Carbohydrate Microarrays

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High-Throughput Carbohydrate Microarray Analysis of 24 Lectins**

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Lectins, non-immunoglobulin proteins that bind carbohydrates, play a central role in a wide range of biological processes such as cell–cell recognition, viral and bacterial pathogenesis, and inflammation.[1,2] Moreover, they are used extensively as research tools, diagnostics, and therapeutics. For example, mistletoe lectin is in clinical trials as an anticancer agent.[3] Therefore, a fundamental understanding of carbohydrate–protein interactions and comprehensive information on lectin specificity is critical. Unfortunately, evaluation of lectin specificity is not trivial. One common method involves measuring the binding of lectins to cells, tissues, and glycoproteins. This approach frequently uncovers interesting and useful binding properties, however, cells, tissues, and glycoproteins display complex mixtures of carbohydrate epitopes. Therefore, it is exceedingly difficult to determine the specific carbohydrate structures being recognized by the lectin. An alternative approach involves measuring binding to structurally defined carbohydrate epitopes through techniques such as isothermal calorimetry (ITC), mono- and oligosaccharide inhibition studies, enzyme-linked lectin assays (ELLA), and surface plasmon resonance assays (SPR). Unfortunately, these methods can be labor intensive, require large amounts of carbohydrates, and/or be difficult to perform in a high-throughput fashion. Moreover, these studies have typically been limited to the small number of carbohydrate epitopes that were readily accessible. Although lectin specificity has been studied often, much more comprehensive information is still needed.

Carbohydrate microarrays are an emerging technology for the high-throughput evaluation of carbohydrate-macromolecule interactions.[4–27] Analogous to DNA and protein arrays, carbohydrate microarrays contain numerous carbohydrate epitopes immobilized on a solid support in a miniaturized fashion. The microarray format allows one to rapidly evaluate many potential interactions with a minimal amount of sample. Our group has recently developed a carbohydrate microarray and a highly sensitive assay to detect binding.[21] To maximize throughput, each slide contained 16 wells with an entire array printed in each well (see Figure 1a). To illustrate the capabilities of our microarray and study lectin sepcificity, 24 lectins were evaluated at eight different concentrations by using the microarray. As one of the largest and most-comprehensive lectin studies ever reported, the results should be a useful resource for scientists conducting basic and applied research with lectins. Interestingly, microarray analysis revealed unexpected ligands for many of the lectins.

Our approach for fabricating arrays involves printing carbohydrate–bovine serum albumin (BSA)/human serum albumin (HSA) conjugates and glycoproteins on epoxide-functionalized glass microscope slides. This strategy permits immobilization of both structurally discrete synthetic carbohydrates as well as natural carbohydrates presented on glycoproteins. Our first array contained 29 components. To increase the diversity, 23 additional glycans were chemically synthesized and 21 were purchased (see the Supporting Information). The array then contained 73 different components: 4 controls, 54 BSA/HSA conjugates, and 15 glycoproteins (see Figure 1c).

Lectins were evaluated by using our previously reported assay. Briefly, slides were incubated with biotinylated lectins in serial dilutions, washed, and then incubated with streptavidin–horseradish peroxidase (HRP). Finally, wells were incubated with a Cy3-labeled tyramide substrate. The fluorescence intensities of each spot were then measured by using

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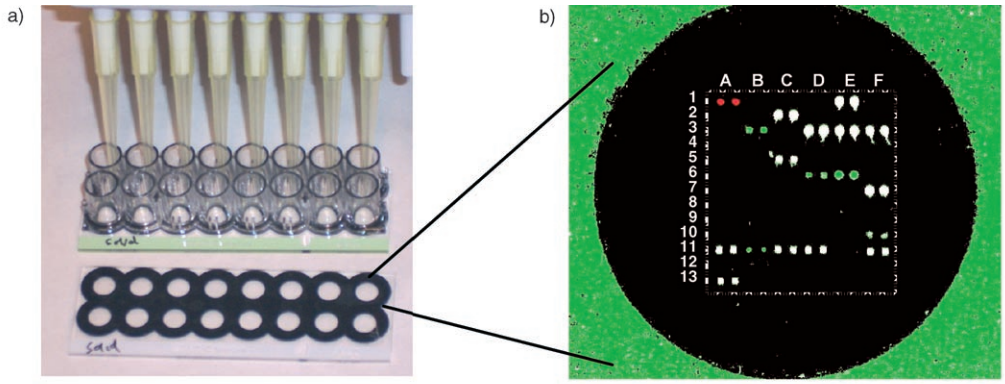
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



c)

