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Methods for measuring exchangeable protons in glycosaminoglycans

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Abstract

Recent NMR studies of the exchangeable protons of GAGs in aqueous solution, including those of the amide, sulfamate and hydroxyl moieties, have demonstrated potential for the detection of intramolecular hydrogen bonds providing insights into secondary structure preferences. GAG amide protons are observable by NMR over wide pH and temperature ranges, however, specific solution conditions are required to reduce the exchange rate of the sulfamate and hydroxyl protons and allow their detection by NMR. Building on the vast body of knowledge on detection of hydrogen bonds in peptides and proteins, a variety of methods can be used to identify hydrogen bonds in GAGs including temperature coefficient measurements, evaluation of chemical shift differences between oligo- and monosaccharides, and relative exchange rates measured through line shape analysis and EXSY spectra. Emerging strategies to allow direct detection of hydrogen bonds through heteronuclear couplings offer promise for the future. Molecular dynamic simulations are important in this effort both to predict and confirm hydrogen bond donors and acceptors.

Keywords: Glycosaminogycan, NMR, Chemical Exchange, Hydrogen Bond, Temperature Coefficient, Chemical Shift Difference, Activation Energy, EXSY

Running title: Measurement of exchangeable protons in glycosaminoglycans

1. Introduction

Glycosaminoglycans (GAGs) are a family of long chain polysaccharides including chondroitin sulfate (CS), hyaluronan (HA), dermatan sulfate (DS), keratin sulfate (KS), heparin (Hp) and heparan sulfate (HS). The inherent microheterogeneity of GAGs is introduced during biosynthesis as various patterns of *O*-sulfation, C5 epimerization and *N*-acetylation or sulfation. While this microheterogeneity enables the GAGs to mediate a variety of biological processes through their ability to bind to many different proteins it also makes the molecular level characterization of intact

GAGs challenging, though advances in mass spectrometry hold promise for GAG sequencing (1,2). While disaccharide analysis can be useful for characterization of the overall composition, most information about GAG microstructure is obtained by depolymerization through enzymatic or chemical means and isolation of individual oligosaccharides using chromatographic methods (3). Advances in the synthesis of GAG oligosaccharides is emerging as an efficient method to obtain well-defined sequences and promises to eliminate the bottleneck of oligosaccharide isolation from GAG digests (4). Whether the oligosaccharide is isolated from a GAG digest or produced synthetically, its primary structure must be determined and mass spectrometry and NMR methods are well established for this purpose (5). In contrast, methods for understanding the solution structure and dynamics of GAG oligosaccharides are still evolving.

Experiments to evaluate solution state GAG secondary structure have primarily relied on analysis of coupling constants and detection of NOEs (6). Though GAG oligosaccharides exhibit a great deal of flexibility in solution, elements of primary structure such as iduronic acid residues (IdoA) are well known to limit conformational flexibility. In this chapter we describe strategies to identify new elements of GAG secondary structure by characterization of the exchangeable protons, which can uniquely report on the presence of intramolecular hydrogen bonds. These exchangeable protons are located on the GAG amine ($N\mathbf{H}_3^+$), amide ($N\mathbf{H}COCH_3$), sulfamate ($N\mathbf{H}SO_3^-$), and hydroxyl ($O\mathbf{H}$) groups. These NH and OH protons are called “exchangeable” because they exchange chemically with the aqueous solvent. The process of chemical exchange can be described by the following reversible reaction, using an *N*-sulfoglucosamine ($GlcNHSO_3^-$) sulfamate proton as an example:

$$HOH + GlcN\mathbf{H}SO_3^- \rightleftharpoons \mathbf{H}OH + GlcNHSO_3^-$$

In an NMR measurement, the exchanging protons, indicated in the chemical reaction above in red, are labeled with different frequencies (chemical shifts) that reflect their local chemical environment before exchange. There are three possible exchange regimes that can be considered in an NMR measurement: slow, fast, and intermediate, defined by the exchange rate (reactions per second) and the

