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Research paper

Expedient assembly of Oligo-LacNAcs by a sugar nucleotide regeneration system: Finding the role of tandem LacNAc and sialic acid position towards siglec binding

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ABSTRACT

Sialosides containing (oligo-)*N*-acetyllactosamine (LacNAc, Galβ(1,4)GlcNAc) as core structure are known to serve as ligands for Siglecs. However, the role of tandem inner epitope for Siglec interaction has never been reported. Herein, we report the effect of internal glycan (by length and type) on the binding affinity and describe a simple and efficient chemo-enzymatic sugar nucleotide regeneration protocol for the preparative-scale synthesis of oligo-LacNAcs by the sequential use of β1,4-galactosyltransferase (β4GalT) and β1,3-*N*-acetylglucosyl transferase (β3GlcNAcT). Further modification of these oligo-LacNAcs was performed in one-pot enzymatic synthesis to yield sialylated and/or fucosylated analogs. A glycan library of 23 different sialosides containing various LacNAc lengths or Lac core with natural/unnatural sialylation and/or fucosylation was synthesized. These glycans were used to fabricate a glycan microarray that was utilized to screen glycan binding preferences against five different Siglecs (2, 7, 9, 14 and 15).

1. Introduction

Glycoconjugates are the class of biomolecules with diverse structures, extensively distributed as glycoproteins and glycolipids in living organisms [1]. They are found over cellular surfaces, extracellular matrix, antibodies, toxins, and hormones. These molecules play critical roles in controlling several pathological and physiological processes, including molecular recognition, cell adhesion, and the initiation of the immune response [1,2]. These glycoconjugates are generally processed in the lumen of endoplasmic reticulum (ER) or Golgi complex by several glycosyl enzymes in multiple glycosylation stages that produce glycosidic linkages between one monosaccharide to another. As whole process is non-template driven and dynamically depends upon metabolism, signal transduction and cellular status, resulting into generation of highly complex and heterogeneous glycoconjugates [1]. Therefore, even with the information of relevant gene expression in a given cell type, the precise calculation of glycosylation outcome is unachievable. Additionally, it is virtually difficult to separate heterogeneous glycoforms. Instead, amalgamation of chemical methods and *in vitro* enzymatic methods are appropriate for the preparation of homogeneous glycan samples.

Immune cells express a wide variety of glycan-binding receptors and they are capable to immediately sense and respond to the glycan signature changes of their environment [3], leading to the initiation of inhibitory or excitatory immune processes. However, tumor cells attain the ability to evade immune responses by decreasing immune recognition via changing glycan microenvironment [3]. Glycans containing sialic acid (NeuAc), or sialoglycans, have been denoted as 'self-associated molecular patterns' (SAMPs) [1,3], which are recognized by intrinsic inhibitory receptors, to regulate immune homeostasis and to downregulate immune responses.

Many Siglecs are inhibitory receptors and are a subfamily of immunomodulatory receptors whose functions are regulated by

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their glycan ligands [3,4]. Most of these proteins are expressed on immune and hematopoietic cells and function as immunomodulatory receptors via binding to specific sialoglycans. The interactions between Siglecs and glycans result in inhibitory or, in some cases, excitatory signal transduction, modulating immunity, inflammation, infection, aging, and cancer [3–6]. Siglecs have been classified into two different categories: evolutionarily conserved Siglecs (Siglec-1, 2, 4, and 15) and rapidly evolving CD33-related Siglecs (3,5–16) [3,5]. The ligand-binding site of Siglecs has been located in the amino-terminal V-set domain [5]. The adjacent C2-set domain also contributes to ligand recognition. The active site of Siglecs contains a conserved arginine residue, which is positively charged at physiological pH [7]. The carboxylic acid of the sialic acid of sialoglycans forms a salt bridge with the guanidine group of the amino acid residue, resulting in a stable interaction. By definition, each Siglec binds a terminal NeuAc residue and generally shows binding affinity (K_D) in the high μM to low mM range for the sialosides. Siglecs have a characteristic distinct specificity for sialylated ligands, where recognition depends particularly upon the NeuAc linkage (α(2,3), α(2,6), or α(2,8)) [6] to the penultimate saccharide. Moreover, each Siglec family member possesses a distinct pattern of expression on immune cells, which leads to unique, non-overlapping, and cell-specific functions. In addition, controlled expression patterns on cells and endocytic activity have made Siglec receptors attractive molecular targets, for e.g. 'Trojan horse strategy" for targeted payload delivery of chemotherapeutics or toxins by attaching them to a ligand or an antibody which binds to the Siglecs and releases them into targeted cells for immune cell-mediated diseases [3,5,8]. Despite with the advancement and development in this field, our understanding of exact biological functions of most Siglecs, and specific ligands in mouse and in human is not well understood. Although efforts have been made and, in fact, few therapeutics as monoclonal antibodies based on targeting of Siglecs are already under clinical trial [9]. However, antibody-based treatment can result in serious side-effects or generate anti-drug antibodies against therapeutic antibody [9,10]. Glycan-based targeting of Siglecs is an alternative approach to antibody-based strategy and, in general, glycans possess lower immunogenicity as compared to proteins [9], which makes them a suitable targeting beacon. Thus, it is clinically essential to explore the glycan binding preferences of different Siglecs. High affinity ligands for Siglecs can be used as agonist or antagonist to regulate Siglec functions and can also be attached to toxins or chemotherapeutics agent for their cell specific delivery [9].

In the nature, *N*-acetyllactosamine (LacNAc, Galβ(1,4)GlcNAc) units on *N*- and *O*-linked glycans or lactose (Lac, Galβ(1,4)Glc) could be further modified by glycosyl transferases (GTs), which transfer sugars to Gal (galactose) or GlcNAc (*N*-acetylglucosamine), generating sialylated and fucosylated structures [11–13]. These glycans with different numbers of LacNAc repeats have a significant influence on the recognition and specificity of receptor binding [14–16]. However, the potential impact of the penultimate internal glycans remains largely unknown in context of Siglecs. Although several research teams [8,17–23] have reported chemical and chemoenzymatic syntheses of sialylated glycans and their binding to Siglecs, the role and effect of internal glycans or tandem epitopes, such as Lac vs LacNAc and the number of LacNAc repeats (oligo-LacNAc) required for efficient binding, were not systematically addressed. To gain better insight into these influences in the context of natural and non-natural glycans, sialosides containing oligo-LacNAc as core structures are required. Furthermore, glycan microarrays utilized to date contain diverse glycans [17–20], systematic studies of the effect of the inner core structure on binding affinity are limited. The creation of a more diverse array of naturally occurring or unnatural glycan structures essentially requires the development of improved chemical or chemo-enzymatic synthesis methods.

