Glycoconjugates

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Differentiation between Structurally Homologous Shiga 1 and Shiga 2 Toxins by Using Synthetic Glycoconjugates**

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Shiga toxins 1 and 2 (Stx1 and Stx2) are major virulence factors of *Escherichia coli* O157:H7 and have been included on the list of Select Biothreat Agents.[1] Of the estimated 70000 *E. coli* O157:H7 cases of disease per year in the United States, 10–15% develop hemolytic uremic syndrome (HUS); 3–5% succumb during the acute phase of the disease; and an equivalent number suffer brain damage and renal failure.[2] Treatment of Shiga-toxin-mediated HUS remains primarily supportive, as postdiarrheal antibiotic treatment is reported to enhance toxin production and progression of HUS.[3] Synthetic toxin neutralizers have proven to be ineffective in affected patients, but could be useful as prophylactics if diagnosis can be made early.[4–6] Furthermore, the presence of Stx genes on the lysogenic bacteriophage[7] allows facile transmission of Stx onto harmless serotypes of *E. coli* and other enteric bacterial species, making it a serious emerging threat to humans.[8]

Shiga toxin is a member of the AB_5 family of toxins. The A subunit is enzymatically active and cleaves a single adenine residue from the 28S ribosomal RNA, rendering the ribosome incapable of protein synthesis. The B, or binding, subunit is a homopentamer that binds to globotriaosylceramide (Gb3) in lipid rafts on the cell surface and ultimately delivers the A subunit to its cytoplasmic target. The two major antigenic variants of Shiga toxin, Stx1 and Stx2, share 55% amino acid homology. Differences in affinity for Gb3 were observed by using surface plasmon resonance spectroscopy; the affinities K_d of Stx1 and Stx2 for Gb3 are 4.6×10^{-8} M and 3.7×10^{-7} M, respectively.[9] However, Stx1 and Stx2 showed similar affinities for Gb3 derivatives using electrospray ionization mass spectrometry.[10] Spacer length, mode of presentation, and assay conditions appear to influence binding profoundly.[11–13] Strains of *E. coli* O157:H7 can produce Stx1, Stx2, or both, but epidemiological studies suggest that Stx2- (and not Stx1-) producing *E. coli* are associated with the development of HUS.[14] These observations have been confirmed by baboon[15] and murine[16] models. Indeed, the lethal dose for Stx1 and Stx2 in mice has been reported to be approximately 1200 and 2.4 ng, respectively.[16] Therefore, in addition to the early and rapid detection of Shiga-toxin-producing *E. coli*, identification of Shiga toxin variants produced by the infecting strain to determine its pathogenic potential is critical.[17]

In previous studies, the O-polysaccharides on several lipopolysaccharide (LPS) serotypes expressed by harmless *E. coli* strains have been shown to bind Stx2 specifically and neutralize its toxicity to mammalian Vero cells in culture.[18] The structure of one neutralizing polysaccharide, corresponding to serogroup O117, had been previously determined.[19] Interestingly, it resembles Gb3; however, there are significant structural differences. Gb3 has a terminal α-1,4-digalactose moiety (Figure 1, top), whereas the neutralizing polysaccharide has a modified terminal digalactose moiety (Figure 1, bottom); the galactose residues possess a bulky *N*-acetyl group at the 2-position. In contrast to Gb3, which binds to both Stx1 and Stx2, the neutralizing polysaccharide was not able to bind Stx1 or to neutralize its effects on Vero cells,

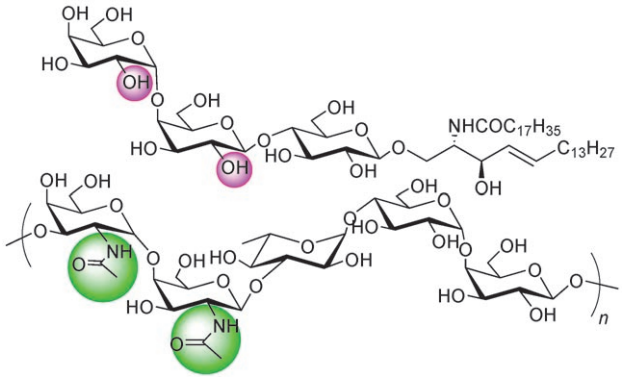

Figure 1. Structure of Gb3 (top) and the O117 LPS (bottom). The hydroxy and *N*-acetyl groups have been shaded for emphasis.