difference in frequency (in Hz) between the two chemical environments (or sites) experienced by the exchanging protons (7). Because of the dependence of the spectrum on the difference in chemical shift difference of the exchanging species, the appearance of the spectrum can be dramatically affected if it is measured at different magnetic field strengths. In slow exchange, the rate of exchange is much less than the frequency difference of the two sites and the protons will be detected in the NMR spectrum as two distinct resonances. In the fast exchange regime, the exchange rate is much greater than the frequency difference and a single exchange-averaged resonance is detected at a chemical shift weighted by the populations of the two species. In a typical solution of a GAG polymer or oligosaccharide, the water protons are present at such a high concentration (55 M) that the chemical shift is essentially that of the water protons. Lying between these two extremes in the intermediate exchange regime, the exchange rate is on the same order of magnitude as the chemical shift difference between the exchanging species and an increase in the resonance line widths (or line broadening) is observed in the NMR spectrum.

If instead of exchange rates, we think about the lifetime of the exchanging proton on the timescale of the NMR measurement, these exchange phenomena become easier to understand. In the slow exchange limit, the lifetime of the water and $GlcNHSO_3^-$ protons is long compared to the time required to measure the spectrum. Almost all of the protons initially labeled with the water or $GlcNHSO_3^-$ frequency at the start of acquisition, remain in that environment for the duration of the acquisition (typically 1 to 5 sec). If the exchange rate is increased, for example by heating the solution, the lifetimes of the protons in the two sites is reduced and the reaction can move into the intermediate exchange regime. In intermediate exchange, a proton that is in a $GlcNHSO_3^-$ molecule at the start of acquisition may exchange with water while the spectrum is being recorded. When the whole population of protons in the sample is considered, the exchange reaction introduces an uncertainty in the chemical shift of the exchanging protons which is reflected by an increase in the resonance line width. In the fast exchange limit, the protons exchange rapidly between $GlcNHSO_3^-$ and water such that many exchanges occur as the spectrum is being measured, producing a single resonance at a chemical shift that represents a weighted average of the two environments.

Evidence for all three exchange regimes is observed in Figure 1, which shows the pH dependence of the $GlcNHSO_3^-$ sulfamate protons (8). At the low and high pH extremes, no resonance is detected for the sulfamate protons. Exchange with water is fast on the NMR timescale and the average chemical shift of the exchanging protons is that of the water resonance because of the high concentration of water in the solution relative to that of the GlcNS monosaccharide. As the pH is raised from 6.67 to 8.54 the resonances of the NH protons appear in Figure 1. Chemical exchange between the α- and β-anomers is slow on the NMR timescale and a separate resonance is detected for the NH resonance of each anomeric form. The resonance of the α-anomer NH appears at a chemical shift of 5.34 ppm while that of the β-anomer is at 5.79 ppm. It is interesting to note that there is apparently a difference in the exchange rate of the protons in the α- and β-anomers; the resonance of the α-anomer NH can be detected over a wider pH regime than the β-anomer. Between pH 7.49 and 7.97 the exchange rate of the α-anomer NH becomes sufficiently slow that the coupling to the adjacent C2 proton becomes observable. Outside of this pH optimum the resonances broaden as the exchange rate increases.

Various NMR experiments can be used to measure exchange rates, several of which are discussed in this chapter. Comparison of the relative exchange rates of protons in different chemical environments can highlight unusually slow exchange, which is typically attributed to participation by the proton in a hydrogen bond. Experiments to detect hydrogen bonding in GAGs are based on the extensive body of work to identify hydrogen bonds involving the amide protons of proteins (9-11). While the hydrogen bond donors can be unambiguously identified through NMR measurements, identifying the hydrogen bond acceptor is more difficult, therefore molecular dynamics (MD) simulations are useful both to predict the existence of hydrogen bonds and to identify the hydrogen bonding pairs. For example, MD simulations by Langeslay et al. for the synthetic Hp pentasaccharide Arixtra (Figure 2) provided additional evidence for the hydrogen bond involving the central $GlcNHSO_3^-$ NH and identified the hydrogen bond acceptor as the adjacent 3-*O*-sulfo group (12). This simulation identified the hydrogen bond in structures in which the

2-*O*-sulfo-iduronic acid (IdoA2S) residue is in either the $^{2}S_{O}$ (Figure 2A) or $^{1}C_{4}$ (Figure 2B) conformation.