1. Cy5-BSA (position A1)
2. BSA
3. HSA
4. Glc-β -BSA
5. GalNAc-α -BSA
6. Glc-α -BSA
7. Man-α -BSA
8. GlcNAc-β -BSA
9. GalNAc-β -BSA
10. Fuc-β -BSA
11. Fuc-α -BSA
12. Rha-α -BSA
13. Rha-β -BSA
14. Gal-α -BSA
15. Gal-β -BSA
16. GalNAcα1-Thr-Gly-BSA (Tn)
17. GalNAcα1-6Galβ -BSA
18. GalNAcα1-3Galβ -BSA (A_{di})
19. GlcNAcα1-4Galβ -BSA
20. Galα1-3Gal-BSA (Bdi)
21. Galβ1-4GlcNAc-BSA (LacNAc)
22. Galβ1-3GalNAc-HSA (TF_{di})
23. Galβ1-4Glcβ -BSA (Lac)
24. Galβ1-3GlcNAcβ -BSA (Le^c)
25. Galβ1-6Man-α -BSA
26. Manα1-6Man-α -BSA
27. GalNAcβ1-4Galβ -BSA ($GA2_{di}$)
28. Glcβ1-4Glcβ -BSA (Cellobiose)
29. Glcα1-4Glcβ -BSA (Maltose)
30. Glcα1-6Glcβ -BSA (Isomalt)
31. Manα1-6[Manα1-3]Manα - BSA (ManT)
32. Galα1-4Galβ -BSA
33. Sialyl2-3Galβ1-4GlcNAc - BSA (3'SLacNAc)
34. GalNAcα1-3(Fucα1-2)Galβ - BSA (BG-A)
35. Galα1-3(Fucα1-2)Galβ -BSA (BG- B)
36. Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ - HSA (BG-H1)
37. Galβ1-3[Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ - BSA (Le^a)
38. Fucα1-2Galβ1-3[Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ - BSA (Le^b)
39. Fucα1-2Galβ1-4[Fucα1-3)GlcNAc - HSA (Le^y)
40. Galβ1-4[Fucα1-3)GlcNAc -HSA (Le^x)
41. SLe^x - BSA
42. Galα1-4Galβ1-4Glc - HSA (Gb3)
43. Xylβ1-4Xylβ1-4Xylβ1-4Xylβ1 - BSA (Xylβ4)
44. Araα1-5Araα1-5Araα1-5Araα1-5Araα1 - BSA (Ara5)
45. Xylα1-6Glcβ1-4(Xylα1-6)Glcβ1-4(Xylα1-6)Glcβ1 - BSA (X3Glc3)
46. 3'Sialyllactose - HSA (GM3)
47. 6'Sialyllactose - HSA (6'SLac)
48. Sia-LeA - HSA (SLe^a)
49. Di-LeX – BSA
50. Manβ1-4Manβ1-4Manβ1-4Manβ1-BSA (Manβ4)
51. Manβ1-4(Galα1-6)Manβ1-4(Galα1-6)Manβ1-4Manβ-BSA (G2M4)
52. Glcα1-6Glcα1-4Glcα1-4Glcβ -BSA
53. Galβ1-3GlcNAcβ1-3Galβ -BSA (LNT)
54. Sia2-3Galβ1-3GlcNAcβ1-3Galβ1-BSA (LSTa)
55. Galβ1-3(Sia2-6)GlcNAcβ1-3Galβ-BSA (LSTb)
56. Sia2-6Galβ1-3GlcNAcβ1-3Galβ-BSA (LSTc)
57. Galβ1-3GalNAcβ1-4Galβ-BSA (GA1)
58. KLH
59. oxKLH
60. Bovine submaxillary mucin (BSM)
61. Asialo-BSM (aBSM)
62. Deacetylated-BSM (deAcBSM)
63. Ovine submaxillary mucin (OSM)
64. asialo-OSM (aOSM)
65. Glycophorin (Gn)
66. asialo-glycophorin (aGn)
67. Prostate Specific Antigen (PSA)
68. Heat Shock Protein 90 (hsp90)
69. Thyroglobulin (Tgl)
70. Alpha fetoprotein (AFP)
71. Carcinoembryonic antigen(CEA)
72. Fatty Acid Binding Prot. (FABP)
73. Cy3-BSA (position A13)

Figure 1. The carbohydrate microarray: a) the 16-well slide format; b) typical assay results for a single well; c) components of the array in the order that they were printed (black = controls, blue = BSA/HSA conjugates, green = glycoproteins).

