J|A|C|S ARTICLES

Published on Web 09/07/2010

Glycan Array on Aluminum Oxide-Coated Glass Slides through Phosphonate Chemistry

Shih-Huang Chang,[†,‡] Jeng-Liang Han,[†] Susan Y. Tseng,[†] Hsin-Yu Lee,[†] Chin-Wei Lin,[†] Yu-Chen Lin,[†,§] Wen-Yih Jeng,[⊥] Andrew H.-J. Wang,[⊥] Chung-Yi Wu,*[,†] and Chi-Huey Wong*[,†]

Genomics Research Center, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 115, Taiwan, Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan, Department of Chemistry, National Taiwan University, Taipei, Taiwan, and Institute of Biological Chemistry, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 115, Taiwan

Received May 28, 2010; E-mail: cyiwu@gate.sinica.edu.tw; chwong@gate.sinica.edu.tw

Abstract: A new type of glycan array covalently or noncovalently attached to aluminum oxide-coated glass (ACG) slides has been developed for studies of enzymatic reactions and protein binding. To prepare the noncovalent array, glycans with a polyfluorinated hydrocarbon ($-C_8F_{17}$) tail are spotted robotically onto the ACG slide surface containing a layer of polyfluorinated hydrocarbon terminated with phosphonate. After incubation and washing, the noncovalent array can be characterized by MS-TOF via ionization/desorption at a low laser energy without addition of matrix. A representive cellotetraose array was developed to study the activity and specificity of different cellulases and to differentiate the exo- and endoglucanase activities. To prepare the covalent array, glycans with a phosphonic acid tail were synthesized and spotted robotically onto the ACG slide surface. After incubation, the slides can be used directly for quantitative protein binding analysis. Compared to the preparation of glycan arrays on glass slides and other surfaces, this method of arraying using phosphonic acid reacting with ACG is more direct, convenient, and effective and represents a new platform for the high-throughput analysis of protein−glycan interactions.

Introduction

Carbohydrates often exist on cell surfaces as glycoproteins or glycolipid conjugates and play important structural and functional roles in numerous biological recognition processes.[1−4] In addition, natural products often require glycosylation for biological activities, and many organisms diversify functions and detoxify harmful exogenous xenobiotics through glycosylation.[5] However, the study of carbohydrate−protein interactions still encounters much difficulty, largely because of the inaccessibility of such structurally complex molecules and their low-affinity interactions with glycan-binding proteins (GBPs). Typically the monomeric dissociation constant (K_D) in a carbohydrate−protein interaction is in the millimolar range; thus, carbohydrate-mediated biological responses are often through multivalent interactions on the cell surface in order to achieve high affinity and specificity.[6] Recently, glycan arrays have been developed to mimic their cell-surface display for the study of carbohydrate−receptor interactions.[7−16] Multiple proteins or glycans can be quantitatively and qualitatively analyzed once in a monovalent or multivalent manner.[17−22] However, most of glycan arrays used today are difficult to characterize and

[†] Genomics Research Center, Academia Sinica.
[‡] Institute of Biochemical Sciences, National Taiwan University.
[§] Department of Chemistry, National Taiwan University.
[⊥] Institute of Biological Chemistry, Academia Sinica.

(1) Apweiler, R.; Hermjakob, H.; Sharon, N. *Biochem. Biophys. Acta* **1999**, *1473*, 4–8.
(2) Raman, R.; Raguram, S.; Venkataraman, G.; Paulson, J. C. *Nat. Methods* **2005**, *2*, 817–824.
(3) Fukuda, M.; Hindsgaul, O. *Molecular and Cellular Glycobiology*; Oxford, University Press: Oxford, 2000.
(4) Seeberger, P. H. *Nature* **2005**, *437*, 1239.
(5) Thorson, J. S.; Vogt, T. In *Glycosylated Natural Products in Carbohydrate-Based Drug Discovery*; Wong, C.-H., Ed.; Wiley-VCH, Weinheim, 2002; pp 685−712.
(6) Weis, W. I. *Annu. Rev. Biochem.* **1996**, *65*, 441–473.
(7) Park, S.; Shin, I. *Angew. Chem., Int. Ed.* **2002**, *41*, 3180–3182.
(8) Houseman, B. T.; Mrksich, M. *Chem. Biol.* **2002**, *9*, 443–454.
(9) Wang, D.; Liu, S.; Trummer, B. J.; Deng, C.; Wang, A. *Nat. Biotechnol.* **2002**, *20*, 275–281.
(10) Fukui, S.; Feizi, T.; Galustian, C.; Lawson, A. M.; Chai, W. *Nat. Biotechnol.* **2002**, *20*, 1011–1017.
(11) Love, K. R.; Seeberger, P. H. *Angew. Chem., Int. Ed.* **2002**, *41*, 3583–3586.
(12) Willats, W. G. T.; Rasmussen, S. E.; Kristensen, T.; Mikkelsen, J. D.; Knox, J. P. *Proteomics* **2002**, *2*, 1666–1671.
(13) Mellet, C. O.; Garciá Fernández, J. M. *ChemBioChem* **2002**, *2*, 819–822.
(14) Paulson, J. C.; Blixt, O.; Collins, B. E. *Nat. Chem. Biol.* **2006**, *2*, 238–248.
(15) Feizi, T.; Fazio, F.; Chai, W.; Wong, C.-H. *Curr. Opin. Struct. Biol.* **2003**, *13*, 637–645.
(16) Shin, I.; Park, S.; Lee, M. *Chem.—Eur. J.* **2005**, *11*, 2894–2901.
(17) Liang, P. H.; Wu, C.-Y.; Greenberg, W. A.; Wong, C.-H. *Curr. Opin. Chem. Biol.* **2008**, *12*, 86–92.
(18) Wu, C.-Y.; Liang, P.-H.; Wong, C.-H. *Org. Biomol. Chem.* **2009**, *7*, 2247–2254.
(19) Liang, C.-H.; Wu, C.-Y. *Expert Rev. Proteomics* **2009**, *6*, 631–645.
(20) Liu, Y.; Palma, A. S.; Feizi, T. *Biol. Chem.* **2009**, *390*, 647–656.
(21) Park, S.; Lee, M.-R.; Shin, I. *Chem. Commun.* **2008**, 4389–4399.
(22) Oyelaran, O.; Gildersleeve, J. C. *Curr. Opin. Chem. Biol.* **2009**, *13*, 406–413.

