Bioconjugates

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Effects of Neighboring Glycans on Antibody–Carbohydrate Interaction**

Chi-Hui Liang, Sheng-Kai Wang, Chin-Wei Lin, Cheng-Chi Wang, Chi-Huey Wong, and Chung-Yi Wu**

Carbohydrate recognition is a crucial event in many biological processes, including the progression of diseases such as AIDS, influenza, and cancer.[1–4] Thus, characterization and reconstruction of carbohydrate epitopes to mimic authentic composition and presentation have become one of the goals in glycoscience that may greatly influence the strategy of drugs design. For example, carbohydrate epitopes on virus or cancer cells represent attractive targets for development of carbohydrate-based vaccines.[5–9] Understanding the presentation of carbohydrate epitopes on cell surface allows us to more closely mimic the natural setting in the context of vaccine design. For instance, the HIV envelope glycoprotein gp120 contains high-mannose clusters on its surface to shield peptides from recognition by the host immune system and facilitate invasion by binding to the C-type lectin DC-SIGN on dendritic cells. Therefore, mimicking the high-mannose clusters on the HIV surface has become a promising approach to develop carbohydrate-based vaccines.[10–12] However, clustered expression patterns of carbohydrates are complicated. Incomplete mimicry of carbohydrate epitopes on cell surface could lead to a failed vaccine design. Recently, Danishefsky and co-workers have shown that the $Man_9GlcNAc_2$-based vaccine elicited a high-titer antibody response that recognizes the $Man_9GlcNAc_2$ epitope but fails to neutralize HIV, thereby suggesting that it is not an optimal mimic of the epitope of gp120.[13] To reach an optimal presentation of the carbohydrate epitope, several research groups have attempted to address the question by modulating antigen density and flexibility, but an optimal method has not yet emerged.[14–19] Furthermore, there is a concern that carbohydrate–protein interactions may be either enhanced by multivalency or suppressed by steric hindrance.[20,21] In addition, though not well understood, the proximity effect by other molecules may be significant. Herein, we investigated the effects of neighboring glycans on carbohydrate–antibody interaction using glycan microarrays. Interestingly, we found that heterogeneous glycans, which were prepared by mixing two distinct oligosaccharides and spotted onto glass slides, provide the superior binding affinity compared to the individual components in the microarray experiments. These results additionally suggest that heterogeneous-ligand glycans can serve as a novel strategy for the development of carbohydrate-based vaccine design.

To understand the effects of neighboring glycans on carbohydrate–antibody recognition, we first employed heterogeneous glycans arrays to study anti-SSEA3 (stage-specific embryonic antigen 3, Gb5) antibody interactions. All glycans were amine-modified and covalently bonded to a glass surface activated by *N*-hydroxysuccinimide. The slides were spotted with 50 μM of Gb5 (5×10^{-14} mole/spot), six heterogeneous glycans (SSEA4/Gb5, Globo H/Gb5, Gb4/Gb5, Gb3/Gb5, Gb2/Gb5, Bb2/Gb5; 1:1 mole ratio by mixing the glycan with 50 μM Gb5), and one mixture of 50 μM Gb5 and 50 μM 5-amino-1-pentanol (the linker). The binding schemes are each shown in Figure 1a. Each sample was printed with 12 replicates horizontally to form an array of 12×8 spots on each subarray. The heterogeneous glycan subarray was repeated through a series of dilution steps with the density of viable glycan samples decreasing by a factor of two in each step. Slide images obtained from a fluorescence scan after anti-Gb5 antibody incubation are shown in Figure 1b, and their fluorescence intensities have been compared in a bar chart (Figure 1c). Because all these samples contained the same amount of Gb5 for anti-Gb5 antibody binding, they would have had equal fluorescence intensities if the structures of neighboring glycans did not affect the antibody recognition. However, our results clearly show that the relative binding strength, based on fluorescence intensities, was SSEA4/Gb5 > Gb5 only≅linker/Gb5 > Gb3/Gb5 > Gb2/Gb5 > Bb2/Gb5 > Gb4/Gb5 > Globo H/Gb5. The highest fluorescence intensity in SSEA4/Gb5 was likely a result of the cross-reactivity and multiligand effects which were caused by one antibody binding with two different structures of glycans simultaneously.[22] Furthermore, the antibody binding avidity in heterogeneous samples seemed to be influenced by the affinity and steric effects of neighboring glycans. As revealed in Figure 1c, a longer length of neighboring glycans

