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ORIGINAL ARTICLE

Development and Immunogenicity of a Brazilian Glycoconjugate vaccine against Meningococcal W in a Pilot Scale

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Abstract

Recent changes in the epidemiology of meningococcal have been reported and meningococcal group W (MenW) has become the third most prevalent group isolated in Brazil in the last 10 years. In this study we have developed a conjugate vaccine for MenW using a modified reductive amination conjugation method through a covalent linkage between periodate-oxidized MenW non-*O*-acetylated polysaccharide and hydrazide-activated monomeric tetanus toxoid. Process control of bulks was done by physicochemical analysis including polysaccharide and protein quantification, high performance liquid chromatography – size exclusion chromatography, capillary electrophoresis, and hydrogen nuclear magnetic resonance. Conjugate bulks were best produced with concentration of polysaccharide twice as high as protein, at room temperature, and pH approximately 6.0. A scaled-up bulk (100 mg scale) was formulated and inoculated intramuscularly in mice in a dose–response study (0.1, 0.5, 1.0 and 10.0 µg of polysaccharide/dose). The immunogenicity of conjugate bulks was determined by serum bactericidal assay and ELISA assays of serum from immunized mice. ELISA and SBA titers revealed high titers of IgG and demonstrated the functionality of the antibodies produced in all doses studied 15 days after the third dose. However, significant differences were observed among them by ELISA. In conclusion, this study established the best conditions to produce MenW conjugate bulks and showed the efficacy of the obtained conjugate bulk in induce a good immune response in mice. Further experiments will need to be done to scale up the conjugation reaction and then allow the use of this conjugate in clinical trials.

Keywords Conjugate vaccine · Meningococcal vaccine · Group W meningococci · Immunological evaluation

Introduction

Neisseria meningitidis (N. meninigitidis) defined as a bacterium that belongs to the commensal microbiota of the human upper respiratory tract. However, some of the clonal lineages can cause invasive disease, in both endemic and epidemic forms. Meningococci are encapsulated bacteria and the antigenic properties of the capsular polysaccharide (CPS) are the basis of its classification into groups. Twelve groups are universally recognized, but only six (A, B, C, W, Y, X) are associated with almost all clinical cases worldwide [1]. Their relative importance as causative agents varying considerably across geographical locations, period and age of the hosts. Frequent unexplained changes in group incidences and emergence of new virulent strain variants and higher levels of invasive disease have been observed for specific groups and clonal complexes globally [2]. In this scenario vaccination is considered as the most effective and

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cost-efficient means of preventing infectious diseases caused by encapsulated bacteria [3].

There are three currently licensed conjugate vaccines targeting meningococcal groups A, C, W and Y (MenACWY vaccines). These vaccines include capsular polysaccharides from each of the four groups individually conjugated to a carrier protein. These quadrivalent conjugate vaccines include MenACWY-DT (Menactra®; Sanofi Pasteur), which uses diphtheria toxoid as the carrier protein; MenACWY-CRM_{197} (Menveo®; GlaxoSmithKline), which uses a non-toxic mutant of diphtheria protein, CRM_{197}; and MenACWY-TT (Nimenrix®; Pfizer Inc), which uses tetanus toxoid [4]. Recently, a MenACWY-TT (MenQuadfi®, Sanofi Pasteur) was licensed in United States to replace Menactra® which contained diphtheria toxoid as carrier protein [4].

Sporadic cases of meningitis caused by *N. meningitidis group W* (MenW) have been reported from the African meningitis belt since 1981. The first large outbreak of MenW disease occurred during the 2000 Hajj in Saudi Arabia, with over 300 cases reported worldwide. During 2001–2003, a major MenW epidemic occurred in Burkina Faso [5].

This group emerged in the South Cone of South America in the mid-2000s and in the United Kingdom in 2009–2010. Strains belonging to sequence type (ST) 11 are the most prevalent associated with hyperinvasive disease [6]. The re-emergence of these MenW strains has been observed globally since 2010 in regions such as the African meningitis belt, France, and Chile [7]. The MenW isolated from the South Cone of South America are showed by whole genome sequencing to be part of ST11 but with some diversity [8, 9]. Since 2009–2010, the United Kingdom has been detecting an increase in disease caused by an endemic hypervirulent strain of MenW that began in older adults and subsequently spread to people in all age groups [10]. In response to these trends national immunization programs have offered tetravalent conjugate vaccines (A, C, W and Y) in the United Kingdom and South America [11, 12]. Invasive meningococcal disease (MD) caused by MenW has been associated with atypical gastrointestinal clinical presentations, including nausea, vomiting and diarrhea mainly in teenagers [13].

