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Elucidating Structural Features of an Entirely Carbohydrate Cancer Vaccine Construct Employing Circular Dichroism and Fluorescent Labeling

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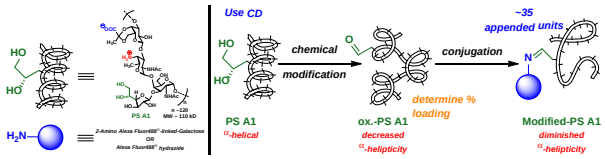

Structural changes of PS A1 does not alter function.

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Elucidating Structural Features of an Entirely Carbohydrate Cancer Vaccine Construct Employing Circular Dichroism and Fluorescent Labeling

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The zwitterionic polysaccharide PS A1 from anaerobe *Bacteroides fragilis* ATCC 25285/NCTC 9343 is known to elicit a T-cell-dependent, major histocompatibility complex class II (MHCII) immune response through a correspondingly similar protein-antigen-based mechanism/pathway. The biological activity of PS A1 is known to arise from alternating charged motifs on adjacent monosaccharides comprising a tetrameric repeating oligomeric unit creating an alpha-helical secondary structure. However, we have learned that this alpha-helical structural characteristic may not play a role in immune activation. Paradoxically, our current knowledge of structure - activity relationships (SARs) with electrostatically charged polysaccharides has become more clearly defined, yet a lack of tools/probes for measuring dynamic structural changes hinders progress in carbohydrate-based vaccine development. Site- and region-specific structural modifications of PS A1, followed by conjugation with a known carbohydrate cancer antigen, the Thomsen-nouveau (Tn = alpha-D-GalNAc-OSer/Thr) antigen, does not alter antibody isotype switching ability and leads to specific IgG3 antibodies in C57BL/6 mice. Circular dichroism (CD) and studies using fluorescently labeled PS A1, described herein, reveal information pertaining to structure - activity relationships and the nature of Tn conjugation to chemically modified PS A1. The CD spectra of a Tn-PS A1 construct at $8.5 \geq pH \leq 3.5$ illustrates complete loss of alpha-helical character while spectra obtained in the $3.6 \leq pH \geq 8.4$ range denotes minimal alpha-helicity in comparison to naturally occurring PS A1. Temperatures exceeding 60 ^{o}C reveal complete loss of helical character. Two methods for Alexa Fluor488® fluorescent labeling studies of chemically oxidized PS A1 have given rise to percent conjugation values (% loading) calculated to be on average 35 Tn molecules bound. Combined, our results argue that altering the structure of PS A1, without chemically modifying the electrostatic charge character, does not alter immune response/recognition in mice. These findings have important implications for the design of entirely carbohydrate-based vaccine constructs.

