Chapter 1

Carbohydrate Microarray

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Additional information is available at the end of the chapter

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1. Introduction

Glycosylation adorns more than one half of the proteins in eukaryotic cells [1,2]. This post-translational modification plays an indispensible role in many important biological events, especially on cell surface [1,3]. Alterations in carbohydrate structures are known to correlate with the changes in protein stability and clearance, as well as various physiological functions including cell-cell adhesion, inflammation, tumor metastasis, and infection of bacteria and viruses [4-8]. Although glycosylation is essential for the formation and progression of various diseases, study of this subject is hampered by lack of effective tools available to date, in addition to structural heterogeneity and complexity of carbohydrates. A number of techniques have been developed to analyze the binding interactions between carbohydrates and proteins [2,9]. For instances, lectin blotting/binding assay has become a routine method to determine the glycan-protein interactions [10], but the relatively low sensitivity and the necessity of multiple wash steps/time-consuming have restricted the sensitivity and application. Surface plasmon resonance is another highly sensitive method which monitors the interactions in real time and in a quantitative manner [11-16]. However, sometimes the sensitivity is relatively low toward the use of low molecular-weight carbohydrates, though the problem can be overcome by labeling sugars with heavy metal ions [17]. In addition, fluorescence polarization and two-photon fluorescence correlation have been applied to study lectin-glycan interactions [18-21]. The most applicable technique is carbohydrate microarrays which immobilize oligosaccharides to a solid supports are developed and widely used to measure the carbohydrate binding properties of proteins, cells, or viruses [22-25]. For example, a high-content glycan microarray is developed by a robotic microarray printing technology in which amine-functionalized glycans are coupled to the succinimide esters on glass slides [26,27]. These microarrays have also been subjected for profiling the carbohydrate binding specificities of lectins, antibodies, and intact viruses.

2. Carbohydrate microarray

In our recent work, we have developed two novel carbohydrate microarrays: solution microarray [28] and membrane microarray [29]. Carbohydrate solution assay is a high-throughput, homogenous and sensitive method to characterize protein-carbohydrate interactions and glycostructures by in-solution proximity binding with photosensitizers (**Figure 1**). The technology, also called AlphaScreen™, is first described by Ullman et al., and has been used to study interactions between biomolecules [30-34]. In these assays, a light signal is generated when a donor bead and an acceptor bead are brought into proximity. This method usually provides good sensitivity with *femto*-mole detection under optimized conditions, relying on the binding affinity between analytes. All the procedures are carried out in 384-welled microtiter plates, thus qualifying the protocol as high-throughput. Two particles of 200 nm are involved in this technology including streptavidin-coated particles (donor beads) and protein A-conjugated particles (acceptor beads). Biotinylated polyacrylamide (biotin-PAA)-based glycans that are immobilized on donor beads can be recognized by lectins or antibodies, and connected with acceptor beads through specific antibodies (**Figure 1**). A number of carbohydrate binding proteins, including eleven lectins and seven antibodies, are profiled for their carbohydrate binding specificity to validate the efficacy of this developed technology. This assay is performed in homogeneous solutions and does not require extra wash steps, preventing the loss of weak bindings that often occur in the repeating washes of glycan microarray. However, antigen/ligand excess effect may happen in the homogeneous solution assay if the concentrations of carbohydrate epitopes, proteins, or antibodies are too high. One mg of biotin-PAA-sugar can be applied for fifty thousand assays because minimal amount of materials are needed in this microarray system (a range of nano-gram is required per well). Although the detection limit of biotin-PAA-sugar is good (2 ng per well), the linear range is too narrow for quantitative application.

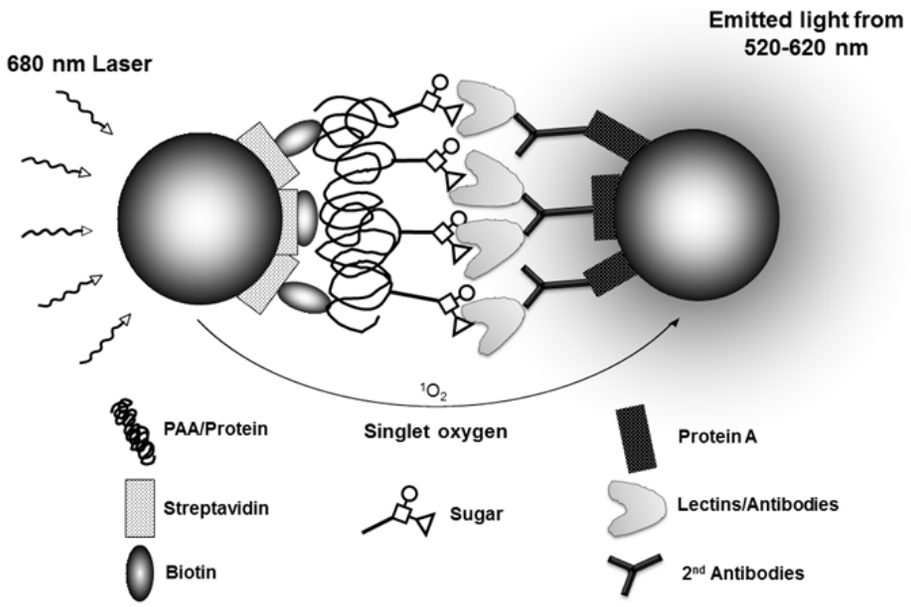


Figure 1. In-solution proximity binding with photosensitizers which was developed to characterize the protein-carbohydrate interactions [28].

Carbohydrate membrane microarray is fabricated by immobilization of the biotin-conjugated PAA-based glycans on aldehyde-functionalized UltraBind via streptavidin. Streptavidin interacted strongly with biotin and formed covalent linkage with membranes after reductive amination, which prevented the loss of glycans from membrane during repeated wash steps. The use of PAA also avoided the nonspecific interactions that take place in other studies between some lectins and non-glycosylated proteins (e.g. HAS or BSA) [35]. The operation of this carbohydrate membrane microarray is similar to that of Western blotting and can be performed easily by anyone without prior intensive training.

3. Applications

3.1. Carbohydrate binding profiles of lectins and antibodies (solution microarray)

Fifty-four biotinylated polyacrylamide backboned glycans (biotin-PAA-glycans) (**Table 1**) are collected in total to examine fifteen carbohydrate-binding proteins, including eight lectins (Con A, DBA, GS-I, PNA, SBA, UEA-1, WFA and WGA), and six antibodies (anti-Le^a, Le^b, Le^x, Le^y, sialyl Le^a and sialyl Le^x). The resulting signals are indicated with bars as relative intensities (**Figures 2 and 3**). The natural carbohydrate ligands for these lectins are listed in **Table 2**. All of the lectins showed nearly the same carbohydrate binding preferences as those in literatures. For example, concanavalin A (Con A) bound preferentially to mannose (No. 3) and biantennary *N*-glycan (No. 53), and very weakly to 3- and 6-sulfated galactosides (No. 19, 23 and 25). DBA, a GalNAc-binding lectin, recognized GalNAcα1-3Gal-containing epitopes (No. 11 and 39). ECA interacted with LacNAc disaccharide, Galβ1-4(6-sulfo)GlcNAc, and Galβ1-4(α1-2Fuc)GlcNAc (No. 17, 24, 31 and 47), and weakly bound to Le^c (Galβ1-3GlcNAc, No. 20). GS-I preferred interacting with Gal/GalNAc that contains α1-3 or 1-4 linkage (No. 11, 13, 14, 16, 40 and 42). MAA, in this study, recognized mainly to 3′-sulfated Galβ1-3GlcNAc, 3′-sulfated Galβ1-4GlcNAc and LacNAc and weakly to 3-sialylated galactosides (No. 26, 37 and 53). PNA interacted with Galβ1-3GalNAc (No. 15) and bound to some galactosides weakly (No. 12, 16, 20, 45 and 46). SBA preferentially interacted with α-linked galactosides (No. 16 and 42) and *N*-acetylgalactosaminoside (No. 11). SNA, a well-known α2-6 sialoside-binding lectin, interacted strongly to 6′-sialyl lactose and sialylated diantennary *N*-glycan (No. 36 and 53). UEA-1 specifically bound with Fucα1-2Gal-containing glycans (No. 18, 31 and 49). Due to weak interaction with PAA, WFA is the only one lectin showing higher background signals than the others. It recognized nearly half of the glycans on the glycan library, such as GlcNAc- and NeuAcα2-3-Gal/NeuAcα2-6-Gal containing saccharides. WGA also bound to terminal Gal or GalNAc epitopes (GalNAcα1-3Gal, No. 11 and Galβ1-4(6HSO_3)GlcNAc, No. 24) according to some minor signals. Interestingly, WGA showed better interactions with chitotriose than with chitobiose and GlcNAc. In addition, the binding specificities of monoclonal anti-carbohydrate antibodies also revealed some interesting features. As shown in **Figure 3**, anti-Le^a antibody bound tightly with Le^x, but less with Le^b and sialyl Le^a. Anti-Le^b antibody represented specificities for both Le^b and