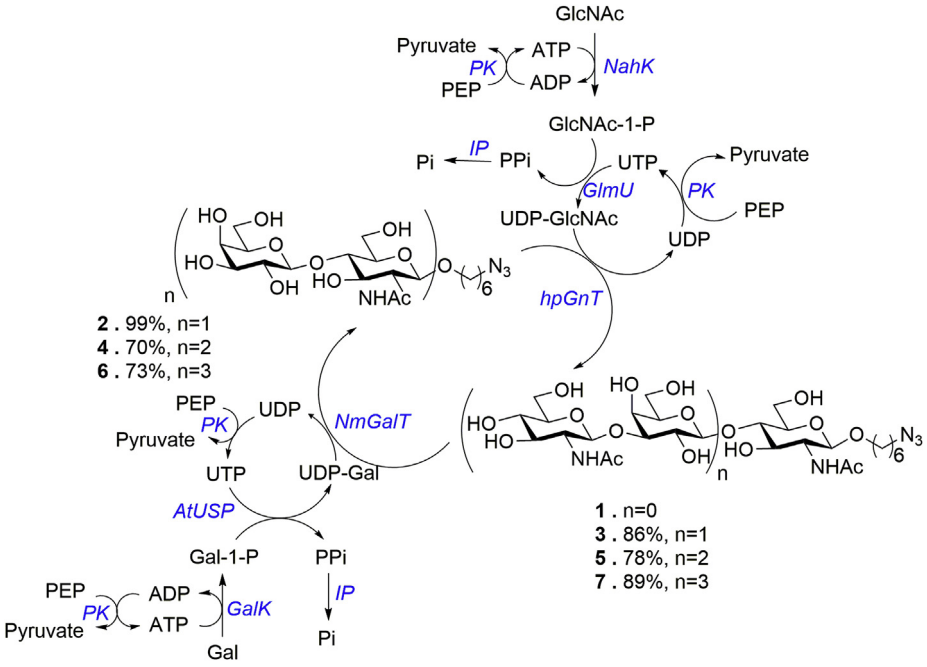
Although LacNAc extension and its biological significance are already well documented [12,14,24,25], interactions at the molecular level are difficult to study due to a lack of robust synthetic routes to produce them. Chemical synthetic strategies have been developed [26–28], yet these methods always involve several tedious protection and deprotection steps. Alternatively, sugar nucleotide-dependent enzymatic route (consecutive use of β(1,4)-galactosyltransferase (β4GalT) and β(1,3)-*N*-acetylglucosaminyltransferase (β3GlcNAcT)) was applied to yield oligo-LacNAc [24,25,29–31]. However, conventional enzymatic reactions require stoichiometric amounts of sugar nucleotides, which are relatively expensive. In addition, the nucleoside diphosphate and diphosphate generated as byproducts during the reaction inhibit the activities of GTs [32], resulting in inefficient yields in large-scale syntheses. Therefore, it is necessary to develop a simplified method to accomplish complex enzymatic reactions in a one-pot reaction with reduced cost and increased ease of production.

Previously, Wong [33], Wang [34], and, later, another group [35] demonstrated a solution to circumvent product feedback inhibition in GT-catalyzed reactions by the *in situ* regeneration of sugar nucleotides. Sugar nucleotide regeneration systems have been developed by mimicking the biosynthetic pathway [33]. Where a catalytic amount of sugar nucleotide (ATP and UTP) is adequate for the continuous generation of sugar donors, kinase and phosphorylase enzymes carry out regeneration by continuous phosphorylation and nucleotide transfer. Inorganic pyrophosphate generated as the byproduct is degraded by inorganic phosphatase (IP) [36] via hydrolysis. Moreover, it is important to choose appropriate enzymes and optimized reaction conditions for nucleotide regeneration and glycosylation. Selection of optimal reaction conditions depends on numerous factors, including the pH, temperature, metal ion, nucleotide concentration, and enzyme stability and compatibility.

Although sugar nucleotide regeneration systems have been used in the production of oligosaccharides [33–35], herein, we report the first sugar nucleotide regeneration system to efficiently construct the oligo-LacNAcs as well as the results of the role and effect of internal glycan length and type on binding affinity against Siglecs. Sialoglycans with tandem epitope were enzymatically synthesized with oligo-LacNAc and Lac core structures, and were further sialylated and/or fucosylated to generate a glycan molecular library. Each glycan was attached with an azido-hexyl linker at the reducing end to facilitate the fabrication of a glycan microarray on a glass slide by copper (I)-catalyzed azide alkyne cycloaddition (CuAAC). The glycan microarray was screened to find preferred binding and recognition patterns against Siglecs (2, 7, 9, 14, and 15).

2. Results and discussion

To achieve efficient hundred-milligram-scale synthesis of oligo-LacNAc, we developed a simple and cost-effective method for regenerating sugar nucleotide donors by multienzyme system. As shown in Scheme 1, two kinases, galactokinase (EcGalK, from *E. coli* K-12) [33,34] and *N*-acetylhexosamine kinase (NahK, from *Bifidobacterium longum* sp.) [30], were applied to generate Gal-1-phosphate (Gal-1-P) and GlcNAc-1P, from Gal and GlcNAc, respectively, by transferring a phosphate group from ATP to the sugar. The byproduct, ADP, was transformed back to ATP by pyruvate kinase (PK), which utilizes PEP as the phosphate source. Then, UDP sugar pyrophosphorylase (AtUSP from *Arabidopsis thaliana*) [37] and GlmU (*N*-acetylglucosamine-1-phosphate uridyltransferase) [38] transfer the nucleoside monophosphate (UMP) onto glycosyl 1-P from UTP to generate UDP-Gal and UDP-GlcNAc, respectively. IP



Scheme 1. Synthesis of oligo-LacNAc by sugar nucleotide regeneration system.

regulates the hydrolysis of pyrophosphate produced during the transfer of UMP from UTP to glycosyl 1-phosphate. Generated glycosyl UDP donors were then transferred to acceptors to form glycosidic bonds by GT-catalyzed reactions. The released UDP byproduct was regenerated to UTP by a PK-catalyzed reaction with PEP as the phosphate source.

2.1. UDP-Gal regeneration system

In the one-pot multienzyme system, UDP-Gal was regenerated and served as the donor for β4GalT (NmGalT, from *Neisseria meningitides*) [39]-catalyzed glycosidic bond formation. ATP is the most frequently used phosphorylating agent and UTP is the UMP source for enzyme-catalyzed formation of UDP-sugars. Because byproduct (PPi and UDP) inhibition slows down the overall reaction rate of the multienzyme-catalyzed reactions, it is necessary to reduce the concentration of nucleoside diphosphate and pyrophosphate in the reaction solution. As shown in Scheme 1, since ATP is the initiator of one-pot multienzyme system, the optimal amount of ATP for *in situ* regeneration is important to minimize byproduct inhibition and thus promote the overall reaction rate. A variety of conditions were screened to determine the optimal amount of ATP by using different concentration ratios ranging from 1/10 to 1/200 (ATP vs Gal), as shown in Table 1. The results showed that the reaction could be performed at all the tested concentration ratios, but in the given 24 h time, 1/50 equivalent of ATP was found to be an ideal concentration to efficiently regulate regeneration, whereas 1/10 equivalent of UTP was found to be adequate. By using an optimized UDP-Gal regeneration system coupled with NmGalT, five-hundred-milligram-scale synthesis of **2** was achieved with 99% isolated yield (502 mg). The desired compound was characterized by ^{1}H NMR: the spectrum shows an anomeric proton at 4.43 ppm with a coupling constant of 7.0 Hz, confirming a β-glycosidic linkage. Similarly, tetrasaccharide (di-LacNAc) **4** and hexasaccharide (tri-LacNAc) **6** were synthesized with isolated yields of 70% (63 mg) and 73% (18 mg), respectively, by UDP-Gal regeneration strategy. It should be noted that the use of manganese ions (Mn^{2+}) inactivated the UDP sugar phosphorylase and the use of excess Gal decreased the product yield and hindered the purification process.

