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Glycan microarrays for screening sialyltransferase specificities

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Abstract Here we demonstrate that glycan microarrays can be used for high-throughput acceptor specificity screening of various recombinant sialyltransferases. Cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) was biotinylated at position 9 of *N*-acetylneuraminic acid (Neu5Ac) by chemoenzymatic synthesis generating CMP-9Biot-Neu5Ac. The activated sugar nucleotide was used as donor substrate for various mammalian sialyltranferases which transferred biotinylated sialic acids simultaneously onto glycan acceptors immobilized onto a microarray glass slide. Biotinylated glycans detected with fluorescein–streptavidin conjugate to generate a specificity profile for each enzyme both confirming previously known specificities and reveal additional specificity information. Human α2,6sialyltransferase-I (hST6Gal-I) also sialylates chitobiose structures (GlcNAcβ1-4GlcNAc)$_n$ including *N*-glycans, rat α2,3sialyltransferase (rST3Gal-III) tolerates fucosylated acceptors such as Lewis^a, human α2,3sialyltransferase-IV (hST3Gal-IV) broadly sialylates oligosaccharides of types 1–4 and porcine α2,3sialyltransferase-I (pST3Gal-I) sialylates ganglio-oligosaccharides and core 2 *O*-glycans in our array system. Several of these sialyltransferases perform a substitution reaction and exchange a sialylated acceptor with a biotinylated sialic acid but are restricted to the most specific acceptor substrates. Thus, this method allows for a rapid generation of enzyme specificity information and can be used towards synthesis of new carbohydrate compounds and expand the glycan array compound library.

Keywords Carbohydrate · Sialyltransferase · Specificity · Enzyme · Glycan array

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Abbreviations

Neu5Ac	*N*-acetylneuraminic acid
CMP-Neu5Ac	cytidine-5′-monophospho-*N*-acetylneuraminic acid
LacNAc	*N*-acetyllactosamine
LacDiNAc	GalNAcβ1-4GlcNAc
ST3Gal-I	Galβ1-3GalNAcα2,3-sialyltransferase
ST3Gal-III	Galβ1-3(4)GlcNAcα2,3-sialyltransferase
ST3Gal-IV	Galβ1-4(3)GlcNAcα2,3-sialyltransferase
ST6Gal-I	Galβ1-4GlcNAcα2,6-sialyltransferase
ST6GalNAc-I	GalNAcα2,6-sialyltransferase
MS	mass spectrometry
NMR	nuclear magnetic resonance
GM1	ganglioside GM1
LSTb	sialyl-lacto-*N*-tetraose b
type 1	Galβ1-3GlcNAc
type 2	Galβ1-4GlcNAc
type 3	Galβ1-3GalNAcα
type 4	Galβ1-3GalNAcβ
core 1	Galβ1-3GalNAcα
core 2	Galβ1-3[GlcNAcβ1-6]GalNAcα
core 3	GlcNAcβ1-3GalNAcα
core 4	GlcNAcβ1-3[GlcNAcβ1-6]GalNAcα
core 6	GlcNAcβ1-6GalNAcα
H-type 2	Fucα1-2Galβ1-4GlcNAc

Introduction

More than 50% of all human proteins carry various glycan chains. Glycans are a large group of compounds consisting of sugars with diverse structures present both inside and on the cell surface. The diversity in glycan structures allows them to play an important role in a variety of biological events that underlie the development and function of multicellular organisms [1–3]. The biosynthesis of glycans takes place in endoplasmic reticulum and Golgi aparatus involving nucleotide sugar donors, nucleotide sugar transporters, glycosyltransferases and their acceptors. The activated nucleotide–monosaccharides (glycosyl donors) are the substrates for the glycosyltransferases in mammals. Each glycosyl donor is accepted by various glycosyltransferases mediating the transfer of the glycosyl group onto different acceptors. *In vivo*, these enzymes are generally specific for the given glycosyl donor, the type of linkage to be formed, the structure of the acceptor and a specific hydroxyl group of the acceptor. Such observed specificity is required to permit the synthesis of biomolecules without a template. The glycosyltransferases, thus, display acceptor specificities as well as the stereo- and regioselectivities in formation of glycosidic linkages [4]. Nevertheless, these enzymes have shown some flexibility with respect to their substrate requirements under *in vitro* conditions, *i.e.* in the absence of their specific substrate and/or when the acceptors are provided in high concentration. Such flexibility has made the glycosyltransferases favorable to be used as biocatalysts in carbohydrate synthesis and several of them are abundantly over-expressed as recombinant enzymes with high activities to make glycans for biological studies and pharmaceutical applications [5, 6].

To generate libraries of various glycans or analogs thereof, it is important to further explore the acceptor specificities of glycosyltransferases and assess their potential applications for *in vitro* synthesis. A variety of methods for evaluation of relative glycosyltransferase acceptor specificities have been developed including radiochemical, spectrophotometric, immunological and chromatographic assays [7]. In this work we have utilized our recently developed glycan microarray [8] to demonstrate enzymatic solid-phase glycosylation reactions using labeled donor substrates to assay 200[1] glycan acceptors, mounted on a glass slide, simultaneously.