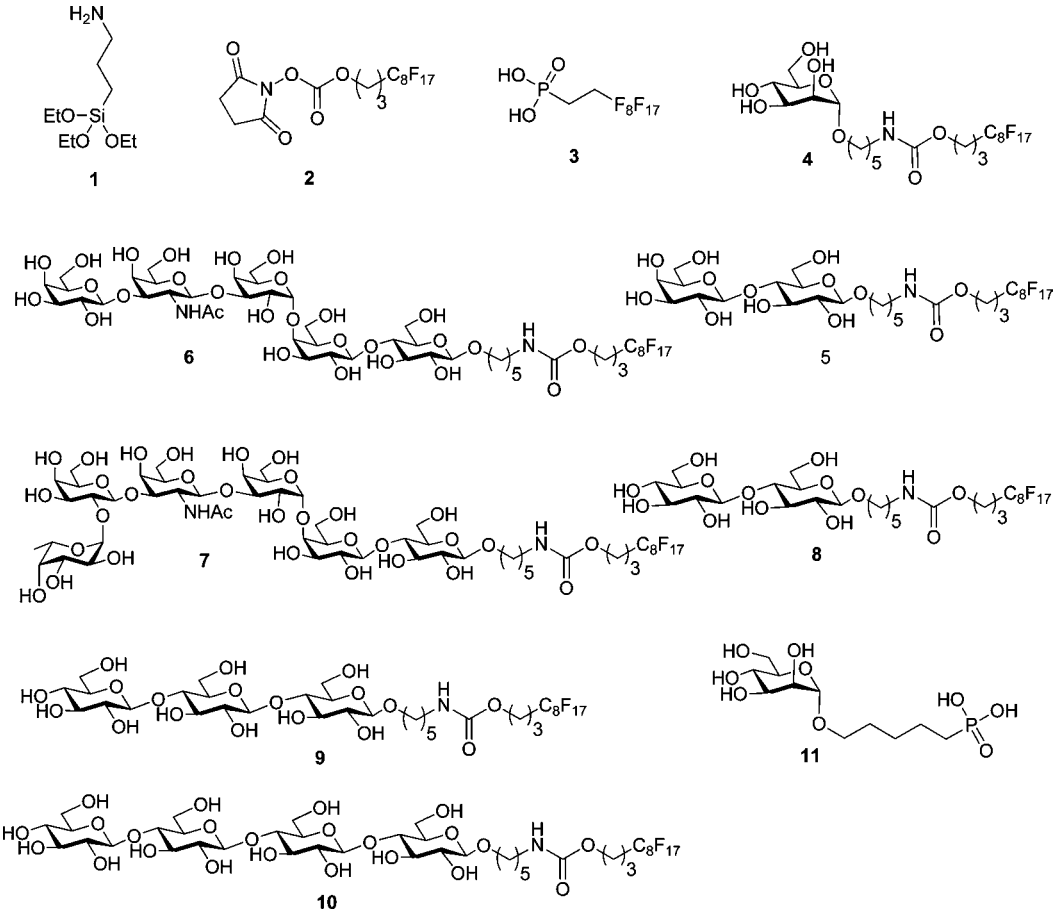


Figure 1. Chemical structures of compounds used in this study. Aminopropyltriethoxysilane (APTES, **1**); *N*-succinimidyl 4,4,5,5,6,6,7,7,8,8,9,9,10,10-,11,11,11-heptadecafluoro-undecyl carbonate **2**; (3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecyl)phosphonic acid (HDFDPA, **3**), **4**−**10** are polyfluorinated derivatives of mannose (**4**), Gb5 (**5**), lactose (**6**), Globo H (**7**), cellobiose (**8**), cellolotriose (**9**), cellotetraose (**10**), and mannose with a phosphonic acid group (**11**). Detailed synthetic procedures of compounds **3**−**11** and their ^{1}H and ^{13}C NMR spectra are shown in the Supporting Information.

quantify and thus lack of quality control. To tackle this problem, a glycan array was developed using 2,6-diaminopyridine (DAP) as a fluorescent linker to sugars and printed on epoxy-activated slides.[23−25] Another approach is to combine the array method and mass spectrometry for label-free analysis. For example, Mrksich's group has developed a method using self-assembled monolayer for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (SAMDI-TOF MS) to observe and optimize synthesis of glycans on gold surface.[26,27] This strategy was further used by the Flitsch group to monitor glycosyltransferases involved in protein O-glycosylation.[28,29] To avoid nonspecific protein adsorption on the gold surface, a tri(ethylene glycol) spacer was used. Since the nature of adducts and the degree of fragmentation of the parent molecular ions were dependent on the laser fluence rate (laser energy),[30,31] addition of matrix is often required to reduce fragmentation of the parent molecular ion. However, the signal of matrix may interfere with the analyst spectrum, especially when the analyte is a small molecule. We have recently developed a desorption/ionization on silicon mass spectrometry (DIOS-MS) method to characterize the oligosaccharides that are covalently bound to porous silicon with a built-in photocleavable linker.[32] In addition, a technique based on nanostructure-initiator mass spectrometry (NIMS) was developed to characterize noncovalent glycan arrays using fluorous tagged substrates that are physically adhered to the fluorohydrocarbon-coated porous silicon surface.[33] However, incorporation of a photocleavable linker to individual glycans is quite tedious, and preparation of porous silicon plates requires corrosive acids which may not generate uniform results. Another disadvantage is that covalent glycan arrays on conventional glass slides cannot be characterized directly by mass spectrometry. We have recently developed a new type of aluminum oxide-coated glass slides (ACG) which can be functionalized by

(23) Xia, B.; Kawar, Z. S.; Ju, T.; Alvarez, R. A.; Sachdev, G. P.; Cummings, R. D. *Nat. Methods* **2005**, *2*, 845–850.
(24) Song, X.; Jia, B.; Lasanajak, Y.; Smith, D. F.; Cummings, R. D. *Glycoconjugate J.* **2008**, *25*, 15–25.
(25) Song, X.; Xia, B.; Stowell, S. R.; Lasanajak, Y.; Smith, D. F.; Cummings, R. D. *Chem. Biol.* **2009**, *16*, 36–47.
(26) Ban, L.; Mrksich, M. *Angew. Chem., Int. Ed.* **2008**, *47*, 3396–3399.
(27) (a) Mrksich, M. *ACS Nano* **2008**, *2*, 7–18. (b) Gurard-Levin, Z. A.; Mrksich, M. *Annu. Rev. Anal. Chem.* **2008**, *1*, 767–800.
(28) Laurent, N.; Voglmeir, J.; Write, A.; Blackburn, J.; Pham, N. T.; Wong, S. C. C.; Gaskell, S. J.; Flitsch, S. L. *ChemBioChem* **2008**, *9*, 883–887.
(29) (a) Laurent, N.; Voglmeir, J.; Flitsch, S. L. *Chem. Commun.* **2008**, 4400–4412. (b) Laurent, N.; Haddoub, R.; Flitsch, S. L. *Trends Biotechnol.* **2008**, *26*, 328–337.
(30) Trevor, J. L.; Lykke, K. R.; Pellin, M. J.; Hanley, L. *Langmuir* **1998**, *14*, 1664–1673.
(31) Gong, W.; Elitzin, V. I.; Janardhanam, S.; Wilkins, C. L.; Fritxch, I. *J. Am. Chem. Soc.* **2001**, *123*, 769–770.
(32) Lee, J.-C.; Wu, C.-Y.; Apon, J. V.; Siuzdak, G.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2006**, *45*, 2753–2757.
(33) Northen, T. R.; Lee, J.-C.; Hoang, L.; Raymond, J.; Hwang, D. R.; Yannone, S. M.; Wong, C.-H.; Siuzdak, G. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 3678–3683.

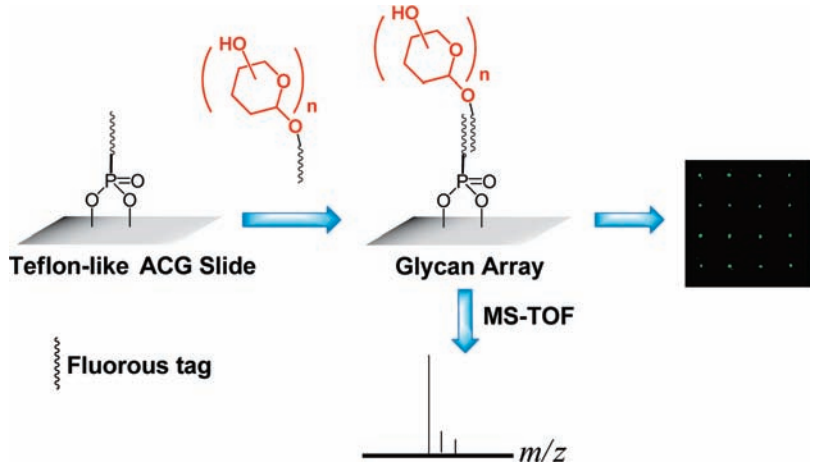
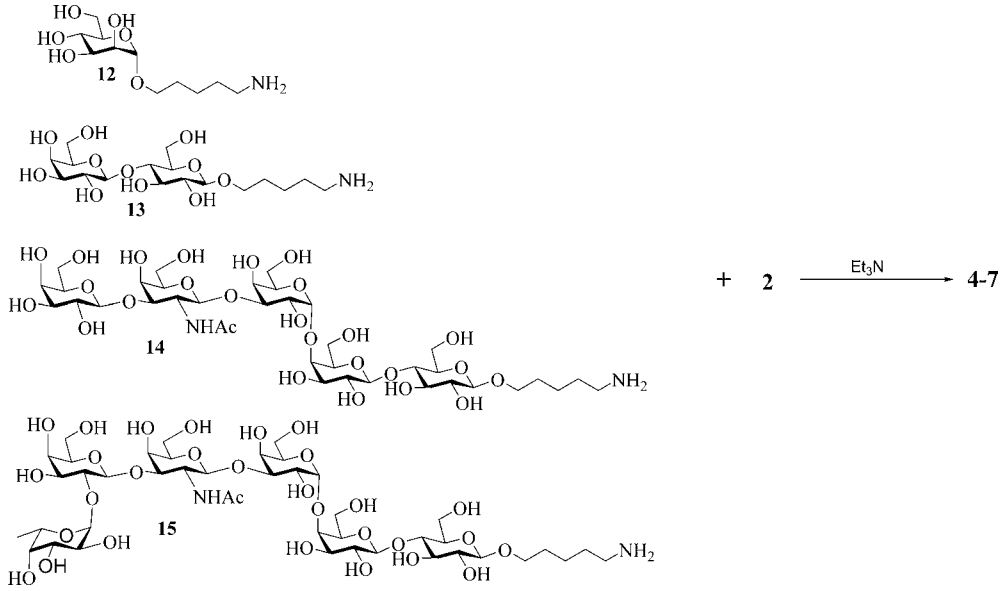


Figure 2. Preparation of noncovalent glycan array on the Teflon-like ACG slides.

