Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Immunological Evaluation of the Entirely Carbohydrate-based Thomsen-Friedenreich - PS B Conjugate

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Electronic Supplementary Information (ESI)

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Experimental:

Culturing *B. fragilis.* The growth of *B. fragilis* has been reported previously.[1]

Purification of PS B (2). 20 L of *B. fragilis* was harvested after 48 h of growth and centrifuged at 4,000 X g for 20 min at 4 °C in 500 mL centrifuge bottles. Cell supernatant was removed and the pellet was re-suspended in 500 mL of 0.15 M NaCl. Liquefied phenol (EMD Millipore) (500 mL) was added to the re-suspended cell pellet and stirred at 70 °C for 30 min. The aqueous layer was removed from the liquefied phenol using a separatory funnel. The aqueous layer was back extracted three times with diethyl ether and dialyzed with SnakeSkin™ dialysis tubing (10K MWCO). Crude bacterial lysate was treated with RNase (Sigma) and DNase (Sigma) in 0.1 M sodium acetate buffer (pH 4.5), followed by Pronase® (Roche) treatment (pH 7.0) and finally dialysis. The crude mixture was purified by size exclusion chromatography (Sephacryl S-300 HR) with elution buffer (0.5% sodium deoxycholate, 50 mM glycine, and 10 mM EDTA (pH 9.8)). Fractions were collected and analyzed using UV-spectroscopy; fractions were pooled if there was no absorbance at 260 and 280 nm. The elution buffer was removed by dialysis and crude samples were analyzed by ^{1}H NMR. The final step in purification required anion-exchange chromatography (DEAE-Sepharose) to separate the zwitterionic polysaccharides using Tris-HCl (pH 7.3) and a salt gradient from 0 M – 2 M NaCl for elution of the polysaccharides was used. Purity of PS B was assessed by ^{1}H and ^{31}P NMR.

Synthesis of anomeric aminooxy TF (3). Synthesis of TF-ONH_2 has previously been reported by us.[2]

Synthesis of TF-PS B (4). Random oxidization of 1.0 mg of PS B using 10 mM of sodium periodate in 0.5 mL 0.1 M sodium acetate buffer, pH 5.0 was accomplished by allowing the reaction to stir for 90 min in the dark followed by quenching with 1 M KCl. TF-ONH_2 (**3**) 2.0 mg was then added to the solution of oxidized PS B and the reaction was allowed to stir overnight. TF-PS B was dialyzed and lyophilized. Conjugation was observed by oxime formation (7.4 - 8.0 ppm) using H^1 NMR (see below for spectral data).

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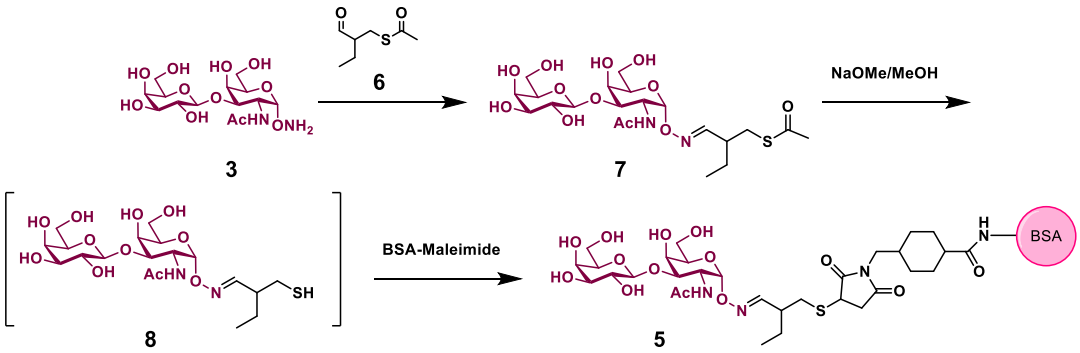
1. De Silva, R. A., Wang, Q., Chidley, T., Appulage, D. K., and Andreana, P. R. Immunological response from an entirely carbohydrate antigen: design of synthetic vaccines based on Tn-PS A1 conjugates. *J. Am. Chem. Soc.*, **2009**, *131*, 9622-9623.
2. Bourgault, J. P., Trabbic, K. R., Shi, M., and Andreana, P. R. Synthesis of the tumor associative α-aminooxy disaccharide of the TF antigen and its conjugation to a polysaccharide immune stimulant. *Org. Biomol. Chem.*, **2014**, *12*, 1699-1695.
3. Wu, J., and Guo, Z. Improving the antigenicity of sTn antigen by modification of its sialic acid residue for development of glycoconjugate cancer vaccines. *Bioconjugate Chem.*, **2006**, 1537-1544.

TF-BSA (5). Aminooxy TF (**3**) 5.0 mg was reacted with mercaptoaldehyde (**6**)[3] for 18 h in sodium acetate buffer (pH 5.5) at room temperature and purified using Sephadex G-10 and deionized/distilled H_2O as the eluent. Fractions containing the TF-linker were lyophilized. 2.5 mg of (**7**) was deacetylated using Zemplen's method consisting of NaOMe in methanol followed by base neutralization with DOWEX 50W x 8-100 ion exchange resin. The solution was then filtered and concentrated under reduced pressure.

Scheme S1. Synthesis of TF-BSA (**5**).



