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Fluorescent Labeling Agents Change Binding Profiles of Glycan-Binding Proteins

Yiyan. Fei[†], Yung-Shin Sun[†], Yanhong Li[‡], Kam Lau[‡], Hai Yu[‡], Harshal A. Chokhawala[‡], Shengshu Huang[‡], James P. Landry[†], Xi Chen[†], and X.D. Zhu^{†,*}

[†]Department of Physics, University of California at Davis, Davis, California, 95616, USA

[‡]Department of Chemistry, University of California at Davis, Davis, California, 95616, USA

Abstract

Interactions of glycan-binding proteins (GBPs) with glycans are essential in cell adhesion, bacterial/viral infection, and cellular signaling pathways. Experimental characterization of these interactions based on glycan microarrays typically involves (1) labeling GBPs directly with fluorescent reagents before incubation with the microarrays, or (2) labeling GBPs with biotin before the incubation and detecting the captured GBPs after the incubation using fluorescently labeled streptavidin, or (3) detecting the captured GBPs after the incubation using fluorescently labeled antibodies raised against the GBPs. The fluorescent signal is mostly measured *ex situ* after excess fluorescent materials are washed off. In this study by using a label-free optical scanner for glycan microarray detection, we measured binding curves of 7 plant lectins to 24 glycans: four β 1-4-linked galactosides, three β 1-3-linked galactosides, one β -linked galactoside, one α -linked *N*-acetylgalactosaminide, eight α 2-3-linked sialosides, and seven α 2-6-linked sialosides. From association and dissociation constants deduced by global-fitting the binding curves, we found that (1) labeling lectins directly with fluorescent agents change binding profiles of lectins, in some cases by orders of magnitude; (2) those lectin-glycan binding reactions characterized with large dissociation rates, though biologically relevant, are easily missed or deemed insignificant in *ex situ* fluorescence-based assays as most captured lectins are washed off before detection. This study highlights the importance of label-free real-time detection of protein-ligand interactions and the potential pitfall in interpreting fluorescence-based assays for characterization of protein-glycan interactions.

Keywords

Carbohydrate; Glycan microarray; Label-free detection; Lectin; Protein-carbohydrate interaction

*xdzhu@physics.ucdavis.edu; Fax: +1 530-752-4717; Tel: +1 530-752-4689.

Supplementary Information

Drawings of 24 oligosaccharides used in this study are depicted in Figure S1. Binding profiles of ECA, PNA, MAA, WFA, and SNA with and without FITC labeling are listed as Figure S2 through Figure S5. Tables of equilibrium dissociation constants of 7 non-labeled and FITC-labeled lectins with 24 glycans are tabulated in Table-WGA, Table-RCA, Table-IgG-RCA, Table-ECA, Table-PNA, Table-MAA, Table-WFA, and Table-SNA. We only list the constants of more tightly bound lectin-glycan complexes, i.e., those corresponding to solid columns in Figure 3 through Figure 5 and Figure S2 through S6. Furthermore we discuss the experimental evidence of multiple presentations of a surface-immobilized glycan target (referred as multiple sites in this work) for a glycan-binding protein and the significance of a small minority of the glycan targets having large association constants with a protein probe while the majority of the same but differently presented glycan targets bind to the probe with much small association constants.

Introduction

Protein interactions with carbohydrates presented on host cellular surfaces are essential in cell adhesion, bacterium/virus-host interaction, and cellular signaling in living systems^{1–4}. Systemwide studies of protein-glycan interaction have followed the heels of genomic and proteomic research. The role of glycans in cellular processes is complex and at same time promising for drug development and health improvement. The complexity is due in part to the structure diversity of glycans in comparison with those of DNA and protein molecules (even considering that the latter are further organized into higher-order structural forms), and partly due to less research that has so far been directed to glycobiology. The recent emergence of glycan microarrays and high-throughput synthesis of glycans presents promising platforms for characterization of protein-glycan, virus-glycan, and bacterium-glycan interactions, and in turn to accelerate the pace of glycomic studies^{5–12}.

Binding profiles of glycan-binding proteins (GBP) are useful for identifying reagents with high specificities for tissue or cell staining to unveil spatial distributions of different glycan types. For example, *Maackia amurensis* Lectin (MAA) highlights the presence of α 2-3-linked sialosides while *Sambucus nigra* Lectin (SNA) maps out the distribution of α 2-6-linked sialosides¹³. Wheat germ agglutinin (WGA) reports densities of GlcNAc and Gal β 1-4GlcNAc. Hemagglutinins (HAs) from seasonal human influenza A viruses prefer to bind α 2-6-linked sialosides, while HAs from seasonal avian influenza A viruses favor α 2-3-linked sialosides⁹. Furthermore, binding profiles of glycan-binding proteins on the membrane surface of a bacterial or a viral particle can be used to distinguish different bacterial or viral strains⁹.