The reported annual incidence of MD in Latin America varies widely, ranging from < 0.1 cases per 100,000 inhabitants in countries such as Bolivia, Cuba, Mexico, Paraguay, and Peru to nearly 2.0 cases per 100,000 inhabitants in Brazil. Despite MD being a mandatory notifiable disease in all Latin American countries, reports are likely to represent underestimates of the true disease burden. In Brazil, MenW is responsible for more than 10% of total MD cases and sporadic cases have been notified mainly in south region [14].

Several multi-step conjugation methods have been employed for covalently linking polysaccharides to proteins. Reductive amination methodology is currently used to prepare licensed conjugate vaccines against encapsulated bacteria like *Haemophilus influenzae* type b, *N. meningitidis* and *Streptococcus pneumoniae* [15]. Our group developed a meningococcal C conjugate vaccine that is in Phase II/III clinical trials with scaled up production [16, 17]. With the increase of MenW cases in Brazil and to use the acquired knowledge from the authors the aim of this study is to develop a Brazilian conjugate vaccine against MenW produced by reductive amination using hydrazide-activated tetanus toxoid as a carrier protein.

Material and methods

Isolation of MenW polysaccharide (PSW)

MenW strain 2467 from Adolfo Lutz, São Paulo, Brazil, was cultured on solid Tryptic Soy Broth for 16–18 h followed by one culture expansion in 2 l flasks of Frantz medium for 4 h at 200 rpm and 37 °C. Culture was transferred to a 15 l bioreactor under stirring with pH and temperature controlled for 4 h and after this period, this culture was used as inoculum for the remaining 150 l culture for 16 h. Samples were collected every 2 h, inactivated using 10% v/v formaldehyde and the growth was measured at 600 nm. At the same time samples were collected, inactivated by temperature, centrifuged and sterile filtered for quantification of polysaccharide and glucose [17, 18]. After 16 h bacterial cells were inactivated during 15 min at 56 °C, centrifuged and the supernatant was concentrated to 10% of the initial volume.

Culture supernatant was precipitated by adding a quaternary ammonium salt and a filtration assistant (Celite) as described by Takagi and coworkers [19]. Elution was done with different salt concentrations to elute differentially polysaccharide contaminants such as protein and nucleic acid. Extractive solution was used to elute polysaccharide portion. PSW was then precipitated with ethanol, submitted to freeze-drying, and stored at -20 °C [20]. The PSW batch obtained by this methodology was used for all experiments and was in accordance with WHO requirements [21]. The strain used in this study produced non-*O*-acetylated polysaccharide.

PSW oxidation studies

PSW is a polymer of galactose and sialic acid that was submitted to oxidation using $NaIO_4$ which simultaneously reduce the polysaccharide length and introduce terminal aldehyde groups in the molecule. PSW was dissolved in water at room temperature, then 23.4 mM $NaIO_4$ was added, and the reaction was conducted in the dark. Excess glycerol was used to quenching the reaction. Dialysis using a 12–14 KDa membrane was done to eliminate the subproducts and residual glycerol. The kinetics of the oxidizing reaction was

done and to follow the reaction steps samples were taken in every two hours to determine that all native PSW was cleaved by $NaIO_4$. Evaluation of oxidized PSW (PSWoxi) in different reaction times was done by aldehyde quantification and high-performance liquid chromatography—size exclusion chromatography (HPLC-SEC) [22–24]. The aldehyde content of the activated PSW was then determined by BCA assay using glucose as a standard [25, 26].

Protein activation

Tetanus toxoid protein (TT) was provided by Instituto de Tecnologia do Paraná, Paraná, PR, Brazil. Purified monomeric Tetanus Toxoid (PMTT) was purified in Bio-Manguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. PMTT was activated by reaction with hydrazine dihydrochloride and carbodiimide, the solution was stirred for 1.5 h at room temperature under acidic conditions [16, 22, 24]. Activated protein was submitted directly to dialysis against phosphate buffer and then concentrated using an Amicon® ultra centrifugal filter. Final protein content was determined by Lowry assay using BSA as a reference [16, 27].