Scheme 1. Synthesis of Polyfluorinated Carbohydrates **4**−**7**



reacting with an alkyl monoethoxysilane and linked to a glycan containing the photocleavable linker.[34] The glycan array with a photocleavable linker on the ACG slide surfaces can be characterized by time-of-flight mass spectrometry (MS-TOF) without matrix. However, the array often gave a relatively low signal-to-noise (*S*/*N*) ratio, most likely due to incomplete photocleavage. Here, we reported a new method for the preparation of stable polyfluorinated ACG slides through phosphonate chemistry, which was then noncovalently arrayed with glycans for the study of cellulase activities by MS-TOF. Cellulases are of current interest because of their potential application to biofuel production.[35] Cellobiose or cellotriose with a fluorogenic or chromogenic group has been commonly used as substrate in the investigation of cellulase activity and specificity.[36] However, the fluorogenic or chromogenic leaving group generated in the enzymatic reaction only showed signal at high pH, but cellulases exhibit their optimum activity at low pH (4−6).[36] In this study, the glycan array is subject to the enzymatic hydrolysis at pH 4−6, and the hydrolyzed products remaining on the slide surface can then be identified by MS-TOF *in situ*. This study has led to the development of a new assay to differentiate the exo- and endocellulase activities using cellotetraose as a substrate. Moreover, a new covalent glycan array using synthetic glycans with a phosphonic acid tail directly reacting with the aluminum oxide surface has been developed.

Results and Discussions

Preparation of Teflon-like ACG Slides. To prepare the Teflon-like ACG slides, triethoxysilane **1** and phosphonic acid derivatives **3** (Figure 1) were synthesized to react with the oxidized aluminum surface. Fabrication of the silane-based slide involves a two-step chemical reaction: functionalization of the aluminum oxide surface using compound **1** as a grafting reagent under a moisture-free environment, followed by the amide bond forma-

(34) Tseng, S. Y.; Wang, C.-C.; Lin, C.-W.; Chen, C.-L.; Yu, W.-Y.; Chen, C.-H.; Wu, C.-Y.; Wong, C.-H. *Chem. Asian J.* **2008**, *3*, 1395–1405.
(35) (a) Zhang, Y. H. P.; Himmel, M. E.; Mielenz, J. R. *Biotechnol. Adv.* **2006**, *24*, 452–81. (b) Nieves, R. A.; Ehrman, C. I.; Adney, W. S.; Elander, R. T.; Himmel, M. E. *World J. Microbiol. Biotechnol.* **1998**, *14*, 301–304.
(36) (a) Coleman, D. J.; Studler, M. J.; Naleway, J. J. *Anal. Biochem.* **2007**, *371*, 146–153. (b) Chernoglazov, V. M.; Jafarova, A. N.; Klyosov, A. A. *Anal. Biochem.* **1989**, *179*, 186–189. (c) Deshpande, M. V.; Eriksson, K. E.; Pettersson, L. G. *Anal. Biochem.* **1984**, *138*, 481–487.

tion between NHS-activated polyfluorohydrocarbon **2** and the amine group on the surface of the slide. On the other hand, since the phosphonic acid group could be conjugated to aluminum oxide directly under mild conditions,[37] a Teflon-like ACG slide was made in a one-step chemical reaction, using an aqueous solution of **3** and a cleaned aluminum oxide surface under sonication to form a monolayer of perfluorophosphonate on the surface.[38] The covalent formation of phosphonic acid was confirmed by FTIR (see Supporting Information (SI) Figure S1.).[35,38] These two types of slides were checked with MS-TOF spectrometry for background test, and both showed a cleaner baseline when compared to that with the previous method.[34] (see SI Figures S2-a and S3-a). The one-step method using **3**, however, was much more convenient and efficient.

Noncovalent Glycan Array on the Teflon-like ACG Slides. With this encouraging result, we used the easily prepared phosphonate slide to create a noncovalent glycan array (Figure 2). Mannose with an amine linker **12**[34] was reacted with compound **2** to form the polyfluorinated ($-C_8F_{17}$) derivative **4**[39] (Scheme 1). A solution of this sugar derivative was spotted robotically[40] onto the Teflon-like ACG slide surface. After incubation, the slides were rinsed repeatedly with distilled water and subjected to MS-TOF analysis. A very clean mass spectrum was obtained, showing the peaks at 806 and 822 as the sodium and potassium adducts, respectively (SI, Figure S3-b). The same slide was then used for protein-binding analysis using Alexa 488-labeled concanavalin A as the protein source (SI, Figure S3-c). Both experiments showed satisfactory results. To further extend the scope of this type of glycan array, compounds **13**−**15**[41] were used for the synthesis of polyfluorinated lactose **5**, SSEA-3 (Gb5) **6**, and Globo H**7** (Scheme 1), which were then immobilized onto the Teflon-like ACG slide surface for mass analysis and protein-binding evaluations.

In the MS analysis without adding additional matrix, no fragmented signal was found, even with the use of such a labile sugar, Globo H (Figure 3A), compared to the MALDI-TOF analysis of Globo H where fragmentation, especially through the cleavage of fucosidic linkage, is often observed.[32] Parts B and C of Figure 3 showed that these glycan arrays had retained their sugar−protein binding patterns[35] when VK-9 (a mouse anti-Globo H monoclonal antibody) and anti-SSEA-3 monoclonal antibody were used for binding studies, respectively.

We further investigated the effect of laser fluence rate (laser energy) and matrix on this new surface. In the absence of matrix, we obtained a high *S*/*N* ratio (22) at a very low laser fluence rate (9%), and no fragmented signal was found (Table 1). When the fluence rate was increased to 10%, the *S*/*N* ratio was enhanced to 40 without any fragmentation (original mass spectrometry was shown in SI, Figure S4). We also added DHB (2,5-dihydroxybenzoic acid) as a matrix to check the matrix effect on this new surface. To our surprise, the *S*/*N* ratio is only 7.3 when the fluence rate is 11%. When the fluence rate was increased to 13%, the *S*/*N* ratio was 61 with 6% fragmentation signal (SI, Figure S5). Apparently, DHB matrix cannot enhance the signal with a low laser fluence rate. However, it can enhance the *S*/*N* ratio and reduce the fragmentation when the fluence rate is over 25% (Table 1).