Experimental measurements of binding profiles of GBPs on glycan microarrays are mostly fluorescence-based^{5–12}. These involve labeling GBPs with fluorescent agents either directly, or indirectly by conjugating the GBPs with biotin or Fc fragments of human IgG or precomplexing the GBPs with anti-GBP antibodies before incubation with glycans and then followed by reactions with fluorescent-labeled streptavidin or secondary antibodies^{4, 8, 9, 12}. The unbound proteins and protein complexes are washed off thoroughly before endpoints of the GBP-glycan reactions are detected with a fluorescence scanner. There are two general problems with such ex-situ fluorescence-based detection. Post-reaction washing removes most captured GBPs with equilibrium dissociation constants K_D in the range of μ M to *sub*-mM. Even for captured GBPs that survive washing, their binding profiles are subject to change due to conjugation with fluorescent agents or precomplex formation with antibodies before reaction with glycans. Often the effect of direct fluorescent labeling or conjugation with Fc fragment of human IgG or precomplex formation with anti-GBP antibodies on the binding profile of a GBP is not fully characterized, if at all⁹. Consequently experimental binding profiles deduced from fluorescence measurements can be significantly inaccurate, and thus subsequent applications of these profiles to glycan distribution mapping should be taken with caution to avoid erroneous interpretation.

We report a direct experimental study of the effect of fluorescent labeling on binding profiles of glycan-binding proteins (GBPs). We measured equilibrium association constants of 7 plant lectins, with and without fluorescein isothiocyanate (FITC) conjugated to the lysine residues or the terminal amino groups of the proteins, to 24 glycans in a glycan microarray immobilized on a streptavidin functionalized glass surface. The glycans include 15 sialosides, eight α 2-3-linked and seven α 2-6-linked. The equilibrium association constants are extracted from the binding curves to the glycans detected using an ellipsometry-based optical scanning microscope.

Results

Fluorescence-labeling of lectins alters the binding curves of the lectins to glycan targets

Figure 1 shows the association-dissociation curves (binding curves) of FITC-labeled and non-labeled WGA and MAA to 24 oligosaccharides as listed in Table 1 (the drawings of these oligosaccharides can be found in Supplementary Information). The solid lines are global fits to a two-site Langmuir reaction model that will be described below. It is immediately clear that the binding profiles of these two plant lectins (glycan-binding proteins) are profoundly changed by FITC labeling. Non-labeled WGA binds to both α 2-3- (OS-10 through OS-17) and α 2-6-linked (OS-18 and OS-21 through OS-24) sialosides, while FITC-labeled WGA only binds to α 2-3-linked sialosides. FITC labeling also reduces the affinity of MAA to four α 2-3-linked sialosides (OS-11 through OS-14) (see Supplementary Information for details). Non-labeled MAA binds to Gal β 1-4GlcNAc6S β (OS-6), while FITC-MAA binds to Gal β 1-4GlcNAc β (OS-5) instead.

Endpoint assays of lectin-glycan reactions depend on how reactions end

Another clearly noticeable feature is that dissociation times of FITC-MAA from four α 2-3-linked sialosides (OS-11 through OS-14), Neu5Ac α 2-3Gal β 1-4Glc β -, Neu5Ac α 2-3Gal6S β 1-4Glc β -, Neu5Ac α 2-3Gal β 1-4GlcNAc β -, Neu5Ac α 2-3Gal β 1-4GlcNAc6S β -, are dramatically shortened so that an endpoint assay of these binding reactions after the lectin solution is removed easily miss the evidence of the reaction. This is best illustrated in Figure 2 which shows the change in surface mass density of the glycan microarray before and after incubation with non-labeled and FITC-labeled MAA. While the evidence of non-labeled MAA binding reactions with four sialosides remains strong, it is essentially missing for FITC-labeled MAA reactions with the four sialosides due to much faster dissociation rates.