Materials and methods

Radioactive labeled sugar nucleotides were from New England Nuclear Life Science Products Inc. (Boston, MA). The radioactive nucleotide–sugars were diluted with unlabeled nucleotide-sugars (Sigma) to the desired specific radioactivity. All other oligosaccharide substrates were purchased from Sigma Chemical Co. (St. Louis, MO). The synthesis of 9-amino-9-deoxy-CMP-Neu5Ac and the method for subsequent biotinylation were described previously [9, 10]. The product purification using size exclusion chromatography gave 9-biotinamido-9-deoxy-CMP-Neu5Ac and verified by mass spectrometry (MS) analysis. Preparative synthetic reactions were monitored by thin layer chromatography (TLC) performed on Silica Gel 60F precoated TLC plates (EMD Chemicals Inc., Gibbstown, New Jersey, USA). After development with appropriate eluants, the spots were visualized by UV light for nucleotides and/or dipping in 5% sulfuric acid in ethanol, followed by charring to detect sugars. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker DRX-600 MHz instruments at 25°C and were referenced to acetone δ 2.225 (^{1}H in D_2O) and δ 30.89 (^{13}C in D_2O). ESI-TOF high-accuracy MS spectra was recorded with an LC MSD TOF (Agilent Technologies, Santa Clara, CA) or a MALDI-TOF (Applied Biosystems, Foster City, CA) using dihydroxybenzoic acid as matrix. Water was purified by NanoPure Infinity Ultrapure water system (Barnstead/Thermolyne, Dubuque, IO) and degassed by vacuum treatment before use. Data images were analysed and the intensity of fluorescence in spots corresponding to the antibodies bound to the individual glycans was quantified using a ScanArray 5000 (Perkin Elmer, Boston, MA) confocal scanner and image analyses were carried out using ImaGene image analysis software (BioDiscovery Inc, El Segundo, CA). No background subtractions were performed and data were plotted using Microsoft Excel software.

Sialyltransferases

All the recombinant sialyltransferases were overexpressed in our laboratory as previously reported (http://www.functionalglycomics.org) in the following expression systems: baculovirus, insect cell (pST3Gal-I, hST6Gal-I, hST6GalNAc-I), Fungus, *Aspergillus Niger* (rST3Gal-III), mammalian, COS-1 cells (hST3Gal-IV). Enzymes were purified on ion-exchange and glycosyltransferase affinity column chromatography (CDP-gel, CalBiochem, San Diego, CA).

Glycan microarray assay

Assays were performed in Tris–HCl buffer (100 mM, pH 7.0) containing CMP-9Biot-Neu5Ac (1 mM) , sialyltransferase (50 mU) and BSA (3%) in a total volume of 0.5 ml. The mixture was applied onto the glycan array surface and gently shaken at room temperature for 90 min. The slide was extensively washed with Tris–HCl buffer (100 mM, pH 7.0)

[1] Current size of the glycan array is approx. 320 glycans (see http://www.functionalglycomics.org).

followed by water and incubated with Tris–HCl buffer (100 mM, pH 7.0) containing streptavidin–Alexa488 conjugate (1:100 dilution) and incubated for 30 min at room temperature. The slide was washed as described above and subject to image analysis using a confocal scanner.

Sialyltransferase ^{14}C assay

Assays were generally performed at 37°C in 100 uL reaction volume with substrate concentrations of 10 mM oligosaccharide acceptor and 1.8 mM CMP-Neu5Ac including 9 nmol ^{14}C-CMP-Neu5Ac (final specific activity 0.7 Ci/mol). Reaction buffer contained cacodylate (50 mM, pH: 6.5 for hST6Gal-I, pST3Gal-I, rST3Gal-III, hST3Gal-IV and pH 7.5 for hST6GalNAc-I and chST6GalNAC-I) containing NaCl (100 mM). Typical sialyltransferase reactions were quenched by the addition of ice-cooled water (0.7 ml). The reaction mixtures were then passed through Dowex columns (1-X8, 200–400 mesh, PO_4^{2-} form, 0.5×3 cm). The columns were washed with water (1.3 ml), the effluents were directly collected in scintillation vials and the radioactivities were assayed by liquid scintillation counting (Beckman LS counter).

Preparative sialylation of chitobiose

Chitobiose (15 mg, 0.035 mmol) and crude CMP-Neu5Ac (0.71 mmol) were dissolved in buffer (1 mL, 100 mM NaCl, 200 mM cacodylate, pH 6.5). hST6Gal-1 (1 U) was added and the reaction mixture was incubated at 37°C for 12 h. The reaction mixture was centrifuged and the supernatant was loaded on a G15 Sephadex column (1.0×120 cm) equilibrated and eluted with 5% *n*-BuOH/H_2O. The appropriate fractions were collected and lyophilized to yield the trisaccharide Neu5Acα2-6GlcNAcβ1-4GlcNAc (10 mg, 38%). Selected ^{1}H NMR (600 MHz, D_2O), *δ*(ppm): 5.18 (d, 0.6H, *J*=3.09 Hz, 4.72 (d, 0.4H, *J*=7.78 Hz, GlcNAc2 H-1β), 4.58 (d, 1H, J=8.5 Hz, GlcNAc H-1), 3.78 (m, 1H, GlcNAc H-2), 3.90 (m, 3H, GlcNAc H-6a), 3.76 (m, GlcNAc H-6b), GlcNAc2 H-1α), 2.69 (dd, 1H, *J*=3.6 Hz, *J*=12.9 Hz), 1.72 (t, 1H, J=12.0 Hz). Selected ^{13}C NMR (600 MHz, D_2O), *δ*(ppm): 102.549, 95.391, 91.179, 82.138, 81.788, 74.915, 74.867, 74.123, 73.214, 72.382, 70.781, 69.021, 68.816, 63.980, 63.284, 60.961, 60.817, 59.977, 56.144, 53.941, 52.550, 40.711, 30.890, 22.685. ESI-TOF high-accuracy MS *m/z* calculated for (M + H), 738.2531, found 738.2531, *m/z* calculated for (M + Na), 760.2359, found 760.2376.