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suggesting the *N*-acetyl group modifies binding specificity towards Stx1.

On the basis of these observations, we hypothesized that analogues of Gb3 could distinguish between Stx variants. We developed a modular synthetic approach that allows variable spacers and recognition elements to be incorporated without considerable alteration of the overall synthetic strategy. An illustration of the tailored glycoconjugate is depicted in Figure 2 along with the structures of the synthetic glycoconjugates. The three components of the generic glycoconjugate are 1) recognition element, 2) flexible spacer terminated in an azide, and 3) dimeric scaffold bearing two alkynes. Using this design, we synthesized biantennary ligands with varying *N*-acetylation patterns. Each of the subunits in the B pentamer of Stx possesses up to three Gb3 binding sites, and, therefore, multiple binding sites are available. For initial studies we chose a dimeric biotinylated scaffold to increase binding affinity. As the length of the spacer has been shown to affect the binding affinity,[11–13] we examined the influence of linker length by synthesizing compounds with 6 (**GC-1, GC-2a** and **GC-3a**) and 12 carbon atom (**GC-2b** and **GC-3b**) spacers. We used biotin because it affords easy access to multivalency as one streptavidin tetramer binds to four biotins and it can be conjugated to commercial streptavidin matrices for toxin capture.[20] The molecule was designed such that the biotin and, hence, streptavidin is attached to the opposite end of the rigid scaffold to minimize biotin interference in the binding event.