NMR and MS analysis for Compound 7

^{1}H NMR (600 MHz, D_2O): (E and Z isomers): **δ** 7.4 (dd, J_1 = 15.1 Hz, J_2 = 8.6 Hz, $1H_E$), 5.3 (dd, J_1 = 13.9 Hz, J_2 = 4.0 Hz), 4.4-4.3 (m, 4 H), 4.2 (dd, J_1 = 12 Hz, J_2 = 2.3 Hz, 1 H), 4.0 (d, J_1 = 5.7 Hz , 1 H), 3.9-3.8 (m, 2 H), 3.8 (d, J = 3.1 Hz, 2 H), 3.6-3.5 (m, 9 H), 3.5-3.5 (m, 4 H), 3.4-3.4 (m, 2 H), 3.1 (q, 3H), 3.0-2.9 (m, 3 H), 2.3-2.2 (m, 4 H), 1.9 (d, 7 H), 1.5-1.4 (m, 5 H), 1.2 (t, 6 H), 0.8-0.8 (m, 6 H).

^{13}C NMR (150 MHz, D_2O): (E and Z isomers): **δ** 200.9, 174.6, 158.3, 104.7, 98.6, 76.9, 75.0, 72.5, 71.0, 68.6, 60.8, 52.1, 47.6, 46.6, 42.4, 42.0, 30.8, 30.0, 24.7, 21.9, 10.9.

LRMS:ESI [M+$(Na)^+$] calcd for 563.19 found 563.1.

Analysis of Compound 5

2.0 mg of BSA-maleimide (Pierce Biotechnology) was dissolved in 0.3 mL of reaction buffer (1 x PBS buffer with 0.1 M EDTA (pH 7.2). Compound **8** was then dissolved in 0.2 mL of reaction buffer and added to a solution containing BSA-maleimide. The reaction proceeded for 24 hr at 4 °C and was extensively dialyzed at 4 °C. Conjugation was analyzed by MALDI-TOF (M/Z 90080.640). Mass loading was calculated using the following equation: (MW of TF-BSA – MW of BSA Mal)/ (MW of TF-linker).

We determined there were 34 molecules of TF-linker conjugated per BSA-maleimide.

Immunizations. Jax C57BL/6 male mice, 6 weeks, were obtained from Jackson Laboratories and maintained by the Department of Laboratory Animal Resources (DLAR). All animal protocols were performed in compliance with the relevant laws and institutional guidelines and have been approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Toledo. Mice were immunized by interperitoneal injections (i.p.) with 10 µg of TF-PS B, PS B and TF-BSA with and without TiterMax® Gold were performed. Injections were performed on Day 0, 7, 14, and 28. Blood was collected and pooled in a BD Vacutainer® SST™ on Day 32 using a cardiac puncture technique to draw blood. Blood was allowed to clot and serum was separated in BD Vacutainer® SST™ using a manufacture protocol.

PS B poly-L-lysine (PS B-PLL) and TF-PS B poly-L-lysine (TF-PSB-PLL). 100 µg of PS B or TF-PS B was added to a test tube containing 0.5 mL of 0.01 M NaOH (0.001% phenolphthalein indicator) and 0.5 mg of cyanuric chloride. The mixture was vortexed for 1 min and 0.1 mL of 0.1 % poly-L-lysine (PLL) was then added to the mixture, vortexed for 1 min and allowed to react for 3 h at 4 °C on a shaker. The conjugate was diluted to 30 mL with 0.1 M carbonate buffer (pH 9.2).

ELISA. Immulon™ 4 HBX 96 well plates (coated with either PS B/TF-PS B-PLL or TF-BSA) and maleic anhydride activated 96 well plates (coated with TF-ONH_2) (Thermo Scientific) were used to determine titers from immunized PS B, TF-PS B, and TF-BSA mice. The Immulon™ 4 HBX plates were coated with TF-BSA or TF-PSB/ or PS B-PLL (3 µg/mL in 0.1 M carbonate buffer (pH 9.2). Maleic anhydride plates were coated with TF-ONH_2 (**3**) as per manufacture instructions. Plates were left at 37 °C for 1 h with shaking and then continued overnight at 4 °C. The plates were then washed three times with washing buffer (1 x TBS, 0.05% Tween 20, pH 7.3) and blocked with blocking buffer (2% BSA, 1 x TBS, pH 7.3) and incubated for 1 h, followed by washing three more times with washing buffer. Anti-sera was initially diluted 1:300 for total antibody titers and 1:100 for IgG isotypes, then serially diluted in half-log_{10} dilutions and incubated for 2 hr at 37 °C followed by washing three times with washing buffer. Alkaline phosphatase secondary antibodies anti- (kappa, IgG) diluted (1:2000) and (IgM, IgG1, IgG2a, IgG2b, and IgG3) were purchased from (Southern Biotech) diluted (1:1000) and incubated for 1 h, followed by washing three times with washing buffer. PNPP tablets (Pierce) were dissolved in diethanolamine substrate buffer (pH 9.8) and then 100 µL was added to each well for 30 min for sufficient color to develop to detect secondary antibodies. The reaction was quenched with 2 M NaOH. Optical density measurements were obtained using a UV plate reader (Bio-Tek PowerWave HT) and the 96 well plates were read at 405 nm using Gen5 2.0 plate reading software. All assays were performed in triplicate. Titers were determined by regression analysis with half-log_{10} dilutions plotted against

absorbance. The titer cutoff value was set at 0.2 for titer determination. Statistically analysis from ELISAs for experimental groups were compared with the controls using paired *t* test using GraphPad Prism 6.