Lectin reactions with immobilized glycan targets are described by Two-site Langmuir reaction model

The third prominent feature revealed in Figure 1 is that most binding curves cannot be fitted to a 1-to-1 Langmuir reaction model¹⁴, unlike association-dissociation curves of monoclonal antibodies to surface-bound antigens. The goodness-of-fit is based on how close the ratio of the fitting residual to the standard variation of the data is to unity. For all the lectins investigated in this work, the two-site model consistently yields a better fit than the one-site model does. This may not be surprising for two reasons: (i) lectins can have more than one binding pocket for glycan receptors; and (ii) a surface-bound glycan can present itself in more than one stereochemical configuration. For fluorescently labeled lectins, they are often inhomogeneous mixtures with an average number of fluorophores per lectin molecule as supplied by the vendors (see Materials and Methods Section). This makes differently labeled lectins another contributing factor to the need of the two-site model. Since sizes of the plant lectins used in this study are comparable to or larger than the individual size of streptavidin tetramers (52 kDa) that are used to immobilize biotin-conjugated oligosaccharides on a glass surface (see Materials and Methods section), each lectin has access to more than one immobilized oligosaccharide (if energetically favorable) so that the lectin binding can enter into a “multi-valence” configuration with multiple glycan targets dominated by one of them. To extract key characteristics of lectin-glycan binding reactions with the fewest number of fitting parameters, we find it necessary and sufficient to use a two-site Langmuir reaction model¹⁴. In this model, a lectin is assumed to bind to surface-bound glycans in two possible configurations (or loosely “sites” of binding), each with a distinct association rate constant (k_{on}), a dissociation rate constant (k_{off}) and in turn an equilibrium association constant $K_a = k_{on}/k_{off} = 1/K_d$ (K_d is the dissociation constant).

The binding curves of a lectin to glycan targets in three lectin concentrations were globally fitted to the two-site Langmuir reaction model, with association and dissociation rate constants and relative coverage of each “site” as fitting parameters. The fits are shown in solid lines in Figure 1 for WGA and MAA. From the association and dissociation rate constants, we compute equilibrium association constants $K_a = k_{on}/k_{off}$ and use K_a to represent affinity constants.

The affinity constant profiles for WGA and RCA, with and without FITC labeling, are shown in Figures 3 and 4. The affinity profiles for the remaining 5 lectins, along with Tables of equilibrium dissociation constants $K_d = 1/K_a = k_{off}/k_{on}$ are listed in Supplementary Information.

WGA-glycan binding profile

The affinity constant profile is changed dramatically by FITC-labeling (Figure 3). Non-labeled WGA binds to GalNAc α (OS-2) and Gal β 1-4GlcNAc β (OS-5) and Gal β 1-4GlcNAc6S β (OS-6) with K_a around 1 nM^{-1} (or $K_d \sim 1 \text{ nM}$). All 8 α 2-3-linked sialosides and 5 out of 7 α 2-6-linked sialosides have K_a around 0.01 nM^{-1} (or $K_d \sim 100 \text{ nM}$)^{15–18}. Most noticeable observation is that FITC-WGA no longer binds to Neu5Ac α 2-6Gal β in the present label-free binding assay which has a detection limit of $K_d \sim 500 \text{ }\mu\text{M}$. Labeling WGA with FITC decreases the binding affinity of WGA to GalNAc α and Gal β 1-4GlcNAc(6S) β by as much as 5 orders of magnitude for the GalNAc α glycoside. Though it is generally expected that fluorescent labels are prone to alter the binding property of a glycan-binding protein, we are not aware of other label-free studies in the literature that directly examine how binding affinity constants of glycan-binding proteins change as a result of adding only fluorescent labels to the proteins.

RCA-glycan binding profile

The affinity profile is also changed significantly by FITC-labeling, more noticeable for some glycan targets (Figure 4). Non-labeled RCA binds to Gal β -(OS-1), Gal β 1-4Glc β (OS-3), Gal6S β 1-4Glc β (OS-4), Gal β 1-4GlcNAc β (OS-5)³¹ and all 7 α 2-6-linked sialosides (OS-18 through OS-24)^{19, 20} with K_d in the range of 1 nM to 100 nM. Non-labeled lectin binds additionally to Gal β 1-3GlcNAc β (OS-7) and Gal β 1-4GalNAc β -(OS-9). Most noticeable change due to FITC labeling is that the labeled protein no longer binds to Gal6S β 1-4Glc β (OS-4), and 2 α 2-6-linked sialosides Neu5Ac α 2-6GalNAc α (OS-18) and Neu5Ac α 2-6Gal β 1-3GlcNAc β (OS-24). Instead, the labeled protein now binds to GalNAc α (OS-2)¹⁴ and Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc β (OS-8).

