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Chemical and Biophysical Approaches for Complete Characterization of Lectin–Carbohydrate Interactions

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Abstract

Lectins are carbohydrate-binding proteins unrelated to antibodies or enzymes. While carbohydrates are present on all cells and pathogens, lectins are also ubiquitous in nature and their interactions with glycans mediate countless biological and physical interactions. Due to the multivalency found in both lectins and their glycan-binding partners, complete characterization of these interactions can be complex and typically requires the use of multiple complementary techniques. In this chapter, we provide a general strategy and protocols for chemical and biophysical approaches that can be used to characterize carbohydrate-mediated interactions in the context of individual oligosaccharides, as part of a glycoprotein, and ending with visualization of interactions with whole virions.

1. INTRODUCTION

Surface-displayed complex carbohydrate structures are ubiquitous in nature. They are displayed on cell and viral surfaces in the form of glycoproteins, glycolipids, and proteoglycans. Interactions with their receptors facilitate recognition and signaling processes throughout biology. To precisely control and regulate myriad physical interactions, lectin–carbohydrate interactions must be extremely specific and precise (Gabijs, Andre, Jimenez-Barbero, Romero, & Solis, 2011). Both lectins and carbohydrates can be viewed as multivalent molecules with lectins often containing more than one carbohydrate-binding site per monomeric subunit as well as assembling as oligomers, and carbohydrates often existing as branched or long-chain polymers. These attributes combined give rise to enormous variability; nonetheless through layers of recognition that start at the monosaccharide level and expand to include factors such as valency, density of surface-displayed glycans or receptors, and distances and orientations of binding interfaces, high degrees of specificity are achieved. To fully understand the chemical and structural basis for carbohydrate-mediated events in biology, it is necessary to characterize each layer of recognition. To achieve this, multiple complementary techniques must be employed.

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Among surface-displayed glycoproteins, the HIV envelope glycoprotein gp120 (120 kDa) is one of the most enigmatic. Asn-linked glycans make up approximately half of its molar mass (60 kDa) with the majority represented by high-mannose oligosaccharides that form a so-called glycan shield. While this glycan coat is necessary for folding and oligomerization of gp120 into fusion-competent trimers, it also appears as a primary epitope of, or is accommodated by, a growing number of anti-HIV antibodies (Burton et al., 2012; Doores, 2015; Stewart-Jones et al., 2016). HIV gp120 represents a logical target for HIV inhibitors as it facilitates virus entry into target cells by a direct association with cellular receptors such as CD4 and CCR5, and viral transport by membrane lectins such as DC- and L-SIGN (Wilen, Tilton, & Doms, 2012), and is the sole target of HIV-neutralizing antibodies (Burton et al., 2012; Doores, 2015). As new approaches to blocking HIV infection remain a priority, interest in carbohydrate-binding agents (including lectins, antibodies, natural products, and synthetic receptors) as antivirals has continued to rise. Carbohydrate-binding agents capable of binding the gp120 glycan shield have been shown to block virus infection, preventing interaction with the host (Acharya, Lusvarghi, Bewley, & Kwong, 2015). In particular, lectins that are specific for high-mannose oligosaccharides are promising candidates for microbicide development as they can block HIV infection with remarkable breadth and potency (Balzarini, 2007).

The mannose-binding lectins cyanovirin-N and griffithsin (GRFT) are among the most potent HIV inhibitors described to date (Boyd et al., 1997; Mori et al., 2005). Their interactions with soluble mannosides have been studied quite thoroughly and three-dimensional structures of those complexes have been solved (Bewley, 2001; Ziolkowska et al., 2006). Detailed descriptions of their interactions with their biological targets, such as Man₉GlcNAc₂Asn and gp120, have been more challenging in part due to limitations that arise from formation of cross-linked products. In this chapter, we use the well-studied model system of HIV-1 envelope glycoprotein gp120 and an HIV-binding therapeutic lectin GRFT to present different strategies and a general workflow employing complementary chemical and biophysical methods that allow for precise characterization of these types of interactions in the context of individual oligosaccharides, as part of a glycoprotein, and ending with visualization of interactions with whole virions (Fig. 1).

2. SELECTION AND PRODUCTION OF THE LECTIN

Many of the anti-HIV lectins described to date are of nonhuman origin and were isolated from algae, cyanobacteria, or bacteria (Hoorelbeke et al., 2010; Ziolkowska & Wlodawer, 2006). These lectins are generally amenable to heterologous expression in well-proven bacterial expression systems utilizing commercial plasmids such as the pET vectors. For many studies, lectins may be expressed by subcloning the encoding gene into an inducible expression vector. To assist with proper folding and/or excretion, solubility, and purification, it may be desirable to fuse the protein to a periplasmic secretion signal, a solubility tag, or an affinity tag, respectively. It is important to note that for structural and biophysical studies discussed here, the presence of a protein fusion tag attached to the lectin may be detrimental to some of the methods due to the introduction of artifacts in binding attributed to the tag, increasing molar masses that effect line widths in NMR studies and sedimentation coefficients in analytical ultra-centrifugation experiments. On the other hand the presence of

an affinity tag can be useful for immobilization of the lectin onto a solid surface for subsequent binding studies. These factors should be considered when choosing the expression system and downstream cleavage of the lectin from the tag.

2.1 Expression of Lectins in Bacteria

2.1.1 Equipment

- Incubator and water bath
- FPLC (GE Healthcare)
- Centrifuges

2.1.2 Protocol

1. Using standard procedures subclone a synthetic gene encoding GRFT or the lectin of interest into a pET15b vector (or equivalent expression vector of your choice). GRFT can be expressed with a hexahistidine tag at the N-terminus followed by a thrombin cleavage site. *Note:* Incorporation of two glycine residues between the thrombin cleavage site and the beginning of GRFT facilitates cleavage of the tag (Fig. 2).
2. Transform the plasmid into the chemically competent BL21(DE3) strain of *Escherichia coli* following the manufacturer's instructions.
3. Unlabeled and ^{15}N -labeled proteins, respectively, can be obtained from bacteria grown in Luria Bertani broth, or M9 minimal media supplemented with $^{15}\text{NH}_4\text{Cl}$ as the sole nitrogen source. Bacteria are grown in the presence of the antibiotic resistance marker of the expression vector (in this case ampicillin) at 37°C until the optical density reaches 0.8 A_{600} .
4. Induce protein expression by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. If the solubility of the protein is a concern, IPTG can be titrated into the media to a final concentration of 0.2–0.5 mM.
5. Harvest cells by centrifugation 5 h after addition of IPTG (ca. $5000 \times g$). *Note:* At this point the bacterial pellet can be stored at -80°C for extended periods of time.
6. Resuspend bacteria in 20 mM Tris-HCl pH 7.4, 10 mM benzamidine (or other protease inhibitor), and 8 M urea. *Note:* This protocol pertains to GRFT expression. Some lectins may not require exposure to denaturation conditions for folding and purification. Sonication or high-pressure lysis can be used to help break the bacterial cells.
7. Centrifuge the lysed cells (60 min, $20,000 \times g$).
8. Load the supernatant containing 6H-GRFT onto a Ni-chelating column equilibrated in 20 mM Tris-HCl, 200 mM NaCl, and 20 mM imidazole, pH 7.4 containing 8 M urea.

9. Wash the bound protein with 10 column volumes of the same buffer and elute with a linear gradient from 20 to 500 mM imidazole.
10. Combine fractions containing 6H-GRFT in a dialysis cassette (<5 kDa MWCO). Place the cassette in an empty beaker containing a magnetic stir bar.
11. Begin protein refolding by dropwise addition of 2 L of 20 mM Tris-HCl, pH 7.4 overnight at 4°C with stirring.
12. Remove the sample from the dialysis cassette and centrifuge the solution (3000 × g or higher) to remove any precipitated protein.
13. The hexahistidine (H6) tag can be removed by treatment with 0.75 U thrombin per mg of GRFT overnight at room temperature. *Note:* The resulting GRFT will contain two extra glycine residues at the N-terminus. NMR and functional studies have shown these to not interfere with the activity of GRFT (Lusvarghi, Ghirlando, Wong, & Bewley, 2015; Xue et al., 2012).
14. Remove any traces of uncleaved H6-GRFT by flowing the solution of protein through a Ni-chelating column. *Note:* The flow-through fraction will contain the protein lacking the H6 tag.
15. Proceed to the second step of purification by applying the protein onto a monoQ 10/100GL column equilibrated in 20 mM Tris-HCl, pH 7.4. Elute using linear gradient from 0 to 1 M NaCl, pH 7.4. Alternatively, the proteins can be purified using gel filtration chromatography with the column equilibrated and eluted in the buffer of choice. *Note:* For some mannose-binding lectins, we have found it to be necessary to add 200 mM mannose to the buffer to prevent retention of the protein on dextran-based size exclusion columns such as the Superdex series (GE Healthcare).
16. Combine GRFT-containing fractions and concentrate them in a 5 kDa molecular weight cutoff concentrator.
17. Measure protein concentrations from A_{280} values (ϵ 11,920 cm⁻¹ M⁻¹ per GRFT subunit).

