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Title: Exploring Protein–Glycan Interactions: Advances in Nuclear Magnetic Resonance

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **10/09/2025**

When you are ready to submit your video files, please contact our Content Manager, Utkarsh Khare.

Current Protocol Length

Number of Steps: 24
Number of Shots: 49

Introduction

1.1. **Ariana Azevedo Vasconcelos:** We study protein–protein and protein–ligand interactions, focusing on integrin–disintegrin complexes. Our model explores structural dynamics and highlights the role of surface forces in mediating complex formation and interaction stability.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.2*

What significant findings have you established in your field?

1.2. **Fabio Ceneviva Lacerda de Almeida:** Our group is dedicated to the study of the role of surface forces in molecular recognition and the evolution of binding sites by studying surface hydrophobic clusters present in proteins.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.6*

What advantage does your protocol offer compared to other techniques?

1.3. **Ariana Azevedo Vasconcelos:** NMR in solution enables the detection of transient interactions. Although weak interactions are essential for life, there is a bias toward higher-affinity complexes, even though they are present in many important biological functions.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

What research questions will your laboratory focus on in the future?

1.4. **Fabio Ceneviva Lacerda de Almeida:** Our group is focusing on using integrative structural biology approaches to study large protein complex. We are using NMR as the main tool to study dynamics and functional aspects.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Mapping Ligand–Protein Interactions Using Chemical Shift Perturbation, Saturation Transfer Difference, and Relaxation NMR Techniques

Demonstrator: Ariana Azevedo Vasconcelos

2.1. To begin, prepare a four millimolar solution of D-mannose in sodium phosphate buffer at pH 7.4 with fifty millimolar sodium chloride and five percent deuterium oxide **[1]**. Divide the solution into two samples, one containing eighty micromolar CVN *(C-V-N)* and the second, without **[2-TXT]**.

2.1.1. WIDE: Talent measuring and mixing sodium phosphate buffer, sodium chloride, deuterium oxide, and D-mannose to produce a four millimolar solution.

2.1.2. Talent aliquoting the prepared solution into two separate tubes. **TXT: CVN: Cyanovirin-N**

2.2. For mapping the ligand binding through chemical shift perturbation, on the NMR spectrometer, set up the HSQCETGPSI *(H-S-Q-C-E-T-G-P-S-I)* pulse sequence for ^{1}H-^{13}C *(H-One-C-Thirteen)* HSQC *(H-S-Q-C)* acquisition **[1]**. Configure the time domain, the spectral width, carrier frequency and number of scans **[2]**.

2.2.1. SCREEN: 68674_screenshot_1 00:00-00:30

2.2.2. SCREEN: 68674_screenshot_2. 00:05-00:44
Video Editor: please speed up the video
AND
TEXT ON PLAIN BACKGROUND:
Time Domain (TD): 1024 × 128 complex points (^{1}H × ^{13}C dimensions)
Spectral Width (SW): 10.0171 ppm (6009.615 Hz) for ^{1}H, 80 ppm (12069.106 Hz) for ^{13}C
Carrier Frequencies: 4.7 ppm for ^{1}H, 75 ppm for ^{13}C
Number of Scans: 448.
Video Editor: Please play both shot side by side

2.3. Calculate chemical shift perturbation using the equation provided **[1]**.

2.3.1. SCREEN: 68674_screenshot_3. 00:21-00:44

2.4. To map ligand binding through saturation transfer difference, first create a new dataset **[1].** Configure the parameters for STD-NMR experiments by either screening different saturation frequencies **[2]** or fixing the frequency while varying saturation times to evaluate buildup **[3]**.

2.4.1. SCREEN: 68674_screenshot_4. 00:00-00:16

2.4.2. SCREEN: 68674_screenshot_5. 00:02-00:20

2.4.3. SCREEN: 68674_screenshot_6. 00:04-00:20

2.5. Set up 1D ^{1}H NMR experiments using the zgpr *(Z-G-P-R)* pulse sequence **[1].** Tune the spectrometer for ^{1}H **[2]**, perform shimming, and measure a hard 90 degree pulse **[3]**.

2.5.1. SCREEN: 68674_screenshot_7. 00:02-00:18

2.5.2. SCREEN: 68674_screenshot_8. 00:19-00:25

2.5.3. SCREEN: 68674_screenshot_9.1. 00:08-00:20

2.6. Center the carrier frequency approximately at 4.7 parts per million corresponding to the water signal **[1]**. Then select the STDDIFFESGP.3 *(S-T-D-D-I-F-F-E-S-G-P-POINT-Three)* pulse sequence and set the acquisition parameters **[2]**.