Table 1
ATP equivalent optimization for UDP-Gal regeneration.

Entry	ATP (eq)	ATP (Cf)	Time (h)	Yields (%)
1	1/10	4.0 mM	24	99
2	1/15	2.7 mM	24	99
3	1/25	1.6 mM	24	99
4	1/50	0.8 mM	24	99
5	1/100	0.4 mM	24	72
6	1/200	0.3 mM	24	65

2.2. UDP-GlcNAc regeneration system

For the synthesis of GlcNAc-LacNAc trisaccharide, β3GlcNAcT (HpGnT, from *Helicobacter pylori*) [40] was used to form a β(1,3)-glycosidic linkage on galactosyl acceptors using UDP-GlcNAc as the donor substrate. Similar as the UDP-Gal regeneration system, a variety of conditions were screened to determine the optimal concentration ratio of ATP to GlcNAc from values ranging from 1/15 to 1/50, as shown in Table 2. Within 22 h of reaction time, the ratios of 1/15 and 1/20 showed complete consumption of the starting material, whereas a 1/50 ratio resulted in a trace of unreacted starting precursors. Although completed conversion was achieved by extending the reaction time to 30 h, 1/50 equivalent of ATP and 1/10 equivalent of UTP was chosen for the subsequent synthesis. We observed that in the sugar nucleotide regeneration system, NahK and HpGnT were stable and exhibited higher substrate

Table 2
ATP equivalent optimization for UDP-GlcNAc regeneration.

Entry	ATP (eq)	ATP (Cf)	Time (h)	Yields (%)
1	1/15	2.7 mM	22	95
2	1/20	2.0 mM	22	95
3	1/50	0.4 mM	22	86

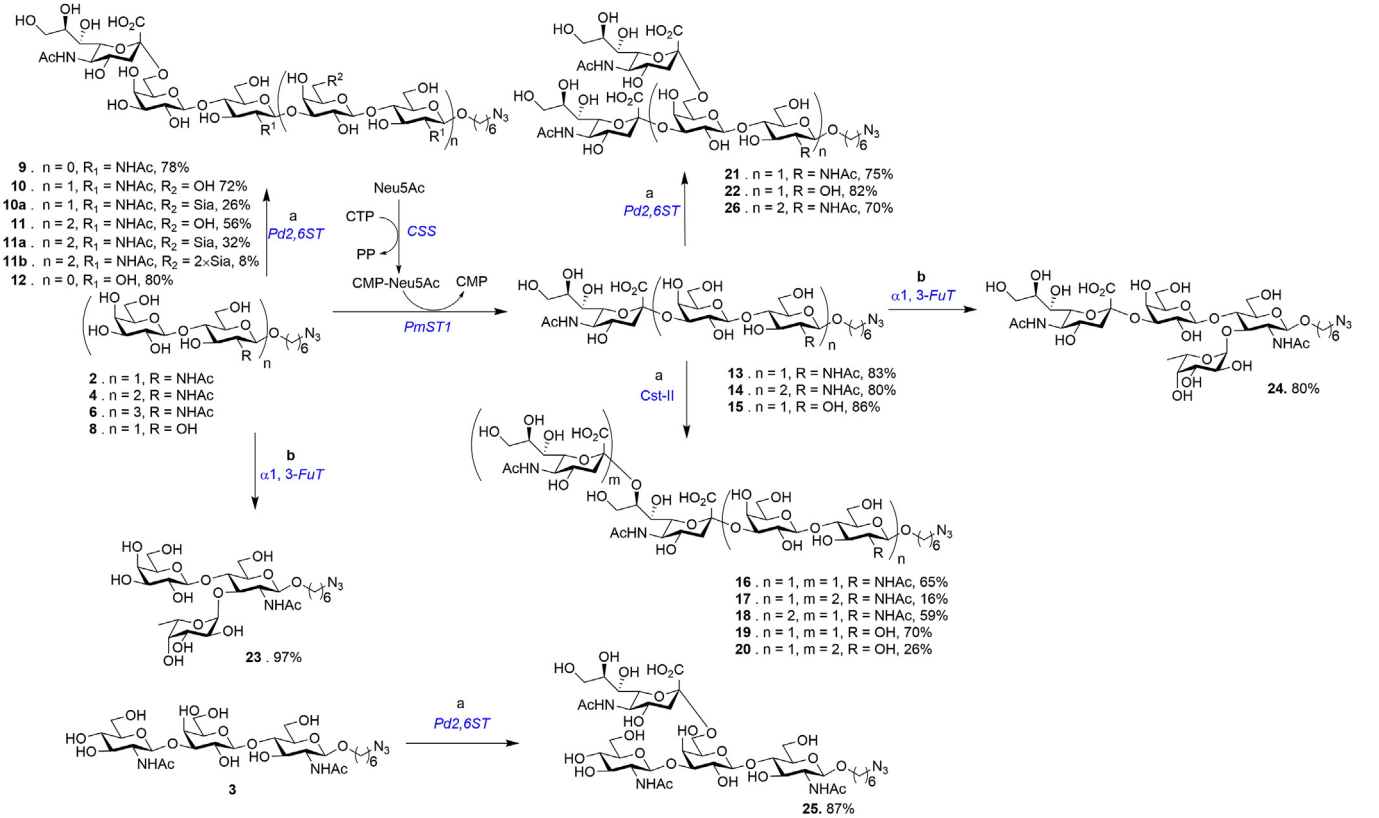
conversion rates, whereas GlmU was less stable and required larger amounts to achieve efficient conversion. The anticipated trisaccharide **3** was purified with an isolated yield of 86% (246 mg). The desired compound was characterized by ^{1}H NMR, which showed an anomeric proton of GlcNAc at 4.71 ppm with a coupling constant of 8.4 Hz, validating the β-glycosidic linkage. Likewise, pentasaccharide **5** was synthesized with 78% yield (29 mg). Notably, due to the poor solubility of $(LacNAc)_3$ **6** in water, glycosylation was carried out in cacodylic acid buffer to obtain heptasaccharide **7** with a significantly higher yield of 89% (5 mg). This enhancement is presumably due to the contribution of cacodylic acid buffer, which provides better solubility of oligo-LacNAcs. It should be noted that the synthetic yields of oligo-LacNAcs were quantitative based on the TLC analysis. However, the desired products were lost during purification processes.

2.3. Enzymatic synthesis of sialosides

Next, modifications (sialylation and fucosylation) of the synthesized LacNAcs and Lac were performed to create a library of naturally occurring and non-natural glycans by sequential one-pot multi-enzyme synthesis for the evaluation of their binding preferences among different Siglecs. The main framework of our glycan library was variation in the length of LacNAc, modification with Neu5Ac and/or fucose (Fuc) or in the replacement of LacNAc with Lac, as shown in Scheme 2. By choosing an appropriate sialyltransferase (ST), sialosides with α(2,3)-, α(2,6)-, α(2,3) and α(2,6)-, and α(2,3)-α(2,8)-linkages were synthesized. To accomplish the synthesis of sialylated glycans, a sequential one-pot two-step process was applied using CMP sialic acid synthetase (CSS, EC 2.7.7.43) and Neu5Ac to generate cytidine 5-phosphate sialic acid (CMP-Neu5Ac), followed by the addition of recombinant enzyme α(2,6)-ST (Pd2,6ST from *Photobacterium damsela*) [25,41] and/or α(2,3)-ST (PmST1 from *Pasteurella multocida*) [42] to yield a regioselective glycosidic linkage on the acceptor (Gal moiety).