[*] Dr. C.-H. Liang,[+] C.-W. Lin, Prof. C.-H. Wong, Prof. C.-Y. Wu
The Genomics Research Center, Academia Sinica, Taiwan
128 Academia Road, Section 2, Nankang, Taipei 115 (Taiwan)
E-mail: chwong@gate.sinica.edu.tw
cyiwu@gate.sinica.edu.tw
Dr. S.-K. Wang,[+] Dr. C.-C. Wang, Prof. C.-H. Wong
Department of Chemistry, The Scripps Research Institute
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)
C.-W. Lin, Prof. C.-H. Wong, Prof. C.-Y. Wu
Chemical Biology and Molecular Biophysics
Taiwan International Graduate Program, Academia Sinica
128 Academia Road, Section 2, Nankang, Taipei 115 (Taiwan)
[+] These authors contributed equally.

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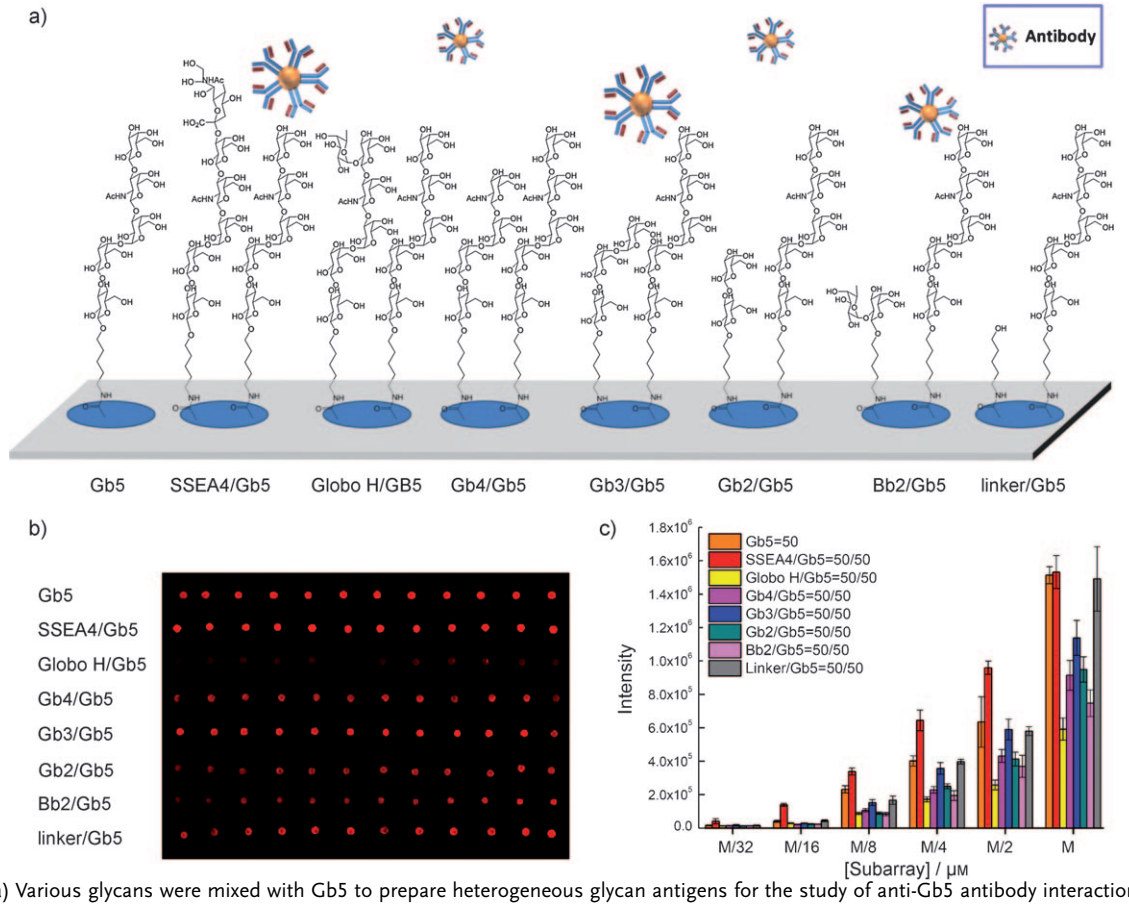