3. SELECTION OF THE GLYCAN TARGET

Prior to performing experiments for analyzing lectin-carbohydrate interactions, the carbohydrate ligand for that lectin should be known. A number of methods can be used for identifying the carbohydrate-binding partner/s of lectins. Examples include screening for lectins binding to a glycan microarray (Rillahan & Paulson, 2011; Smith, Song, & Cummings, 2010) or glycans binding to a lectin microarray (Pilobello & Mahal, 2007); NMR-based titrations where full-length carbohydrates or fragments thereof are titrated into lectin solutions and detected by NMR (Bewley & Shahzad-ul-Hussan, 2013). Similarly, as discussed later, carbohydrate binding can be detected by surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) (Dam, Talaga, Fan, & Brewer, 2016; Giannetti, 2011).

3.1 Purification of Man₉GlcNAc₂ From Recombinant HIV-1 Glycoprotein gp120

Oligomanosides varying in structure and composition from Man₃GlcNAc₂ up to Man₉GlcNAc₂ (Man-9) can be difficult to obtain commercially in quantities necessary for structural and biophysical studies. Moreover, they can be prohibitively expensive. The protein soybean agglutinin, which contains one Man-9 unit per monomer, is an often-used source for obtaining quantities of Man-9. Purification requires processing large amounts of soybean flour, enzymatic digestion of the protein, and multistep chromatographic separations (Evers, Hung, Thomas, & Rice, 1998). Recently, the group of Kato and coworkers engineered a yeast cell line to produce Man-9 (Kamiya, Yamamoto, Chiba, Jigami, & Kato, 2011). For the studies described here, we developed a reliable method for purifying milligram quantities of Man-9 from recombinant gp120 produced in the presence of the A-mannosidase inhibitor kifunensine (Sastry, Bewley, & Kwong, 2015; Shahzad-UI-Hussan et al., 2017).

3.1.1 Equipment

- Centrifuges
- FPLC (GE Healthcare)
- HPLCs (Agilent Technologies)

3.1.2 Protocol

1. Denature recombinant gp120 in a solution containing 0.25% SDS and 20 mM dithiothreitol at 95°C for 10 min.
2. Adjust buffer composition by adding 0.1 vol. sodium phosphate solution (0.5 M, pH 7.5) and NP-40 detergent to a final concentration of 0.25%. Add PNGase F (NEB) to the sample (10 U nmol⁻¹ gp120).
3. Digest the protein at 37°C for 18 h, checking progress by the change in mobility of gp120 in SDS-PAGE upon deglycosylation.
4. Separate the glycan fraction from the protein and buffer components using a Sephadex 75 column (GE Healthcare), eluting in 20 mM sodium phosphate and 150 mM sodium chloride, pH 7.5. Monitor the eluent by UV absorption at 210 nm for maximum glycan sensitivity.
5. Identify fractions containing Man-9 using LC-MS. Dilute aliquots of fractions in 1 vol. acetonitrile and inject on the TSKgel Amide-80 column (TOSOH, 2 × 150 mm, 3 μm), eluting under isocratic conditions with 55% acetonitrile (0.1% TFA).
6. Combine and lyophilize fractions containing Man-9. Redissolve the sample in water.
7. Purify Man-9 from the remaining small molecule contaminants on a Superdex Peptide column (GE Healthcare), eluting in 10% acetonitrile in water with 0.1% TFA.
8. Combine fractions containing pure Man-9, determined using LC-MS as detailed in step 5, and lyophilize to obtain a pure sample of Man-9.

3.2 Strategy to Design and Synthesize Glycopeptide Oligomannose Mimics

Another approach that can be used for probing specificity, stoichiometry, and recognition of individual saccharide units by lectins entails the use of glycan mimetics. Such synthetic constructs can be extremely useful because factors such as valency, carbohydrate density, and distance between individual carbohydrate units can be designed into the final product and therefore precisely controlled. Using this approach with mannosylated glycopeptides, we were able to construct and characterize glycopeptides that recapitulated GRFT binding and kinetics with Man-9 and demonstrate that the spacing between individual mannose residues strictly controlled stoichiometric vs intermolecular GRFT-glycan binding as well as dissociation rates.

We and others have described methods that allow for the synthesis of glycopeptides that bear individual terminal mannoses (Ordanini et al., 2015; Talaga et al., 2014; Wang et al., 2008, 2011; Woller & Cloninger, 2001). We used a peptide-based scaffold; however, the scaffold can be tuned to the desired synthetic route and downstream application. In general, any scaffold containing terminal amines can be used for this purpose. A general protocol outlining the synthetic scheme used to generate designer glycopeptides containing varying numbers and displays of mannose residues is shown in Scheme 1 and Section 3.2.2. Detailed synthetic procedures can be found in Lusvarghi et al. (2015).

3.2.1 Equipment

- Chemical fume hood
- Solid-phase peptide synthesizer (CEM Liberty Blue microwave synthesizer)
- HPLCs (Agilent Technologies)

3.2.2 Protocol: Synthesis of Mannose-Containing Glycopeptides That Mimic Man-9

1. Design the peptide scaffold so that mannose units can be incorporated and chemical features including valency, intermannose distances, flexibility, and presentation can be systematically varied. Scheme 1 illustrates a linear peptide display containing three mannose units on an alternating Lys-Gly-Lys-Gly-Lys backbone. The spacing between sugar units can be varied by including or omitting glycine residues, and incorporating lysine, ornithine, or diaminopropanoic acid, for example.
2. Carry out solid-phase synthesis using *N*- α -fluorenylmethyloxycarbonyl (Fmoc)-protecting groups to build the desired scaffold. The free amine of the amino acid side chains that will be coupled to the sugar units must bear a protecting group, such as 4-methyltrityl (Mtt), that will not be cleaved during the piperidine treatments used for removal of the Fmoc-protecting groups but can be selectively deprotected before cleavage from the resin. The N-terminus of the peptide can be terminally acetylated or coupled to a desired linker or an affinity tag such as biotin (shown in Scheme 1).

3. Prepare carboxymethyl 2,3,4,6-tetra-*O*-acetyl- α -D-mannopyranose for coupling to free amino groups (Cheaib, Listkowski, Chambert, Doutheau, & Queneau, 2008). Starting with commercially available peracetylated mannose, removal of the acetate group at C-1 with hydrazine, followed by alkylation with *tert*-butyl bromoacetate, yields the protected intermediate. Hydrolysis of the *tert*-butyl ester with 50% vol. trifluoroacetic acid in methylene chloride gives carboxymethyl 2,3,4, 6-tetra-*O*-acetyl- α -D-mannopyranose in quantitative yields (Grandjean et al., 2004).
4. Remove the Mtt-protecting groups from the ω -amino group by treating the peptide resin from Step 2 with a solution containing 65% CH₂Cl₂, 20% hexafluoroisopropanol, 10% trifluoroethanol, and 5% triisopropylsilane.
5. Install the terminal mannose residues by combining the free amine peptide resin with 2.5 equiv. of carboxymethyl 2,3,4,6-tetra-*O*-acetyl- α -D-mannopyranose per unit of free primary amino group, 2.5 equiv. of HBTU, and 5 equiv. of DIEA. Under a N₂ stream allow coupling to proceed overnight.
6. Cleave the acetylated glycopeptide from the resin using trifluoroacetic acid containing 0.1% water (2 h, rt) and precipitate with cold diethyl ether. Centrifuge at 4°C and dry crude peptide in vacuo.
7. Purify the peptides using RP-HPLC eluting with a gradient of acetonitrile in 0.05% TFA in water.
8. Remove the acetyl groups from the mannose hydroxyl groups using saturated K₂CO₃ in MeOH for 2 h at rt. The fully deprotected glycopeptides can be purified directly using RP-HPLC followed by lyophilization. HR-MS and NMR confirm the composition of the final products.

