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Factors Affecting Protein–Glycan Specificity: Effect of Spacers and Incubation Time

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Cell surface glycans regulate several essential intercellular communication processes.[1] Unlike most protein–DNA and protein–protein interactions that lead to "on" or "off" downstream signals, glycan–protein interactions result in a range of responses—for example, from one "on" state to a different "on" state or a completely "off" signal.[2] The complex nature of these interactions requires the development of novel analytical and biochemical tools, such as metabolic engineering, or the incorporation of synthetic glycans into cells through exogenous insertion and glycan microarrays. Glycan microarrays, in particular, have gained increased prominence in recent years because of high-throughput screening capabilities. This widely used technology has been utilized to profile the broad binding preferences of numerous analytes.[3] However, several questions regarding the effects of different immobilization chemistries, glycan density and display, on protein binding remain unanswered. Surface chemistry has become increasingly important because several groups have shown that glycan display[4,5] and density[6] are involved in glycan–protein recognition events that can affect binding outcomes. In this report, we addressed the following questions: First, how important is the length of the spacer that separates the glycan from the imprinted surface? While it is generally accepted that polyvalency leads to increased cooperativity, does varying glycan surface densities influence specificity by increasing the binding of less avid molecules? What role does protein concentration play in determining binding? Does incubation time influence binding? Answering these questions is increasingly relevant because this technology is rapidly moving from the basic research (screening for binding preferences) to the more advanced clinical (detection of cancers,[7] pathogens,[8,9] etc.) stage. Certainly, false positives and negatives in a clinical setting are unacceptable and could prove detrimental to this widely used technology.

Here, we studied the factors that govern glycan specificities using influenza hemagglutinin (HA) and chemically defined *S*-sialosides. By using our previously described synthetic strategy, which has been successfully used to develop ligands for the capture of toxins,[10] viruses[11] and bacteria,[12] we constructed a panel of biotinylated *S*-sialosides that could adopt a variety of topologies (Figure 1). The biotinylated *S*-sialosides, unlike naturally occurring *O*-sialosides, are impervious to cleavage by viral neuraminidase (NA) and therefore, can be used to analyze HA, NA, and intact virus. All glycans possess the same recognition element, sialic acid α2,6-galactose thiodisaccharide, but differ