Preparative sialylation of LacDiNAc

LacDiNAc (275 mg, 0.55 mmol) and crude CMP-Neu5Ac (0.72 mmol) were dissolved in buffer (5 mL, 40 mM $MnCl_2$, 200 mM cacodylate, pH 7.5). hST6Gal-1 (3.8 U) was added and the reaction mixture was incubated at 37°C for 18 h. The reaction mixture was centrifuged and the supernatant was loaded on a G15 Sephadex column (1.0× 120 cm) equilibrated and eluted with 5% *n*-BuOH/H_2O. The appropriate fractions were collected and lyophilized to yield the trisaccharide Neu5Acα2-6GalNAcβ1-4GlcNAc-β$OCH_2CH_2N_3$ (200 mg, 47%). Selected ^{1}H NMR (600 MHz, D_2O), *δ*(ppm): 4.61 (d, 1H, *J*=8.3 Hz), 4.50 (d, 1H, *J*=8.5 Hz), 2.66 (dd, 1H, *J*=4.6 Hz, *J*=12.4 Hz), 2.072/2.067 (overlapping singlets, 6H), 2.028 (s, 1H), 1.71 (t, 1H, *J*=12.2 Hz). Selected ^{13}C NMR (600 MHz, D_2O), *δ*(ppm): 175.599, 175.361, 175.333, 174.178, 102.912, 101.514, 81.859, 74.854, 74.355, 73.353, 73.231, 72.345, 71.316, 69.419, 69.053, 68.798, 68.035, 63.994, 63.306, 60.944, 55.176, 53.083, 52.586, 51.010, 40.783, 23.057, 22.864, 22.681. ESI-TOF high-accuracy MS *m/z* calculated for (M + H), 785.3047, found 785.3061.

Sialylation of biantennary agalacto-*N*-glycopeptide

The agalacto-glycopetide (0.02 mg, 0.022 umol) was dissolved in cacodylate buffer (0.1 mL, 100 mM, pH 6.5) containing CMP-Neu5Ac (0.8 mg, 0.875 umol), $MnCl_2$ (10 mM), alkaline phosphatase (10 U), bovine serum albumin (BSA) (20 μg) and hST6Gal-I (2.2 mU). After 17 h the reaction mixture was analyzed by mass spectrometry and Dionex. MALDI-TOF MS *m/z* calculated for (M + H) 1904, found 1904.

Sambucus nigra agglutinin (SNA) lectin analysis

Biotinylated SNA-I (10 μg/mL) diluted in phosphate saline buffer (PBS) containing tween-20 (0.05%) (1 mL) was applied onto the slide and incubated in a humidified chamber at 20–22°C for 1 h with gentle shaking. Washing in PBS (+Tween-20, 0.01%, 10 dips) and PBS (10 dips) was followed by immediate incubation with labeled Streptavidin–Alexa488 1/100 (1 mg/mL) in 1 mL PBS (+3% BSA, 0.05% tween-20) for 30 min, which was followed by washing with PBS (+Tween-20, 0.01%, 10 dips), PBS (10) dips and deionized water (2×3 dips), finally the slides were dried in a stream of air, followed by immediate analysis using a confocal slide array laser scanner.

Results and discussion

Many of the known glycosyltransferases utilize a limited repertoire of nucleotide sugars as donors for the glycosylation reaction and extensive information regarding structural key reporter groups on these nucleotide sugars for various glycosyltransferases have been studied [11, 12]. Since many sialyltransferases tolerate various modifications at the C9-position on Neu5Ac, we initially targeted

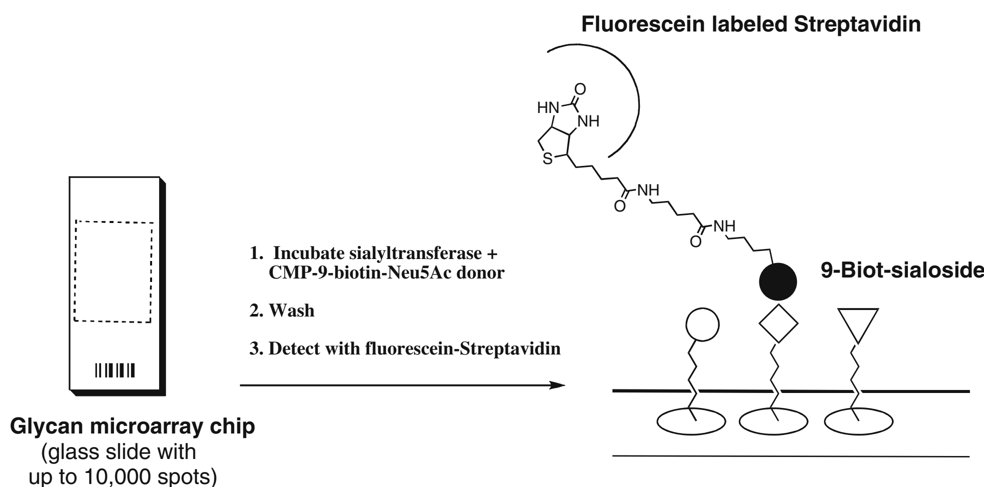


Fig. 1 Principle of evaluating sialyltransferase acceptor specificities on the glycan microarray. Enzymatic transfer of biotinylated nucleotide sugar donor followed by detection with fluorescently labeled Streptavidin–Alexa Fluor488 conjugate