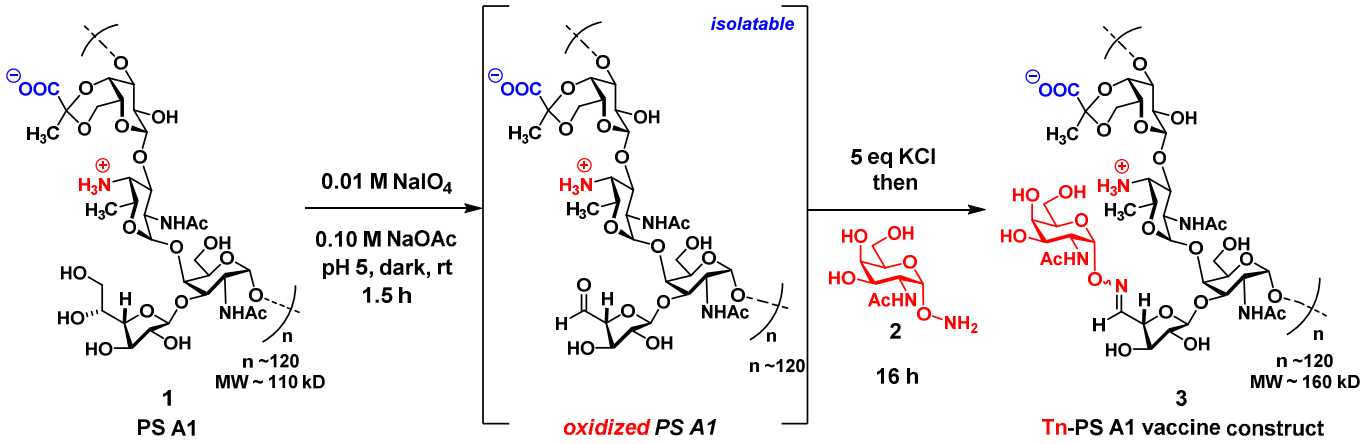
Introduction

Vaccines are powerful tools for disease prevention and therapy. Aberrant carbohydrates on cancer cell surfaces are important strategic targets for the development of cancer vaccines. However, as single entities, void of lipids/peptides/proteins, carbohydrates have long been known to only invoke the B-cell humoral arm of the immune system in a *T-cell-independent* manner, by crosslinking to B-cell receptors producing low-affinity IgM antibodies (Abs).[1,2] For effective immunity a strong and long-term response must be generated through both *cellular and humoral arms* of the immune system involving MHCII proteins, $CD4^+$ T-cells and B-cells in a *T-cell-dependent* cascade.[3] Recently, Kasper isolated $CD4^+$ cell clones binding to capsular polysaccharides presented by MHCII illustrating immune activation with T-cells.[4] It was Kasper et. al. who also discovered a new class of bacterial polysaccharides characterized by an alternating zwitterionic charge motif on adjacent monosaccharides and showed them capable of stimulating T- and B-cell immune responses.[5] These molecules have been appropriately termed zwitterionic polysaccharides (ZPSs) and current research endeavors focus on their ability to induce immunity through antigen presenting cell (APC) processing to activate T-cells directly through the α,βT-cell receptor of MHCII-ZPS complexes.[5] ZPSs have also been linked to activating APCs and up-regulating MHCII co-stimulatory molecules and cytokines generating conditions that favor the activation of T-cells.[5,6] It has been convincingly shown that a natural ZPS from the commensal anaerobe, *Bacteroides fragilis* ATCC 25285/NCTC 9343, capsular PS A1 (**1**) (Scheme 1), is also a toll-like receptor 2 (TLR-2) agonist.[7] The biological validation of **1** challenges the current paradigm of carbohydrate-immune processing[8-10] by illustrating that ZPSs alone elicit T-cell immune responses; the implications of this could have an enormous impact on the treatment of various diseases most likely through vaccine development.

The primary structure of the polysaccharide PS A1 (**1**) consists of a zwitterionic tetrasaccharide repeating core unit containing the sugars 2,4-dideoxy-4-amino-D-*N*-acetylfucose, D-*N*-acetylgalactosamine, D-galactopyranose, and D-galactofuranose with a 4,6-pyruvate acetal attached to the galactopyranose.[11] Valuable information about the solution structure of PS A2 *Bacteroides fragilis* 638R, (similar sequence to PS A1) has been resolved by total correlation spectroscopy (TOCSY) and nuclear Overhauser spectroscopy (NOESY).[12] This information provides a model for ZPS processing highlighting a polysaccharide with extended alpha-helical character over two (tetrasaccharides) repeating units per turn (pitch of 20 Å) and exposed alternating zwitterionic motifs on the outside.[12]

In addition to using NMR-based TOCSY or NOESY, circular dichroism (CD) has been employed to determine secondary structure of polysaccharides, such as for the capsule of group B *meningococci*, or carrageenan due to their unique ionic distributions.[13,14] In 2007, Cobb and co-workers discovered that PS A1 helical structure produced a CD spectrum similar to proteins containing a high degree of alpha-helical content.[15] This contributed to an immunogenic model in which the activity of PS A1 is proposedly dependent on the presence of the electrostatically charged character motif and overall size (~110 kDa).[15] It has been shown that removal of either or both zwitterionic charged motifs can annul the secondary structure and the ability to bind MHCII molecules.[6]

Scheme 1. Synthesis of the Tn-PS A1 glycoconjugate immunogen.[16]