Figure 1. a) Various glycans were mixed with Gb5 to prepare heterogeneous glycan antigens for the study of anti-Gb5 antibody interaction. b) Slide images from a fluorescence scan after antibody incubation with anti-Gb5 antibodies. c) Binding of anti-Gb5 antibody to each of the mixtures was evaluated through a series of diluted steps to determine the effect of structure on recognition. The concentration in μM for each component of the mixture is given in the legend (upper left corner). The x axis shows the dilutions of each subarray concentration M.

had a greater impact on steric contribution. A comparison of the steric interference on the Gb2 and Bb2 carbohydrate showed that the large angle bicyclic rigid structure of Bb2 exerts more steric hindrance. The difference in the neighboring glycans effect between Gb2 and Bb2 on Gb5 may result from the steric effects of Bb2 influencing the arrangement of densely arrayed Gb5 glycans which are critical for antibody recognition. In a control experiment, the result of linker/Gb5 suggested that the linker had no influence in antibody binding avidity. A notable exception to this steric masking effect is the case of Gb3/Gb5, which is capable of binding antibody with high avidity, but the reason is unclear. Taken together, our observation suggests that the presence of neighboring glycans affects binding.

To study the cross-reactivity in anti-Gb5 antibody recognition, seven carbohydrates including Gb5 and its analogues as well as the linker were spotted in 100 μM concentration with 12 replicates and probed with anti-Gb5 monoclonal antibody. Slide images obtained from a fluorescence scan (Figure 2a) confirmed that anti-Gb5 antibody, which was considered to be specific for Gb5, could actually cross-react with SSEA4. It also showed that anti-Gb5 antibodies could tolerate the addition of sialic acid to Gb5 but not fucose.

To explore the multiligand effects, three samples (100 μM Gb5, mixture of 50 μM Gb5 and 50 μM SSEA4, and 100 μM SSEA4) were spotted on the slide with 12 replicates in a 12×3 pattern. A series of dilution steps was performed, and their fluorescence signals were measured for each dilution (Figure 2b), and their fluorescence intensities were compared in a bar chart (Figure 2c). The intensity of the mixture of Gb5/SSEA4 is higher than the intensity of the average of Gb5 and SSEA4. Therefore, the binding avidity of Gb5/SSEA4 mixture ($B_{Gb5\text{-}SSEA4}$) is the strongest among the three samples (see the Supporting Information for the calculation). A schematic drawing of this multivalent binding and three types of possible multiligand binding modes were described in a multivalent manner (Figure 2d). It should be noted that competition between Gb5 ligands and SSEA4 during immobilization should be considered when the concentration of glycans reached saturation point. It may be that the observed fluorescence is not completely due to the contribution of the mixed glycan, but to the kinetics and the spacial arrangement of amide bond-formation. To prevent preferential immobilization of one of the two glycans, all glycan samples were serially diluted under unsaturated condition. By doing this, all glycans were able to immobilize on the glass surface, and free NHS groups on the surface were still available for conjugation. Owing to the possibility of different reaction rates between ligands, a longer incubation time was also used to ensure high binding efficiency. Furthermore, an aldehyde-