synthesis of the 9′-biotinylated CMP-Neu5Ac (CMP-9Biot-NeuAc) donor analog by chemoenzymatic synthesis. Briefly, 9′-azido-9′-deoxy-CMP-NeuAc was prepared using a microbial CMP-Neu5Ac synthetase as previously described [9]. The azide moiety was further reduced to amine with triphenylphosphine, reacted with *N*-hydroxysuccinimide-activated spacered biotin (NHS-LC-LC-Biotin, Pierce) [10] and isolated by size exclusion chromatography. Using various sialyltransferases, biotinylated Neu5Ac was efficiently transferred from the nucleotide sugar analog (CMP-9Biot-NeuAc) to sialylate an array of 200 immobilized glycans [8]. The biotinylated glycans were detected with fluorescently labeled streptavidin followed by fluorescence detection using a confocal slide scanner (Fig. 1). Slide image was converted to a readout bar graph and the signal pattern illustrates the preferred acceptor specificities for each enzyme.

We analyzed five mammalian sialyltransferases: human ST6Gal-I (E.C.2.4.99.1) [13, 14], human ST6GalNAc-I (E.C.2.4.99.3) [15, 16], porcine ST3Gal-I (E.C.2.4.99.4) [17], rat ST3Gal-III (E.C.2.4.99.6) [18–20] and human ST3Gal-IV (E.C.2.4.99.6) [21, 22]. All enzymes tolerated the biotinylated donor analog which was readily transferred to acceptors on the glycan array. A titration experiment with increasing concentrations of the CMP-(9Biot)-Neu5Ac (0.001–0.5 mM) at maximum glycan surface density (saturation at >50 μM printing concentration [8]) using hST6Gal-I, gave saturation of donor concentration >0.1 mM for the ***N***-acetyllactosamine (LacNAc) acceptor (**70**). This is in agreement with previous published donor analogs [17, 23] (Fig. 2a). The transfer of 9Biot-Neu5Ac from CMP-9Biot-Neu5Ac could be inhibited with addition of non-labeled CMP-Neu5Ac at a total donor concentration of 0.1 mM (Fig. 2b) and as low as 0.1 mU enzyme/reaction assay was readily detected (not shown). All five sialyltransferases gave the expected acceptor specificity profile. The extensive collection of immobilized acceptor molecules of various terminal natural glycan structures from glycoproteins and glycolipids, and additional acceptor specificity information was also gathered as discussed below.

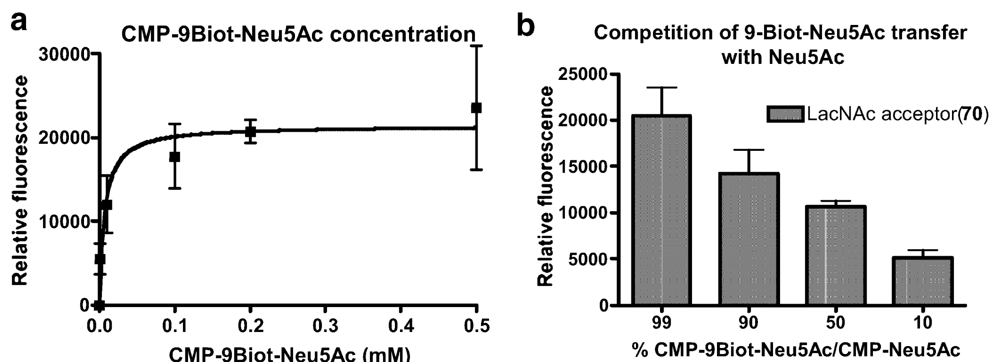


Fig. 2 Analysis of labeled donor CMP-9Biot-Neu5Ac with hST6Gal-I on the array. **a** Increasing concentrations of the CMP-(9Biot)-Neu5Ac (0.001–0.5 mM) using hST6Gal-I (50 mU) followed by detection of biotinylated LacNAc acceptor (**70**) with Streptavidin–Alexa Fluor488. **b** Competition with increasing concentration of non-labeled CMP-Neu5Ac at total donor concentration 0.1 mM using hST6Gal-I and immobilized LacNAc (**70**) acceptor substrate