Results

The enormous yet understudied potential for entirely carbohydrate-based vaccine development inspired our group to hypothesize that the carrier proteins/peptides/lipids of current glycoconjugate vaccines could be replaced with PS A1. In previous work, we conjugated the Thomsen-nouveau (Tn)[16,17] and Thomas-Friedenreich (TF)[18] tumor associated carbohydrate antigens (TACAs) to **1** and discovered an *antigen specific* immune response.[16,17] The Tn TACA was chosen for two main reasons: 1) it represents the simplest structure of all the known TACAs[19] rendering it readily accessible through synthetic strategies and 2) a number of reports exist detailing Tn as a glycoprotein conjugate that provides data points for comparison purposes in our own studies. Our approach involved conjugating the

Tn aminooxy sugar (**2**) to PS A1 (**1**) *via* selective oxidative cleavage of the vicinal diol (1° 6-OH, 2° 5-OH) found on the bacterial-exclusive D-galactofuranose using 0.01 M $NaIO_4$ (Scheme 1). The Tn-PS A1 (**3**) immunogen has been characterized using NMR showing distinct oxime doublets of the *Z* and *E* isomers at 6.3 and 7.21 ppm respectively in ~ 1:1 ratio.[16]

With our Tn-PS A1 immunogen, we sought, in the current study, to understand the impact of Tn coupling on the helical content of PS A1 through

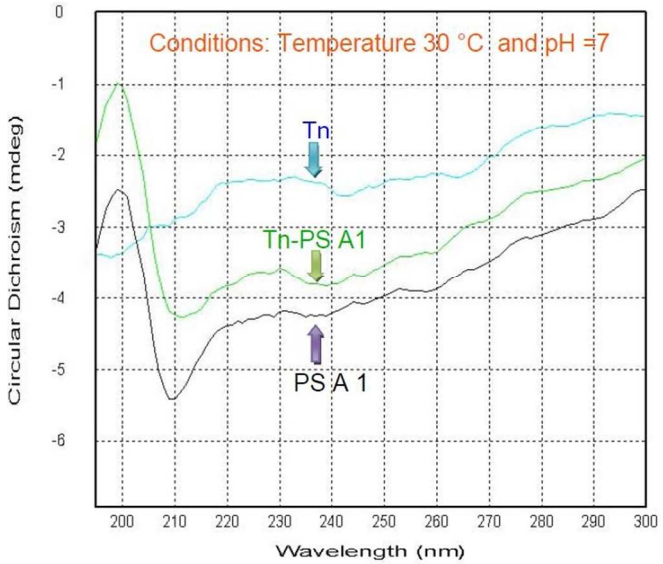


Figure 1. CD spectra of PS A1 (**1**), Tn (**2**) and Tn-PS A1 (**3**) at 30 ^{0}C and pH = 7.

circular dichroism (CD) studies; a common technique applied to monitor optical properties of the solution structure of molecules which can reveal information about secondary structure. These spectra are known to vary with protein conformation upon ligand binding or thermodynamic unfolding.[20] It is well-known that CD spectroscopy uses optical rotation and changes in ellipticity to determine the difference of asymmetric monosaccharides in solution, which can provide structural information such as conformations and generation of dipole moments.[21,22]

PS A1 is naturally substituted with specific chromophores containing defined optical activity, such as the *N*-acetate groups, a common post-glycosylational marker. The conformational changes of PS A1 may affect the polarizability, static field contributions, and orientation of the chromophores, resulting in changes in optical activity. The optically active absorption bands of the substituted chromophores arise from the $n–\pi^*$ transitions located in the range 200-250 nm.[23-25] Therefore, CD provides a convenient method of investigating the conformational change of polysaccharide substituents. In light of our promising biological results and what is based on the aforementioned, we elected to characterize and compare structural integrity of PS A1 in our Tn-PS A1 vaccine construct utilizing CD.

To this end, we sought to determine whether the Tn antigen conjugated to PS A1 would affect the helical character by comparing the CD data of pure PS A1 with Tn-PS A1 at room temperature (Figure 1). CD spectra of Tn-PS A1 showed a distinct decrease in ellipticity as compared to that of pure PS A1 at wavelength 217 nm in a 0.1 mg/mL concentration. That loss of helical character resulting from Tn conjugation to PS A1 could be due to a change in conformation and/or a direct result of the oxime chromophore.