Precomplexed GBPs with primary antibodies change the GBP-glycan interaction. 4, 8, 9, 12

Since the conjugation of FITC (400 Daltons) to lectins has a profound effect on the lectin-glycan binding profile, we expect that precomplexing lectins with anti-lectin IgGs (150 kDaltons) or the Fc-fragments of IgGs (60 kDaltons) should have the similar effect, even though precomplexing lectin is one of the commonly used methods in characterizing lectin-glycan interaction. As an initial attempt to investigate the effect of Precomplexing, we mixed RCA (0.67 μM) and goat anti-RCA IgG (1.34 μM) (Vector Laboratories, Burlingame, CA) and performed the binding assays on the glycan microarray under otherwise the same conditions. Figure 5 shows the affinity constant profiles of RCA and the IgG-RCA precomplex. Precomplexed RCA with IgG substantially has changed the binding profile. Notably, the IgG-RCA precomplex does not bind to Gal β 1-3Glc/GalNAc β (OS-7 and OS-9) and Neu5Ac α 2-6Gal β 1-3GlcNAc β (OS-24) but still binds to Gal β 1-4GlcNAc β (OS-5) and Neu5Ac α 2-6Gal β 1-4GlcNAc β (OS-22). Table of the equilibrium dissociation constants (Table-S3) is listed in Supplementary Information.

Discussion

Accurate information on binding profiles and ligand specificities of glycan-binding proteins (GBPs) is crucial for understanding mechanisms of cellular interactions at molecular level, particularly for those related to bacterial and viral infection and cellular signaling. It is the starting point of developing effective means to enhance those interactions that are beneficial and to interrupt or deter those that disrupt normal cellular functions. Despite the routine use of fluorescence-based detection for characterizing protein-glycan interactions, the effect of such detection method on experimentally determined binding profiles is not well characterized. Our present study illustrates some key aspects of how an endpoint assay and the use of direct or indirect fluorescence labeling can lead to incomplete or in some cases grossly inaccurate binding profiles of GBPs.

Our present experimental study shows that labeling a glycan-binding protein alters the equilibrium association constant of the protein with glycan receptors by as much as orders of magnitude, and the alteration is not easily predictable for different glycans even if the latter are close analogs of each other. A case in point is that WGA binds to α 2-6-linked sialosides Neu5Ac α 2-6Gal β (OS-18, and OS-21 through OS-24) as shown in Figure 3 while FITC-WGA does not. As described in Materials and Methods Section, there are on average 2 FITC molecules conjugated to one WGA at its accessible primary amine sites, and 5 FITC molecules conjugated to one RCA with the same conjugation chemistry. The observed modification of glycan-binding profiles of WGA and RCA by FITC is most likely due to a steric occlusion of the glycan-binding pocket by the physical presence of FITC molecules instead of significant changes in the lectin structure in response to the conjugation. This proposition is supported by our recent finding that WGA and RCA conjugated with biotin using the same conjugation chemistry (from the same vendor) have essentially the same glycan-binding profiles as the un-conjugated (unlabeled) WGA and RCA, respectively. This means that binding profiles obtained with fluorescence-labeled GBPs can be problematic and the use of these profiles for subsequent purposes need caution. Though tagged recombinant lectins may better retain binding properties of unlabeled (unconjugated) lectins than amine-based fluorescent labeled lectins may be constructed far from the glycan-binding pocket of interest, that conclusion still needs confirmation against unlabeled lectins by a label-free method under same assay conditions.

Due to high dissociation rates of many GBP-glycan complexes at room temperatures, endpoints of GBP-glycan binding reactions measured by ex-situ fluorescence-based methods vary with how the reactions are ended and post-reaction processing before fluorescence read-out. As illustrated in MAA and FITC-MAA binding to four α 2-3-linked sialosides, different conclusions can be drawn from an endpoint-type assay, depending on when the endpoint is taken. Vigorous washing would have removed most or all lectins captured by the glycans (Figure 2d and Figure 1d).

Real-time association-dissociation curves, enabled by the label-free detection method, reveal the evidence that more than one GBP-glycan complex form between a lectin and surface-bound glycans. As a result, it can be problematic to describe the steady-state endpoint of a GBP-glycan reaction as being proportional to $[c]/([c] + K_d) = K_a[c]/(K_a[c] + 1)$ with K_a being the sole equilibrium association constant¹⁴. Such a relationship is routinely used to extract K_a or K_d for protein-glycan interaction in fluorescence-based studies. The potential pitfall is that depending upon the protein concentration $[c]$ and incubation time, the apparent K_d is dominated by different protein-glycan complexes (Figure 1a and Figure 1b), rendering the information not a suitable characteristic of the interaction.