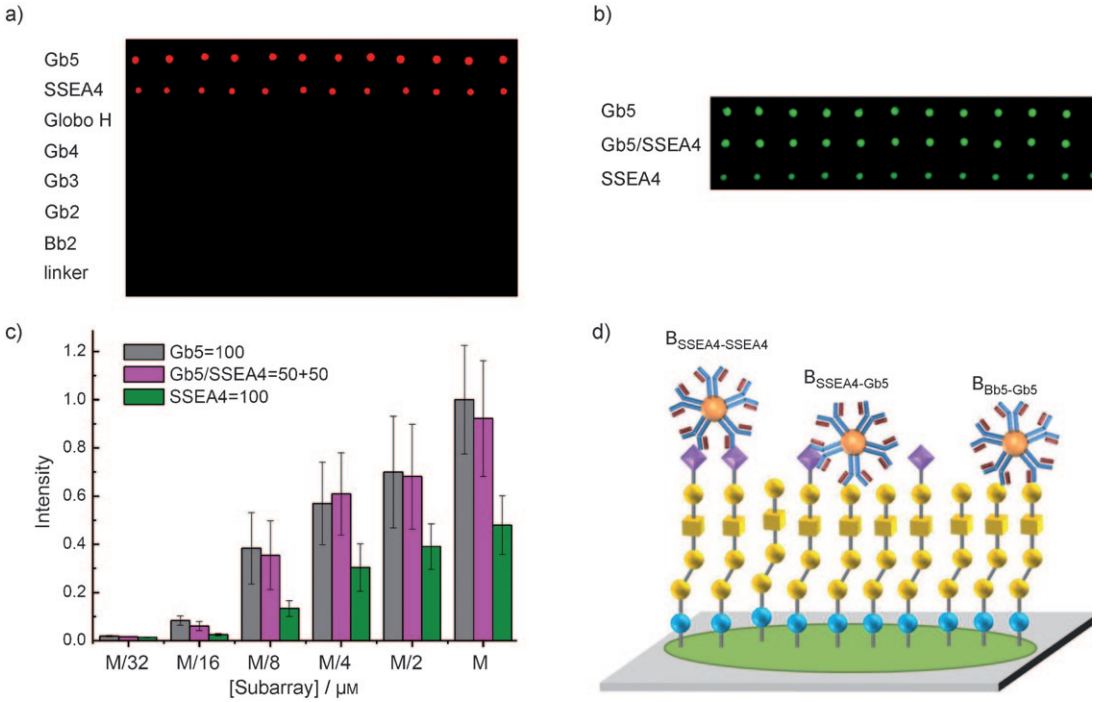

Figure 2. a) Cross-reactivity study of Gb5 and its analogues with anti-Gb5 antibody. b) A slide image and c) binding of antibody to glycan antigens. The concentration in μM for each component of the mixture is given in the legend (upper left corner). The x axis shows the dilutions of each subarray concentration M. d) Schematic description of multi glycan interaction with antibodies. $B_{SSEA4-SSEA4}$: antibody binds to homogeneous SSEA4. $B_{Gb5-Gb5}$: antibody binds to homogeneous Gb5. $B_{SSEA4-Gb5}$: antibody binds heterogeneously to SSEA4 and Gb5. The Figure is not drawn to scale.

derivatized surface was also used to repeat this experiment. Not surprisingly, the results of the multiligand effect from the aldehyde immobilization method had the same trend as that of NHS-derivatized glass slides (see aldehyde slide experiments in the Supporting Information). Taken together, our initial investigations revealed that some antibodies were capable of heteroligation between various glycan binding sites and heterotypic glycan clusters can provide stronger binding avidity than homotypic glycan clusters for antibody recognition.

To evaluate the density effects of the neighboring glycan on antibody recognition, we measured binding properties of anti-Gb5 antibodies from a heterogeneous glycan microarray composed of nine samples of Gb3 and Gb5 in different ratios (5:1, 4:1, 3:1, 2:1,1:1, 1:2, 1:3, 1:4, 1:5, mole/mole; Gb5 was fixed at 5×10^{-14} mole/spot for each sample) and one pure 50 μM sample of Gb5 as schematically shown in Figure 3a. In this case, Gb3 was acting as neighbor glycans and showed no reactivity with anti-Gb5 antibodies. A control experiment of the density effects of the neighboring glycan using the mixture of the linker and Gb5 under the same conditions was carried out, and was considered to be the baseline (see the Supporting Information). The relative binding affinities of these nine Gb3/Gb5 heterogeneous glycan samples to anti-Gb5 antibody were pure Gb5≅1:5≅1:4≅1:3≅1:2 > 1:1 > 2:1 > 3:1 > 4:1 > 5:1 (Figure 3b). Variations in the density of the neighboring glycan (Gb3) revealed significant differences in anti-Gb5 antibody recognition. The higher density of neighboring glycans, the weaker the observed intensity. In this case, the