Pd2,6ST was first examined on LacNAc oligomers with varying lengths, as shown in Scheme 2. Initially, LacNAc acceptor **2** was sialylated in the presence of CMP-Neu5Ac as a donor to give **9** with 78% yield. Likewise, Lac **8** [43] was sialylated to yield α(2,6)-linked **12** in 80% yield. Similar conditions were applied to di-LacNAc **4**, and the α(2,6) monosialyl $(LacNAc)_2$ **10** was obtained in lower yield due to the compete sialylation of internal Gal, giving **10a**. Previously, we achieved the synthesis of oligo-LacNAcs with different degrees of sialylation by controlling the amount of donor Neu5Ac in the reaction [30]. Thus, compounds **10**, **10a**, **11**, **11a**, and **11b** were obtained. Similar to the previous report [30] PmST1 was applied to exploit the formation of α(2,3)-sialyl linkages on acceptors **2**, **4**, and **8** to afford **13**, **14**, and **15**, respectively.

Cst-II [44], a bifunctional enzyme from *C. jejuni*, transfers Neu5Acs on terminal Gal residue to form α(2,3)-linkage and subsequently assembles Neu5Acs on α(2,3)-linked sialosides by forming α(2,8)-disialic acid linkages to give GD3, GT3, GQ3, and sialylated oligomers [43,45]. The major limitation associated with Cst-II is that high concentration of the enzyme or prolonged reaction time often leads to sialidase activity, although it can be controlled by continuous monitoring of the reaction and a low enzyme concentration. We envisioned that the extent of Neu5Ac condensation by Cst-II could be controlled by varying the amount of ST and donor to provide disialosides. The use of Cst-II for the synthesis of α(2,8)-disialic acid were reported previously [45,46], the desired GD3 (**19**) was obtained by using GM3 (**15**) as the acceptor with little excess of Neu5Ac to provide 70% yield (Table 3, Entry 1), consistent with reported result [46]. Increasing Neu5Ac amount to 5 equivalents furnished a mixture of GD3 (48%) and GT3



Scheme 2. Modification of oligo-LacNAcs and (LacNAc-)Lactoses. a) Neu5Ac, CTP, $MgCl_2$, CSS, Tris-HCl, pH 8.0.37 °C. b) Fuc, GTP, ATP, $MnCl_2$, $MgCl_2$, Tris-HCl, FKP, pH 7.5, 37 °C.

Table 3
Optimization of sialylation conditions for Cst-II.

Acceptor ——Neu5Ac + CTP, CSS, CSt–II→ GM3(**15**) + GD3(**19**) + GT3(**20**)

Entry	Acceptor	Neu5Ac (eq)	time (h)	GM3	GD3	GT3	GQ
1	**15**	1.2	5	N.D	70%	N.D	N.D
2	**15**	5	2	31%	48%	12%	N.D
3	**8**	10	8	10%	53%	26%	2%
4	**8**	5	8	5%	33%	17%	N.D
5	**8**	2.5	16	N.D	44%	5%	N.D

N.D: not detected, "Yield is based on acceptor".

(12%) within 2 h of incubation (Entry 2). Thus, higher order sialylated compounds can be prepared by using excess amount of Neu5Ac. The remaining GM3 acceptor in the reaction mixture indicates that, in the excess amount of Neu5Ac, GD3 can compete with GM3 to serve as the acceptor. In addition, multi-sialylation was directly performed on acceptor **8** (1.0 equiv) with Neu5Ac in varying ratios as the donor precursor using CSS and Cst-II in a one-pot two-enzyme system. In the presence of a large excess of Neu5Ac (10 equivalents), Cst-II exhibited extensive α2,8-linked multiple sialylation activity to give GD3 **19** (53%), a substantial amount of trisialylated glycan GT3 **20** (26%), and traces of polysialylated structure GQ3 (~2%) (Entry 3). When the amount of Neu5Ac was restricted to 5 equivalents, the formation of GT3 was suppressed to 17%, and the GD3 yield dropped to 33% (Entry 4). By further decreasing the molar ratio of the donor precursor Neu5Ac to 2.5 equivalent, sialylation was driven to the complete consumption of GM3 to produce higher amounts of GD3 **19** (44%). However, the formation of trisialoside cannot be completely eliminated and small amounts of GT3 (5%) were still isolated (Entry 5), even when the reaction time was extended from 8 h to 16 h. Moreover, these results reflect that the sialylation product distribution was affected by varying the molar ratio of Neu5Ac, and GM3 is a better acceptor than Lac for Cst-II. Similarly, as shown Scheme 2, using 1.2 equivalents of Neu5Ac with GM3 analog, **13** (Neu5Acα2,3LacNAc), afforded a mixture of GD3 **16** and GT3 **17** in 65% and 16% yields, respectively. Similarly, acceptor **14** (Neu5Acα(2,3)di-LacNAc) was α(2,8)-sialylated to give **18** in 59% yield. The α(2,3)-linked monosialosides can also serve as acceptors for further α(2,6)-sialylation by Pd2,6ST. Sialosides **13**, **15**, and **14** were sialylated using Pd2,6ST to yield branched α(2,3)α(2,6)-linked disialosides **21**, **22**, and **26** with 75%, 82%, and 70% yield, respectively, under the aforementioned conditions. It is worth mentioning that α(2,6) monosialosides, **9** and **12**, cannot serve as acceptors for PmST1 to produce disialosides **21** and **22**, indicating that PmST1 preserves high acceptor specificity. As mentioned in early studies, we found that Pd2,6ST can assemble α(2,6)-Neu5Acs at both the terminal and internal Gal units. Thus, Pd2,6ST was tested for the sialylation of trisaccharide **3**, which contains an internal Gal. As expected, the internally sialylated compound **25** was obtained in good yield (87%).

2.4. Synthesis of fucosides

L-Fucokinase/GDP-fucose pyrophosphorylase (FKP) [47] from *Bacteroides fragilis* is a bifunctional enzyme and was used to generate GDP-Fuc (GDP-fucose) directly from Fuc. GDP-Fuc was then taken up by a recombinant *Helicobacter pylori* α1,3-fucosyltransferase (HPα3FuT) [48] to form an α(1,3)-linkage on GlcNAc. Thus, Le^x-trisaccharide **23** was produced by the fucosylation of LacNAc acceptor **2** in the sequential one-pot synthesis using Hpα3FuT in the presence of ATP, guanosine 5-triphosphate (GTP), and FKP to give 97% yield. We observed that once α(1,3) Fuc was present on LacNAc, α(2,3) sialylation could not be performed by PmST1, which is consistent with previous reports [49,50]. Thus, SLe^x **24** was synthesized by using sialoside **13** as an acceptor and fucosylation by Hpα3FucT to give tetrasaccharide **24** in 80% yield. Overall, as shown in Schemes 2 and 23 different glycans were obtained by the developed enzymatic synthesis strategy.

