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Disentangling Glycan-Protein Interactions: Nuclear Magnetic Resonance (NMR) to the Rescue

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Abstract

The interactions of glycans with proteins modulate many events related to health and disease. In fact, the establishment of these recognition events and their biological consequences are intimately related to the three-dimensional structures of both partners, as well as to their dynamic features and their presentation on the corresponding cell compartments. NMR techniques are unique to disentangle these characteristics and, indeed, diverse NMR-based methodologies have been developed and applied to monitor the binding events of glycans with their associate receptors. This protocol outlines the procedures to acquire, process and analyze two of the most powerful NMR methodologies employed in the NMR-glycobiology field, ^{1}H-Saturation transfer difference (STD) and ^{1}H,^{15}N-Heteronuclear single quantum coherence (HSQC) titration experiments, which complementarily offer information from the glycan and protein perspective, respectively. Indeed, when combined they offer a powerful toolkit for elucidating both the structural and dynamic aspects of molecular recognition processes. This comprehensive approach ~~not~~ enhances our understanding of glycan-protein interactions and contributes to advancing research in the chemical glycobiology field.

Introduction

Molecular recognition of glycans is essential for many processes related to health and disease. The specificity and selectivity of biological receptors (lectins, antibodies, enzymes) for glycans heavily depend on adjusting the precarious balance between the diverse components of enthalpy (CH-π and van der Waals, hydrogen bonds, electrostatics) and entropy (hydrophobicity, dynamics, solvation-desolvation)[1].

Given the large chemical diversity and dynamic nature of glycans, NMR methods have been widely employed to dissect glycan interactions for more than 25 years[2], since these methodologies afford superb information on molecular recognition events with precise details, at atomic

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resolution[3,4], even when the required interaction evidence cannot be retrieved by employing other methodologies. As key point, NMR is versatile and allows studying dynamic events, at the atomic level, at different time scales, constituting the best technique by far for studying the structure, conformation, and dynamics of glycans in solution. Nevertheless, disentangling this information may be a rather complex process that requires the employment of well-defined strategies together with careful data analysis[5].

NMR techniques are diverse and, indeed, there are many methodologies that can be employed to unravel glycan-protein interactions[6]. We herein describe two basic NMR approaches that are currently employed to decipher glycan-receptor interactions[7,8], making emphasis on how to untangle the presentation of the key glycan epitope as well as the protein binding site[9].

In any molecular recognition event, when a receptor binds to a given ligand, there is a chemical exchange process that affects many NMR parameters of the participants in the binding[10]. Therefore, from the NMR perspective, the interaction can be monitored either from the point of view of the glycan ligand or from that of the protein receptor[11]. Generally speaking, the protein receptor is a large biomolecule (slow rotational motion, with rates in the ns timescale, and therefore, fast transverse relaxation), while the interacting glycan can be considered as a small-medium size molecule (fast rotational motion, with rates in the ps timescale, and slow transverse relaxation)[12]. From a standard perspective, the NMR signals of the glycan are narrow, while those of the receptor are broad[13].

Ligand-based NMR methods rely on the dramatic change that many glycan NMR parameters experience when passing from the free to the bound state[14]. STD-NMR is the most employed experimental NMR technique to assess diverse glycan binding features[15], from deducing the existence of binding in the solution state to the determination of the glycan binding epitope; that is, the atoms of the ligand that are in contact with the protein receptor[16].

Alternatively, receptor-based NMR methods monitor the changes that take place in the signals of the protein receptor in the presence of the glycan with respect to those recorded for the apo state[17]. These are mainly focused on screening the chemical shift perturbations of the protein signals between both states. The most commonly employed experiment is ^{1}H-^{15}N HSQC, or its TROSY alternatives[18].

The combination of both approaches allows applying NMR to many diverse systems that display a wide range of affinities. However, for the receptor-based NMR methods, in contrast to those based on the ligand, a relatively large amount of soluble, non-aggregated, stable isotope-labelled (^{15}N) protein must be available.

We herein describe both methods, highlighting their strengths, and weaknesses. Note that the basic steps described in the protocol serve as examples for the use of Bruker spectrometers. Consequently, commands and parameters names align with those utilized in TopSpin (Bruker's spectrometers control software).

Protocol

1. Saturation transfer difference NMR (STD-NMR)

NOTE: The subsequent lines outline fundamental procedures to acquire, process, and analyze STD-NMR experiments. These steps serve to exemplify the technique's utility for detecting ligand binding and for elucidating the ligand binding epitope. For a more profound understanding of the design

and acquisition of NMR experiments, please refer to the corresponding manufacturer's manual provided with the NMR instrument.