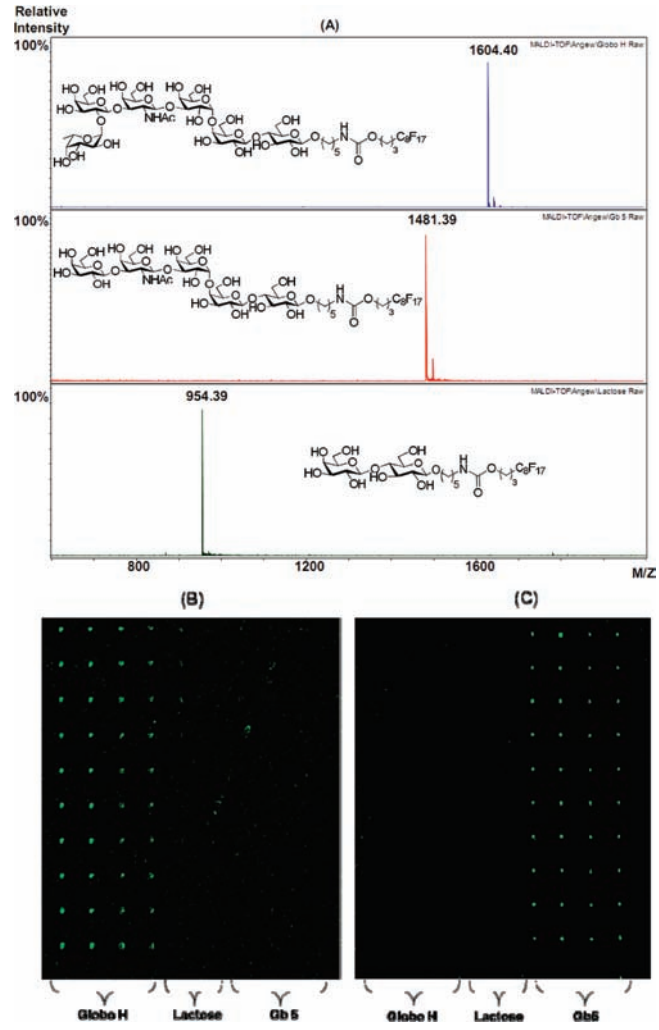


Figure 3. Two types of assays for noncovalent glycan array on the Teflon-like ACG slide. (A) MALDI mass spectrometric analysis of polyfluorinated Globo H **7** (MW: 1604.40), Gb5 **6** (MW: 1458.39), and lactose **2** (MW: 932.21) immobilized on a Teflon-like ACG slide as their sodium adducts $[M + Na]^+$ at 1627.44, 1481.39, and 954.39 respectively. The fluence rate is 12% without matrix addition. Protein-binding assay of (B) Globo H/VK9/anti-VK9-Cy3 and (C) Gb5/anti-SSEA3-A488. The matrix was a 10 × 10 (100 spots) array of perfluorinated Globo H (left four columns), lactose (fifth and sixth columns, served as the negative control), and Gb5 (right four columns). VK9 is a monoclonal antibody against Globo H, and anti-SSEA3 is a monoclonal antibody against Gb5. Both Cy3 and A488 are fluorescent probes. The ACG with polyfluorinated phosphate surface was used for arraying.

Cellulase Activity Studies. From the above studies, we have found that polyfluorinated carbohydrates immobilized on the Teflon-like ACG slide can be easily characterized by ionization/desorption at low laser energy and the signals can be obtained with high *S*/*N* ration without fragmentation. These results encouraged us to further study enzyme specificity and activity: enzymatic hydrolysis of the immobilized polyfluorinated cellobiose **8** was conducted on the phosphonic acid slide surface. Three commercially available cellulases, *Aspergillus niger* (*A.*

(37) Grundmeier, G.; Schmidt, W.; Stratmann, M. *Electrochin. Acta* **2000**, *45*, 2515–2533.
(38) McNatt, J. S.; Morgan, J. M.; Farkas, N.; Ramsier, R. D.; Young, T. L.; Rapp-Cross, J.; Espe, M. P.; Robinson, T. R.; Nelson, L. Y. *Langmuir* **2003**, *19*, 1148–1153.
(39) (a) Ko, K. S.; Jaipuri, F. A.; Pohl, N. L. *J. Am. Chem. Soc.* **2005**, *127*, 13162–13163. (b) Mamidyala, S. K.; Ko, K. S.; Jaipuri, F. A.; Park, G.; Pohl, N. L. *J. Fluorine Chem.* **2006**, *127*, 571. (c) Jaipuri, F. A.; Collet, B. Y.; Pohl, N. L. *Angew. Chem., Int. Ed.* **2008**, *47*, 1707–1710. (d) Chen, G. S.; Pohl, N. L. *Org. Lett.* **2008**, *10*, 785–788.
(40) Blixt, O.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17033–17038.
(41) Wang, C.-C.; Huang, Y.-L.; Ren, C.-T.; Lin, C.-W.; Hung, J.-T.; Yu, J.-C.; Yu, A.-L.; Wu, C.-Y.; Wong, C.-H. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 11661–11666.

Table 1. *S/N* Ratio of Globo H under Different Laser Fluence Rate with or without Matrix (DHB) Addition

matrix-assisted desorption/ionization			matrix-free desorption/ionization		
fluence rate (%)[a]	GH *S/N*[b]	frag. *S/N*[c]	fluence rate (%)[a]	GH *S/N*[b]	frag. *S/N*[c]
11	7.3	0	9	22	0
13	61	6	10	40	0
14	157	13	12	218	5
15	316	25	16	275	10
18	1690	87	18	375	17
19	1956	108	19	741	64
25	3128	168	25	2184	356
35	1445	125	35	1512	373

[a] Fluence: the laser power (or fluence rate) applied to the slide surface. [b] GH *S/N*: the signal/noise ratio for Globo H. [c] Frag. *S/N*: the signal/noise ratio for the peak of fragmentation of Globo H.

niger), *Trichoderma reesei* (*T. reesei*), and *Trichoderma viride* (*T. viride*) were prepared separately at 5 U/mL in a sodium acetate (25 mM) buffer solution (pH 5), and aliquots were loaded onto the functionalized slide divided into 16 wells using Fast Frame reaction chambers. For comparison, an aliquote of enzyme solution (100 μL) was added to the solution of fluorinated cellobiose (100 μL of 0.5 mM) in Eppendorfs. After reaction, the solution was transferred to the empty wells of the same slide. Each well was rinsed separately three times with deionized water, and the slide was dried again under high vacuum before MS-TOF assay.

In the MS-TOF analysis, the enzyme from *T. viride* functioned the best for the hydrolysis of cellobiose derivative in solution, and that from *T. reesei* hydrolyzed disaccharide most effectively among the three enzyme sources on the slide. In solution, the enzyme from *A. niger* seemed to also act as a synthetase, and the overall reaction produced 8% of cellotriose (MW 1093) which was detected as sodium adduct at *m*/*z* of 1116.3 $[M + Na]^+$ (SI, Figure S6A). The enzyme from *A. niger* was characterized as a typical endo-type cellulose[42] which cleaved five glucose units in length at a time. It did not hydrolyze either cellobiose or *p*-nitrophenyl-β-D-glucoside.[43] To further understand the mode of action, polyfluorinated ($-C_8F_{17}$) cellotriose **9** was subjected to enzymatic hydrolysis on the Teflon-like ACG slide. Using the same analytical procedure, the results (Figure 4) indicated that the enzyme from *T. reesei*