1.1. Observing exchangeable protons by NMR

To be able to characterize the exchangeable protons of GAGs in solution, they must be detectable by NMR. The different types of exchangeable GAG protons undergo chemical exchange with the aqueous solvent at different rates and therefore different solution conditions may be required for their detection. For example, there are currently no conditions known that allow detection of GAG primary amines in aqueous solution because their exchange is too rapid. As is the case for peptides and protons, the GAG amide protons exchange fairly slowly with water and can be detected over a wide range of pH values, with 90% H_2O/10% D_2O and pH 6.0 typical of conditions used for measurement of NMR spectra. Although lower temperatures are often used to slow the exchange rate and sharpen the resonances of exchangeable protons, amide protons, like those of HA, can be detected well above room temperature (13,14). Recently, Langeslay and coworkers reported solution conditions that slow the rate of solvent exchange of the Hp and HS sulfamate protons and allow their detection by NMR (8). The optimum solution pH for detection of the sulfamate protons is between 7.5 and 8.5, with more negatively charged oliogsaccharides preferring a more basic pH (8,12,15). The resonances of the sulfamate protons can be detected at room temperature, but become sharper at lower temperatures because the solvent exchange rate is reduced.

The GAG hydroxyl protons exchange more rapidly than either the amide or sulfamate protons. Avoiding buffer anions and removing dissolved CO_2, both of which catalyze the solvent exchange of hydroxyl protons, is essential. Hydroxyl proton resonances are observable by NMR at lower temperatures and are sharpest below the freezing point of water (16-19). This requires the addition of deuteroacetone or another solvent to reduce the freezing point of water ideally without affecting its solution structure (16). With sufficient oligosaccharide or salt content, however, solutions could be supercooled

without the addition of aprotic solvent providing solution conditions that are more biologically relevant (19).

An important consideration in the measurement of NMR spectra for exchangeable protons is the selection of a method for solvent suppression. Spectra should be acquired with the minimum amount of D_2O required to maintain a stable lock signal, typically 5 or 10%. Suppression of the intense H_2O resonance should be accomplished using a method such as WATERGATE (20) or excitation sculpting (21) that avoid transfer of saturation from the water resonance to the signals of the exchangeable protons. Resonance assignments for the exchangeable protons can be accomplished using standard homonuclear two-dimensional NMR experiments such as COSY, TOCSY, NOESY and ROESY, however care should again be taken in the choice of the solvent suppression method employed to avoid saturation of the resonances of interest.

1.2. Temperature coefficient measurements

A well-established and simple method to identify protons involved in hydrogen bonds is through the measurement of the temperature dependence of their chemical shift ($\Delta\delta/\Delta T$), a parameter known as the temperature coefficient (12-14,17,18). Exchangeable protons with smaller temperature coefficients are considered to be protected from solvent exchange, most likely through participation in a hydrogen bond. Langeslay et al. used temperature coefficients to provide evidence for a hydrogen bond involving the internal sulfamate proton of Arixtra (Figure 3A) (12). As shown in Figure 3B, the Arixtra sulfamate NH resonances broaden and shift upfield towards the water resonance as the solution temperature is raised. The sulfamate protons at the reducing (V) and non-reducing (I) ends have similar temperature coefficients, while the NH resonance labeled III is hardly affected by the increase in temperature, suggesting its involvement in a persistent hydrogen bond. Blundell et al. used temperature coefficients of the amide ^{1}H and ^{15}N resonances of HA to demonstrate that the amide groups participate

in transient rather than persistent hydrogen bonds as previously proposed (13,14). In addition, Nestor et al. used temperature coefficients as evidence for structural stabilization of HA through the participation of hydroxyl protons in hydrogen bonds (18). Though a reduced temperature coefficient is consistent with the presence of a hydrogen bond, this parameter alone does not provide definitive evidence for its existence and most studies rely on multiple lines of evidence.

