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MicroPlate Sialyltransferase Assay (MPSA): a rapid and sensitive assay based on an unnatural sialic acid donor and bioorthogonal chemistry

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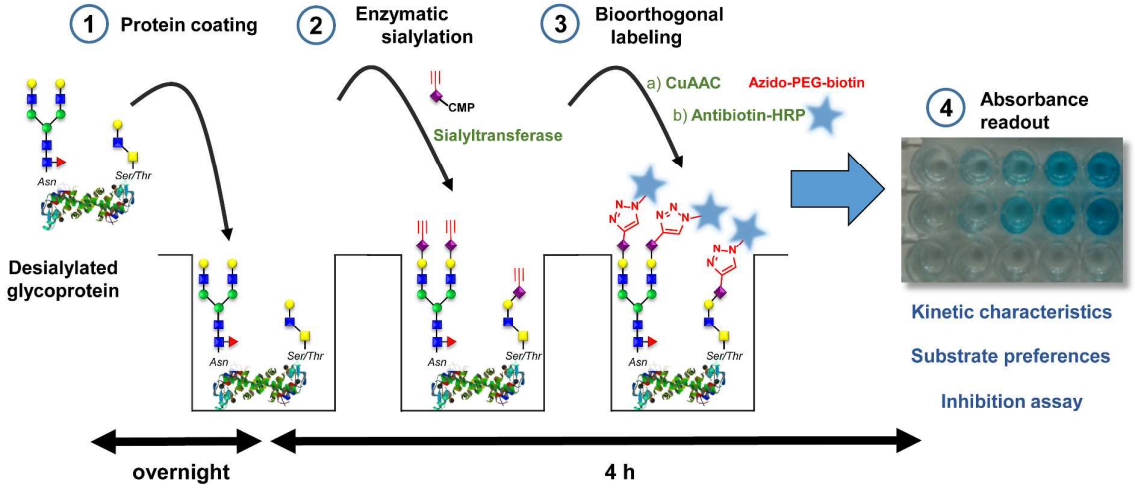
ABSTRACT

Mammalian sialyltransferases transfer sialic acids onto glycoproteins and glycolipids within the Golgi apparatus. Despite their key role in glycosylation, the study of their enzymatic activities is limited by the lack of appropriate tools. Herein, we developed a quick and sensitive sialyltransferase microplate assay based on the use of the unnatural CMP-SiaNAl donor substrate. In this assay an appropriate acceptor glycoprotein is coated on the bottom of 96-well plate and the sialyltransferase activity is assessed using CMP-SiaNAl. The alkyne tag of SiaNAl enables subsequent covalent ligation of an azido-biotin probe via CuAAC and an anti-biotin-HRP conjugated antibody is then used to quantify the amount of transferred SiaNAl by a colorimetric titration. With this test, we evaluated the kinetic characteristics and substrate preferences of two human sialyltransferases, ST6Gal I and ST3Gal I toward a panel of asialoglycoprotein acceptors, and identified cations that display a sialyltransferase inhibitory effect.



INTRODUCTION

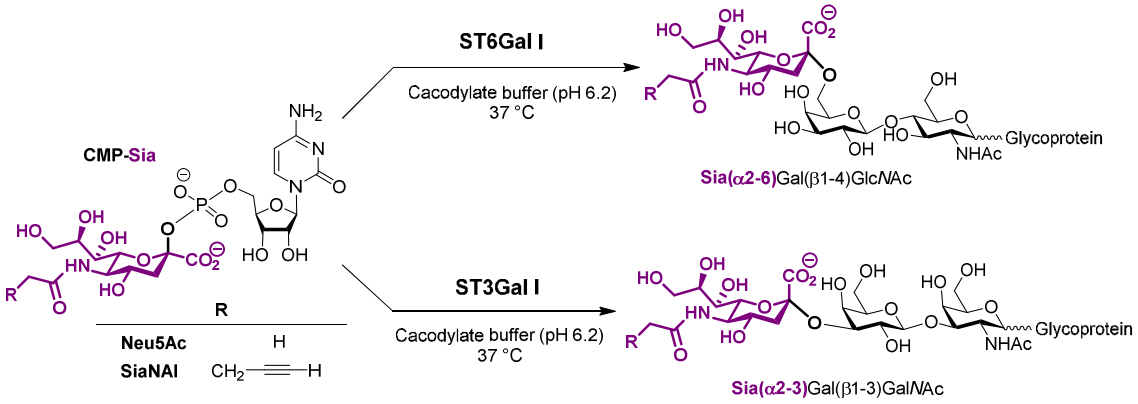
In the world of glycobiology, the sialic acids family occupies a quite unique position. Comprising more than 50 natural members, this family of nine carbon carboxylated monosaccharides is often found at the non-reducing extremity of glycans.[1] Due to their terminal position and negatively charged nature, sialic acids are involved in many biological phenomena such as cell-cell adhesion, host-pathogen interactions, or regulation of pathophysiological events.[2] Sialyltransferases (STs) of the CAZy GT-29 family are found in all eukaryotic branches and in alpha-proteobacteria and represent a subset of glycosyltransferases responsible for the introduction of sialic acid residues onto glycoconjugates.[3] In the past two decades, a growing interest for STs has been observed in various fields such as clinical biomarkers, biocatalysis, natural products synthesis or biotherapeutics.[4–6] Twenty different STs have been identified in mammalian genomes.[7] Mainly human and mouse STs have been biochemically characterized up to now and were shown to catalyze the transfer of a sialic acid residue from its activated form, cytidyl mono-phosphate sialic acid (CMP-Sia), onto galactose (Gal), *N*-acetylgalactosamine (Gal*N*Ac) or another sialic acid (Sia) residue of glycoproteins or glycolipids defining four families of STs (ST3Gal, ST6Gal, ST6GalNAc and ST8Sia). All characterized STs use CMP-Sia as a donor substrate, each enzyme presents its own specificity with regards to acceptors and type of glycosidic linkage formed.[8] Even though sialylation diversity encountered in Nature mainly relies on the activity, specificity and regulation of STs, these enzymatic properties are not completely understood yet. Methods and specific tools are therefore needed for the study of sialylation reactions and a complete understanding of the associated biochemical processes. Commercial radiolabeled CMP-[^{14}C]Neu5Ac or synthetized CMP-[^{14}C]Neu5Gc have been widely used for the characterization of ST activity since the work of Weinstein and Paulson.[9] Although very sensitive, these approaches are expensive and the use of radiolabeled substrates necessitates access to highly controlled and specific facilities. As an alternative, the structural and quantitative analysis of the acceptor glycan before and after sialylation provides valuable information about STs enzymatic characteristics. Mass spectrometry combined to liquid chromatography (LC-MS) has long been used for the analysis and characterization of sialylated glycans[10] and we recently described the use of micro-LC/ESI-MRM-MS[3] analysis to quantify transfer rates of STs, using various CMP-Sia donors

and various acceptor substrates.[11] Despite being precise, sensitive, and quantitative, such analyses are time consuming and require specific equipment and know-how. Some efforts have been made in the development of simple and accessible methods to open sialylation assessment to non-specialists. For instance, a method relying on indirect colorimetric detection of inorganic pyrophosphate has been developed with Malachite green reagent.[13] First developed for soil and water analyses, this colorimetric assay has been used to probe ST6Gal I and ST6GalNAc V activities.[14,15] However, this assay presents several limitations such as high background when non-purified enzymes are used, impossibility to use phosphate buffers or interferences with detergents. Besides, the Malachite green reagent assay has been shown to interact with arsenate, a major component of the cacodylate buffer, the gold standard buffer for *in vitro* assays with STs.[16]