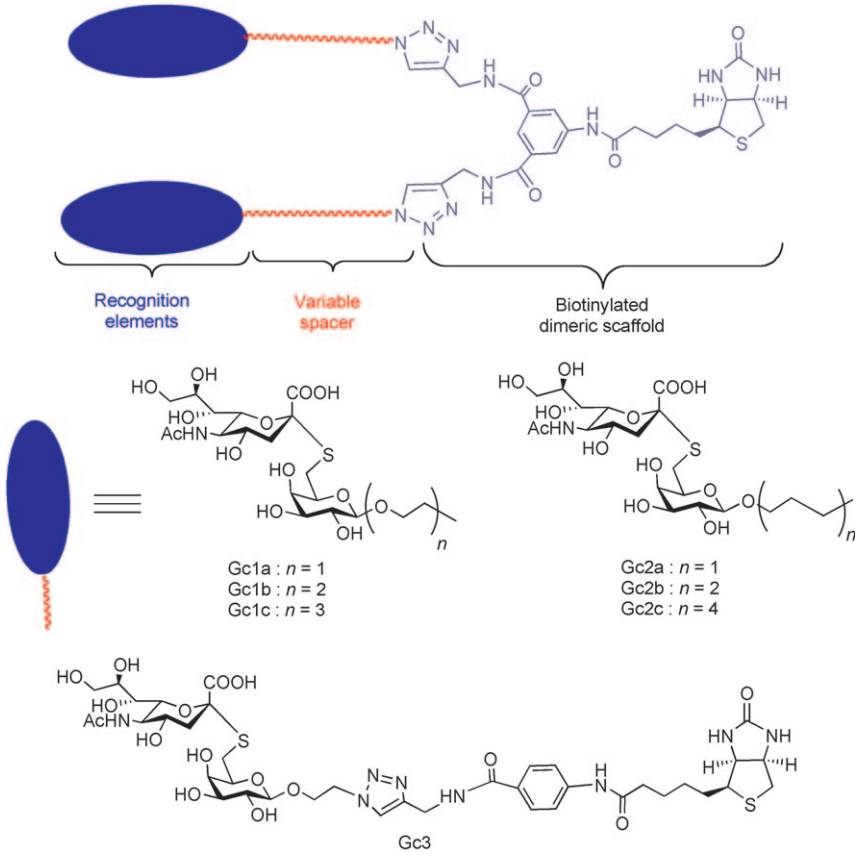

Figure 1. Representation of the synthetic biotinylated S-sialosides and the structures of the seven glycans. The blue ellipse is the glycan recognition element, the biotinylated scaffold and the spacer are colored purple and red, respectively.

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in their presentation by virtue of the biotin and spacers. The biotin affords a controlled spatial arrangement of the glycans because the distance between the binding sites in a streptavidin is fixed and the spacers position the glycans at defined distances from the surface. We chose short or long oligoethylene glycol (**Gc1 a**–**c**) and alkyl spacers (**Gc2 a**–**c**) to compare hydrophobic and hydrophilic effects. We also synthesized a monoantennary ligand, **Gc3**, to study the effect of mono versus biantennary architectures (see the Supporting Information).

We assessed binding by ELISA analysis using HA (A/New Caledonia/20/99). This recombinant soluble HA is a trimer with three binding sites and therefore is expected to increase the binding affinity when compared to a monomeric HA. The results of HA binding to the panel of glycans at a concentration of 12 μg mL^{-1} are shown in Figure 2 A. The monoantennary glycan, **Gc3**, exhibits weak binding, presumably because the short carbon spacer prevents the glycan from adopting the appropriate topology. In the hydrophobic alkyl **Gc1 a**–**c** and hydrophilic oligoethylene glycol **Gc2 a**–**c** series, binding is directly proportional to the length of the spacer; increase in spacer length leads to an increase in the signal strength. This dramatic increase in binding affinities underscores the importance of orientation (or topology) effects. Indeed, **Gc1 a**, the glycan with the shortest spacer, exhibits no binding, possibly because the glycan is too close to the surface. The hydrophobic alkyl **Gc2 a**–**c** compounds bind more tightly than the hydrophilic oligoethylene glycol **Gc2 a**–**c** compounds. **Gc2 b** and **Gc2 c** are the best binders for this particular HA. This suggests that the nature of the spacers (hydrophobic or hydrophilic) plays a significant role in determining the binding affinities. Presumably, hydrophobic regions of HA could interact with the hydrophobic spacers of **Gc2 b**–**c.** Alternatively, the hydrophilic oligoethylene glycol spacers of **Gc1 b**–**c** could be too floppy to orient the carbohydrate component appropriately into the binding pockets. Thus, modulation of the glycan presentation can dramatically influence the binding affinity.