4. CHARACTERIZATION OF LECTIN–CARBOHYDRATE OR LECTIN–GLYCOPEPTIDE INTERACTIONS

To obtain a complete picture of how lectins achieve affinity and specificity, it is necessary to determine their carbohydrate specificity, the stoichiometry with which they bind their carbohydrate ligands, and the rates at which binding and dissociation occur. There is no single method from which all of these molecular details can be obtained; however employing a strategic combination of the following techniques can provide a complete picture.

4.1 Isothermal Calorimetry

Isothermal calorimetry can be used to determine the thermodynamic parameters of the binding including the enthalpy, entropy, and free energy of binding, and the stoichiometry. These in turn yield equilibrium association constants calculated from the equation:

$$\Delta G = \Delta H - T \Delta S = -RT \ln K_a \quad (1)$$

where G , H , and S , respectively, are the changes in free energy, enthalpy, and entropy of binding. T is the absolute temperature and $R = 1.98 \text{ cal mol}^{-1} \text{ K}^{-1}$. One advantage of ITC is that it is a label-free approach and does not require attachment of the lectin or carbohydrate ligand to a surface. On the other hand, one disadvantage of ITC is the relatively large amounts of material it requires. This has been reduced in recent years with the development of microcalorimeters with cells having an approximate volume of 200 μL .

4.1.1 Instrumentation

- MicroCal ITC 200 (ca. 200 μL cell volume)
- Origin software

4.1.2 Protocol

1. Prepare the protein and ligand solutions in buffer containing 10–20 mM Tris–HCl, pH 7.4. *Note:* It is essential that the protein and ligand solutions have identical buffer composition, since small changes in the buffer can result in changes in the heat release during the titration, affecting the data. Extensive dialysis of the protein in the desired buffer is recommended.
2. Before every titration, it is recommended to routinely run a buffer-to-buffer titration. *Note:* This will ensure that the system is clean and working properly. Extensive cleaning of the cell and the syringe is very important to have reliable data and not to waste valuable samples.
3. You may use the MicroCal software experimental design feature for calculating the optimal concentrations for experiments. This software requires some knowledge of thermodynamic parameters such as the stoichiometry, the equilibrium affinity constant, and the associated change in heat that are not always available prior to running the first experiments. If some knowledge of the system is available, the selection of an acceptable “ C ” parameter helps determine the correct range of concentrations calculated from Eq. (2)

$$C = K \cdot M \cdot n \quad (2)$$

where K is the equilibrium association constant, M is the concentration of the macromolecule in the cell, and n is the stoichiometry parameter. The parameter C defines the shape of the experimental curve. Ideally the curve should be sigmoidal and values of C typically range between 10 and 100. For most lectins, we have found that the concentration of the protein in the cell should be 5–50 times the expected K_d , whereas the ligand in the syringe should be 10–20 times the concentration of the protein in the cell. Weaker interactions may require higher protein and ligand concentrations to obtain optimal curves.

4. Prepare at least 220 μL of the protein solution at 8–20 μM (at least 10% greater volume than that of the cell). Place the protein solution in the sample cell. To fill the cell, use a syringe with a blunt end. After drawing up the protein solution

remove all bubbles from the syringe and insert it into the cell until it touches the bottom, and then pull it slightly above the base. Dispense the sample into the cell very slowly until the surface of the solution can be seen outside the cell. The syringe is removed very slowly by making small circles touching the sides of the cell aperture. Once the end of the syringe appears, it is placed to rest at the small ridge at the opening of the cell. Excess solution can then be removed by pulling up on the plunger of the syringe. *Note:* It is very important to avoid the presence of bubbles in the cell as well as in the injector because they can cause large spikes in the data. Samples should be degassed prior to performing the titrations by placing under vacuum with stirring.

5. Prepare 50 μL of glycopeptide or oligosaccharide solutions at 100–400 μM and load into the injector using the syringe fill command under the instrument control menu. *Note:* The optimal concentration might change depending on the stoichiometry and affinity of binding.
6. Set up the experimental parameters: After inserting the injector into the cell, the advanced experiment design option allows the user to set the number of injections, the volume of each injection, the temperature, the time between injections, the initial delay, and the reference power. For titrations of glycopeptides or oligomannosides, 25 $1.5 \mu\text{L}$ injections made every 120 s were used, with an initial delay of 180 s. The reference power was set to 5–6. Stirring is always set at 1000 rpm.
7. Start the experiment. *Note:* It is important to observe the baseline at the beginning and during the experiment. Before the first injection the system should be equilibrated, with the baseline and reference power within $0.2 \mu\text{Cal s}^{-1}$ of one another. The baseline should remain stable during the entire experiment for good fitting of the data.
8. Analyze the data using the Origin software interface. The experimental data can be fit to a standard one-site model where H (enthalpy change, kcal mol^{-1}), K_a (association constant, M^{-1}), and n (number of binding sites) are set as variables. The quality of the fitting can be evaluated by the chi-squared parameter and the standard deviation where smaller values correspond to better fits. If the stoichiometry is greater than one, the fits will provide the stoichiometry if n is allowed to vary. Alternatively, n can be set to 2, for example, and the fitting rerun. Both protocols should give the same stoichiometry.
9. Titrations should be performed in triplicate to obtain statistically significant data with errors.

Expected outcome: ITC data will provide the stoichiometry and the binding affinity (K_a). These values are necessary for proposing a model for binding and interactions.

4.2 Surface Plasmon Resonance

SPR is a label-free detection method that allows analysis of molecular interactions on a surface. SPR detects changes in mass (concentration), and therefore binding, with no need

for radioactive or fluorescence labeling. The interaction kinetics data are collected in real time, enabling determination of rates of association and dissociation of interacting molecules.

Direct binding SPR methods can be used to determine equilibrium binding constants. Measurements are made using a Biacore T100 (GE Healthcare) system with a CM5 chip for immobilization. *Note:* Selection of the chip will depend on the application; however, CM5 chips bearing a thick dextran layer with free carboxyl groups are generally preferred. The lectin is first covalently linked to the carboxymethylated dextran layer using standard 1-ethyl-3-(3-dimethylamino) propyl carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) coupling strategy. It is desirable to immobilize the protein or the ligand in two neighboring flow cells (flow cells 2 and 3) at different densities, reported as RUs (response units). This can be accomplished by immobilizing two solutions whose concentrations may vary by 5- to 10-fold, giving RUs of <400 and >2000, to be used for comparative runs. It is essential that the buffer used to prepare the protein solutions and to run over the flow cells does not contain amines since they will react with the activated carboxylates on the surface of the chip and reduce the available sites for coupling. Extensive dialysis or buffer exchange columns can be used if the protein is dissolved in amine-containing buffer such as Tris.

4.2.1 Instrumentation

- Biacore T100 (GE Healthcare) was used for the experiments described later.

4.2.2 Protocol

1. Prepare two to four solutions of the protein at 2–200 $\mu\text{g mL}^{-1}$ at different pH values ranging from 4 to 6, using sodium acetate or sodium tetraborate buffers. The optimal pH for immobilization is determined using the pH scouting method in the Biacore software, where the solution that gives preconcentration values similar to the desired RUs will be optimal for coupling. (This scouting routine is based on noncovalent binding.) If two solutions with different pH values lead to comparable curves, we generally couple the solution with the higher pH because these conditions favor amine coupling vs association only. The concentration of the protein may have to be varied in order to increase or decrease the RU values to achieve optimal binding.
2. Once the optimal pH has been determined, prepare a solution of the protein (approximately 150 μM) and follow the immobilization wizard. Use EDC/NHS coupling to couple the free amines from the protein to the chip. Modify the reference cell in position 1 with ethanolamine. This flow cell will be used as a control for nonspecific binding and refractive index changes.
3. Once the immobilization is finished, prepare PBS or TBS containing 0.05% surfactant P20, pH 7.4 to run binding assay experiments.
4. Prepare glycopeptide and/or glycan samples in filtered and degassed buffer identical to the running buffer by preparing serial dilutions from stock solutions (0–10 μM).

