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Exploring the Effect of Conjugation Site and Chemistry on the Immunogenicity of an anti-Group B *Streptococcus* Glycoconjugate Vaccine Based on GBS67 Pilus Protein and Type V Polysaccharide

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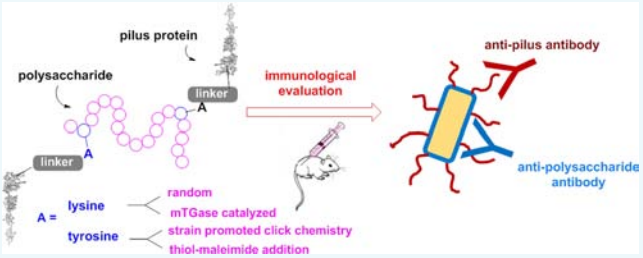
Supporting Information

ABSTRACT: We have recently described a method for tyrosine-ligation of complex glycans that was proven efficient for the site selective coupling of GBS capsular polysaccharides (PSs). Herein, we explored the effect of conjugation of type V polysaccharide onto predetermined lysine or tyrosine residues of the GBS67 pilus protein with the dual role of T-cell carrier for the PS and antigen. For the preparation of a conjugate at predetermined lysine residues of the protein, we investigated a two-step procedure based on microbial Transglutaminase (mTGase) catalyzed insertion of a tag bearing an azide for following copper-free strain-promoted azide−alkyne [3 + 2] cycloaddition (SPAAC) with the polysaccharide. Two glycoconjugates were obtained by tyrosine-ligation through the known SPAAC and a novel thiol-maleimide addition based approach. Controls were prepared by random conjugation of PSV to GBS67 and CRM_{197}, a carrier protein present in many commercial vaccines. Immunological evaluation in mice showed that all the site-directed constructs were able to induce good levels of anti-polysaccharide and anti-protein antibodies inducing osponophagocytic killing of strains expressing individually PSV or GBS67. GBS67 randomly conjugated to PSV showed carrier properties similar to CRM_{197}. Among the tested site-directed conjugates, tyrosine-directed ligation and thiol-malemide addition was elected as the best combination to ensure production of anti-polysaccharide and anti-protein functional antibodies (in vitro opsonophagocytic killing titers) comparable to the controls made by random conjugation, while avoiding anti-linker antibodies. Our findings demonstrate that (i) mTGase based conjugation at lysine residues is an alternative approach for the synthesis of large capsular polysaccharide−protein conjugates; (ii) GBS67 can be used with the dual role of antigen and carrier for PSV; and (iii) thiol-maleimide addition in combination with tyrosine-ligation ensures the production of anti-polysaccharide and anti-protein functional antibodies while maintaining low levels of anti-linker antibodies. Site-specific conjugation methods aid in defining conjugation site and chemistry in carbohydrate−protein conjugates.



INTRODUCTION

Glycoconjugate vaccines, prepared by covalent linkage of carbohydrates to proteins, have allowed tackling of diverse life-threatening infections.[1] Emergence of antibiotic resistance renders vaccination an even more attractive approach for the prevention of a number of infectious diseases. Conjugation of glycans to a protein carrier is key to engaging a T cell memory response against the sugar, susceptible to boost effect.[2] Different parameters have been found to influence the immunogenicity of glycoconjugates vaccines, such as the sugar length, the amount of incorporated sugar, the type of sugar or charge at the nonreducing terminal end, and even the chemistry and the linker used for conjugation.[3] To date, the effect of the conjugation site has seldom been systematically investigated. This is due to the fact that glycoconjugate vaccines are generally obtained by targeting with unspecific methods lysines or glutamates/aspartates on the surface of the protein.[3] The regioselectivity of these approaches is poor, due to the high accessibility of the conjugation sites. Novel methods for site selective conjugation of glycan antigens are now available.[4−6] Our group has demonstrated that the triazolidinone-ene insertion of bifunctional linkers onto the tyrosine residues of carrier protein in combination with Cu(I) catalyzed azide−alkyne [3 + 2] cycloaddition (CuAAC)[7,8] of glycans can facilitate the preparation of glycoconjugates with defined sugar and connectivity.[9,10] These novel tools can now aid deciphering the contribution of glycan, conjugation site, and linker on the immunogenicity of glycoconjugates.[11]

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Gram-positive *Streptococcus agalactiae* or Group B *Streptococcus* (GBS) is a major cause of morbidity and mortality among neonates, and the causative agent of invasive infections also in elderly people.[12] Vaccination of pregnant women has the potential to protect newborns from sepsis and meningitis associated with GBS.[13]The capsular polysaccharide (PS) is known as a major virulence factor, and based on its structure, ten different PS serotypes have been characterized.[14,15] Five of them, namely, Ia, Ib, II, III, and V are responsible for the vast majority of the disease in North America and Europe.[16] A trivalent GBS vaccine, composed of serotypes Ia, Ib, and III capsular polysaccharide−protein conjugates, is currently under phase II evaluation among pregnant women.[16]

Among the GBS surface antigens, pilus proteins have been identified through the *reverse vaccinology*[17] approach, as promising vaccine candidates.[18] We have recently proposed that conjugation of a polysaccharide to a protein of the same pathogen with the dual role of antigen could be a general approach to broaden the coverage of glycoconjugate vaccines.[19]

While doing so, we have shown that the use of strain promoted azide−alkyne [3 + 2] cycloaddition (SPAAC)[20,21] is more efficient than CuAAC for the incorporation of charged high-molecular-weight polysaccharides from the GBS capsule.[19] This two-step procedure ensures a highly consistent site-directed conjugation, and the ability to ascertain the integrity of the modified protein before conjugation to the polysaccharides.[19] These are considered two important features when preservation of the protein epitopes is mandatory. Based on genetic diversity, three different groups of GBS pili can be distinguished, namely, type 1, type 2a, and type 2b.[22,23] The developed method was applied to the conjugation of GBS PSII with Pl-1 protein GBS80.[24] When compared to a conjugate obtained by a classic random conjugation, the biomolecule made by the tyrosine ligation elicited comparable anti-polysaccharide and anti-protein antibodies that were effective in mediating opsonophagocytic killing of strains expressing exclusively PSII or GBS80 pilus protein and protecting the newborn mice against GBS infection after vaccination of the dams.[24] This demonstrated that tyrosine-ligation allows creation of more homogeneous vaccines, correlation of the immune response to defined connectivity points, and fine-tuning of the conjugation site in glycan−protein conjugates. Some levels of anti-linker antibodies were, however, generated by the linker employed for the copper-free click chemistry.[24]

In this context, we have undertaken work to explore the effect of the conjugation site and chemistry on the immunological activity of anti-GBS vaccine candidate prepared by conjugation of PSV to GBS67. Type V polysaccharide has accounted, together with types Ia and III, for the majority of isolates from infants and adults with invasive GBS disease in the United States.[21] Conjugation of this polysaccharide to TT has been proposed as a way to prevent infections caused by this emerging serotype.[25,26] GBS67 is an ancillary highly conserved three-domain protein of pilus 2a, which contributes to bacterial adherence to respiratory and intestinal epithelial cells, and to biofilm formation.[27,28]

