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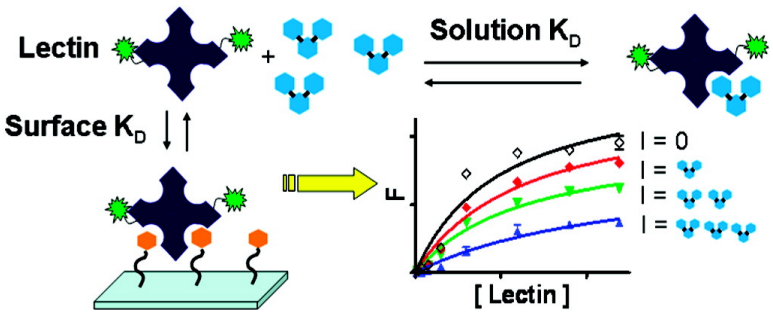

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Quantitative Analysis of Carbohydrate−Protein Interactions Using Glycan Microarrays: Determination of Surface and Solution Dissociation Constants

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Abstract: Carbohydrate−protein interactions on surface and in solution were quantitatively measured by a glycan microarray. Assessing carbohydrate affinities is typically difficult due to weak affinities and limited sources of structurally complex glycans. We described here a sensitive, high-throughput, and convenient glycan microarray technology for the simultaneous determination of a wide variety of parameters in a single experiment using small amounts of materials. Assay systems based on this technology were developed to analyze multivalent interactions and determine the surface dissociation constant ($K_{D,surf}$) for surface-coated mannose derivatives with mannose binding lectins and antibodies. Competition experiments that employed monovalent ligands in solution yielded K_D and K_i values in solution similar to equilibrium binding constants obtained in titration microcalorimetry and surface plasmon resonance experiments.

Introduction

Carbohydrates, present as free oligosaccharides or as glycoconjugates, play an important role in many biological events, particularly those involving cell surfaces.[1,2] Specific interactions between carbohydrates and proteins are often essential in viral and bacterial infection, the immune response, differentiation and development, and the progression of tumor cell metastasis.[3−5] Therefore, an understanding of carbohydrate−protein interactions at the molecular level would lead to a better insight into the biological process of living systems and assist the development of therapeutic and diagnostic strategies.

Despite the ubiquity and importance of carbohydrates in biology, difficulties in the study of carbohydrate−protein interactions have hindered the development of a mechanistic understanding of carbohydrate structure and function.[6] The structural complexity of carbohydrates is a major obstacle: although the other two classes of biopolymers, nucleic acid and proteins, have a linear arrangement of repeating units, carbohydrate building blocks have multiple points of attachment, leading to highly branched and stereochemically rich structures. In addition, binding affinities are weak typically in the $\sim 10^{-3}$ to 10^{-6} M range of dissociation constants, compared with antigen−antibody interactions (10^{-8} to 10^{-12}).[7−9] Although techniques such as isothermal titration calorimetry (ITC),[10] affinity capillary electrophoresis,[11] surface plasmon resonance (SPR),[12] and frontal affinity chromatography[13] are all significant advances, they are often limited by the amount of available materials. Hence, the design of sensitive and high-throughput technologies for characterizing carbohydrate−protein interactions remains a challenge. Inspired by the success of DNA and protein microarrays, the chip-based approach has been advanced as a useful tool in the emerging field of glycomics.

Glycan microarray technology is a promising approach for the investigation of carbohydrate−protein interactions. Surface-based carbohydrate arrays can facilitate the study of lectin recognition; the presentation of carbohydrates in an array provides a method to simultaneously monitor multiple binding events and the effects of multivalency.[14] To date, most publications have focused on array fabrication and interaction profiling.[14−25] The most advanced development is an array of

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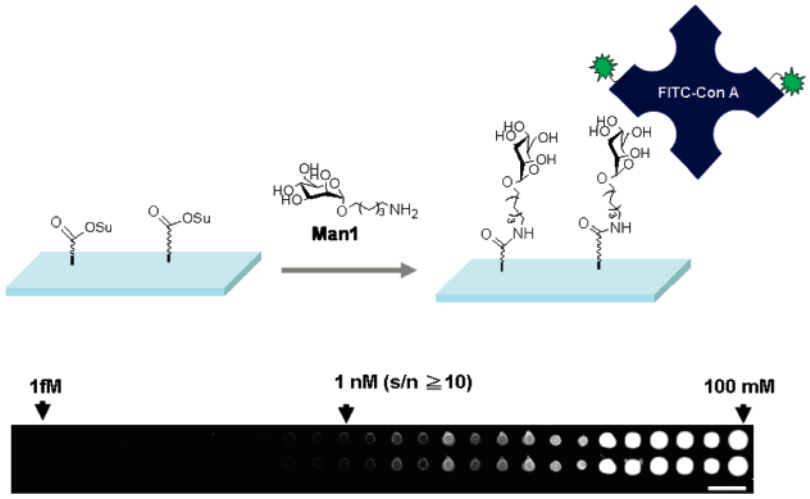