Conjugation studies

Activated PMTT (PMTTH) and PSWoxi were covalently linked by reductive amination as follow. PSWoxi was dissolved in purified water overnight. PMTTH was added to the solution and the reaction occurred in the presence of sodium cyanoborohydrate. The reaction occurs with subsequent hydrazones formation and the linkage was further stabilized by reduction with sodium cyanoborohydrate. Adipic acid dihydrazide was used to quenching the reaction by blocking unreacted aldehyde groups [15, 21–24, 28]. The conjugates were purified by dialysis against phosphate buffer under basic and neutral conditions using Amicon ultracentrifugal filters. For the purification of pilot batches was used tangential flow filtration in Centramate system (Pall Corporation®) with the same buffers described above.

Reactions were first performed on a 20 mg scale. Different reaction times, reactant concentration, temperature, buffer composition and pH were evaluated to compare the ratio among obtained conjugates batches (PSW-TTH). Afterwards, to confirm the best reaction conditions, pilot batches scaled-up for 100 mg were obtained.

Quality control assays required by WHO were done to evaluate produced batches. All conjugate batches were analyzed by HPLC-SEC and total sugar and protein contents in final conjugates were determined by resorcinol and Lowry methods [18, 27], respectively, and the results were used to calculate polysaccharide-protein ratio. Pilot batches were also analyzed by hydrogen nuclear magnetic resonance (^{1}H NMR) 1D spectroscopy using the same conditions described by Jones & Lemercinier, 2002 [29]. The amount of free polysaccharide was evaluated by a standardized and validated method using capillary zone electrophoresis according to Souza and coworkers (data not shown) [30].

High performance liquid chromatography – size exclusion chromatography (HPLC-SEC)

Different fractionation range of size exclusion chromatography columns were necessary for the evaluation of protein, polysaccharide, and conjugate samples. In order to compare the variety of molecules that participate in the conjugation process on the same scale, the TSK® 6000 PWxL, TSK® 5000 PWxL and TSK® 4000 PWxL (Tosoh Bioscience, King of Prussia, PA, USA) were used individually. Samples were loaded onto the gel filtration column and eluted isocratically for 35 min in 0.2 M NaCl as the mobile phase at a flow rate of 0.5 mL min^{-1} and UV detection at 206 nm and 254 nm.

Hydrogen nuclear magnetic resonance (^{1}H NMR)

The hydrogen resonance spectroscopy was used for monitoring native PSW, oxidized PSW and conjugated PSW providing identity, structure, and purity. Analysis of dry samples (10 mg) dissolved in Deuterium oxide (D_2O) (D, 99.9% + 0.01% DMSO-D6 (w/w) + 0.01% DSS-D6, Cambridge Isotope Laboratories Inc.) were recorded at 400 MHz at 37 ºC using a Bruker Avance spectrometer, as previously described [17, 22, 29].

Immunization procedures

A hundred Swiss mice (six weeks old) were separated in four equal groups between male and female. These animals were immunized intramuscularly in dose–response studies using concentrations varying from 0.1 µg/dose up to 10 µg/dose (0.05 mL) of conjugate bulks with aluminum hydroxide (Alhydrogel/Brenntag®; 35 µg of Al^{+3}/dose) as adjuvant, three times with a 15-day interval. Blood was collected by retro-orbital vein puncture before the first dose and 15 days after the third dose. Sera were isolated and stored at − 20 °C until use.

Enzyme linked immunosorbent assay (ELISA)

Antibody levels in sera were determined by ELISA. To detect anti polysaccharide IgG antibodies 96-well plates (Greiner Bio-one Ref. 655,061) were coated with 100 µL of equal quantities of PSW and methylated human serum albumin (5 µg/ml) and kept overnight at 4 °C. After washing six times with 200 µL washing buffer (Tris Buffer Saline; TBS with 0.05% Tween 20), antiserum samples

and in-house standard serum were added to each well at a serial two-fold dilution starting from 1/400 and 1/600 respectively. All sera were titrated in duplicate. After overnight incubation, the plates were washed four times and incubated with 100µL goat anti-mouse IgG whole molecule conjugated with alkaline phosphatase (1/3000 dilution in TBS pH 7.4) for 2 h. After washing p-nitrophenyl phosphate (1 mg/mL) was added and plates were incubated for 20 min. Absorbance was read at 405 nm. ELISA titers were calculated using arbitrary unit of ELISA in reference to a standard serum obtained of mice immunized with a tetravalent conjugate vaccine (MenACWY-CRM_{197}) (1000 U/mL) and expressed by Ln transformed values (Ln U/mL) [16, 17, 31].