An alternative to the protein chemical modification is represented by chemo-enzymatic approaches.[29,30] In particular, Transglutaminases (TGase) are a family of enzymes that catalyze the formation of a covalent bond between the γ-carbonyl amide group of glutamines and the primary amine of lysines.[31] We anticipated that TGase would allow the introduction of a functional group onto the lysine residues of the pilus protein for chemoselective condensation of the glycan.[32]

In the present study we synthesized a set of conjugates obtained by two different site-selective approaches and with diverse linkers to compare their in vivo immunological activity and the anti-linker antibody levels. To this end, we first synthesized a new linker for comparison of the tyrosine-selective conjugation of the polysaccharide through thiol-maleimide[33] addition and the previously reported copper-free azide−alkyne [3 + 2] cycloaddition.[19] Although it has been shown that the product of sulfhydryl addition to maleimides can undergo retro Michael side reactions,[34] glycoconjugates made by this method have been proven sufficiently stable for vaccine development.[35] A two-step conjugation strategy at lysine residues based on the microbial Transglutaminase (mTGase) catalyzed insertion of an azide linker bearing a Cbz-Gln-Gly (ZQG) tag was also tested for the coupling of the large-molecular-weight streptococcal polysaccharides. The potential of the conjugates to elicit anti-polysaccharide and anti-protein IgGs that recognize specifically the two antigens on the bacterial surface and mediate opsonophagocytic killing of strains expressing either PSV or GBS67, and the levels of anti-linker antibodies were finally evaluated in animal model, using as controls random conjugates of PSV with GBS67 and the genetically detoxified diphtheria toxin CRM_{197}, a carrier used in a number of commercial vaccines.[3,36]

RESULTS AND DISCUSSION

Synthesis of the Glycoconjugates. PSV is a complex anionic polysaccharide and Figure 1 illustrates its repeating unit, composed of seven monomers.

To explore the effect of the conjugation site and the linker on the immunogenicity of GBS67 conjugates with PSV, the set of novel biomolecules depicted in Figure 2 was designed. In constructs **1** and **2**, PSV was bound to the protein via tyrosine-ligation through SPAAC and thiol-maleimide addition, respectively, to compare the effect of different linkers on the

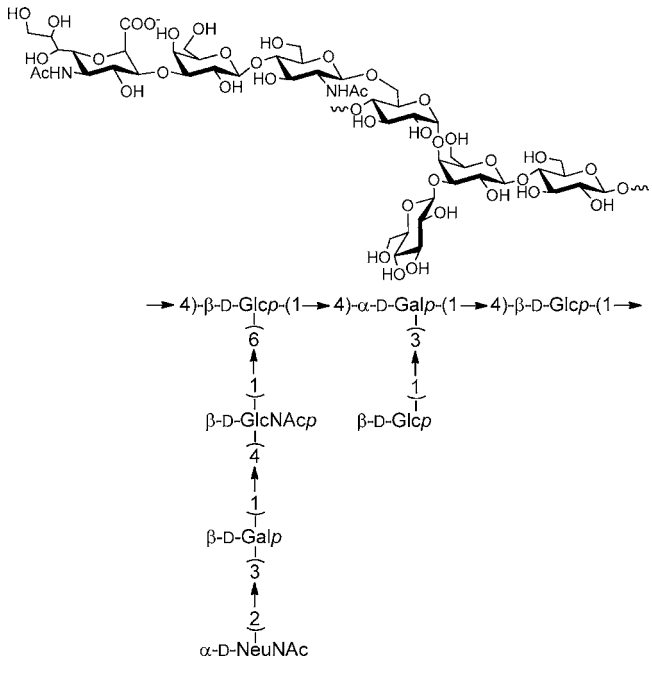

Figure 1. Chemical structure of PSV.

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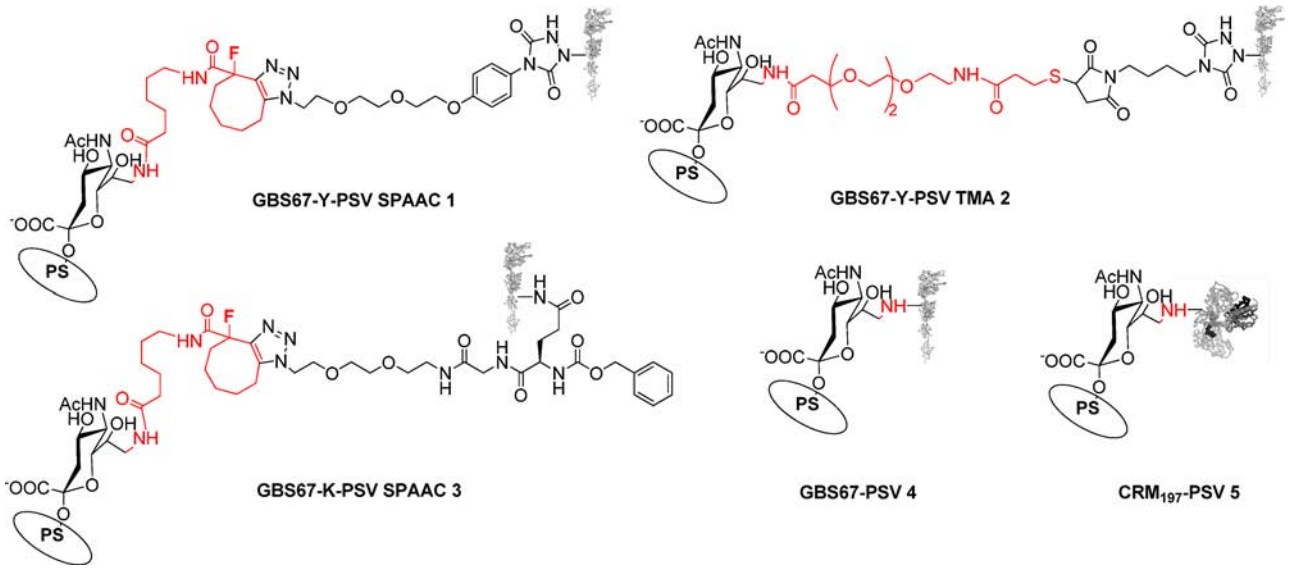


Figure 2. Set of synthesized glycoconjugates **1**−**5**.