2.6.1. SCREEN: 68674_screenshot_10. 00:00-00:11

2.6.2. SCREEN: 68674_screenshot_11. 00:07-00:23

2.7. Set the spectral width as required, interscan delay d1 *(d-One)* to four seconds, and define saturation time d20 *(d-Twenty)* as per experiment goals **[1]**. Load the FQ2LIST *(F-Q-Two-List)* with off-resonance frequency at minus 40 parts per million and on-resonance frequencies to saturate protein only **[2]**.

2.7.1. SCREEN: 68674_screenshot_12. 00:10-00:30

2.7.2. SCREEN: 68674_screenshot_13. 00:05-00:22

2.8. Test the on-resonance frequencies of minus 0.59, 0.73, and 8.1 parts per million, to determine optimal conditions **[1]**.

2.8.1. SCREEN: 68674_screenshot_14. 00:04-00:28

2.9. Use the optimal frequency to acquire STD spectra at saturation times of 0.5, 1, 1.5, 2, 2.5, 3, and 4 seconds **[1]**. Plot the corresponding A_{STD} *(A-S-T-D)* values as a function of saturation time **[2].**

2.9.1. SCREEN: 68674_screenshot_15. 00:44-00:58

2.9.2. SCREEN: 68674_screenshot_16. 00:04-00:18,00:50-00:54,01:06-01:09

2.10. Now set number of scans to 64 and average the experiment loop four times **[1]**. Then calculate the total number of scans **[2-TXT]**.

2.10.1. SCREEN: 68674_screenshot_17. 00:03-00:11

2.10.2. SCREEN: 68674_screenshot_17. 00:12-00:20

TXT: Use 32,768 complex points in the direct dimension (TD), Spectral width: 10.0171 ppm (6,009.615 Hz)

AND

TEXT ON PLAIN BACKGROUND:
Total scans = ns x l4
Video Editor: Please play both shot side by side

2.11. Set interscan delay to 4 seconds and acquisition time to 2.7262976 seconds **[1]**. Configure saturation pulse by setting the duration to 50 milliseconds and control of the Gaussian shape via the shaped program nine SP9 **[2]**.

2.11.1. SCREEN: 68674_screenshot_18. 00:00-00:08

2.11.2. SCREEN: 68674_screenshot_19. 00:00-00:13

2.12. Apply a $T_1\rho$ *(T-One-rho)* filter in STD-NMR variant then set the spin-lock time d29 *(d-Twenty-nine)* based on protein size **[1-TXT].**

2.12.1. SCREEN: 68674_screenshot_20. 00:00-00:11
TXT: Use smaller times for larger proteins and vice versa

2.13. To map the ligand binding ^1H-R_2, first select the CPMG_ESGP2D *(C-P-M-G-E-S-G-P-2-D)* pulse program from the Bruker standard library **[1].** Set the experiment as a 2D acquisition **[2]**.

2.13.1. SCREEN: 68674_screenshot_21. 00:00-00:22

2.13.2. SCREEN: 68674_screenshot_21. 00:23-00:32

2.14. Configure the acquisition parameters as shown **[1].** Then run the experiment for 200 cycles of 8 scans each **[2].** Adjust the variable counter list according to the desired total time of the CPMG cycle **[3].**

2.14.1. TEXT ON PLAIN BACKGROUND:
Time domain (TD): 32,768 complex points in the direct ^1H dimension
(TD1): 2 points
Spectral width (SW): 7.9932 ppm (4,795.396 Hz)
Interscan delay (d1): 4 s
Carrier frequency (o1): 4.7 ppm (centered on water signal)
Number of scans (ns): 8
Number of dummy scans (ds): 4
CPMG delay (d20): ≤1 ms (i.e., shorter than 1⁄3 J_HH)
Number of averages (TDav): 200
Video Editor: Please play both shot side by side

2.14.2. SCREEN: 68674_screenshot_22. 00:00-00:05

2.14.3. SCREEN: 68674_screenshot_23. 00:00-00:11

2.15. Run two experiments, one for the sample with the protein and one for the ligand-only sample **[1]**.

2.15.1. SCREEN: 68674_screenshot_24. 00:03-00:26

2.16. Plot the ^{1}H spectra for each T_{CPMG} *(T-C-P-M-G)* using the command efp *(E-F-P)* or sinm *(SINE-M)* followed by fp (f-p)**.** Then adjust the window function according to the best processing strategy **[1]**. Calculate the CPMG quotient Q using the equation **[3]**.