1.3. Chemical shift difference measurements

Comparison of the chemical shifts of the exchangeable protons of a residue within an oligosaccharide to their value in the corresponding monosaccharide, expressed as $\Delta\delta = \delta_{oligo} - \delta_{mono}$, can also be an indication of hydrogen bonding. Exchangeable protons experience a downfield shift when they are in close proximity to an electronegative atom. The larger and more negative the value of $\Delta\delta$, the closer the proton is to the electronegative atom and the more likely it is participating in a hydrogen bond (17,18). This parameter has been used as evidence for weak hydrogen bonds involving the HA hydroxyl protons (18). Although $\Delta\delta$ values can provide evidence for hydrogen bonds, they do not provide conclusive evidence of a hydrogen bond because the differences in chemical shift reflect a balance of hydration and proximity to an electronegative atom (17,22). Chemical shift differences should considered together with other experimental results, such as temperature coefficients and exchange rates.

1.4. Line shape analysis for the evaluation of exchange rates and determination of activation energies.

As shown in Figure 3B, as the solution temperature is raised, the NH resonances of Arixtra broaden due to an increase in the rate of the solvent exchange reaction. Line shape analysis allows this resonance broadening to be quantified. Figure 4 illustrates this process using least squares fitting in Mathematica to simulate the measured spectrum with a Lorenzian and determine the width at half-height of the Arixtra sulfamate protons. The

resonance width at half-height is proportional to the exchange rate constant, *k*, and can be used to calculate the energy barrier, $\Delta G^{\ddagger}$, for chemical exchange using the Eyring-Polanyi equation shown below (7,12,23,24).

$$k = \frac{k_b T}{h} e^{\frac{-\Delta G^{\ddagger}}{RT}}$$

A smaller value of $\Delta G^{\ddagger}$ suggests a low barrier to solvent exchange. A larger value of $\Delta G^{\ddagger}$ reflects the energetic penalty necessary to break a hydrogen bond to allow solvent exchange to occur. The energy difference typically expected for an NH or OH proton involved in a hydrogen bond is roughly 2 – 3 kcal/mol. Comparison of the $\Delta G^{\ddagger}$ values determined for the three sulfamate protons of Arixtra by Langeslay et al. provided conclusive evidence for the involvement of the internal glucosamine sulfamate proton in a hydrogen bond, the first $NHSO_3^-$ hydrogen bond observed in aqueous solution (12).

A library of mono- and oligosaccharides was prepared by enzymatic depolymerization of amide-containing polysaccharides and by chemical modification of heparin and heparan sulfate saccharides including members that contain a 3-O-sulfated glucosamine residue. The systematic evaluation of this saccharide library facilitated assessment of the effects of structural characteristics, such as size, sulfation number and site, and glycosidic linkage, on amide proton solvent exchange rates. Charge repulsion by neighboring negatively charged sulfate and carboxylate groups was found to have a significant impact on the catalysis of amide proton solvent exchange by hydroxide. This observation leads to the conclusion that solvent exchange rates must be interpreted within the context of a given chemical environment. On their own, slow exchange rates do not conclusively establish the involvement of a labile proton in a hydrogen bond and additional supporting experimental evidence such as reduced temperature coefficients is required.

1.5. EXSY measurements for evaluation of relative exchange rates

The faster exchange rates of the hydroxyl protons allows for evaluation of their relative exchange rates through measurement of EXSY spectra (25,26). The EXSY spectra measured for the α- and β-glucuronic acid (GlcA) hydroxyl protons as a function of mixing time are shown in Figure 5. The cross peaks at the chemical shift of the water resonance are observed to increase as the mixing time is incremented. A slower rate of cross peak intensity build-up indicates that the proton is protected from solvent exchange through participation in a hydrogen bond. Sandström and coworkers used this method to find hydrogen bonds in 3,4-disubstituted methyl α-D-galactopyranosides (17).

1.6. Direct detection of hydrogen bonds through heteronuclear coupling

Battistel et al. identified hydroxyl proton hydrogen bonds in aqueous solutions of sucrose, a topic that has been previously debated in the literature (16,27), through direct observation of heteronuclear coupling (19). A fully aqueous solution containing no organic modifiers was used to measure [^{1}H, ^{13}C] HSQC-TOCSY cross peaks (Figure 6) for 300 mM sucrose at natural abundance levels of ^{13}C, unambiguously identifying the hydrogen bond donors (^{1}H) and acceptors (^{13}C). MD simulations confirmed the presence of intramolecular inter-ring hydrogen bonds between the fructose OH1 and glucose OH2, which act as both donors and acceptors in ‘flip-flop’ hydrogen bonds. MD simulations also confirmed the presence of intermolecular hydrogen bonds between both glucose OH3 (donor) and fructose OH3 (acceptor), which was also detected by cross peaks arising from the $^{2}J_{OH\text{-}OH}$ coupling using selective COSY experiments, demonstrating the potential of this experiment to studying interactions between GAGs. Though this work used concentrations (50 mM and above) that are not generally feasible for GAG oligosaccharides, this limitation could be overcome through the use of isotopically labeled compounds (28).