5. Set up the experiment so glycopeptide or glycan samples are injected in a range of concentrations over the lectin surface at a flow rate of $20 \mu\text{L min}^{-1}$ for 2 min followed by flowing running buffer through the cell for 3 min. Maintain the temperature throughout the experiment at 25°C . A duplicate injection and several buffer injections (blanks) are used for a positive control and double referencing, respectively. Regeneration of the lectin ideally can be performed by injection of $10 \mu\text{L}$ of a high concentration solution ($>200 \text{ mM}$) of the monosaccharide or disaccharide that binds to the lectin. Alternatively, the sensor chip can be regenerated using a low pH solution of glycine-HCl, pH 1.5–3.5, or a high pH solution containing 10 mM NaOH , followed by running buffer. The gentlest conditions should be used for regeneration of the chip.
6. The experimental data are then analyzed using equilibrium affinity and fitted to a Langmuir 1:1 binding. Association and dissociation rates are calculated using exponential data fits based on a 1:1 binding model (BiAevaluation software).

Expected outcome: SPR data will provide measures of the association and dissociation rates. In our experience, intermolecular cross-linking plays an important role in the dissociation rates and can result in very slow, and even immeasurable, dissociation rates. Lectins that display these types of binding profiles have opposing carbohydrate-binding faces tend to aggregate viral particles and are generally the most potent HIV entry inhibitors (Lusvarghi et al., 2016).

4.3 Sedimentation Velocity Analytical Ultracentrifugation

Analytical ultracentrifugation is a label-free method that allows for the characterization of noninteracting species, such as the individual lectins, as well as interacting systems (Schuck, Zhao, Brautigam, & Ghirlando, 2016). In sedimentation velocity AUC, the sample is subjected to high centrifugal forces, typically $\sim 200,000 \times g$, and the combined processes of sedimentation and diffusion are monitored in real time through the ultraviolet–visible absorbance and/or Rayleigh interference optical detections systems. The sedimentation–diffusion process for a single noninteracting species is governed by the Lamm equation (Correia & Stafford, 2015), and combinations of this equation will describe the observed sedimentation velocity boundary for an ensemble of species (Brown & Schuck, 2006; Schuck et al., 2016). The continuous $c(s)$ distribution model, implemented in SEDFIT, is used to analyze sedimentation velocity data—here the experimental data are fitted numerically in terms of a series of Lamm equation solutions providing a signal distribution of sedimenting species, in which all species are assumed to have the same frictional ratio (Schuck, 2000). Standard protocols have been developed for sedimentation velocity data collection and these have been the subject of many reviews (Schuck et al., 2016; Zhao, Brautigam, Ghirlando & Schuck, 2013); we briefly describe the methodology used and refer the reader to these references for further details. Sedimentation experiments on lectins and their complexes with carbohydrates are usually performed in PBS at concentrations where both the absorbance (230 or 280 nm) and interference signal contributions can be monitored. The samples are prepared for sedimentation velocity analytical ultracentrifugation (SV-AUC) by a final purification step in PBS, or by dialysis against PBS, with the matching PBS used as a reference.

4.3.1 Instrumentation

- ProteomeLab Analytical Ultracentrifuge (Beckman Coulter)
- An50-Ti rotor
- Microfuge

4.3.2 Protocol

1. Prior to setting up the experiment the ProteomeLab Analytical Ultra-centrifuge (Beckman Coulter), together with the An50-Ti rotor and the monochromator arm, is equilibrated under vacuum at 20°C.
2. For each sample to be analyzed, 400 µL of the PBS reference buffer and 400 µL of the sample are loaded at room temperature in 12 mm path length, two-channel sector shaped, Epon-charcoal centerpiece cells equipped with sapphire windows. The volumes of the reference and the sample need to match, and this is achieved by loading 2×200 µL of the PBS reference and the sample sequentially, using the same pipettor and capillary tip. 3 mm path length cells are used for higher sample concentrations, and when these are used, 100 µL of reference and sample solutions are used. The cells have been cleaned and assembled prior to use, and once the reference and the sample are loaded, the cell is sealed with a plug gasket and the housing plug (Balbo, Zhao, Brown, & Schuck, 2009).
3. The rotor is removed from the analytical ultracentrifuge. The filled cells are loaded into the rotor, aligned with the rotor score marks, and the whole assembly is placed into the analytical ultracentrifuge. Thermal equilibration is critical for SV-AUC, and the system is now allowed to equilibrate under vacuum at 20°C by “running” the centrifuge at 0 rpm. The temperature on the console is observed, and once stable at 20.0°C, the system is allowed to equilibrate for an additional 1–2 h.
4. Meanwhile the ProteomeLab graphical user interface is set up to collect absorbance and interference sedimentation data for each cell. The absorbance wavelength is set to 280 or 230 nm, depending on the loading concentration, and absorbance data are set to be collected as a single replicate from 6.0 to 7.2 cm in a continuous mode with a spacing of 0.003 cm. Interference data are collected from 5.9 to 7.2 cm using laser delays and durations that are set up during rotor acceleration. A sedimentation method is programmed to collect scans continuously.
5. At thermal equilibrium, the rotor is accelerated to 50,000 rpm from the analytical ultracentrifuge console. During this time the interference laser parameters are set using the graphical user interface on the Windows computer. Once the final rotor speed is reached, the data collection method is started and allowed to proceed overnight.
6. Data collection is stopped once the sample has completely sedimented and a baseline scan is present in all cells. The folder containing all the data is transferred to another computer for analysis.

7. Sedimentation data are first processed in REDATE 1.0.1 (Ghirlando et al., 2013; Zhao, Ghirlando, Piszczek, Curth, Brautigam et al., 2013) to correct for time-stamp errors and remove truncated scans.
8. Prior to an analysis in SEDFIT 15.01c (Schuck, 2000), the protein partial specific volume is calculated based on the amino acid composition in SEDNTERP (Cole, Lary, Moody, & Laue, 2008), or the calculator utility in SEDFIT. The solvent density and viscosity are determined based on the buffer composition in SEDNTERP. These parameters are used for the subsequent $c(s)$ analysis.
9. All sedimentation scans describing the complete process of sedimentation are loaded into SEDFIT and analyzed in terms of a continuous $c(s)$ distribution. The sedimentation coefficient range is set to describe all the sedimenting materials, and in the case of lectins with carbohydrate, we set this to 0–12 S. A resolution of 0.05 S is typically used, and a maximum entropy regularization confidence level of 0.68 is set. The positions of the sample meniscus and bottom are set, along with the data-fitting limits, and a “run” is carried out to evaluate the initial values for the meniscus and frictional ratio. In the analysis, the meniscus position and the frictional coefficient are refined, along with the appropriate radially invariant and time-independent noise corrections.
10. Data refinement is carried out using a combination of both Marquardt–Levenberg and Simplex algorithms, until the best fit is obtained. The quality of the fit is evaluated using the root mean square deviation, the bitmap representation of the residuals, and the residuals histogram. The observed value of the root mean square deviation should approach a value typical for the noise in data collection.
11. The best-fit $c(s)$ distribution is evaluated, and in the case of noninteracting systems, the best-fit frictional ratio allows for a determination of the molar masses for the species observed. Sedimentation coefficients are then corrected to standard conditions at 20°C in water.

Expected outcome: The sedimentation velocity $c(s)$ profile describes the behavior of the sedimenting species in solution. The method has high resolving power and can be used to characterize a lectin preparation and determine the oligomeric state of that lectin (Fig. 3; Lusvarghi et al., 2016). It can also be used to qualitatively assess lectin binding to carbohydrates and glycopeptides (Lusvarghi et al., 2015). Through a series of experiments where the lectin and carbohydrate concentrations are changed, the method can be used to determine the binding affinities of the interaction.

4.4 Nuclear Magnetic Resonance of Lectin–Glycan Interactions

Nuclear magnetic resonance (NMR) is one of the most versatile and powerful techniques for studying protein–carbohydrate interactions. Binding can be observed at a range of timescales and affinities and can give atomic-level detail about the binding site/s on the protein, and interaction interfaces on the ligand.