Flow Cytometry. MCF-7 and HCT-116 cells lines were provided by (Dr. Frederick Valeriote, Henry Ford Health Systems). Anti-sera was diluted to 1:200 with FACS buffer (1X PBS, 2% FBS, and 0.001% azide) and incubated with the cell lines (1 x 10^6 cells) for 30 min on ice. Cells were washed with FACS buffer three times and incubated with secondary antibodies using either AlexaFluor® 488/647 and washed three times. Cells were analyzed by flow cytometry using BD FACSCalibur™ and data analysis obtained using FlowJo software.

Synthesis of STn-PS B (9). Random oxidization of 1.0 mg of PS B using 10 mM of sodium periodate in 0.5 mL 0.1 M sodium acetate buffer pH 5.0 was accomplished by allowing the reaction to stir for 90 min in the dark, followed by quenching with 1 M KCl. 2.0 mg of STn-ONH_2 was then added to the solution of oxidized PS B and the reaction was allowed to stir overnight. TF-PS B was dialyzed and lyophilized. Conjugation was observed by oxime formation (7.4 - 8.0 ppm) using H^1 NMR (see below for spectral data).

Periodate-Rescorcinol Assay for Sialic Acid. A linear gradient of sialic acid, *N*-acetyl galactose, and galactose amine was generated from 40, 35, 30, 25, 20, 15, 10, 7.5, 5, 2.5, 1, and 0.5 µg. STn-PS B (**9**) and PS B (**2**) were added in triplicate in separate wells at 50 µg per well. 40 µL was placed in triplicate for each concentration in a 96 well plate. 10 µL of 5 mM $NaIO_4$ was placed in each well and incubated for 35 min at 4 °C making a final concentration of 1 mM. 100 µL of rescorinol solution (0.6 g of resorcinol in 100 mL of 17% HCl solution and 0.0025 mM of $CuSO_4$) was added to the well-plate and incubated for 60 min at 90 °C. The unknowns were determined from the sialic acid concentration at 580 nM.

Percent loading of STn-PS B. Sialic acid by weight was determined from the periodate rescorinol assay and STn percent loading was calculated by the following equation.

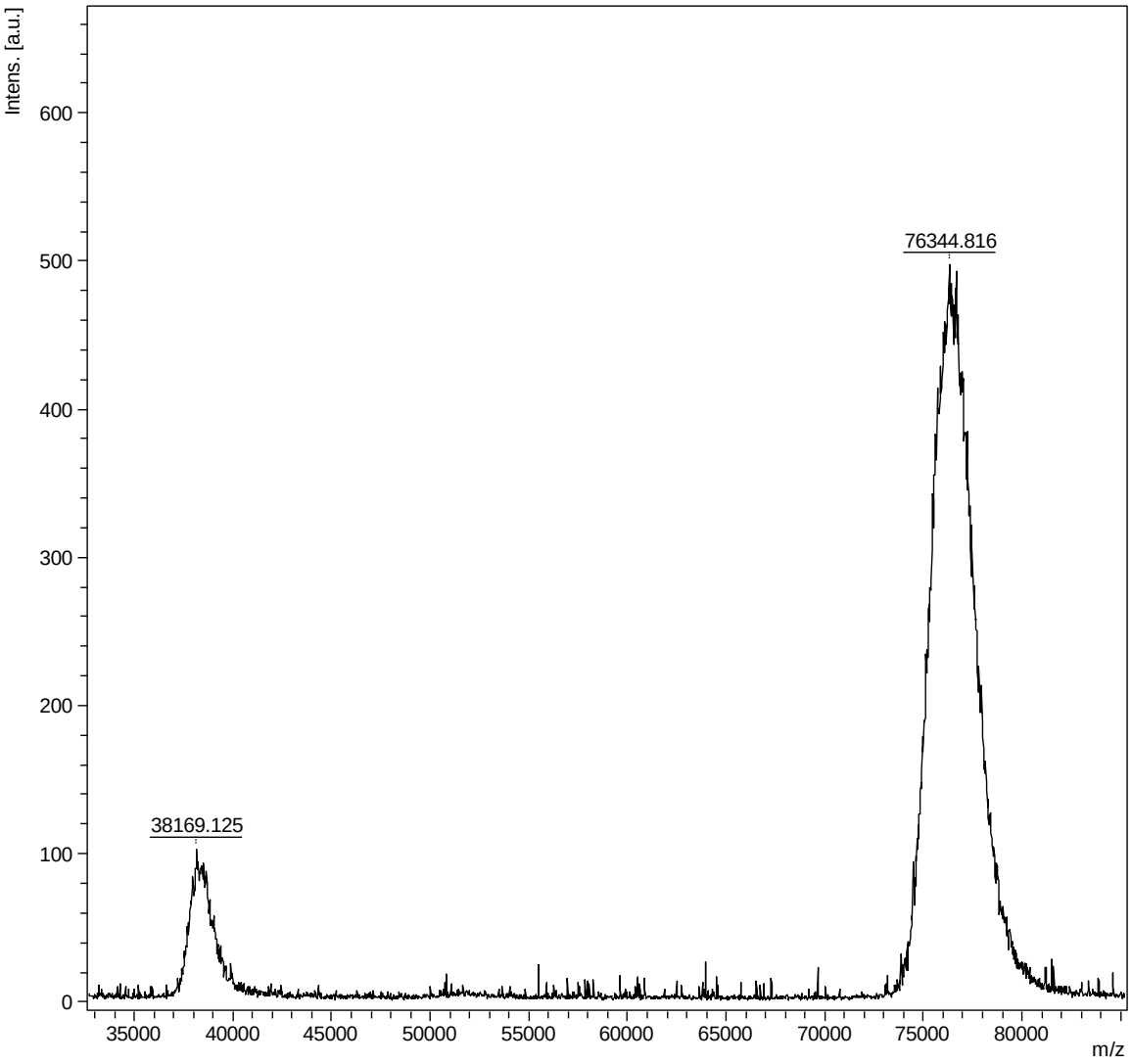
Amount of sialic acid (µg) from assay (weight of glyconjugate) x (molecular weight STn/molecular weight of sialic acid) x 100%.[3]

