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Combining carbochips and mass spectrometry to study the donor specificity for the *Neisseria meningitidis* β1,3-*N*-acetylglucosaminyltransferase LgtA

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ABSTRACT

A library of 11 UDP-*N*-acetylglucosamine analogs were rapidly screened for their activities as donors for the *Neisseria meningitidis* β1,3-*N*-acetylglucosaminyltransferase (LgtA) by direct on-chip reaction and detection with SAMDI-TOF mass spectrometry. Six of the analogs were active in this assay and were analyzed by SAMDI to characterize the kinetics toward LgtA. The analysis revealed that substitutions on C-2, C-4, and C-6 affect the activity of the donors, with bulky groups at these positions decreasing affinity of the donors for the enzyme, and also revealed that activity is strongly affected by the stereochemistry at C-3, but not C-4, of the donor. The study is also significant because it demonstrates that SAMDI can be used to both profile glycosyltransferase activities and to provide a quantitative assessment of enzyme activity.

Neisseria meningitidis is a heterotrophic gram-negative diplococcal bacterium best known for its role in meningitis and other forms of meningococcal disease such as meningococcemia. Lipopolysaccharide (LPS) is a component of the cell wall of *N. meningitidis* that acts as an endotoxin. Its structure mimics the mammalian poly-*N*-acetyllactosaminoglycans and is widely used as a tool to study tumor initiation in humans.[1] The UDP-GlcNAc:Gal β1,3-*N*-acetylglucosaminyltransferase encoded by the *lgtA* gene is responsible for the biosynthesis of LPS by forming the GlcNAcβ1-3Gal linkage.[2] Recombinant LgtA is known to display broad acceptor specificity towards glycoconjugates bearing galactosides at the non-reducing end and also accepts UDP-GalNAc[3]—yet the donor specificity of LgtA remains unclear. In this paper we describe a study that uses biochips and mass spectrometry to characterize the activities of eleven sugar nucleotide analogs in an LgtA-catalyzed glycosylation reaction. We identify several non-natural sugar donors using the on-chip assay and we also combine solution-phase reactions with mass spectrometry to quantitatively characterize the activities of these donors.

The most common routes that are now used to characterize glycosyltransferase (GT) activity rely on radiometric, chromatographic and fluorometric assays, and have the limitations that they require labels and are tedious. For example, in the common radiometric assays, the donor must be labeled with ^{32}P, ^{14}C or ^{3}H, which entails a significant effort. The labeled reagents pose a hazard and also require that the products be separated from the donor prior to analysis. Here, we use a label-free solid phase assay that is based on self-assembled monolayers (SAMs) presenting immobilized carbohydrates that serve as the acceptors for GT-catalyzed glycosylation reactions.[4] We used SAMs that present a lactoside as the LgtA acceptor and at a density of 10% against a background of tri(ethylene glycol) groups (Fig. 1**a**). The latter prevents non-specific interactions of the enzyme with the surface and maintains the activity of immobilized carbohydrates.[5] The assay is performed by applying a drop of solution containing LgtA and a sugar donor to the monolayer, allowing the mixture to react for a short time, and then analyzing the monolayer using matrix assisted laser desorption-ionization mass spectrometry (in a technique termed SAMDI MS) to reveal the masses of the substituted alkanethiols.[6] Hence, this method can directly observe both the substrate and the product of the LgtA-catalyzed reaction, erasing the need for labels and also providing a measure of the yield for the glycosylation reaction. The assay is also notable because it requires only two microliters of sample and therefore minimizes the use of expensive reagents.

We prepared eleven UDP-GlcNAc analogs[7] and used the SAMDI assay to first identify those donors that were accepted by LgtA