Therefore, alternative methods are needed to probe ST activity; one of them is to monitor the reaction through quantification of the transferred sialic acid. Toward this aim, the activated sialic acid (CMP-Sia) has to be conjugated with a molecule allowing its detection. In the '80s, Brossmer and collaborators synthesized unnatural CMP-Sias donors that proved to be suitable for enzymatic sialylation using purified STs,[17,18] and more recently fluorogenic or chromogenic substrates were designed to monitor sialylation reactions by FRET or HPLC.[19,12] Synthesis of activated CMP-Sias conjugated with a fluorescent probe is however hard to achieve. The chemical reporter strategy, pioneered in the '90s, successively by the groups of Reutter and Bertozzi,[20,21] has opened the way for new tools to study and visualize sialoglycoconjugates. In this strategy, a small bioorthogonal moiety is chemically introduced onto a monosaccharide which can subsequently enter the cell and hijack its metabolism. Once introduced into glycoconjugates, the chemical modification is ligated to a specific complementary probe through bioorthogonal ligation, allowing the detection of the target molecule. In a seminal study, Mbua *et al.* combined chemical reporter strategy and activated sialic acids with the use of recombinant ST6Gal I to transfer an azide analog of sialic acid onto living cells.[22] Subsequent strain-promoted azide-alkyne cycloaddition (SPAAC) with a cyclooctyne-functionalized fluorophore allowed them to specifically detect cell surface α2-6-sialylated glycoproteins. Such chemoenzymatic glycan labeling has been increasingly applied to detect glycoproteins over the past few years through

the use of unnatural CMP-activated sialic acids[23–27] and other nucleotide monosaccharide donors.[28] Based on this method, we developed sensitive assays that can be used to study the biochemical properties of STs, to screen potential ST inhibitors, or to characterize new enzymatic activities. Recently, we reported a simple chemoenzymatic procedure that provides synthetically modified CMP-Sias without any purification steps[29] and the joint use of LC-MS, radioactive assays, and enzymatic labelling with non-natural sialic acids analogs to probe the activity and specificity of two human STs (ST3Gal I and ST6Gal I).[11] In this study, we propose a rapid and sensitive microplate sialylation assay (**MPSA**) that allows easy monitoring of ST activity in various conditions using a alkynyl analog of CMP-Neu5Ac (*i.e.*, CMP-SiaNAl).

RESULTS AND DISCUSSION



Scheme 1: Sialylation reaction and enzymes preferential acceptor substrates with ST6Gal I on *N*-glycans and ST3Gal I on *O*-glycans. Sialic acid is represented in purple.

Two human recombinant STs were chosen for the development of our sialylation assay (Scheme 1): whereas ST6Gal I exclusively transfers sialic acid with an α2-6 glycosidic linkage on the galactose residue of Galβ1-4GlcNAc of *N*-glycans, ST3Gal I specifically targets *O*-glycans for the α2-3 transfer of sialic acid on Galβ1-3GalNAc. ST3Gal I and ST6Gal I are the two most widely known and studied sialyltransferases. In addition to their respective specificity toward either *N*- or *O*-glycans, this made them relevant models to set up and illustrate the versatility of MPSA. Recombinant human ST6Gal I and ST3Gal I deleted of their first 56 amino acids (*i.e.* cytoplasmic tail, transmembrane and stem domains) and bearing a 3XFlag tag at their N-terminus were therefore produced into transfected HEK293 (Human Embryonic Kidney) cell culture medium. We previously reported the tolerance of both ST6Gal I and ST3Gal I toward CMP-SiaNAl, the alkynyl analog of CMP-Neu5Ac, and showed that they exhibit similar kinetic parameters with natural and unnatural donor substrates.[11] A simple four step procedure was set-up: (1) the acceptor glycoconjugate is coated overnight into a 96-well plate. (2) After saturation of wells with oxidized Bovine Serum Albumine (BSA), crude enzymatic solution of either ST6Gal I or ST3Gal I and freshly prepared CMP-SiaNAl (Figure **1A**) are incubated in the wells at 37 °C. (3) Azido-PEG-biotin is covalently attached to the alkyne of each transferred sialic acid through copper catalyzed alkyne-azide cycloaddition (CuAAC). (4) Biotin is then specifically detected by an anti-biotin antibody coupled with Horse Radish Peroxydase (HRP). The

readout can be readily achieved with a simple measure of each well absorbance with TMB (3,3′,5,5′-Tetramethylbenzidine) substrate and a microplate spectrophotometer at 620 nm (Figure **1B**).

To assess the specificity of MPSA for sialylation reaction we set up the standard procedure described above using fetuin, a model glycoprotein containing 3 sialylated *N*-glycans and 3 sialylated *O*-glycans.[30,31] Before the coating, fetuin was chemically desialylated (yielding asialofetuin) with trifluoroacetic acid (TFA). Culture medium containing crude ST6Gal I expressed and produced in transfected HEK cells or an ST-free cell culture medium (mock medium) and 0-100 μM CMP-SiaNAl were incubated with the coated asialo-glycoprotein. Subsequently, re-sialylated glycoproteins were reacted with azido-PEG-biotin in presence of $CuSO_4$ and sodium ascorbate to perform a CuAAC ligation. After washing steps, the covalently linked biotin was finally detected with anti-biotin-HRP antibody and the absorbance measured at 620 nm. Figure **1B** illustrates the significant increase of the absorbance with the concentration of donor-substrate, whereas only a slight increase of the absorbance was detected in mock medium. Therefore, in the subsequent experiments, signal obtained with the mock medium were systematically subtracted from the one obtained with sialyltransferases in order to only consider specific signal.