Further experimental evidence of multiple presentations of a surface-immobilized glycan target (referred as multiple sites in this work) for a glycan-binding protein

From the association-dissociation curves that we have reported here, one can see clearly that a glycan-binding protein probe forms more than one type of complexes with the “same” immobilized glycan targets, and each complex has characteristic association and dissociation rate constants. The question is whether these different probe-target complexes are formed primarily (a) by the only presentation of the immobilized targets with different binding pockets on the protein probe or (b) by different presentations of the immobilized targets with the same pocket on the probe. In scenarios (a), as long as one of the binding pockets on the protein probe and the immobilized target can form a complex with an equilibrium dissociation constant $K_d (= 1/K_a)$ comparable to or smaller than the probe concentration $[c]$, the immobilized targets should capture nearly a full monolayer of the protein probes by the end of the association phase. In scenarios (b), on the other hand, even when one presentation of the immobilized targets bind to the pocket on the protein probe with high affinity so that K_d is much less than the probe concentration $[c]$ and thus all the immobilized targets of such presentation capture protein probes successfully, the target surface may not capture a full monolayer of the probes if other presentations of the immobilized targets form complexes with the protein probe with K_d larger than $[c]$. Based on these considerations, we find that Scenario (b) is supported by the experiment as follows.

The surface mass density of a monolayer of protein probes and the corresponding optical signal can be estimated from the size and molecular weight of the protein. Alternatively, since we record binding curves of a protein probe to many glycan targets simultaneously under the same condition, the optical signal for a monolayer of the captured probes can be estimated as the largest optical signal $\Delta\delta_{\max}$ by the end of the association phase across the entire target microarray, regardless the type of the target that produces $\Delta\delta_{\max}$. We use $\Delta\delta_{\max}$ to examine whether Scenario (a) or (b) is supported. For example Figure 1c shows the binding curves of unlabeled MAA to 24 glycans from which identified OS-3, OS-6, and OS-11 through OS-14 as ligands of the proteins (since we use the same scale for all 24 binding curves, the association-dissociation curves to OS-3 are not obvious and yet become clear when we zoom in). As displayed in Figure S4, the equilibrium dissociation constants K_d of the more stable MAA-glycan complexes (Site 1) for all 6 glycans are below 100 nM (i.e., K_a 's are above 0.01 nM^{-1}) which is much smaller than the MAA concentration of $[c] = 3 \text{ }\mu\text{M}$. If Scenario (a) dominated, we would expect the optical signals from all 6 targets by the end of association phase to reach values comparable to the maximum value $\Delta\delta_{\max}$ in Figure 1c. But this does not happen. The optical signals for 4 sialosides (OS-11 to OS-14) do reach a comparable level of 8 units corresponding to a surface mass density of $4 \times 10^{-7} \text{ g/cm}^2$ for the captured MAA. By contrast the optical signals for OS-3 and OS-6 reach much lower levels, indicating that the surface mass density of MAA captured by Gal β 1-4Glc β (OS-3) is only $6 \times 10^{-9} \text{ g/cm}^2$ or 1/16 of those by any one of 4 sialosides. This can only mean that the immobilized OS-3 targets have more than one presentation for MAA. Only the presentations of OS-3 with dissociation constants comparable or less than the MAA concentration will be recorded. The fact that the maximum surface mass density of MAA captured by OS-3 is only 1/16 of MAA captured by OS-12 or OS-13 indicates that the surface number density of those OS-3 with association constants displayed in Figure S4 is at least 16-fold less than the surface number density of OS-12 and OS-13. As a result, we can conclude that 94% of the immobilized OS-3 has equilibrium association constants above 500 μM (the detection limit of our present real-time binding assay). This finding applies to reactions of all 7 lectins to the 24 glycan targets.

Significance of the minor presentation of glycan targets with large association constants in a binding assay

An *in vitro* binding assay between a protein probe and a ligand (e.g., a glycan in the present study) always involves at least one of the two reactants being situated in a setting different from the *in vivo* environment. As a result the presentations of the protein and/or the ligand are altered from those *in vivo*. Which complex formed by the protein and the ligand most closely represents the *in vivo* one is a difficult question to answer. It is thus important that one acquires information on fractional make-ups of different protein-target complexes and the corresponding association constants. As illustrated in the present study, the simultaneous measurement of binding curves of a protein to multiple immobilized targets enables not only recording binding reactions with a wide range of association constants but also determining whether the recorded reactions are those between the proteins with major or minor fractions of the immobilized target. We need both types of information to understand the relevance of the affinity profiles obtained by such a binding assay to the corresponding *in vivo* events involving the “same” protein and the “same” ligands.