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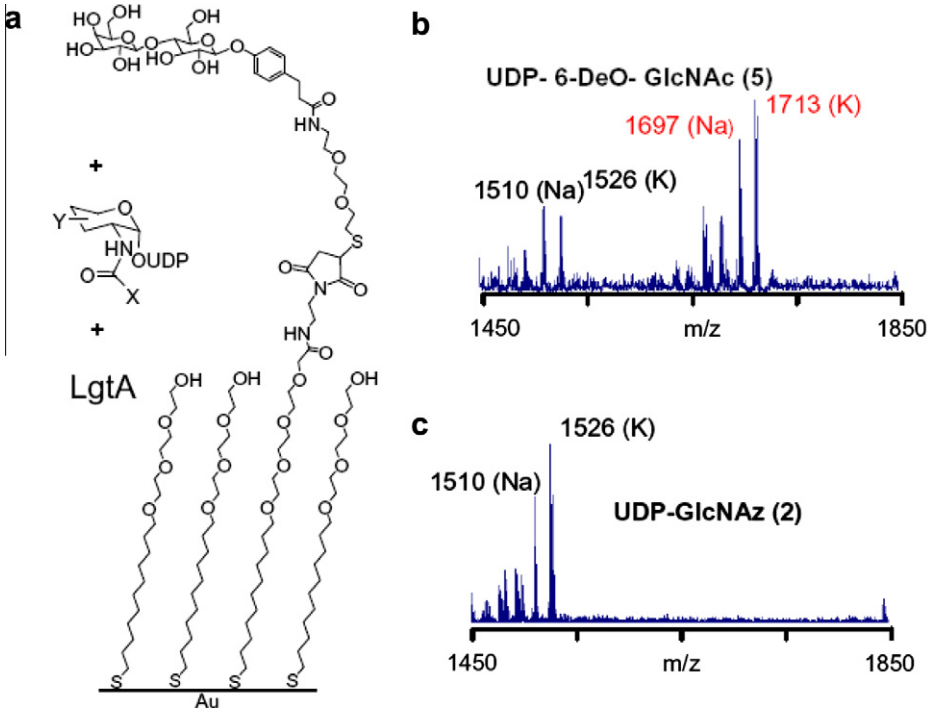

Figure 1. This work used monolayers presenting the lactoside to assay LgtA-catalyzed glycosylation activities. (a) The lactose-presenting SAMs were treated with LgtA and one of the eleven donor analogs. The reaction products were characterized by SAMDI; (b) A mass spectrum is shown for a glycosylation reaction using the donor UDP-6-DeO-GlcNAc (**5**). Black and red numbers correspond to the mass to charge ratio (*m*/*z*) of the disulfide peaks of the lactose substrate and the product, respectively. The spectrum shows a difference of *m*/*z* 187 between the two groups of peaks, indicating the addition of one 6-DeO-GlcNAc residue to the acceptor substrate; (c) A mass spectrum for the glycosylation reaction using the inactive donor UDP-GlcNAz (**2**) shows product peaks at *m*/*z* 1755 and 1771, indicating that this analog has no reactivity. Letters in parenthesis correspond to different ion adducts.

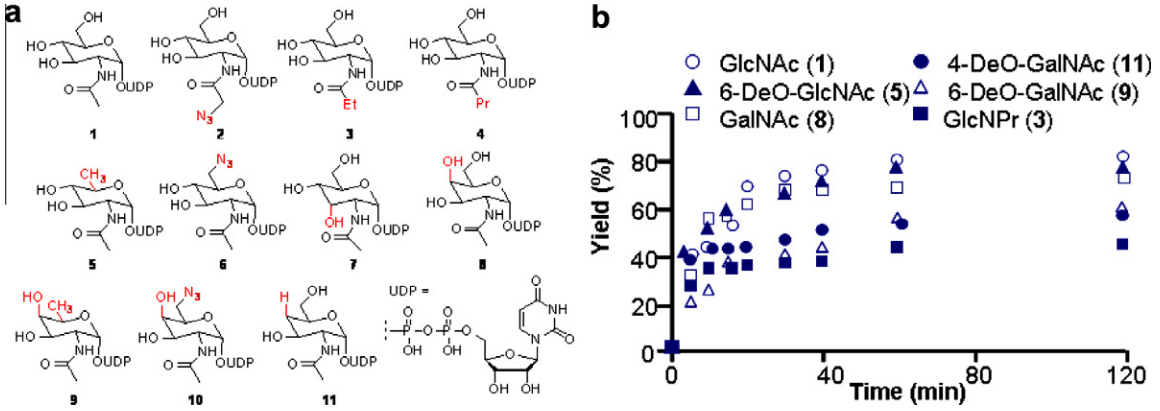

Figure 2. (a) This work assayed the activities of 11 UDP-sugar analogs as donors in the LgtA-catalyzed glycosylation reaction; (b) A time course for the six active donors was determined by SAMDI. Relative amount of glycosylation products at eight times (5, 10, 15, 20, 30, 40, 60 and 120 min) are shown for the six donors. Some overlapping points were offset horizontally for clarity.