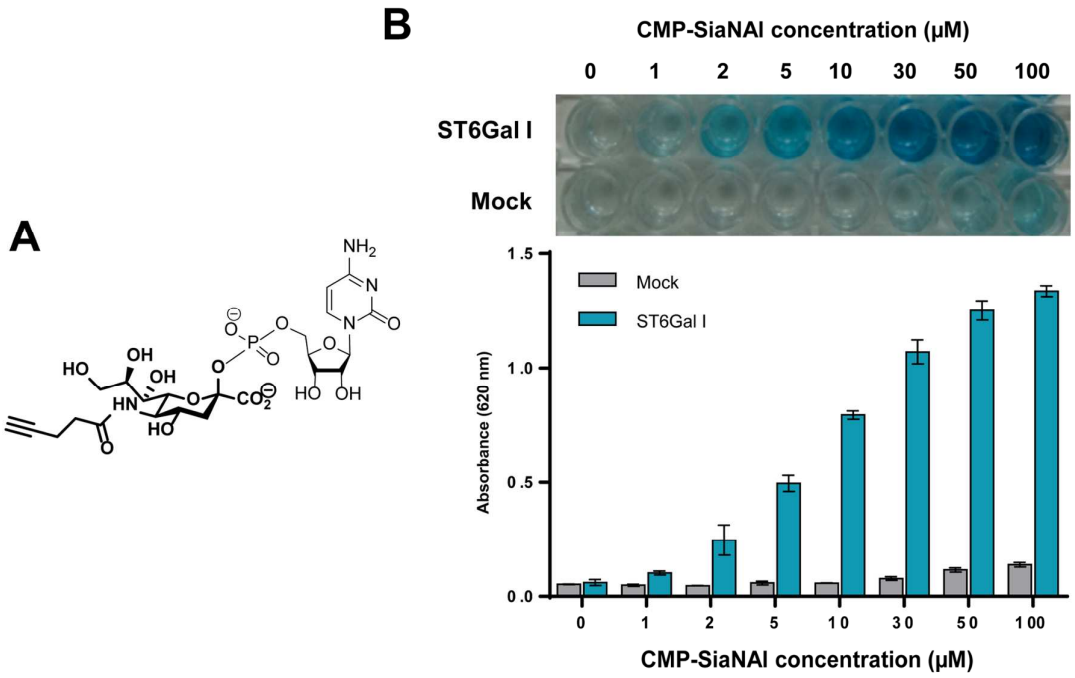


Figure 1: **(A)** Chemical structure of the alkynyl analog of CMP-Neu5Ac: CMP-SiaNAl. **(B)** MPSA experiment after incubation with either ST6Gal I or mock medium and various concentrations of CMP-SiaNAl (0-100 µM). All the wells have been treated with azido-PEG-biotin with CuAAC conditions and finally revealed with an anti-biotin antibody HRP. Absorbance measured at 620 nm for all CMP-SiaNAl concentrations. Error bars represent SEM (n = 2).

To assess the robustness of our test and to set up the essential conditions of an enzymatic assay as simple and fast as possible, we use directly the transfected cell culture medium as an enzyme source for both human ST6Gal I and ST3Gal I. As previously reported, low levels of Δ56ST6Gal I were consistently obtained in the cell culture medium of transfected cells, whereas Δ56ST3Gal I was expressed at much higher level.[11] Consistent with this data, we observed a dependence of the reaction velocity on the enzyme amount used in the MPSA (Figure 2**A**). To keep the amount of enzyme as low as possible and a reaction velocity in the same range for the two recombinant enzymes, the Δ56ST3Gal I enzymatic source was diluted 5 times while ST6Gal I was used undiluted in the subsequent assays. Since the enzyme amount is directly related with the reaction time, the best time for sialylation reaction within the initial velocity was then determined (Figure 2 **B**). For both enzymes, absorbance value reaches a plateau after 1h30 of reaction and to perform all reactions in conditions of initial velocity, the sialylation reaction was therefore fixed to 1 h. The concentration of both donor and acceptor substrates involved in the sialylation reaction should be saturating so that no substrates will

be rate limiting. We thus evaluated the kinetics parameters of each enzyme preparation toward donor substrate (CMP-SiaNAl) and acceptor substrate (asialofetuin). To that aim, either ST6Gal I or ST3Gal I were incubated with 400 ng of coated asialofetuin in presence of various concentrations of CMP-SiaNAl (Figure 2, **C**). Values of 28.2 ± 7.8 µM and 14.8 ± 2.9 µM were calculated for Km toward CMP-SiaNAl for ST6Gal I and ST3Gal I respectively. We fixed the donor substrate concentration to 100 µM in order to obtain non-limiting substrate conditions. Similar experiments were performed to determine the kinetics of enzymes toward asialofetuin. Sialylation reactions were performed with variable quantities of coated asialofetuin, in presence of 100 µM CMP-SiaNAl (Figure 2, **D**). We determined that ST6Gal I had an arbitrary Km toward asialofetuin of 319 ± 151 ng whereas an arbitrary Km of 88.1 ± 41.1 ng was found for ST3Gal I, indicating a higher affinity of this latter enzyme toward asialofetuin. The plateau reached with 400 ng of coated asialofetuin shows that this quantity of acceptor substrate provides the maximal absorbance for both ST6Gal I and ST3Gal I. These results are in accordance with others found in the literature with more traditional methodologies.[11,32]

