78-linked GlcNAc Residues has a Significant Enhancing Effect on the Stability of the Alpha Subunit of Human Chorionic Gonadotropin', *Biochem. Biophys. Res. Commun.*, **232**, 117-120 (1997).
22. C.W.E.M. Thijssen-van Zuijlen, T. De Beer, B.R. Leeflang, R. Boelens, R. Kaptein, J.P. Kamerling, J.F.G. Vliegenthart, 'Mobilities of the Inner Three Core Residues and the Man (alpha 1-6) branch of the Glycan at Asn78 of the Alpha-subunit of Human Chorionic Gonadotropin are Restricted by the Protein', *Biochemistry*, **37**, 1933-1940 (1998).
23. G.M. Clore, A.M. Gronenborn, 'Theory of the Time-dependent Transferred Nuclear Overhauser Effect: Application to Structural Analysis of Ligand-Protein Complexes in Solution', *J. Magn. Reson.*, **53**, 423-442 (1983).
24. F. Ni, 'Recent Developments in Transferred NOE Methods', *Prog. NMR Spectrosc.*, **26**, 517-606 (1994).
25. C.P.J. Glaudemans, L. Lerner, G.D. Daves, Jr, P. Kovàc, R. Venable, A. Bax, 'Significant Conformational Changes in an Antigenic Carbohydrate Epitope upon Binding to a Monoclonal Antibody', *Biochemistry*, **29**, 10 906-10 911 (1990).
26. D.R. Bundle, H. Baumann, J.-R. Brisson, S.M. Gagné, A. Zdanov, M. Cygler, 'Solution Structure of a Trisaccharide-Antibody Complex - Comparison of NMR Measurements with a Crystal Structure', *Biochemistry*, **33**, 5183-5192 (1994).
27. V.L. Bevilacqua, D.S. Thomson, J.H. Prestegård, 'Conformational Aspects of Methyl Lactoside Bound to the Risin B-Chain Interpretation of Transferred Nuclear Overhauser Effects Facilitated by Spin Simulation and Selective Deuteration', *Biochemistry*, **29**, 5529-5537 (1990).
28. V.L. Bevilacqua, Y. Kim, J.H. Prestegård, 'Conformation of Methylmebioside Bound to the Risin B-Chain, as Determined from Transferred Nuclear Overhauser Effects', *Biochemistry*, **31**, 9339-9349 (1992).
29. R. Kaptein, 'Photo-CIDNP of Proteins', *Biol. Magn. Reson.*, **4**, 145-191 (1982).
30. P.J. Hore, R.W. Broadhurst, 'Photo-CIDNP of Biopolymers', *Prog. NMR Spectrosc.*, **25**, 326-345 (1993).
31. K. Hård, J.P. Kamerling, J.F.G. Vliegenthart, 'Application of Laser Photo-CIDNP for an Intact Glycoprotein in Solution', *Carbohydr. Res.*, **236**, 315-320 (1992).
32. H.-C. Siebert, S. André, G. Reuter, H.-J. Gabius, R. Kaptein, J.F.G. Vliegenthart, 'Effect of Enzymatic Desialylation of Human Serum Amyloid P Component on Surface Exposure of Laser Photo CIDNP (Chemically Induced Dynamic Nuclear Polarization)-Reactive Histidine, Tryptophan and Residues Tyrosine', *FEBS Lett.*, **371**, 13-16 (1995).
33. H.-C. Siebert, C.-W. Von der Lieth, R. Kaptein, J.F.G. Vliegenthart, C.S. Wright, H.-J. Gabius, 'Knowledge-based Homology Modeling and Experimental Determination of Amino Acid Side Chain Accessibility by the Laser Photo CIDNP. Approach in Solution: Lessons from the Small Sialidase of *Chlostridium perfringens*', *J. Mol. Modeling*, **2**, 351-353 (1996).
34. R.K. Harris, *Nuclear Magnetic Resonance Spectroscopy: A Physicochemical View*, Longman, Oxford, 1986.
35. R.R. Ernst, G. Bodenhausen, A. Wokaun, *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*, Clarendon Press, Oxford, 1987.

36. A.A. Derome, *Modern NMR Techniques for Chemistry Research*, Pergamon Press, Oxford, 1987.
37. F.J.M. van der Ven, *Multidimensional NMR in Liquids, Basic Principles and Experimental Methods*, VCH Publishers, New York, 1995.
38. S. Braun, H.-O. Kalinowski, S. Berger, *100 and More Basic NMR Experiments; A Practical Course*, VCH Publishers, New York, 1996.
39. H. Kessler, M. Gehrke, C. Griesinger, 'Two-dimensional NMR Spectroscopy: Background and Overview of the Experiments', *Angew. Chem., Int. Ed. Engl.*, **27**, 490 (1989).
40. S.W. Homans, 'Oligosaccharide Conformation: Application of NMR and Energy Calculations', *Prog. NMR Spectrosc.*, **22**, 55 (1990).
41. A. Bax, S.W. Sparks, D.A. Torchia, 'Homonuclear Hartman–Hahn Experiments', in *Methods in Enzymology*, eds. N.J. Oppenheimer, T.L. James, Academic Press, San Diego, 151–168, Vol. 176, 1989.
42. H. Van Halbeek, 'NMR of Complex Carbohydrates', in *Frontiers of NMR in Molecular Biology*, eds. D. Live, I.M. Armitrage, D. Patel, Wiley-Liss, New York, 195–213, 1990.
43. D. Neuhaus, M. Williamson, *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, VCH Publishers, New York, 1989.
44. L.R. Brown, B.T. Farmer, II, 'Rotating-frame Nuclear Overhauser Effect', in *Methods in Enzymology*, eds. N.J. Oppenheimer, T.L. James, Academic Press, San Diego, 199–216, Vol. 176, 1989.
45. T.-L. Hwang, A.J. Shaka, 'Cross Relaxation Without TOCSY: Transverse Rotating-frame Overhauser Effect Spectroscopy', *J. Am. Chem. Soc.*, **114**, 3157–3159 (1992).
46. H. Desvaux, P. Berthault, N. Birlirakis, M. Goldman, M. Piotto, 'Improved Versions of Off-resonance ROESY', *J. Magn. Reson., Ser. A*, **113**, 47–52 (1995).