Alexa Fluor® 488 percent loading. 100 µg of oxidized PS B was reacted with 100 µg of Alex Fluor® 488-hydrazide (Molecular Probes) for 24 h in PBS buffer pH 7.4 followed by dialysis. The solution was lyophilized and re-dissolved with 100 µl of PBS. Optical density measurements were obtained using a UV plate reader (Bio-Tek PowerWave HT) and then the 96 well plates were read at 495 nm using Gen5 2.0 plate reading software.

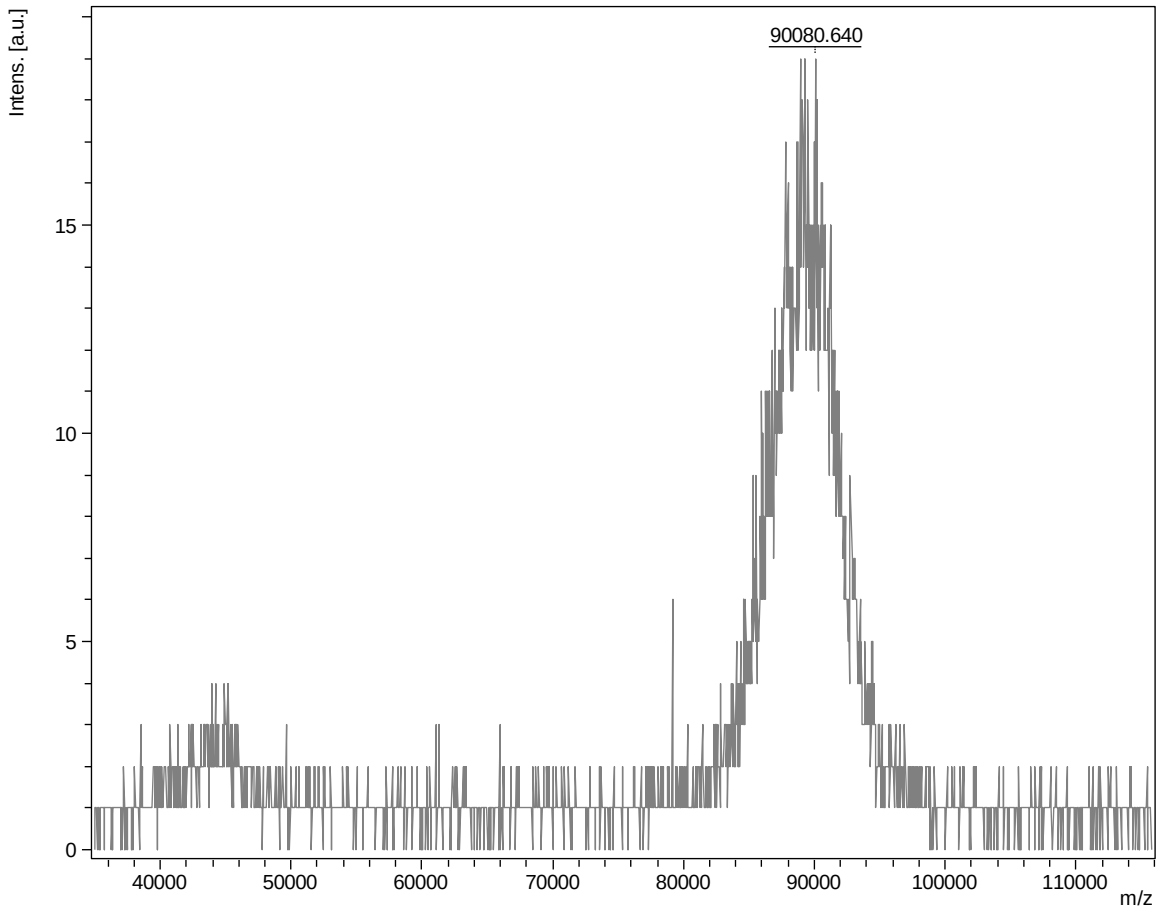
The percent loading of Alexa Fluor® 488 was determined using the manufacturer protocol(s) (Invitrogen Alex Fluor® 488 Protein labeling Kit). The following equation was used:

Moles of dye per mol of PS B = A_{494} / (71000 $cm^{-1}M^{-1}$ X PS B concentration)