1. Acquisition

    1. Prepare the sample with the protein-ligand complex. Employ glycan:lectin molar ratios between 10:1 and 100:1 with protein concentrations ranging between 0.01 and 0.2 mM. For the interaction of *h*Galectin-7 with LacNAc, use 50:1 protein: ligand ratio in deuterated phosphate-buffered saline at pH 7.4.
    NOTE: The protein receptor should be pure and be soluble in the buffer of choice (in the case of STD-NMR experiments, deuterated versions of the corresponding buffer are preferable to reduce the possible ^{1}H NMR signal interference). The concentration of the protein is checked in advance using a spectrophotometer to measure the absorbance at 280 nm.
    2. From the prepared solution, transfer a total volume of 0.6 mL to a 5 mm NMR tube using a pipette.
    3. Prepare the NMR instrument at the required temperature (common experiment temperatures fall between 10 °C and 45 °C). Open the temperature control monitor using the ***edte*** command and set the desired temperature. For the *h*Galectin-7/LacNAc study, the temperature was set to 25 °C.
    4. Generate a new dataset containing the ***zg*** pulse sequence.
        1. For a straightforward operation, open an existing experiment and type the ***edc*** command. A dialog box appears, define the title, the characteristics (sample specifications, solvent) and some parameters of the experiment.
        2. If a change from the original pulse sequence is required, navigate through the ***ased*** (parameters) and ***AcquPars*** (acquisition parameters) windows. At this point, choose the desired pulse program from the spectrometer's library.
        3. For a standard ^{1}H NMR spectrum, select the ***zg*** pulse sequence from the available list.
        NOTE: In the case of samples with increased water content the use of water suppression schemes may be required to increase signal-to-noise ratio. The use of pulse sequences such as zgesgp, which excitation sculpting modules that render excellent suppression but controlling the phase of the remaining signals, are desirable. Please refer to manufacturers' NMR tutorial for additional information on types of water suppression schemes and their main characteristics.
    5. Insert the NMR sample into the probe by activating the sample lift air. Use the ***ej*** command, position the sample on the top of the magnet, and deactivate the sample lift by using the ***ij*** command.
    6. Lock on the solvent signal by typing the command ***lock*** and then selecting the appropriate solvent from the menu.
    7. Once the sample is inserted into the probe, complete the tuning and matching process by using the automatic module ***atma*** or the manual module ***atmm***.

8. Start the automatic shimming through the ***topshim gui*** command. This will open a graphical interface where shim dimension 1D will be selected and started.

   NOTE: To minimize shimming instabilities due to subtle field or temperature variations, ***autoshim*** can be activated for the experiment acquisition. This can be performed by accessing the ***BSMS*** control window and clicking on ***autoshim***. The turning into a green highlight indicates that ***autoshim*** has been activated. Please be aware that when using ***autoshim***, potential sample instability issues must be unnoticed. Therefore, caution is advised when ***autoshim*** is employed.

9. Determine the ^{1}H 90° pulse. This can be carried out automatically through the **pulsecal** command.

10. Modify various parameters in the ***AcquPars*** window. For a regular ^{1}H NMR spectrum, set the number of scans (NS) at 32, and the desired spectral window (SW) at *ca*. 12 ppm.

    NOTE: The ***zgesgp*** pulse sequence includes a module for solvent suppression to eliminate the residual HDO signal, which should be centered in the middle of the spectrum. For this purpose, O1 must be accurately defined in ***AcquPars***.

11. Set the receiver gain to avoid overflow with the automatic command ***rga***.

12. Now, acquire the standard ^{1}H NMR spectrum using the ***zg*** command.

13. Once the acquisition is finished, process the spectrum through the ***efp*** command. Apply the baseline and phase corrections using the ***TopSpin*** menu bar.

    NOTE: ^{1}H NMR signals deriving from the glycan and the protein are observed (**Figure 1**). The detailed analysis of the acquired NMR spectrum is recommended for the implementation of the STD NMR experiment, as outlined in section 1.1.14.

14. Create a new dataset and upload the STD NMR pulse sequence to be employed in the same way described for the ^{1}H NMR experiment in section 1.1.4. In Bruker instruments, different pulse sequences are available in the pulse program catalog, all named ***stddiffXXX***. The simplest one (***stddiff***) does not include any water suppression scheme or protein suppression filter.

    1. For samples with significant H_2O content, select either the ***stddiffgp19*** or ***stddiffesgp*** sequences, which include a watergate or excitation sculpting module. In the case of a spectrum with intense protein NMR signals as background, select the ***stddiffXXX.3*** sequences. In each case, optimize the corresponding specific parameters for each water suppression module (i.e., d19 in watergate schemes).

15. Define the off- and on-resonance frequencies for the STD NMR experiment. Find the frequency list in the AcquPars parameters of the ***ased*** window under the ***FQLIST*** entry. The defined on-resonance and off-resonance frequencies in Hertz must be manually written into the list and saved under a new name. This new list will be used in the STD-NMR experiment.

    1. Choose the on-resonance frequency at a spectral region devoid of glycan signals, usually

around δ(^{1}H) 0 or 6.6 ppm, for typical glycans (**Figure 1**). Set the off-resonance frequency at a region that does not show any ligand or protein proton. It can be safely set at +18000 or -18000 Hz.

16. Define the shaped pulse to be used during the saturation time in the ***AcquPars*** parameters of the ***ased*** window.
    NOTE: There are many possibilities. The Gaussian or Eburp shapes can be safely employed, with a 90° width of the selective pulse of 50 ms.
17. Set the corresponding parameters in the ***AcquPars*** section.
    1. Set the ^{1}H 90° pulse length.
    2. Set the power value for the shaped pulse (estimated through the ***shape*** tool).
    3. Set the total saturation time. Values between 1 s and 4 s can be regularly employed.
    4. Set the relaxation delay to 3 s.
    5. Set the number of scans (NS) to a multiple of 8. Usually, it is set at 256, 512, or 1024 to get the proper signal-to-noise ratio in sets of 2 at each frequency.
    6. Set the number of dummy scans (DS) to 8.
    7. Set the number of points in F2 to 16k, 32k, or 64k.
       NOTE: An increased number of points in F2 will result in improved resolution and signal-to-noise ratio. For that reason, using a minimum of 16k data points is strongly advisable.
    8. Set the number of points in F1. This is the number of frequencies to be used, in this case, 2 (the on-resonance and the off-resonance).
       NOTE: By convention, F2 refers to the direct dimension, the dimension along which the free induction decay (FID) is sampled directly, while F1 denotes the indirect dimension.
    9. Set the receiver gain (RG) to avoid overflow with the automatic command ***rga***.
18. Calculate the time of the total experiment using the command ***expt***.
19. Send the experiment for acquisition through the ***zg*** command.
20. Always check that the experiment is running properly after a few minutes.