4.4.1 Instrumentation

- High-field NMR. A Bruker Avance 600 was used in the experiments described here.
- Gradient shielded cryoprobes (Bruker Biospin)
- Shigemi tubes matched for H₂O/D₂O

4.4.2 Protocol

1. Prepare 150 μ M ¹⁵N-lectin (300 μ L) in 20 mM NaH₂PO₄/Na₂HPO₄, 50 mM NaCl, 10% D₂O, pH 6.8 (NMR buffer) in a Shigemi tube.
2. Record two-dimensional ¹H–¹⁵N HSQC spectra with spectral widths of approximately 12 ppm (¹H) and 37 ppm (¹⁵N) on a 500 MHz or higher field NMR spectrometer at 303K.
3. Perform NMR titrations by addition of 0.5–10.0 μ L aliquots of a 2–10 mM stock of glycan or glycopeptide. This is most easily accomplished by quickly adding, with a pipettor or a long-needled syringe, the titrant directly to the protein in the NMR tube. If using a Shigemi tube, avoid dispersing the titrant on the walls of the tube by positioning the tip in the center of the tube and dispensing rapidly. Record the spectrum after every addition. *Note:* Each experiment should be recorded with identical parameters. Therefore the smaller the volume used for titrating, the better, so as to not increase the total volume of the sample, which in turn decreases the intensity in spectra recorded with the same number of scans.
4. Use the NMRPipe software package (Delaglio et al., 1995) to process spectra and integrate peaks of the free lectin.
5. Use the autofit module of NMRPipe to pick and integrate peaks in subsequent spectra. This automated protocol will correct for slight changes in volume that occur during the titration.
6. Plot the change in intensity of each peak as a function of amino acid sequence number. For lectins that may exhibit different modes of binding, some of which may lead to aggregation, step plots (Fig. 4) that show the change in intensity normalized to free protein as a function of amino acid sequence can be advantageous compared to changes in chemical shift values that may not be observed. Overlay the step plots for each titration. For systems that are monodisperse step plots will reveal the location of the binding sites. (Backbone assignments are needed.) Amino acids close or near the binding site will have lower intensity due to a change in their chemical shift upon binding to the ligand.

Expected outcome: Depending on whether the carbohydrate is in fast or slow exchange, the cross peaks corresponding to residues involved in carbohydrate binding will either show a slow and continuous shift to the bound form or disappear and reappear at the bound form during the titration. The presence of these free and bound resonances will reveal the location of the binding site and can provide measures of affinity, exchange rates, and stoichiometry. Alternatively, if intermolecular cross-linking occurs between lectin and carbohydrate, a

decrease in intensity of all the peaks will be observed. This is due to formation of larger molecular weight species that have rapid longitudinal relaxation rates, leading to disappearance of peaks in the HSQC spectrum. For this latter example, a change in chemical shift cannot be plotted, but the decrease in intensity as a function of amino acid can be viewed with the step plots.

5. CHARACTERIZATION OF LECTINS BINDING TO HIV ENVELOPE GLYCOPROTEIN GP120

The gp120 glycoprotein can be purchased from several commercial vendors. Alternatively, different labs have been able to create scaffolds bearing high-mannose oligosaccharides that mimic this glycoprotein (Doores et al., 2010; Stewart-Jones et al., 2016; Wang et al., 2008).

5.1 An SPR Capture Technique to Distinguish Modes of Lectin Binding to HIV-1 gp120

The interaction of the lectins with gp120 can be assessed using SPR (see above). SPR can be used to determine the kinetic parameters of the binding as well as the binding constant. To obtain a direct measure of the mode of binding of different lectins, we designed a sandwich SPR experiment that reveals the ability of a lectin to cross-link individual gp120 units (Lusvarghi et al., 2016).

5.1.1 Instrumentation

- Biacore T100 (GE Healthcare) system

5.1.2 Protocol

1. Prepare the following buffer for the experiments: PBS containing 0.05% surfactant P20, pH 7.4. All samples should be prepared in filtered and degassed buffer.
2. Immobilize on one flow cell recombinant gp120 or trimeric gp140 by covalently linking the protein to a carboxymethylated dextran matrix (CM5 chip) using EDC/NHS coupling to a final loading of <250 RU. *Note:* For the SPR capture experiments a low protein density is critical to prevent proximity-based intermolecular cross-linking on the chip from occurring.
3. Prepare the reference cell by modifying it with ethanolamine. This flow cell is used as a control for nonspecific binding and refractive index changes.

Direct binding assays

4. Prepare twofold serial dilutions of lectins ranging from 2 μM to 0.25 nM.
5. Inject lectin solutions over gp140 surface at a flow rate of 20 $\mu L \text{ min}^{-1}$ for 1 min followed by flowing running buffer through the cell for 3 min. *Note:* A duplicate injection and several buffer injections (blanks) are used for a positive control and double referencing, respectively.
6. Regenerate the sensor chip by injecting 2 M D-mannose and running through the cell for 30 s, followed by running buffer. *Note:* Other buffers, such as those with

low or high pH (see Section 4.2.2), can be tested if high concentrations of mannose do not regenerate the surface.

Capture binding assays

7. Inject a 100 nM solution of lectin over the chip for 60 s at a flow rate of 20 $\mu\text{L min}^{-1}$. This will form the immobilized gp140:lectin complex.
8. Allow unbound lectin to dissociate for 120 s by flowing running buffer at 20 $\mu\text{L min}^{-1}$.
9. Carry out a second injection of gp140 over the gp140:lectin surface for 1 min using twofold serially diluted gp140 solutions ranging from 0 to 40 nM.
10. Once the second injection is finished, continue to flow the running buffer over the chip for 3 min to measure the dissociation of the second injection of gp140 from the chip surface. *Note:* Again, a duplicate injection and several buffer injections (blanks) are used for a positive control and double referencing, respectively. The overall binding is calculated as the difference of the curve with and without the second gp140 injection.
11. Regenerate the sensor chip by injecting 2 M D-mannose (Sigma) for 30 s, followed by running buffer.
12. Association (k_{on}) and dissociation (k_{off}) rates of the lectins can be determined using a 1:1 binding curve. You may find that the fitting is not optimal due to the complexity of the system and/or extremely slow dissociation rates. Relative responses obtained by comparing lectins among one another nevertheless provide valuable information about the modes of binding.
13. For these multistep binding assays, it is always recommended to repeat experiments at higher and lower gp140 densities. This allows one to determine whether binding artifacts are occurring and to select the experimental conditions that are artifact free. Similar sensorgrams obtained with different RU values will indicate that no rebinding or mass transfer effects were taking place on either chip.

Expected outcome: The ability of lectins to cross-link individual gp120 units is assessed in this SPR capture experiment. Cross-linking of gp120 units can occur within the same viral surface or between different viral particles. These types of data were invaluable for showing that the geometry of glycan-binding sites plays an important role in this process and controls whether envelope spikes are clustered upon the viral membrane, or aggregation of viral particles mediated by lectin tethering occurs (Lusvarghi et al., 2016; Fig. 5).

6. CHARACTERIZATION OF LECTINS BINDING TO AND THEIR EFFECTS ON WHOLE VIRIONS

To perform experiments with HIV virions, either purified inactivated whole HIV virions or pseudotypes can be used for experiments.

6.1 Dynamic Light Scattering

Dynamic light scattering is a label-free method that allows for a measure of the hydrodynamic radius of a single species, or the hydrodynamic radius distribution of an ensemble of macromolecules. Fluctuations in the intensity of scattered light, occurring on the timescale of micro- to milliseconds, are analyzed to produce an autocorrelation function that reports on the translational diffusion of the species under study (Inagaki, Ghirlando, & Grisshammer, 2013). The translational diffusion coefficient is related to the hydrodynamic diameter through the Stokes–Einstein relation, allowing for an analysis in terms of a hydrodynamic diameter.

6.1.1 Instrumentation—DLS measurements were performed on a Brookhaven Instruments Corp. BI-200 goniometer equipped with a BI-9000AT digital autocorrelator and a Spectra Physics Stabilite 2017 argon ion laser operating in the TEM₀₀ mode at 488 nm and constant power.