2.16.1. SCREEN: 68674_screenshot_25. 00:00-00:35

2.16.2. SCREEN: 68674_screenshot_26. 00:02-00:19

3. Mapping the binding of D-mannose on CVN by chemical shift perturbation of the protein

Demonstrator: Fabio C. L. Almeida

3.1. Acquire $^{1}H–^{15}N$ *(H-One-N-Fifteen)* HSQC spectra of ^{15}N-labeled CVN at 298 Kelvin **[1].** Titrate with D-mannose to reach the final concentrations of 0, 1, 2, 5, 10, 20, 40, and 60 millimoles **[2]**.

3.1.1. SCREEN: 68674_screenshot_27. 00:02-00:16

3.1.2. Talent performing titrating with D-Mannose.

3.2. Use phase-sensitive FHSQCF3GPPH *(F-H-S-Q-C-F-3-G-P-P-H)* Fast-HSQC pulse sequence from the Bruker standard library **[1]**. Then calculate the chemical shift perturbation using the formula **[2].**

3.2.1. SCREEN: 68674_screenshot_28. 00:10-end
AND
TEXT ON PLAIN BACKGROUND:
Use parameters:
Time domain (TD): 1024 × 140 complex points in the ^{1}H and ^{15}N dimensions
Spectral width (SW): 16.0274 ppm (9615.385 Hz) in the ^{1}H dimension and 34 ppm (2067.119 Hz) in the ^{15}N dimension
Carrier frequency: 4.7 ppm (^{1}H) and 119 ppm (^{15}N)
Number of scans: 128
Video Editor: Please play both shot side by side

3.2.2. SCREEN: 68674_screenshot_29. 00:03-00:15

3.3. Plot CSP *(C-S-P)* values for each residue as a function of D-mannose concentration to determine dissociation constant K_D *(K-D)* using equation five **[1]**. Then fit the resulting data to a single-binding isotherm using the formula **[2].**

3.3.1. SCREEN: 68674_screenshot_30. 00:06-00:32

3.3.2. SCREEN: 68674_screenshot_31. 00:04-00:20, 00:46-00:48
AND
TEXT ON PLAIN BACKGROUND:

$$CSP = \{(\frac{CSPmax - CSPmin}{2[P_T]})\left(([P_T] + [L_T] + K_D) - \sqrt{(([P_T] + [L_T] + K_D)^2 - 4[P_T][L_T])}\right\} + CSPmin$$

$[P_T]$: protein concentration used in the titration (CVN)
$[L_T]$: ligand concentration (D-mannose)
$CSPmax$: CSP at the saturation concentration of the ligand

$CSPmin$: CSP in the absence of the ligand
Video Editor: Please play both shot side by side

4. Mapping the binding of D-mannose on CVN *via* the $^{15}N–R_2$ position of the protein

Demonstrator: Ariana Azevedo Vasconcelos

4.1. Use phase-sensitive HSQCT2ETF3GPSI3D *(H-S-Q-C-T-2-E-T-F-3-G-P-S-I-3-D)* pulse sequence and configure parameters as shown to measure the ^{15}N-R_2 values of the individual residues of CVN residues in absence and presence of 60 millimolar D-mannose at 298 Kelvin **[1]**.
NOTE: Shots merged and VO edited accordingly

4.1.1. SCREEN: 68674_screenshot_32. 00:00-end

AND
TEXT ON PLAIN BACKGROUND:
Time domain (TD): 1024 × 128 complex points in the ^1H and ^{15}N dimensions, with 8 points in the pseudo dimension
Spectral width (SW): 16.0274 ppm (^1H; 9615.385 Hz) and 34 ppm (^{15}N; 2067.119 Hz).
Carrier frequency: 4.7 ppm (^1H) and 119 ppm (^{15}N).

Number of scans: 32. (v) Variable counter list: 1, 8, 2, 6, 3, 5, 4, and 7, corresponding to relaxation delays (T_{relax}) of 16.96, 135.68, 33.92, 101.76, 50.88, 84.96, 67.84, and 118.72 ms, respectively
Video Editor: Please play both shot side by side

4.2. Process pseudo-three-dimensional spectra using NMRPipe *(N-M-R-Pipe)* to generate HSQC-like spectra for each relaxation delay **[1]**. Import the processed spectra into an analysis platform **[2]**.