Le^a, but less for Le^x and sialyl Le^x. Anti-Le^y antibody not only binding to Le^y, but also recognized lactose, Le^x, sialyl Le^x and H type 2 structures. We also compared the binding patterns of lectins with the results reported by Blixt and coworkers at CFG in which 264 different glycans are studied by using the printed microarray (Ver. 2) (http://www.functionalglycomics.org/glycomics/publicdata/ primaryscreen.jsp). There are forty-seven glyco-epitopes are found to be identical in both analyses. Even the different principles and procedures of the two systems, the binding patterns of eight lectins are nearly the same, except for a few minor differences. For example, our characterized patterns of WFA and WGA show 90% similarity to the CFG data. Nevertheless, the interactions of SBA, WFA and WGA to β-GalNAc (No. 2) in the CFG's printed microarray are not observed in our system. Both of our method and the printed microarray indicate that MAA preferentially binds to sulfated glycans [36]. Because of the observed consistency shown by the two very different methods, we conclude the protein-glycan binding interactions are not affected by the PAA linker, the assay procedure (washing vs. non-washing) and the interacting microenvironment (2D for printed microarray vs. 3D for our solution microarray).

No.	Glycan Name	No.	Glycan Name
1	PAA-biotin	28	GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ, sp=-NHCOCH2NH-
2	β-GlcNAc	29	GlcNAcβ1-3Galβ1-4GlcNAcβ
3	α-Mannose	30	Fucα1-2Galβ1-3GlcNAcβ, Le^d (H type1)
4	β-GlcNAc	31	Fucα1-2Galβ1-4GlcNAcβ (H type2)
5	β-GalNAc	32	Galβ1-3(Fucα1-4)GlcNAcβ (Le^a)
6	α-Fuc	33	Galβ1-4(Fucα1-3)GlcNAcβ (Le^x)
7	α-NeuAc	34	3-HSO_3-Galβ1-4(Fucα1-3)GlcNAcβ (3'sulfate Le^x)
8	α-NeuGc	35	NeuAcα2-3Galβ1-3GlcNAcβ (3'Sialyl Le^c)
9	Glcα1-4Glcβ	36	NeuAcα2-6Galβ1-4Glcβ (6'Sialyl Lactose)
10	GlcNAcβ1-4GlcNAcβ	37	NeuAcα2-3Galβ1-4Glcβ (3'Sialyl Lactose)
11	GalNAcα1-3Galβ	38	NeuAcα2-3(NeuAcα2-6)GalNAcα
12	Galβ1-4Glcβ (Lactose)	39	GalNAcα1-3(Fucα1-2)Galβ (Blood Group A)
13	Galα1-3Galβ	40	Galα1-3(Fucα1-2)Galβ (Blood Group B)
14	Galα1-3GalNAcβ	41	3-HSO_3-Galβ1-3(Fucα1-4)GlcNAcβ (3'sulfate Le^a)
15	Galβ1-3GalNAcβ	42	Galα1-4Galβ1-4Glcβ
16	Galα1-4GlcNAcβ (αLacNAc)	43	NeuAcα2-3Galβ1-4GlcNAcβ
17	Galβ1-4GlcNAcβ (LacNAc)	44	NeuAcα2-3Galβ1-3GalNAcα
18	Fucα1-2Galβ	45	Galβ1-3(NeuAcα2-6)GalNAcα
19	3-HSO_3-Galβ1-4GlcNAcβ	46	Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ
20	Galβ1-3GlcNAc (Le^c)	47	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ
21	NeuAcα2-6GalNAcα	48	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ (Le^b)
22	NeuGcα2-6GalNAcα	49	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ (Le^y)
23	3-HSO_3-Galβ1-3GlcNAcβ	50	NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ (sialyl Le^a)
24	Galβ1-4(6-HSO_3)GlcNAcβ	51	NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ (sialyl Le^x)
25	6-HSO_3-Galβ1-4GlcNAcβ	52	$(NeuAc\alpha2\text{-}8)_{5\text{-}6}$
26	NeuAcα2-3Gal	53	$(NeuAc\alpha2\text{-}6Gal\beta1\text{-}4GlcNAc\beta1\text{-}2Man)_2\alpha1\text{-}3,6Man\beta1\text{-}4GlcNAc\beta1\text{-}4GlcNAc\beta$
27	NeuAcα2-3GalNAcα	54	H_2O

Table 1. List of biotin-PAA-glycans (fifty-two) used in glycan solution microarray [28].

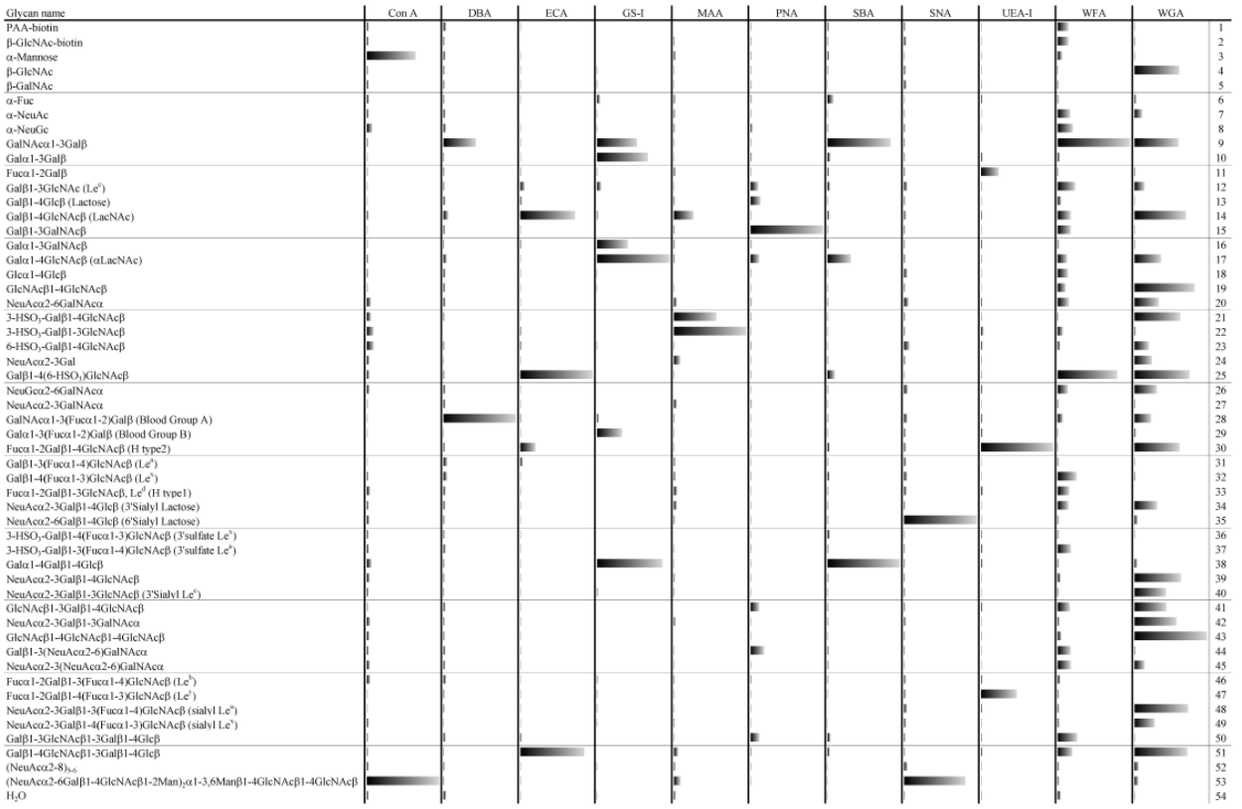


Figure 2. Carbohydrate binding specificities of eleven lectins characterized by glycan solution microarray [28].