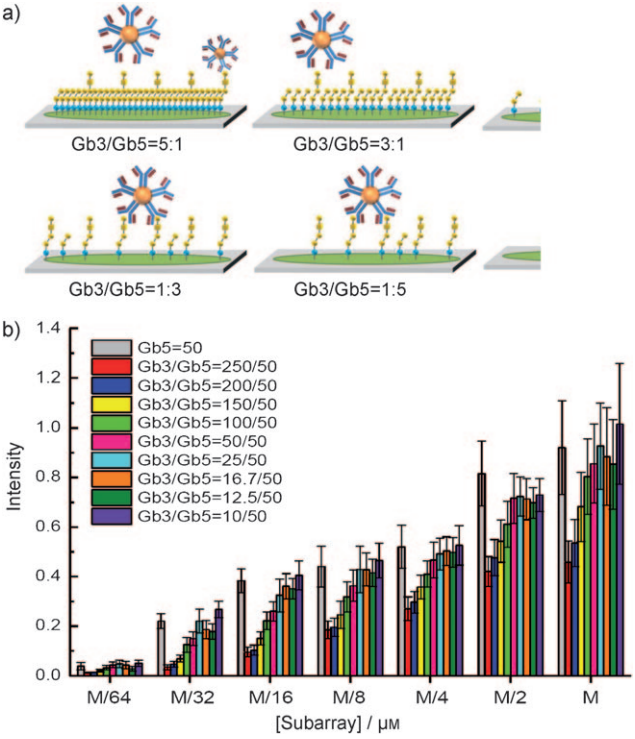

Figure 3. a) Schematic drawing and b) binding properties of anti-Gb5 antibodies to Gb5 antigens with different Gb3 densities; bars represent background subtracted fluorescence values. A series of dilutions was performed to determine the effects of Gb3 density on recognition under various glycan concentrations. Error bars represent the average percentage error for all data points reported.

Gb3/Gb5 ratio over one is considered to affect the anti-Gb5 antibody recognition. Since antibodies do not bind antigen in the presence of high density neighboring glycans, the absence of binding observed does not necessarily mean the absence of the antigen. This so called "glycan shield" mechanism of recognition escape with changes in glycan density and packing was also postulated in the study of HIV-1.[23] Until now, little is known about the effect of the density of neighboring glycan on cancer or virus antigens.

To control the ratio of mixed glycans more precisely, we attached heterogeneous glycans to an AB3-type second-generation dendrimeric scaffold. Two glycans, Man_4 and Man_9, were conjugated to this scaffold at different ratios to give a set of five constructs (I, II, III, IV, V; Figure 4a).