2.5. Fabrication of glycan microarray

The glycan microarray was then used to evaluate the interactions of 23 glycans with five recombinant Fc-fused human Siglecs, Siglec-2, -7, -9, -14, and −15. Since the synthesized glycans contain an azide group at the reducing end, CuAAC [51] was applied to immobilize glycan on the alkynylated glass slide. As shown in Fig. 1, to fabricate an alkynylated glass slide, silyl derivative **27** was synthesized by coupling propargylamine with 3-cyanatopropyltriethoxysilane [52]. The application of a zwitterion, a compound containing both positive and negative charges, makes the slide surface more hydrophilic, resulting in the suppression of nonspecific adsorption [53]. To optimize the suppression of nonspecific protein interactions on the microarray, 10%, 20%, or 50% (v/v) zwitterionic silanes **28** and **27** were co-coated on a piranha-washed silicon slide. The binding results showed that the use of 10% zwitterionic silane provided the best S/N ratio (Fig. S1). Then, azido-glycans were conjugated onto the surface by CuAAC using copper sulfate, the copper(I)-stabilizing ligand THPTA, and sodium ascorbate. THPTA was selected as the Cu(I)-stabilizing ligand due to its good water solubility [54]. To further prevent nonspecific protein interactions on the glycan slide surface, azido-oligoethylene glycol (OEG) **29** [55] was subsequently used to cap the unreacted alkynes, followed by blocking the slide surface with 3% BSA. Next, the slide was incubated with a solution containing Fc-fused Siglec (5 μg/mL) and stained with Fc-specific anti-human-IgG-Cy3 (5 μg/mL) to visualize the binding signals.

Initially, the glycan microarray was used to evaluate the glycan binding behavior with Siglec-2-Fc. The results are shown in Fig. 2. In agreement with previously reported results [25], Siglec-2-Fc exhibits strong affinity to the α(2,6)-Neu5Ac-containing glycans **9**,

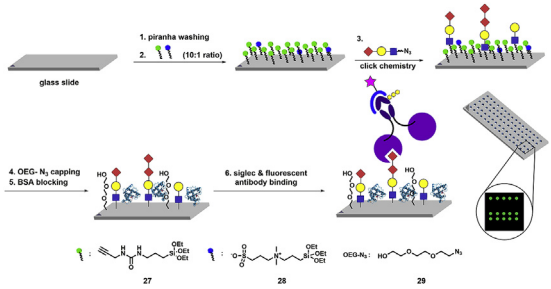


Fig. 1. Flowchart for the immobilization of glycans on an alkyne-zwitterion functionalized chip. Evaluation of Siglec-Glycan interaction by Glycan microarray analysis.

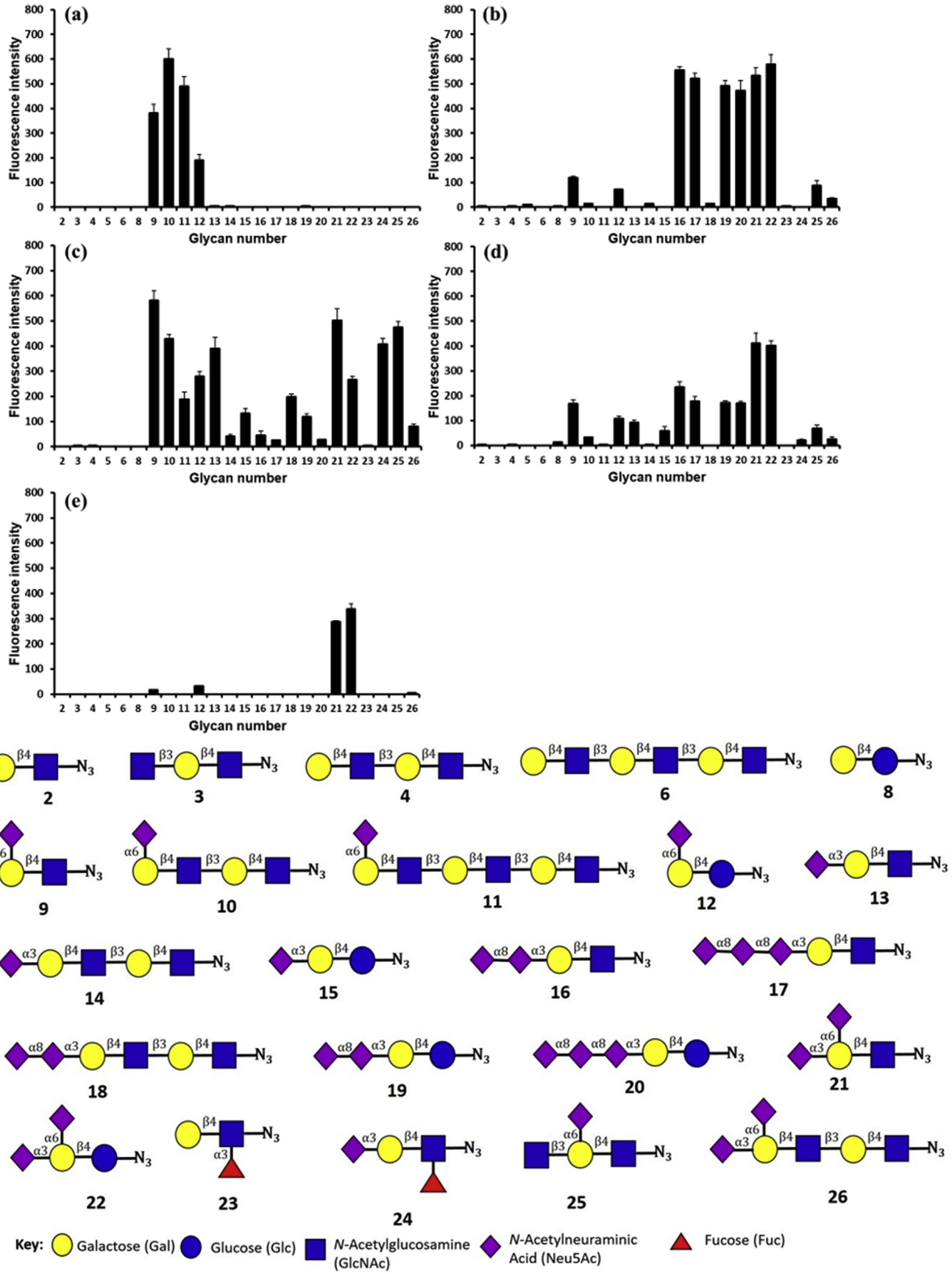


Fig. 2. Glycan binding specificities on microarray as measured by fluorescence intensity for (a) Siglec-2-Fc (b) Siglec-7-Fc (c) Siglec-9-Fc (d) Siglec-14-Fc (e) Siglec-15-Fc.

10, **11**, and **12**, showing high affinity for the di-LacNAc over the tri-LacNAc core structure, suggesting di-LacNAc is optimum length for high affinity recognition (Fig. 2a). It appears to be surprising; as Siglec-2 is the most extensively studied siglec yet, this dramatic enhancement in binding affinity with change in internal glycan length has never been reported before. Interestingly, the presence of Lac as an inner core structure results in lower binding affinity (**12** vs **9**), indicating that the acetamido group of GlcNAc stabilizes binding by additional interactions or that such a structure may provide a topology that affects the orientation of sialic acid, resulting in enhanced affinity. In addition, the addition of GlcNAc to Neu5Acα(2,6)-LacNAc (**25**) completely abolishes the binding affinity. Moreover, the addition of α(2,3)-Neu5Ac (as in **21**, **22**, and **26**) causes the loss of affinity to Siglec-2-Fc (see Fig. 2a).