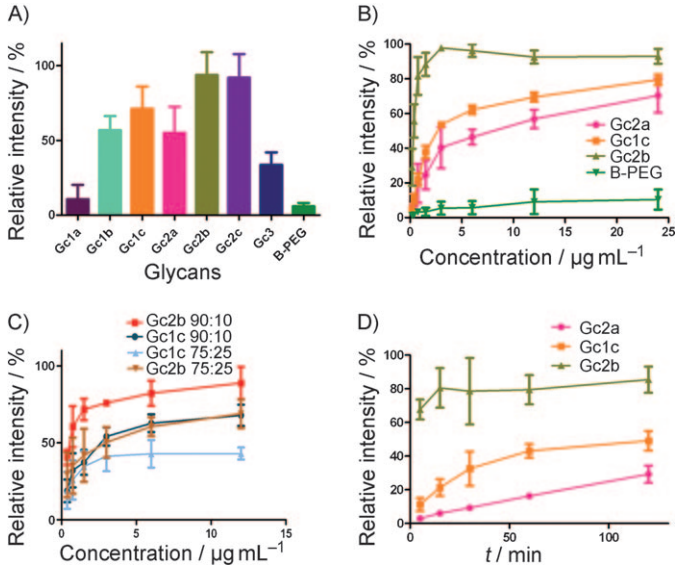

Figure 2. A) Binding of HA (A/New Caledonia/20/99) to synthetic glycans. Biotinylated glycans were added to high-binding capacity streptavidin-coated microtiter wells and incubated with HA at 12 μg mL^{-1} for 2 h. B) Dose-dependent differential binding of HA to **Gc1 c**, **Gc2 a** and **Gc2 b**. The synthetic glycans or B-PEG (negative control) were added to streptavidin-coated microtiter wells and incubated with decreasing concentrations of HA. C) Effect of decreasing glycan surface density on the binding of HA (A/New Caledonia/20/99) to **Gc1 c**and **Gc2 b**. Varying ratios of glycan/ B-PEG were added to high-binding capacity streptavidin-coated microtiter wells and incubated with HA at various concentrations. D) Effect of contact time on the binding of HA to **Gc1 c**, **Gc2 a** and **Gc2 b**. The synthetic glycans were added to streptavidin-coated microtiter wells and incubated with 10 μg mL^{-1} of HA for different time periods. In all experiments, binding was determined by using rabbit anti-HA polyclonal antibody and HRP conjugated goat antirabbit secondary antibody. The binding signals are expressed as percentage of maximum signal for all series in a given set of experiments. Results are the average of three independent trials. (Please see the Supporting Information for details).

Next, we examined the effect of protein concentration on binding affinities. We expected to differentiate between weak and strong binders by monitoring binding over a range of concentrations. Only **Gc2 b** binds to HA at all concentrations, whereas the other glycans do not exhibit any binding at lower concentrations. (Figure 2 B) To quantify the observed results, we used a linearized Hill plot to model the binding data to obtain the apparent binding constant K_ds and the cooperativity factor n.[4] (Figure S1 and Table S1). The value of n is 0.96 for **Gc2 a**; this indicates negative cooperativity. In contrast, the values for n are greater than 1 for **Gc2 b** and **Gc1 c**; this indicates positive cooperativity. A range of K_ds—micromolar (**Gc2 a**, short spacer, 1.42×10^{-7} M), nanomolar (**Gc1 c**, hydrophilic spacer, 0.24×10^{-9} M), and picomolar (**Gc2 b**, hydrophobic, 1.44×10^{-12} M)—are observed. These results clearly demonstrate that protein concentration is a critical determinant; at high concentrations of HA, all ligands bind, however, specificity is clearly established at lower concentrations. We suggest that protein binding to glycan microarrays, which are typically performed using only one, typically high, protein concentration, be performed over a concentration range to more accurately define specificity.

To explore how surface densities affect binding affinities, we premixed the glycans with biotinylated polyethyleneglycol (B-PEG) at various ratios and subjected them to previous dose dependence ELISA protocol. As shown in Figure 2 C for two glycan/B-PEG (90:10 and 75:25) ratios, decreasing the surface density of ligands *does not play* a role in determining specificities, as decreasing concentrations of HA leads to reduced signal strength for all ligands and ratios. Similar results were observed when different ratios of glycan/B-PEG (ranging from 0:100 to 100:0) are used. We note that these results contrast reports published by Whitesides et al., in which the authors have demonstrated that changes in glycan surface density can reverse receptor specificity of *Bauhinia purpurea*.[13] However, it is important to note that these authors use gold-thiol terminated glycan coupling chemistries, which allow the glycans to be packed more densely on the surface. (Figure S2) In contrast, we use the biotin-streptavidin system, in which glycan surface density is limited by the amount of streptavidin on the surface. Our data indicate that topology of presentation plays a predominant role over glycan surface density, and in other systems, glycan surface densities predominate over topology

effects. Overall, the presentation of the glycan (streptavidin-biotinylated glycan versus gold-thiol terminated glycan) in combination with glycan surface density is critical to obtain selectivity.