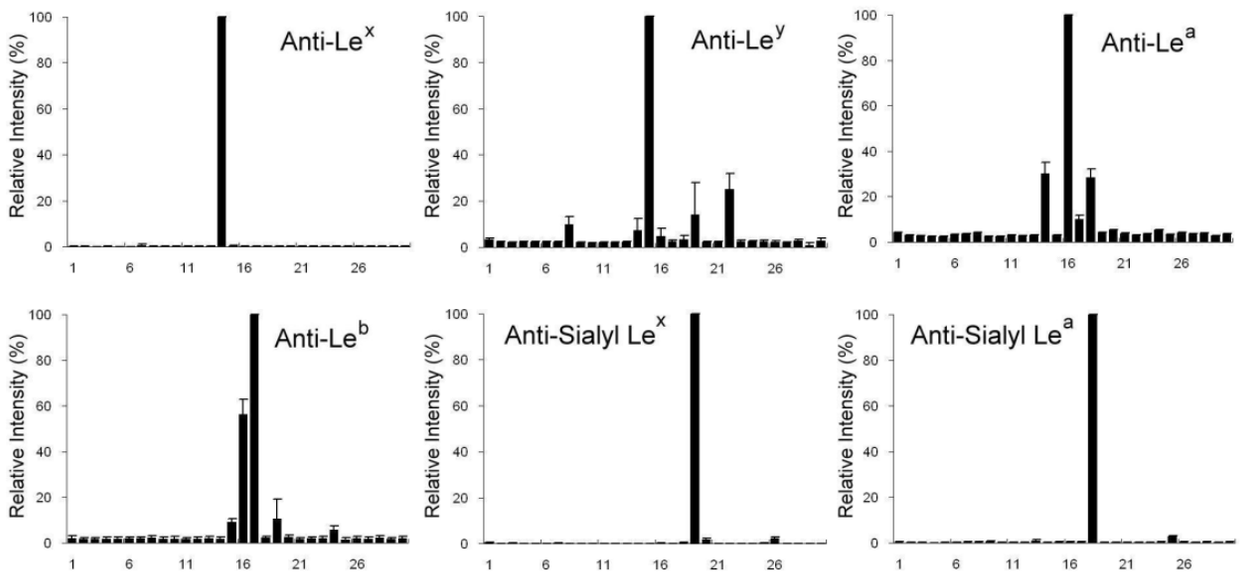


Figure 3. Carbohydrate binding specificities of six antibodies characterized by glycan solution microarray [28].

Lectins	Binding specificities	Lectins	Binding specificities
Con A	Man, Glc, GlcNAc	**WGA**	GlcNAcβ1-4GlcNAc, Neu5Ac
DBA	GalNAc	**ECA**	Galβ1-4GlcNAc
PNA	Galβ1-3GalNAc	**MMA**	Gal
SBA	GalNAc/Gal	**GS-I**	Gal
UEA-1	Fuc	**SNA**	Neu5Acα2-6
WFA	GalNAc		

Table 2. Carbohydrate ligands of commercial available lectins.

3.2. Binding patterns of seventeen lectins and four antibodies (membrane microarray)

The principle and procedures of carbohydrate membrane microarray are showed in **Figure 4**. The western blotting like procedures not only reduces the time and interference, but also increases the application of this platform. In order to look deep inside the carbohydrate binding preferences of proteins and microorganisms, the collections of biotin-PAA-glycans were to increased eighty-eight different structures (**Table 3**). The glycan binding specificities of sixteen lectins (six alkaline phosphatase (AP)-conjugated lectins, four FITC-conjugated lectins, six unconjugated lectins) and four Lewis blood-group antibodies are evaluated and showed in **Figures 5 and 6**. All the lectins recognized the glycans that are consistent with the literature. For instance, ECA preferentially interacted with LacNAc, lactose, GalNAc, and Gal terminal sugars; PNA specifically bound to the Galβ1-3GalNAc structure; SBA dominantly recognized α-linked GalNAc epitopes; 3-sulfate LacNAc is ligand for MAA [36]. Compare the patterns of unconjugated lectins with conjugated lectins (AP- or FITC-attached) indicated that the glycan preferences of ECA and PNA are not interfered by conjugation. More binding signals are observed in the binding profiles of AP-conjugated MAA, SBA and WGA compared with unconjugated or FITC-attached ones. Additionally, the binding patterns of DBA, ECA, GS-I, MAA, SBA and VVA are highly consistent with those reported by Consortium for Functional Glycomics (CFG, printed microarray Ver. 2,). However, few inconsistencies are also observed in the study of MPA, PNA, UEA and WGA. Furthermore, the binding patterns of four Lewis blood group antibodies represented very high specificities (**Figure 6**).