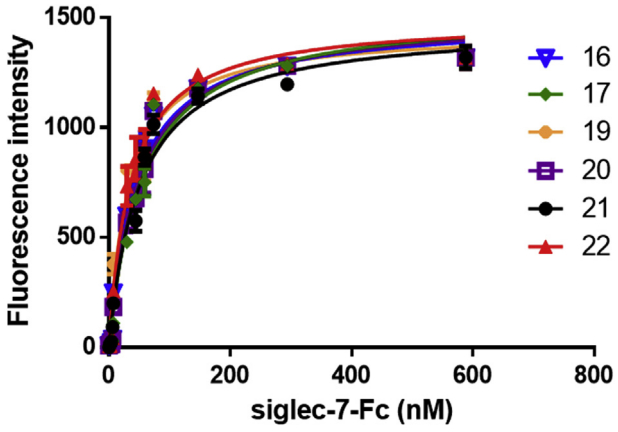
Human Siglec-7 preferentially binds to Neu5Acα(2,8)Neu5Ac-linked GD3 glycans and internally branched α(2,6)-linked sialylated glycans over terminal α(2,6)- or α(2,3)-linked Neu5Ac structures [56]. The binding results (Fig. 2b) revealed that Siglec-7-Fc bound strongly to α(2,8)-linked disialylated/trisialylated LacNAc **16** and **17** and Lac **19** and **20** [21,57]. Moreover, the addition of one more Neu5Ac at the α(2,8)-linked glycan does not enhance binding affinity (**16** vs **17** and **19** vs **20**), and the compounds with the LacNAc core show comparable affinity to those with the Lac core (**16** and **17** vs **19** and **20**). It should also be noted that branched α(2,3)α(2,6)-linked disialylated glycans (**21** and **22**) provide similar affinity as to linear disialosides (**16** and **19**), indicating these novel branched disialic acid structures can mimic linear α(2,8)-linked disialic acid and could be the ligand based probe for Siglec-7 targeting. Surprisingly, extension of the LacNAc chain of **16** to give **18** results in the loss of Siglec-7 binding activity. This activity loss was also observed in branched disialyl di-LacNAc (**21** vs **26**). Moreover, Siglec-7-Fc exhibited significantly decreased affinity for α(2,6)-sialylated LacNAc **9** and α(2,6)-sialyl lactoside **12** and no affinity for α(2,3)-sialylated LacNAc **13** and Lac **15**. Likewise, Siglec-7 fails to recognize α(2,6)-sialyl di-LacNAc **10** and tri-LacNAc **11**, further indicating that the chain length of LacNAc exerts an intrinsic effect on the binding affinity. It can be concluded that elongation of the backbone LacNAc structure will strongly influence the binding affinity to Siglec-7.

Siglec-9 is highly homologous to Siglec-7 but shows strikingly different binding specificities to sialylated glycans, such as GD1a, which is poorly recognized by Siglec 7 [21]. Previous studies have demonstrated that human Siglec-9 specifically recognized 6-*O*-sulfo-sialyl Lewis X (6-*O*-sulfo-sLe^x), ganglioside GD1a, and sialyl-lacto-*N*-tetraose c (LSTc) oligosaccharide [21,58]. As shown in Fig. 2c, sLe^x **24** without a sulfate group displayed good binding affinity for Siglec-9. Siglec-9 also recognizes glycans with Neu5Ac and either α(2,3)- or α(2,6)-linkage to Gal [58]. Sialylated glycans with an α(2,3)-linkage (**13**, **15**, and **24**) or α(2,6)-linkage (**9**, **10**, **11**, **12**, and **25**) bind to Siglec-9-Fc, as expected, but with lower affinity to the disialoglycans (**16**, **17**, **19**, and **20**). However, α(2,6)-sialylated LacNAc **9** provides superior binding affinity than that of α(2,3)-sialylated LacNAc **13**. In general, glycans containing α(2,6)-sialic acid (**9** and **12**) show higher affinity than α(2,3)-linked glycans (**13** and **15**). In addition, the branched α(2,3)α(2,6)-linked disialylated glycans with a LacNAc core (**21**) strongly binds Siglec-Fc-9 compared with a similarly branched disialylated Lac core (**22**), and both **12** and **22** (disialylated lac) show significantly lower affinity than α(2,6)-sialylated LacNAc **9**. These results indicate that glycans with LacNAc core residues (**9**, **13**, and **21**) possess higher binding affinity to Siglec-9-Fc than those with Lac cores (**12**, **15**, and **22**). Hence, our studies suggest that the α(2,6)-sialyl LacNAc (**9**) is likely to be the best ligand for Siglec-9-Fc in this assay platform. In addition, the LacNAc chain length also provides an intrinsic effect on the binding affinity, with the affinity decreasing with increasing chain length (**9** vs **10** and **11**). Notably, increasing the number of linear sialic acid units in an α(2,3)-linked glycan (**13** vs **16** and **17**, **15** vs **19** and **20**) decreases the binding affinity.

It has been demonstrated that Siglec-5 and Siglec-14 both recognize a common set of sialylated ligands on pathogenic bacteria, such as nontypeable *Hameophilus influenzae* and group B *Streptococcus* (GBS), because of their near-identical ligand-binding domains. However, they provide opposing functions to immune cells because of their differences in intracellular signaling motifs [59]. Siglec-14 it is known to have a strong binding preference for a linear α(2,3)α(2,8)-disialylated structure [60]. As shown in Fig. 2d, Siglec-14-Fc prefers to bind linear α(2,3)α(2,8)-disialyl glycans (**16** and **19**) rather than monosialylated glycans (**9**, **12**, **13**, and **15**). In a comparison of α(2,6)- and α(2,3)-linked monosialylated glycans, the glycans with α(2,6)-Neu5Ac show higher affinity than α(2,3)-glycans (**9** vs **13**, **10** vs **14**, and **12** vs **15**), and extension of the LacNAc chain results in a significant decrease in binding affinity for both α(2,6)-linked glycans (**9** vs **10** and **11**) and α(2,3)-linked glycans (**13** vs **14** and **16** vs **18**). Interestingly, the branched α(2,3)α(2,6)-linked disialylated glycans **21** and **22** show higher binding affinity than the linear α(2,3)α(2,8)-disialic acids **16** and **19** and the trisialylated glycans **17** and **20**. This increase in affinity might be because of cumulative effect, arising due to presence of both α(2,3) and α(2,6)-Neu5Ac linkage. Moreover, the LacNAc backbone (**9** and **13**) provides more favorable binding to the Siglec-14-Fc than does the Lac backbone (**12** and **15**). However, in the branched α(2,3)α(2,6)-linked disialylated glycans **21** and **22**, the Lac and LacNAc backbones provide similar skeletons to present sialic acid for interaction with Siglec-14. The results suggest that the branched α(2,3)α(2,6)-linked disialylated glycans **21** and **22** are the best ligands for Siglec-14-Fc.