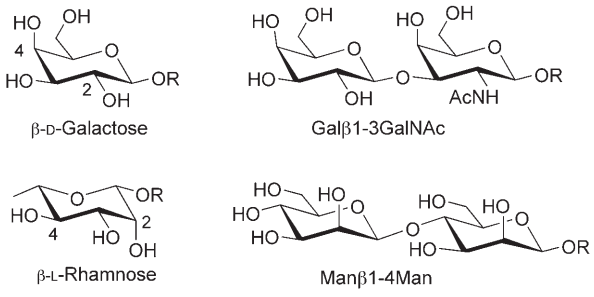
a standard DNA microarray scanner (for an image of a typical well, see Figure 1b). At any single concentration of lectin, some spots may be saturated while others are barely distinguishable from the background signal. By evaluating binding over a broad range of lectin concentrations, one can determine the relative affinities for many ligands. Relative binding has been expressed in terms of the detection limit (DL), which is the lowest concentration of lectin that produced a signal five-times higher than the background for a given epitope. Better ligands have lower detection limits.

Results for each of the 24 lectins are summarized in Table 1.[28] Several findings are of special interest. First, many lectins that bound epitopes would not have been predicted based on their designated specificity.[29] For example, BPL is known as a GalNAc/Galβ1–3GalNAc-binding lectin and RCA120 is considered a Gal/GalNAc-binding lectin. Interestingly, both lectins bound tightly to rhamnose (DL = 80 pM for BPL and 21 pM for RCA120). Furthermore, soluble rhamnose competitively inhibited the binding of these lectins to lactose (see the Supporting Information). From a chemical perspective, recognition of both galactose and rhamnose is remarkable given the significant structural differences (i.e. stereochemistry of the hydroxyl groups at the 2- and 4-positions, oxygenation compared with deoxygenation at the 6-position, and overall D compared with L stereochemistry; see Scheme 1). Recognition of rhamnose may also be important biologically as rhamnose is found in a variety of plant and bacterial carbohydrates. ACL, a Galβ1-3GalNAc binding lectin, was found to bind Manβ1-4Manβ1-4Manβ1-4Man (DL = 29 nM), a plant oligosaccharide. Again, Galβ1-3GalNAc and Manβ1-4Man contained substantial structural differences (see Scheme 1). As a third example, a lectin that binds sialic acid, SNA, demonstrated good binding to some glycans that lack sialic acid such as blood group B (DL =

Table 1: Lectin specificity profiles.