Materials and Methods

Lectins with and without FITC Labeling

Ricinu communis Agglutinin (RCA and FITC-RCA with fluorophore-to-protein ratio F/P = 5.4, 120 kDa), *Maackia amurensis* Agglutinin (MAA and FITC-MAA with F/P = 7.1, 130 kDa), *Sambucus nigra* Bark Agglutinin (SNA and FITC-SNA with F/P = 6.9, 150 kDa), and *Erythrina cristagalli* Agglutinin (ECA and FITC-ECA with F/P = 2.5, 54 kDa) were purchased from Vector Laboratories (Burlingame, CA). Wheat Germ Agglutinin (WGA and FITC-WGA with F/P = 1.7, 36 kDa); Peanut Agglutinin (PNA and FITC-PNA with F/P = 2.3, 110 kDa), and Wisteria Floribunda Agglutinin (WFA and FITC-WFA with F/P = 2.3, 120 kDa) were purchased from EY Laboratories (San Mateo, CA). FITC were conjugated to primary amines (on lysine residues or at the N-terminals) on the lectins to form amide bonds using a proprietary protocol by the vendor. The lectins were diluted with 1× phosphate buffered saline (PBS) to concentrations in the range of μM for real-time binding affinity measurement.

Glycan Synthesis and Biotinylation

Table 1 lists 24 biotinylated oligosaccharides used in the present study^{21–29}. The drawings of these oligosaccharides are available in Supplementary Information. The galactosides^{22, 24–26}, *N*-acetylgalactosaminide²⁷, α 2-3-^{24, 28} and α 2-6-linked^{27, 29} sialosides with a 3-azidopropyl aglycon were synthesized as reported previously. Biotinylation of these glycans was carried out by reducing the azido group in azidopropyl aglycon to amine and coupled to *N*-hydroxy succinamide (NHS) ester of hexa(ethylene glycol) (HEG)-linked biotin (Biotin-HEG-NHS) as reported previously²². All 24 biotinylated oligosaccharides were dissolved in 1×PBS to the concentration of 50 μM for glycan microarray fabrication.

Glycan Microarray on Streptavidin-Coated Glass Slide

The oligosaccharides listed in Table 1 were dissolved in 1× PBS (pH 7.4, 0.22 μm filtered) to concentrations of 50 μM for subsequent microarray fabrication. Using an OmniGrid 100 contact-printing robot (Genomic Solutions, Ann Arbor, MI), we printed 4 replicates of each of the 24 carbohydrates on a streptavidin-functionalized slide (ArrayIt, Sunnyvale, CA) to form a 96-spot carbohydrate microarray over an area of 2.6 mm $\times$ 3.2 mm. The averaged diameter of the printed spots is 130 μm and the center-to-center spot separation is 250 μm . After the excess printed material was washed off the printed surface was left with a layer of oligosaccharides enough to capture a monolayer of lectins. Since the size of a streptavidin

tetramer on the glass slide is between 5.5 nm and 8 nm, we expect the surface density of streptavidin tetramers to be between $1.6 - 3.3 \times 10^{12} \text{ cm}^{-2}$. Assuming that each tetramer has two biotin-binding pockets available and both pockets successfully capture printed biotinylated oligosaccharides, we estimate the surface density of the immobilized oligosaccharides to be between 3.3 and $7 \times 10^{12} \text{ cm}^{-2}$. This estimate was close to what we observed from the captured lectins by the immobilized oligosaccharides. The printed glass slides were stored at room temperature in a slide box for at least 24 hours before further processing for lectin-glycan binding experiments.

Conditions for Lectin-Glycan Binding Assays using Glycan Microarray

The printed glass slide was assembled with a fluidic system containing 8 chambers (each has 30 μL active volume), each housing one of the 8 glycan microarrays. The printed surface was washed *in situ* with a flow of $1 \times \text{PBS}$. The unprinted part of the surface was then blocked with biotin-conjugated bovine serum albumin to prevent non-specific binding of subsequent lectins to streptavidin. For lectin-binding assays, a lectin in $1 \times \text{PBS}$ solution was flown through a chamber for a few seconds with a flow rate of 2.5 mL/min. The flow rate was then decreased to 0.01 mL/min during the association phase of the binding (20 minutes). At the end of the association phase, the flow rate was immediately changed back to 2.5 mL/min and after a few seconds the rate was decreased again to 0.01 mL/min for the remaining duration of the dissociation phase of the binding process (60 minutes).