To confirm that the change in ellipticity observed with Tn-PS A1 was due to a direct effect of the Tn monosaccharide attached to oxidized PS A1, CD of Tn was compared with PS A1 and Tn-PS A1 (Figure 1). Tn alone did not give any significant CD spectrum. Therefore, it was clear that the α-helical character of Tn-PS A1 came entirely from PS A1.

Thermal stability of Tn-PS A1

In determining the thermal stability of Tn-PS A1, CD spectra of Tn-PS A1 in PBS buffer were recorded at different temperatures (Figure 2). It was noted that Tn-PS A1 completely lost α-helical secondary structure at high temperature.

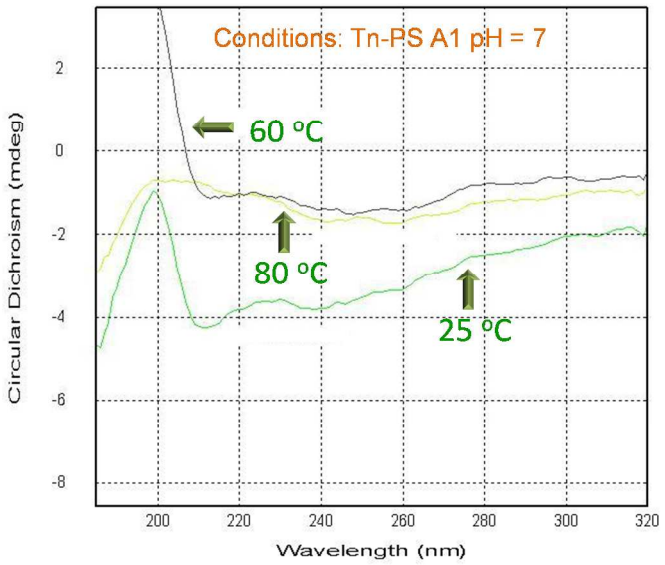


Figure 2. CD spectra of Tn-PS A1 (**3**) with constant pH = 7.0.

pH stability of Tn-PS A1

According to Kasper, proper activation of T-cells by ZPS molecules must be phagocytized by APCs (dendritic cells, B-cells, and macrophages).[9] Once in the endosome, the pH environment of the vesicle is reduced by ATPase proton pumps which activates acidic proteases.[9,26] Unfortunately, ZPSs are not processed by an enzymatic degradation but rather are partially degraded through an oxidative burst pathway.[9,26] These fragments are able to facilitate binding to MHCII proteins in a reduced pH environment that allows presentation to the T-cell.[9] However, the positive MHCII mediated immune responses against our Tn-PS A1 suggests that the construct retains the ability to induce production of IgG antibodies[16,17] even when there is depletion of helical character due to changes in conformation induced by reduced pH and oxidative depolymerisation. Based on the data found within, we now believe that it is most critical to retain the alternating charge character of Tn-PS A1. In order to further validate our data, the CD spectra of Tn-PS A1 were collected at varying pH values. The results showed identical spectra for Tn-PS A1 at pH 5 and 7, while decreasing the pH showed a gradual loss of α-helical content (Figure 3).

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These results indicate that the degree of α-helicity of Tn-PS A1 did not change when the pH of the solution was adjusted from 7 to 5. A better way to compare secondary structure at pH 7 and 5 is to carry out the coupling reactions at the respective pH and compare the CD of those with conjugates. Additionally the pH at 3.9 showed greatly diminished helical character, which suggests that associated zwitterionic charges play a larger role than helical character itself. However, for method validity, the percent loading of Tn to oxidized PS A1 (Scheme 1) at pH 7 and 5 must be equal. Therefore we devised simple but reliable methods to determine the Tn loading to PS A1.