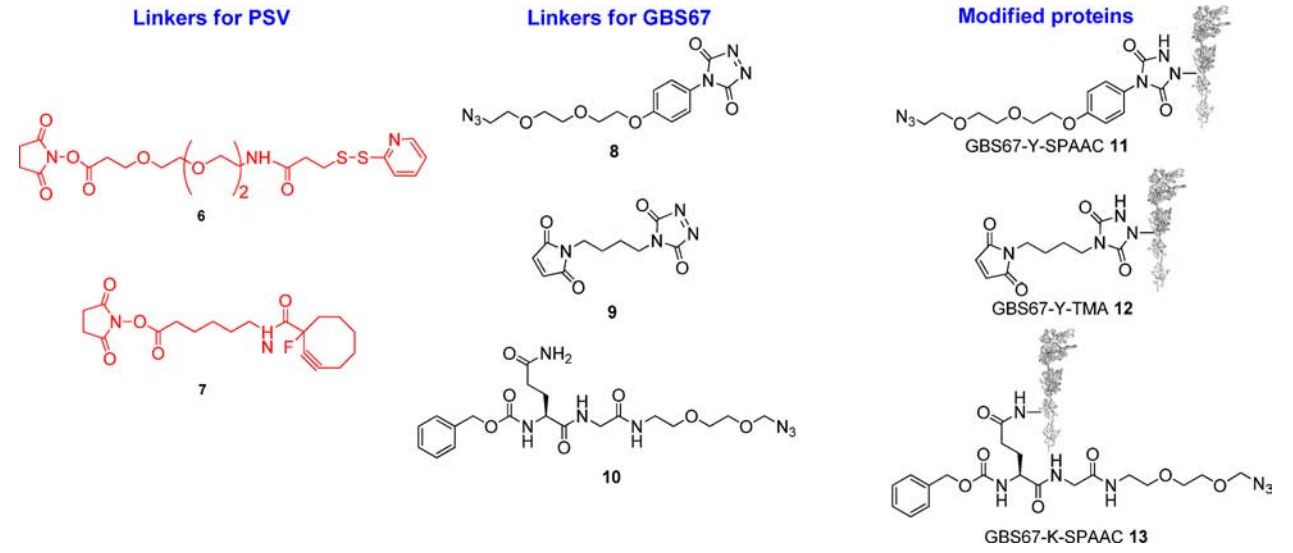


Figure 3. Linkers used for protein and polysaccharide modification.

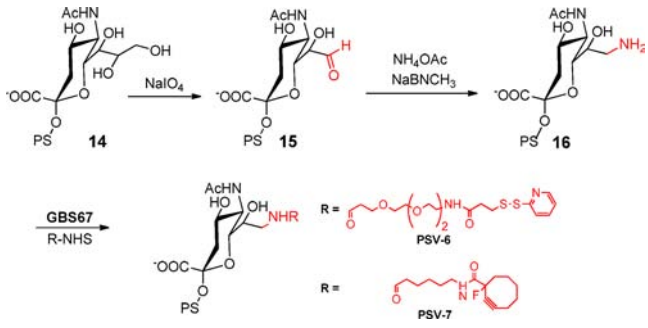
same site. Conjugate **3** was chosen to test the polysaccharide conjugation via SPAAC to predetermined lysine residues of the protein labeled with a ZQC-PEG-azide arm. Biomolecules **4** and **5** were made through classic random conjugations with GS67 and CRM_{197}.

To achieve the polysaccharide and protein modifications needed for site-specific conjugation, the linkers shown in Figure 3 were selected. The preparation of the conjugates commenced with a controlled periodate oxidation of PSV **14** that targeted 20% of the available diol system of NeuAc glycerol chain (Scheme 1).[37]

Following the reductive amination of the generated aldehyde intermediate **15**, PSV derivative **16** was coupled with linkers **6** or **7** to yield the products ready for condensation to the modified GBS67. Tyrosine-modified protein **11** and **12** were prepared by tyrosine-selective amination of GBS67 via triazolidinone-ene reaction in Tris buffer[9,10] with linkers **8**[18] and **9** (Scheme 2), respectively.

The synthesis of the novel linker **9** initiated from condensation of commercial 4-nitrophenyl chloroformate **17** with ethyl carbazate, and then N-Boc-1,4-butanediamine to give **18**. After basic promoted cyclization to obtain **19**, the Boc group was removed in HCl-dioxane. The hydrochloride **20** was precipitated to be next reacted with *N*-methoxycarbonylmaleimide, affording **21**. Oxidation with silica supported nitric acid afforded the target compound **9**.

Scheme 1. Synthesis of Modified PSV



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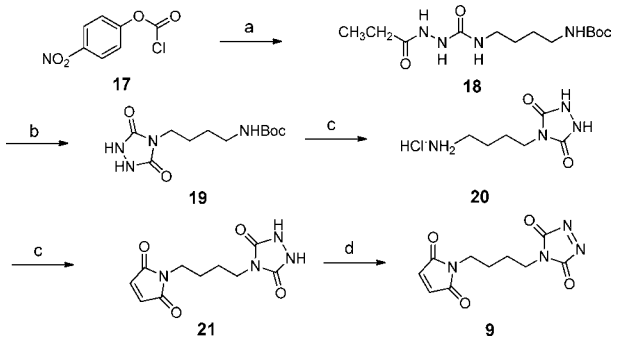
Scheme 2. Reactions Leading to Linker 9[a]



[a]Reagents and conditions: a. $EtOCONHNH_2$, Et_3N; $BocNHCH_2CH_2NH_2$, 66%; b. K_2CO_3, 65 °C; 43%; c. HCl, >95%; d. *N*-methoxycarbonylmaleimide, $NaHCO_3$, 20%; e. SiO_2−HNO_3, >95%.

As we have previously reported, ESI MS analysis of the digested peptides from CRM_{197} modified with triazolidinones **8** or **9** showed the insertion of 3.5 linkers, and the occurrence of the labeling primarily at Y744, while Y282/283, Y336/337, and Y403 were modified to a lesser extent (Figure 4).[19]

Figure 4. GBS67 pilus protein sequence. D1, D2, and D3 domains are colored in red, blue, and green, respectively. Modified tyrosine and lysine residues are yellow and green highlighted, respectively.

To label GBS67 at predetermined lysine residues, the protein and linker **10** (prepared as described in SI) were incubated in the presence of mTGase. An incorporation of an average of 2.5 adducts was observed at ESI MS. After combined tryptic, chymotryptic, trypsin-GluC, trypsin-AspN, and trypsin-chymotrypsin digestion, sequence coverage of 95% was accomplished. The generated peptides were analyzed by LC ESI MS, and semiquantitative estimation of the labeled sites identified K320, K340, K558, and K812 as the most heavily modified residues (Figure 4).

Albeit the crystal structure of GBS67 has not been resolved, the finding that both tyrosine-directed and mTGase-mediated conjugation targeted the D2 and D3 protein domains suggests that these are likely the more accessible portions of the protein.

For the preparation of the conjugates **1** and **3** via SPAAC, the polysaccharide PSV-7 was incubated overnight with the modified proteins **11** or **13** at the saccharide/protein ratios (w/w) reported in Table 1. Conjugate **2** was obtained by thiol-maleimide addition of PSV functionalized with **6**, following deprotection of the sulfhydryl groups with TCEP. While the copper-free conjugation showed good efficiency as compared to the reductive amination in terms of the amount of sugar loaded onto the protein, a moderate degree of incorporation was achieved by the thiol-maleimide addition.

The two controls **4** and **5** were obtained by direct reductive amination of oxidized PSV **15** with GBS67 and CRM_{197}, respectively, according to literature procedures.[37] The glycosylation ratios for the conjugates **1**−**5** were all in the range of 2−3 saccharide/protein (w/w).