Label-Free Detection of Lectin-Glycan Association-Dissociation Curves Using a Real-Time Ellipsometry-Based Scanning Microscope

Details of the ellipsometry-based scanning microscope, i.e., an oblique-incidence reflectivity difference (OI-RD) scanner, have been described previously^{14, 30–32}. In essence, the presence of a layer of molecular targets such as glycans on a solid surface such as that of a glass slide and subsequent binding of protein probes to these targets cause changes in the phase and amplitude of an optical beam reflected from the surface. These changes arise from differences in the refractive index of the target-probe layer, the solid support, and the aqueous ambient, and depend on the polarization of the optical beam. The ellipsometry-based scanner measures in real time the differential phase change $\Delta\delta$ between the p-polarized component and the s-polarized component of the optical beam, in much of the same way a surface-plasmon resonance sensor (SPR) measures the resonance angle shift $\Delta\theta_{\text{SPR}}$. $\Delta\delta$ is proportional to the surface mass density (gm/cm^2) of immobilized glycan targets and subsequent protein-binding complexes, as does $\Delta\theta_{\text{SPR}}$. The ellipsometry-based measurement of a target layer and binding of protein probes to the layer is highly reproducible. The only weakness of such an ellipsometry-based label-free technique is that it is not as sensitive as the fluorescence-based technique. By scanning the focused optical beam across the glycan microarray, we read out the surface mass densities for 96 targets in every 1.8 seconds during association and dissociation processes of the binding. In the present experiment, one unit of the OI-RD optical signal $\Delta\delta$ corresponds to a surface mass density of $5 \times 10^{-8} \text{ g}/\text{cm}^2$, assuming that the protein volume mass density is $1.35 \text{ g}/\text{cm}^3$. For each lectin, the association-dissociation curves (surface mass density vs. time) were measured at three lectin concentrations in the order of μM and varied over a factor of 4 ~ 5. The set of 3 binding curves was globally fit to yield dissociation constants $K_d = k_{\text{off}}/k_{\text{on}}$ or association constants $K_a = k_{\text{on}}/k_{\text{off}}$ (the inverse of K_d)¹⁴. The images of the surface mass density before and after the dissociation phase of the binding were also recorded.

Conclusions

Glycan-GBP binding affinity profiles reported on the literatures have been the main information source towards the understanding of cellular interactions and to use GBPs with

high receptor specificities in mapping the distributions of specific glycans in biological systems. Since most Glycan-GBP binding affinity profiles have been obtained using fluorescence-based detection, one needs to exert caution before relying heavily on the specifics of these binding affinity profiles for crucial conclusions on cell signaling and cell-cell interaction mechanisms. For example, in tissue staining studies involving fluorescence-labeled GBPs (directly or indirectly through antibodies), dubious binding affinity profiles of the labeled proteins can lead to incorrect mapping of different glycan types in various tissues. The same issue applies to efforts using the reported GBP-glycan affinity profiles without additional confirmation to quantify protein or cellular glycosylation patterns.

By the same token, the binding affinity profile of a free-form protein is often used to infer the binding behavior of a virus or a bacterium that expresses such a protein on its surface. Since labeling agents with molecular weights of hundreds of daltons can already alter the binding affinity profile of a protein profoundly, one should expect that the same protein but attached to a viral or bacterial surface can have a significantly altered affinity profile to its ligands. It is thus normal that the binding affinity profile of a free-form protein sometime may not explain the behavior of a bacterial or viral particle that expresses the “same” protein on its surface.

Real-time binding curve measurements over a wide range of protein concentrations are necessary to appropriately determine binding affinity profiles of non-labeled glycan-binding proteins and to quantitatively evaluate the effect of fluorescent labeling agents such as FITC on the affinity profiles as we show in this work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Association-dissociation curves of WGA and MAA binding to 24 oligosaccharides. One unit of the optical signal corresponds to a protein surface mass density of $5 \times 10^{-8} \text{ g/cm}^2$, assuming that the protein volume mass density is 1.35 g/cm^3 . (a) Non-labeled WGA in $1 \times \text{PBS}$ at a concentration of $5.7 \mu\text{M}$ (solid squares), $11.4 \mu\text{M}$ (open circles), and $22.7 \mu\text{M}$ (solid triangles); (b) FITC-WGA in $1 \times \text{PBS}$ at a concentration of $5.7 \mu\text{M}$, $11.4 \mu\text{M}$, and $22.7 \mu\text{M}$; (c) non-labeled MAA in $1 \times \text{PBS}$ at a concentration of $0.75 \mu\text{M}$ (solid squares), $1.5 \mu\text{M}$ (open circles), and $3 \mu\text{M}$ (solid triangles); (d) FITC-MAA in $1 \times \text{PBS}$ at a concentration of $0.75 \mu\text{M}$, $1.5 \mu\text{M}$, and $3 \mu\text{M}$. Vertical lines mark the starts of association and dissociation phases of the binding event, respectively. The solid lines through the curves are global fits to a two-site Langmuir reaction model to yield equilibrium association constants.