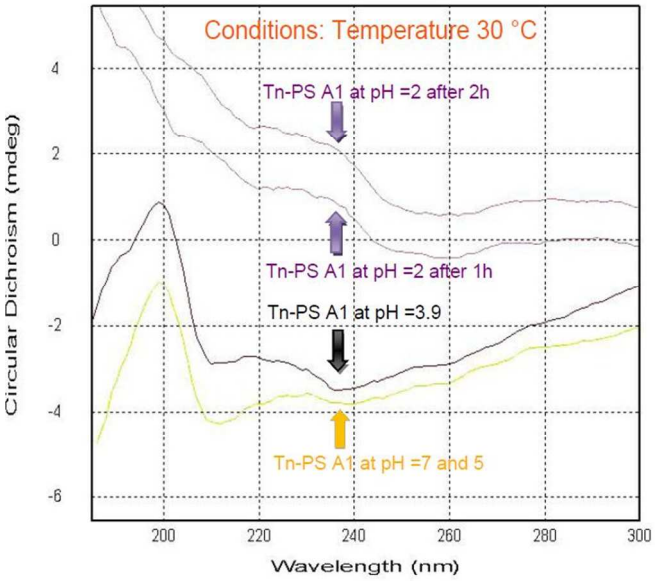


Figure 3. CD spectra of Tn-PS A1 (**3**) at varying pH values.

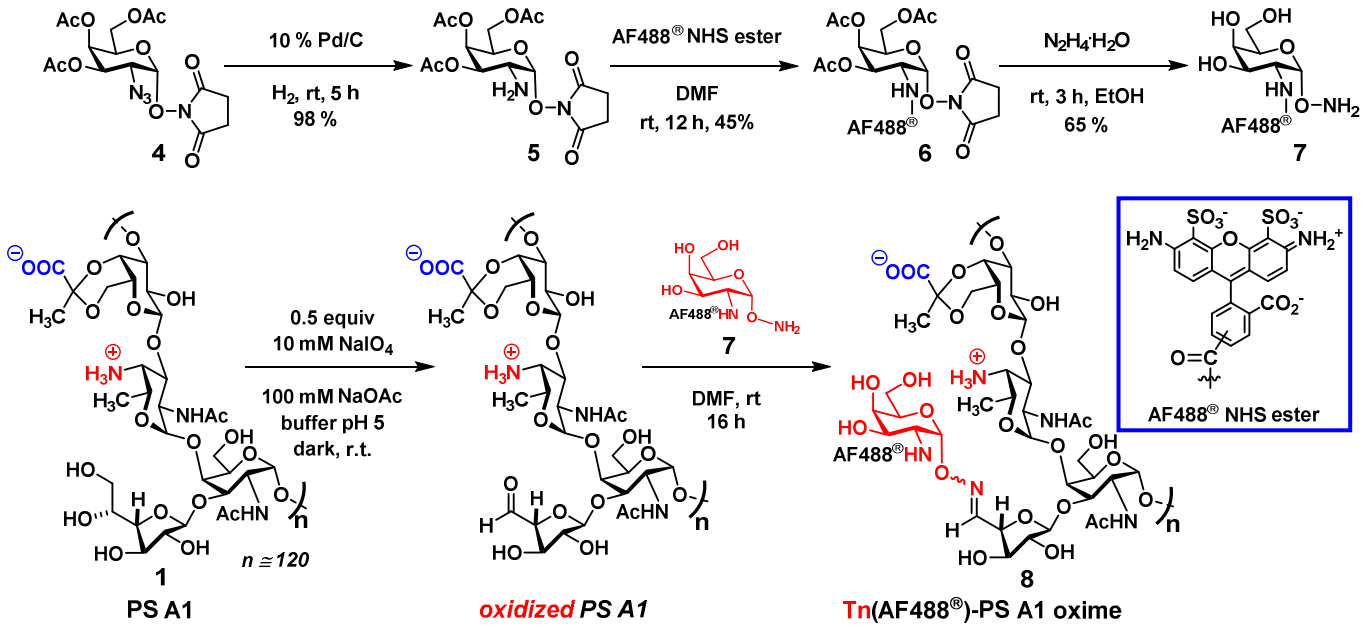
Determination of percent Tn loading onto oxidized PS A1.