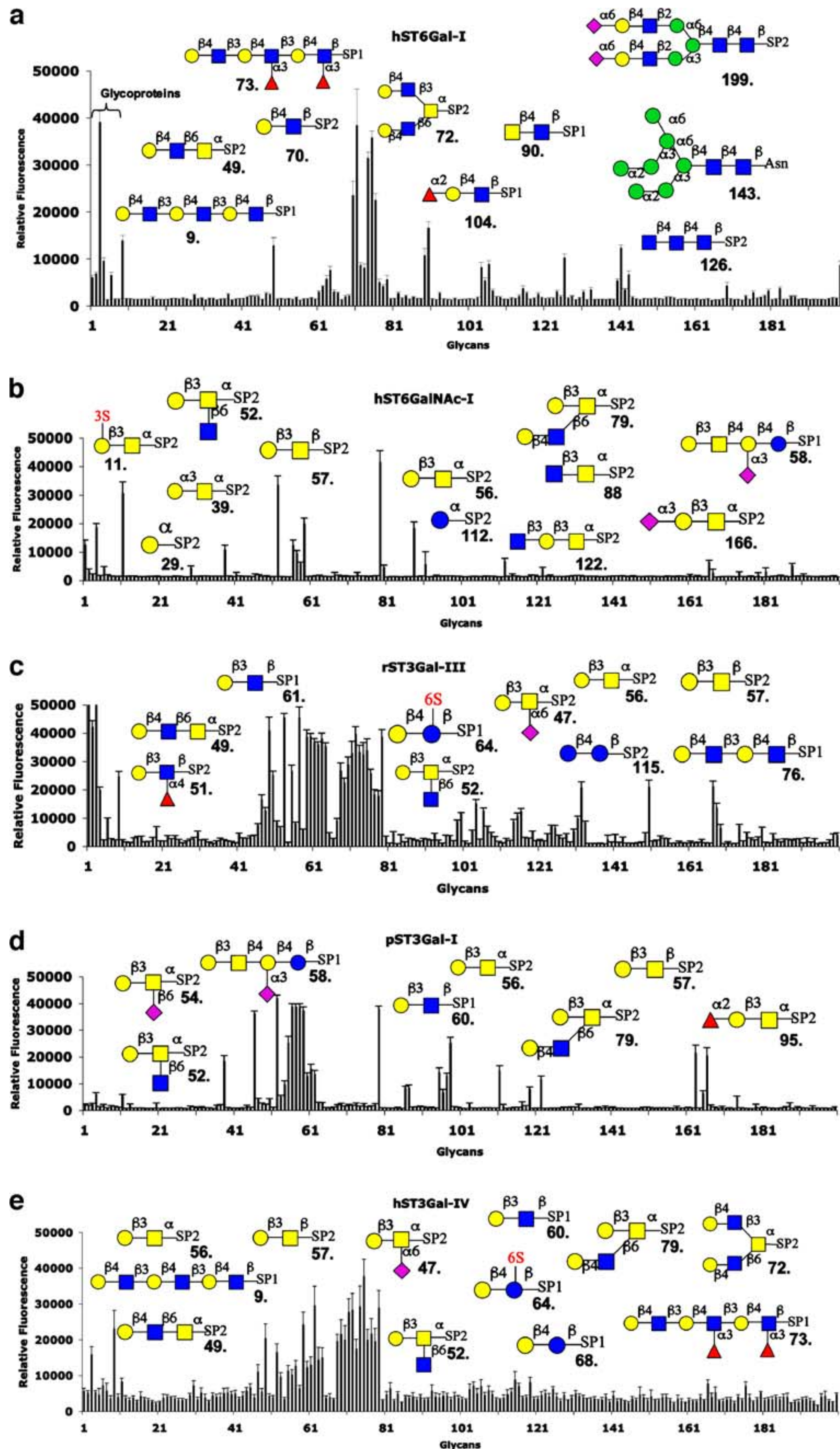
Human ST6Gal-I

Human ST6Gal-I demonstrated the expected acceptor specificity towards various terminal LacNAc (type 2) structures (**9**, **49**, **70**–**79**) [19] including several glycoproteins containing these terminal structures (Fig. 3a). Significant activities towards GalNAcβ1-4GlcNAc (LacDiNAc) (**89**, **90**) and GlcNAcβ1-4GlcNAcβ1-4GlcNAc (chitotriose) (**126**) structures were found and there were less activities with fucosides Fucα1-2Galβ1-4GlcNAc, (H-type 2 (**104**)). To confirm some of the results, preparative synthesis of the α2-6-sialosides Neu5Acα2-6GalNAcβ1-4GlcNAc (6′SLDN) and Neu5Acα2-6GlcNAcβ1-4GlcNAc (6′sialo-chitobiose) were performed and characterized by NMR and MS (see Materials and methods). Interestingly, glycan array signals were also found for various *N*-glycans (**140**–**143**, **199**) containing the core chitobiose. Indeed, the sialylation of agalacto-biantennary glycopeptide with the structure (GlcNAcβ1-2Manα)2,3-Manβ1-4GlcNAcβ1-4GlcNAcβ1-GEN (gly-glu-asn) using hST6Gal-I gave quantitatively a monosialylated product according to Dionex column chromatography and MS data. The same *N*-glycan but with the peptide sequence GENR (gly-glu-asn-arg) did not show any sialylation product (not shown) indicating that hST6Gal-I is sensitive to the peptide backbone for activity. In addition, weak signals could also be detected for acceptor substrates of lower affinity such as type 1 (Galβ1-3GlcNAc) oligosaccharides (**61**, **62**) [19, 23] and are perhaps not unexpected since the branched Galβ1-3(Neu5Acα2-6)GlcNAc (LSTb) motif is present on common milk oligosaccharides structures. Preparative oligosaccharide synthesis was still feasible with low affinity acceptors such as lactose [9].

To initially confirm the data obtained with the biotinylated donor, we investigated whether we could accomplish indirect specificity studies with lectins and performed glycan array staining with *Sambucus nigra* (SNA-I) lectin before and after surface glycosylation with hST6Gal-I. The lectin binds exclusively to α2-6sialylated glycans with type 2 (LacNAc) structures but also to 6′O-sulfated LacNAc (data not shown, for details see http://www.functionalglycomics.org). As indicated in Fig. 4, hST6Gal-I sialylated linear LacNAc or LacDiNAc structures (**70**, **9**, **92**), branched, Galβ1-4-extended core 2 and core 6 O-glycans (**49** and **79**) but not Galβ1-4-extended core 3 (Galβ1-4GlcNAcβ1-3GalNAcα) at these assay conditions (Materials and methods). This is also in agreement with the biotinylation assay (Fig. 3a) showing less incorporation of 9Biot-Neu5Ac into acceptor **77** compared to **49**. Interestingly, we could also see a significant higher transfer of 9Biot-Neu5Ac to biantennary *N*-glycans with terminating LacNAc (**201**) at lower enzyme concentrations (<0.5 mU) which confirms previous studies that branched *N*-glycans are preferred substrates for this enzyme [24, 25]. Although we can not exclude that SNA might have preferences for sialylated biantennary *N*-glycans compared to linear glycans at certain glycan concentrations. In addition, hST6Gal-I sialylates sulfated lactose (**202**) and H-type 2 glycans (**203**), also indicated in Fig. 3a (compounds **104**–**106**). We have shown that in principle, indirect enzyme specificities can be performed by comparing binding of various lectins before and after enzymatic glycosylation reactions on the microarray surface.