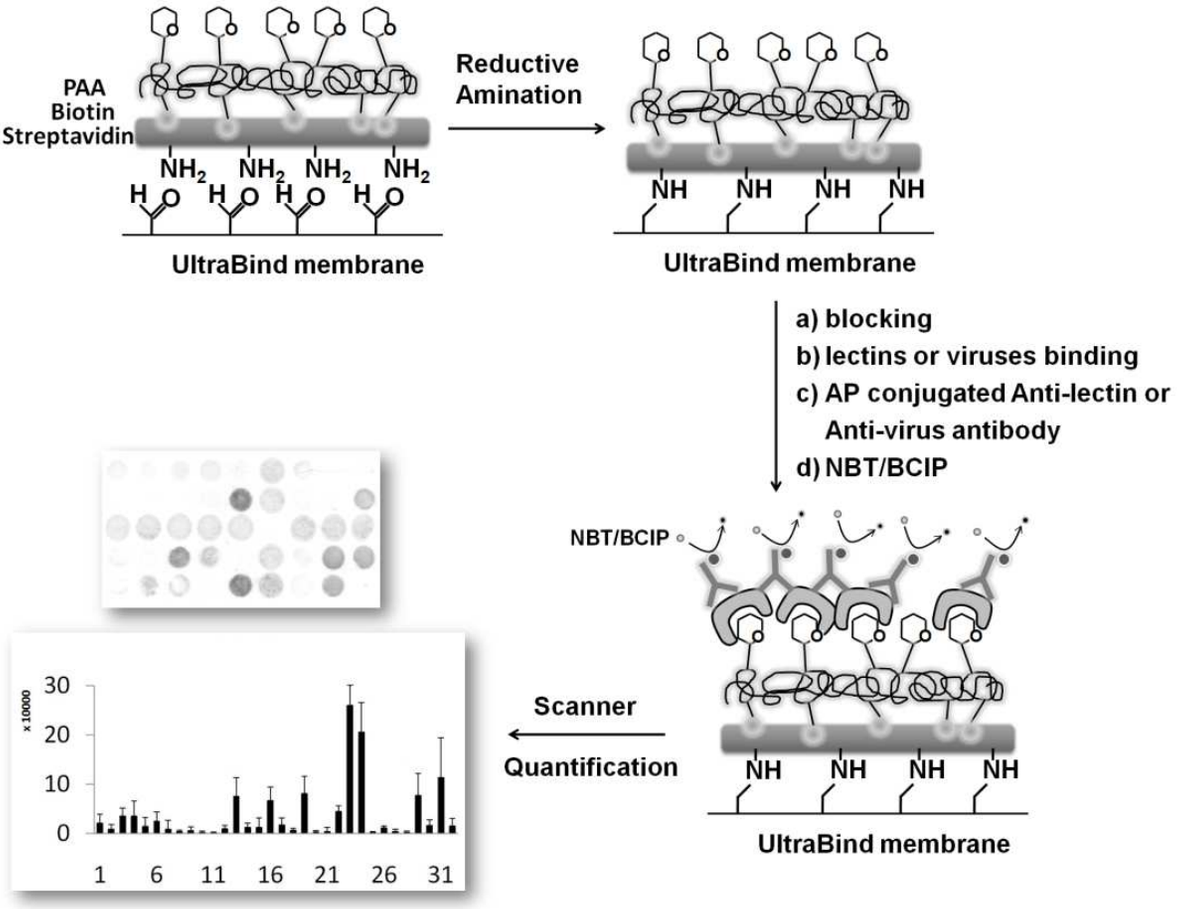


Figure 4. Fabrication, principle, and procedures of carbohydrate membrane microarray [29].

S-1	Blank-PAA-biotin	S-46	Neu5Acα2-6Galβ-PAA-biotin
S-2	β-GlcNAc-sp-biotin	S-47	Neu5Gcα2-6GalNAc-PAA-biotin
S-3	α-Mannose-PAA-biotin	S-48	Neu5Acα2-3GalNAcα-PAA-biotin
S-4	β-GlcNAc-PAA-biotin	S-49	Blood Group A-tri-PAA-biotin
S-5	β-GalNAc-PAA-biotin	S-50	Blood Group B-tri-PAA-biotin
S-6	α-L-Fuc-PAA-biotin	S-51	H(type2)-PAA-biotin
S-7	α-Neu5Ac-PAA-biotin	S-52	Le^a-PAA-biotin
S-8	α-Neu5Ac-$OCH_2C_6H_4$-p-$NHCOOCH_2$-PAA-biotin	S-53	Le^x-PAA-biotin
S-9	MDP(muramyl dipeptide)-PAA-biotin	S-54	Le^d(H type1)-PAA-biotin
S-10	α-Neu5Gc-PAA-biotin	S-55	3'Sialyl-Lactose-PAA-biotin
S-11	β-D-Gal-3-sulfate-PAA-biotin	S-56	6'Sialyl-Lactose-PAA-biotin
S-12	β-D-GlcNAc-6-sulfate-PAA-biotin	S-57	3-HSO_3-Le^x-PAA-biotin
S-13	GalNAcα1-3Galβ-PAA-biotin	S-58	3-HSO_3-Le^a-PAA-biotin
S-14	Galα1-3Galβ-PAA-biotin	S-59	Galα1-4Galβ1-4Glcβ-PAA-biotin
S-15	Fucα1-2Galβ-PAA-biotion	S-60	Galα1-3Galβ1-4Glcβ-PAA-biotin
S-16	Le^c(Galβ1-3GlcNAc)-PAA-biotin	S-61	GlcNAcβ1-2Galβ1-3GalNAcα-PAA-biotin
S-17	Galβ1-4Glcβ-PAA-biotin (Lactose)	S-62	Neu5Acα2-3Galβ1-4GlcNAcβ-PAA-Biotin
S-18	LacNAc-PAA-biotin	S-63	3'Sialyl-Le^c-PAA-biotin
S-19	Fucα1-3GlcNAcβ-PAA-biotin	S-64	Galα1-3Galβ1-4GlcNAcβ-PAA-biotin, sp=-$NHCOCH_2NH$-
S-20	Fucα1-4GlcNAcβ-PAA-biotin	S-65	GlcNAcα1-3Galβ1-3GalNAcα-PAA-biotin
S-21	GalNAcα1-3GalNAcα-PAA-biotin	S-66	GlcNAcβ1-3Galβ1-3GalNAcα-PAA-biotin
S-22	Galα1-3GalNAcα-PAA-biotin	S-67	Galβ1-3(GlcNAcβ1-6)GalNAcα-PAA-biotin
S-23	Galβ1-3GalNAcβ-PAA-biotin	S-68	Blood type A (tri)-PAA-biotin, sp=$(CH_2)_3NHCO(CH_2)_5NH$-
S-24	Galα1-3GalNAcβ-PAA-biotin	S-69	Blood type B (tri)-PAA-biotin, sp=$(CH_2)_3NHCO(CH_2)_5NH$-
S-25	Galβ1-3Galβ-PAA-biotin	S-70	GlcNAcb1-3Galβ1-4GlcNAc-PAA-biotin
S-26	GlcNAcβ1-3Galβ-PAA-biotin	S-71	Neu5Acα2-3Galβ1-3GalNAcα-PAA-Biotin
S-27	αLacNAc-PAA-biotin	S-72	GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα-PAA-biotin
S-28	Glcα1-4Glcβ-PAA-biotin	S-73	Galα1-4Galβ1-4GlcNAcβ-PAA-biotin
S-29	Galβ1-3GalNAcα-PAA-biotin, sp=-p-OC_6H_4-	S-74	GlcNAcβ1-4GlcNAcβ1-4GlcNAc-PAA-biotin
S-30	Galα1-2Galβ-PAA-biotin	S-75	Galβ1-3(Neu5Acα2-6)GalNAcα-PAA-biotin
S-31	GlcNAcβ1-4GlcNAc-PAA-biotin	S-76	Neu5Acα2-3(Neu5Acα2-6)GalNAc-PAA-biotin
S-32	GlcNAcβ1-4GlcNAcβ-PAA-biotin, sp=-$NHCOCH_2NH$-	S-77	Galβ1-4GlcNAcβ1-3GalNAcα-PAA-biotin
S-33	Neu5Acα2-6GalNAc-PAA-biotin	S-78	Le^b-PAA-biotin
S-34	H(type 3)-PAA-biotin	S-79	Le^y-PAA-biotin
S-35	3-HSO_3-Galβ1-4GlcNAc-PAA-biotin	S-80	Sialyl Le^a-PAA-biotin
S-36	3-HSO_3-Galβ1-3GlcNAcβ-PAA-biotin	S-81	Sialyl Le^x-PAA-biotin
S-37	Galα1-6Glcβ-PAA-biotin (melibiose)	S-82	GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glcβ-PAA-biotin
S-38	Neu5Acα2-8Neu5Acα-sp**-PAA-biotin, $(Neu5Ac)_2$	S-83	Galα1-3(Fucα1-2)Galβ1-4GlcNAc-PAA-biotin
S-39	Galβ1-2Galβ-PAA-biotin	S-84	Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-PAA-biotin
S-40	6-HSO_3-Galβ1-4GlcNAc-PAA-biotin	S-85	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-PAA-biotin
S-41	Neu5Acα2-3Gal-PAA-biotin	S-86	(NeuAcα2-8)5-6-PAA-biotin
S-42	Galβ1-4(6-HSO_3)GlcNAcb-PAA-biotin	S-87	Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα-PAA-biotin
S-43	3-HSO_3-Galβ1-3GalNAcβ-PAA-biotin (sulfate-TF)	S-88	α2-6 sialylated diantennary N-glycans-PAA-biotin
S-44	GlcNAcβ1-3GalNAcα-PAA-biotin	S-89	GalNAc-α-Ser-PAA-biotin
S-45	GlcNAcβ1-6GalNAcα-PAA-biotin	S-90	H_2O

α2-6 sialylated diantennary N-glycans :$(NeuAc\alpha2\text{-}6Gal\beta1\text{-}4GlcNAc\beta1\text{-}2Man)_2\alpha1\text{-}3,6Man\beta1\text{-}4GlcNAc\beta1\text{-}4GlcNAc$

Table 3. List of biotin-PAA-glycans (eighty-eight) used in carbohydrate membrane microarray [29].