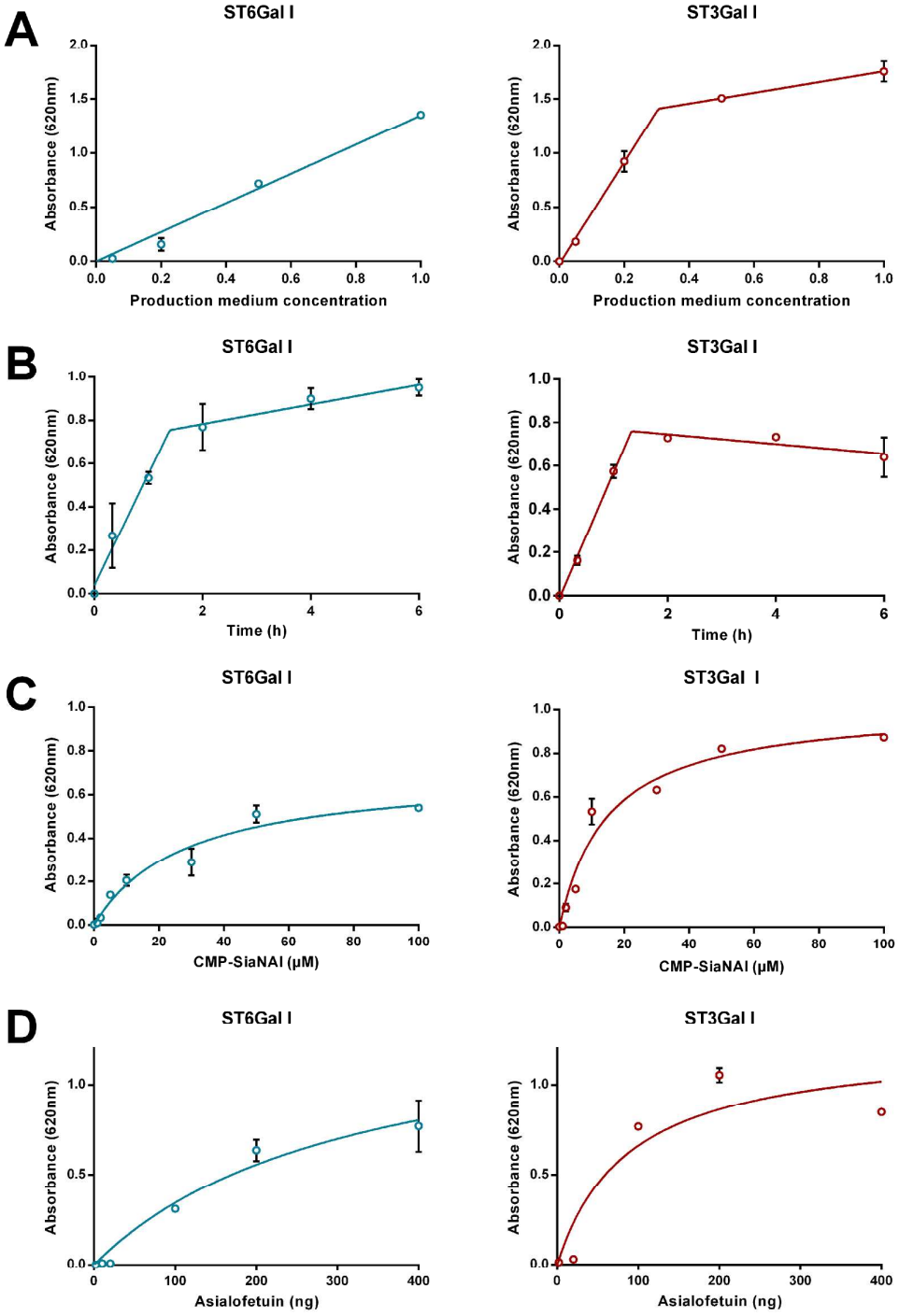


Figure 2. Determination of kinetic parameters with MPSA. Otherwise specified, 100 µM of CMP-SiaNAl and 400 ng of asialofetuin were incubated for 1 h with either Δ56ST6Gal I and Δ56ST3Gal I. (**A**) Activity in function of the dilution factor of the enzymatic source (**B**) Reaction time. MPSA absorbance readout for both ST6Gal I and ST3Gal I in function of time. (**C**) Km determination of both Δ56ST6Gal I and Δ56ST3Gal I toward CMP-SiaNAl (0 - 100 µM) (**D**) and coated asialofetuin (0 – 400 ng). Error bars represent SEM, (n = 2).

The specificity of STs toward acceptor substrates is rather difficult to assess as it usually requires the ability to detect the acceptor glycan. On one hand, biologically relevant synthetic acceptor are hardly available and on the other hand, available short di- or tri-saccharides do not fully reflect the structural complexity of STs' substrate and are therefore not representative of the specificity of these enzymes. To assess the ability of MPSA to reflect the preferences of both enzymes toward *N*- and *O*-glycans, several glycoproteins were used as specific acceptors: Bovine Serum Albumin (BSA) is not glycosylated and was used as a negative control; Porcine gastric mucin (PGM) bears multiple core 1 and 2 *O*-glycans;[33] orosomucoïd, also known as alpha-1-acid glycoprotein, bears five tri- and tetra-antennary *N*-glycans chains;[34] and, finally, fetuin exhibits both *N*- and *O*- glycans. Acceptor glycoproteins were compared either under their native form or after removal of sialic acids in presence of trifluoroacetic acid (TFA). Each acceptor was coated in the microplate overnight, incubated for 1 h with either ST3Gal I, ST6Gal I or mock medium in presence of 100 µM of CMP-SiaNAl. As expected, none of the native glycoproteins was efficiently used by recombinant sialyltransferases because of the preexisting sialic acid residues that occupy the sialylation sites (Figure 3). No significant difference between mock or any ST was observed for BSA, even after TFA treatment. ST3Gal I was found more active toward desialylated PGM whereas ST6Gal I showed more activity onto desialylated orosomucosoid and as already observed, asialofetuin was sialylated by the two recombinant enzymes. These results highlight the versatility of MPSA for acceptor preferences determination. Indeed, in only a few hours, the specificity of any ST can potentially be assessed toward any glycoproteins including bearing several glycosylation motifs. In the last decade, *in vitro* promiscuity toward substrate modifications of *N*-acyl chain has been reported for ST6Gal, ST3Gal, ST6GalNAc, and ST8Sia families, with both mammalian and bacterial systems.[27,35–38] This promiscuity should allow to extend MPSA to other STs in the future.

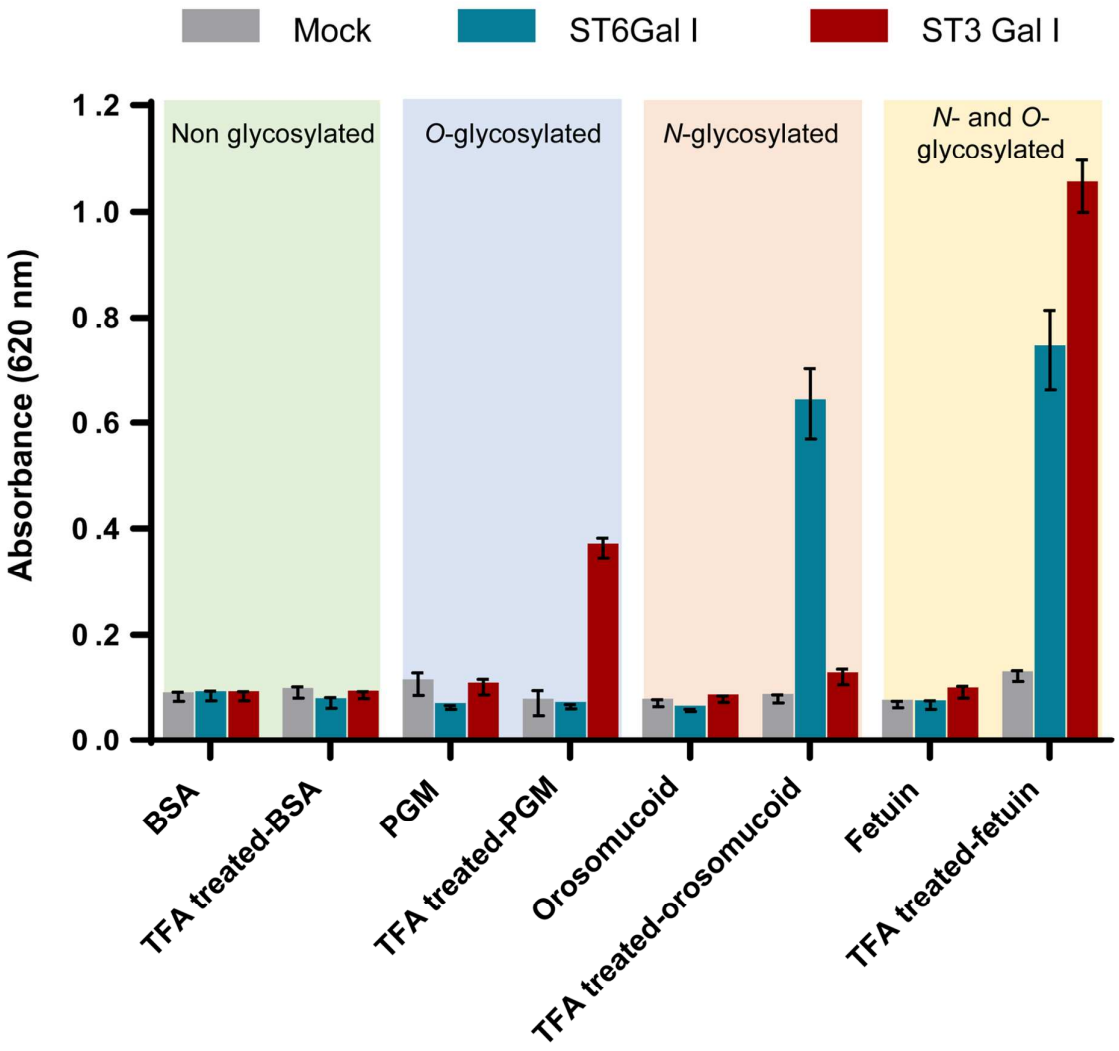


Figure 3. Preference of Δ56ST6Gal I and Δ56ST3Gal I toward different acceptor glycoproteins. Either mock, Δ56ST6Gal I or Δ56ST3Gal I were incubated with 100 μM of CMP-SiaNAl for 1 hour with 400 ng of the indicated coated protein. Error bars represent SEM, (n = 4).