Accurate assessment of the molar ratio of carbohydrate antigen to PS A1 carrier is important for conducting dose-response immunization studies. There are numerous methods to determine the number of antigens coupled to various carrier proteins. For example, the number of STn carbohydrate antigens bound to a carrier protein can be determined by either a resorcinol assay or fluorescence detection of 1,2-diamino-4,5-methylenedioxybenzyl (DMB) derivatives of sialic acid.[27,28] Based on available models for the determination of protein conjugations, three general techniques exist: radioisotopic labeling, spectrophotometry and peptide sequence analysis.[29]

In radioassays, a small of amount of ^{14}C is incorporated during the synthesis. Alternatively, radiolabeling can be performed using iodine. However, the accuracy of this method depends on the extent of labeling, and suffers from the instability of commonly used radiolabeled derivatives. Therefore the radiolabeling approach to Tn-PS A1 conjugate by reducing oxime bond by $[H^3]$-$NaBH_4$ was avoided. The amino acid analysis method, although robust, was unsuitable for our completely carbohydrate based Tn-PS A1 conjugate. Ultimately, we focused on the development of a new coupling reaction monitoring technique based on spectrophotometric method.

Alexa Fluor® dye conjugates provide a powerful and simple method employing UV absorbance. Since oxime bond formation with aldehydes generated by oxidation of D-galactofuranose units of PS A1 proved to be a very reliable technique, we decided to couple the Alexa Fluor488® dye to PS A1 through indirect oxime and direct hydrazone linkages. We used two methods to couple the dyes to PS A1 (Scheme 2 and 3). In Method I, the Alexa Fluor488® NHS ester was coupled to the amine of modified Tn antigen **8** (Scheme 2) forming an amide, whereas in Method II, the Alexa Fluro®488 hydrazide was directly attached to PS A1 (Scheme 3) forming a hydrazone. After purification using dialysis, the UV absorbances of the pure conjugates were recorded at 495 nm. Percent loading was determined using Beer's law.

Scheme 2. Method I: Synthesis of Tn(AF488®)-PS A1 oxime (**8**) for determining percent Tn loading.



Scheme 3. Method II: Synthesis of AF488®-PS A1 hydrazone (**9**) for the determination of percent Tn loading to oxidized PS A1.

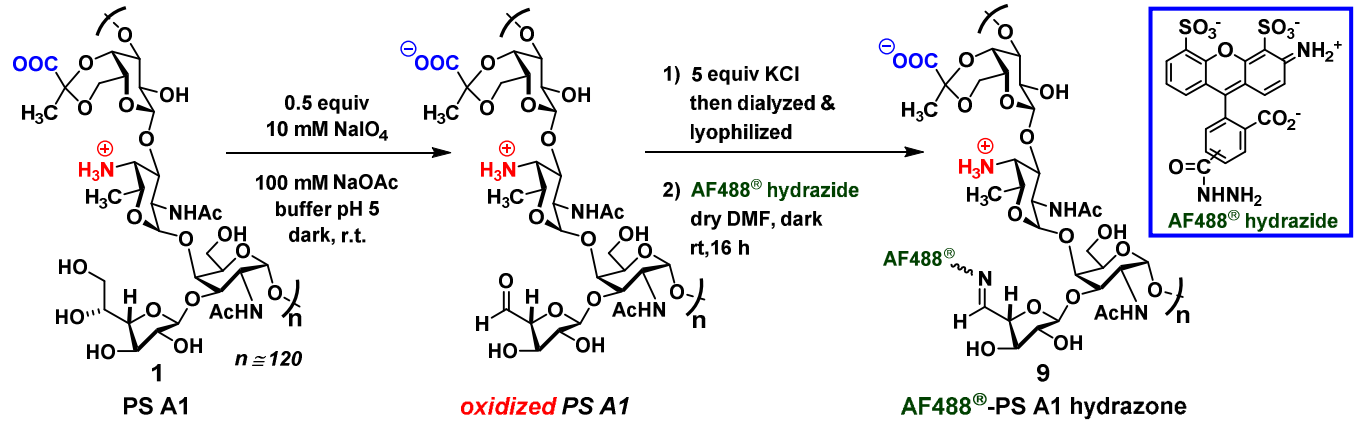


Table 1 shows the degree of Tn loading onto PS A1. Our results indicate that when 1 equivalent of $NaIO_4$ is used for the oxidation of PS A1, greater than 100% loading of AF488® occurs. This implies that over-oxidation can occur with other vicinal diols in the PS A1 repeating unit such as those of D-galactofuranose (the secondary 2-OH and 3-OH). When 0.50 equivalents of $NaIO_4$ is used to oxidize PS A1, 32% loading with Tn(AF488®) aminooxy (**7**) and 37% loading with AF488® hydrazide were found. Using less than 0.50 equivalents of $NaIO_4$ gave relatively low percent loading values.