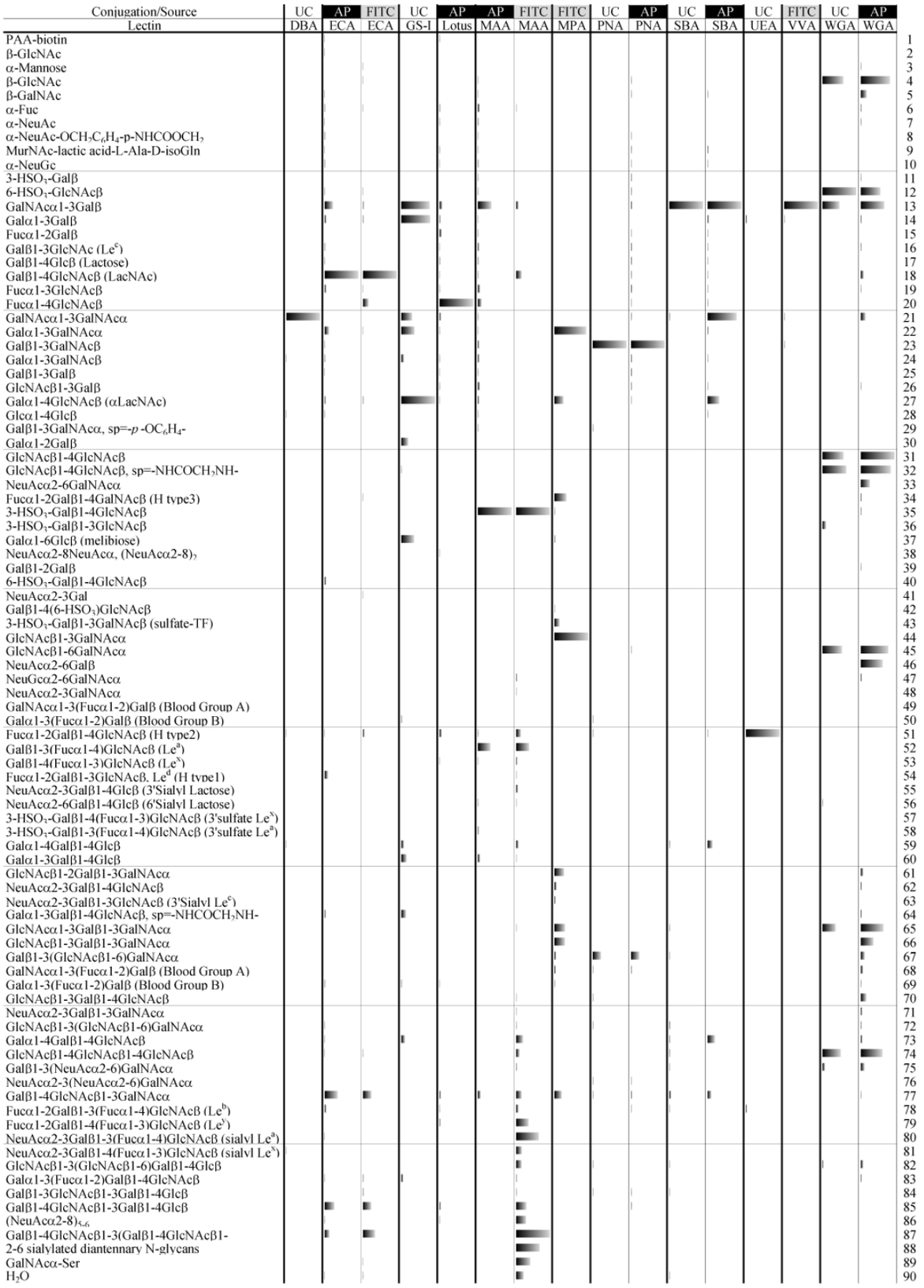


Figure 5. Carbohydrate binding specificities of sixteen lectins (six alkaline phosphatase-conjugated lectins, four FITC-conjugated lectins, and six unconjugated lectins) characterized by carbohydrate membrane microarray [29].

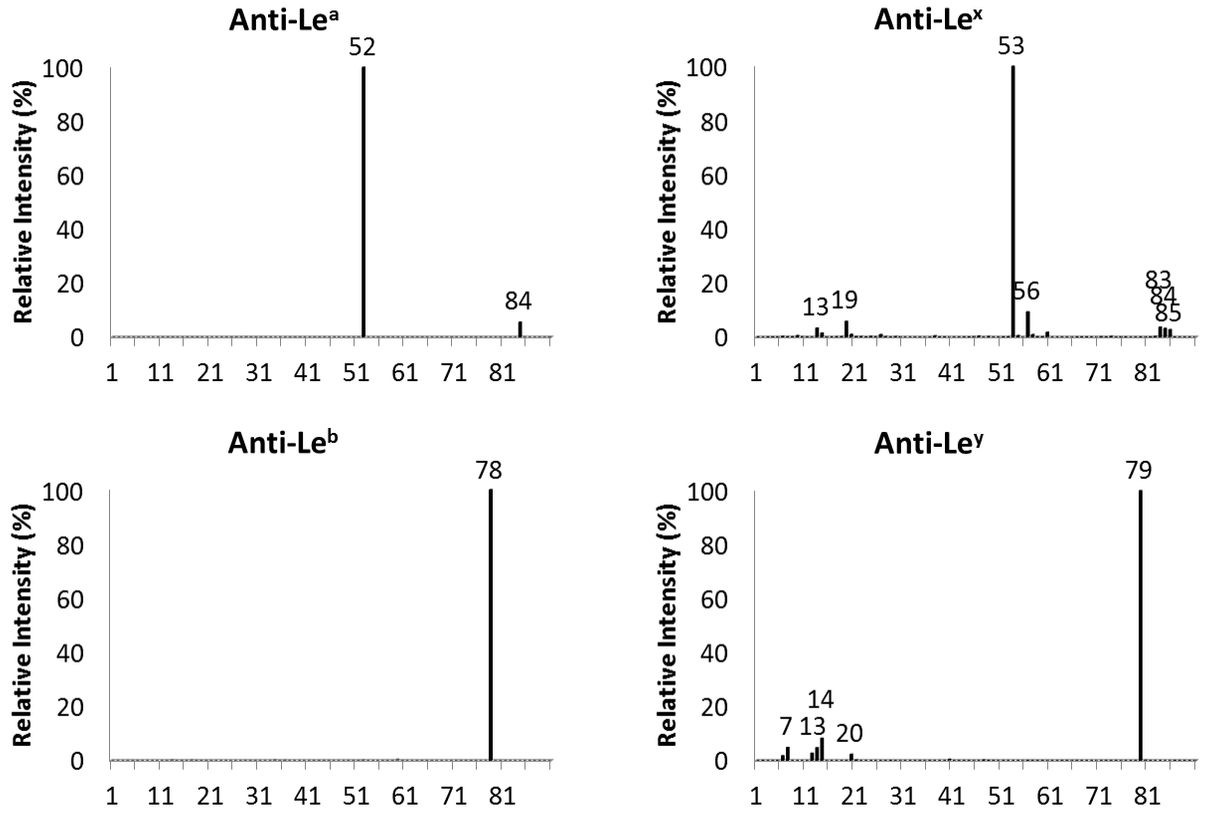


Figure 6. Carbohydrate binding specificities of four antibodies characterized by carbohydrate membrane microarray [29].