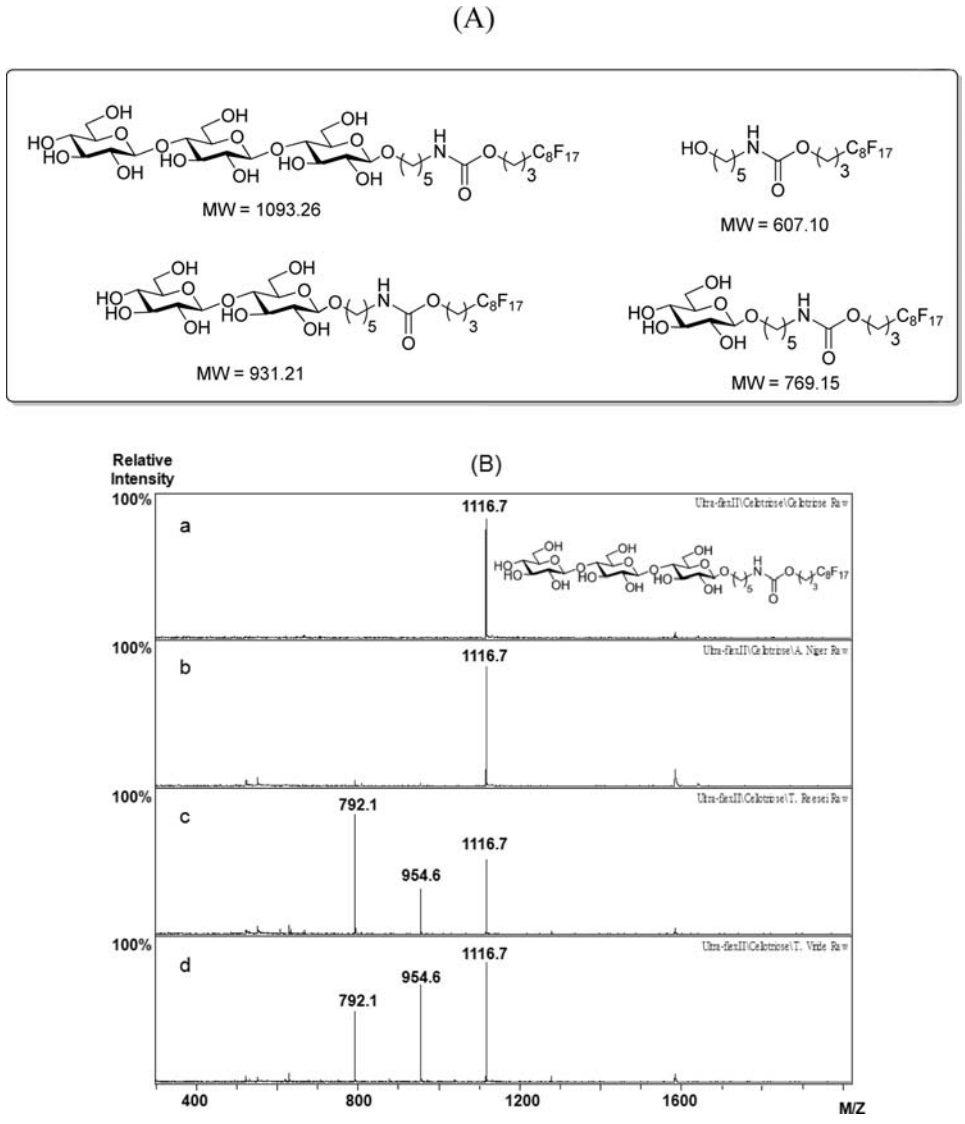


Figure 4. Study of cellulase activity using glycan array combined with mass spectrometry. (A) Hydrolyzed fragments of cellotriose derivatives remained on the Teflon-like ACG slide surface. (B) MS-TOF data of enzymatic hydrolysis of polyfluorinated cellotriose of the control run without enzymes (a), and with the cellulase proteins from *A. niger* (b), *T. reesei* (c), and *A. viride* (d).

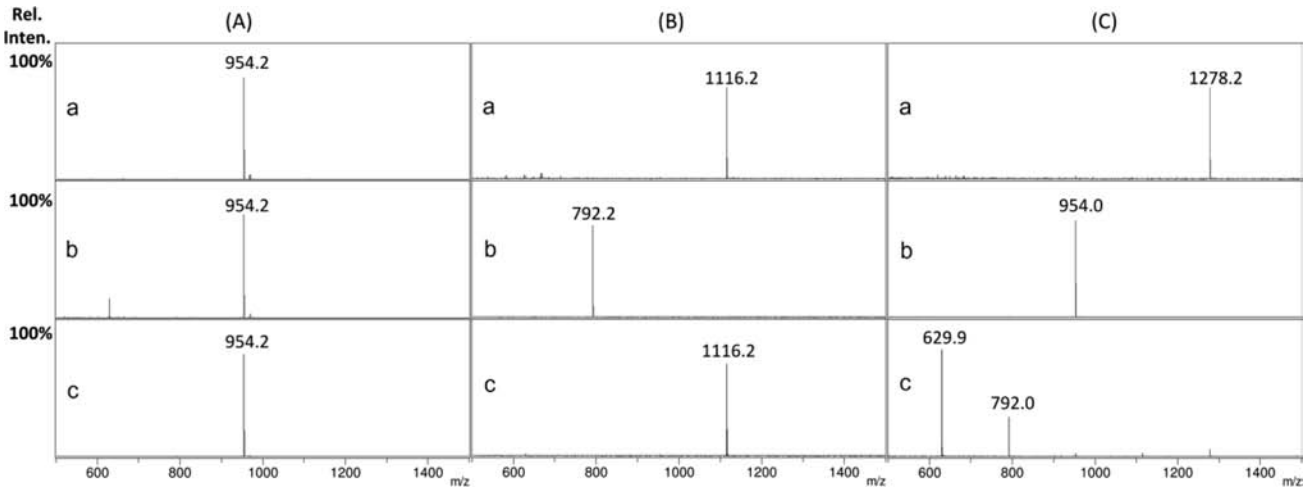


Figure 5. MS-TOF data of enzymatic hydrolysis of different polyfluorinated oligo-cellulose in solution. Polyfluorinated cellobiose **8** (A), polyfluorinated cellotriose **9** (B), and polyfluorinated cellotetraose **10** (C); the control run without enzymes (a), with exoglucanase (L3) (b), and with endoglucanase 44A (c).

hydrolyzed the cellotriose substrate most efficiently among the enzymes from three different species. The results (Figure 4B-b and SI, Figure S6) also indicated that *A. niger* does not function well in hydrolyzing cellobiose or cellotriose on the Teflon-like ACG slide surface. However, in solution, *A. niger* hydrolyzed polyfluorinated cellobiose **8** and cellotriose **9** with one glucose unit at a time at a very slow reaction rate (SI, Figure S6, A-b). Since this commercial enzyme is not pure, these phenomena may be due to the contamination of a small quantity of β-glucosidase in the mixture.

The cellulase from *T. viride* was known to effectively degrade the newspaper material,[44] and *T. reesei*[45] was known to hydrolyze the crystalline form of cellulose. In general, the enzymatic hydrolysis on the ACG slide surface is more site-specific but much slower than that in solution.

Study of Cellulase Specificity Using Glycan Array Combined with Mass Spectrometry. Cellulases are usually divided into several subclasses of isozymes on the basis of their function: β-glucosidases (EC 3.2.1.21]) cleave cellobiose into individual glucose units,[46] exoglucanases (1,4-β-D-glucan cellobiohydrolase [EC 3.2.1.91]) cleave cellobiose units from the nonreducing end of the cellulose chain,[47,48] and endoglucanases (*endo*-1,4-β-D-glucanase [EC 3.2.1.4]) cleave the chain randomly at internal positions[46−48] creating new ends for *exo*-glucanases.[48] HPLC analysis of the products of hydrolysis from MUF-glucosides is often used to determine the hydrolytic specificity of these purified enzymes.[36b] From the above results, the glycan array appeared to be a convenient platform for studying the specificity of various types of cellulases.

In order to develop a better method to study enzyme specificity, we purified the exoglucanase (L3) and endoglucanase (44A) according to the literature methods with minor modifications,[49,50] and enzymatic hydrolysis reactions were conducted in solution with the purified enzymes using substrates **8**, **9** and **10**. At the completion of the reaction, the solution mixtures were transferred to the Teflon-like ACG slide and subjected to MS-TOF assay after washing. Exoglucanase (L3) cleaved the cellobiose unit from the end of cellobiose substrate **8**, but the reaction was very slow (Figure 5A-b). However, the enzyme cleaved cellobiose quickly when cellotriose substrate **9** or cellotetraose **10** was used as the substrate (Figure 5B-b and 5C-b), consistent with the definition of exoglucanase. Endoglucanase 44A could not accept cellobiose substrate **8** or cellotriose substrate **9** (Figure 5A-c and 5B-c) but could cleave the trisaccharide or tetrasaccharide unit of cellotetraose substrate **10** (Figure 5C-c). For comparison, we also immobilized substrates **8**, **9**, and **10** on the Teflon-like ACG surface and carried out the enzymatic hydrolysis on the surface directly. After the same washing procedures, these slides were subjected to MS-TOF assay. Exoglucanase (L3) only weakly cleaved the cellobiose substrate **8** after 24 h incubation (Figure 6A-b) but would cleave the cellobiose unit quickly with **9** (Figure 6B-b) or **10** (Figure 6C-b). The endoglucanase 44A could not accept cellobiose **8** or cellotriose **9** as a substrate (Figures 6A-c and 6B-c). However, it cleaved cellotetraose quickly when **10** was used as substrate (Figure 6C-c). In contrast to the hydrolysis reaction in solution, endoglucanse 44A cleaved cellotetraose, when enzymatic hydrolysis was carried out on the surface, but could not cleave cellotriose, when cellotetraose **10** was used as substrate. These results suggested that cellotetraose **10** can be used to create an array on the Teflon-like ACG surface to identify endo- and exocellulase activities. Comparing to the HPLC method, this array method is more effective and convenient and should facilitate the discovery of new endo- and exocellulases.