Lectin (specificity)[a]	Epitopes bound by the lectin (detection limits)[b]
ACL (Galβ1-3GalNAc)	a-Gn, Di-Le^x (1 nM); Le^x, SLe^x, Gn (4 nM); aOSM, PSA, HSP90, AFP (14 nM); Manβ4, aBSM (29 nM)
BPL (TF, GalNAc)	a-Gn, aOSM, aBSM, Le^X, Di-Le^X (40 pM); GalNAc-β, Rha-β, Tn, GalNAcα1-6Galβ, A_{di}, LNT (80 pM); TF_{di}, Lac, Le^c, LSTb, GA1 (164 pM); FABP (320 pM); Galα1-3Gal, $GA2_{di}$, KLH, OSM (1 nM); GalNAc-α (3 nM); BG-A, BG-B, Le^a, BSM, deAcBSM, Gn, PSA, HSP90, Tgl, AFP, CEA (10 nM)
Con A (Manα, Glcα)	ManT (51 pM), Tgl (102 pM), Man-α (200 pM); KLH, CEA (410 pM); Glc-α (820 pM); BSM, AFP, aBSM, deAcBSM, OSM (3 nM); Maltose, Glc4 (5 nM); oxKLH, aOSM, PSA (10 nM); GlcNAcα 1-4Galβ, Manα1-6Manα (12 nM)
DBL (GalNAcα)	BG-A (892 pM); Tn , GalNAcα1-6Galβ, A_{di} (4 pM); BG-B (7 nM)
ECL (LacNAc)	aGn (9 nM); Lac (11 nM); LacNAc (22 nM); aBSM, aOSM (44 nM); Gal-α, Tgl (88 nM)
GS-I (Gal/GalNAcα)	BG-B (55 pM); Gal-α (110 pM); Gb3 (440 pM); Galα1-3Gal (880 pM); aOSM (4 nM)
GS-II (GlcNAc)	GlcNAcα1-4Galβ (110 pM); a-Gn, aBSM (440 pM); aOSM (880 pM); GlcNAc-β (9 pM); Le^y, Le^X, Di-Le^X, BSM, DeAcBSM (18 nM)
HAA (GalNAcα)	Tn, GalNAcα1-6Galβ, A_{di}, BG-A (7.9 nM); BG-B, aGn, GlcNAcα1-4Galβ (16 nM); GalNAc-α, OSM, aOSM (32 nM)
HPL (GalNAcα)	aBSM, aOSM (13 pM); Tn, GalNAcα1-6Galβ, A_{di}, GlcNAcα1-4Gal, BG-A, OSM, aGn (26 pM); GalNAc-α (53 pM); BG-B (110 pM); BSM (210 pM); DeAcBSM (790 pM); Gn (2 nM); GalNAc-β (3 nM); GlcNAc-β (6 nM)
LBA (BG-A)	BG-B (25 nM); BG-A (100 nM)
LTL (Fuc, Le^x)	Fuc-α (170 pM); Le^y, Le^x (340 pM); Di-Le^x, CEA, Fuc-β (680 pM)
MPL (GalNAcα)	aBSM, aOSM, aGn (780 pM); Tn (6 nM); BG-B, GalNAc-α, Gal-α, GalNAcα1-6Galβ, OSM (25 nM)
PNA (Galβ1-3GalNAc)	a-Gn (11 pM); GA1 (1 nM); TF_{di} (2 nM); Lac (5 nM); BG-B (6 nM); aBSM (11 nM)
PTL (Gal/GalNAc)	BG-A, BG-B (1 nM); A_{di} (6 nM); Tn, GalNAcα1-6Galβ, aOSM (11 nM); aBSM (29 nM); GalNAc-α (57 nM)
RCA120 (Gal/GalNAc)	Rha-β, a-Gn (21 pM); Lac, Tgl (330 pM); LacNAc, BG-B, LSTc, aBSM, OSM, aOSM, Gn, FABP (1 nM); Gal-α, GalNAcα1-6Galβ, Galα1-3Gal, TF_{di}, Galβ1-3GlcNAcβ, Gb3, LNT, LSTb, BSM, CEA (2 nM); Galα1-4Galβ, BG-H1, KLH, deAcBSM, PSA, AFP (4 nM); HSA, (5 nM); GalNAc-β, GA1 (10 nM)
SBA (GalNAc)	aBSM, aOSM (33 pM); Tn, Gb3 (65 pM); GalNAcα1-6Galβ, A_{di} (130 pM); GalNAc-α (260 pM); OSM (520 pM); GalNAc-β (1 nM); aGn (4 nM); $GA2_{di}$ (5 nM); BG-A, BG-B, deAcBSM (10 nM); Gal-α, BSM (21 nM); Gn (42 nM)
SNA (Siaα2-6Gal)	AFP (420 pM); LSTc (830 pM); BG-B, aOSM, Tgl (3.3 nM); TF_{di}, GM3, 6′SLac, BSM, aBSM, deAcBSM, OSM, Gn, PSA, CEA, FBP (6.7 nM); HSA, GalNAc-α, Man3, aGn (13 nM)
SSA (Tn)	aBSM, aOSM (100 nM)
UEA-I (Fucα)	Le^y (6 pM); Fuc-α, Fuc-β (590 pM); BG-B, aGn (6 nM); BG-H1, BSM (12 nM)
VAA (Galβ)	aOSM, a-Gn (17 μM)
VFA (Manα, Glcα)	Tgl (200 nM)
VVL-B4 (Tn)	aOSM (8 pM); aBSM (15 pM); Tn (60 pM); GalNAcα1-6Galβ, A_{di}, OSM (240 pM); GalNAc-α, DeAcBSM, a-Gn (480 pM); GalNAc-β, $GA2_{di}$, BSM (719 pM)
WFA (GalNAcα/β)	aOSM (11 pM); aBSM (23 pM); Tn (46 pM); GalNAcα1-6Galβ, A_{di} (92 pM); GalNAc-β, aGn (180 pM); GalNAc-α, $GA2_{di}$ (730 pM); Lac, Le^c, LNT, LSTb (2 nM); OSM, Gn, Tgl (3 nM) BSM, deAcBSM (29 nM)
WGA (GlcNAc)	GalNAc-α, GalNAc-β, GlcNAc-β, A_{di}, GlcNAcα1-4Gal (36 pM); BG-A, aBSM (140 pM); 3′SLacNAc, aOSM, Gn, (290 pM); Tn, GalNAcα1-6Gal (580 pM); SLe^a,OSM, a-Gn (1 nM); SLe^x, GM3, 6′Slac, LSTa, oxKLH, BSM, deAcBSM (5 nM); PSA, Tgl, AFP, CEA (17 nM); LacNAc (35 nM)