3.3. Surveillance of seasonal and pandemic H1N1 influenza A viruses (membrane microarray)

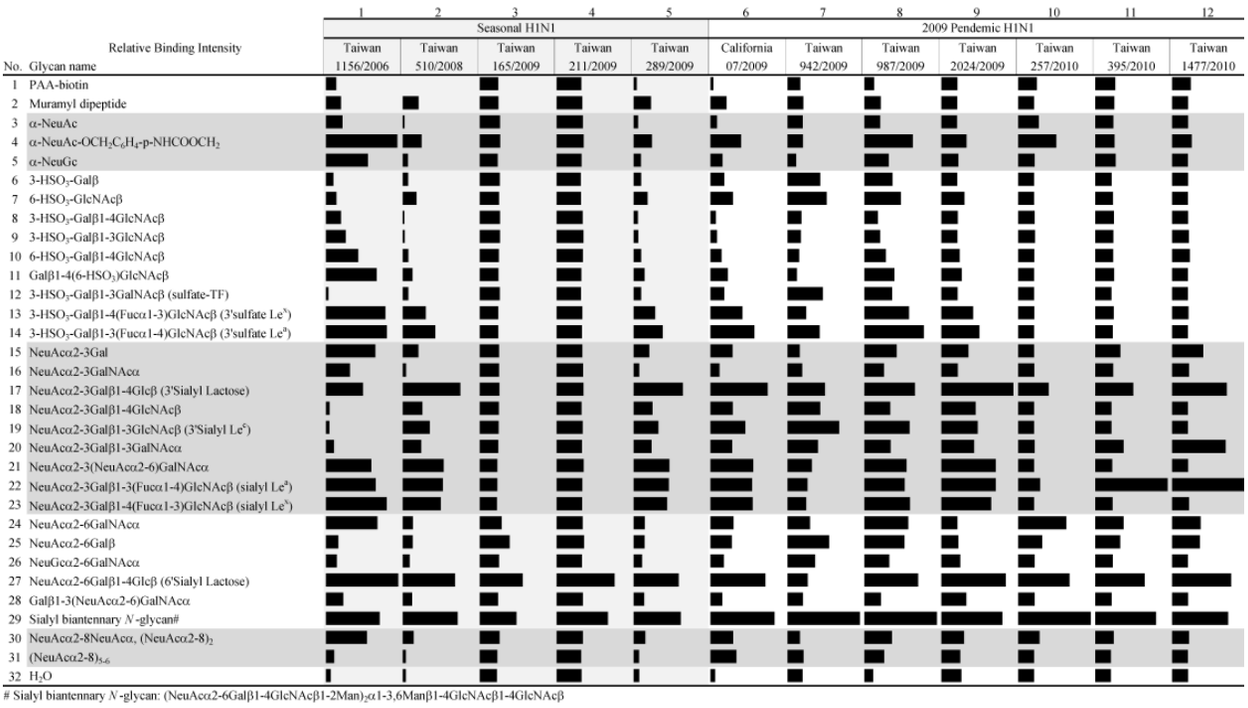
Several methods are applied for exploring the carbohydrate-binding preference of influenza viruses or recombinant HA, including cell-based assays [37-39], immobilization of virus [40-46] and glycan microarray technology [47-49]. In our study, 30 biotin-PAA-glycans (**Table 4**, most of them were sulfated and sialylated) were selected and fabricated on membrane and applied for the carbohydrate ligand surveillance of influenza clinical isolates. We subjected this influenza membrane microarray to characterize the glycan-binding features of five seasonal influenza A (H1N1) and seven A(H1N1)pdm09 clinical isolates. The binding patterns of all studied viruses are successfully profiled, with each virus exhibiting different and clear patterns, thereby enabling characterization (**Figure 7, Lane 1 to 12**). The majority of the seasonal H1N1 viruses bound strongly to 6'-sialyl lactose and sialyl biantennary *N*-glycan (**Figure 7, Lane 1 to 5**). A/Taiwan/1156/2006, A/Taiwan/510/2008, and A/Taiwan/289/2009 isolates interacted with the two glycans, and with numerous α2-3 sialylated structures, including 3'-sialyl lactose, NeuAcα2-3(NeuAcα2-6)GalNAc, sialyl Le^a, and sialyl Le^x. In addition, A/Taiwan/1156/2006 isolate also recognized NeuAc (linked to *p*-aniline), NeuGc, some of the sulfated glycans (3'-sulfated Le^a, 3'-sulfated Le^x, 6-sulfate LacNAc and 6'-sulfate LacNAc), NeuAcα2-6GalNAc, and a NeuAcα2-8 dimer. Contrary to seasonal H1N1 isolated in 2009, pandemic (H1N1) isolated in 2009 accepted more substrates

(**Figure 7, Lane 6 to 9**). California/07/2009 and A/Taiwan/2024/2009 bound with 6-sulfate GlcNAc, 3'-sulfated Le^a, 3'-sulfated Le^x, all of the 2-3 sialylated glycans with the exception of NeuAcα2-3GalNAc, 6'-sialyl lactose, sialyl biantennary *N*-glycan, and α2-8 oligomers. A/Taiwan/942/2009 favored four sulfated glycans (3-sulafe Gal, 6-sulfate GlcNAc, 3'-sulfated Le^a and 3'-sulfated Le^x), most of the 2-3 sialylated glycans (except NeuAcα2-3Gal and NeuAcα2-3GalNAc) and three 2-6 sialylated glycans (NeuAcα2-6Gal, 6'-sialyl lactose and sialyl biantennary *N*-glycan). A/Taiwan/987/2009 recognized most of the PAA-sugar substrates tested, with the exception of the PAA backbone, muramyl dipeptide, αNeuAc, 3-sulafe Galβ1-4GlcNAc, 3-sulafe Galβ1-3GlcNAc and Galβ1-3(NeuAcα2-6)GalNAc. In addition, we found that pandemic (H1N1) viruses isolated in 2009 represented broader substrate specificities than the pandemic viruses isolated in 2010, especially to sulfated sugars. A/Taiwan/395/2010 and A/Taiwan/1477/2010 recognized NeuAcα2-3Gal, 3'sialyl lactose, NeuAcα2-3Galβ1-3GlcNAc, sialyl-Le^a, NeuAcα2-6GalNAc, NeuAcα2-6Gal, 6'-sialyl lactose, and sialyl biantennary *N*-glycan (**Figure 7, Lane 11 to 12**). A/Taiwan/257/2010 accepted only six glycans including NeuAc (linked to *p*-aniline), 3'sialyl lactose, NeuAcα2-6GalNAc, NeuAcα2-6Gal, 6'-sialyl lactose, and sialyl biantennary *N*-glycan (**Figure 7, Lane 9**).

I-1	Blank-PAA-biotin	I-17	Neu5Gcα2-6GalNAc-PAA-biotin
I-2	α-Neu5Ac-PAA-biotin	I-18	Neu5Acα2-3GalNAcα-PAA-biotin
I-3	α-Neu5Ac-$OCH_2C_6H_4$-p-$NHCOOCH_2$-PAA-biotin	I-19	3'Sialyl-Lactose-PAA-biotin
I-4	MDP(muramyl dipeptide)-PAA-biotin	I-20	6'Sialyl-Lactose-PAA-biotin
I-5	α-Neu5Gc-PAA-biotin	I-21	3-HSO_3-Le^x-PAA-biotin
I-6	β-D-Gal-3-sulfate-PAA-biotin	I-22	3-HSO_3-Le^a-PAA-biotin
I-7	β-D-GlcNAc-6-sulfate-PAA-biotin	I-23	Neu5Acα2-3Galβ1-4GlcNAcβ-PAA-Biotin
I-8	Neu5Acα2-6GalNAc-PAA-biotin	I-24	3'Sialyl-Le^c-PAA-biotin
I-9	3-HSO_3-Galβ1-4GlcNAc-PAA-biotin	I-25	Neu5Acα2-3Galβ1-3GalNAcα-PAA-Biotin
I-10	3-HSO_3-Galβ1-3GlcNAcβ-PAA-biotin	I-26	Galβ1-3(Neu5Acα2-6)GalNAcα-PAA-biotin
I-11	Neu5Acα2-8Neu5Acα-sp**-PAA-biotin, $(Neu5Ac)_2$	I-27	Neu5Acα2-3(Neu5Acα2-6)GalNAc-PAA-biotin
I-12	6-HSO_3-Galβ1-4GlcNAc-PAA-biotin	I-28	Sialyl Le^a-PAA-biotin
I-13	Neu5Acα2-3Gal-PAA-biotin	I-29	Sialyl Le^x-PAA-biotin
I-14	Galβ1-4(6-HSO_3)GlcNAcb-PAA-biotin	I-30	(NeuAcα2-8)5-6-PAA-biotin
I-15	3-HSO_3-Galβ1-3GalNAcβ-PAA-biotin (sulfate-TF)	I-31	α2-6 sialylated diantennary N-glycans -PAA-biotin
I-16	Neu5Acα2-6Galβ-PAA-biotin	I-32	H_2O