Figure 1. Glycan microarray fabrication and detection. The *N*-hydroxysuccinimide (NHS) activated glass slide was printed with Man1 at a range of concentrations between 100 mM and 0.5 fM. Fluorescent images were then probed with FITC-labeled Con A. The detection limit was determined to be at 1 nM printing concentration and attomole quantities of sugars per spot. The arrow refers to the printing concentration. The white bar (bottom right) equals 0.5 mm length.

more than 200 carbohydrates on a slide used to analyze the specific binding of mammalian, plant, viral, and bacterial lectins, antibodies, and intact viruses.[15,16] More recently, our group has used Globo H and its truncated analog microarrays to profile the binding specificity of its monoclonal and polyclonal antibodies.[17] However, little attention has been paid to the systematic kinetic and thermodynamic investigation of the interactions using glycan microarrays. Recently, MacBeath and co-workers reported a quantitative analysis of protein−peptide interactions using a protein microarray; in this work the interactions of Src homology 2 and the phosphotyrosine binding domain of phosphopetides were measured, and this study provided a better understanding of the tyrosine phosphorylation network for the epidermal growth factor receptor.[26,27] Inspired by this result, we have used a glycan microarray to develop a method to quantify carbohydrate−protein interactions. To the best of our knowledge this article constitutes the first report of such a strategy.

To demonstrate the capability of the system to quantitatively measure carbohydrate−protein interactions, we used the well-characterized, mannose/glucose-specific lectin concanavalin A (Con A)[28] with a fluorescence label to interact with mannose and oligomannose carbohydrate arrays on glass surfaces. Subsequently, interactions were measured using a fluorescence scanner to monitor the binding of fluorescence-labeled lectins. Array imaging measurements demonstrated that the immobilized carbohydrates were accessible to proteins in solution and that binding affinities could be detected. With the use of this data, Langmuir isotherms[29,30] for the binding of proteins to the surface were constructed to determine the dissociation constants ($K_{D,surf}$, functional affinities) on the surface. By using competition binding experiments, in which carbohydrates or inhibitors were present in solution with proteins, it was possible to calculate solution equilibrium dissociation constant (K_D or K_i). This provided an excellent method for rapid generation of inhibition data for proteins, with small low-affinity monovalent lectins ligands (i.e., the carbohydrates).

Results and Discussion

Arraying and Detection Limit. Our strategy for covalently attaching a defined glycan to a glass slide is based on the standard micorarray robotic printing technology using *N*-hydroxysuccinimide (NHS) activated glass surface, to which glycans containing an amine linked to the anomeric position were covalently attached. Prior to producing the slides, we first explored the scope of printing concentrations. Because carbohydrates do not fluoresce and modified carbohydrates bearing fluorescent groups might interact differently with the protein, we used fluorescein isothiocyanate (FITC) cadaverine as a model. FITC cadaverine was printed in concentrations ranging from 100 mM to 1 fM, and the slide was scanned before and after washing. The surface coverage of FITC was measured in a fluorescence wash-off experiment, and the density of maximum loading was found to be 10^{14} molecules/cm^2 (see the Supporting Information for the calculation), a similar value to most peptides or sugars attached to an SPR biosensor surface.[31,32] More importantly, at concentrations below 100 μM, the fraction of surface covered by each molecule varied in proportion to its concentration, whereas over 500 μM, the

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surface seemed to be saturated (Figure S1, see the Supporting Information). Next, monomannose derivative bearing primary amine (Man1) was printed on the glass slide in concentrations ranging from 100 mM to 0.5 fM (Figure 1) and incubated with FITC-labeled Con A (100 nM). The limit of detection was found to be in the nanomolar printing concentration, when the ratio of signal-to-noise was more than 10. This result demonstrates that microarrays require only a very small quantity of carbohydrate; in our assay the loading of the spot is 0.6 nL, and therefore the minimum amount for the detection is attomoles (10^{-18} mol) per spot, allowing several experiments to be carried out on a single glass slide. Assay miniaturization through the construction of high-density microarrays is thus well suited for the investigation of carbohydrate−protein interaction.

Multivalent Carbohydrate−Protein Interaction on the Surface. FITC-labeled Con A was incubated with different printing concentrations of Man1 on the surface; after washing the slide was scanned to get the fluorescence intensities. We created a binding curve based on printing concentrations and fluorescence intensities, and we found that the binding curve reached saturation (in the case of FITC−Con A and Man1, the curve became saturated at 10 μM printing concentration), which was independent of surface density (surface saturated when printing concentration was over 100 μM). This saturated curve is an indication of multivalent interaction between protein and printed carbohydrates. However, accurate measures of surface coverage of carbohydrate on each spot were not available by current technique. As the signal intensity in an array depends on the surface density of the immobilized carbohydrate, it is essential to normalize carbohydrate concentrations prior to printing. In order to determine the dissociation constant on surface, we plotted protein concentrations against fluorescence intensity at different concentrations of printed sugar. Figure 2a depicts photographs of glass slides printed at 16 different concentrations with a 16 × 16 pattern of Man1 from 100 μM (first left column) to 0.1 μM (first right column). The arrays were probed with 10 concentrations of protein−FITC-labeled Con A, ranging from 800 to 25 nM. Con A concentrations were plotted against median fluorescence intensities of replicate spots to give a set of curves (Figure 2b). The curves were analyzed as Langmuir isotherms, assuming that the system reached equilibrium during incubation,