Serum bactericidal activity (rSBA) assay

Serum samples were assayed by SBA to determine antibodies effectiveness as previously described by Cedré et al., 2012 [32]. In this procedure four different strains (2467, 3856, 2460, S4383) of MenW were tested against rabbit baby complement but only strain S4383 showed low lysis of bacteria cells. This strain was plating on Columbia blood agar and incubated at 37 °C in 5% CO_2 overnight. Some colonies were subcultured on another Columbia blood agar plate and incubated in the same conditions for 4 h. Cells were suspended in Hanks buffer and bacterial quantity was adjusted to 8×10^4 CFU.ml^{-1}. Sera from mice immunized with MenW conjugate bulks were serially diluted and incubated with a suspension of meningococcal strain S4383. Pel-Freez® baby rabbit complement lacking bactericidal activity was added. After incubation 10 µl of the mixture from each well was plated out on Columbia blood agar plate by the tilt method [33]. Bactericidal activity was determined based on efficiency of bacterial killing compared with controls with SBA titers defined as the highest dilution of sera at which > 50% killing occurred.

Statistical analysis

ELISA data were expressed in Ln U.ml^{-1}. The IgG titers were compared for each dose–response concentration by using a multifactor analysis of variance (ANOVA). SBA titers were log-transformed, described as the mid-value antibody titer, and expressed as geometric mean [21]. Differences in antibody levels and SBA titers among sera from immunized mice were assessed by the non-parametric Kruskall–Wallis test. A p-value < 0.05 was considered significant. The statistical analysis was performed using GraphPad Prism® ver. 6.01 (GraphPad Software, Inc., La Jolla, CA, USA).

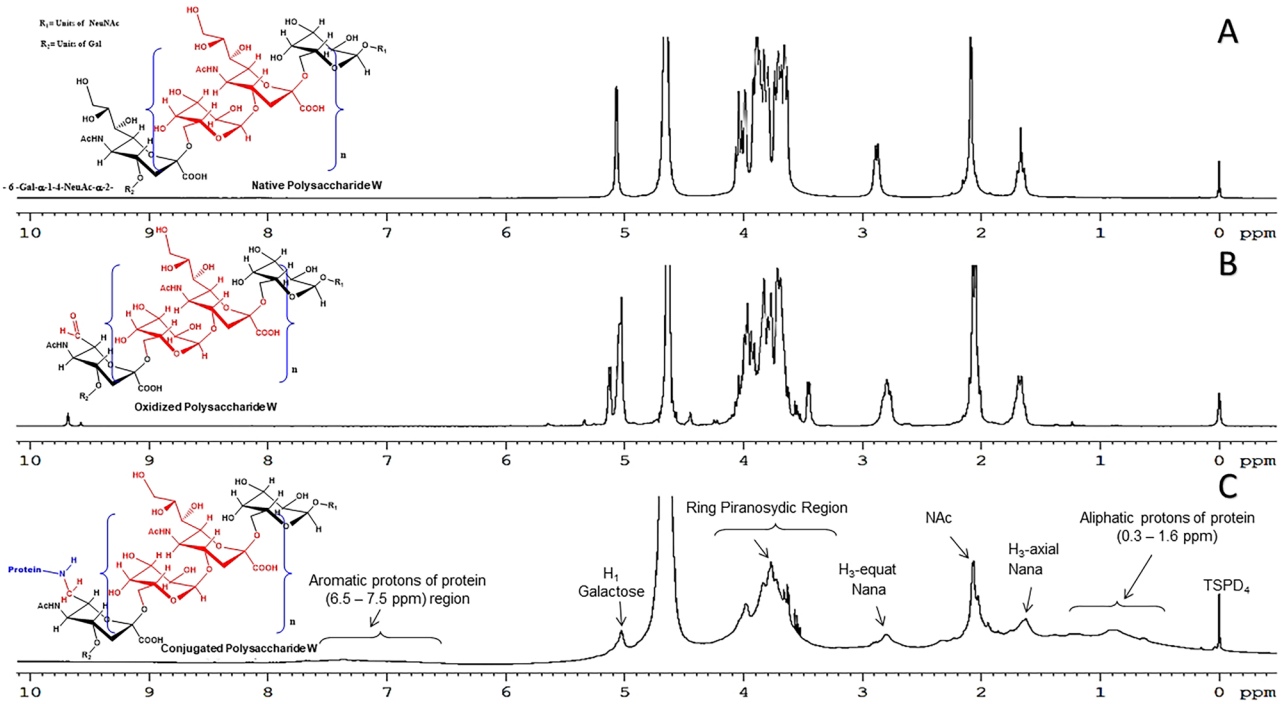

Fig. 1 Comparison of 400 MHz ^{1}H NMR spectra of **A**, Native PSW, **B**, Oxidized PSW and **C**, conjugate batch PSW-TTH 15 with 264 scans highlighted characteristic assignments of hydrogen of galactose, Nana, *N*-acetyl, protein aliphatic and aromatic groups