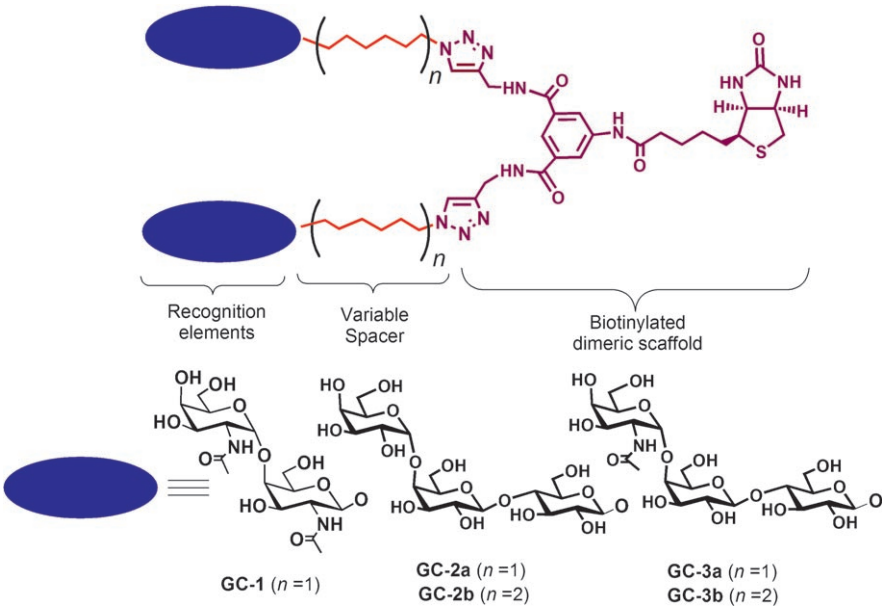
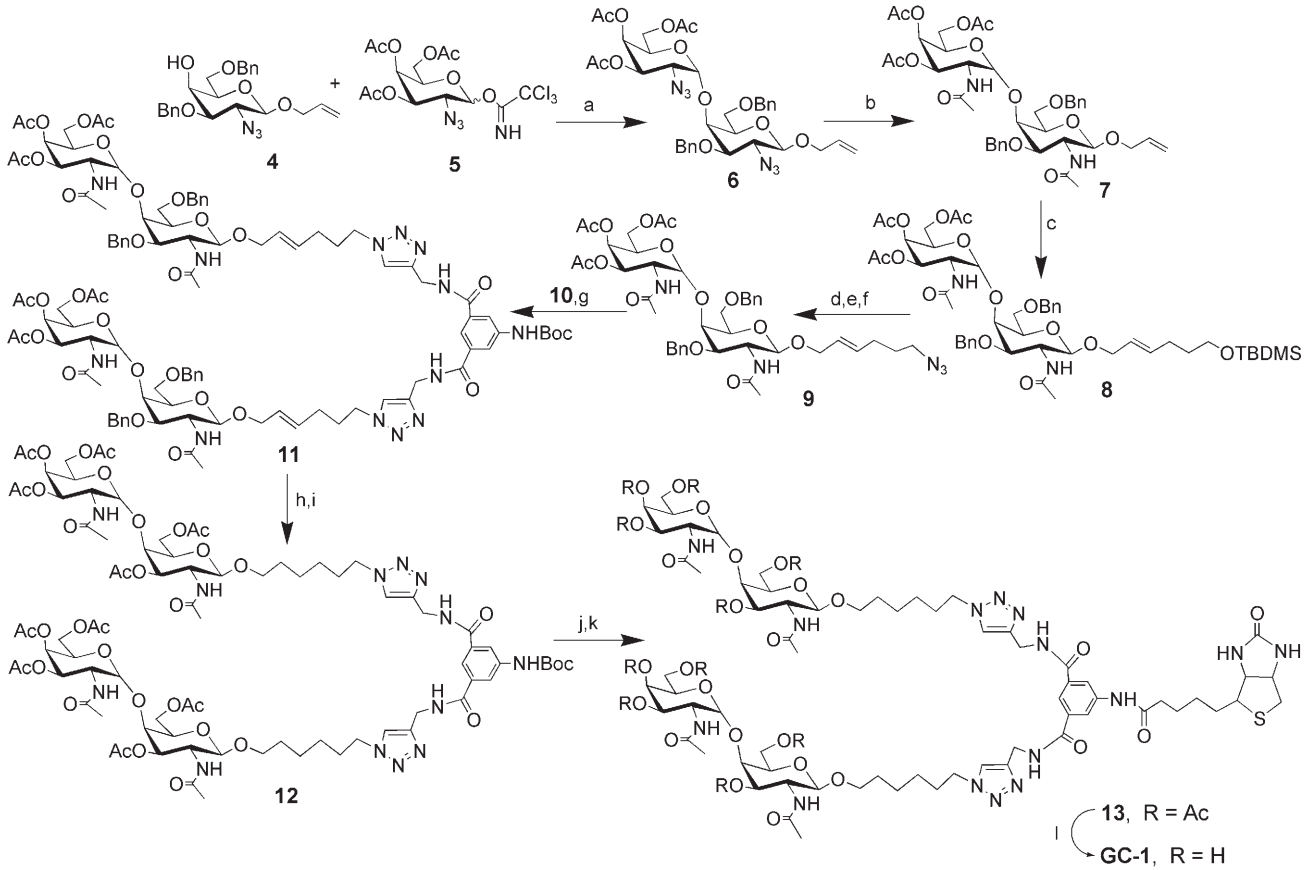


Figure 2. Representation of the tailored biotinylated glycoconjuate and the structures of the five molecules. The blue ellipse is the carbohydrate recognition element, the biotinylated scaffold and the spacer are colored purple and red, respectively.

We choose 2-deoxy-2-azidogalactosamine derivatives as suitable starting materials as these derivatives yield α isomers with high stereoselectivity. The synthesis of a representative glycoconjugate, GalNAc(α-1,4)GalNAc, coupled to biotin is shown in Scheme 1. Briefly, coupling of acceptor **4**, obtained by a two-step procedure from the known allyl-2-azido-4,6-benzylidene-2-deoxy-β-D-galactopyranoside,[21] with trichloroacetimidate donor **5**[22] in the presence of catalytic amount of TMSOTf yielded disaccharide **6** in reasonable yields (see the Supporting Information). The azide functionalities were reduced to the *N*-acetyl groups by using thiolacetic acid to give **7**. Extension of the allyl group by using a cross-metathesis procedure was achieved with Grubbs catalyst to yield the *E*,*Z*-isomers **8a,b** as an inseparable mixture. Cleavage of the silyl group and subsequent mesylation and conversion to an azide yielded **9a,b** in 68% yield over the three-step sequence. The azide can easily be coupled by 1,3-dipolar addition to dimeric scaffold **10**, bearing two alkynes and a protected amine previously synthesized in our group.[23] Thus, reaction of 2.1 equivalents of the azide-bearing glycoconjugate with **10** yielded **11**. Hydrogenation in the presence of Pearlman's catalyst, and reacylation yielded **12** in quantitative yield. The final steps involved cleavage of the Boc protecting group and coupling to biotin to yield **13**, which was subjected to Zemplén conditions to give **GC-1**. The final product **GC-1** was purified by using a Biogel P-2 column and lyophilized to yield a white foam. Similarly, **GC-2a/b** and **GC-3a/b**, in which the terminal galactose of Gb3 is replaced by an *N*-acetyl galactosamine derivative, were synthesized (see the Supporting Information).