Immunogenicity of the Glycoconjugates. To test the impact of the different conjugation site and chemistries in eliciting specific anti-PSV and anti-GBS67 antibodies, two groups of eight CD-1 mice were immunized with three subcutaneous injections of the prepared conjugates, at the dose of 0.5 μg in terms of polysaccharide content, and 1 μg based on protein content, respectively. Based on our previous experience with these antigens, these dosages are in a range that avoids saturation of the immune response against the polysaccharide and the protein, respectively, and allow better discrimination of possible differences among the conjugates.[24]

PSV conjugated with GBS67 and CRM_{197}, and unconjugated GBS67 were used as controls. Booster doses of the glycoconjugates were administered at 3 and 5 weeks after the initial vaccination.

The generated sera following the third administration were analyzed by ELISA for the levels of anti-polysaccharide and anti-protein IgG antibodies, respectively. To assess the antibody functionality the pooled sera from vaccinated mice were tested by in vitro killing-based opsonophagocytosis assay (OPKA).[38] This is a well-established assay that mimics in vivo bacterial killing by host effector cells, following opsonization by specific antibodies,[38] and therefore considered a robust surrogate of the protection induced by GBS vaccines.[39] To clearly determine the individual contribution of the polysaccharide and the protein in the three glycoconjugates, sera were assayed against two different strains (Table 2): 2603, which is heavily coated with PSV, and 515, expressing Pl-2a in association with a GBS type Ia polysaccharide capsule. An additional strain, CJB1111, that is a producer of both the polysaccharide and the protein, was used to determine additive effects of the antibodies against both PS and protein.

Table 1. Characteristics of the Synthesized Glycoconjugate 1−5

glycoconjugate	saccharide:protein stoichiometry[a] (w/w)	saccharide:protein in conjugate[b] (w/w)	free saccharide[c] (%)	conjugation efficiency[d] (%)
GBS67-Y-PSV SPAAC 1	3:1	2.5	<4.8	86
GBS67-Y-PSV TMA 2	6:1	3.0	14.7	50
GBS67-K-PSV SPAAC 3	3:1	2.0	<6.1	67
GBS67-PSV 4	2:1	1.8	<6.6	90
CRM_{197}-PSV 5	0.7:1	1.9	<6.3	40

[a]Amount of reagents used in the conjugation reaction. [b]Carbohydrate:protein ratio in the purified glycoconjugate. [c]Conjugated and unconjugated PS as estimated by HPAEC-PAD quantification of GlcNAc. [d]Amount of conjugated polysaccharide vs amount of polysaccharide used for conjugation.

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Table 2. Functional Activity of the Glycoconjugates 1−4 and Corresponding Controls

	Anti PSV activity		Anti PSV/GBS67 activity	Anti GBS67 activity	
glycoconjugate	Anti-PSV IgG Median titers[a]	OPKA titers (2603)	OPKA titers (CJB111)	Anti-GBS67 IgG Median titers[a]	OPKA titers[e] (515)
dose	*0.5 μg PSV*	*0.5 μg PSV*	*0.5 μg PSV*	*1 μg GBS67*	*1 μg GBS67*
Alum	<10	<30	<30	<10	<30
GBS67-Y-PSV SPAAC 1	354 (23−4396)	197	493	162621 (122197−183447)	260
GBS67-Y-PSV TMA 2	956 (30−4035)	731	1497	542567 (423899−639251)	323
GBS67-K-PSV SPAAC 3	145 (94−214)	294	628	163709 (119336−324948)	174
GBS67-PSV 4	3596 (811−9430)[b]	2465	4626	381001 (261968−492158)	336
CRM_{197}-PSV 5	2785 (1543−8275)[c]	1307	2372	-	-
GBS67	-	-	-	691079 (442301−1290000)[d]	529

[a]Median titers with 25−75% percentile range; statistical analysis was calculated according to the Mann−Whitney test. [b]4 vs 3 $p = 0.0006$. [c]5 vs 3 $p = 0.0003$. [d]GBS67 vs 1 $p = 0.001$; GBS67 vs 3 $p = 0.002$; GBS67 vs 4 $p = 0.03$. [e]OPKA titers from duplicates.