Results

Characteristics of the purified polysaccharide After fermentation and purification steps, native PSW was obtained in accordance with World Health Organization Requirements [18]. It contains 58.48% sialic acid, 0.16% nucleic acids, 3.96% protein and 99.88% of PSW recovery before distribution coefficient (Kd) of 0.5. ^{1}H NMR presented a PSW profile as observed in literature with characteristic assignments of hydrogen linked to carbon 1 of galactose and the hydrogen linked to carbon 3 of sialic acid [34] (Fig. 1A).

Comparison between size exclusion columns Since PSW polysaccharide was obtained by a different methodology of purification without use of phenol extraction, some differences in polysaccharide and bulk conjugate length were expected. Analysis of meningococcal conjugates previously obtained by our group were done by using TSK® 4000 PWxL column [16, 17]. For the molecules used in this study, we needed a previous test with different fractioning ranges to permit further evaluation of the bulks that would be produced (data not shown). Only with TSK® 5000 PWxL were observed peaks between the void volume (Vo) and the total elution volume (Vt) of the column. For this reason, in this paper this column was used in HPLC-SEC assays (Figs. 2 and 3).

Attributes of oxidized polysaccharide. Polysaccharide oxidation is necessary to introduce aldehyde groups and to reduce its length to facilitate the conjugation reaction. For this, it is important to study different concentration of the oxidizing reagent and/or the reaction time. The easiest way to observe the length reduction of the polysaccharide is by size exclusion chromatography. Kinetics of oxidizing reaction was done by injecting each two hours' reaction samples. As observed in Fig. 2, after 10 h of reaction the aldehyde concentration of oxidized polysaccharide was 29.73 nM and PSWoxi eluted at a higher elution volume as compared with native polysaccharide (Table 1). The good resolution between peaks allowed evaluation of reaction kinetics and monitoring the purification process (Fig. 2). The introduction of aldehyde was evaluated by BCA assay. The results showed an increase in concentrations of aldehyde during the reaction course (Table 1). By performing ^{1}H NMR analysis it was possible to verify the integrity of molecule since the oxidized PSW showed characteristic assignments related to polysaccharide moiety, as well as the aldehyde group