Human ST6GalNAc-I

This enzyme transfers Neu5Ac of CMP-Neu5Ac to the 6-hydroxyl group on internal GalNAc on *O*-glycans with strict specificities towards the *O*-glycan GalNAcα- (Tn-antigen), Galβ1-3GalNAcα- (T-antigen) and Neu5Acα2-3Galβ1-3GalNAcα- (sialyl T-antigen) structures [4, 15]. It has demonstrated activities towards O-linked oligosaccharides attached to peptides of glycoproteins. Weak activities towards synthetic non-amino acid aglycons of *O*-glycans have also been reported for hST6GalNAc-I [16]. In contrast, chicken ortholog of this enzyme (chST6GalNAc-I) requires at least one amino acid for activity [26]. Since this version (v1.0) of our glycan array only displays O-linked glycans with ethylamino-spacer and no O-linked glycopeptides, hST6GalNAc-I was used in this study. On the glycan array, as expected, hST6GalNAc-I showed specific activities towards T-antigen (**56**) and sialyl T-antigen (**166**) glycans. In addition structures with type 4 (Galβ1-3GalNAcβ) including the GM1 oligosaccharide (**57**–**59**), and other 3′-substituted T-antigens (**11**, **122**) were also sialylated. The core 3 glycan GlcNAcβ1-3GalNAcα (**88**) was also acceptor but not when extended with a terminal Galβ1-4 (**77**). Surprisingly, hST6GalNAc-I showed strong activities with core 2 glycans (Galβ1-3GalNAcβ[GlcNAcβ1-6]GalNAc) (**52**, **79**) which is unexpected since the 6-hydroxyl group on the penultimate GalNAc is occupied by the GlcNAcβ1-6-substitutions and is not available for sialylation at the penultimate GalNAc. Core 2 *O*-glycan structures lacking the terminal galactose such as core 6 (GlcNAcβ1-6GalNAcα- (**128**) or substituted with other monosaccharides such as core 4 (GlcNAcβ1-3[GlcNAcβ1-6]GalNAcα- (**120**) did not give any signal indicating that hST6GalNAc-I possibly also sialylated the terminal galactose of core 2 glycans. To further study this we first performed standard radioactive enzyme assay using selected T-antigen and core 2 acceptors. Relative specificities for the acceptors, Galβ1-3GalNAcαO*p*NP (23%), Galβ1-3[GlcNAcβ1-6]GalNAcOα*p*NP (12%), Galβ1-3GalNAcOαThr (100%) and Galβ1-3[GlcNAcβ1-6]GalNAcαThr (100%) were obtained. No activities were detected with Galβ1-3[Neu5Acα2-6]GalNAcOαThr (α2-6 sialyl T-antigen) or Neu5Acα2-6GalNAcOαThr (α2-6 sialyl Tn-antigen) (Table 1). We also assayed the


a
hST6Gal-I
Glycoproteins
Relative Fluorescence
Glycans
b
hST6GalNAc-I
c
rST3Gal-III
d
pST3Gal-I
e
hST3Gal-IV

◀ **Fig. 3 a–e** Evaluation of sialyltransferase acceptor specificities on the glycan microarray. Biotinylated sugar nucleotide (1 mM) was transferred to acceptor substrates on the array surface with sialyltransferases (50 mU) and subsequently overlay with fluorescently labeled Steraptavidin–Alexa-488. Image analysis of each enzyme using a confocal scanner generated a bar chart describing relative enzyme acceptor substrate affinities. Symbol structures, *dotted black circles*: Glc, *dotted light circles* Gal, *dotted black squares*: GlcNAc, *dotted light squares*: GalNAc, *dotted black diamonds*: Neu5Ac, *dotted black triangles*: Fuc, *dotted gray diamonds*: KDN, *dotted gray circles*: Man, *half-filled diamonds*: GlcA, *dotted light diamonds*: Neu5Gc, SP1=–$(CH_2)_2$–NH–, SP2=–$(CH_2)_3$–NH–

chST6GalNAc-I using the same acceptor substrates above. Relative specificities of the acceptors, Galβ1-3GalNAcαO*p*NP (0%), Galβ1-3[GlcNAcβ1-6]GalNAcOα*p*NP (0%), Galβ1-3GalNAcOαThr (100%) and Galβ1-3[GlcNAcβ1-6]GalNAcαThr (19%) were found which were in line with our previous finding that chST6GalNAc-I requires the α-threonine aglycon for activity. Even as the ST6GalNAc-I enzyme was purified on a CDP-hexanolamine agarose affinity column [26], we considered the possibility of the presence of small amounts of an exoglycosidase that removes GlcNAc from the internal GalNAc of core 2 to provide the natural substrate, *i.e.* core 1, for ST6GalNAc-I to form α2-6-sialylT-antigen. To address this issue, we carried out a preparative incubation of hST6GalNAc-I with a commercial *p*-nitrophenyl (pNP)-core 2 (Galβ1-3[GlcNAcβ1-6]GalNAcα-pNP) acceptor substrate and CMP-Neu5Ac for 48 h. The reaction mixture was purified on a C-18 reverse phase column to recover the hydrophobic pNP materials and characterized by thin layer chromatography TLC and MS. The result of TLC, consistent with the MS analysis, showed carbohydrate spots in two migration levels corresponding to sialyl-core1 and core 1 structures. However, the MS results, indeed, showed a minor peak at 998.33 corresponding to the mass of sialylated core 2, but the major peaks at 504.15 and 795.25 corresponding to the masses of core 1 and sialyl core 1, respectively, were readily detected. The results implied that part of the detected product in radioactive assay should be related to sialyl core 1, generated from core 2 degradation, providing the conventional core 1 substrate for the enzyme. We also verified that the printed core 2 glycans were not detected with the *Maclura Pomifera* lectin (MPL) that readily detects core 1 but not core 2 glycans (http://www.functionalglycomics.org), indicating that there were no traces of degraded core 2 that appeared at the array surface (data not shown).