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Table 1. Percent loading of AF488® on oxidized PS A1.

Equivalents of $NaIO_4$[a]	Percent Loading using AF488®[b] Method I / Method II
1	110[d]
0.75	42[d]
0.50	32[c]
0.50	37[d]
0.25	12[d]
0.10	4[d]

[a]equivalents of $NaIO_4$ = Moles PS A1 x 120. The number 120 is derived based on the number of repeating tetrameric oligosaccharide units composing PS A1.[9,16] [b]Number of repeating units coupled with AF488® as a percentage. [c]Method I. [d]Method II

Discussion

We have established that the structure of Tn-PS A1 has less α-helical content then PS A1, as determined by CD, and the percent loading of Tn on PS A1 can be determined by using spectrophotometric methods. Our results suggest that it is not the α-helical nature of the polysaccharide but the alternating charges that contribute to MHC class II activation.[17] Our data also shows how the α-helical content is diminished under physiological conditions using pH and temperature. This leads us to believe the Tn-PS A1 construct binds MHCII through electrostatics and that structural aspects do not play a critical role, contrary to what has been previously proposed.[9] The successful addition of 35 molecules of Tn per oxidized unit of PS A1 reduces the α-helical content of PS A1 but leaves the alternating charge character on adjacent repeating units, however, immune activation has been evidenced.[16,17] The synthesis of carbohydrates with zwitterionic motifs involving adjacent units may lead to substantial advances in understanding the immune response against carbohydrates utilizing MHCII pathways for antigen presentation to $CD4^+$ cells and ultimately to carbohydrate-based vaccine development.

Experimental

Bacterial Growth and Isolation

B. fragilis (ATCC 25285/NCTC 9141) was purchased from *Presque Isle Cultures*. To begin the initial growth procedure, the bacteria were streaked on blood agar plates containing BBE. The plates were prepared in an anaerobic glove bag in a CO_2 environment. After the cultures were initiated, the plates were transferred to an anaerobic jar with gas packs in the presence of O_2 indicator strips and placed in an incubator at 37 °C. *Note:* Freeze-dried samples were initially purchased directly from ATCC but after several growth attempts we deemed the samples to be non-virulent.

PYG broth was used for the large-scale growth of *B. fragilis*. Proteose-peptone (20 g), yeast extract (5 g), NaCl (5 g), and 0.001 g of reazurin per 1 L of nanopure H_2O were autoclaved. Glucose 25% (2 mL), potassium phosphate 25% (2 mL), cysteine 5% (1 mL), 0.5% of hemin in 1 N NaOH (100 μL), and 0.5% Vitamin K1 in absolute ethanol (50 μL) were filtered using a 0.22 μm filter and added to the autoclaved PYG broth.

Anaerobic conditions were achieved by degassing solutions for 30 min under an atmosphere of 80% N_2, 10% CO_2, 10% H_2. A resazurin indicator was used to assure an anaerobic environment. Once the media was no longer pink in color, either the agar plates or liquid media were readied for inoculation. The agar plates were cut in sections and placed into the degassed media under an inert atmosphere. For liquid media transfer, 5 mL of culture was seeded in a degassed jar via cannulation. Every 24 hrs the pH of the media was tested and adjusted to 7.2. During the first 24 h of growth, the pH would drop to 5 and 5 M NaOH was used to adjust the pH in 1 mL portions until pH 7.2 was noted. A total of 20 L of bacteria fermentation was accomplished. Purification of PS A1 and coupling of Tn to PS A1 were carried out according to the procedure reported by De Silva *et al*.[16]