6.1.2 Protocol

1. Incubate solutions containing 2×10^{11} virions mL⁻¹ and 15 μ M lectin at room temperature for 30 min. *Note:* We have found that concentration as well as incubation time can affect the degree of aggregation of the viral particles.
2. DLS autocorrelation functions can be accumulated in batch mode for 2–4 min at an angle of 90°, and 20°C. The photomultiplier tube aperture is set to 200 μ m, and the laser intensity is adjusted such that the count rate does not exceed 10^6 counts per second. Autocorrelation delay times are set from 5 μ s to 50 ms to report on all the species in solution, and the count rate history and values of the measured and calculated baselines are monitored during data collection to ensure data quality. Samples for DLS are prepared in disposable, clean glass test tubes (12 $\times$ 75 mm, Fisher).
3. The intensity autocorrelation functions obtained can be analyzed in terms of a regularized CONTIN distribution of hydrodynamic radii using the Brookhaven Instruments 9KDLSW 2.12 software (Fig. 6; Provencher, 1982a, 1982b).

Expected outcome: DLS experiments complement sandwich SPR experiments, since they directly address the issue of whether or not a lectin can cross-link gp120 units within the same virus or between different viruses. Formation of virus aggregates will be an indication of the lectin's ability to cross-link gp120 units on different viruses.

6.2 Transmission Electron Microscopy

Electron microscopy can be used to directly visualize the effects that different lectins have on viral particles. It can also be used for immunostaining of viral particles to provide ancillary evidence that morphological changes are mediated by lectin binding.

6.2.1 Instrumentation—Transmission electron microscopy (TEM) experiments were performed on an FEI Morgagni transmission electron microscope.

6.2.2 Protocol for TEM

1. Incubate 2×10^{10} virions mL^{-1} combined with 1–10 μM lectin at room temperature for 30 min.
2. Prior to beginning the adsorption and staining process, prepare all reagents in advance and have tweezers and lint-free wipes (such as KimWipes) setup.
3. Absorb 5 μL aliquots of lectin-treated virions onto a Lacey carbon film on a 300-mesh copper grid (Ted Pella).
4. Remove solution by touching the side of the grid with a tissue paper. *Note:* When wicking excess liquid from the grid, use a lint-free wipe and avoid touching the surface where viral particles are absorbed.
5. Rinse with 5 μL of PBS two to three times. *Note:* Work quickly, the grid cannot be allowed to dry between any step.
6. Expose lectin-treated virions to 5 μL 0.5% uranyl acetate for 1 min for negative staining. Staining can be optimized by varying the concentration of uranyl acetate and staining time.
7. Acquire images with an FEI Morgagni transmission electron microscope, operating at 80 kV, and equipped with an ATM Advantage camera. Images were recorded at $11,000 \times$ magnification.

6.2.3 Protocol for Immunostaining

1. Perform steps 1–5 from Protocol 6.2.2.
2. To the particles adsorbed to the grid, add 5 μL of a primary antilectin antibody at a 1:1000 dilution (the dilution may have to be adjusted depending on the source and purity of the antibodies).
3. Incubate for 1 h at room temperature in a humidified chamber.
4. Wash three times with 5 μL PBS.
5. Add secondary antibodies labeled with 6 nm gold nanoparticles (Nanoprobes, Yaphank, NY).
6. Allow particles to bind to virions for 1 h at room temperature.
7. Wash two times with 5 μL PBS and one time with 5 μL water.
8. Expose immunostained virions to 5 μL of 0.5% uranyl acetate for 1 min for negative staining.
9. Acquire images with an FEI Morgagni transmission electron microscope, operating at 80 kV, and equipped with an ATM Advantage camera. Images were recorded at $11,000 \times$ magnification.

Expected outcome: These experiments allow direct visualization of the effects of lectins on viral particles. When treated with lectins that can cross-link HIV Env trimers aggregated viral particles may be observed; on the contrary for particles treated with lectins whose

glycan-binding sites are cofacial, clustering of viral spikes on the surfaces of individual virions can be observed.

6.3 HIV Neutralization Assays

When performing any biophysical or structural studies on a protein of interest, especially if it was produced recombinantly, the protein should be tested in a functional assay to ensure that it is correctly folded and active. Single-round HIV neutralization assays have been used for years by dozens of laboratories making them a well-proven ancillary method for establishing protein inhibition and/or function. Protocols for preparing viral particles pseudotyped with the desired HIV strains have been published (Li et al., 2005), as have protocols for performing single-round HIV, SIV, and SHIV infectivity assays (Montefiori, 2005). For those new to this assay, a brief protocol and templates appear below that should be helpful for setting up the plate and optimizing the assay. Most reagents have been made available at no cost by the NIH AIDS Reagent Program (www.aidsreagents.org).

6.3.1 Instrumentation

- Biosafety cabinet
- CO₂ incubator
- Benchtop centrifuge
- Luminescence plate reader

6.3.2 Protocol

1. Culture and split TZM-bl cells (that constitutively express HIV receptors and coreceptors), and prepare a solution 0.5×10^6 cells mL⁻¹.
2. Prepare the 96-well plate as described in Fig. 7. In brief, dispense the appropriate volume of media into each well, and add solvent (or buffer) or lectin dissolved in solvent (or buffer) to wells 2 and 11, respectively. Pipette up and down to mix. Perform serial dilutions by removing 10 µL of media containing lectin from well 11 and adding to well 10; mix by pipetting solution up and down, and repeat until well 3 is reached. After mixing the solution in well 3, discard 10 µL of solution from well 3. *Note:* Rows A and H should not be used since uneven evaporation and/or heating can occur in these cells. Perform each experiment in triplicate for each sample.
3. Add viruses and cells.
4. Incubate overnight at 37°C in a 5% CO₂ atmosphere.
5. After 18–24 h add 150 µL of cDMEM to each well.
6. After 18–24 h aspirate media from cells being careful not to remove or disturb cell layers.
7. Add 50 µL of Glo-lysis buffer.
8. Shake for 15 min.

9. Take 30 μL from each well and place it in a clean black plate used for luminescence plate readers.
10. Add 50 μL of substrate per well.
11. Read luminescence.
12. Plot the percentage of infection as a function of lectin concentration (use a logarithmic scale for the concentration).
13. To obtain the 50% inhibitory dose (IC_{50}) the data can be fit to the equation

$$y = a(1 + x/b)^{-1} \quad (3)$$

where a is the percentage of infection in the absence of any lectin and b is the IC_{50} value.

Expected outcome: The neutralization assays are used to confirm function and activity of recombinant lectins and give numerical values of potency for each.

7. CONCLUDING REMARKS

The glycosciences have enjoyed a renaissance in recent years, due to enabling tools such as lectin and glycan microarrays, and technologies including genomics, proteomics, transcriptomics, and mass spectrometry. Nevertheless, the complexity of lectin–glycan interactions is inherent and careful characterization of these complexes will continue to pose challenges, especially if detailed properties such as affinity, stoichiometry, and higher order interactions are to be dissected. In this chapter, we have provided strategies in the way of chemical and biophysical approaches, along with general workflows for researchers wishing to navigate any or all levels of lectin–glycan interactions. Though the approaches vary in the skill required to perform them, many universities and research institutions now have core facilities designed to help with many of the experiments described here. Given the increasing appreciation for the roles glycans play in biology, our ability to define and even visualize these interactions makes this an exciting time for researchers working in this area.