$$F = \frac{F_{\max}[\mathrm{P}]}{[\mathrm{P}] + K_{\mathrm{D,surf}}} \qquad (1)$$

where $F_{\max}$ is the maximum fluorescence intensity, a measure of the amount of active carbohydrate on the surface, [P] is the total lectin concentration, and $K_{\mathrm{D,surf}}$ is the equilibrium dissociation constant for surface carbohydrate and lectin (see the Experimental Section for eq 1 derivation). Although the printed concentrations of monomannose vary by up to 10-fold from 100 to 10 μM, the $K_{\mathrm{D,surf}}$ values obtained from these individual curves, as well as from replicate experiments, are narrowly distributed (mean $K_{\mathrm{D,surf}}$ = 83 nM; SD = 4.7 nM; Table 1). However, at lower printing concentration (ca. 1 μM), the surface reaches a critical density, at which point the binding affinity is lower, probably due to the increased distance between the carbohydrates on the surface. This is because Con A is capable of forming two attachment points to the surface and the distance between these points is approximately 65 Å.[28] At the printing

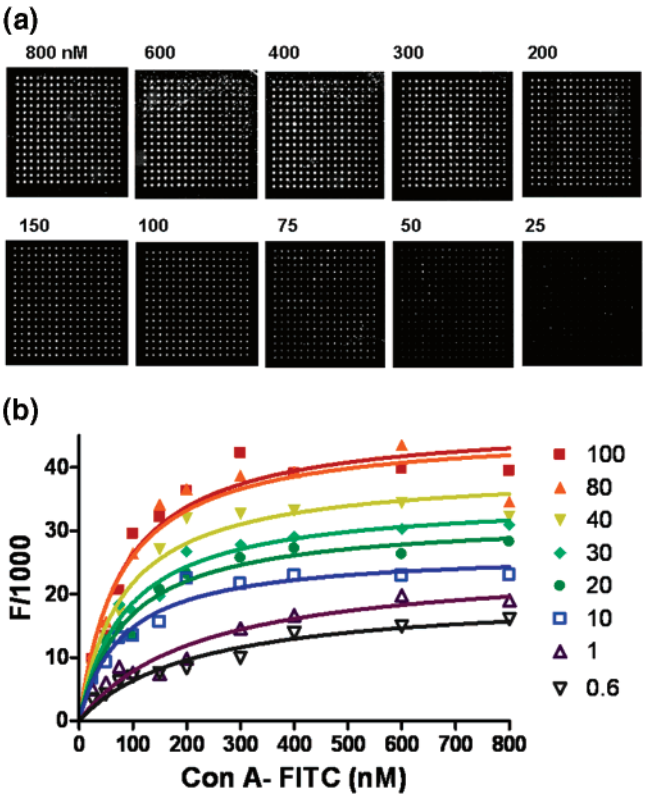

Figure 2. (a) Man1 with concentrations of 100 (first left column), 80, 60, 40, 30, 20, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, and 0.1 μM (first right column). The images were obtained from slides incubated with different concentrations of FITC-labeled Con A (from 800 to 25 nM as indicated above each square). (b) Binding curves for Man1 printed at different concentrations are shown. The curves were obtained using FITC-labeled Con A. The $K_{\mathrm{D,surf}}$ values were obtained by fitting the curves to eq 1.

Table 1. Functions of Different Printing Concentrations and the Corresponding Fluorescence Intensities ($F_{\max}$) and the Dissociate Constants on the Surface ($K_{\mathrm{D,surf}}$)

printing concn μM	$F_{\max}$	$K_{\mathrm{D,surf}}$, nM
100	40950	80.4
80	40030	76.8
40	34050	81.7
30	29490	88.7
20	26910	90.6
10	22670	81.8
1	18250	221
0.6	14250	214

concentration of over 10 μM, the distance between mannose residues on the surface is close enough such that on average, an adsorbed Con A can bind to two mannose residues. However, when the printing concentration is below 10 μM, the average distance between immobilized mannose residues is too far for a multivalent interaction with Con A. The increase in binding strength shown in the $K_{\mathrm{D,surf}}$ values for high surface densities of a carbohydrate is the result of multivalent interactions.[33] It is well-known that carbohydrate-binding proteins interact weakly with monovalent ligands but strongly with multivalent carbohydrates.[34] The FITC washing-off experiment indicated that the average space between each sugar is about 100 Å at a printing