Creation of Covalent Glycan Array on ACG Slides. Several types of functionalized glass slides are commercially available and have been used for covalent glycan array preparation, including glass slides coated with amine, carboxylate, *N*-hydroxysuccinimide (NHS), avidin, epoxy, aldehyde, chelating

(42) Uzcategui, E.; Ruiz, A.; Montesino, R.; Johansson, G.; Pettersson, G. *J. Biotechnol.* **1991**, *19*, 271–285.
(43) Okada, G. *Agric. Biol. Chem.* **1985**, *49*, 1257–1265.
(44) van Wyk, J. P. H.; Mogale, M. A.; Seseng, T. A. *Biotechnol. Lett.* **2000**, *22*, 491–494.
(45) Fadel, M.; Kahil, T. *Egypt. J. Biotechnol.* **2004**, *16*, 74–94.
(46) Boschker, H. T. S.; Cappenberg, T. E. *Appl. Environ. Microbiol.* **1994**, *60*, 3592–3596.
(47) Hilden, L.; Johannson, G. *Biotechnol. Lett.* **2004**, *26*, 1683–1693.
(48) Muñoz, I.; Ubhayasekera, W.; Henriksson, H.; Szabo, I.; Pettersson, G.; Johansson, G.; Mowbray, S.; Stahlberg, J. *J. Mol. Biol.* **2001**, *314*, 1097–1111.
(49) Schubot, F. D.; Kataeva, I. A.; Chang, J.; Shah, A. K.; Ljungdahl, L. G.; Rose, J. P.; Wang, B. C. *Biochemistry* **2004**, *43*, 1163–1170.
(50) Kitago, Y.; Karita, S.; Watanabe, N.; Kamiya, M.; Aizawa, T.; Sakka, K.; Tanaka, I. *J. Biol. Chem.* **2007**, *282*, 35703–35711.

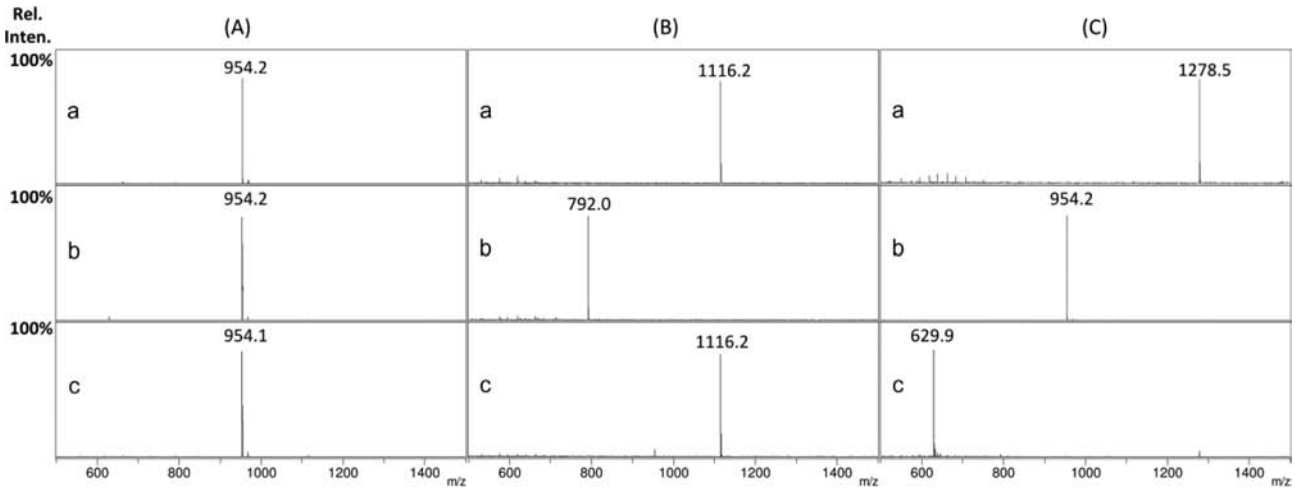
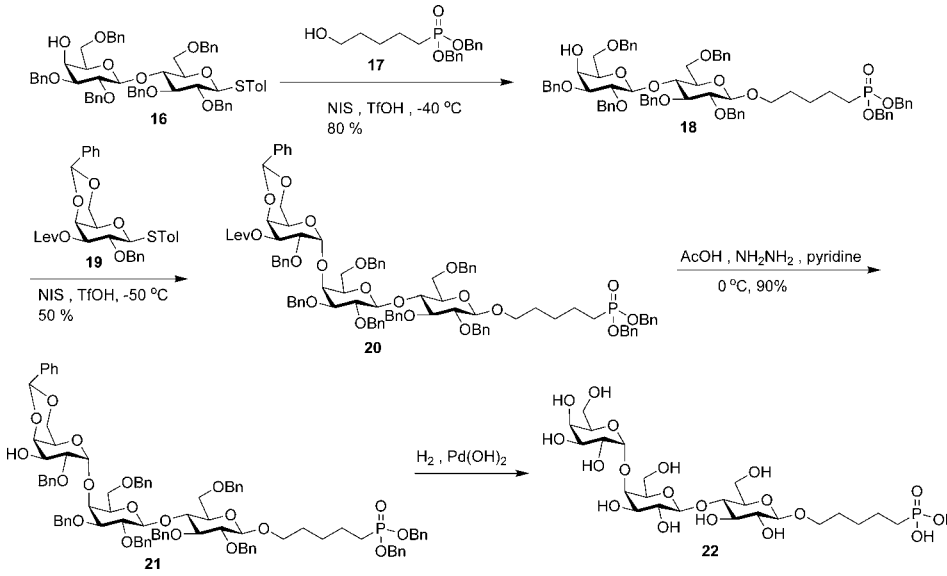


Figure 6. MS-TOF data of enzymatic hydrolysis of different polyfluorinated oligo-cellulose on Teflon-like ACG slide. Polyfluorinated cellobiose **8** (A), polyfluorinated cellotriose **9** (B), and polyfluorinated cellotetraose **10** (C) immobilized on Teflon-like ACG slide; the control run without enzymes (a), with exoglucanase (L3) (b), and with endoglucanase 44A (c) for 24 h incubation.

Scheme 2. Synthesis of Gb3 with Phosphonic Acid Linker Compound **22**



nickel group, and others.[19] When creating a glycan array on these surfaces, a suitable buffer and repeated blocking and washing steps are needed. Our previous studies showed that phosphonic acid could react with the ACG slide directly and the resulting array could tolerate repeating washing steps. We therefore tried to use this property to develop covalent glycan arrays using mannose phosphonic acid derivative **11** (Figure 1, see Scheme S5 (SI), for detailed synthetic procedures) as an example. It was found that the coupling reaction was much faster than on the traditional *N*-succinimidyl glass slide surface, with the incubation time reduced from 12 h to less than 15 min. In addition, the ACG microarray showed great improvement on signal-to-noise ratio since there was less light scattering to interfere with the fluorescence signal (Figure S7 (SI)).