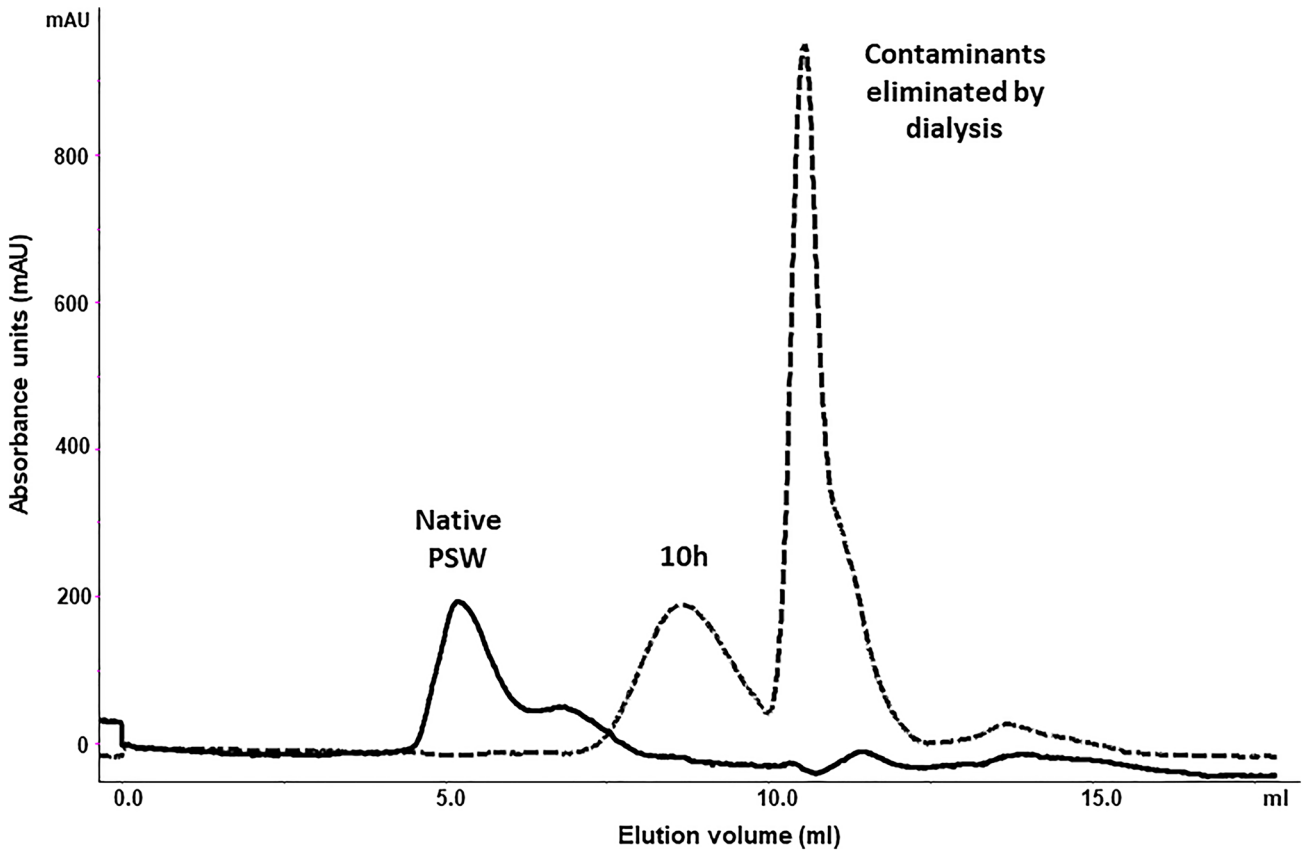

Fig. 2 Chromatographic elution differences between native PSW and oxidized PSW after 10 h of reaction. Analysis with a flow rate of 0.2 mL min − 1 using isocratic elution of 0.2 M NaCl monitoring at 206 nm. (Vo = 4.5 ml and Vt = 12.2 ml)