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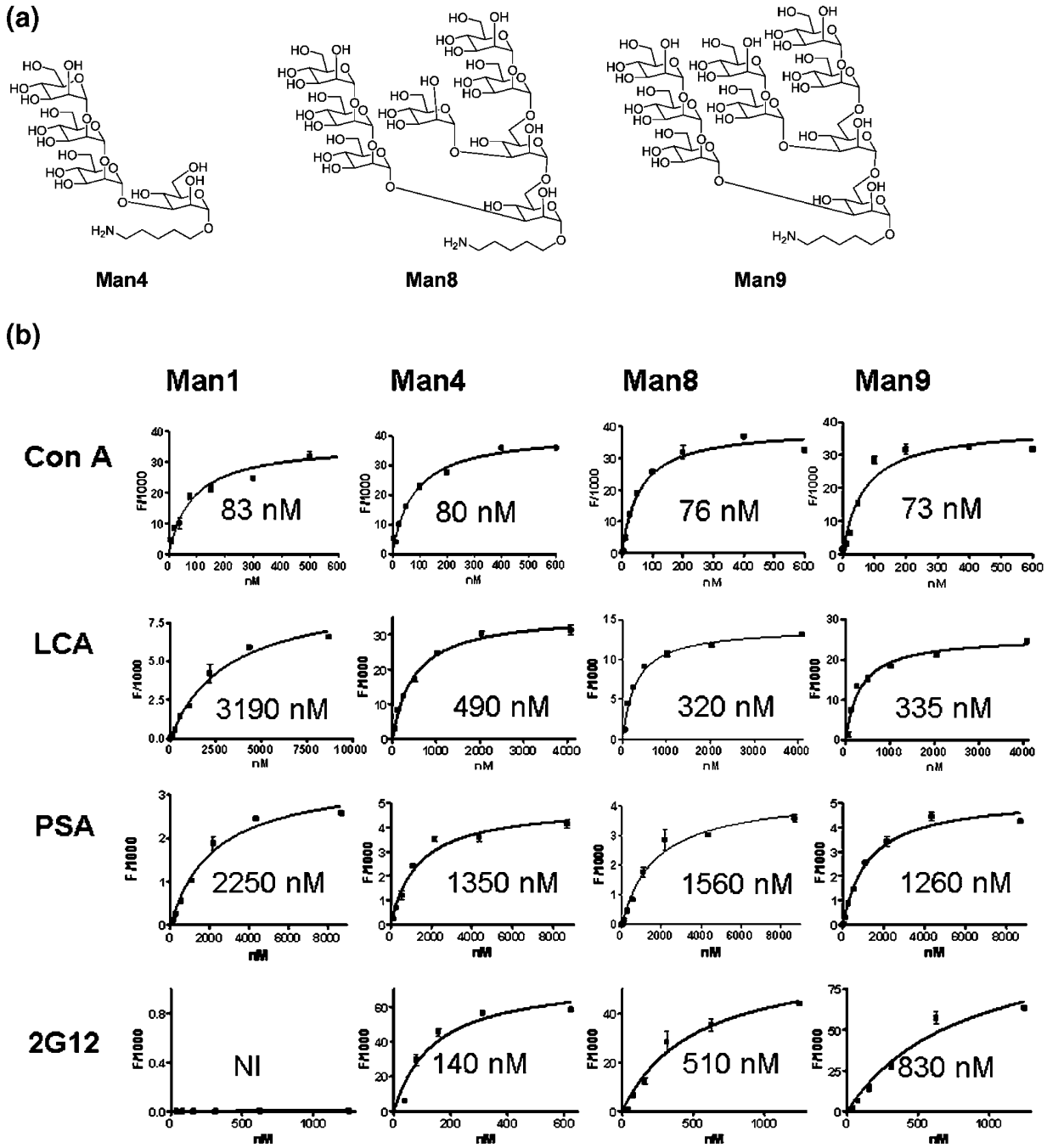

Figure 3. (a) Structures of Man4, Man8, and Man9. (b) The binding curves were obtained from the function of lectins or antibody concentration and fluorescence intensity determined from array images. $K_{D,surf}$ values were obtained by fitting the curves to eq 1. The error bars indicated in the figures show the average percentage error for all data points reported in the figures.

concentration of 1 μM (see the Supporting Information for the calculation). This result verifies FITC to be an appropriate model for the determination of sugar density and suggests this method be used as a tool to predict the distance of two binding sites within one protein.

Using the same method, we printed different carbohydrates[35] (Man1, Man4, Man8, Man9—see Figure 3a) at 100 μM and measured their binding to different proteins at different concentrations. The mannose binding lectins Con A, *Lens culinaris* agglutinin (LCA), *Pisum sativum* agglutinin (PSA), and the human monoclonal antibody 2G12 were each incubated with sugar arrays in different concentrations. The model developed for binding fitted the data quite well, and $K_{D,surf}$ values were obtained using eq 1 (Figure 3b). We observed that the relative binding affinities of these lectins to surface mannose were Con A > LCA > PSA. The binding affinities of Con A to the four

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carbohydrates were, however, close, all about 80 nM and consistent with the values determined by SPR.[31] The relative binding specificity of LCA was Man9 ≈ Man8 ≈ Man4 > Man1, and this strongly supported that LCA preferentially binds to polymannose structure. The garden pea lectin PSA is thought to have the same binding specificity to LCA,[36] but in this experiment we found that the binding trend of PSA to these oligosaccharides was similar to that of Con A, albeit weaker (up to 2 orders of magnitude). The human monoclonal antibody 2G12 against the mannose epitope of gp120 on HIV was reported to be in favor of the Manα1−2 Man structure.[37] From this study, Man1 has no interaction and Man4 displayed the strongest interaction with K_D of 140 nM. These values were consistent with our previous measurements from the microtiter plate assay.[37] Overall, the method demonstrated here showed that the apparent binding mode and strength of carbohydrate−protein interaction on cell surfaces can be mimicked and quantitatively analyzed by a glycan array in a rapid and convenient manner with a very small amount of carbohydrate.