2. Processing
   NOTE: A pseudo-2D spectrum is obtained after applying the protocol described above. The number of rows corresponds to the number of employed frequencies, typically two: the on-resonance and the off-resonance.
   1. Process the fid for the first experiment.
      1. Make the Fourier transform of the fid number 1 (through the ***efp*** command) and select the destination of the processed spectra (select procno number). Alternatively, use the ***rser 1*** command to read the first fid.
      2. Then, adjust the line broadening factor through ***lb*** command (usually 3-5 Hz), and the phase. To manually phase, click on the ***Process*** tab and then ***adjust phase*** submenu. Perform zero and first-order corrections by clicking and dragging on the corresponding button. Save the phasing

results. In addition, perform baseline correction through the command ***abs***.

2. Read the fid for the second experiment, and make the Fourier transform (through the ***efp*** command) with the same line broadening factor. Adjust the phase with the same phase parameters and baseline correction and save the processed spectrum with a different code.

3. Read the two processed spectra with the multiple function (command: ***.md***) and subtract them (off-resonance - on-resonance) using the button available in the multiple visualization (Δ). The new spectrum is the STD NMR spectrum, which is saved with a different code.

4. Make a superimposition of the STD NMR spectrum with the off-resonance spectrum.

    1. Open the off-resonance spectrum (fid 1) and type the ***.md*** command to open the multiple display window. Then, upload the STD spectrum.

    2. Compare the frequencies and intensities (automatically displayed on the top right side) of the signals in the STD NMR spectrum. This provides the desired information about those protons that are close to the protein and their relative proximity. The higher the relative intensity, the closer to the protein they are (**Figure 2**).

5. Measure the intensities (integrals) in the off-resonance experiment using the corresponding software. In TopSpin, go to ***Process*** **>** ***Integrate***. Define the regions and write the integrals in a file (I_0).

6. Measure the intensities (integrals) in the STD NMR experiment using the same parameters and write them in a file (I_{sat}).

7. ~~For each proton NMR signal, calculate the difference between its intensity in the off-resonance spectrum and the STD NMR spectrum (I_0- I_{sat}).~~

8. Calculate the STD value for each proton signal using the following equation:

    $STD = (I_0 - I_{sat})/I_0$.

9. Calculate the relative STD as a percentage. To do this, give a 100% value to the proton that exhibits the maximum difference between the intensities in the off-resonance and the STD NMR spectrum. Calculate the relative STD intensities for the other protons accordingly.

    NOTE: Proper analysis of STD data, especially for determining ligand binding epitope, requires the complete assignment of ^{1}H signals of the ligand. Therefore, it is strongly recommended that this task be completed before the acquisition of the STD spectra.

2. ^{1}H-^{15}N HSQC experiments

NOTE: The following lines detail the employment of ^{1}H-^{15}N HSQC experiments to monitor the changes in the chemical shifts of the ^{1}H and ^{15}N NMR resonances of the receptor (lectin) in response to the presence of increasing amounts of the ligand (oligosaccharide)[19]. The Chemical Shift Perturbation (CSP) analysis based on the extracted data is highly valuable for the identification of binding partners but also for mapping the protein binding interface and determining binding affinities. For a more profound understanding of the design and acquisition of NMR experiments, please refer to

the corresponding manufacturer's manual provided with the NMR instrument.

1. Acquisition and processing

    1. Prepare the sample with the lectin of interest. Ensure that the receptor is fully ^{15}N-labelled in every amino acid residue, both in the backbone and side chains. Typically, to detect the water-exchangeable HN cross peaks in the spectrum, employ a 90:10 mixture of H_2O: D_2O to prepare the buffered solution. The required lectin concentrations range between 0.05 and 0.2 mM, depending on the availability of the ^{15}N-labelled receptor and the necessary signal-to-noise ratio.

        NOTE: The protein should be stable during the whole experimental time without visible generation of precipitate in the NMR tube. Moreover, it should be pure and soluble in the selected buffer. The full ^{1}H and ^{15}N assignment of the HSQC cross-peaks should have been previously carried out so that every cross-peak in the HSQC spectrum is identified with a label corresponding to the specific amino acid residue.

    2. From this preparation, transfer a total volume of 0.6 mL to a 5 mm NMR tube.

    3. Set the NMR instrument at the required temperature. See step 1.1.3 and follow the same operations.

    4. Create a new dataset. See step 1.1.4 and repeat the operations.

    5. Insert the NMR sample into the probe as described in step 1.1.5.

    6. Lock on the solvent signal. To start the locking procedure, use the command ***lock*** and select the appropriate solvent from the menu. The lock signal can be traced in the lock window. Set the lock gain so the lock signal is visible in the lock window.