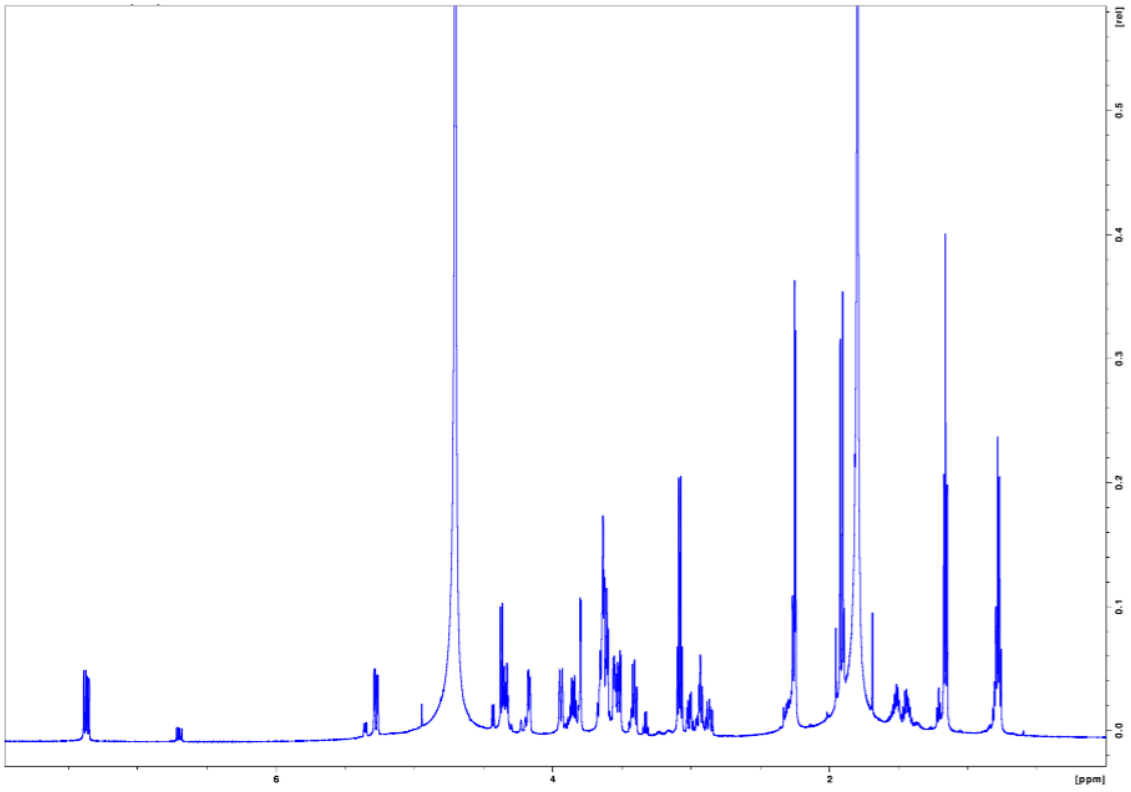
MALDI-TOF of BSA-Maleimide (Thermofisher)



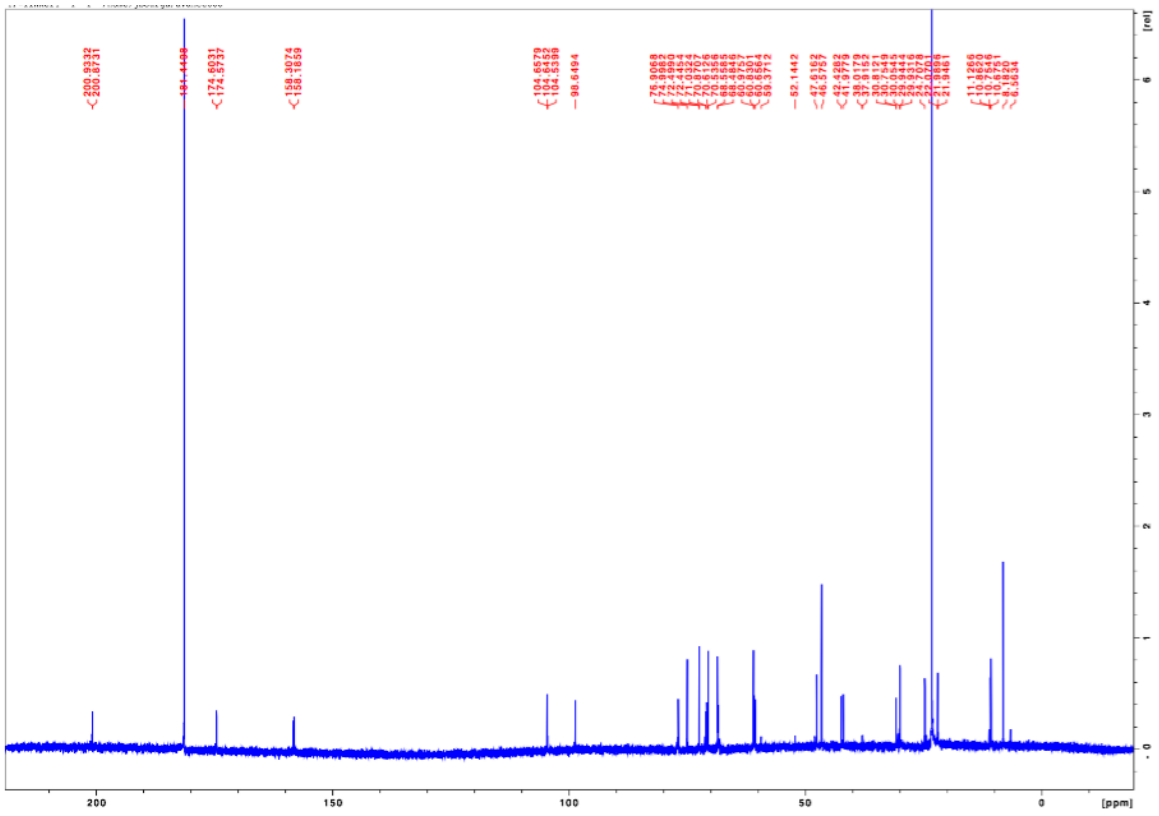
MALDI-TOF of TF-BSA (5)