To assess the relevance of MPSA as a screening assay for potential inhibitors of STs activities, we measured the relative activity of Δ56ST6Gal I and Δ56ST3Gal I in presence of 10 mM of various compounds. Activity of STs is usually assessed in Cacodylate buffer containing 10 mM of $MnCl_2$. In these experiments, we used a Cacodylate buffer with no $MnCl_2$ as a control and STs activities were subsequently expressed as percentage of this control condition (Figure 4A). Cytidyl-mono-phosphate (CMP, 10 mM) was used as a positive control of inhibition as it has been shown to be a competitive inhibitor of STs' activity.[39,40] As expected, 10 mM of CMP strongly inhibited the activity of both enzymes. It has long been known that most glycosyltransferases require the presence of a divalent cation to be active. MPSA was therefore conducted in presence of 10 mM of EDTA, a strong divalent cations chelating reagent leading to reduced activities of ST6Gal I and ST3Gal I. A first hypothesis would be that EDTA chelates divalent cations from the buffer and from the culture medium containing the enzyme. Therefore, the resulting lower STs activity suggests that divalent cations play an important role in sialyltransferase activity, in contradiction with works of Shah *et al.*[41] Another hypothesis is that cations' role is to stabilize the CMP-Sia as previously proposed by Kolter and Sandhoff.[42] We then investigated whether commonly used cations could exhibit any increasing or decreasing effect on STs' activity. MPSA was realized with 10 mM of $MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$ and $ZnCl_2$ (Figure 4**A**). Magnesium and calcium appear to have no major effect on ST6Gal I nor ST3Gal I. Although not statistically significant, manganese appeared to inhibit ST6Gal I activity as previously reported for serum sialyltransferase[41,43] and to slightly stimulate ST3Gal I activity. At the opposite, we observed that 10 mM of cobalt or zinc strongly inhibited the two sialyltransferase activities. Although inhibition effects have been reported with polyoxometalate complexes,[44] this is the first time that sialyltransferases' inhibition is reported with these cations. We therefore studied the dose-response inhibition for cobalt and zinc for ST6Gal I and ST3Gal I (Figure 4**B**). We found that ST6Gal I is more sensitive to $ZnCl_2$ and $CoCl_2$ inhibition than ST3Gal I. Indeed, in presence of 100 µM of $ZnCl_2$ or $CoCl_2$, ST6Gal I activity strongly decreases (approximatively 25 %) whereas ST3Gal I activity remains high (80 %). Although these first findings would deserve further investigations, they highlight the potential of MPSA for interesting advances in the study of STs inhibition.

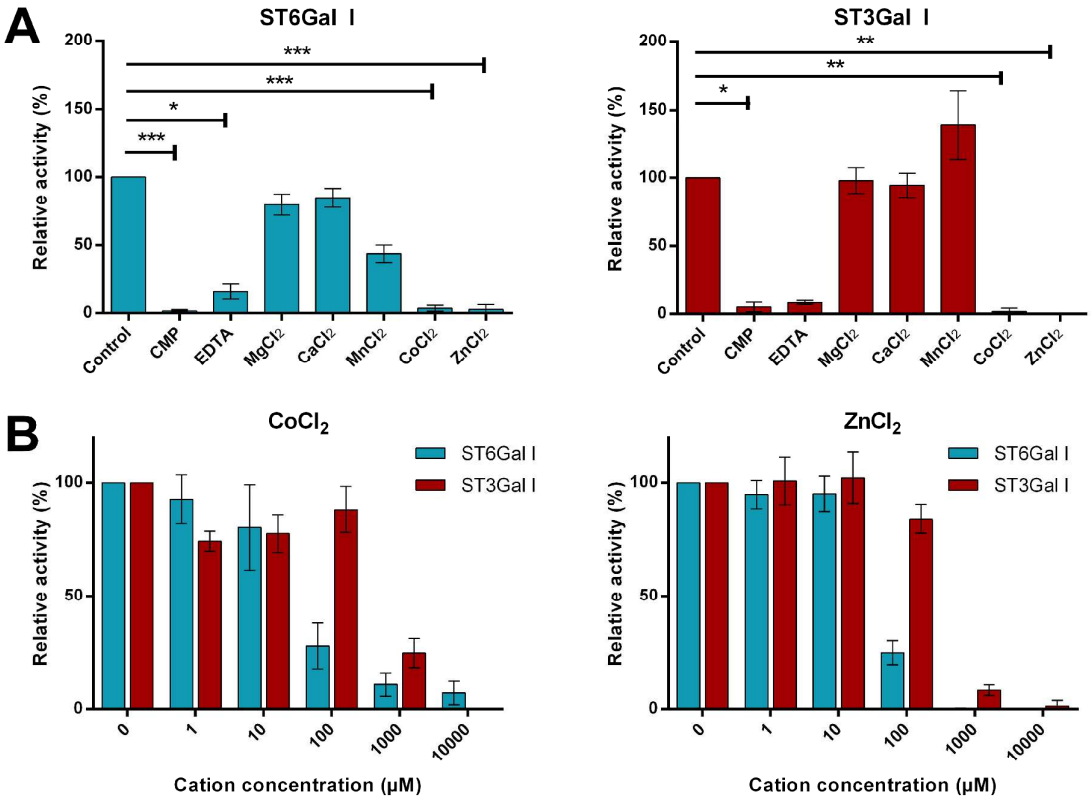


Figure 4. Inhibition effects on Δ56ST6Gal I and Δ56ST3Gal I activities. **(A)** Relative activity of Δ56ST6Gal I (left) and Δ56ST3Gal I (right) in presence of 10 mM of CMP, EDTA, $MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$. Cacodylate buffer (10 mM) was used as control and set at 100 %. Enzymes were incubated for 1 h with CMP-SiaNAl (100 µM) and asialofetuin (400 ng). Error bars represent SEM, (n = 3), p-value = 0.0001. **(B)** Dose-effect relationship of $CoCl_2$ (left) and $ZnCl_2$ (right) on Δ56ST6Gal I and Δ56ST3Gal I activity. Enzymes were incubated for 1 h with CMP-SiaNAl (100 µM) and asialofetuin (400 ng) in presence of either $CoCl_2$ or $ZnCl_2$ (1 µM – 10 mM). Error bars represent SEM, (n = 6).

CONCLUSION

We presented here a new fast, sensitive, versatile and easy to set up MPSA that can be applied to probe ST activities without any dedicated facilities or equipment. With the use of two recombinant ST preparations (the human Δ56ST6Gal I and Δ56ST3Gal I), we showed that MPSA can be applied to different investigations such as kinetic parameters determination, substrate specificity assessment and inhibitors screening. MPSA using CMP-SiaNAl should be applicable to other sialyltransferases and will provide a powerful and simple method for future advances in sialylation research.