    7. Complete the tuning and matching process automatically (through the command ***atma***) or manually (the ***atmm*** command will open the ATM control window to adjust the wobble curve).

    8. Set the optimal shims by using the TopShim tool. Use the command ***topshim gui***. See instructions in step 1.1.8.

    9. Determine the ^{1}H 90° pulse length (as described in step 1.1.9) and the offset frequency (the command ***o1calib*** will run an interactive O1 calibration routine, retrieving the offset frequency). This later parameter is extremely important when experiments with solvent suppression schemes are employed.

    10. Create a new dataset as described in section 1.1.4. To reduce or eliminate the interference of the H2O signal, use the pulse sequence ***zgesgp***.

    11. Set up the experiment by modifying various parameters in the ***AcquPars*** window.

        1. Introduce the ^{1}H 90° pulse length and offset (o1) as previously determined, and set the number of scans (NS) at 32 and the spectral window (SW) at around 12 ppm.

        2. Determine the power level of the shaped pulse using the ***Shape*** tool available in the Topspin menu bar.

        3. Set the receiver gain with the automatic command ***rga***.

12. Acquire the experiment using the ***zg*** command and process the resulting FID to obtain the ^{1}H NMR spectrum.

13. Create a new dataset to be used for acquiring the ^{1}H-^{15}N HSQC NMR experiment. In the ***AcquPars*** tab, select the pulse program ***hsqcetfpf3gp*** available in the pulse program catalog.

14. Set up the experiment. Load the default shapes, powers, and times using the command ***getprosol***. Then, update the values of the ^{1}H 90° pulse length and offset.

15. Define the following parameters.
    1. Set the relaxation delay at 1-5 s.
    2. Set the number of scans to a multiple of 4. Usually, it is set to 8, 16, 32, or 64 to get the proper signal-to-noise ratio.
    3. Set the number of dummy scans to 128.
    4. Set the number of points in F2 to 1k, 2k, or 4k.
    5. Set the number of points in F1: the number of t_1 increments to be used. Depending on the spectral window, this is between 128 and 256.
    6. Adjust the center of the spectral window in the ^{15}N dimension to δ 117 ppm and set up the corresponding spectral width to 36 ppm. These values need to be optimized for each particular system.
    7. Set the receiver gain to avoid overflow (by using the ***rga*** command)

16. Calculate the time of the total experiment. A typical experimental time is about 1 h.

17. Type ***zgto*** send the experiment for acquisition.

    NOTE: Always check that the experiment is running properly after a few minutes.

18. Process the FID using the command ***xfb***. Carry out the baseline correction by using the command ***abs2*** and phase corrections in the ***Process*** tab. To manually phase, click on the ***adjust phase*** submenu and then select several cross-peaks of the 2D spectra. Afterwards, sequentially apply zero and first-order corrections to both rows and columns by clicking and dragging on the corresponding button. Save the phasing results.

19. Save the resulting 2D spectrum.

20. Prepare a highly concentrated stock solution of the ligand. Typical values are 50-100 mM.

21. From the highly-concentrated stock solution of the glycan, transfer the corresponding volume (a few microliters) to the NMR tube containing the receptor to get the desired protein: ligand molar ratio and record the spectra.

    NOTE: This step initiates the titration series, where the ligand is titrated into the protein sample. The appropriate protein-to-ligand ratios must be determined for each particular case. If the binding affinity is completely unknown, it is recommended to use substoichiometric amounts of the ligand in the initial points.

22. Perform steps 2.1.1 to 2.1.19 for the newly prepared sample.

23. Repeat steps 2.1.21 and 2.1.22 for samples with increasing protein-to-ligand ratios.

    NOTE: The accurate fitting of the titration series data requires the acquisition of multiple ^{1}H-^{15}N-HSQC experiments, covering a wide range of protein-to-

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ligand ratios, including those needed to achieve protein saturation.

2. Analysis

1. Visualize the processed 2D HSQC spectrum for the apo species using the appropriate software: TopSpin, MestReNova, and CCPNMR are all suitable programs for handling NMR data.

NOTE: This is the fingerprint spectrum of the protein. The observed ^{1}H and ^{15}N chemical shifts depend on the corresponding chemical environment of every amino acid, which strongly depends on the 3D structure of the protein. This spectrum is called the protein fingerprint spectrum. A well-dispersed 2D ^{1}H-^{15}N HSQC spectrum in which all the cross peaks display uniform intensities strongly suggests the presence of a well-folded protein[19].

2. Generate the list of ^{1}H and ^{15}N frequencies for all the cross peaks. The use of supplementary software, such as the CCPNMR program[20], can aid in the process.

3. Superimpose the spectrum for the first or second titration points onto that for the apo protein.

1. To do that, open the 2D spectrum corresponding to the apo state, click on the ***Multiple display*** tab and then add the second 2D spectrum. The visual inspection of both spectra provides information on the existence of interaction between the ligand and the protein.

NOTE: From the protein's perspective, the existence of binding provides changes in the chemical environment of the amino acids directly involved in the recognition event, with the concomitant chemical shift perturbations (CSP).

4. Repeat steps 2.2.2 and 2.2.3 for every titration point, generating lists of the ^{1}H and ^{15}N frequencies for all the cross peaks in the different spectra, corresponding to different protein-ligand molar ratios.