[a] Nominal specificities listed by the commercial suppliers. [b] The detection limit (DL) is the lowest concentration of lectin that produced a signal 5-times higher than the background. Abbreviations (highest concentration tested): ACL = *Amaranthus caudatus* lectin (29 nM), BPL = *Bauhinia purpurea* lectin (10 nM), Con A = concanavalin A (12 nM), DBL = *Dolichos biflorus* agglutinin (7 nM), ECL = *Erythrina cristagalli* lectin (88 nM), GS-I = *Griffonia simplicifolia* I (4 nM), GS-II = *Griffonia simplicifolia* II (18 nM), HAA = *Helix aspersa* agglutinin (130 nM), HPL = *Helix pomatia* lectin (13 nM), LBA = Lima bean agglutinin or *Phaseolus lunatus* (100 nM), LTL = *Lotus tetragonolobus* lectin (7 nM), MPL = *Maclura pomifer* lectin (50 nM), PNA = peanut agglutinin (46 nM), PTL = *Psophocarpus tetragonolobus* lectin (57 nM), RCA120 = *Ricinus communis* agglutinin I (10 nM), SBA = soybean agglutinin (42 nM), SSA = *Salvia sclarea* agglutinin (400 nM), SNL = *Sambucus nigra* lectin or eldeberry lectin (13 nM), UEA-I = *Ulex europaeus* agglutinin I (12 nM), VAA = *Viscum album* agglutinin or mistletoe lectin (17 mM), VFA = *Vicia faba* agglutinin (29 nM), VVL-B4 = *Vicia villosa* lectin B4 (14 nM), WFL = *Wisteria floribunda* lectin (29 nM), WGA = wheat germ agglutinin (140 nM).



Scheme 1. Structures of selected carbohydrates.

3.3 nM) and the T disaccharide (Galβ1-3GalNAc, DL = 6.7 nM). Interestingly, binding to LSTc, a carbohydrate with a sialic acid at the nonreducing end, could be inhibited with the T disaccharide but not with blood group B. Perhaps one of the most unexpected results was that SNA and RCA120 bound to HSA (non-glycosylated protein used as a control in our array).[30] HSA competitively inhibited carbohydrate binding. These results highlight how easily proteins, cells, and tissues could be mischaracterized when using lectins.

Several other features of lectin binding should also be mentioned. First, lectins frequently exhibit binding requirements beyond simple mono/disaccharide specificity. For

example, RCA120 showed little or no binding within the concentration range tested to a number of Gal and GalNAc terminal carbohydrates such as GalNAcα1-4Galβ, GalNAcα1-3Galβ, Galβ1-6Manα, GalNAcβ1-4Gal, BG-A, Le^a, and Le^x. Although this aspect of lectin recognition is known, detailed information on secondary binding requirements is not available for most lectins. Carbohydrate microarrays provide rapid access to comprehensive binding profiles. Second, lectin specificity can be difficult to predict based on binding (or lack of binding) to closely related structures. For example, the GalNAc binding lectin SBA showed little or no binding to the Galα monosaccharide and the Galα1-3Gal disaccharide. However, SBA bound very well to Gb3 (DL = 65 pM, Galα1-4Galβ1-4Glc). Therefore, it is critical to evaluate binding to a wide range of carbohydrates.

In summary, a carbohydrate microarray was used to evaluate over 1700 potential lectin–ligand interactions over a range of lectin concentrations. The format allowed for very rapid analysis with a minimal amount of expensive and difficult to obtain carbohydrates. For comparison, an ELLA assay carried out in 96-well plates would have required at least 150 plates and 100-fold larger quantities of each BSA conjugate and protein. The unexpected binding properties uncovered in this study and the extensive binding information obtained highlight the utility of carbohydrate microarrays for rapid evaluation of carbohydrate–protein interactions.

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[28] Different lectins contain different levels of biotin incorporation. As the biotin/lectin ratio will affect the detection limit, the results for different lectins should be compared qualitatively rather than quantitatively.
[29] Some epitopes contain a thioglycoside linker at the reducing end of the sugar. Although the linker can potentially affect recognition, inhibition was also observed with sugar lactols (see the Supporting Information).
[30] Although possible, several lines of evidence suggest that the observed binding is not due to a minor glycoprotein impurity. First, the manufacturer states that the HSA is non-glycosylated, greater than 99% pure, and essentially free of globulins. Second, binding to HSA was not observed with other lectins with similar specificity. Recognition of proteins/peptides by carbohydrate-binding proteins has been reported previously; for examples, see A. Pashov, M. Perry, M. Dyar, M. Chow, T. Kieber-Emmons, *Curr. Top. Med. Chem.* **2005**, *5*, 1171 and K. R. Oldenburg, D. Loganathan, I. J. Goldstein, P. G. Schultz, M. A. Gallop, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 5393.