EXPERIMENTAL PROCEDURES

Material. Fetuin, orosomucoid, mucin from porcine stomach Type III, TMB and azido-PEG3-biotin were purchased from Sigma. Anti-biotin HRP conjugated was purchased from Jackson Immunoresearch. CMP was from TCI chemicals. BTTAA was synthesized in our laboratory.[28] Sodium cacodylate was from Prolabo, EDTA, magnesium acetate, potassium chloride and calcium chloride were from Acros organics, sodium chloride and magnesium chloride were from euromedex, lithium chloride is from Bio Basic Canada Inc., manganese chloride from Riedel-deHaen and cobalt chloride from Merck and zinc chloride from Fluka Chemika.

Production of recombinant enzymes. Preparation of expression plasmids for human Δ56ST6Gal I and Δ56ST3Gal I was reported recently.[11] For production of sialyltransferases in cell culture medium, seventy percent confluent HEK293 cells were transiently transfected using lipofectAMINE reagent (InVitrogen) and ultraMEM (LONZA), following the instruction of the manufacturer. Thirty-six hours post-transfection, cell culture medium was collected, centrifuged to eliminate cell debris and used as crude enzyme fraction. The cell culture medium containing Δ56ST6Gal I was used undiluted and Δ56ST3Gal I was diluted by 5.

Synthesis of CMP-SiaNAl. Synthesis of CMP-SiaNAl was carried out from D-mannosamine as previously described.[29] D-mannosamine hydrochloride (1 eq.) and pentynoic acid NHS ester (1 eq.) were dissolved into DMF under nitrogen atmosphere. Triethylamine (3 eq.) was added and reaction was stirred overnight. Solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica column (CH_2Cl_2/EtOAc/MeOH; 45 : 45 : 10). After

removing of the solvents, ManNAl was obtained as a white crystalline solid (95 %). ManNAl (1 eq.), sodium pyruvate (2 eq.) and Neu5Ac aldolase (Sigma Aldrich, EC 4.1.3.3) from *E. Coli* K12 (5 U) were dissolved into 1.5 mL of phosphate sodium buffer containing 20 mM of $MgCl_2$. The reaction was stirred overnight at 37 °C. The reaction was diluted with water and loaded onto anion-exchange resin column (Dowex 1X8). Alkynyl acid was eluted with 0.2 M of NH_4HCO_3. Salts were subsequently removed through a gel filtration resin (P2). SiaNAl (0.028 mmol, 1 eq.) and cytidine 5'-triphosphate disodium salt (15 mg, 0.028mmol, 1 eq.) were dissolved into Tris-HCl buffer (100 mM, pH 8.8) containing 20 mM $MgCl_2$. Then, CMP-Sialic acid synthetase from *Neisseria Meningitidis* group B (Sigma Aldrich, EC 2.7.7.43), 1U, and inorganic pyrophospatase from baker's yeast (*S. cerevisiae*), (Sigma Aldrich, EC 3.6.1.1), 1U were added to the mixture. Reaction was incubated for 1 h at 37 °C. The solution was then cooled down to 4 °C and directly diluted at the right concentration for subsequent sialylation assays with no further purification in order to avoid the hydrolysis of the product which is quite unstable in solution. CMP-SiaNAl was characterized by ^{1}H, ^{13}C and ^{31}P NMR.

NMR ^{1}H (400 MHz, D2O): δ = 7.51 (d, *J* = 7.6, 1H, **H_{20}**), 5.72 (d, *J* = 7.6, 1H, **H_{21}**), 5.58 (d, *J* = 3.9, 1H, **H_{19}**), 4.00 – 3.87 (m, 4H, **ribose**), 3.85 (d, *J* = 5.5, 1H, **H_{15}**), 3.76 (d, *J* = 10.4, 1H), 3.74 – 3.66 (m, 1H), 3.61 (d, *J* = 10.3, 1H, **H_5**), 3.56 (dd, *J* = 10.2, 3.0, 1H), 3.51 (d, *J* = 12.2, 1H, **$H_{9\ a}$**), 3.26 (dd, *J* = 14.2, 7.1, 1H), 3.20 (dd, *J* = 10.8, 6.0, 1H, **H_9 b**), 2.22 – 2.03 (m, 5H, **H_{11}, H_{12}, H_3 eq**), 1.99 (s, 1H, **H_{14}**), 1.26 (ddd, *J* = 13.0, 11.7, 5.8, 1H, **H_3 ax**). ^{13}C (101 MHz, D2O): δ = 175.24 (**C_1**), 174.38 (**C_{10}**), 165.92 (**C_{22}**), 157.54 (**C_{23}**), 141.42 (**C_{20}**), 99.90 (**C_2**), 96.53 (**C_{21}**), 88.98 (**C_{19}**), 83.22 (**C_{14}**), 82.31, 73.94, 71.65, 70.31 (**C_{13}**), 69.18, 69.01, 68.78, 66.57, 64.75 (**C_{15}**), 62.90 (**C_9**), 51.63 (**C_5**), 40.98 (**C_3**), 34.65 (**C_{11}**), 14.56 (**C_{12}**). ^{31}P (162 MHz, D2O): δ = -4.63

Preparation of cations solutions. Each salt was dissolved in 100 mM of cacodylate buffer at concentration of 25 mM before sialylation. For the sialylation reaction, cacodylate was at 40 mM and salts at 10 mM once diluted with CMP-SiaNAl and sialyltransferase-containing medium.

Desialylation of glycoproteins. Glycoproteins were desialylated using 0.1 M of trifluoroacetic acid 2 hours at 80 °C, and free sialic acid was eliminated using dialysis membrane MWCO 3500 spectra/por3

(18 mm flat width; Spectrum Laboratories) during 24 hours. Content was then transferred in glass-tube and lyophilized.

Oxidation of BSA. Four grams of BSA were dissolved in 100 mL of sodium acetate 0.1 M pH 4,5 and 10 mM of sodium metaperiodate. After 6 hours incubation at room temperature, periodate was neutralized with 10 mM of glycerol and solution was dialyzed in membrane membra-cel MC30x500CLR against distilled water for 24 h. Then, the solution was lyophilized.

MPSA general procedure. 400 ng of asialofetuin in 100 µL of sodium bicarbonate buffer (20 mM pH 9,6) were coated in 96-well plate (F8 MaxiSorp Loose Nunc-Immuno Module ThermoScientific) overnight at 4 °C. After 3 washes with 150 µL of PBST- 0,05 % (Phosphate Buffer Saline-Tween) saturation was made 1 hour at room temperature using 100 µL of oxidized BSA at 0.05% dissolved in sodium bicarbonate buffer. After 3 washes with 150 µL of PBST-0.05%, sialylation step was realized in 100 µL containing 40 µL of 40 mM cacodylate buffer (pH 6.4), 55 µL of medium containing sialyltransferase and 5 µL of freshly prepared CMP-SiaNAl (to limit any degradation of the donor substrate) at 2 mM. After 1 hour incubation at 37 °C, wells were washed with PBST-0,05 % and 100 µL of mix for CuAAC was added (300 µM of $CuSO_4$, 600 µM of BTTAA, 2.5 mM of sodium ascorbate (Sigma), 250 µM of azido-biotin completed with PBS). After 1 hour incubation at 37 °C, washes were made with PBST-0.05 % and wells were incubated with 100 µL of anti-biotin antibody HRP-conjugated diluted to 1/25000 in PBST-0.05% for 1 hour at 37 °C. After washes, 100 µL of TMB (3,3',5,5'-tetramethylbenzidine) was added and incubated 20 min at room temperature in dark. Finally, absorbance was quantified at 620 nm using spectrophotometer (SpectroStar Nano; BMG Labtech). Data were then analyzed in GraphPad Prism 5 using Kruskal-Wallis test statistic between samples.