Binding of Stx1, Stx2, and Stx2c to the synthetic glycoconjugates was assessed by ELISA analysis as described previously.[18] Toxin-containing culture supernatants, sterilized by filtration, were prepared from C600:H19B (Stx1), C600:933W (Stx2), and O157 strain C394-03 (Stx2c).[24] The biotinylated compounds were diluted in phosphate-buffered saline (PBS) and added in excess to commercially available precoated streptavidin microwell plates with a binding capacity of around 125 pM per microwell. Binding was allowed to proceed at room temperature for 2 hours. The wells were washed three times with PBS and incubated with PBS (negative control) or Stx-containing culture supernatant at room temperature for 2 hours. The color was developed by using commercially available polyclonal antibody to Shiga toxin (Meridian Bioscience, Inc., Cincinnati, OH), alkaline phosphatase conjugated goat antirabbit IgG, and *p*-nitrophenyl phosphate (Meridian Bioscience, Inc. or Sigma, St. Louis, MO). The absorbance ($\lambda = 405$ nm) was read by an ELX800 microplate reader (Bio-Tek Instruments, Inc).

The results are shown in Figure 3. Similar to observations for neutralizing LPS, Stx2 bound to the di- and mono-*N*-acetyl substituted galactosamine **GC-1** and **GC-3a**, respectively (Figure 3A), whereas Stx1 failed to bind to either compound



Scheme 1. Synthesis of the biotinylated dimer of disaccharides. Reagents and conditions: a) TMSOTf, DCM, −20 °C→RT, 63 %; b) AcSH, RT, 12 h, 57 %; c) (1,1-dimethylethyl)dimethyl(4-pentenyloxy)silane, $[((Cy)_3P)_2RuCl_2(CHPh)]$, DCM, 16 h, 87 %; d) TBAF, THF, RT, 3 h, 87 %; e) MsCl, $(iPr)_2EtN$, DCM, −10 °C→RT, 5 h; f) NaN_3, DMF, 65 °C, 5 h; 67 % over two steps; g) $CuSO_4$, sodium ascorbate, THF/H_2O, RT, 2 days, 86 %; h) H_2, $Pd(OH)_2$, MeOH, RT, 1 atm, 12 h; i) Ac_2O, pyridine, DMAP, 88 % over two steps; j) TFA, TIPS, DCM, RT, 8 h; k) (+)-biotin, CDMT, NMM, THF/DMF, 0 °C→RT, 32 h, 61 % over two steps; l) NaOMe, MeOH, RT, 16 h, 86 %. TMS = trimethylsilyl; Ac = acetyl; Bn = benzyl; Tf = trifluoromethanesulfonyl; DCM = dichloromethane; Cy = cyclohexyl; TBDMS = *tert*-butyldimethylsilyl; TBAF = tetrabutylammonium fluoride; Ms = methanesulfonyl; DMF = *N*,*N*-dimethylformamide; Boc = *tert*-butoxycarbonyl; DMAP = 4-dimethylaminopyridine; TFA = trifluoroacetic acid; TIPS = triisopropylsilyl; CDMT = 2-chloro-4,6-dimethoxy-1,3,5-triazine; NMM = *N*-methylmorpholine.