Synthesis of compounds 5, 6, and 7

Compound 5. Compound **4** (0.20 g, 0.47 mmol) was dissolved in nanopure H_2O and 10% Pd-C (0.015 g, 0.094 mmol) was added to the solution under an atmosphere of H_2 for 16 h. The reaction was filtered through a layer of Celite®. The crude mixture was concentrated under a

reduced atmosphere and purified using silica gel chromatography (10:1 Hex:EA to 1:1 Hex:EA) giving compound **5**. Yield = 0.15 g (80%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.51 (s, 1H), 5.43 (d, J = 3.0 Hz, 1 H), 5.14 (dd, J_1 = 11.5 Hz, J_2 = 3.0 Hz, 1H), 5.03 (dt, J_1 = 6.5 Hz, J_2 = 1.0 Hz, 1H), 3.93 - 4.25 (m 2H), 2.78 (s, 4H), 2.77 (s, 2H), 2.13 (s, 3H), 2.07 (s , 3H), 2.06 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.94, 170.94, 170.70, 170.61, 170.29, 105.56, 69.34, 69.34, 67.66, 61.82, 61.82, 25.69, 25.69, 21.01, 21.01, 20.87; HRMS: EIMS [(M + H) $^+$] cacld for $C_{16}H_{23}N_2O_{10}$ 403.1353 found 403.1356.

Compound 6. Alexa Fluor488® NHS ester (Invitrogen, USA) (0.001 g, 1.50 x 10^{-3} mmol) was dissovled in 1 mL of anhydrous DMF *in the dark*. Compound **5** (0.063 g, 1.56 x 10^{-3} mmol) was added to the solution and the reaction was allowed to stir for 16 h *in the dark* under argon. Preparatory TLC (CH_3CN with 20% H_2O) was used to separate unreacted Alexa Fluor488® NHS ester. Yield = 45 % (determined by UV absorbance); HRMS: EIMS [(M + Na)]$^+$ calcd for $C_{37}H_{32}N_4O_{20}S_2Na$ 939.0967 found 939.0971.

Compound 7. Compound **6** was dissolved in absolute ethanol (0.5 mL) and hydrazine hydrate (0.01 mL, 0.002 mmol) was then added to the solution and stirred for 6 h *in the dark* at room temperature under argon. The crude mixture was concentrated and used in the next step without purification. HRMS: EIMS [(M + Na)]$^+$ calcd for $C_{27}H_{24}N_4O_{15}S_2Na$ 731.0580 found 731.0600.

Compound 8. Compound **7** was dissolved in anhydrous DMF (20-50 μL) *in the dark* and oxidized PS A1 was added to the dye solution at a molar ratio of 1 to 120 (PS A1: compound **7**, assuming one mole of PS A1 had 120 repeating tetrasaccharide units). Oxime formation was carried out *in the dark* at room temperature for 16 h. The Alexa Fluor488® labelled PS A1 was purified by size exclusion column (sephacryl S-300 HR). Beer's law (equation 1) was used to calculate percent loading using the UV wavelength of the Alexa Fluor488® dye.

$A = \varepsilon c l$ (eq. 1)

A = UV absorbance at wave length 495 nm

ε is the molar absorptivity with units of L mol^{-1} cm^{-1}; for Alexa Fluor488® dye at 495 nm ε = 71,000

l = part length = 1 cm

c = concentration in mol L^{-1}

From equation 1; The concentration of Alexa Fluor488® attached to PS A1 ($C_{AF488^®}$) = A_{495}/(71,000 L mol^{-1} cm^{-1} *1 cm). By measuring absorbance of the Alexa Fluor488® labeled PS A1 at 495 nm, the concentration was determined and the number of moles of the dye attached to PS A1 (PS A1 does not absorb at wave length 495 nm) was then calculated. Since the number of moles for PS A1 added for the reaction is known, the molar ratio between Alexa Fluor488® dye and PS A1 can be calculated (equation 2).

Molar ratio = Alexa Fluor488® dye moles/ Alexa Fluor488®-PS A1 dye moles (eq. 2)

Under the assumption that PS A1 has 120 repeating units, the percent loading is given by the following equation 3:

Percent Loading = Alexa Fluor488® dye moles x 100/(Alexa Fluor488®-PS A1 dye moles x 120) (eq. 3)

Circular Dichroism

All CD measurements were carried out on a Chirascan CD Spectrometer (Applied Photophysics, Kingston Rd, Leatherhead, UK) equipped with a temperature controlled cuvette holder. All samples were analyzed in PBS. The concentration of all the samples were 2.5 mg/mL and a quartz cuvette with 0.2 cm path length was used to collect the data.

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Notes and references

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