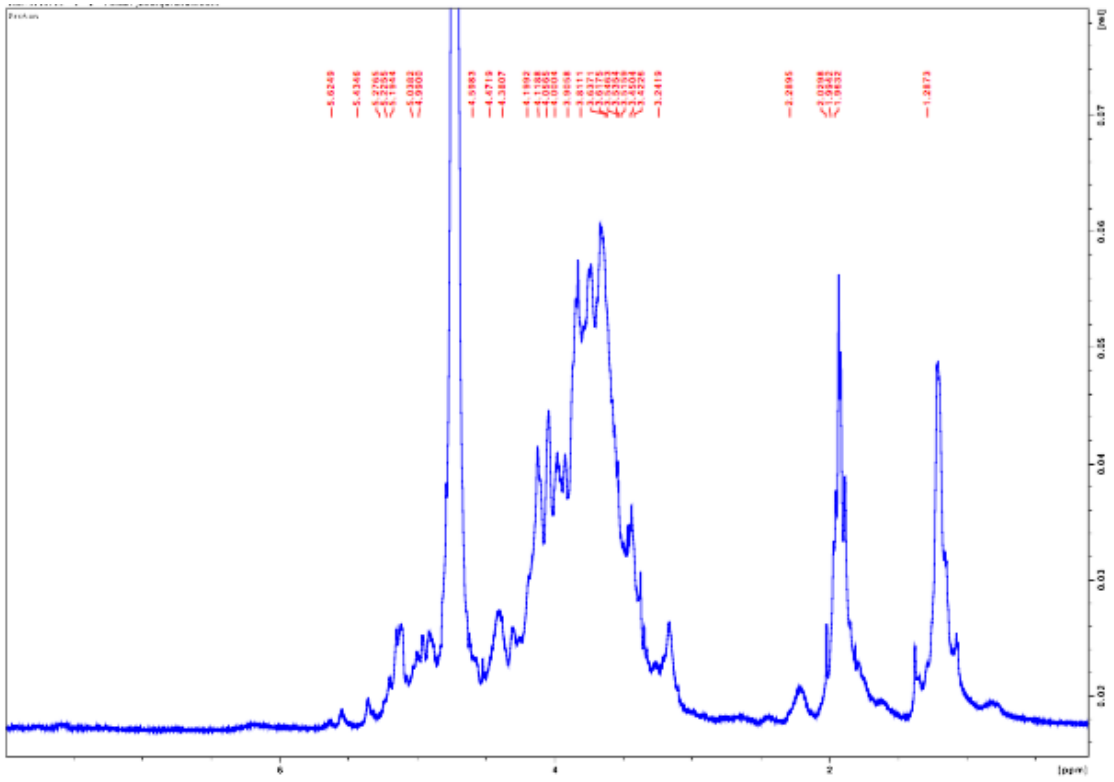
^{1}H NMR of TF-linker (7)



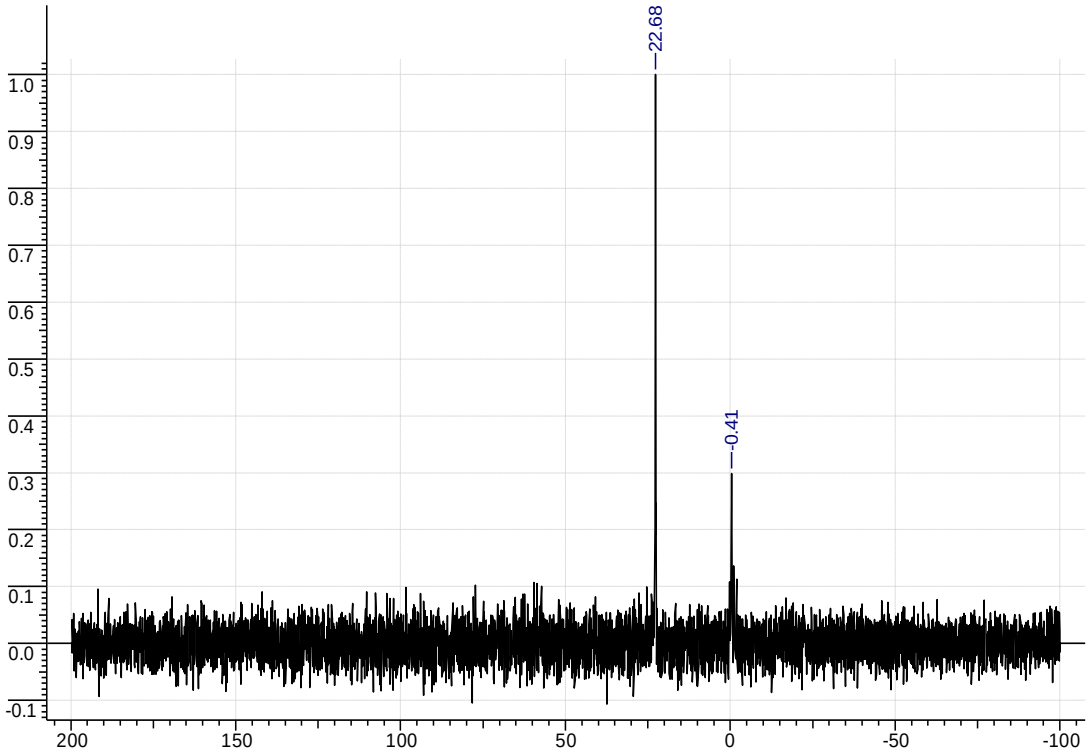
^{13}C NMR of TF-linker (7)