α2-6 sialylated diantennary N-glycans :$(NeuAc\alpha2\text{-}6Gal\beta1\text{-}4GlcNAc\beta1\text{-}2Man)_2\alpha1\text{-}3,6Man\beta1\text{-}4GlcNAc\beta1\text{-}4GlcNAc$

Table 4. List of biotin-PAA-glycans (thirty) fabricated on carbohydrate membrane microarray for carbohydrate binding surveillance of influenza clinical isolates [29] [50].



Sialyl biantennary *N*-glycan: (NeuAcα2-6Galβ1-4GlcNAcβ1-2Man)$_2$α1-3,6Manβ1-4GlcNAcβ1-4GlcNAcβ

Figure 7. Carbohydrate binding profiles of twelve influenza A clinical isolates characterized by carbohydrate membrane microarray [50].

3.4. Surveillance of influenza B clinical isolates (membrane microarray)

The influenza carbohydrate membrane microarray is subsequently subjected for the surveillance of twelve influenza B (IB) clinical isolates [50]. All of the viruses are collected in the Clinical Virology Laboratory of National Cheng Kung University Hospital between 2001 and 2004. The clinical isolates are amplified in MDCK cells and purified by a sucrose gradient. The binding patterns of the influenza B viruses are investigated and are all found to exhibit clear binding profiles (**Figure 8**). Three common glycans, including α-Neu5Ac (*p*-anilinyl linked), 6'sialyl-lactose, and α2-6 sialylated biantennary *N*-glycans are bound by all of the influenza B viruses. Furthermore, some additional bindings are also observed. For instance, B/Taiwan/314/2001 and B/Taiwan/1729/2002 also recognized sulfated sugars (3-sulafe Gal, 6-sulfate GlcNAc, Galβ1-4(6-HSO$_3$)GlcNAc, and 3'-sulfated Le^a) and two α2-6 sialosides (NeuAcα2-6GalNAc and NeuAcα2-6Gal). B/Taiwan/288/2002 and B/Taiwan/1902/2004 portrayed minor interactions with α2-3 (3'-sialyl lactose, NeuAcα2-3Galβ1-4GlcNAc, 3'-sialyl Le^c and sialyl Le^x) and α2-6 sialylated sugars (NeuAcα2-6GalNAc and NeuAcα2-6Gal). Although the signal is relatively low, B/Taiwan/872/2004 and B/Taiwan/913/2004 also interacted with sulfated sugars. Surprisingly, seven viruses showed weak bindings to α2-8 linked sialic acids, including B/Taiwan/314/2001, B/Taiwan/174/2002, B/Taiwan/288/2002, B/Taiwan/1729/2002, B/Taiwan/872/2004, and B/Taiwan/913/2004.

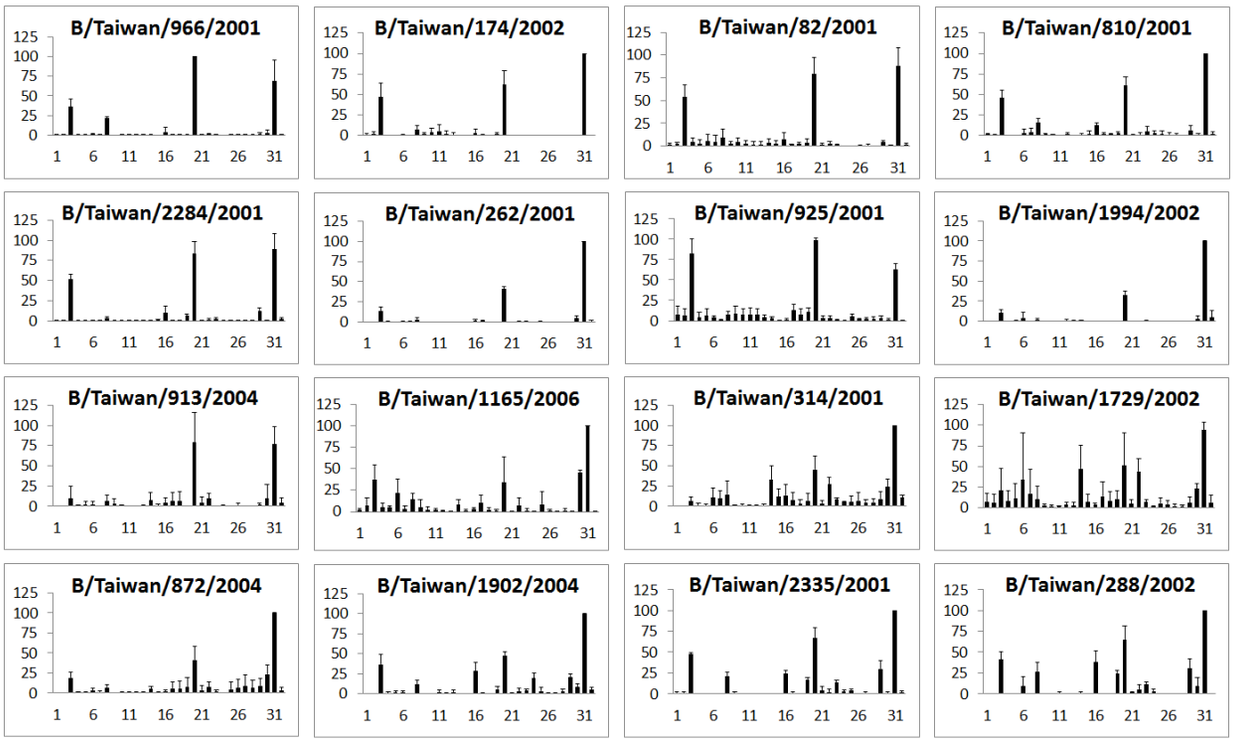


Figure 8. Carbohydrate binding profiles of sixteen influenza B clinical isolates characterized by carbohydrate membrane microarray [50].

The DNA sequences of hemagglutinin of the twelve influenza B clinical isolates were analyzed, translated into sequences of amino acids, and aligned with B/HongKong/8/73, Victoria (B/Victoria/02/87), and Yamagata (B/Yamagata/16/88) lineages using online software EBI ClustalW2. Surprisingly, the twelve influenza B clinical isolates could all be categorized into two groups depending on protein sequence alignment. Group 1 contained eight clinical isolates (B/Taiwan/314/2001, B/Taiwan/174/2002, B/Taiwan/288/2002, B/Taiwan/1729/2002, B/Taiwan/1994/2002, B/Taiwan/872/2004, B/Taiwan/913/2004 and B/Taiwan/1902/2004, **Figure 8, Table 5**) and Group 2 contained four viruses (B/Taiwan/262/2001, B/Taiwan/925/2001, B/Taiwan/966/2001, B/Taiwan/2284/2001). We found that all of the viruses in group 2 were isolated in 2001. In addition, there were twelve amino acid differences between the two groups. Most of the amino acids were located at the chain region and subunit interfaces, while four amino acids were located within the antigenic site (loop 160, amino acid number of B/HK/73) [51,52]. Additionally, four amino acids changed the charge property from group 1 to group 2 viruses (**Table 5**, No. 56, 116, 181 and 182, amino acid number of B/Yamagata/16/88). Different carbohydrate binding properties were also observed between some viruses of the two groups. Specifically, group 2 viruses interacted with three major sialylated glycans, while some of group 1 viruses bound not only strongly to the three glycans, but also weakly to sulfated and α2-3 sialylated glycans. Additionally, the number of charged amino acids was higher in group 2 than in group 1 viruses and B/HongKong/8/73 (**Table 5**).