Next, we studied the effect of incubation time on the binding event. The previously described ELISA studies were performed with one difference, the time of exposure of glycans to HA prior to the wash step was varied. HA binds to **Gc2b** within 5 min (Figure 2D). In contrast, **Gc2a** and **Gc1c** require longer incubation times. Since incubation time is a critical factor, we used surface plasmon resonance (SPR) to monitor binding in real time. The same biotinylated ligands were attached to the commercial streptavidin coated Biacore chips. Binding affinity was assessed using a 2.5 min injection of HA (10.75 $\mu g\,mL^{-1}$). As expected from the contact dependent time ELISA study, we found that HA bound only to **Gc2b** and **Gc2c** and not the other glycans (Figure 3A). To obtain apparent K_ds,

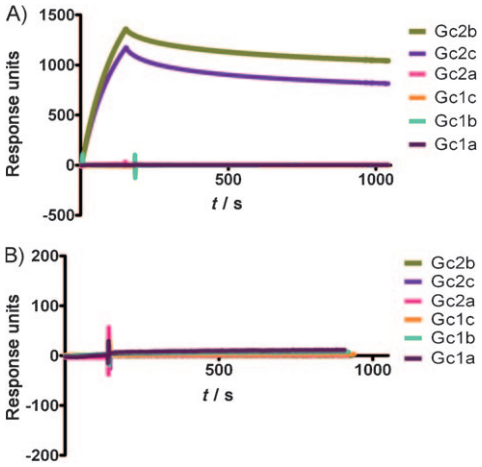

Figure 3. Differential binding of HA to synthetic glycans. SPR response curves for the binding of A) HA (A/New Caledonia/20/99) and B) (A/Solomon Islands/03/2006) binding at 10.75 $\mu g\,mL^{-1}$ using a Biacore 2000. Only **Gc2b** and **Gc2c** bind to HA (A/New Caledonia/20/99). Biotinylated glycans were coated onto a streptavidin-coated Biacore chip (SA chip) until saturation was achieved. Biotinylated PEG (B-PEG) was the control. (Please see the Supporting Information for details)

HA was injected at different concentrations over the chips. A representative set of SPR response curves for **Gc2b** is shown in Figure 3B. The apparent K_ds obtained by using a bivalent model for HA binding to **Gc2b** and **Gc2c** are 2.95 nM and 2.56 nM, respectively. (Table S2) The nanomolar binding affinities indicate that these ligands are excellent binders of HA. The picomolar K_d for **Gc2b** from the ELISA studies (1.44×10^{-12} M) is very different from the nanomolar K_d obtained by SPR. We attribute these differences to different assay conditions. Unlike the ELISA studies in which the incubation time was 2 h, in SPR, the HA is continuously flowed over the glycans for 2.5 min; this minimizes incubation time. Nevertheless, the SPR studies corroborate the ELISA studies, and glycan specificities are dependent on the incubation time. We also tested the binding affinities of a different H1 hemagglutinin (A/Solomon Islands/03/2006), which has 96% homology to the New Caledonia strain. Interestingly, we observed no detectable binding using SPR. (Figure 3B).

In summary, we have demonstrated that glycan specificity is dependent on several factors that include structure, topology and density of glycans. Additionally, binding is assay dependent. Static systems (ELISA) are very different from dynamic systems.(SPR). Thus, this study could have significant implications for glycan microarray technology, an invaluable high-throughput screening resource for establishing binding preferences. Most glycan microarray assays employ only one (typically high) protein concentration AND only one incubation time.[14] Thus, those assays have been performed under conditions that maximize binding and therefore, yield limited information regarding selectivity. Results presented here indicate that varying protein concentration and reducing incubation time could potentially lead to the identification of extremely high affinity glycans for a particular analyte. We are currently using this approach to identify high affinity glycans for other toxins and pathogens.

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