4.2.1. SCREEN: 68674_screenshot_33. 00:05-00:20,01:52-01:56

4.2.2. SCREEN: 68674_screenshot_34. 00:12-00:21, 00:35-00:40

4.3. Then select all spectra and apply the **Follow Intensity Changes** tool **[1].** Plot the intensity of each cross-peak as a function of T_{relax} *(T-Relax)* and fit the decay to a mono-exponential function to extract ^{15}N-R_2 values for each residue **[2]**.

4.3.1. SCREEN: 68674_screenshot_35. 00:14-00:24

4.3.2. SCREEN: 68674_screenshot_35. 00:25-00:31

4.4. Calculate the experimental error from the signal-to-noise ratio of the HSQC-like spectra at 67.84 milliseconds **[1]**. Process a region of the spectrum containing only noise and convert it to a text file using the given command **[2-TXT]**.

4.4.1. SCREEN: 68674_screenshot_36. 00:04-00:28

4.4.2. SCREEN: 68674_screenshot_36. 00:47-00:55, 01:14-01:16
TXT: Command: pipe2txt.tcl ./PROCs/ft/R2_67_84.ft2 > noise67_84.txt

4.5. Then determine the standard deviation of the noise region or noise intensity, using statistical software **[1].** Compute the experimental error using the given equation **[2]**.

4.5.1. SCREEN: 68674_screenshot_37. 00:04-00:08, 00:13-00:23

4.5.2. SCREEN: 68674_screenshot_37. 00:28-00:43
AND
TEXT ON PLAIN BACKGROUND:

$$R_2^{error} = \frac{1}{T_{relax}} \frac{I_{noise}}{I_{each\_residue}}$$

Video Editor: Please play both shot side by side

Results

5. Results

5.1. Chemical shift perturbations were observed for both anomeric forms of D-mannose in the presence of CVN *(C-V-N)*, with greater shifts seen in β *(beta)*-D-mannose, indicating preferential binding **[1]**. Two distinct peaks were observed for most D-mannose hydrogens in the presence of CVN, corresponding to free and bound states **[2]**.

5.1.1. LAB MEDIA: Figure 1C.

5.1.2. LAB MEDIA: Figure 1C. *Video editor: Sequentially highlight blue and green points*

5.2. The hydrogen nuclei exhibiting the highest ASTD *(A-S-T-D)* values were associated with β-D-mannose, consistent with strong dipolar interactions with CVN **[1]**.

5.2.1. LAB MEDIA: Figure 2C *Video editor: Sequentially highlight the four plots with red hydrogen labels*

5.3. In the transverse relaxation rate analysis, $H_{\beta 4}$ *(H-Beta-Four)* was the only proton that exhibited a notable increase in relaxation upon binding to CVN **[1]**.

5.3.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the position labeled Hβ4 in the "D-mannose + CVN" red spectrum*

5.4. The ^{1}H and ^{15}N chemical shift perturbations, induced by the addition of a ligand was highest at ligand concentration up to 10 millimoles **[1].**

5.4.1. LAB MEDIA: Figure 4A *Video editor: Highlight the top graph labelled "D-mannose=10 mM"*

5.5. Residues I40*(I-Forty)*, E41 *(E-Forty-One)*, N42 *(N-Forty-Two)*, V43 *(V-Forty-three)*, D44 *(D-Forty-Four)*, and G45 *(G-Forty-Five)* within the β-strand showed significant chemical shift perturbations, identifying them as the high-affinity binding site for D-mannose **[1]**. Binding isotherms showed residue D44 had the highest affinity among the β-strand residues, with a dissociation constant of approximately 1 millimolar **[2]**.

5.5.1. LAB MEDIA: Figure 4A and 4B. *Video editor: Highlight the columns labelled I40, E41, N42, V43, D44, and G45 in 4A*

5.5.2. LAB MEDIA: Figure 4C. *Video editor: Highlight the D44 plot*

5.6. Measurement of ^{15}N-R_2 *(N-Fifteen-R-Two)* relaxation rates showed both increases and decreases in ΔR_2 *(Delta-R-Two)* values upon D-mannose binding, suggesting a mix of conformational stabilization and exchange processes **[1]**.