REFERENCES

- Acharya P, Lusvarghi S, Bewley CA, & Kwong PD (2015). HIV-1 gp120 as a therapeutic target: Navigating a moving labyrinth. *Expert Opinion on Therapeutic Targets*, 19(6), 765–783. [PubMed: 25724219]
- Balbo A, Zhao H, Brown PH, & Schuck P (2009). Assembly, loading, and alignment of an analytical ultracentrifuge sample cell. *Journal of Visualized Experiments*, 33, e1530.
- Balzarini J (2007). Carbohydrate-binding agents: A potential future cornerstone for the chemotherapy of enveloped viruses? *Antiviral Chemistry & Chemotherapy*, 18(1), 1–11. [PubMed: 17354647]
- Bewley CA (2001). Solution structure of a cyanovirin-N:Man alpha 1–2Man alpha complex: Structural basis for high-affinity carbohydrate-mediated binding to gp120. *Structure*, 9(10), 931–940. [PubMed: 11591348]
- Bewley CA, & Shahzad-ul-Hussan S (2013). Characterizing carbohydrate-protein interactions by nuclear magnetic resonance spectroscopy. *Biopolymers*, 99(10), 796–806. [PubMed: 23784792]

- Boyd MR, Gustafson KR, McMahon JB, Shoemaker RH, O'Keefe BR, Mori T, et al. (1997). Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: Potential applications to microbicide development. *Antimicrobial Agents and Chemotherapy*, 41(7), 1521–1530. [PubMed: 9210678]
- Brown PH, & Schuck P (2006). Macromolecular size-and-shape distributions by sedimentation velocity analytical ultracentrifugation. *Biophysical Journal*, 90(12), 4651–4661. [PubMed: 16565040]
- Burton DR, Ahmed R, Barouch DH, Butera ST, Crotty S, Godzik A, et al. (2012). A blueprint for HIV vaccine discovery. *Cell Host & Microbe*, 12(4), 396–407. [PubMed: 23084910]
- Cheah R, Listkowski A, Chambert S, Doutheau A, & Queneau Y (2008). Synthesis of new mono- and disaccharidic carboxymethylglycoside lactones (CMGLs) and their use toward 1,2-bisfunctionalized carbohydrate synthons. *Tetrahedron: Asymmetry*, 19(16), 1919–1933.
- Cole JL, Lary JW, Moody TP, & Laue TM (2008). Analytical ultracentrifugation: Sedimentation velocity and sedimentation equilibrium. *Methods in Cell Biology*, 84, 143–179. [PubMed: 17964931]
- Correia JJ, & Stafford WF (2015). Sedimentation velocity: A classical perspective. *Methods in Enzymology*, 562, 49–80. [PubMed: 26412647]
- Dam TK, Talaga ML, Fan N, & Brewer CF (2016). Measuring multivalent binding interactions by isothermal titration Calorimetry. *Methods in Enzymology*, 567, 71–95. [PubMed: 26794351]
- Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, & Bax A (1995). NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *Journal of Biomolecular NMR*, 6(3), 277–293. [PubMed: 8520220]
- Doores KJ (2015). The HIV glycan shield as a target for broadly neutralizing antibodies. *The FEBS Journal*, 282(24), 4679–4691. [PubMed: 26411545]
- Doores KJ, Fulton Z, Hong V, Patel MK, Scanlan CN, Wormald MR, et al. (2010). A nonself sugar mimic of the HIV glycan shield shows enhanced antigenicity. *Proceedings of the National Academy of Sciences of the United States of America*, 107(40), 17107–17112. [PubMed: 20852065]
- Evers DL, Hung RL, Thomas VH, & Rice KG (1998). Preparative purification of a high-mannose type N-glycan from soy bean agglutinin by hydrazinolysis and tyrosinamide derivatization. *Analytical Biochemistry*, 265(2), 313–316. [PubMed: 9882408]
- Gabius HJ, Andre S, Jimenez-Barbero J, Romero A, & Solis D (2011). From lectin structure to functional glycomics: Principles of the sugar code. *Trends in Biochemical Sciences*, 36(6), 298–313. [PubMed: 21458998]
- Ghirlando R, Balbo A, Piszczek G, Brown PH, Lewis MS, Brautigam CA, et al. (2013). Improving the thermal, radial, and temporal accuracy of the analytical ultracentrifuge through external references. *Analytical Biochemistry*, 440(1), 81–95. [PubMed: 23711724]
- Giannetti AM (2011). From experimental design to validated hits a comprehensive walk-through of fragment lead identification using surface plasmon resonance. *Methods in Enzymology*, 493, 169–218. [PubMed: 21371592]
- Grandjean C, Santraine V, Fardel N, Polidori A, Pucci B, Gras-Masse H, et al. (2004). Efficient preparation of carbohydrate and related polyol-amphiphiles by hydrazone ligation. *Tetrahedron Letters*, 45(17), 3451–3454.
- Hoorelbeke B, Huskens D, Ferir G, Francois KO, Takahashi A, Van Laethem K, et al. (2010). Actinohivin, a broadly neutralizing prokaryotic lectin, inhibits HIV-1 infection by specifically targeting high-mannose-type glycans on the gp120 envelope. *Antimicrobial Agents and Chemotherapy*, 54(8), 3287–3301. [PubMed: 20498311]
- Inagaki S, Ghirlando R, & Grishammer R (2013). Biophysical characterization of membrane proteins in nanodiscs. *Methods*, 59(3), 287–300. [PubMed: 23219517]
- Kamiya Y, Yamamoto S, Chiba Y, Jigami Y, & Kato K (2011). Overexpression of a homogeneous oligosaccharide with ¹³C labeling by genetically engineered yeast strain. *Journal of Biomolecular NMR*, 50(4), 397–401. [PubMed: 21698488]
- Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, et al. (2005). Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for

- standardized assessments of vaccine-elicited neutralizing antibodies. *Journal of Virology*, 79(16), 10108–10125. [PubMed: 16051804]
- Lusvarghi S, Ghirlando R, Wong CH, & Bewley CA (2015). Glycopeptide mimetics recapitulate high-mannose-type oligosaccharide binding and function. *Angewandte Chemie International Edition*, 54(19), 5603–5608. [PubMed: 25776945]
- Lusvarghi S, Lohith K, Morin-Leisk J, Ghirlando R, Hinshaw JE, & Bewley CA (2016). Binding site geometry and subdomain valency control effects of neutralizing Lectins on HIV-1 viral particles. *ACS Infectious Diseases*, 2(11), 882–891. [PubMed: 27669574]
121117.).
- Mori T, O'Keefe BR, Sowder RC, 2nd, Bringans S, Gardella R, Berg S, et al. (2005). Isolation and characterization of griffithsin, a novel HIV-inactivating protein, from the red alga *Griffithsia* sp. *The Journal of Biological Chemistry*, 280(10), 9345–9353. [PubMed: 15613479]
- Moulaei T, Shenoy SR, Giomarelli B, Thomas C, McMahon JB, Dauter Z, et al. (2010). Monomerization of viral entry inhibitor griffithsin elucidates the relationship between multivalent binding to carbohydrates and anti-HIV activity. *Structure*, 18(9), 1104–1115. [PubMed: 20826337]
- Ordanani S, Varga N, Porkolab V, Thepaut M, Belvisi L, Bertaglia A, et al. (2015). Designing nanomolar antagonists of DC-SIGN-mediated HIV infection: Ligand presentation using molecular rods. *Chemical Communications*, 51(18), 3816–3819. [PubMed: 25648900]
- Pilobello KT, & Mahal LK (2007). Lectin microarrays for glycoprotein analysis. *Methods in Molecular Biology*, 385, 193–203. [PubMed: 18365713]
- Provencher SW (1982a). CONTIN—A general purpose constrained regularization program for inverting noisy linear algebraic and integral equations. *Computer Physics Communications*, 27(3), 229–242.
- Provencher SW (1982b). A constrained regularization method for inverting data represented by linear algebraic or integral equations. *Computer Physics Communications*, 27(3), 213–227.
- Rillahan CD, & Paulson JC (2011). Glycan microarrays for decoding the glycome. *Annual Reviews in Biochemistry*, 80, 797–823.
- Sastry M, Bewley CA, & Kwong PD (2015). Effective isotope labeling of proteins in a mammalian expression system. *Methods in Enzymology*, 565, 289–307. [PubMed: 26577736]
- Schuck P (2000). Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. *Biophysical Journal*, 78(3), 1606–1619. [PubMed: 10692345]
- Schuck P, Zhao H, Brautigam CA, & Ghirlando R (2016). *Basic principles of analytical ultracentrifugation*. Boca Raton: CRC Press, Taylor & Francis Group.
- Shahzad-UI-Hussan S, Sastry M, Lemmin T, Soto C, Loesgen S, Scott DA, et al. (2017). Insights from NMR spectroscopy into the conformational properties of man-9 and its recognition by two HIV binding proteins. *Chembiochem*, 18(8), 764–771. [PubMed: 28166380]
- Smith DF, Song X, & Cummings RD (2010). Use of glycan microarrays to explore specificity of glycan-binding proteins. *Methods in Enzymology*, 480, 417–444. [PubMed: 20816220]
- Stewart-Jones GBE, Soto C, Lemmin T, Chuang GY, Druz A, Kong R, et al. (2016). Trimeric HIV-1 Env structures define glycan shields from clades A, B, and G. *Cell*, 165(4), 813–826. [PubMed: 27114034]
- Talaga ML, Fan N, Fueri AL, Brown RK, Chabre YM, Bandyopadhyay P, et al. (2014). Significant other half of a glycoconjugate: Contributions of scaffolds to lectinglycoconjugate interactions. *Biochemistry*, 53(27), 4445–4454. [PubMed: 24950232]
- Wang SK, Liang PH, Astronomo RD, Hsu TL, Hsieh SL, Burton DR, et al. (2008). Targeting the carbohydrates on HIV-1: Interaction of oligomannose dendrons with human monoclonal antibody 2G12 and DC-SIGN. *Proceedings of the National Academy of Sciences of the United States of America*, 105(10), 3690–3695. [PubMed: 18310320]
- Wang X, Matei E, Deng L, Ramstrom O, Gronenborn AM, & Yan M (2011). Multivalent glyconanoparticles with enhanced affinity to the anti-viral lectin cyanovirin-N. *Chemical Communications*, 47(30), 8620–8622. [PubMed: 21720651]
- Wilens CB, Tilton JC, & Doms RW (2012). HIV: Cell binding and entry. *Cold Spring Harbor Perspectives in Medicine*, 2(8), a006866. [PubMed: 22908191]