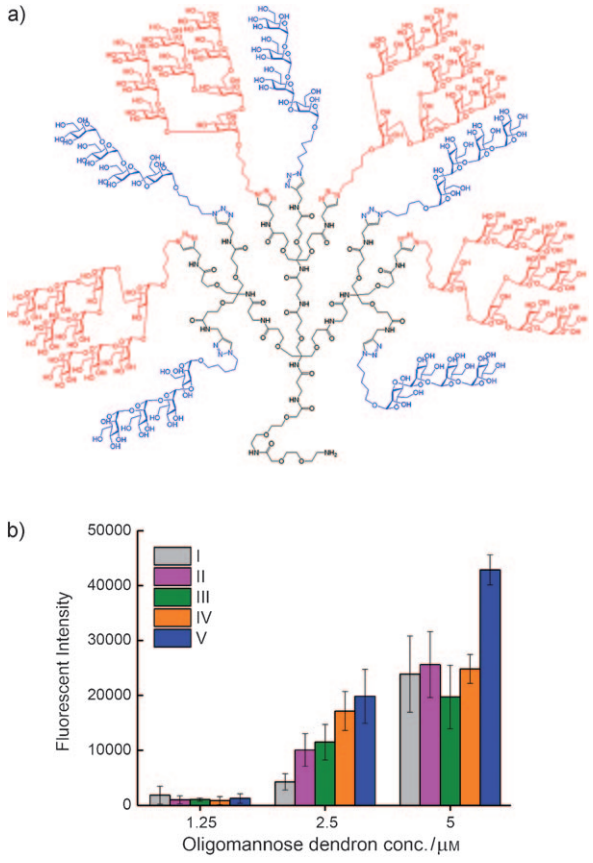

Figure 4. a) Representative oligomannose dendron constructs. The ratio of Man4/Man9 is 5:4. Man_4 is marked in blue and Man_9 is marked in red. b) Binding intensities of fluorescent 2G12 complex with oligomannose dendrons arrayed on glass slide at different printing concentrations, ranging from 1.25 μM to 10 μM.

Synthesis of oligomannose derivatives and conjugation reaction have been described previously by us.[14] Conjugation ratios were determined using MALDI-TOF MS and the composition of oligomannose were listed in Table 1 (see the mass spectra in the Supporting Information). These oligomannose dendrons were then printed onto a NHS-activated glass slide to form an array of conjugates with various densities. From our previous experience, we used a print solution of 5 μM which provides an excess of oligomannose dendron relative to the surface capacity and additional 0.005% Tween 20 to facilitate surface immobilization.[14,24] Under these conditions, we were able to obtain consistent signal intensities of the spots for both low- and high-density conjugates to evaluate 2G12 antibody binding. Fluorescence intensities of the set of oligomannose dendron samples were measured after incubation with various concentrations of Cy3-anti-Fc antibody labeled 2G12 (Figure 4b). The relative binding affinities of these five oligomannose dendrons at 5 μM to 2G12 antibody were V > II > IV > I > III and the surface dissociation constants ($K_{D,surf}$) were calculated (Table 1; see details in the Supporting Information). Heterogeneous-oligomannose glycodendron V, has the strongest binding affinity to 2G12 among the five oligomannose dendrons. Importantly, the density-dependent binding properties observed in 2G12 antibodies should be taken into account. Our previous study showed that increases in the number and density of synthetic Man_9 or Man_4 on dendrons directly correlated with increase in 2G12 binding.[14] Here, we produced oligomannose dendrons by combining clusters of both the Man_4 and Man_9 antigens within a single dendrimertic scaffold, allowing 2G12 to bind with a higher avidity. These studies also suggested that heterogeneous glycans may be a useful approach to carbohydrate-based vaccine designs.

Table 1: Construct of oligomannose dendrons.

	Average oligomannose number in dendron[a]		$K_{D,suf}$ with 2G12 [nM]
	Man_4	Man_9	Glycodendron at 5 μM
I	9	0	34.9 ± 2.57
II	0	9	30.34 ± 2.54
III	6	3	47.4 ± 4.75
IV	3	6	33.25 ± 1.93
V	5	4	13.47 ± 0.25

[a] Numbers of Man_4 and Man_9 were estimated by MALDI-MS analysis.

In summary, this new study showed that antibody binding was affected by the density and structures of neighboring glycans in carbohydrate antigen recognition. The immunologically inert glycan, Gb3, simultaneously immobilized on glycan array slides reduced anti Gb5-antibody interactions in a density-dependent manner. Moreover, antibodies are capable of forming multivalent interactions with different glycans simultaneously, and the overall binding avidity increased in the presence of heterogeneous glycans. Of course, cell surfaces present a complex mixture of various molecules, and their environments are much more complicated than a simple mixture of two or three glycans. Although the observed neighboring effects in this study need further study, the strategy of making heterogeneous glycans should be applicable to better mimic complex epitope presentation. Our results should benefit the future design of carbohydrate-based vaccines by using a combinatorial approach.

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