NOTE: The chemical shifts at each titration point can be measured without the need to perform any new cross-peak assignment. In the case of fast exchange regime, commonly observed in lectin:glycan interactions, one can simply follow the progressive movement of peaks throughout the titration.

5. Check that, for the last titration point, there are basically no chemical shift perturbations with respect to the previous addition. This fact is indicative that the protein binding site has been saturated with the ligand, which is in high excess.

6. Calculate the maximum chemical shift perturbations (maxCSP) using the equation below:

$$\delta_{maxCSP} = \sqrt{\frac{1}{2}[\Delta\delta_H^2 + (0.14\Delta\delta_N)^2]}$$

ΔH and $\Delta\delta_N$ are the chemical shift differences in the ^{1}H and ^{15}N frequencies between the apo state and the last titration point, respectively.

7. Plot the maximum chemical shift perturbations (maxCSP) in the vertical y-axis of a 2D plot versus the corresponding amino acid residue (in the horizontal x-axis).

8. Make a visual inspection of the amino acid residues that display the maximum CSP between the bound and the apo states of the protein. It is highly likely

that they belong to the binding site or are neighbors to it.

9. If the 3D structure of the protein is available, open the corresponding PDB with the appropriate software such as PyMOL or BIOVIA Discovery studio. These molecular visualization programs are widely used in structural biology applications. Select the residues that display the highest maxCSP (above twice the standard deviation) with a particular color to localize the putative binding site.

10. In the case of a fast exchange regime, estimate the dissociation constant (K_D) from a non-linear least-squares fit of the observed CSP for the ^1H-^{15}N HSQC cross-peaks at each point ($\Delta\delta_{obs}$) *versus* the particular protein [P] and ligand [L] concentration at that point:

$$\Delta\delta_{obs} = \Delta\delta_{\infty}\left(\frac{([P]+[L]+K_D)-\sqrt{([L]+[P]+K_D)^2-4[P][L]}}{2[P]}\right)$$

NOTE: This equation can be applied to those cross peaks that display clear isolated signals. The obtained values are averaged to provide the estimation of K_D.

Representative Results

Herein, we present a protocol for the exploitation of ^1H-STD NMR and ^1H-^{15}N HSQC experiments to unravel the details of the binding interaction between lectins and small oligosaccharides. The results obtained in the analysis of the molecular recognition of LacNac by *h*Galectin-7 (*h*Gal-7) are included, serving as an illustrative example of the successful implementation of the protocol and the effectiveness of these NMR methodologies to study the fine details of the molecular recognition process. **Figure 3** shows the ^1H-STD NMR spectrum for the interaction of LacNAc with *h*Gal-7. The existence of STD NMR signals indicates binding (**Figure 3A**). Moreover, only those signals belonging to protons in close contact with the protein show up, allowing the delineation of the binding epitope (**Figure 3B**). **Figure 4** highlights how the ^1H-^{15}N HSQC spectrum of a protein can be used as its fingerprint, and **Figure 5** illustrates the application of ^1H-^{15}N heteronuclear single quantum coherence (HSQC) titration experiments to define the chemical shift perturbation of *h*Galectin-7 backbone amide groups upon LacNAc binding. These data not only reveal the existence of interaction but also delineate the lectin's binding interface. **Figure 6** demonstrates how the analysis of the titration data enables the estimation of the binding affinity of LacNAc by *h*Galectin-7, which falls in the high micromolar range. This finding is consistent with results obtained using alternative techniques.

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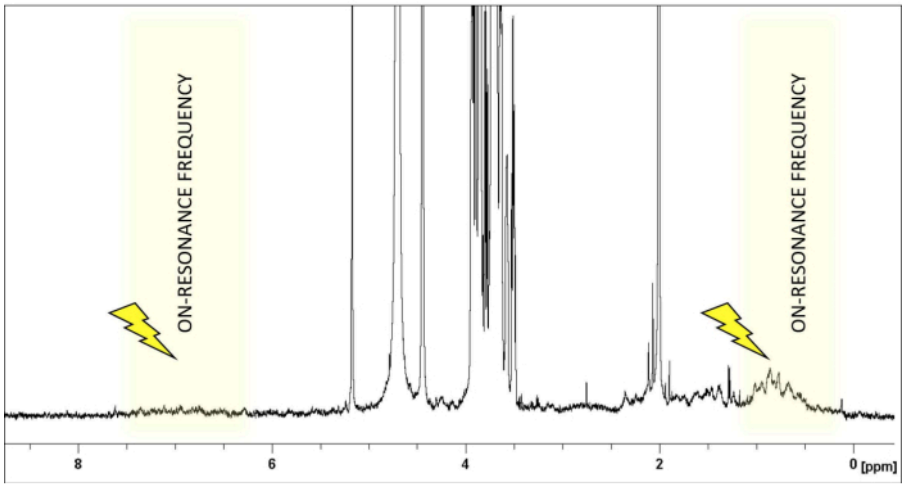

Figure 1: The selection of the on-resonance frequency. ^{1}H-NMR spectrum of LacNAc:*h*Gal-7 50:1 ratio in deuterated phosphate-buffered saline at pH 7.4 is shown. Signals of the ligand (LacNAc) are confined in the region between 2.0-5.2 ppm. The saturation frequency is carefully selected to ensure the absence of ligand protons within a 1-2 ppm range, allowing the selective irradiation of the protein's protons. Please click here to view a larger version of this figure.