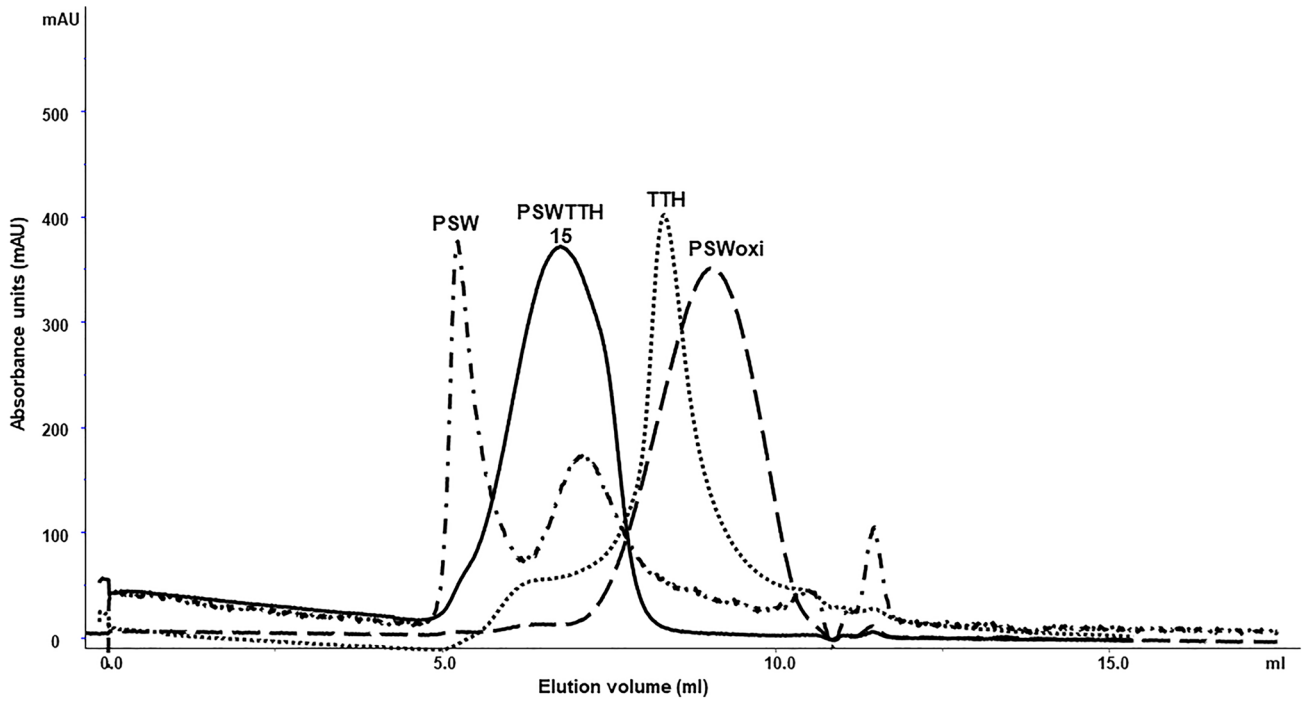

Fig. 3 HPLC-SEC comparative profiles of a representative MenW native polysaccharide (PSW), MenW conjugate bulk (PSW-TTH 15), activated PMTT (PMTTH) and oxidized PSW (PSWoxi). Analysis of all molecules were done with a flow rate of 0.2 mL min − 1 using isocratic elution of 0.2 M NaCl monitoring at 206 nm. (Vo = 4.6 ml and Vt = 12.3 ml)

assignment at 9.7 and its hydrated forms in 5.1 e 5.3 ppm (Fig. 1B), suggesting success of oxidation reaction.

Physicochemical characteristics of glycoconjugates Conjugate ratio was used to evaluate conjugation reaction efficacy. In general, immunogenic bacterial conjugate vaccines had polysaccharide:protein ratio close to 0.5 [16, 35–38]. Table 2 shows the effect of some parameter's variations in the conjugation ratio. As observed reactant proportion did not present significant differences among the batches studied (PSW-TTH 01, PSW-TTH 02 and PSW-TTH 03). Then, duplicating the concentration of the reactants and maintaining their proportions, we observed an increase in the reaction conjugate ratios (PSW-TTH 04, PSW-TTH 05, PSW-TTH 06 and PSW-TTH 07). The study of the influence of buffers with different pH values in conjugates ratio demonstrated that when the reaction occurs with 2-morpholinoethanesulfonic acid buffer at pH 6.1 a higher conjugation ratio than the observed with buffers at pH values 7.0, 7.5 and 9.0. was observed (PSW-TTH 09, PSW-TTH 10, PSW-TTH 11 and PSW-TTH 12). Differences in reaction's temperature did not show important variations in the conjugation ratio (PSW-TTH 13 and PSW-TTH 14).

Table 1 Kinect study of oxidized PSW obtaining. KD was calculated for each time using (Vo = 4.5 ml and Vt = 12.2 ml). Percentage of aldehyde represents the ratio of the number of aldehyde groups generated in the PSW molecule

Kinetics study of oxidized PSW		
Time	K_D	Aldehyde Concentration (mM)
2h	0.30	10.4
4h	0.35	17.3
6h	0.39	16.6
8h	0.42	17.9
10h	0.45	19.2
12h	0.47	21.3

After optimization of reaction parameters two conjugate bulks were produced using five-fold increase for polysaccharide to allow the development of other analysis techniques and to perform quality control assays. These batches were named as pilot batches. A proportion of reactants (polysaccharide:protein) of 2:1 had been chosen. The two batches (PSW-TTH 15 e PSW-TTH 16) obtained showed a conjugation ratio of 0.52 and 0.49, respectively (Table 2). The HPLC-SEC profile of PSW-TTH 15 showed a single peak with a K_D of 0.16 with higher molecular weight than the PMTTH ($K_D = 0.43$) and oxidized PSW ($K_D = 0.45$)

Table 2 Influence of main reaction parameters in the final conjugation reaction ratio. Conjugate bulks produced to establish the best conditions of conjugation reaction. This table summarizes the influence of reactant proportion and concentration, pH and temperature in conjugate ratios. *Conjugate ratio = PS concentration / Protein concentration. Room temperature: 22–25 ºC. MES—2-morpholinoethanesulfonic acid