Human Siglec-15 has been reported to bind preferentially to sialyl Tn (sTn, Neu5Acα(2,6)GalNAc) [61]. Consistent with the previous report, the screening results of the glycan microarray (Fig. 2e) show that human Siglec-15-Fc does not recognize α(2,3)-linkage whereas α(2,6)-sialylated LacNAc and Lac glycans **9** and **12** both show very little signal. Surprisingly, branched α(2,3)α(2,6)-disialylated LacNAc and Lac (**21** and **22**) exhibit good binding affinity. The increase in affinity might be attributed to the presence of additional α(2,3)-linked Neu5Ac unit. However, increase in core length (**26**) completely abolishes binding. In contrast, α(2,3)α(2,8)-disialylated and α(2,3)α(2,8)α(2,8)-trisialylated glycans do not show binding affinity. The results indicate that the presence of GalNAc as the core sugar with a reducing end α linkage may be important for the desired orientation of sialic acid, resulting in a "correct structure" for interaction with Siglec-15. However, more diverse sialylated glycans are needed to understand the SAR of this interaction. Based on the glycan microarray binding results, it may be concluded that the branched α(2,3)α(2,6)-disialylated glycans (**21** and **22**) exhibit the best binding affinity for Siglec-15-Fc.

Siglec-7 is expressed on the surface of natural killer (NK) cell and serves as an inhibitory receptor of NK cell cytotoxicity. Small molecular ligands for Sigelc-7 may be potential immunomodulators [56]. Thus, the binding activity of the glycan microarray was further evaluated by measuring the surface dissociation constant values (K_D) of sialylated glycans (**16**, **17**, **19**, **20**, **21**, and **22**) to Siglec-7 as shown in Fig. 3. The K_D values were determined by plotting lectin concentrations against the fluorescence intensity of binding with different glycans. GD3 (**19**) is a known high-affinity ligand for Siglec-7 with a solution dissociation constant in the nanomolar range (K_D 60 nM) [56]. Due to the high glycan density on the slide, the K_D measured by the fabricated glycan microarray for **19** with Siglec-7 is 32.7 ± 4.1 nM, indicating the reliability of the binding propensity of Siglecs measured by the glycan microarray. The K_Ds of **16**, **17**, **20**, **21**, and **22** with Siglec-7 are in the nM range,



Glycan no.	K_D + SD (nM)
16	43.3 ± 3.7 nM
17	54.1 ± 5.7 nM
19	32.7 ± 4.1 nM
20	48.7 ± 4.5 nM
21	38.7 ± 4.7 nM
22	26.8 ± 4.0 nM

Fig. 3. Binding curves of Siglec-7 with surface-immobilized glycans 16, 17, 19, **20**, **21**, and **22**. The arrays were incubated with Siglec-7 from 0.367 nM up to 588 nM, and then *anti*-hIgG-cy3 was added.

and Lac is a better core structure than LacNAc to present sialic acids to provide higher binding affinities. However, trisialosides **(17** and **20**) showed lower binding affinities than disialosides (**16** and **19**). Among the six assayed glycans, **22** was the best ligand, similar as GD3 (**19**), for Siglec-7 with $K_D = 26.8 \pm 4.0$ nM.

3. Conclusion

In this study, we developed an expedient enzymatic synthesis of oligo-LacNAc on a preparative scale, where expensive sugar nucleotides were regenerated by a set of kinases and phosphorylases. This regeneration method significantly reduces the cost of production and enables the facile purification of complicated oligosaccharides. Defined-length oligo-LacNAc and Lac were further modified with Neu5Ac or Fuc via sialyltransferases or fucosyltransferases. Integrating these methods, a glycan library with 23 different analogs containing variations in chain length, internal glycan, and modification with Neu5Ac and/or Fuc was successfully synthesized. Our robust yet flexible synthetic strategy not only delivered a route to access complex glycans but also led us to the possibility of finding high-affinity ligands for Siglecs. The orderly analysis of Siglec specificity towards the sialoside library indicates that internal glycan length and core type significantly influence and affect binding affinity. Binding results of glycans **9, 10**, and **11** against Siglec-2 and Siglec-9 shows prominent effect of internal glycan length in recognition. Siglec-2 and -9 show higher affinity for the LacNAc than the Lac core, whereas Siglec-7 shows the opposite affinity. The branched α(2,3)α(2,6)-disialic acid-containing glycans **21** and **22** show enhanced binding affinity towards Siglec-7, 9, 14, and 15.

3.1. Experimental procedures

Synthesis of LacNAc unit by sugar nucleotide regenerating system.

Assembly of Gal. The sugar nucleotide regenerating system for the synthesis of the LacNAc unit was constructed from the assembly of Gal (200 mg, 1.11 mmol) using ATP (12.25 mg, 0.022 mmol), UTP (29.32 mg, 0.055 mmol), phosphoenolpyruvate (502.98 mg, 2.44 mmol, monopotassium salt), $MgCl_2$ (10 mM) and 6-azidohexyl-GlcNAc acceptor (**1**, 345 mg, 0.99 mmol) in HEPES buffer (22 mL, 100 mM, pH 7.5). An enzymatic solution containing the enzymes EcGalK (3 μg/mL), AtUSP (40 μg/mL), NmGalT (93 μg/mL), PK (100 U), and iIP (10 U) was added to the above solution and then incubated at 37 °C for 24 h with shaking. The reaction was monitored by TLC (v/v, EtOAc:MeOH:H_2O:HOAc = 3:2:1:0.5, $R_f = 0.6$). When the reagents were completely converted to LacNAc **2**,[28] the same volume of 99% ice-cold ethanol was added to the reaction mixture and incubated at 4 °C for 30 min. The reaction mixture was centrifuged, and the supernatant was evaporated and purified by reversed-phase chromatography. The fractions containing the product were collected and concentrated by a high-vacuum evaporator. The product was further purified by a Bio Gel P-2 gel column and produced by lyophilization to give **2**[28] in 99% (502 mg) yield. ^{1}H NMR (400 MHz, D_2O) δ 4.44 (d, $J = 7.7$ Hz, 1H), 4.39 (d, $J = 7.7$ Hz, 1H), 3.92 (dd, $J = 1.9$, 12.2 Hz, 1H), 3.84–3.83 (m, 2H), 3.77 (dd, $J = 4.6$, 12.2 Hz, 1H), 3.72–3.60 (m, 6H), 3.57 (dd, $J = 3.2$, 10.1 Hz, 1H), 3.49–3.45 (m, 3H), 3.26 (m, 2H), 1.97 (s, 3H), 1.54–1.51 (m, 4H) 1.32–1.30 (m, 4H); HRMS (ESI) *m/z* calcd for $C_{20}H_{37}N_4O_{11}$ $[M+H]^+$ 509.2459; found 509.2451.