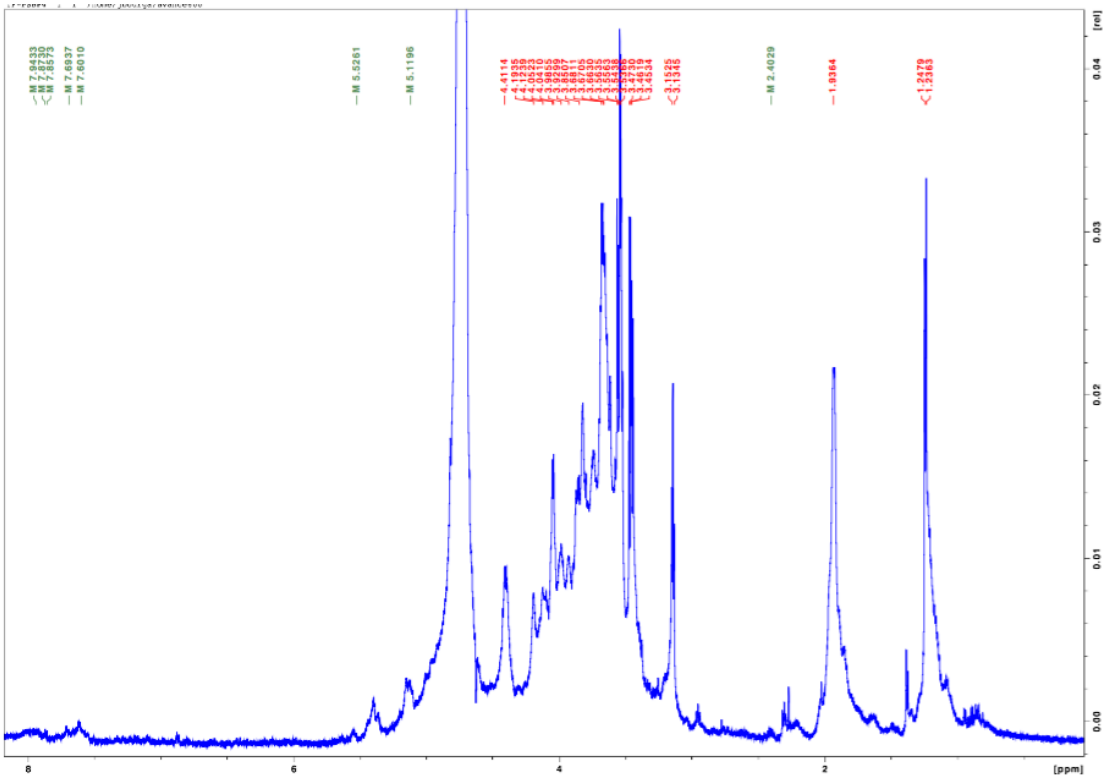
^{1}H NMR of Purified PS B (2)



^{31}P NMR of PS B (2)



^{1}H NMR of TF-PS B (4)



Expansion of ^{1}H NMR of TF-PS B (4) from 6.8-8.0 ppm

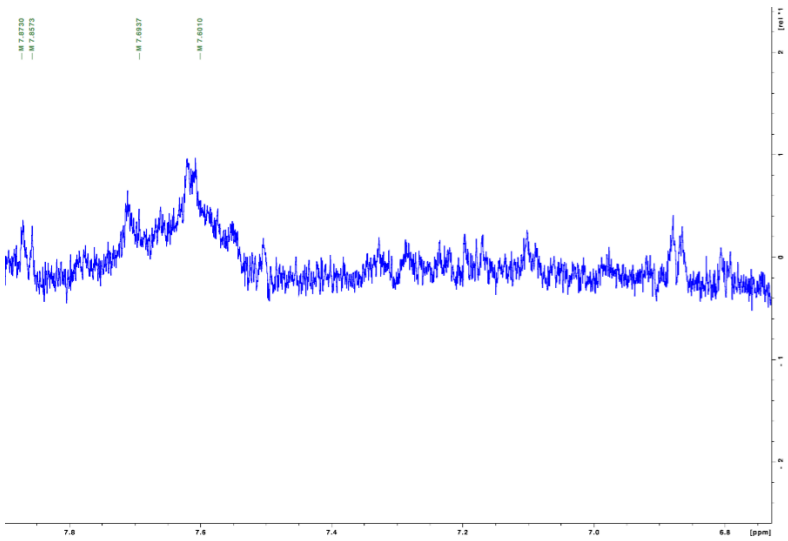


Table S1. Comparison of TF-PS B (**4**) and TF-BSA (**5**) isotypes on ELISA

Immunizing Antigen	Plate Coating	IgG1	IgG2b	IgG3
TF-BSA (TiterMax Gold)	TF-MA	4	-	-
TF-BSA	TF-MA	-	-	-
TF-PSB (TiterMax Gold)	TF-MA	448	52	-

ELISA with TF-maleic anhydride (TF-MA) coated plates were used to determine the titer of TF-PS B (**4**) and TF-BSA (**5**). TF-maleic anhydride plates were used because TF-ONH_2 (**3**) could be conjugated to the maleic anhydride coated plates without the need for protein conjugates or linkers, therefore allowing us to obtain true recognition of the TF-antigen.

N.B. Another method for screening TF was used (TF-KLH) and it was constructed using similar conditions to TF-BSA. However, the linker portion of **8** was exactly the same as seen in TF-BSA (**5**) and contributed to augmented binding data due to the immunogenicity of the linker.