We compared the binding strength of lectins or antibodies to mannose derivatives at one or two concentrations of these proteins and obtained a ranking order for binding specificities. For example, the relative binding strength, based on maximal fluorescence intensities, is Man4 (F_{max} = 36 070) > Man9 (F_{max} = 25 780) > Man8 (F_{max} = 13 940) > Man1 (F_{max} = 9458), but that based on dissociation constants is Man8 (K_D = 320 nM) > Man9 (K_D = 335 nM) > Man4 (K_D = 490 nM) > Man1 (K_D = 3190 nM). To date, most studies of carbohydrate−protein interactions have used a threshold-based, one-step qualitative analysis, i.e., interaction or noninteraction. The threshold varies from one carbohydrate to another and is based on how well the carbohydrate behaves in the assay. Even when closely related glycans are studied under ideal conditions, they vary with respect to the surface density of active carbohydrates. Since the intensity of a spot depends both on $K_{D,surf}$ (which results from binding affinity), and F_{max} (which results from surface active carbohydrate density and protein binding), the information obtained by probing an array with a single concentration of analytes may not be accurate.[27,38] This study shows that quantitative measurements can be carried out to accurately study the nature of carbohydrate−protein interaction on surface.

Solution Dissociation Constant. The solution equilibrium dissociation constant (K_D) for carbohydrate−lectin interactions can be determined using microarrays in a competitive assay. This analysis allows for the direct comparison between microarray affinities measurements to those obtained from solution-based affinity measurements. In a competitive binding experiment, carbohydrates in solution compete with immobilized carbohydrate ligands for the binding sites on the lectin, establishing a coupled equilibrium between the binding of protein to the immobilized species and to the species present in solution. With the use of array imaging signals, the unbound protein concentration [P] can be obtained via Langmuir isotherms (eq 1). Once the concentration of P has been measured,

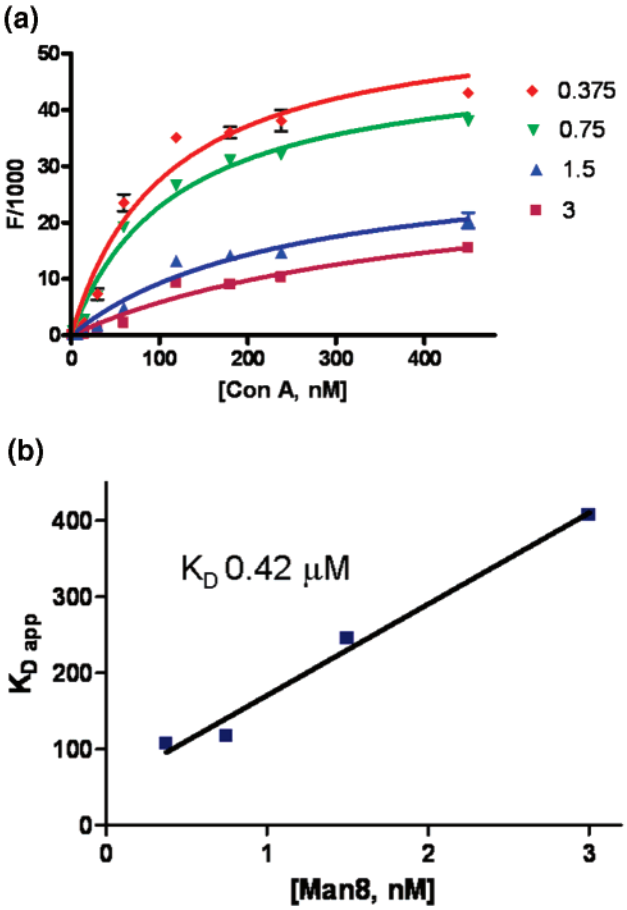

Figure 4. (a) Competition experiment between solution and surface Man8 for FITC−Con A. At different concentrations of the competitor, binding curves were obtained from the bound Con A concentrations and fluorescence intensities. (b) The K_D values were determined from a replot of the K_{Dapp} vs free Man8 concentrations according to eq 2.