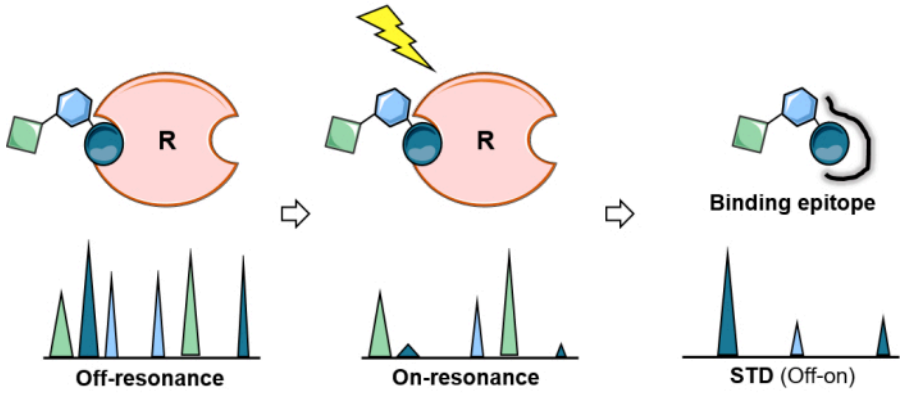

Figure 2: The STD NMR experiment. Schematic representation of the STD experiment: the first spectrum (off-resonance) serves as a reference while in the second (on-resonance), protein saturation is performed. The saturation is efficiently propagated across the entire protein and transferred to the ligand protons in close contact with the protein. The resulting difference spectrum (STD spectrum) yields only those resonances that have experienced saturation. The analysis of the STD experiment allows the epitope mapping of the binding sugar. Please click here to view a larger version of this figure.

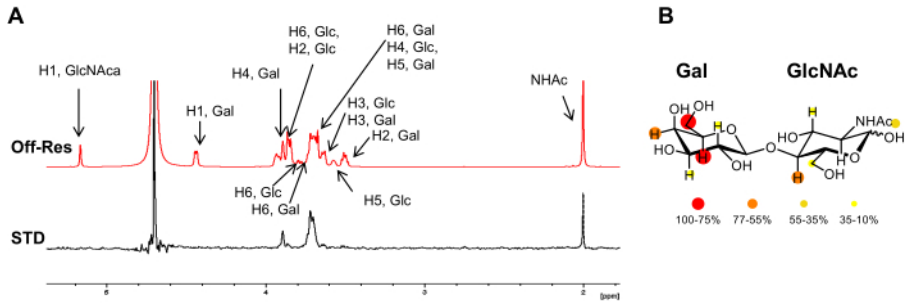

Figure 3: Binding analysis from the ligand's perspective. (**A**) Superimposition of the off-resonance and ^{1}H STD-NMR spectra for the interaction of LacNAc with *h*Gal-7. In the STD spectrum, only those signals belonging to protons in close contact with the protein show up. The annotation of the ^{1}H resonances of the ligand is reported in the off-resonance spectrum. (**B**) The relative STD intensities were colored-mapped into the chemical structure of LacNAc. Please click here to view a larger version of this figure.

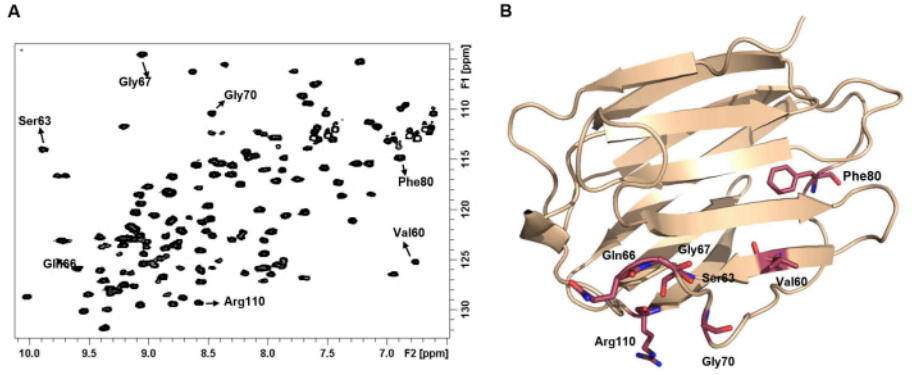

Figure 4: The ^{1}H-^{15}N HSQC spectrum of a protein represents its fingerprint. (**A**) ^{1}H-^{15}N HSQC spectrum of 100 µM of *h*Gal-7 in the apo form. The spectrum was recorded at 25 °C. Some NH cross-peaks were annotated with the label of their corresponding amino acid. (**B**) Each NH pair displays a unique chemical shift that depends on the chemical environment and consequently, on the 3D structure of the protein. Please click here to view a larger version of this figure.