2. Materials

1. HA amide buffer: 5% (v/v) D_2O, 0.02% (w/v) NaN_3, pH 6.0 (14).
2. Sulfamate proton buffer: 20 mM sodium phosphate buffer, 2 mM EDTA-d_{16}, 1 mM DSS in 90% H_2O/10% D_2O at pH 8.2 (8).
3. Hp hydroxyl proton solution: 85% H_2O/15% acetone-d_6, pH 8.1.
4. Methanol standard for temperature measurements.
5. Program for calculating line widths and exchange rates (i.e. Mathematica).

3. Methods

3.1. Preparing solutions

NH protons

1. Dissolve oligosaccharides in 600 µL of 90% H_2O/10% D_2O in an Eppendorf tube.
2. Measure pH directly in the Eppendorf tube using a microelectrode.
3. Make pH adjustments with small amounts of 0.1 M HCl and 0.1 M NaCl in 90% H_2O/10% D_2O to pH 8.2.

OH protons

1. Boil HPLC grade water to remove any dissolved CO_2. Allow water to cool to room temperature in a sealed container.
2. All solution preparation must be performed in a glovebox to prevent absorption of CO_2.

3.2 Resonance assignments

1. Record TOCSY, COSY, and ROESY spectra to assign the carbon-bound proton resonances (29). This measurement can be performed using a D_2O solution to allow detection of protons that are close in chemical shift to the water resonance.
2. Measure TOCSY and COSY spectra in 90 or 95% H_2O solution using a solvent suppression method such as WATERGATE (20) or excitation sculpting (21) that avoids saturation of the exchangeable protons.

3. The TOCSY spectrum will show cross peaks between the exchangeable proton and the other carbon-bound protons within the same ring system.
4. Cross peaks arising from 3-bond couplings between the exchangeable proton and neighboring carbon-bound protons will be observed in the COSY spectrum, confirming the resonance assignment.

3.3 Temperature coefficient measurements

1. Cool the NMR sample temperature to 5 ºC for amide and sulfamate protons and -15 ºC for hydroxyl protons.
2. Measure the initial temperature using the methanol standard. Allow the NMR tube to sit in the probe for at least 10 min for the solution to come to a uniform temperature.
3. Measure the spectrum and calculate the temperature based on the Van Geet equation (30).
4. Place the sample in the probe and allow it to come to a uniform temperature for at least 10 min.
5. Measure the spectrum and determine the chemical shift of the exchangeable protons. For doublets, the chemical shift is calculated by measuring the chemical shifts of the two resonances that make up the doublet and calculating the average.
6. Spectra should be recorded for at least 7 different temperatures to accurately determine the temperature coefficient value.

3.4 Chemical shift difference measurements

1. Prepare separate solutions of the oligosaccharide and of each corresponding monosaccharide contained within the larger oligosaccharide sequence.
2. Measure the chemical shifts of the exchangeable protons at the temperature of interest.
3. Calculate the chemical shift differences by subtracting the chemical shift of the proton within the monosaccharide from the chemical shift of the corresponding proton within the oligosaccharide ($\Delta\delta = \delta_{oligo} - \delta_{mono}$).

3.5 Line shape analysis for the evaluation of exchange rates and determination of activation energies.

1. Repeat procedure for temperature coefficient measurements. These calculations require 15-20 different temperature points for accurate fits. Spectra should be recorded with high digital resolution. All spectra must be acquired and processed using exactly the parameters.
2. For TopSpin users, type the command “tojdx” in the command line to extract as a JCAMP file for Mathematica. Use the parameters “JCAMP-FIX,” “RSPEC,” and version “5.0” for each ^{1}H NMR spectrum.
3. Determine the resonance line widths of the exchangeable protons by non-linear least-squares fitting of a Lorentzian peak shape to the experimental data using a program such as Mathematica (12,31,32).
4. Plot these resonance line widths as a function of temperature to the Eyring-Polanyi equation to calculate the activation energy of proton exchange, $\Delta G^{\ddagger}$ using a program such as Mathematica (7,23,24).