it is possible to determine the K_D using eq 2, which is derived from the multivalent Scatchard formula (see the Experimental Section for the derivation of eq 2):

$$F = \frac{F_{max}[Po]}{[Po] + K_{D,surf}\left(1 + \frac{[Lo]}{K_D}\right)} \qquad (2)$$

where [Lo] is the ligand (carbohydrate) concentration applied to the system and K_D is the solution equilibrium dissociation constant. The derivation of this equation makes four assumptions: (1) the nonspecific binding of protein to the slide surface is negligible compared to the total amount of protein in the system; (2) the binding sites in the protein bind to the ligand independently; (3) the initial concentration of ligand is much greater than the initial concentration of protein so that the concentration of unbound ligand is approximately equal to the total concentration of ligand (i.e., [Lo] ≈ [L]); (4) the initial protein concentration for the system is greater than the initial concentration of protein−ligand complex (i.e., [Po] ≈ [P]). A competition binding experiment was performed by treating Con A to various oligomannoses, followed by incubation with corresponding oligomannoses surface. Binding curves, representing different concentrations of competitors, were obtained as the function of FITC−Con A concentrations and fluorescence intensities from the Man8 surface (Figure 4a). The data was

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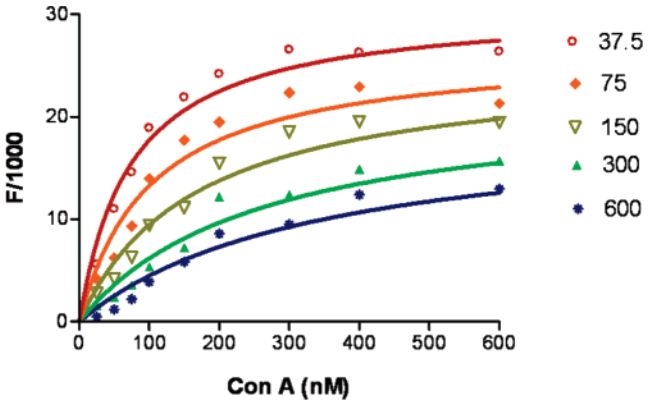

Figure 5. The binding curves were obtained from the surface bound Man1−Con A concentrations and fluorescence intensities. Different curves mean different concentrations of competitor (α-MeMan) in the solution.

Table 2. Competitors and Solution K_i Values for the Interaction with Con A

competitors	array (mM)	ITC (mM)[a]	SPR (mM)[b]
α-MeMan	0.16	0.12	0.09
α-MeGlc	0.69	0.52	0.29
α-MeGal	−[c]	−	−
mannose	1.2	−	−
glucose	25	−	−
galactose	80	−	−

[a] Ref 41. [b] Ref 12. [c] 20% inhibition at 100 mM. "−" not determined.

analyzed according to eq 2, to afford apparent K_D values, which were then replotted against competitor concentration to afford the solution K_D values (Figure 4b). With the use of this analysis, the K_D values for Man1, Man4, Man8, and Man9 for Con A were found to be 250, 55, 0.42, and 0.13 μM, respectively. These values agree well with the solution dissociation constants of 0.3 to ~1.0 μM for either Man8 or Man9 derivatives obtained from SPR followed by HPLC analysis.[39] This analysis clearly shows that Man9 is more than 10^3-fold stronger than Man1 in binding to Con A. This is because Man8 and Man9 are bivalent ligands containing the α(1,6) and α(1,3) arms of the core residue, where the α(1,6) was identified as the high-affinity or primary site and α(1,3) arm as the low-affinity or secondary site.[40] A comparison of the solution K_D (ex 250 μM for the monovalent Man1) and the $K_{D,surf}$ (83 nM) values provide the extent of multivalent effect.

Competitive Inhibitors of Carbohydrate-Binding Proteins in Solution. When different inhibitors (such as α-methyl mannose (α-MeMan), α-methyl glucose (α-MeGlc), etc.) are applied to the system, binding curves can be analyzed using eq 10 (see the Experimental Section) and the inhibition constant K_i can be obtained. Different concentrations of inhibitors were incubated with the slide bound with a protein of interest (Figure 5), the fluorescent intensities were monitored, and then the K_i values were determined (Table 2). The values agree well with the K_i of 92 and 290 μM (for α-MeMan and α-MeGlc, respectively) obtained by SPR[12] and with the K_i of 120 and 520 μM (for α-MeMan and α-MeGlc, respectively) obtained by microcalorimetry measurements.[41] The relative affinity value of α-MeMan to α-MeGlc (K_i (α-MeGlc)/K_i (α-MeMan) = 4.3) is consistent with the result obtained from microcalorimetry measurements[41] and dextran precipitation induced by Con A.[42] These results indicate that this competition assay can reproduce the binding constants determined by well-tested solution methods. In addition, this method has an advantage in that only one surface is needed to rapidly measure a variety of inhibitors. Moreover, the microarray competition assay can illuminate the molecular features important for carbohydrate−protein complexation and will provide a basis for optimizing inhibitor structure.

Conclusion

The glycan array system described here offers several features that make it attractive as a tool for glycomics: it requires small quantities of materials (10^{-18} mol) for high-throughput analysis and can be used for quantitative analysis of carbohydrate−protein interaction on surface and in solution. The system is considered to be a good mimic of cell-surface arrays of carbohydrates in which the dissociation constants of multivalent interactions[34] can be determined for comparison with the monovalent, solution dissociation constants determined through the competition analysis. We envision the method to be useful for the characterization of sugar-binding specificities of proteins and for the high-throughput discovery of inhibitors of carbohydrate-binding proteins with therapeutic value.