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Notes
The authors declare no conflicts of interest.

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ABBREVIATIONS

BSA, Bovine Serum Albumin; CMP, Cytidine mono-phosphate; CMP-Sia, Cytidine-5'-monophosphate-*N*-acetylneuraminic acid; CuAAC, Copper Catalyzed Alkyne Azide Cycloaddition: EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; FRET, Forster resonance energy transfer; Gal, Galactose; GalNAc, *N*-acetyl galactosamine; HEK, Human embryonic kidney; HPLC, High performance liquid chromatography; HRP, Horse radish peroxidase; LC-MS, Liquid chromatography combined to mass-spectroscopy; MPSA, Microplate sialylation assay; MRM: Multiple reaction monitoring; PGM, Porcine gastric mucin; Sia, sialic acid; SPAAC, Strain promoted azide alkyne cycloaddition; ST, sialyltransferase; TFA, trifluoroacetic acid; TMB, 3,3′,5,5′-Tetramethylbenzidine.

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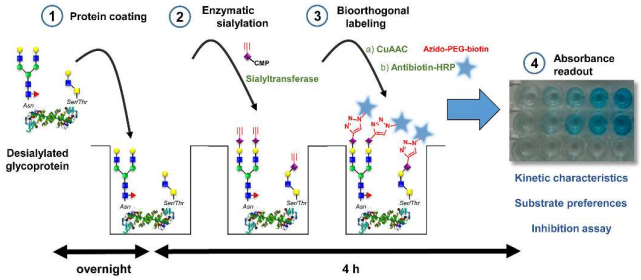
TOC graphic (table of content graphic)



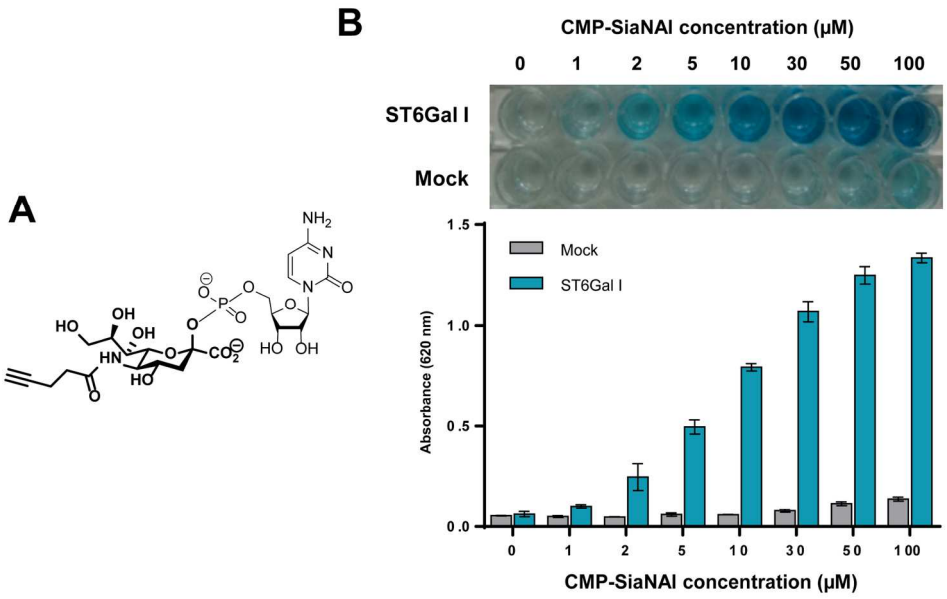


Figure 1: (A) Chemical structure of the alkynyl analog of CMP-Neu5Ac: CMP-SiaNAl. (B) MPSA experiment after incubation with either ST6Gal I or mock medium and various concentrations of CMP-SiaNAl (0-100 μM). All the wells have been treated with azido-PEG-biotin with CuAAC conditions and finally revealed with an anti-biotin antibody HRP. Absorbance measured at 620 nm for all CMP-SiaNAl concentrations. Error bars represent SEM (n = 2).

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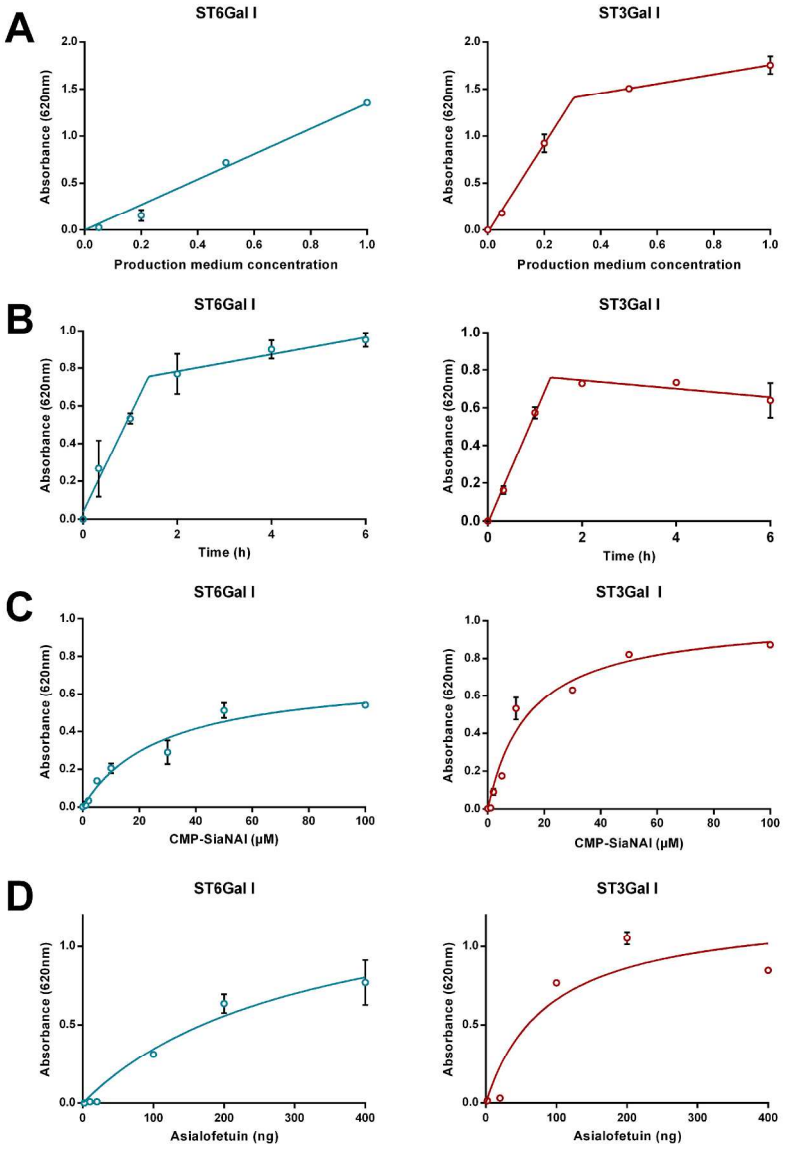

Figure 2. Determination of kinetic parameters with MPSA. Otherwise specified, 100 μM of CMP-SiaNAl and 400 ng of asialofetuin were incubated for 1 h with either Δ56ST6Gal I and Δ56ST3Gal I. (A) Activity in function of the dilution factor of the enzymatic source (B) Reaction time. MPSA absorbance readout for both ST6Gal I and ST3Gal I in function of time. (C) Km determination of both Δ56ST6Gal I and Δ56ST3Gal I toward CMP-SiaNAl (0 - 100 μM) (D) and coated asialofetuin (0 – 400 ng). Error bars represent SEM, (n = 2).