(Fig. 2**a**). We prepared a reaction plate having a 10 × 5 array of gold-coated circular islands, each 2 mm in diameter and modified with a monolayer that was terminated with lactose groups and tri(ethylene glycol) groups with the former present at a density of 10% (Supplementary data). We prepared cocktails containing LgtA and one of the analogs (2 mM) in the assay buffer and applied each sample to individual islands in the array, and then incubated the reactions in a humidified chamber at 30 °C. After a defined period of time, we rinsed the array with distilled water, ethanol and then applied matrix and analyzed the monolayers using SAMDI (Supplementary data). Figure 1b and c show representative spectra for assays performed with an active and an inactive donor. In the reaction that used the donor UDP-6-DeO-GlcNAc (**5**), the lactose substrate was converted to the trisaccharide product (with peaks at 1697 and 1713 corresponding to the sodium and potassium adducts, respectively) in high yield. For the reaction that used the donor UDP-GlcNAz (**2**), in contrast, there is an absence of the product peaks at 1754 and 1770 demonstrating that the donor is not active. Of the eleven donors we assayed, five were inactive and six displayed varying degrees of activity.

To characterize the kinetics of the reaction for the active donors, we performed assays in solution to avoid perturbations that may arise from presentation of the ligands at the surface.[5,8] In this 'pull-down' format, the reactions were performed in solution with an azido-modified lactose as acceptor and one of the active analogs as donor. The reactions were stopped by adding cold ethanol and

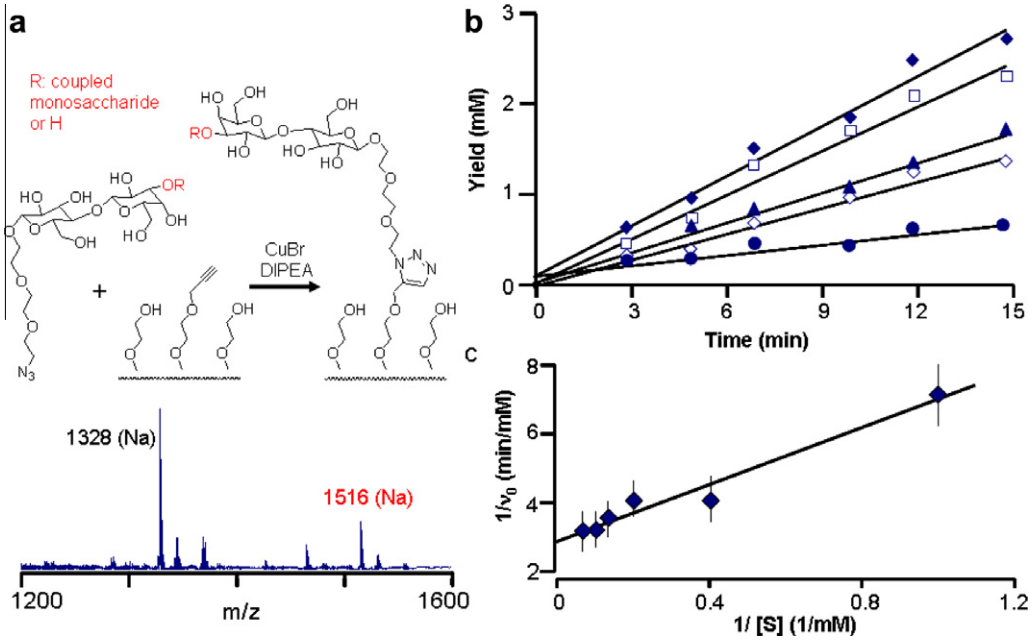

Figure 3. Glycosylation reactions were performed in solution using a lactose azide substrate in order to determine kinetic parameters for the six active donors. (a) Reactions were terminated at different times and then applied to the monolayers to allow immobilization and SAMDI characterization of both the lactose substrate and product. The lower panel shows the spectrum from a 15 min reaction of UDP-6-DeO-GlcNAc (5) at the concentration of 7.5 mM. The peak at *m*/*z* 1516 indicates the partial conversion of substrates to the product, and the peak intensities are used to calculate the yield of the reaction; (b) The glycosylation yield was plotted versus reaction time for each donor concentration, and the data were fit with least-square linear regression. The initial velocities were determined from the slopes of the fitted lines for five concentrations of UDP-6-DeO-GlcNAc: 10 mM (filled diamond), 7.5 mM (empty square), 5 mM (filled triangle), 2.5 mM (empty diamond), 1 mM (filled circle); (c) Kinetic parameters for UDP-6-DeO-GlcNAc were determined from the reciprocal plot of the five donor concentrations versus the initial velocities. The linear regression produced a straight line with an equation of $y = 3.8556x + 2.9155$. The apparent K_m and V_{max} can be calculated from the slope and intercept. Each error bar represents the standard error of 10 replicas.

then spotted onto a monolayer that presented a terminal alkyne group among tri(ethylene glycol) groups (Fig. 3**a**). In this way, a copper-catalyzed click reaction between the azido sugars and the monolayer serves to immobilize both the trisaccharide product and disaccharide substrate, so that SAMDI analysis can then determine the extent of glycosylation.