(Figure 3B). The trisaccharide **GC-3a** appears to be a better substrate for Stx2 than the disaccharide **GC-1**, as the former exhibited enhanced toxin binding and was more sensitive (toxin binding less than 100 ng per well). In contrast, **GC-2a**, a trisaccharide analogue of Gb3, bound exclusively to Stx1 and not to Stx2. The limit of detection was 10 ng of toxin per microwell. We attribute the exclusive selectivity of **GC-2a** towards Stx1 to the architecture of the biantennary analogue; possibly, the shorter spacer permits binding to Stx1, but constrains binding to Stx2.[10] Interestingly, increase in the spacer length leads to loss of sensitivity; **GC-2b**, the Gb3 analogue with a 12-carbon-atom spacer bound to Stx1 with lower affinity than **GC-2a**. The binding studies involving the *N*-acetylated analogue **GC-3b** were very intriguing. Increase in spacer length from 6 (**GC-3a**) to 12 carbon atoms (**GC-3b**) led to loss of selectivity and sensitivity; **GC-3b** bound to both Stx2 and Stx1 with equal affinity. Clearly, the role of the spacer in the binding event remains to be determined.

Next, we tested the ability of **GC-2a** to capture Stx1 in clinical applications. Stx1 was spiked into a stool, diluted as recommended for the commercially available diagnostic Premier EHEC ELISA (Meridian Bioscience, Inc.), and the assay was carried out as described before. As seen in Figure 3C, Stx1 is efficiently captured without any interference from a complex sample, and similar results were seen for Stx2 (data not shown). We are particularly excited by these results because the ligands could readily be applied to any streptavidin-based biosensor platform to detect toxin serotypes during the narrow window of opportunity of around 3 days between the onset of watery diarrhea and HUS.[1] We also tested the ability of these glycoconjugates to bind to Stx2c as this variant has been found in human clinical samples.[24,25] Stx2c binds to **GC-1** with higher affinity than Stx2 (Figure 3D). Unlike Stx1 and Stx2, which are 55 % homologous, the emerging Stx2c variant differs from Stx2 by only a few amino acids,[24,26] and the ability of these glycoconjugate derivatives to recognize small changes is remarkable.

In summary, our results indicate that it is feasible to develop highly selective and sensitive synthetic glycoconjugate-based Shiga toxin detection reagents by introducing simple manipulations in the structure of known saccharide

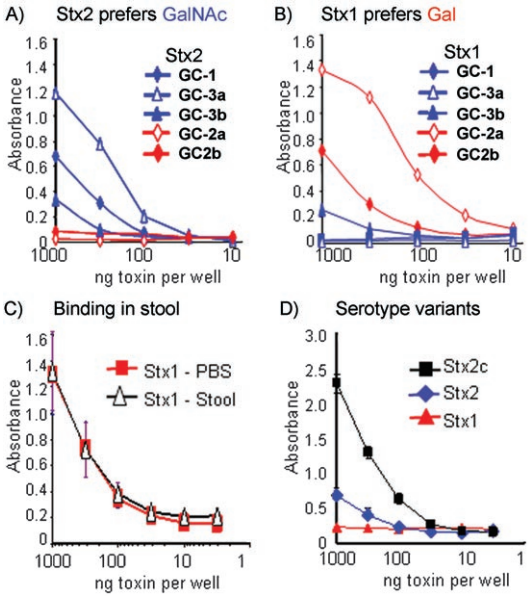


Figure 3. A,B) Differential binding of Shiga toxin variants to synthetic glycoconjugates. C) Ability of **GC-2a** to capture Stx1 in human stool. D) Differential binding to **GC-1**. Biotinylated glycoconjugates were added to streptavidin-coated microtiter wells and incubated with decreasing concentrations of Stx. Binding was determined by using anti-Stx polyclonal antibody and alkaline phosphatase conjugated goat antirabbit secondary antibody. Results are the average of three independent trials.

receptors. These glycoconjugates are highly robust, inexpensive, require no refrigeration, can be scaled up, applied to any sensor platform, and bind to toxins present in real-world samples. Finally, the strategy described herein relies on receptor-mediated recognition events, as opposed to antigen-based recognition. Receptor-binding strategies are particularly useful to detect antigenic variants since amino acid changes may change antibody binding without changing function; however, receptor binding cannot be varied without loss of function. Developing a focused glycoconjugate library and correlating binding to toxicity is the subject of our current endeavors and will be reported soon.

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