From (**Table S1**), the TF-MA plates were used as a common platform to compare the titer data from TF-BSA and TF-PS B. The data from TF-PS B was similar to what was seen in (**Table 1; entry E**) but anti-TF-BSA antibodies had minimal recognition of IgG to TF.

^{1}H NMR of STn-PS B (9)

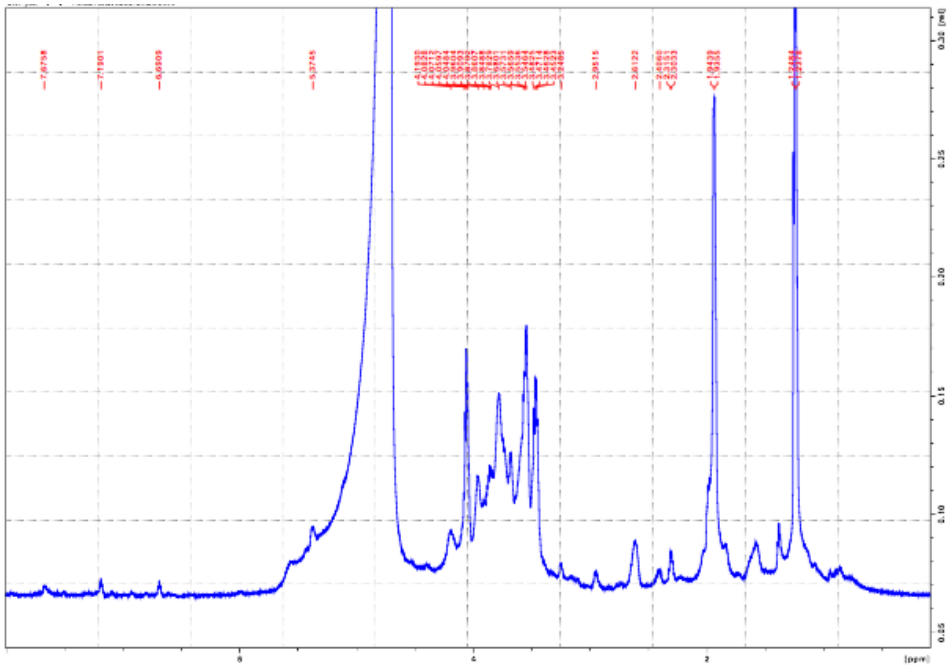
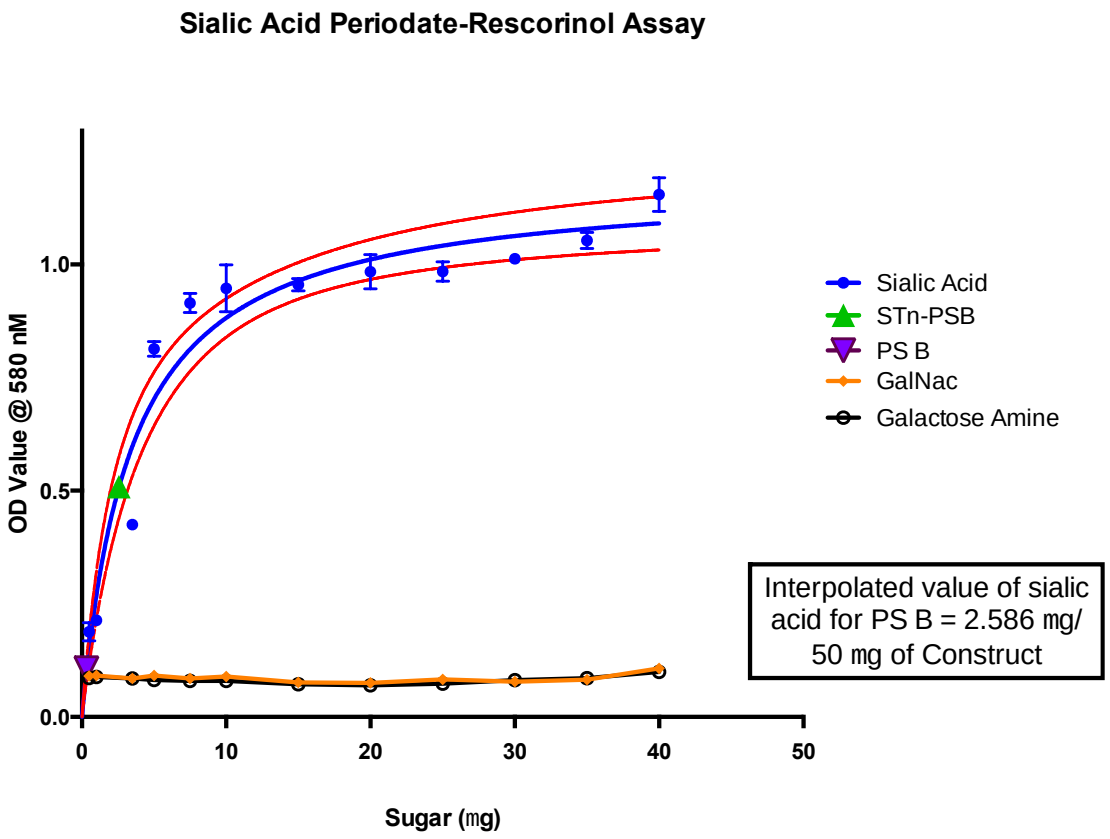


Figure S1. Sialic acid determination using periodate-rescorinol assay.[4]



4. Svennerholm, L. Quantitive estimation of sialic acids: II. A colorimetric resorcinol-hydrochloric acid method, *Biochimica et Biophysica Acta* **1957**, *24*, 604-611.