To characterize K_m and V_{max} values for LgtA-catalyzed transfer of 6-Deo-GlcNAc from UDP-6-DeO-GlcNAc (**5**) to the lactose substrate, we prepared reaction mixtures in 96 well plates, where each well had LgtA, the lactose azide (10 mM) and UDP-6-DeO-GlcNAc at concentrations ranging from 1 to 10 mM (in a total volume of 5 μL). We also investigated the time course of the reactions by aliquoting identical sets of the solutions in multiple wells, and stopping each set of reactions at different times. In this way, we evaluated reactions for five concentrations of donor and 12 reaction times to give 60 data points for each donor. At the end of the reaction, we combined an aliquot of the reaction mixture, copper bromide and diisopropylethylamine (DIPEA) and applied the samples to individual islands on the SAMDI plate. We confirmed that the immobilization reaction was complete in 20 min and that both the product and lactose substrate reacted with the monolayers with similar kinetics. The monolayer was then analyzed by SAMDI, and the yield of glycosylation was measured from the ratio of peak intensities of the product to the sum of the intensities for the product and the lactose substrate (Fig. 3**a**). The yields for each donor concentration were plotted against the reaction time. Initial velocities were determined by obtaining the slopes from least-square linear regression (Fig. 3**b**). A Lineweaver–Burk plot was then constructed by plotting the inverse of the initial velocities against the inverse of the five concentrations, and apparent K_m and V_{max} were calculated by taking the slope and intercept from the line that was fit by linear regression (Fig. 3**c**). We repeated this process to determine the kinetic constants for the other five active donors and summarized the results in Table 1.

Table 1
Kinetic parameters determined for the active sugar donors in the LgtA-catalyzed glycosylation of lactose

Sugar donor	K_m[a] (mM)	V_{max} (mM min^{-1})	V_{max}/K_m (min^{-1})
UDP-GlcNAc (**1**)	0.40	0.456	1.15
UDP-GlcNPr (**3**)	21.1	0.110	0.005
UDP-6-DeO-GlcNAc (**5**)	1.32	0.343	0.259
UDP-GalNAc (**8**)	2.09	0.135	0.065
UDP-6-DeO-GalNAc (**9**)	3.05	0.115	0.038
UDP-4-DeO-GalNAc (**11**)	3.28	0.097	0.029

[a] The apparent K_m values were measured at 30 °C with 10 mM lactose azide as acceptor.

The set of sugar donors we used in this work contained modifications at *N*-acyl, C-3, C-4, and/or C-6 positions of UDP-GlcNAc and therefore permitted an analysis of the structural requirements for activity in the LgtA-catalyzed glycosylation reaction. We draw several conclusions from this panel. First, the donor must have an equatorial C-3 hydroxyl group, as the analog (**7**) having an axial substituent at this position displayed no activity. LgtA is less sensitive to changes in stereochemistry at C-4 as shown by the activity of UDP-GalNAc (**8**). We also found that the activity of the donor decreased for larger *N*-acyl groups, as evidenced by the decreasing reactivity from a methyl group (**1**) to the ethyl group (**3**) and the fact that even larger azidomethyl (**2**) and propyl groups (**4**) led to a complete loss of activity. We also found that removal of the hydroxyl groups at C-6 or C-4 did not have a significant effect on the activity of these donors (Entry 3, 5, and 6 in Table 1), suggesting that these hydroxyl groups do not participate in critical hydrogen bonding interactions with the enzyme.

This work is significant for two reasons. First, we provide a characterization of the donor substrate specificity of LgtA using a set of structurally modified sugar nucleotides. Because GTs are

important in the enzymatic synthesis of complex carbohydrates, this knowledge provides a guide to the donors that can be used in LgtA-catalyzed construction of oligosaccharides. Second, we take advantage of the combination of self-assembled monolayers and MALDI MS to perform high throughput assays of GT function. This SAMDI assay offers the benefits that it can perform true label-free assays; it requires only small quantities of enzyme and donor reagents and it is compatible with solid phase arrays, and the high throughput that they offer. We believe this combination will make this method important for identifying and characterizing a broad range of enzyme activities directed toward carbohydrate substrates.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.100.

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