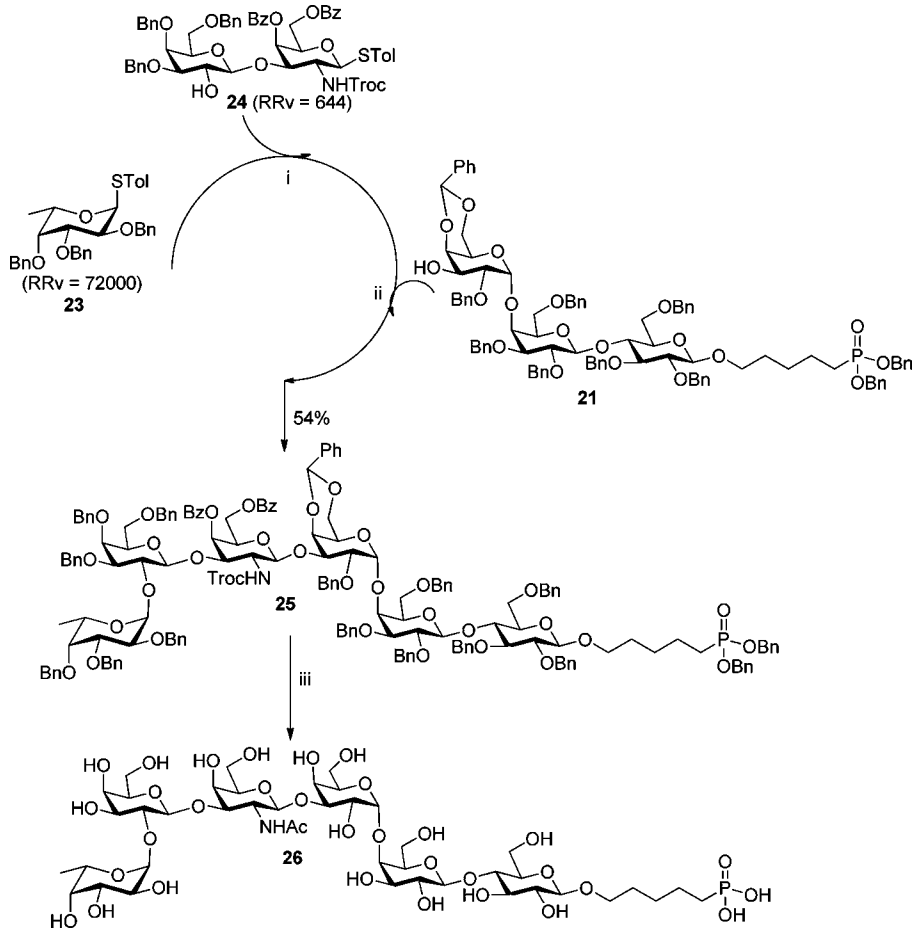
After the mannose compound with different concentrations (Figure S8 (SI)) was spotted on the slides, they were rinsed repeatedly with distilled water and used for protein binding analysis, with Alexa 488-labeled concanavalin A. The incubation process was continued for another hour; then, it was washed again to prevent nonspecific binding between the lectin and the slide surface.

Encouraged by this results, we then established a procedure for the synthesis of complex oligosaccharides, including Gb3 and Globo H with a phosphonic acid group in the tail. As illustrated in Scheme 2, condensation of the lactosyl thiocresol **16** with the phosphate linker **17** using NIS/TfOH as a promoter[51] afforded β-lactosyl derivative **18** in 80% yield. Compound **18** was then glycosylated with galactosyl thiocresol donor **19** to give the α-(1→4)-linked trisaccharide **20** in 50% yield. After deprotection, Gb3 with phosphonic acid tail (**22**) was obtained in 54% yield.

With the trisaccharide acceptor **21** in hand, a programmable [1 + 2 + 3] one-pot approach[52] was used to construct the entire Globo H hexasaccharide compound **25** in 54% yield. Deprotection of **25** began with removal of the trichloroethoxycarbonyl group by using activated Zn particles with acetic acid, followed by acetylation of the amine group with acetic anhydride and

(51) Chao, C.-S.; Li, C.-W.; Chen, M.-C.; Mong, K.-K. T. *Chem.—Eur. J.* **2009**, *15*, 10972–10982.
(52) Huang, C.-Y.; Thayer, D. A.; Chang, A. Y.; Best, M. D.; Hoffman, J.; Head, S.; Wong, C.-H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 15–20.

Scheme 3. One-Pot Synthesis of Globo H with Phosphonic Acid Tail, **26**[a]



[a] Reagents and conditions: (i) NIS, TfOH, −50 °C. (ii) NIS, TfOH, −30 °C, 54% from **23**. (iii) (1) Zn/AcOH, (2) Pyridine/Ac_2O/Cat. DMAP, (3) NaOMe/MeOH, (4) H_2/$Pd(OH)_2$.

pyridine. Zemplén debenzoation and deprotection of the benzylidene acetal and benzyl group were accomplished by using Pd-black in 5% formic acid/methanol under 1 atm hydrogen, to give the fully deprotected Globo H hexasaccharide with phosphonic acid tail (**26**) (Scheme 3).

Gb3 **22** and Globo H **26** were then spotted on the aluminum oxide-coated glass slide for Mbr1 (a mouse anti-Globo H monoclonal antibody) binding experiments. The printing procedure was the same as for mannose compound **11**. Since the properties of solvents will affect the spot size of the printed oligosaccharides, we tested several solvents for **26**. We began with water only; however, the coupling process and the dehydration reaction proceeded slowly, resulting in large printed spots. Then, more viscous glycerol was tested. Although the spot size was small enough to gain strong signal intensity, the high boiling point of glycerol made the printed spot impossible to dry. Ethylene glycol was finally chosen due to its high viscosity, medium boiling point, and small size of printed spots. As expected, the monoclonal antibody MBr1 can only recognize the Globo H glycan but not Gb3 (Figure 7A). To demonstrate the sensitivity of this new type of glycan array, various concentrations of Globo H **26** from 250 μM (1.5×10^{-13} mol) to 0.4 μM (2.4×10^{-16} mol) were spotted onto the aluminum oxide-coated glass slide, and the same binding experiment with MBr1 was carried out to study the sensitivity of this new type of glycan array. The array could detect the presence of antibody down to the attomole level (a and b of Figure S8 (SI)) that is equivalent to the sensitivity of a traditional glass slide.[53] The dissociation constant of Mbr1 binding to Globo H was determined[17] (Figure 7B,C), and found to be consistent with the number generated from the NHS slides. These experiments demonstrated that glycans with a phosphonic acid tail can be easily prepared and covalently arrayed on ACG for use in binding analysis. Now, we are synthesizing more glycans with phosphonic acid tail to create a glycan array with more structure diversities on ACG slide.

For comparison, the dissociation constant of Mbr1 binding to Globo H noncovalently bound on the fluorinated ACG was also tested (Figure S9 (SI)). Due to the highly hydrophobic property of fluorinated ACG slide surface, the printing conditions should be optimized to print diluted solutions with small volumes. To reduce the hydrophobicity, ethylene glycol was used to rinse the surface and then was removed by compressed dry air. After that treatment, the solution of compound **7** was adjusted to 5 μM in ethylene glycol and printed on the fluorinated ACG slide surface. From the surface K_D determination, we found that the dissociation constant calculated from the noncovalent binding type of fluorinated ACG slide is larger than that of the covalent binding type. Consistent with the study

(53) Liang, P.-H.; Wang, S.-K.; Wong, C.-H. *J. Am. Chem. Soc.* **2007**, *129*, 11177–11184.