Experimental Section

Materials. NHS-coated glass slides (Nexterion H slide, SCHOTT North America; high-density amine binding slide, Amersham bioscience), FITC-labeled concanavalin A (Con A, Sigma), FITC-labeled *L. culinaris* agglutinin (LCA, Sigma), FITC-labeled *P. sativum* agglutinin (PSA, Sigma), α-methyl mannose (α-MeMan, Vector laboratory), α-methyl glucose (α-MeGlc, Acros), and α-methyl galactose (Sigma) were used. Mannose derivatives (Man1, Man4, Man8, Man9) were synthesized as previously described.[35] Other standard chemicals were purchased from commercial suppliers, and used as received.

Microarray Fabrication. Microarrays were printed (Genomic Solutions, Gene Machine) by robotic pin (SMP2B, TeleChem International Inc.) deposition of ~0.6 nL of various concentrations of amine-containing glycans in print buffer (300 mM phosphate, pH 8.5 containing 0.005% Tween-20) from a 384 well plate onto slides. The slide for (1) the scope of printing concentration studies was prepared as follows: NHS-coated glass slides were printed with Man1 or FITC cadaverine at 30 concentrations between 0.5 fM and 100 mM from left to right with 16 replicates vertically placed in each subarray. Ten identical subarrays were fabricated in a 5 × 2 pattern, and each subarray consisted of a 30 × 16 pattern of spots, with a 0.25 mm pitch. After 1 h of reaction, the slide's surface was divided by drawing with permanent marker to avoid contamination for later protein incubation. (2) The slide for Figure 2 was printed with Man1 with concentrations of 100 (first left column), 80, 60, 40, 30, 20, 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, and 0.1 μM (first right column, 16 different concentrations) from left to right, with 16 replicates vertically placed in each grid, and totally 24 replicates (8 × 3 pattern) subarrays in one slide. (3) The slides for $K_{D,surf}$ and K_D determination were prepared as follows: the slides were printed of Man1, Man4, Man8, and Man9 with a concentration of 100 μM from right to left, 16 replicates vertically placed in each grid, and totally 24 subarrays replicates in one slide. After 1 day of reaction, the slides were washed with PBST buffer (0.05% Tween-20) for 30 min

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(40) Dam, T. K.; Oscarson, S.; Sacchettini, J. C.; Brewer, C. F. *J. Biol. Chem.* **1998**, *273*, 32826−32832.
(41) Mandal, D. K.; Kishore, N.; Brewer, C. F. *Biochemistry* **1994**, *33*, 1149−1156.
(42) Scott, J. K.; Loganathan, D.; Easley, R. B.; Gong, X.; Goldstein, I. J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5398−5402.

and then blocked with blocking solution (superblock blocking buffer in PBS, Pierce) for another 1 h. The slides were dried by purging with Ar gas and then stored at room temperature in a desiccator. The slides were washed with PBS buffer (pH 7.4) before use.

Fluorescence Wash-Off Measurements. FITC cadaverine with concentrations from 100 mM to 0.5 fM was printed onto the slide and read at a 488 nm laser by an ArrayWorx microarray reader (Applied Precision). After 12 h of reaction at dark, the slide was washed with PBST (0.05% Tween-20) buffer. The slide was dried by purging with Ar gas, then read again at A488 by the array scanner. The median fluorescence intensity from each printing concentration was obtained. Since the printing concentration, $C = (100\ \mu M, ..., 0.1\ \mu M)$, and the volume, $V = 0.6$ nL, of FITC cadaverine are known, the number of FITC that remain bound to the surface (Np) is the product of the number of FITC printed and the ratio of prequench (Qpre) to postquench (Qpost) spot intensities, where N_A is Avogadro's number. Each spot in the array is around 0.1 mm in diameter.

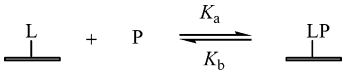
$$\mathrm{Np} = \frac{CVN_A\mathrm{Qpost}}{\mathrm{Qpre}} \tag{3}$$

Direct Binding Assay. FITC-labeled Con A (4 mg/mL), FITC-labeled PSA (2 mg/mL), and FITC-labeled LCA (2 mg/mL) were diluted in phosphate−BSA buffer (50 mM, pH 6.5; 1 mM $CaCl_2$, 1 mM MnCl, 0.9% NaCl (w/v), 1% BSA (w/v)). Human monoclonal antibody 2G12 was used in PBST buffer with 1% BSA. For all incubations, 10−25 μL of protein solution was applied to each subarray using a 24 well bottomless incubation chamber (The Gel Company). Humidifying incubation was performed under foil and using a shaker for 1 h at room temperature. The slide was washed three times with incubation buffer, three times with PBST buffer (0.05% Tween-20), three times with distilled water, and then centrifuged at 200*g* for 5 min to ensure complete dryness. The array was then imaged at a resolution of 5 Å m with an A488 laser using an ArrayWorx microarray reader to visualize fluorescence. The method for the interaction of human monoclonal antibody 2G12 with sugars was similar to lectin. 2G12 in PBST buffer with 1% BSA was precomplexed with Cy3-labeled goat antihuman IgG (Jackson ImmunoResearch) and then placed to the slide and incubated for 1 h. The images were read using a 595 nm laser with the array reader.