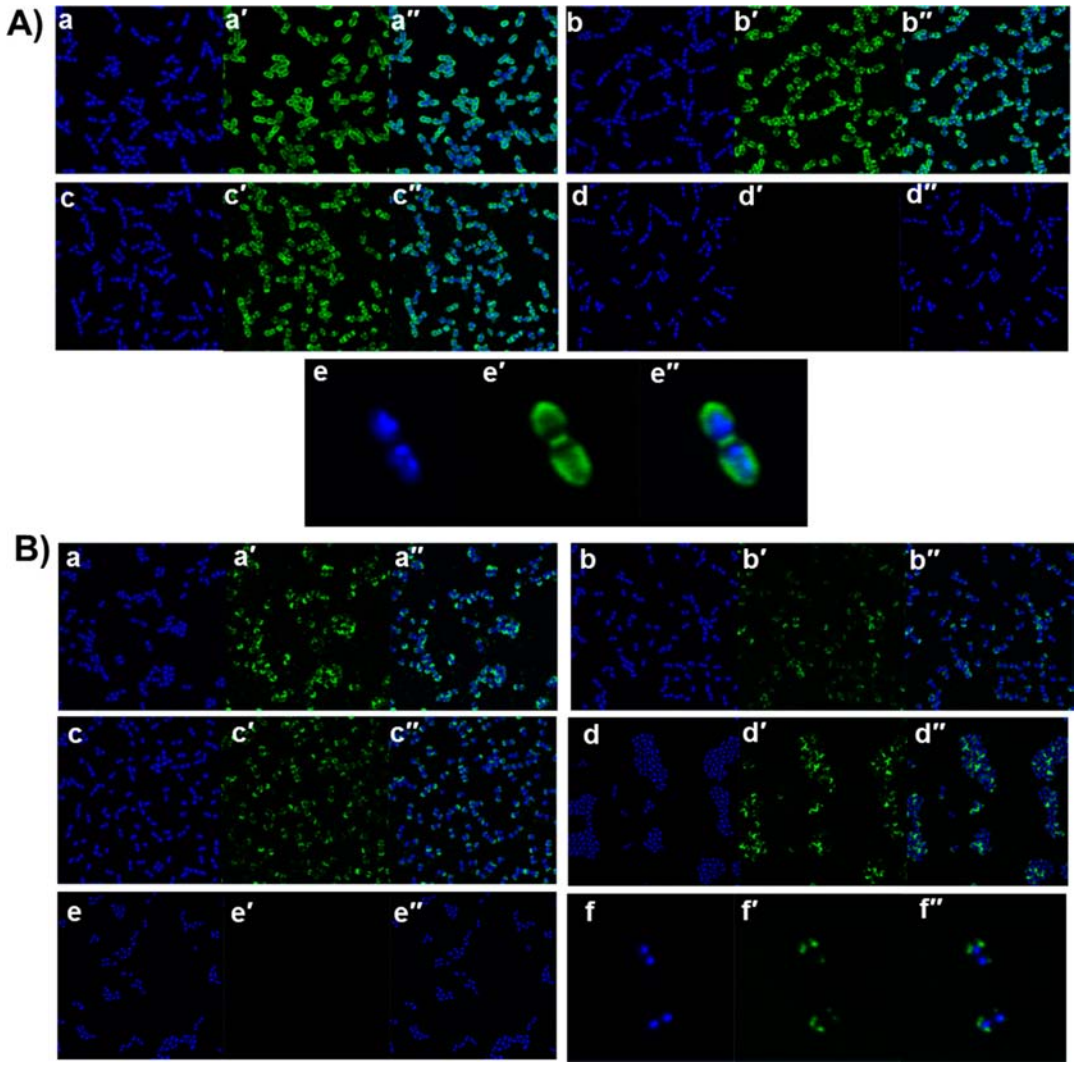

Figure 5. Immunofluorescence staining by confocal miscroscopy of PSV (A) and GBS67 (B) on the surface of GBS strain 2603VR and 515, respectively, with sera from conjugates GBS67-Y-PSV SPAAC **1** (a), GBS67-Y-PSV TMA **2** (b), GBS67-K-PSV SPAAC **3** (c). Sera against unconjugated GBS67 and PSV (Ad) and the conjugate GBS80-PSII (Be) were the negative controls for the polysaccharide and protein detection, respectively. Anti-GBS67 serum was the positive control for the protein (Bd). (Ae) and (Bf) are the magnifications of (Aa) and (Ba), respectively. Sera were diluted 1:500. In panels a−f staining of the bacteria with DNA counterstain DAPI (4′,6-Diamidino-2-Phenylindole Dihydrochloride) is shown; in panels a′−f′ is the staining in the presence of anticonjugate serum; panels a″−f″ are superimpositions of the other two panels.

By immunofluorescence staining experiments of sera collected after the third dose, we observed that our glycoconjugates **1**−**3** were all able to induce antibodies binding the surface of GBS strains expressing either PSV or GBS67 (Figure 5). Staining of the capsule and the pili are enlarged in Figure 5Be and Bf, respectively.

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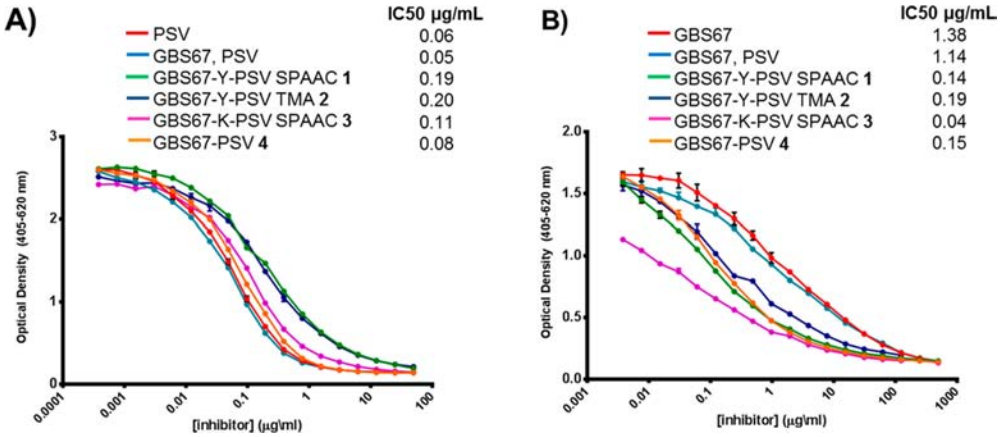

Figure 6. (A) Competitive ELISA of anti-PSV polyclonal sera generated from mice immunized with PSV conjugated to a nonpilus protein, using HSA-PSV as coating reagent. PSV and its mixture with GBS67 were the controls. (b) Competitive ELISA of anti-GBS67 polyclonal sera using the protein as coating reagent. GBS67 and its mixture with PSV were the controls.

As shown in Table 2, all the conjugates elicited anti-PSV antibody levels which mediated opsonophagocytic killing of type V GBS. We observed some differences among the anti-polysaccharide IgG titers induced by the diverse conjugates made by site-selective chemistry. In particular, higher titers were achieved with conjugate **2** made by thiol-maleimide addition onto tyrosine, which differed only 2−3-fold (not significant by statistical analysis) from the random conjugates **4** and **5**, followed by conjugates **1** and **3**, which gave titers from 5- to 20-fold lower than the controls. In agreement with the elicited anti-PS IgG levels, the OPKA titers measured against strain 2603 showed that the sera from conjugates **1**−**3** were able to induce opsonophagocytic killing of the capsulated strain. This indicated that the antibody levels raised by conjugates **1**−**3** were sufficient to induce an effective functional activity. Taking in considerations that 3-fold differences are within the assay variability, conjugate **2** exhibited an OPKA titer comparable to that of controls **4** and **5**. Sera from conjugates **1** and **3** induced OPKA titers from 4- to 10-fold lower than the random conjugates **4** and **5**. The same trend was found when sera were tested against strain CBJ111, which expressed both PSV and GBS67, except that the titers were shifted to higher levels.

These data suggested that, irrespective of the conjugation site and chemistry, GBS67 behaves as a carrier for PSV. Among the tested site-directed conjugates, the combination of thiol-maleimide addition and tyrosine-ligation appeared more similar to controls **4** and **5** in terms of antibody levels and functionality.

When an identical protein dose was supplied, all the conjugates induced high levels of anti-protein antibodies. Construct **2** prepared by thiol-maleimide at Y gave IgG titers comparable to that of the unconjugated protein. These IgG levels were significantly higher than those achieved with the conjugates **1** and **3** prepared by click chemistry at Y or K, respectively.

OPKA titers measured against strain 515 (Table 2) showed that compounds **1**−**3** were all endowed with the same levels of opsonophagocytic activity with respect to random conjugate **4**. Hence, the incorporation of PSV did not impair the functional activity of the antibodies generated against the pilus protein, regardless of the conjugation site and chemistry.

These results indicated that tyrosine-directed conjugation through thiol-maleimide addition resulted in the highest anti-protein antibody levels in the set, and the amounts of antibodies elicited by the three constructs were in all cases sufficient to induce efficacious bacterial killing.

To analyze the binding of the recognition of polysaccharide and protein epitopes in the set of prepared vaccines, glycoconjugates **1**−**4** were assayed as competitors of the binding of anti-PSV and anti-GBS67 serum to HSA-PSV and GBS67, respectively (Figure 6). All the conjugates were equipotent inhibitors of the binding of the anti-polysaccharide serum to PSV. When the anti-protein serum was employed, products **1**−**4** were better inhibitors of the protein, as previously observed with a similar analysis on GBS80.[24] This is due to the multivalent exposition of protein along the polysaccharide chain.[24] The conjugates **2** and **3** prepared on Y with two different chemistries exhibited no difference as inhibitors, indicating that the protein epitopes were identically exposed in both constructs. The conjugate **3** made at K via mTGase was a 4−5-fold stronger inhibitor than products **1** and **2** obtained by conjugation at Y. Overall, in agreement with observed immunogenicity, no remarkable difference was found on the exposition of the saccharide and protein epitopes on the glycoconjugates.