	Amino acid number of B/HK/8/73	29	48	56	75	116	162	*	*	176	180	181	217	Number of charged amino
	B/HK/8/73	V	Q	N	T	N	K			Y	K	G	V	2
	B/Victoria/02/87	V	K	K	T	N	K	D	N	N	E	G	V	5
	B/Yamagata/16/88	V	K	N	T	N	R	D		Y	K	G	V	4
Group1	B/Taiwan/314/2001	V	R	D	A	N	K	D	N	H	E	G	V	5
	B/Taiwan/174/2002	V	R	D	T	N	K	D	N	Y	E	G	V	
	B/Taiwan/288/2002	V	R	D	T	N	K	D	N	Y	E	G	V	
	B/Taiwan/1729/2002	V	R	D	T	N	K	D	N	Y	E	G	V	
	B/Taiwan/1994/2002	V	R	D	T	N	K	D	N	Y	E	G	V	
	B/Taiwan/872/2004	V	R	D	T	N	K	D	N	Y	E	G	V	
	B/Taiwan/913/2004	V	R	D	T	N	K	D	N	Y	E	G	V	
	B/Taiwan/1902/2004	V	R	D	T	N	K	D	N	Y	E	G	V	
Group2	B/Taiwan/262/2001	A	K	T	I	K	R	E	N	H	K	E	I	7
	B/Taiwan/925/2001	A	K	T	I	K	R	D	N	H	K	E	I	
	B/Taiwan/966/2001	A	K	T	I	K	R	E	N	H	K	E	I	
	B/Taiwan/2284/2001	A	K	T	I	K	R	E	N	H	K	E	I	
	Amino acid number of B/Yamagata/16/88	29	48	56	75	116	162	163	*	177	181	182	218	

Table 5. Critical amino acid differences between the two groups of influenza B clinical isolated viruses [50].

According to the binding preference for α2-6 and α2-3 sialylated oligosaccharides displayed by these clinical isolated influenza viruses, these clinical isolates might infect not only the surface of nasal mucosa, pharynx, larynx, trachea and bronchi (that are resided in the upper respiratory tract to express α2-6 sialosides predominantly), but also alveoli, bronchi (that are located in the lower respiratory tract), eyes, and tissues in the gastrointestinal tract (that contain both α2-3 and α2-6 sialosides) [53,54]. After we surveyed more clinical isolates, factors which affect the binding preference of IB could be dissected. We believe that application of the carbohydrate microarray for detailed analysis of sugar-binding structures will eventually build the connection between clinical symptoms and the genetic specification of influenza viruses.

3.5. Characterization the glycan structure of glycoproteins (solution microarray)

The aforementioned lectins are further applied to characterize the glycan structures of six biotinylated proteins including ovalbumin, porcine mucin, human serum albumin, human transferrin, fetuin and asialofetuin. Distinctive glycopatterns are generated in accordance with the analysis of lectins (**Figure 9**). Porcine mucin is known to have *O*-linked fucosylated glycans with terminal GalNAc, GlcNAc and NeuAc residues. Our results showed positive signals in the tests with the ECA, DBA, UEA-1 and WGA lectins, indicating that the mucin contains the determinants of Galβ1-4GlcNAc, GalNAc, Fucα1-2Gal and GlcNAc/NeuAc, respectively. Manα1-3(Fucα1-6)GlcNAc and NeuAcα2-6Galβ1-4GlcNAc containing biantennary or triantennary *N*-glycans have been reported as the major glyco-structures of human transferrin [55-58]. Strong Con A and SNA signals are shown in our binding assay,

but the relative intensity of GlcNAc-binding lectins is not as good as those of Con A and SNA, which is the contribution of the interference by the terminal sialic acids [59]. All of the results are similar to the analyses from lectin microarray or dot blot analysis [55-58].

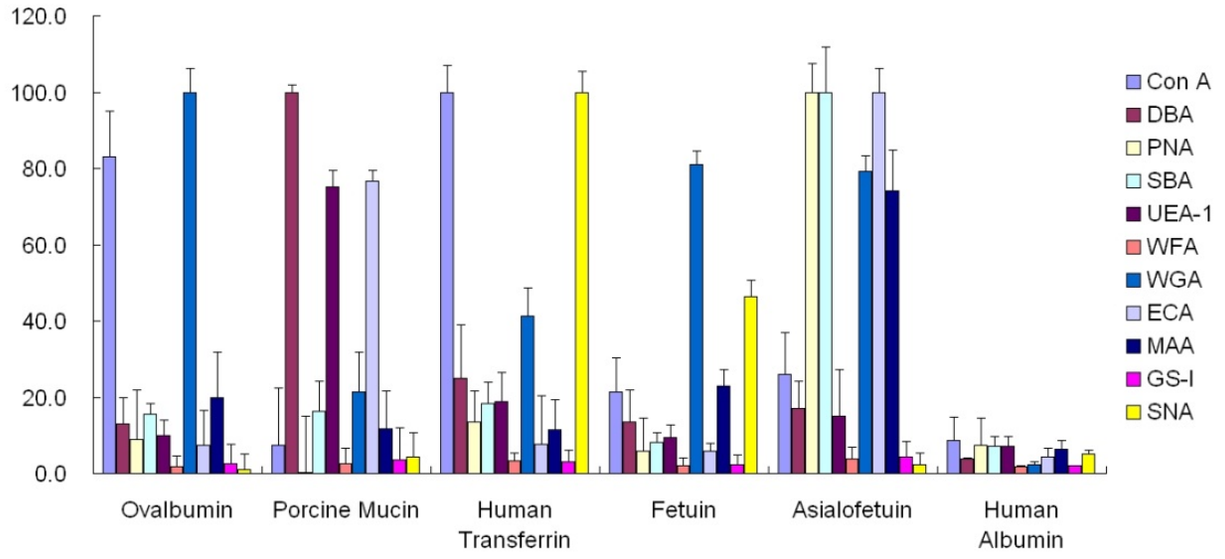


Figure 9. Glycopattern profiling of six biotinylated proteins with ten lectins by glycan solution microarray. The six proteins include ovalbumin, porcine mucin, human serum albumin, human transferrin, fetuin and asialofetuin [28].

4. Perspective

In summary, carbohydrate-protein interactions are the key steps for many physiological and pathological evens. Hence, development of new carbohydrate microarrays is important for detecting these activities. Our studies have demonstrated a rapid, highly sensitive, and reliable method to characterize carbohydrate-protein interactions with minimized materials (in the range of ng per well). Lectins and antibodies are evaluated and most of the results are coherent to previous reports, including those of CFG. Solution microarray is the first homogeneous and washless carbohydrate microarray which offers a reliable alternative for characterizing sugar-binding features of lectins and proteins, as well as antibodies. In addition, membrane microarray is also easy handled with low cost (USD$0.25 for one membrane microarray). The easy-handling feature avoids the necessity of repetitive training and accelerates the screening efficiency. Both of the microarrays represent a convenient and reliable way to examine the carbohydrate-binding features of various proteins, high-throughput drug screening, and the glycan binding surveillance of influenza viruses.

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