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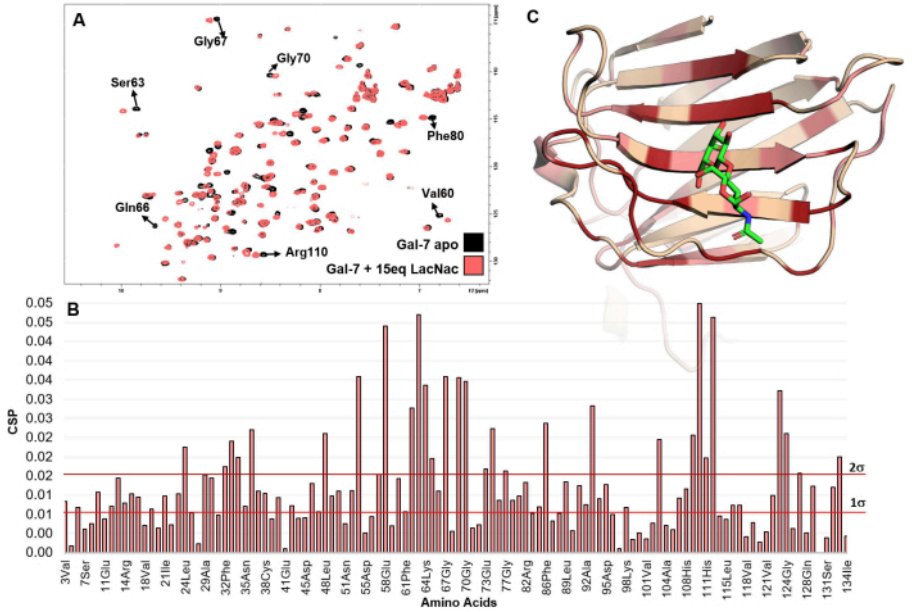

Figure 5: Binding analysis from the protein's perspective. (**A**) Superimposition of the ^{1}H-^{15}N HSQC spectra recorded for the titration of LacNAc into *h*Gal-7 solution is shown. Inspection of the spectra, where several cross-peaks experience chemical shift changes, clearly indicates interaction. (**B**) The plot of the maximum chemical shift perturbations (maxCSP) of the backbone amide signals deduced from the titration of LacNAc (15 equivalents) with *h*Gal-7. (**C**) The most perturbed amino acids of *h*Gal-7, according to the CSP analysis are mapped into the 5gal PDB structure. In the 3D model, the red coloration refers to CSP value over 2σ, whereas the pink ones to values between 1σ and 2σ. The colored region likely represents the binding site. Please click here to view a larger version of this figure.

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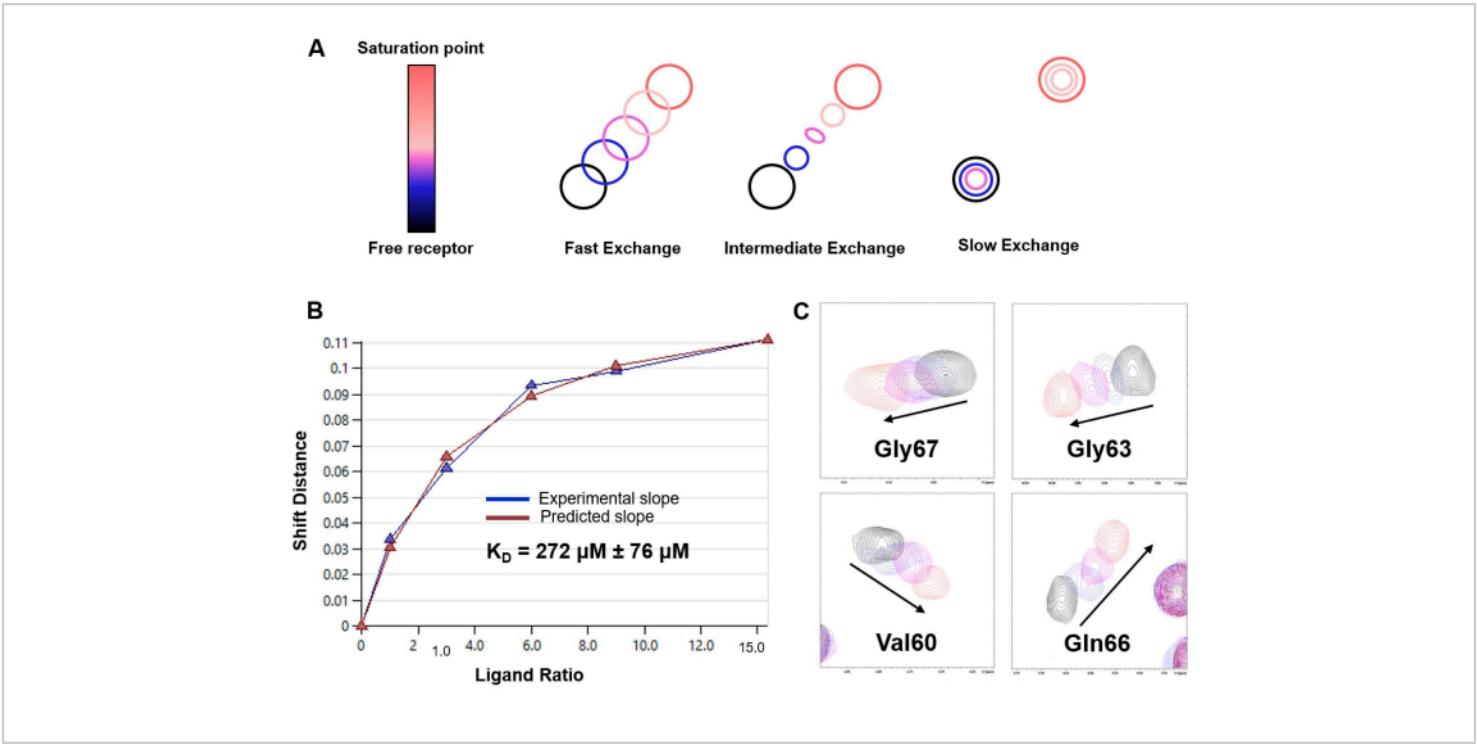