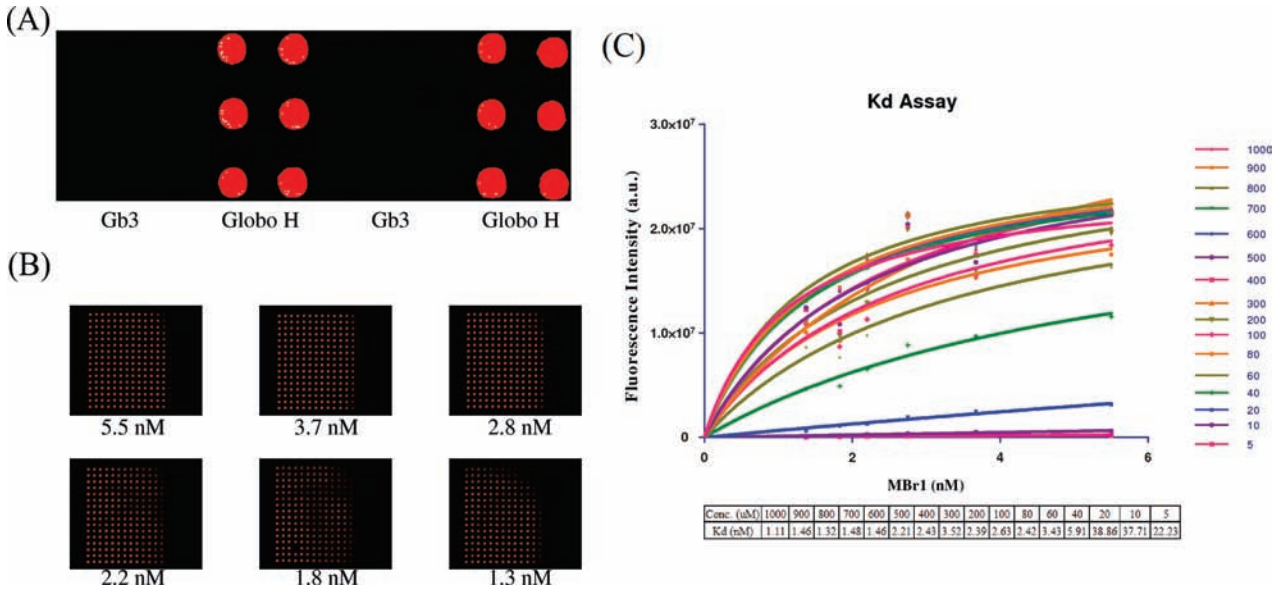


Figure 7. Binding of monoclonal antibody MBr1 to Gb3 and Globo H on the aluminum oxide-coated glass slide. (A) Scanometric image and corresponding analysis of glycan array after probing with monoclonal antibody MBr1, followed by incubation with a fluorophore-tagged mouse anti-IgM secondary antibody. The slide contained Globo H and Gb3 printed at 100 μM concentration. (B) and (C) show the dissociation constants of Mbr1 and Globo H. ACG slides were printed with Globo H of 16 concentrations ranging from 10^{-12} to 10^{-15} mol. The $K_{D,surf}$ values were calculated from the corresponding plot of intensity versus the concentrations of Globo H and Mbr1.

of mannose binding to ConA on various surfaces,[53,54] the covalent array on ACG provides a very suitable platform for protein binding study.

Conclusion

In summary, we have developed novel platforms of covalent and noncovalent glycan arrays on aluminum oxide-coated glass slides. Combining with mass spectrometry, the noncovalent glycan array can be used to characterize glycans directly without fragmentation and also to identify and differentiate various types of cellulases and their efficiency. The unique properties of aluminum oxide-coated glass slides allow for the very convenient and direct preparation of covalent glycan arrays via phosphonic acid chemistry. Both ACG-based glycan arrays, especially the covalent glycan array, have been shown to be particularly useful for the study of protein−sugar interactions, and the noncovalent glycan array has been shown to be useful for the study of enzymatic reactions using mass spectrometry in the absence of matrix. Synthesis of glycans with a polyfluoro or phosphonic acid tail are ongoing in our laboratory (Table S1 (SI)).

Materials and Methods

Compounds 1−26. The detailed procedures for the synthesis of compound **1**−**26** are shown in the Supporting Information.

Fabrication of the Phosphonic Acid-Based Teflon-like ACG Slides. The aluminum-coated glass slide was washed by acetone and water for three times and then dried by dry clean air. The clean slide was then activated and cleaned by oxygen plasma (Harrick plasma, PDC-32G) for 15 min. After activation, the slide was immersed into 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecylphosphonic acid (HDFDPA) **3** solution (1 M, 65% 2-propanol in H_2O, pH = 6.17) immediately. The solution was gently sonicated (50 W) for 15 min.[8] Following the ultrasonic treatment, the slide was removed from the solution and then immersed into another pure 2-propanol solution for 15 min. The solution was also sonicated to remove excess phosphonic acid on the slide surface. The slide was dried by nitrogen under reduced pressure. Upon completion of the reaction, the slide was washed thoroughly with IPA and purge dried by nitrogen. Water contact angle ($\geq$115°) measurement was quickly checked for the completion of the slide fabrication.

MS-TOF Analysis and Glycan Array Preparation of 4. MS-TOF Analysis of the Polyfluorinated Mannose Derivative Adsorbed on the Teflon-like ACG Slides. Compound **4** was dissolved in a methanol/water (6/4) solvent mixture at approximately 10 mM, 1 mM, and 100 μM in series. The slides were spotted manually with 1 μL of solution and also microarrayed with the BioDot AD3200 instrument (Agilent Technology) by robotic pin (Array It, SMP4) with a deposition of approximately 1.1 nL of the solution per spot of the array. The slides were stored in 30% humidity chamber overnight and then analyzed by mass spectrometry. The blank and polyfluorinated mannose slides, including both silane-based and phosphonic acid-based ACG slides, were analyzed with Bruker Ultraflex MALDI-TOF mass spectrometer equipped with a nitrogen pulsed laser (355 nm). An equal volume of BSA trypsin digested (1 pmol/μL) solution was mixed homogeneously with DHB (dihydroxybenzoic acid, 10 mg in 1:1 acetonitrile/water) solution and was used as the standard for MS-TOF mass calibration. Each data point was collected at the average of 500 shots of the laser beam with laser fluence between 2 to 20%. Most of the experiments were carried out under positive polarized electrical field.

Fluorescence-Tagged Con A/Mannose Binding of the Polyfluorinated Mannose Adsorbed on the Teflon-like ACG Slides. A 100-μL portion of Alex 488-tagged concanavalin A (Con A) in phosphate−BSA buffer (25 μg/mL, pH 6.5) were applied on the ACG slide surface immobilized with mannose derivative. These slides with Con A solution were incubated at room temperature for approximately 2 h. After incubation, the slides were washed three times each with 12 mL of phosphate−BSA buffer, PBST buffer, and deionized water in Petri dishes with gentle swirling and then were purge dried by nitrogen and analyzed by Array WoRx (Applied Precision) in reflective mode using the fluorescence light scanner at 530 nm.

(54) Jaipuri, F. A.; Collet, B. Y.; Pohl, N. L. *Angew. Chem., Int. Ed.* **2008**, *47*, 1707–1710.

On-Chip Analysis by MALDI-TOF (Ultra-Flex II) (Reaction in Eppendorf). Cellulase was prepared (5 U/mL in 25 mM pH 5.05 NaOAc buffer solution), and a substrate was also dissolved in NaOAc buffer solution (25 mM, pH 5.05) to give 0.5 mM substrate solution. A cellulase solution (100 μL) was added to 100 μL substrate solution in Eppendorf to give a 2.5 U/mL cellulase solution with 0.25 mM substrate. This solution was incubated at 37 °C for 18 h. 100 μL of this incubated solution were added onto the slide loaded in FAST Frame. The slide was put in a drybox to remove water, and then trace water was removed by high vacuum. Each well of the FAST Frame multislide plate was rinsed by 100 μL of water to dissolve and remove the buffer solution. After removal of any residual water on the plate by high vacuum, the slide was analyzed by MALDI-TOF Ultra-Flex II.

On-Chip Analysis by MALDI-TOF (Ultra-Flex II) (Reaction on Chip Directly). A solution of cellulase was prepared (5 U/mL in 25 mM pH 5.05 NaOAc buffer solution), and a substrate was dissolved in NaOAc buffer solution (25 mM, pH 5.05) to give 0.5 mM substrate solution. A 50-μL portion of the cellulase solution was added to 50 μL of the substrate solution onto the glass slide which was loaded in FAST Frame multislide plate to have a 2.5 U/mL cellulase solution and 0.25 mM substrate. The well of the plate was sealed, and the whole assembly was incubated at 37 °C for 18 h. The slide was put in a drybox to remove the water, and then high vacuum was applied to remove trace water. Each well of the FAST Frame multislide plate was rinsed by 100 μL of water to dissolve and remove the buffer solution. After removal of any residual water on the plate by high vacuum, the slide was analyzed by MALDI-TOF Ultra-Flex II.

Acknowledgment. We thank Mr. Cheng-Lung Chen for his help to grow the anodized ACG slide, Mr. Yung-Fung Lin for his help with NMR analysis of synthetic compounds, and Mrs. Jennifer Chu for critically reading the manuscript. We thank the Academia Sinica, Taiwan, and National Science Council, Taiwan, for financial support. (Grant NSC 97-2113-M-001-009-MY2 to C.-Y.W).

Supporting Information Available: Complete reference 40, Figures S1−S9, Table S1, detailed experimental procedures and characterization, and ^{1}H and ^{13}C NMR spectra of compounds **1**−**26**. This material is available free of charge via the Internet at http://pubs.acs.org.

JA1046523