Rat ST3Gal-III

This sialyltransferase utilizes Galβ1-3/4GlcNAc (type-1/2), lactose and Galβ1-3GalNAcα- (core 1) acceptors [4, 19]. In our study we also found significant activities towards the type 4 (Galβ1-3GalNAcβ-) structures (**57**) including asialo-GM1 (**59**), core 2 antigens (**52**) and α2-6 sialyl T-antigen (**47**) but not β2-6 sialyl T-antigen (**54**). In addition, we report that this sialyltransferase can utilize the fucosylated acceptor Lewis[a] (Galβ1-3[Fucα1-4]GlcNAc-) oligosaccharide (**50**, **51**). To confirm these findings, we selected

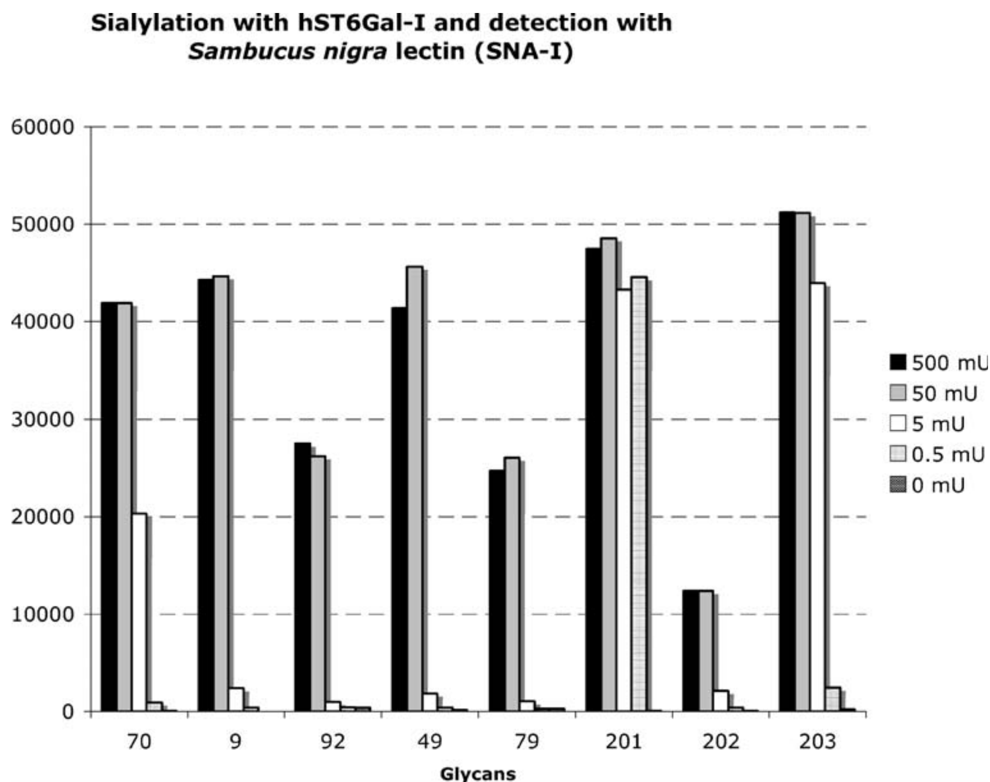


Fig. 4 Detection of unlabeled sialylated acceptors with SNA-I lectin after incubations with hST6Gal-I (0–500 mU). For compound identification see text and supporting information

Table 1 Relative acceptor specificities of hST6GalNAc, chST6GalNAc-I and rST3Gal-III

Acceptors (5 mM)	Relative sialyltransferase rate (%)		
	hST6GalNAc-I	chST6GalNAc-I	rST3Gal-III
Galβ1-3GalNAcαOpNP	23	0	–[a]
Galβ1-3[GlcNAcβ1-6]GalNAcOαpNP	12	0	–
Galβ1-3GalNAcOαThr	100	100	–
Galβ1-3[GlcNAcβ1-6]GalNAcαThr	100	19	–
Galβ1-3[Neu5Acα2-6]GalNAcOαThr	0	–	–
Neu5Acα2-6GalNAcOαThr	0	–	–
Galβ1-4GlcβSp	–	–	100
Galβ1-4GlcNAcβSp	–	–	150
Galβ1-3[Fucα1-4]GlcNAcβSp	–	–	50
Galβ1-4[Fucα1-3]GlcNAcβSp	–	–	5
Fucα1-2Galβ1-3GlcNAcβSp	–	–	<2
Fucα1-2Galβ1-4GlcNAcβSp	–	–	8