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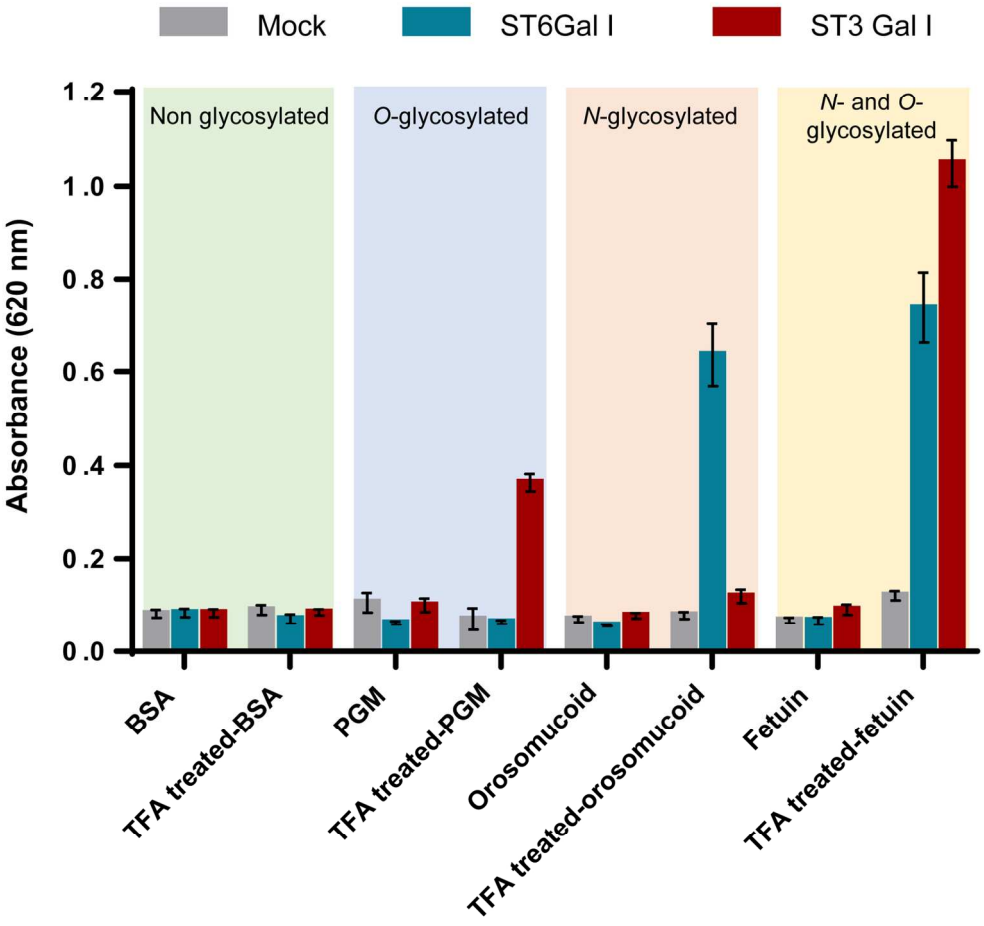

Figure 3. Preference of Δ56ST6Gal I and Δ56ST3Gal I toward different acceptor glycoproteins. Either mock, Δ56ST6Gal I or Δ56ST3Gal I were incubated with 100 μM of CMP-SiaNAl for 1 hour with 400 ng of the indicated coated protein. Error bars represent SEM, (n = 4).

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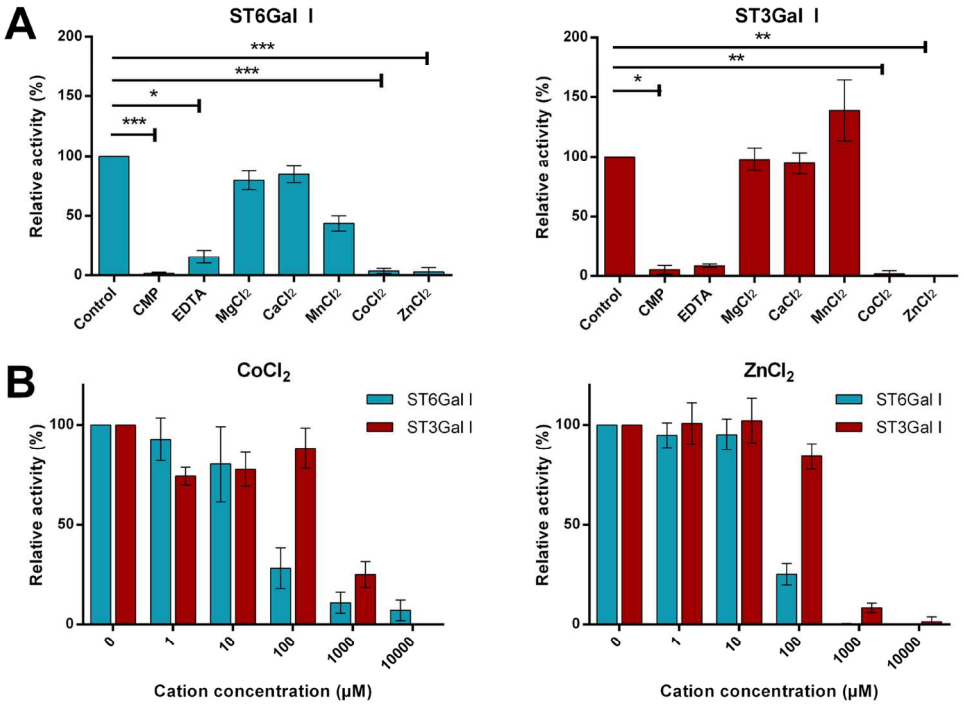

Figure 4. Inhibition effects on Δ56ST6Gal I and Δ56ST3Gal I activities. (A) Relative activity of Δ56ST6Gal I (left) and Δ56ST3Gal I (right) in presence of 10 mM of CMP, EDTA, MgCl2, CaCl2, MnCl2, CoCl2, ZnCl2. Cacodylate buffer (10 mM) was used as control and set at 100 %. Enzymes were incubated for 1 h with CMP-SiaNAl (100 µM) and asialofetuin (400 ng). Error bars represent SEM, (n = 3), p-value = 0.0001. (B) Dose-effect relationship of CoCl2 (left) and ZnCl2 (right) on Δ56ST6Gal I and Δ56ST3Gal I activity. Enzymes were incubated for 1 h with CMP-SiaNAl (100 µM) and asialofetuin (400 ng) in presence of either CoCl2 or ZnCl2 (1 µM – 10 mM). Error bars represent SEM, (n = 6).

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