Figure 6: K_D determination based on ^{1}H-^{15}N HSQC titration experiments. (**A**) Representation of the pattern of ^{1}H-^{15}N HSQC-based titration depending on the chemical exchange rate in the NMR time scale of the system in the study (fast, intermediate, or slow). A fast exchange regime was observed in the case of LacNAc/*h*Gal-7 interaction. (**B**) Fitting curve and K_D estimation obtained from the CSP analysis at varying ligand concentrations for the model system of *h*Gal-7 and LacNAc disaccharide. The estimated K_D is reported with the corresponding error as an average of the data for 20 different amino acids; (**C**) Snippets of the ^{1}H,^{15}N-HSQC spectra displaying the shift of selected cross-peaks during the titration. Please click here to view a larger version of this figure.

Discussion

Saturation transfer difference NMR (STD-NMR) has become the most used and versatile NMR method for studying ligand-protein interactions. As shown above, it relies on the saturation transfer phenomenon, and the experimental setup involves the acquisition of two one-dimensional (1D) ^{1}H spectra: the on-resonance¬ and the ¬off-resonance spectra. During the on-resonance experiment, saturation of specific protons of the protein is achieved by applying a train of low-power radiofrequency pulses during a certain period (saturation time typically ranges from 1-3 s). To avoid direct saturation of the ligand, the frequency and length of the saturation pulses are optimized for selectively irradiating specific protons of the protein; i.e., they must be applied at a frequency vacant of any ligand signals and with an appropriate length (**Figure 1**). As a rule of thumb for 50 ms saturation pulses, 1 ppm difference should be kept from the saturation region to the closest ligand signals. Generally, selective saturation pulses applied on the aliphatic region of the protein provide increased saturation effects. Alternatively, aromatic protons (6-7 ppm) can also be irradiated if the ligand molecule does not contain any aromatic signals. This

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is very useful for naturally occurring glycans, as they do not bear aromatic groups. Once a certain region of the protein is selectively irradiated, the saturation propagates along the protein via dipolar ^{1}H-^{1}H cross-relaxation (spin diffusion). Eventually, the saturation reaches the protein protons at the binding site, which is then transferred to the sugar protons that are in close contact (r < 5 Å) with the receptor via intermolecular ^{1}H-^{1}H NOEs. Obviously, the intensity of the signals of the saturated ligand protons decreases. After receiving the saturation, due to the binding kinetics, the transiently bound ligands (fast exchange is required) dissociate and the saturation information is accumulated in the free state. Due to this process, the NMR on-resonance spectra present diminished signals (**Figure 2**).

To clearly exhibit this intensity perturbation of the ^{1}H nuclei of a binding glycan, a control proton NMR spectrum (off-resonance) is acquired in which the saturation is applied far away from any receptor or carbohydrate signal (usually between 40-100 ppm), under the same conditions. The subtracted 1D spectrum between the off-resonance and on-resonance exclusively shows the signals of the ^{1}H nuclei of the ligand that have modified intensities: those that were close enough to the receptor binding site to receive the magnetization (**Figure 2**).

Nevertheless, not all the ^{1}H nuclei of the bound carbohydrate receive the same amount of saturation. Theoretically, the magnetization transfer from the receptor to the bound ligand is distance-dependent (1/r6). This means that the intensities of transferred saturation among the glycan ^{1}H nuclei contain information on the spatial proximities between the protons of the ligand and those of the receptor, and the STD NMR intensities are larger for those protons that are closer to the receptor. Accordingly, the STD NMR experiment also allows determining the binding epitope of the carbohydrate (**Figure 2** and **Figure 3**) since protons of the ligand sitting closer to the protein surface show higher intensities than those that do not directly participate in the binding.

The experiment can be applied to systems with weak-medium affinity, rarely to systems with strong affinities in the low µM or nM range. Indeed, it requires that the dissociation rate be fast in the relaxation time scale. Otherwise, the saturation transfer information is lost through relaxation before the ligand dissociates.

On the other hand, protein-based NMR experiments are unique to unraveling ligand-protein interaction with amino acid level accuracy without solving the atomic resolution structures. It directly examines molecular recognition phenomena in solution without the need for co-crystallization. CSP analysis mapping is exceptionally powerful for discovering ligands and mapping the protein binding site (**Figure 4** and **Figure 5**). This method is applicable to any range of affinities between the mM and nM range, even for systems where the exchange rate is slow in the chemical shift time scale[21].

Nevertheless, this approach will probably not work for proteins with molecular weights above 30-40 kDa due to relaxation issues. The TROSY alternative[18] can then be used, being particularly powerful when coupled with protein deuteration. Moreover, the protein should be uniformly labeled with ^{15}N (and another sample double labeled with ^{13}C and ^{15}N to be able to complete the required backbone assignment). Therefore, protein expression conditions, including the corresponding expression system should be optimized to be able to obtain milligram amounts of protein. Proteins that display a tendency to oligomerize or aggregate are also not suitable for this analysis. The instrument used

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herein to record the NMR data is a Bruker 800 MHz spectrometer equipped with a TCI cryoprobe. It would be highly challenging to use this methodology using instruments below 600 MHz or without a cryogenic probe.

Disclosures

The authors have nothing to disclose.

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