pNp Para nitrophenyl, *Sp* 2-azidoethyl, *Thr* threonine
[a] Not tested

a few substrates for radioactive assays as described above. Relative activities at 5 mM acceptor concentration for lactose (100%), LacNAc (150%), Lewis[a] (50%), Lewis[x] (6%), H-type 1 (1.4%) and H-type 2 (7.8%) were found and in good agreement with the signal pattern on the glycan array experiment (Table 1). Unexpectedly, we also detected a signal for the type 1 structures Neu5Ac/Neu5Gc/KDNα2-3Galβ1-3GlcNAc- (**132**, **150**, **167**) which were already sialylated at the 3′Gal-position. One possible explanation is that the enzyme, to a certain degree, performs an exchange reaction by first hydrolyzing the α2-3-sialoside followed by transfer of the biotinylated Neu5Ac. Indeed, in a standard radioactive assay (10 mM acceptor, 90 μM CMP[C^{14}]Neu5Ac), rST3Gal-III transferred/exchanged [C^{14}]Neu5Ac to **167** (Neu5Acα2-3Galβ1-3GlcNAc) at a >50% relative efficiency compared to the type 1 disaccharide **60** (data not shown). No signals to other Neu5Acα2-3Gal-structures (type 2, type 3) were detected indicating that exchange reactions on the array only took place with high affinity acceptor substrates. No asialo impurities have been identified in printed materials or detected on the microarray using MS and lectin analysis respectively re-assuring that signals are not due to sialylation of printed galactosides.

Porcine ST3Gal-I

Porcine ST3Gal-I is known to have strict activities towards core 1 *O*-glycans with terminal linear galactose [4, 27] which was confirmed here as well (**56**) (Fig. 3d). In addition our results showed a very strong selectivity towards a selection of branched oligosaccharides such as core 2 *O*-glycans (**52**, **79**) but not the core 6 (**120**), core 4 (**49**, **128**) or core 3 (**78**, **88**) structures lacking the terminal Galβ3-moiety. Also ganglioside of type 4 (Galβ1-3GalNAcβ-) epitopes (**57**) including GM1 and asialo-GM1 (**58**, **59**) were readily sialylated. Even H-type 3 (Fucα1-2Galβ1-3GalNAcα-) structures (**95**–**98**) were well recognized by the pST3Gal-I as well as some simple monosaccharides Galβ- (**46**) and GalNAcβ- (**86**) but not their α-anomeric counterparts (**29**, **80**). Also for this enzyme, some degree of exchange reaction takes place specifically for the Neu5Acα2-3Galβ1-3GalNAc structures (**163**, **165**, **166**).

It has been suggested that ST3Gal-I competes with core 2 β1-6GlcNAcT-I for the same acceptor which could affect further elongations of the synthesized *O*-glycans [28–30]. Recently, Matta and coworkers demonstrated that ST3Gal-I had equal or better affinity towards core 2 compared to the T-antigen [31], a result that we now also confirmed with our solid-phase array results. Thus, the possibility exists that sialylation *in vivo* also occurs after β1-6GlcNAcT formation on mucins.

Human ST3Gal-IV

Human ST3Gal-IV has been cloned independently from human placenta [21] and a human lymphoma cell line [22]. Analysis of recombinant ST3Gal-IV have shown that this enzyme used either glycoproteins or glycolipids containing Galβ1-3GlcNAc- (type 1) or Galβ1-4GlcNAc- (type 2) sequences [22, 29, 32]. Indeed, we also showed specificity towards arrayed oligosaccharides with type 1 terminus including sialyllacto-*N*-tetraose b (LST b) (Galβ1-3[Neu5Acα2-6]GlcNAcβ1-3Galβ1-4Glc-) (**53**), various type 2 termini including poly-LacNAc (**9**, **73**) as well as lactose (**68**). *O*-glycans such as the T-antigen (**56**) and core 2 structures (**52**, **79**) are good acceptors and some activities towards the α2-6 sialyl T-antigen (**47**). Extended type 2 structures such as poly-LacNAc (**9**, **73**) were good acceptors but not LacDiNAc or any other substitutions were tolerated. Some activities towards type 4 (Galβ1-3GalNAcβ-) epitopes (**57**–**59**) were also seen. Nevertheless that some mixed results regarding activity to type 1 structures have been reported the hST3Gal-IV seems to have strong bias for the type 2 structures *in vivo* and *in vitro*. In fact, our

data show that all type 1, 2, 3 and 4 sequences are tolerated by the enzyme at least for glycans attached to a surface presumably at high concentrations.

We have demonstrated that our glycan microarray can rapidly evaluate hundreds of acceptor substrates simultaneously using small amount of enzymes. A broad spectrum of methods have been developed to assay glycosyltransferase activities during the enzyme preparation and to assess the final products. Depending on the method used, different problems impede the accurate evaluation of the enzyme activity. Separation of the synthesized product from un-reacted donor, and in some cases acceptor, is the major problem in solution-based assays. Nonspecific binding of lectins or antibodies, as well as the low-throughput acceptors are limitations facing the solid-phase immunoassays [7]. Increased information of donor specificities and advancements in chemoenzymatic synthesis has enabled synthesis of various labeled sugar nucleotides to be used as donor substrates by different sialyltransferases. In this study, we tested the substrate specificity of a series of sialyltransferases on 200+ acceptors mounted on the microarray, using a chemoenzymatically synthesized biotinylated CMP-NeuAc donor substrate. This technique may prevail over the typical problems facing enzyme activity and substrate specificity studies. Detecting new acceptor substrates for sialyltransferases is not only useful in *in vitro* glycan synthesis but it may also shed light on regulatory mechanisms involved in biosynthesis of specific carbohydrate ligands.

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