Anti-Linker Antibodies. The triazole ring introduced by the click chemistry has been shown in some examples to induce specific antibodies.[40] A possible drawback connected with the presence of rigid rings in the spacer used for conjugation is that the immune response could be directed to the linker rather than to the glycan.[41] We have previously observed that, while the triazole system is not particularly immunogenic *per se*,[11] antibodies were generated against the cyclooctyne employed for the SPAAC.[24] The presence of these condensed rings, however, was not detrimental for the efficacy of a GBS80-PSII conjugate vaccine.[24] To evaluate the presence of antibodies when the thiol-maleimide addition was used as alternative coupling chemistry, construct **2** was designed. We, therefore, evaluated the possible presence of anti-linker antibodies in our set of biomolecules. For this purpose, the sera generated against conjugates **1**−**3** were assayed against a reagent presenting the triazole ring generated by the azide−alkyne [3 + 2] cycloaddition and a series of constructs reproducing the portion of each conjugate included between the modified sialic acid of the polysaccharide and the amino acidic residue targeted for conjugation.

For the incorporation of the sialic acid, we employed a tetrasaccharide [α-D-Glcp(1 → 4)-α-D-NeuNAc-(2 → 6)]$_2$

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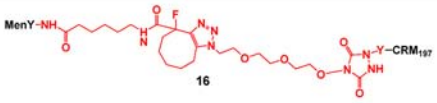
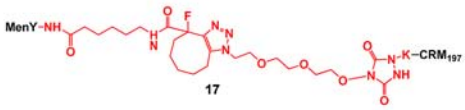
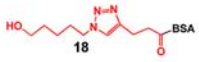
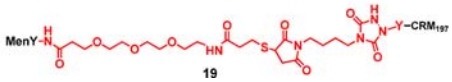
Table 3. ELISA Analysis of Anti-Linker Antibodies[a]

Coating reagent	Anti GBS67-Y-PSV SPAAC 1 IgG median titer	Anti GBS67-Y-PSV TMA 2 IgG median titer	Anti GBS67-K-PSV SPAAC 3 IgG median titer
 16	5971 (1600-12800)	<10	nd[b]
 17	nd	<10	1131 (50-6400)
 18	27 (10-100)	nd	21 (10-100)
 19	<10	47 (25-100)	nd

[a]Serum against the different conjugates **1**−**3** was tested against the ELISA plate coated with the indicated reagent. Titers were expressed as median with 25−75% percentile range. [b]Not done.

(degree of polymerization 2; DP2) fragment from meningococcal serogroup Y (MenY) capsular polysaccharide, possessing only the terminal end sialyl moiety in common with GBS polysaccharide.[24] In this manner, any interference from the carrier and the other sugar residues could be rolled out. Screened sera showed the presence of relatively high levels of anti-linker IgGs directed against the cyclooctene (Table 3). By contrast, almost no detectable amount of antibodies was found to be elicited against the triazole rings present in the structures **1** and **3**, respectively. Notably, the linkers used for thiol-maleimide addition induced an extremely low anti-linker response compared to the cyclooctene ring generated by SPAAC.

CONCLUSIONS

While among the different interdependent variables that affect the immunogenicity of glycoconjugate vaccines, large focus has been addressed to the sugar part, the contribution of the conjugation site and linkers is almost unexplored.[11] The use of site-selective chemistry ensures a high consistency in the synthesis of glycoconjugates and correlation of the immunogenicity with determined attachment sites.[9] We have recently developed a method for tyrosine-ligation of complex glycans based on strain-promoted azide−alkyne [3 + 2] cycloaddition (SPAAC) that we have proven efficient for the site-selective coupling of GBS capsular polysaccharides.[19,24] We have also shown that tyrosine-directed conjugation of GBS PSII results in high levels of functional anti-carbohydrate and anti-protein antibodies compared to random classic conjugation.[24] However, antibodies against the cyclooctene ring generated by the copper-free click chemistry were produced.

Chemo-enzymatic approaches are emerging as alternatives to chemical modification of native residues.[30] Herein, we exploited lysine or tyrosine-directed conjugation approaches to investigate the effect of conjugation of type V polysaccharide onto the predetermined site of GBS67 pilus protein that was used with the dual role of carrier and antigen. To do so, we synthesized two glycoconjugates by the triazolidinone-ene reaction of the tyrosine residues of GBS67 with bifunctional linkers that we subsequently coupled to PSV by two click reactions, SPAAC and thiol-maleimide addition, respectively.[9,19] For preparation of a conjugate at predetermined lysine residues of the protein, we used an approach based on mTGase catalyzed coupling of a Cbz-Gln-Gly (ZQC) tag bearing an azide for following SPAAC with the polysaccharide. Conjugation by SPAAC occurred at higher efficiency of polysaccharide incorporation onto the protein when compared to thiol-maleimide addition. mTGase based conjugation at lysine residues was demonstrated here as a feasible approach for the synthesis of glycoconjugate vaccines from large bacterial polysaccharides.

To evaluate the effect of the connectivity and linker used for conjugation, the three constructs were tested in mice for their capability to induce anti-PSV and anti-GBS67 functional antibodies, using as controls PSV conjugated to the pilus protein and CRM_{197}, a well-known protein carrier. We found that, irrespective of the conjugation site and chemistry, all the constructs were able to elicit good levels of anti-polysaccharide and anti-protein antibodies that induced osponophagocytosis in strains individually expressing PSV or GBS67. GBS67 can, therefore, be used with the dual role of antigen and carrier for PSV. Among the tested site-directed conjugates, tyrosine-directed ligation and thiol-malemide addition was elected as the best combination to ensure production of anti-polysaccharide and anti-protein functional antibodies avoiding anti-linker antibodies. Site-specific conjugation methods are, therefore, important tools to define linker and conjugation site in carbohydrate−protein conjugates.

EXPERIMENTAL PROCEDURES

Bacterial Strains. The purification process of GBS serotype V strain 2603 was based on previously described procedures. Briefly, the bacterial pellet was recovered by centrifugation at 4000 rpm for 20 min and incubated with 0.8 N NaOH at 37 °C for 36 h. After centrifugation at 4000 rpm for 20 min, 1 M Tris buffer (1:9, v/v) was added to the supernatant and diluted with 1:1 (v/v) HCl to reach a neutral pH.

GBS67 Expression. Pilus protein was expressed in *E. coli* as described in the literature.[28]

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Tyrosine-Ligation on GBS67. As reported in the literature,[9] to the protein (0.001 μmol) in Tris 0.5 M pH 7.4 (67 μL) at 4 °C was added four portions of linker **8** or **9** in acetonitrile every minute (total of 20 equiv). The mixture was agitated at 4 °C for 30 min. The mixture was desalted and buffer-exchanged to PBS pH 7.4 two times using Zeba 7 kDa cutoff spin columns (Thermo Fisher).

GBS67 Labeling with *Z-Q-G-NH-(PEG)$_3$-N$_3$* 10 by Microbial Transglutaminase (mTGase). 250 uL GBS67 protein (4.27 mg/mL) was added to 1.25 mL Z-Q-G-NH-(PEG)$_3$-N$_3$ **10** (8 mg/mL) in 10 mM Tris pH 8 and 50 μL of MTGase (50 mg/mL in PBS) was added. Reaction was incubated overnight at 37 °C. LC MS showed addition of an average of 2.5 adducts. Reaction was then passed through a 50 kDa Amicon concentrator column and washed 3× with PBS. Recovered material was analyzed by LC MS giving modified GBS67 **13** in 43% overall yield.