Competitive Binding Assay. A volume of 15 μL of series dilutions of competitor was incubated with different concentrations of protein (15 μL). The mixture was then loaded onto the slides by using a 24 well incubation chamber and incubated for 1 h under a humidifying container at room temperature. The following procedure is the same as the direct binding assay.

Data Analysis. ArrayVision 8.0 (Applied Precision) was used for the fluorescence analysis and extraction of data. Equilibrium binding data were analyzed by fitting the data to the appropriate equation, assuming that ligands bound to one or two independent sites, using the commercial nonlinear regression program GrapPad PRISM (GraphPad). The error bars indicated in the figures show the average percentage error for all data points reported in the figures.

Calculation. The formation of surface-bound complex (LP) on the slide between analyte protein (P) and surface-bound ligand (L) can be generally considered to be the simple bimolecular reversible reaction scheme.[43]



(43) Attie, A. D.; Raines, R. T. *J. Chem. Ed.* **1995**, *72*, 119−124.

The observed rate of complex formation may be written

$$\frac{\mathrm{d[LP]}}{\mathrm{d}t} = K_a[\mathrm{L}][\mathrm{P}] - K_b[\mathrm{LP}] \tag{4}$$

The concentration of unoccupied ligand [L] is the difference between the total amount of ligand [Lo] and the amount of [LP]. Substituting [Lo] − [LP] for [L] in eq 4 gives

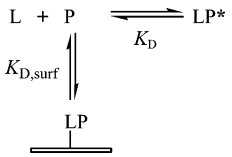
$$\frac{\mathrm{d[LP]}}{\mathrm{d}t} = K_a[\mathrm{P}]([\mathrm{Lo}] - [\mathrm{LP}]) - K_b[\mathrm{LP}] \tag{5}$$

If the total amount of ligand [Lo] is expressed in terms of maximum analyte binding capacity of the surface, all concentration terms can then be expressed as a binding signal response (F).

$$\frac{\mathrm{d}[F]}{\mathrm{d}t} = K_a[\mathrm{P}](F_{max} - F) - K_bF \tag{6}$$

When at equilibrium $\mathrm{d}[F]/\mathrm{d}t = 0$ and $K_{D,surf} = K_b/K_a$, the dissociation constant of ligand and protein complex is obtained as shown in eq 1.

The interaction between a monovalent ligand (L) in the solution, a monovalent ligand (L) on the surface, and a multivalent protein (P) can be represented as



The expression for the equilibrium solution dissociation constant for this interaction is

$$K_D = \frac{[\mathrm{L}][\mathrm{P}]}{[\mathrm{LP}^*]} \tag{7}$$

where [LP*] is the concentration of protein/ligand complexes, [P] is the concentration of free protein, and [L] is the concentration of free ligand. Since a multivalent protein (P) may have q binding sites (B), the concentration of free binding sites [B] is equal to q[P]. Likewise, the formation of a binding site/ligand complex [BP*] is equal to q[LP*]. From eq 7, both numerator and denominator multiply q value. The interaction of one acceptor binding site with ligand can be represented as

$$K_D = \frac{q[\mathrm{L}][\mathrm{P}]}{q[\mathrm{LP}^*]} = \frac{[\mathrm{L}][\mathrm{B}]}{[\mathrm{LB}^*]} \tag{8}$$

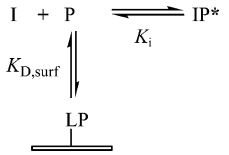
Since the binding sites in the protein bind to the ligands independently, the individual dissociation constant is therefore the same as the protein dissociation constant.

Then, the α value is defined as ratio of free protein and total protein which is then substituted [LP*] value by eq 7 and rearranged to give eq 9.

$$\alpha = \frac{[\mathrm{P}]}{[\mathrm{P}] + [\mathrm{LP}^*]} = \frac{1}{\dfrac{[\mathrm{Lo}]}{K_D} + 1} \tag{9}$$

To determine K_D, the unbound protein in this system is calculated to become α[Po] and which is substituted to eq 1 and rearranges to yield eq 2.

The interaction between inhibitors (I) in the solution, a ligand (L) on the surface, and a multivalent protein (P) can be represented as



The array imaging data is used to measure [P] and K_i can be determined by the eq 10.

$$F = \frac{F_{max}[Po]}{[Po] + K_{D,surf}\left(1 + \frac{[I]}{K_i}\right)} \quad (10)$$

The fraction of inhibition (f) is equal to $1 - F/F_{max}$; eq 10 can be rearranged to give eq 11.

$$1 - \frac{F}{F_{max}} = f = \frac{[I]}{[I] + K_i\left(1 + \frac{[Po]}{K_D}\right)} \quad (11)$$

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Supporting Information Available: The figures for fluorescence wash-off measurement of surface densities, calculation of surface densities, and complete ref 15. This material is available free of charge via the Internet at http://pubs.acs.org.

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