Figure 2.

Surface mass density changes on the 24-target glycan microarray after first incubation in (a) non-labeled WGA; (b) FITC-labeled WGA; (c) non-labeled MAA; (d) FITC-labeled MAA, for 30 minutes, and then dissociation in $1\times$ PBS for 60 minutes. One unit of OI-RD signal corresponds to a protein surface mass density of $5 \times 10^{-8} \text{ g/cm}^2$. The numbers in the panels are glycan IDs as defined in Table 1. Due to significant dissociation over 60 minutes, many binding events evident in binding curve measurements (Figure 1) appear missing or less pronounced in the endpoint mass density change measurement (Figure 2), best illustrated in FITC-MAA binding to α 2-3-linked sialosides (Figure 2d vs. Figure 1d).

Figure 3. Equilibrium association constants of WGA (top panel) and FITC-WGA (bottom panel) binding to 24 glycans. For each glycan target, the solid column shows the constant to the one of the two sites with higher affinity, and the shaded column shows the constant to the other site with lower affinity. OS-3 through OS-6 have terminal Gal β 1-4. OS-7 through OS-9 have terminal Gal β 1-3. OS-10 through OS-17 are α 2-3-linked sialosides. OS-18 through OS-24 are α 2-6-linked sialosides. Unlike non-labeled WGA, FITC-WGA does not bind to α 2-6-linked sialosides.

Figure 4. Equilibrium association constants of RCA (top panel) and FITC-RCA (bottom panel) binding to 24 glycans. FITC labeling significantly changes the affinity profile of RCA, but responding to more detailed glycan structural modifications (e.g., OS-1 vs. OS-2, OS-3 vs. OS-4, OS-7 vs. OS-8, and OS-23 vs. OS-24).

Figure 5. Equilibrium association constants of RCA (top panel) and goat IgG-RCA (bottom panel) binding to 24 glycans. The precomplex of RCA with goat anti-RCA IgG changes the affinity profile of RCA. Most notably, the affinities to Gal β 1-3Glc/GalNAc β (OS-7 and OS-9) and Neu5Ac α 2-6Gal β 1-3GlcNAc β (OS-24) are reduced by 5 orders of magnitude to below the detection limit. Yet the binding affinities to similar structures such as Gal β 1-4GlcNAc β and Neu5Ac α 2-6Gal β 1-4GlcNAc β remain essentially unchanged.

A list of 24 biotinylated oligosaccharides and assigned identification numbers used for lectin binding profile measurements in the present work.

Table 1

Glycan I.D.	Glycan Structures	
OS-1	Gal	β-Biotin
OS-2	GalNAc	α-Biotin
OS-3	Gal	β1-4Glc β-Biotin
OS-4	Gal6S	β1-4Glc β-Biotin
OS-5	Gal	β1-4GlcNAc β-Biotin
OS-6	Gal	β1-4GlcNAc6S β-Biotin
OS-7	Gal	β1-3GlcNAc β-Biotin
OS-8	Gal	β1-3GlcNAc β1-3Galβ1-4Glc β-Biotin
OS-9	Gal	β1-3GalNAc β-Biotin
OS-10	Neu5Aca2-3 Gal	β-Biotin
OS-11	Neu5Aca2-3 Gal	β1-4Glc β-Biotin
OS-12	Neu5Aca2-3 Gal6S	β1-4Glc β-Biotin
OS-13	Neu5Aca2-3 Gal	β1-4GlcNAc β-Biotin
OS-14	Neu5Aca2-3 Gal	β1-4GlcNAc6S β-Biotin
OS-15	Neu5Aca2-3 Gal	β1-3GlcNAc β-Biotin
OS-16	Neu5Aca2-3 Gal	β1-3GlcNAc β1-3Galβ1-4Glc β-Biotin
OS-17	Neu5Aca2-3 Gal	β1-3GalNAc β-Biotin
OS-18	Neu5Aca2-6 GalNAc	α-Biotin
OS-19	Kdna2-6 Gal	β1-4Glc β-Biotin
OS-20	Neu5Gca2-6 Gal	β1-4Glc β-Biotin
OS-21	Neu5Aca2-6 Gal	β1-4Glc β-Biotin
OS-22	Neu5Aca2-6 Gal	β1-4GlcNAc β-Biotin
OS-23	Neu5Aca2-6 Gal	β1-4GlcNAc6S β-Biotin
OS-24	Neu5Aca2-6 Gal	β1-3GlcNAc β-Biotin