Polysaccharide Modification. Oxidation of GBS type V PS was targeted to 20% of sialic acid residues.[37] The native polysaccharide (100 mg) was stirred with 151 μL 0.1 M of sodium periodate in 9.8 mL of 10 mM sodium phosphate buffer in the dark, for 2 h at RT. The mixture was purified by tangential flow filtration (Sartocon 30 kDa filter, Sartorius).

Insertion of Linker 6 on PSV. For the preparation of GBS67-PSV glycoconjugate through random chemistry at lysine, the oxidized polysaccharide (20 mg) was dissolved in a 200 mM sodium phosphate/2 M NaCl buffer pH 7.2 and mixed with the protein (10 mg) with a final concentration of PSV of 4 mg/mL and for GBS67 2 mg/mL, the following procedure was followed. The oxidized PS (80 mg) dissolved in H_2O milli-Q at a concentration of 5 mg/mL, was subjected to reductive amination by treatment with ammonium acetate (8.8 g) and sodium cyanoborohydride (1.6 g). After incubation for 5 days at 37 °C, the crude mixture was filtered by Tangential Flow Filtration (TFF, Sartocon 30 kDa filter, Sartorius). Quantification of polysaccharide and amino groups was performed by colorimetric assays. Partially aminated polysaccharide (30 mg, corresponding to 4.5 × 10^{-3} mmol of oxidized sialic acid) was dissolved in 3 mL of DMSO, MFCO-*N*-hydroxysuccinimide **5** (Berry & Associates, 10 equiv) and 50 μL of triethylamine was added. Reaction was stirred for 3 h at RT. The crude was precipitated and washed (8 × 30 mL) in EtOAc at 4 °C in order to remove the excess unreacted linker. Then the PS was lyophilized and sialic acid was quantified by colorimetric assay.

Insertion of Linker 7 on PSV. Aminated PS (50 mg corresponding to 7.5 × 10^{-3} mmol of oxidized sialic acid) was dissolved in 5 mL of DMSO, PEG4-SPDP **7** (Thermo-Scientific 10 equiv) and 50 μL of triethylamine was added. Reaction was stirred for 3 h at RT. The crude was precipitated and washed (8 × 30 mL) in ethyl acetate at 4 °C in order to remove the excess unreacted linker. Then reduction of the 2-pyridyldithio was performed adding a Tris(2-carboxyethyl)-phosphine hydrochloride solution (Sigma-Aldrich, 3 equiv) stirring 3 h at RT. The polysaccharide was dialyzed against water in a 30 kDa Amicon centriguge filter, and sialic acid was estimated.

Conjugation of Modified GBS67 and PSV. Modified GBS67 (3.5 mg) and activated polysaccharide (10.5 mg, or as described in Table 1) were mixed in PBS at pH 7.2 and incubated overnight at RT in the dark.

Conjugation was monitored by SDS-PAGE 3−8% of polyacrylamide in Tris acetate. The conjugates were purified by a CHT hydroxyapatite column (Biorad laboratories). In the first step of purification, unreacted protein was removed by elution with 2 mM sodium phosphate pH 7.2 (90 mL, 1 mL/min) followed by 400 mM sodium phosphate pH 7.2 (90 mL, 1 mL/min). Then the conjugate was purified from free polysaccharide by another run on the same column with 2 mM sodium phosphate/550 mM NaCl pH 7.2 (50 mL, 0.5 mL/min), 10 mM sodium phosphate pH 7.2 (50 mL, 1 mL/min), 35 mM sodium phosphate pH 7.2 (40 mL, 1 mL/min), 400 mM sodium phosphate pH 7.2 (40 mL, 1 mL/min). The conjugate is detected by measuring UV absorption at 215, 254, and 280 nm. Protein content in the purified glycoconjugates was determined by micro-BCA (Thermo-scientific).

Total Saccharide Was Quantified by HPAEC-PAD. A standard sample with five increasing concentrations of GBS PSV ranging between 1.2 and 24.0 μg/mL (as saccharide powder/mL) in saccharide content was prepared for building a calibration curve. Four GBS PSV samples were prepared targeting final concentrations in the calibration curve range. The reference and analytical samples for GBS PSV were prepared in 4 M trifluoroacetic acid, incubated at 100 °C for 2 h, dried under vacuum (SpeedVac Thermo), and suspended in water All analytical samples were filtered with 0.45 μm Acrodisc (Pall) filters before analysis. HPAEC-PAD analysis was performed with a Dionex ICS3000 equipped with a CarboPac PA1 column (4 × 250 mm; Dionex) coupled with PA1 guard to column (4 × 50 mm; Dionex). Samples (50 μL injection volume) were run at 1 mL/min, using isocratic elution with 24 mM NaOH, followed by a washing step with 0.5 M NaOH. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A quadruple-potential waveform for carbohydrates was applied. Unconjugated saccharide was separated by SPE C4 hydrophobic interaction column (0.5 mL resin, Bioselect, Grace Vydac) and subsequently estimated by HPAEC-PAD analysis. The resulting chromatographic data were processed using Chromeleon software 6.8 (Dionex).

Preparation of PSV-HSA for ELISA Coating. To HSA (Sigma), at a concentration of 10 mg/mL in 100 mM MES (Sigma) buffer pH 5, EDAC (1.5 equiv relative to HSA carboxylic groups) was added, and the mixture was gently mixed to allow complete solubilization; then, ADH (13.4 eq relative to HSA carboxylic groups) was added to the solution. The reaction was kept under gentle stirring for 1 h at RT. Next, the reaction was quenched by adding 1/6 of the reaction volume of 400 mM sodium phosphate buffer, pH 7.2.

The conjugation reaction was performed at GBS PSV concentration of 5 mg/mL in 100 mM MES/250 mM NaCl buffer pH 5.0 EDAC (1:1, mol PS/mol EDAC) and the SulfoNHS (1:1, mol PS/mol SulfoNHS) was added to GBS PSV solution.

After the reagents were dissolved, HSA-ADH (2:1 protein/polysaccharide w/w) was added and the reaction was incubated at RT under gently stirring for 12−15 h, at which time the reaction was quenched by adding 1/5 of the reaction volume of 400 mM NaPi buffer pH 7.2. The crude reaction was purified by size-exclusion chromatography (SEC) on a Sephacryl S400 resin (G&E Healthcare). The chromatographic step was performed on Akta system (G&E Healthcare) at 2 mL/min flow rate, detecting the conjugate by measuring UV absorption at 215, 254, and 280 nm.