Assembly of GlcNAc. UDP-GlcNAc was prepared by a sugar nucleotide regenerating system using 9.8 mL of Tris buffer (50 mM, pH 7.5) containing GlcNAc (87.0 mg, 0.39 mmol), ATP (10.8 mg, 0.03 mmol), UTP (26.0 mg, 0.05 mmol), phosphoenolpyruvate (184.1 mg, 0.79 mmol, monopotassium salt), $MgCl_2$ (10 mM) and LacNAc **2** (200 mg, 0.39 mmol). After adjusting the pH to 7.5, enzymes including NahK (20 μg/mL), GlmU (40 μg/mL), HpGnT (0.2 mg), PK (200 U), and IP (10 U) were added to the above solution and then incubated at 37 °C for 30 h with shaking. The reaction was monitored by TLC (v/v, EtOAc:MeOH:H_2O:HOAc = 4:2:1:0.50.5, $R_f = 0.5$). When the reagents were completely converted to the product, the same volume of 99% ice-cold ethanol was added, and the resulting solution was incubated at 4 °C for 30 min. The reaction mixture was centrifuged at 10,000×*g* for 15 min, and the supernatant was evaporated. The resulting residue was purified by reversed-phase silica column chromatography (Sep-Pak C18 20 cc Vac cartridge) using gradient elution from 0% to 30% methanol. The product-containing fractions were pooled together and then lyophilized to give **3**[28] in 86% (246 mg) yield. ^{1}H NMR (400 MHz, D2O) δ 4.71 (d, $J = 8.4$ Hz, 1H), 4.54 (d, $J = 7.3$ Hz, 1H), 4.48 (d, $J = 7.8$ Hz, 1H), 4.17 (d, $J = 3.2$ Hz, 1H), 4.01 (m, 1H), 3.95–3.91 (m, 2H), 3.84 (dd, $J = 5.2$, 12.2 Hz, 1H), 3.80 (d, $J = 3.8$ Hz, 1H), 3.77–3.63 (m, 8H), 3.62–3.57 (m, 4H), 3.52–3.48 (m, 2H), 3.35 (t, $J = 6.8$ Hz, 2H), 2.06 (s, 3H), 2.05 (s, 3H), 1.63–1.59 (m, 4H), 1.39–1.38 (m, 4H). HRMS (ESI) *m/z* calcd for $C_{28}H_{49}N_5O_{16}Na$ [M+Na]+734.3072; found 734.3064.

α(2,3)α(2,8)-Sialylation catalyzed by Cst-II. To a 3.2 mL solution of 100 mM Tris-HCl (pH 8.0) containing Neu5Ac (5 eq), acceptor (15 mM), and CTP (10 eq) were dissolved in with 20 mM of MgCl2, CSS (50 μg/mL) and Cst-II (9.6 μg/mL). The pH of the solution was monitored and adjusted to 8.5 with 2 N NaOH (aq). The reaction mixture was incubated in a shaker at 37 °C for 5 h. The progress of the reaction was monitored by TLC (v/v, EtOAc:CH_3OH:AcOH:H_2O = 3:2:1:0.5). The reaction was stopped by adding the same reaction volume of cold ethanol and the solution was kept on ice for 30 min. The mixture was centrifuged at 10,000×*g* for 15 min.

The supernatant was concentrated and purified by Bio-Gel P-2 gel filtration column using deionized distilled H_2O as eluent. The fractions with product were pooled and lyophilized to give final product.

α(2,3)α(2,6)-Sialylation catalyzed by PmST1 and Pd2,6ST. To a 5.3 mL solution of 100 mM Tris-HCl (pH 8.0) containing 1.5 eq of Neu5Ac, acceptor glycan (15 mM), CTP (2 eq) and 20 mM of MgCl2 was added CSS (50 μg/mL) and of PmST1 (50 μg/mL). The solution was incubated in a shaker at 37 °C and the progress of the reaction was monitored by TLC. The reaction was workup by adding the same amount of cold ethanol and kept on ice for 30 min. The mixture was centrifuged at 10,000×*g* for 20 min. The supernatant was concentrated and then purified by Bio-Gel P-2 gel filtration column. Lyophilized product was re-dissolved to a 2.6 mL solution of 100 mM Tris-HCl buffer (pH 8.0) containing 1.5 eq of Neu5Ac, 2 eq of CTP, and 20 mM of $MgCl_2$ was added CSS (50 μg/mL) and of P. damsela Pd2,6ST (26 μg/mL). The solution was incubated in a shaker at 37 °C. The reaction solution was adjusted to pH 8.0 with 1 N NaOH (aq). The progress of the reaction was monitored by TLC (v/v, EtOAc:CH_3OH:AcOH:H_2O = 3:2:1:0.5). The reaction was workup by adding the same amount of cold ethanol and kept on ice for 30 min. The insoluble precipitates were removed by centrifugation and the supernatant was concentrated by vacuum. The desired product was purified by Bio-Gel P-2 using deionized distilled H_2O as eluent. Lyophilized product was characterized by NMR spectrometry and mass spectrometry.

Fabrication of alkyne-functionalized microarray. Commercial microscope glass slides were washed with piranha solution (H_2SO_4/H_2O_2 = 3:1) at room temperature for 1 h and then rinsed with ddH_2O and EtOH. The slides were immersed in a DMSO solution with a mixture of **27** and **28** (both were prepared as 10 mM solutions) in ratios of 10:1, 5:1, and 1:1 (V/V), respectively, at room temperature for 6 h. The solution was removed, and the slides were washed with DMSO and ddH_2O three times each to give alkyne-functionalized slides.

Glycan printing and Siglec binding assay. Glycans were printed by robotic contact arraying machine (Biodot; AD1500 arrayer; robotic pin SMP3). Approximately 0.6 nL of glycan printing solution (500 μM of glycan, $CuSO_4$, THPTA, and sodium ascorbate in ddH_2O+10% glycerol) was spotted onto alkynylated glass slides. Each glycan was spotted with five replicates. Immediately after printing, slides were placed in a chamber at 80% humidity and incubated at room temperature (rt) for 12 h. The remaining alkyne groups on the surface were capped by immersing the slides in a 50 mM azido-ethylene glycol in ddH_2O with 10 mM of $CuSO_4$, THPTA, and sodium ascorbate at rt for 2 h. Slides were rinsed in water, dried by low-speed centrifugation, and stored in desiccators at room temperature before use.

Glycan slides were blocked with blocking buffer (3% BSA in PBS buffer) for 0.5 h. After rinsed in 0.05% PBST buffer and water, the slide was incubated with a pre-mix solution of Fc-fused Siglec (10 μg/mL) and Fc-specific anti-human-IgG-cy3 (10 μg/mL) (1:1 v/v prepared in PBS buffer with 0.1% BSA) at room temperature for 1 h. Solutions were removed and the slide was washed with 0.05% PBST and ddH_2O, dried by low-speed centrifugation prior to image acquisition. Fluorescence intensity was measured using a VIDAR Revolution 4550 and image analysis was performed using ArrayEase software. The average over the printed replicates was calculated using Array Vision Version 8.0 (Imaging Research, Inc.).

Measurement of K_D. Various concentrations of Siglec-7, namely, 588, 294, 147, 73.5, 58.8, 44.1, 29.4, 7.35, 5.88, 4.41, 2.94, 1.47, and 0.735 nM, were incubated with a glycan microarray containing **16**, **17**, **19**, **20**, **21**, and **22** at room temperature for 1 h. The solution was removed, and the slide was washed with 0.05% PBST and ddH_2O and dried by low-speed centrifugation. The slide was then incubated with Fc-specific anti-human-IgG-cy3 (10 μg/mL) at room temperature for 0.5 h followed by washing with 0.05% PBST and ddH_2O. Finally, the slide was dried by centrifugation, and the fluorescence intensity was measured using a VIDAR Revolution 4550. Image analysis was performed using ArrayEase software. The raw data obtained from the microarray scanner were processed, and the surface dissociation constant K_D was calculated by GraphPad Prism using the following equation:

$$y = \frac{B_{max} \cdot x}{K_D + x}$$

x: The concentration of Siglec, y: the fluorescent intensity, B_{max}: predicted maximum binding, K_D: surface dissociation constant.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.07.046.

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