5.6.1. LAB MEDIA: Figure 5C. *Video editor: Highlight the red and blue lines*

5.7. Residues C58*(C-Fifty-Eight)*, R59 *(R-Fifty-Nine)*, K74 *(K-Seventy-Four)*, and R76 *(R-Seventy-Six)* demonstrated increased ΔR_2 values, confirming their involvement in the high-affinity binding site **[1]**, while residues F4 *(F-Four)*, C8 *(C-Eight)*, R24 *(R-Twenty-Four)*, and G27*(G-Twenty-Seven)* represented the low-affinity region **[2]**.

5.7.1. LAB MEDIA: Figure 5D. *Video editor: Highlight the data points corresponding to C58, R59, K74, and R76*

5.7.2. LAB MEDIA: Figure 5D. *Video editor: Highlight the data points labeled F4, C8, R24, and G27*

Pronunciation Guide:

1. Glycan
Pronunciation link:
https://www.merriam-webster.com/dictionary/glycan
IPA: /ˈglaɪˌkæn/
Phonetic Spelling: gly-kan

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2. Integrin
Pronunciation link:
https://www.merriam-webster.com/dictionary/integrin
IPA: /ˈɪntəgrɪn/
Phonetic Spelling: in-tuh-grin

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3. Disintegrin
Pronunciation link:
https://www.merriam-webster.com/dictionary/disintegrin
IPA: /dɪsˈɪntəgrɪn/
Phonetic Spelling: dis-in-tuh-grin

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4. Hydrophobic
Pronunciation link:
https://www.merriam-webster.com/dictionary/hydrophobic
IPA: /ˌhaɪdrəˈfoʊbɪk/
Phonetic Spelling: hy-droh-foh-bik

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5. Ligand
Pronunciation link:
https://www.merriam-webster.com/dictionary/ligand
IPA: /ˈlɪgənd/ or /ˈlaɪgænd/
Phonetic Spelling: lig-and (common) or lie-gand

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6. Mannose
Pronunciation link:
https://www.merriam-webster.com/dictionary/mannose
IPA: /ˈmænoʊs/
Phonetic Spelling: man-ohs

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7. Cyanovirin
Pronunciation link:
https://www.howtopronounce.com/cyanovirin
IPA: /saɪˌænoʊˈvɪrɪn/
Phonetic Spelling: sigh-an-oh-veer-in

8. NMR (Nuclear Magnetic Resonance)
Pronunciation link:
https://www.merriam-webster.com/dictionary/nuclear%20magnetic%20resonance
IPA: /ˈnuːkliər mægˈnɛtɪk ˈrɛzənəns/
Phonetic Spelling: new-klee-er mag-net-ik reh-zuh-nuhns

9. HSQC (Heteronuclear Single Quantum Coherence)
Pronunciation link:
No confirmed link found
IPA: /ˌhɛtərəˈnuːkliər ˈsɪŋgəl ˈkwɑntəm koʊˈhiːrəns/
Phonetic Spelling: het-er-oh-new-klee-er sin-guhl kwahn-tum koh-heer-ens

10. CPMG (Carr–Purcell–Meiboom–Gill)
Pronunciation link:
No confirmed link found
IPA: /ˈkær pɜrsəl ˈmaɪbuːm gɪl/
Phonetic Spelling: care per-suhl my-boom gill

11. Isotherm
Pronunciation link:
https://www.merriam-webster.com/dictionary/isotherm
IPA: /ˈaɪsəˌθɝm/
Phonetic Spelling: eye-so-therm

12. Resonance
Pronunciation link:
https://www.merriam-webster.com/dictionary/resonance
IPA: /ˈrɛzənəns/
Phonetic Spelling: reh-zuh-nuhns

13. Conformational
Pronunciation link:
https://www.merriam-webster.com/dictionary/conformation
IPA: /ˌkɑnfɔrˈmeɪʃənəl/
Phonetic Spelling: con-for-may-shuh-nuhl

14. Anomeric
Pronunciation link:
https://www.merriam-webster.com/dictionary/anomeric
IPA: /ˌænəˈmɛrɪk/
Phonetic Spelling: an-uh-mer-ik

15. Dipolar
Pronunciation link:
https://www.merriam-webster.com/dictionary/dipolar
IPA: /daɪˈpoʊlər/
Phonetic Spelling: dye-poh-lur