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Periodate Oxidation of MenY DP2. MenY DP2 was obtained by controlled acidic hydrolysis of MenY polysaccharide and purifications of different size oligosaccharide by anionic exchange chromatography on Mono Q column as described in the literature.[42] 50% of sialic acid residues were targeted for oxidation. MenY DP2 (100 mg) was stirred with 491 μL 0.1 M of sodium periodate in 9.5 mL of 10 mM sodium phosphate buffer in the dark, for 2 h at RT. The mixture was purified by gel filtration chromatography on a Sephadex G-10 column (G&E Healthcare) using 10 mM NaCl pH 7.2 as elution buffer (190 mL, 1 mL/min). Subsequently, reductive amination and linker insertion were performed with the same conditions reported for GBS PSV. CRM_{197} modified at tyrosine and lysine residues (CRM_{197}-Y-N_3, CRM_{197}-Y-mal, and CRM_{197}-K-N_3) was prepared similarly as described above for GBS67.

Preparation of Reagents for Analysis of Anti-Linker Antibodies. For the conjugation of CRM_{197}-Y-N_3 and CRM_{197}-K-N_3 to modified MenY DP2, to 1.5 mg of each protein (2.5×10^{-5} mmol) in 10 mM of sodium phosphate buffer pH 7.2, the saccharide (2.1 mg, 60 equiv) was added at a final concentration of 2 mg/mL in terms of protein. The mixture was incubated overnight at RT. Conjugation was monitored by SDS-PAGE 4−8% of polyacrylamide in MOPS. Unreacted saccharide was removed by 10 cycles of filtration with Vivaspin (30 kDa MW cutoff). The loading of saccharide was determined by MALDI-TOF MS analysis (Bruker Daltonics). For the conjugation of CRM_{197}-Y-mal to modified MenY DP2, MenY tetrasaccharide was activated with PEG4-SPDP as described for PSV. Conjugation was carried out as described above.

Immunization Studies in Mice. All animal studies were carried out in compliance with current Italian legislation on the care and use of animals in experimentation (Legislative Decree 116/92) and with the Novartis Animal Welfare Policy and Standards. Protocols were approved by the Italian Ministry of Health (authorization 110/2012-B) and by the local Novartis Animal Welfare Body (authorization AWB 201114). Groups of 10 CD-1 mice were immunized by intraperitoneal injection of 1 μg in protein content of each glycoconjugate using alum hydroxide as adjuvant. Alum hydroxide, GBS67 and GBS67-PSV, and CRM_{197}-PSV were used as controls. Mice received the vaccines at days 1, 21, and 35. Sera were bled at days 1, 35, and 49.

ELISA. Direct enzyme-linked immunosorbent assay titers of PS-specific and protein antibody were determined using as coating reagents PSV-HSA, GBS67, or constructs with different linkers. Microtiter plates (NUNC Polysorp; Nalge Nunc International Corp., Rochester, NY) were coated by adding 100 μL per well of coating reagent (1−1.5 μg/mL) in PBS 1× pH 7.2. The plates were incubated overnight at 4 °C and were washed with PBS containing 0.05% Tween 20 (PBS-T), then blocked with 0.5% bovine serum albumin in PBS for 1.5 h at 37 °C. The wells were then filled with 100 μL of serum at dilution 1:400 in PBS and incubated at 37 °C for 1 h. After 3 washes, 100 μL per well of peroxidase-labeled goat anti-mouse was added (Sigma-Aldrich) and plates incubated for 1.5 h at 37 °C. The plates were again washed 3 times with PBS-T, and finally 100 μL of peroxidase substrate (1 μg/mL in diethanolamine pH 9.8) was added to each well, following incubation of the plates for 30 min at room temperature. The reaction was stopped by the addition of 100 μL of a solution of EDTA 7% and the plates were read immediately at 405 nm.

Competitive ELISA. 96-well Microtiter plates (NUNC Polysorp; Nalge Nunc International Corp., Rochester, NY) were coated by adding 100 μL per well of GBS67 or PSV (1.5 μg/mL) in PBS 1× pH 7.2. The plates were incubated overnight at 4 °C and were washed three times with PBS containing 0.05% Tween 20 (PBS-T), then blocked with 0.5% bovine serum albumin in PBS for 1.5 h at 37°. A pool of antiserum versus GBS67 or PSV (dilution 1:1500 in PBS 1× pH 7.2) was incubated 15 min at RT with different concentrations of the protein used as inhibitor in a final volume of 100 μL and then transferred into coated plates. After 2 h of incubation at 37 °C, plates were washed three times and of peroxidase-labeled goat anti-mouse diluted 1:10 000 (100 μL for well, Sigma-Aldrich) was added and incubated for 1.5 h at 37 °C. The plates were again washed 3 times with PBS-T, and finally peroxidase substrate (100 μL of a 1 μg/mL solution in diethanolamine pH 9.8) was added to each well, following incubation of the plates for 30 min at room temperature. Then the plates were read immediately at 405 nm. The statistical and graphical analysis was performed using GraphPad Prism 6 software.

Confocal Microscopy. To verify GBS67 and polysaccharide capsule type V localization on the cell surface, bacterial cultures of GBS 515 and 2603VR strains were grown in THB at 37 °C to late logarithmic phase (OD_{600} = 1), washed once with PBS, suspended in the same volume of 4% formaldehyde, and incubated 15 min at room temperature on Polysine slides (Thermo Scientific). After 4 washes with PBS, bacteria were incubated at room temperature for 30 min in 100 μL PBS with mouse polyclonal antibodies against pilus components or PS capsule (1:500). After two washes with PBS, bacteria were incubated for 15 min at room temperature with goat anti-rabbit−Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) at 1:1000. After three washes with PBS, the slides were mounted with ProLong Gold antifade reagent containing DAPI (4′,6′-diamidino-2-phenylindole; Molecular Probes). Images were acquired by using a Plan-Apochromat/1.40 oil 100× objective mounted on a Zeiss LSM 710 confocal microscope (Carl Zeiss).

Opsonophagocytosis Killing Assay (OPKA). The functional activity of the sera was determined in OPKA assay. HL-60 cells were grown in RPMI 1640 with 20% fetal calf serum. Incubation was at 37 °C with 5% CO_2. HL-60 cells were differentiated to neutrophils with 0.78% dimethylformamide (DMF) and after 4−5 days were used as source of phagocytes.

Serum antibodies serially diluted in HBSS red were mixed with 6×10^4 CFU per well of GBS type V strain 2603 cells or CJB111. HL-60 cells (2×10^6 cell/well) and rabbit complement (diluted at 2−10% in water) were added and incubated at 37 °C for 1 h under shaking. Before (T_0) and after (T_{60}) incubation, the mixtures were diluted and plated in blood agar plates. (NUNC Polysorp; Nalge Nunc International Corp., Rochester, NY) Each plate was then incubated overnight at 37 °C with 5% of CO_2 counting CFUs the next day. OPA titer was expressed as the reciprocal serum dilution leading to 50% killing of bacteria and the % of killing is calculated as follows

$$\text{killing}\,\% = \frac{T_0 - T_{60}}{T_0}$$

where T_0 is the mean of the CFU counted at T_0 and T_{60} is the average of the CFU counted at T_{60} for the two replicates of each serum dilution.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00365.

Synthesis of linkers **9** and **10**, ESI MS spectra of modified GBS67, mapping of labeled sites (PDF)

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Notes

The authors declare no competing financial interest.

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