- Woller EK, & Cloninger MJ (2001). Mannose functionalization of a sixth generation *dendrimer*. *Biomacromolecules*, 2(3), 1052–1054. [PubMed: 11710009]
- Xue J, Gao Y, Hoorelbeke B, Kagiampakis I, Zhao B, Demeler B, et al. (2012). The role of individual carbohydrate-binding sites in the function of the potent anti-HIV lectin griffithsin. *Molecular Pharmaceutics*, 9(9), 2613–2625. [PubMed: 22827601]
- Zhao H, Brautigam CA, Ghirlando R, & Schuck P (2013). Overview of current methods in sedimentation velocity and sedimentation equilibrium analytical ultracentrifugation. *Current Protocols in Protein Science*, 71, 20.12.1–20.12.49.
- Zhao H, Ghirlando R, Piszczek G, Curth U, Brautigam CA, & Schuck P (2013). Recorded scan times can limit the accuracy of sedimentation coefficients in analytical ultracentrifugation. *Analytical Biochemistry*, 437(1), 104–108. [PubMed: 23458356]
- Ziolkowska NE, O’Keefe BR, Mori T, Zhu C, Giomarelli B, Vojdani F, et al. (2006). Domain-swapped structure of the potent antiviral protein griffithsin and its mode of carbohydrate binding. *Structure*, 14(7), 1127–1135. [PubMed: 16843894]
- Ziolkowska NE, & Wlodawer A (2006). Structural studies of algal lectins with anti-HIV activity. *Acta Biochimica Polonica*, 53(4), 617–626. [PubMed: 17128290]

Fig. 1.

Schematic showing the increasing scale of intermolecular interactions covered in this chapter. They range from detecting and characterizing a single sugar bound to a lectin, up to complex macromolecular interactions between networks of lectins and viral particles, all mediated by protein–carbohydrate interactions.

Fig. 2.
Construct used for recombinant expression of GRFT from *Escherichia coli* using a pET vector.

Fig. 3. Analytical ultracentrifugation of lectin and lectin–glycan complexes. (A) Absorbance (280 nm) sedimentation velocity data for a solution of 100 μ M GRFT in PBS. Data were collected at 50,000 rpm and 20°C in a 3-mm path length cell and analyzed in terms of a $c(s)$ distribution with a sedimentation coefficient range of 0–5 S. The *top panel* shows the experimental data—for clarity every third data point and every third scan from 1 to 140 are shown. This range of scans describes the complete sedimentation process. The *solid lines* show the best-fit $c(s)$ model describing the data. Residuals are overlaid in the *bottom panel* and depicted in the bitmap form in the *gray image* where each pixel depicts the residual in the *gray scale format* for each data point, with scans (time) on the ordinate axis, and radius on the abscissa. Large positive and negative residuals will be depicted as *white* or *black pixels*. In this experiment a value of 3 milli-absorbance units is obtained for the root mean square deviation. (B) The corresponding $c(s)$ distribution for 100 μ M GRFT (*red curve*) showing the presence of a single species at 2.62 S with a molar mass of 26.5 kDa, indicative of a dimer. Studies on mGRFT at 38 μ M (*blue curve*) show the presence of a single species at 1.83 S with a mass of 14.0 kDa, consistent with a monomer. All data were plotted in GUSSE. From Brautigam, C. A. (2015). *Calculations and publication-quality illustrations for analytical ultracentrifugation data*. *Methods in Enzymology*, 562, 109–133.

A Stoichiometric binding

B Intermolecular cross-linking

Fig. 4.

NMR titrations and step plots showing different modes of binding for GRFT to glycopeptide and Man-9. (A) Left panel shows ^1H - ^{15}N HSQC spectra for free GRFT (*black*) and GRFT bound to a trimannosyl linear glycopeptide. Stoichiometric binding is observed, and there is no aggregation (confirmed by AUC and ITC). Step plots (*middle*) showing the change in intensity upon binding for each residue, and revealing binding of all three carbohydrate-binding sites per GRFT monomer. Note that a change in intensity may correspond to loss of signal or change in chemical shift. The data support a model of stoichiometric binding depicted in the *cartoon at right*. (B) *Left panel* shows ^1H - ^{15}N HSQC spectra for free GRFT (*black*) and GRFT bound to Man-9 (*blue*). Loss of cross peaks is observed with substoichiometric addition of Man-9 (*center step plot*), indicating inter-molecular cross-linking and aggregation. In contrast to stoichiometric trivalent binding, a model where two arms of Man-9 engage two of three GRFT-binding sites, while a separate arm engages the third site. This leads to an intermolecular cross-linked network for GRFT binding to Man-9 or branched glycopeptides (Lusvarghi et al., 2015).

Fig. 5. SPR capture assay. (A) *Top panel* illustrates the experimental design for introducing soluble lectin (*blue*) over immobilized trimeric gp140 (*gray*), followed by introducing a second injection of gp140 that is captured by lectins that are capable of mediating viral aggregation. SPR data are shown in (B) and (C) for GRFT and its monomeric variant mGRFT, (Moulaei et al., 2010) that poorly neutralized and does not support viral aggregation.

Fig. 6.

Dynamic light scattering of GRFT free and in the presence of viral particles. (A) Dynamic light scattering intensity autocorrelation functions for solutions containing 2×10^{11} virions mL^{-1} in the absence (*blue*) and presence (*red*) of $15 \mu\text{M}$ GRFT. Note the shift to increased autocorrelation times. (B) The autocorrelation functions were transformed into a regularized distribution of hydrodynamic radii using the program CONTIN. These intensity distributions illustrate the propensity of GRFT to bring about virion aggregation.

Fig. 7.

(A) Representation of the distribution of samples and controls in a 96-well plate. Column 1: cells treated with pseudotypes but not lectin (positive control); Column 2: buffer control; Columns 3–11: lectin in increasing concentrations from 3 to 11; Column 12: cells with no virus, negative control. Rows A and H have media to minimize evaporation in wells on the inside of the plate. (B) Volumes of each component added to the different wells on the 96-well plate.

Scheme 1.

Overview of the synthesis of linear mannosylated peptides. Conditions: (A) dichloromethane:hexafluoroisopropanol:trifluoroethanol:triisopropylsilane (DCM:HFIP:TFE:TIS) (12:4:2:1) 3 times 30 min each; (B) carboxymethyl 2,3,4,6-tetra-*O*-acetyl- α -D-mannopyranose, 0.5 M coupling reagent (2-(1H-benzotriazol-1-yl)-1, 1,3,3-tetramethyluronium hexafluorophosphate) in dimethylformamide, 2 M diisopropylethylamine in *N*-methylpyrrolidinone, overnight coupling; (C) 0.1% H₂O in TFA 2 h at room temperature; (D) saturated K₂CO₃ in MeOH 2 h at room temperature.