3.6 EXSY measurements for evaluation of relative exchange rates

1. Cool NMR sample temperature to -15 ºC to -10 ºC to obtain sharper hydroxyl proton resonances.
2. Acquire EXSY spectra using the NOESY pulse sequence with excitation sculpting. Use the largest anticipated mixing time (e.g. 24 ms) to set the receiver gain (RG).
3. Using the same RG for all spectra, acquire EXSY spectra varying the mixing time from 0 – 21 ms.
4. Measure the cross peak volume at the water chemical shift for each of the exchangeable protons at each mixing time.
5. Normalize the cross peak volumes to the diagonal peak volume of each resonance.
6. Plot the normalized cross peak volume as a function of mixing time.

7. Calculate the initial build-up exchange rate constant using the modified Bloch equations (33).

4. Notes

1. The pH that minimizes the exchange rate is compound dependent. More highly charged oligosaccharides give sharper peaks in a somewhat more basic pH than neutral compounds.
2. If sufficient compound is available it is possible to supercool the solution without adding aprotic solvents.
3. For measurements of the OH resonances, remove the CO_2 from the solvent used to prepare solutions, including the NaOH and HCl solutions used to adjust the solution pH, by boiling and/or bubbling with N_2.
4. Prepare solutions and perform pH adjustments inside a glovebox filled with N_2 to avoid absorption of CO_2.
5. Parafilm around the NMR tube will help keep CO_2 from absorbing into the solution during the measurements.
6. Between experiments store NMR tubes containing solutions for OH measurements in a jar full of N_2 in the refrigerator to reduce CO_2 absorption and to help avoid evaporation of deuteroacetone.
7. When calibrating the temperature using the methanol standard, only one scan is needed with the lock and sweep turned off. Measure the chemical shift between the two methanol resonances and convert to temperature using a calibration plot (30).
8. Acquiring spectra at a large number temperatures (e.g. 15-20) will provide more accurate fits to the Eyring- Polanyi equation.
9. Including ^1J coupling constants for the NH protons measured at low temperature in the spectral simulation produces more accurate fits of the experimental spectra when the resonances become very broad.

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Figure Captions

Fig. 1. The line widths of the sulfamate protons of GlcNS vary with solution pH. The exchange rate of the protons is reduced between pH 7.2-8.0 allowing them to be observed by NMR (8). Copyright 2011 American Chemical Society.

Fig. 2. MD predictions of a sulfamate proton hydrogen bond in Arixtra in the IdoA2S(IV) in the $^{2}S_{O}$ (A) and $^{1}C_{4}$ (B) conformations (12). In each IdoA conformation, the hydrogen bond occurs between the $NHSO_{3}^{-}$ and the adjacent 3-*O*-sulfate group. Published with permission of Oxford University Press.

Fig. 3. (A) Arixtra residues are labeled with a Roman numeral, starting with the non-reducing end. (B) The sulfamate protons of Arixtra broaden and experience an upfield shift with increasing temperature (12). At the lowest temperature shown here, the hydroxyl protons are visible as very broad resonances. Published with permission of Oxford University Press.

Fig. 4. Example of Arixtra sulfamate proton resonance peak fitting using Mathematica; (A) simulated spectrum, (B) measured spectrum, and (C) residuals shown at a 20 times the intensity of the spectra (12). Published with permission of Oxford University Press.

Fig. 5. EXSY plots of the α- and β-GlcA hydroxyl protons at mixing times of (A) 6 ms, (B) 12 ms, (C) 18 ms, and (D) 24 ms. As the mixing time increases, the intensity of the exchange cross peaks between the hydroxyl protons and the water resonance increases.

Fig. 6. (A) An HSQC-TOCSY spectrum of 300 mM sucrose. The red squares indicate those cross peaks corresponding to the hydrogen bonds shown in (B) (19). Copyright 2011 American Chemical Society.