Conjugate Samples	PSW:Protein Proportions	Temperature of the Reaction	Buffer/pH	Conjugate Ratios*	Conjugate K_D
PSW-TTH 01	1:2	45 °C	MES buffer pH 6.1	0.20	0.11
PSW-TTH 02	1:1	45 °C	MES buffer pH 6.1	0.27	0.13
PSW-TTH 03	2:1	45 °C	MES buffer pH 6.1	0.24	0.16
PSW-TTH 04	1:2	45 °C	MES buffer pH 6.1	0.27	0.15
PSW-TTH 05	2:1	45 °C	MES buffer pH 6.1	0.44	0.17
PSW-TTH 06	1:1	45 °C	MES buffer pH 6.1	0.34	0.12
PSW-TTH 07	2.5:1	45 °C	MES buffer pH 6.1	0.51	0.18
PSW-TTH 09	2:1	45 °C	MES buffer pH 6.1	0.56	0.15
PSW-TTH 10	2:1	45 °C	Phosphate buffer pH 7.0	0.47	0.14
PSW-TTH 11	2:1	45 °C	Hepes buffer pH 7.5	0.31	0.25
PSW-TTH 12	2:1	45 °C	Borate pH 9.0	0.32	0.25
PSW-TTH 13	2:1	Room temperature	MES buffer pH 6.1	0.61	0.18
PSW-TTH 14	2:1	45 °C	MES buffer pH 6.1	0.59	0.17
PSW-TTH 15	2:1	Room temperature	MES buffer pH 6.1	0.52	0.16
PSW-TTH 16	2:1	Room temperature	MES buffer pH 6.1	0.49	0.15

clearly differentiating a conjugate formation (Fig. 3). The content of free components, protein and polysaccharide of theses bulks were below the limits suggested for bacterial conjugate vaccines, corresponding to 20% for free polysaccharide and 5% for free protein [39].

^{1}H NMR experiments showed the integrity of molecule demonstrated by the presence of characteristic assignments of hydrogen linked to carbon 1 of galactose and the hydrogen linked to carbon 3 of sialic acid (Fig. 1). The formation of conjugate is also clearly demonstrated due to the presence of assignments at 6.5–7.5 and 1.6–0.3 ppm corresponding to the aromatic and aliphatic amino acids from the protein, respectively (Fig. 1C). PSW-TTH 15 conjugate bulk was used for immunological evaluation in dose–response studies.

Immunogenicity of group W glycoconjugates Serum samples of mice immunized with four different concentrations of PSW-TTH 15 pilot conjugate bulk were analyzed for specific IgG antibodies and serum bactericidal activity. ELISA was used to detect IgG antibodies in sera from all groups. As expected, the IgG titer of the serum samples obtained from mice immunized with native PSW showed no increase after three injections (data not shown). On the other hand, all concentrations of conjugate vaccine elicited significant antibody but only 15 days after the third dose we observed a significant increase between pre- and post- immunization of anti-PS IgG titers (Table 3). Differences were observed comparing concentration of 0.1 with 1.0 and 10.0 µg/dose. In addition, comparison between 0.5 and 10.0 µg/dose was also significant ($p = 0.005$).

The serum bactericidal activity of antibodies in sera was determined using baby rabbit complement. SBA titers above 1:8 are considered a protective threshold when using this complement source. Animals immunized with different concentrations of PSW-TTH 15 conjugate bulk showed

Table 3 Antibody evaluation by ELISA and SBA titers with different concentrations doses of PSW-TTH 15 conjugate bulk. Total IgG titers measured by ELISA and SBA assays in immunized mice. Antibody levels in animal serum obtained after immunization with PSWTTH 15 bulk conjugate expressed by geometric mean titer (GMT)*. SBA results were obtained with sera 15 days post third dose using Strain S4383

PSW (µg/dose)	ELISA (Ln U.ml^{-1}) (GMT)*	SBA Titers (GMT)*
0.1	12.0 ± 0.1	1:262.5
0.5	12.5 ± 0.1	1:615.5
1.0	13.2 ± 0.1	1:742.2
10.0	13.5 ± 0.1	1:724.1

detectable rSBA values after the third injection and there were no significant differences among them ($p = 0.2387$), although they were slightly higher for doses above 0.5 µg and reached a plateau (Table 3). Unfortunately, we observed unspecific lysis of strains 3856, 2460 and 2467 face eighteen complement batches available in our laboratory and this made it impossible to conduct SBA with these strains.

Discussion

Many disease outbreaks caused by MenW in Brazil and worldwide in the last few years have been documented [40–42]. Bio-Manguinhos/Fiocruz has been developing a conjugate vaccine by covalent attachment of capsular polysaccharide of *N. meningitidis* group C to tetanus toxoid. This vaccine has been evaluating in Phase II/III clinical studies [17]. Conjugation was known to improve immunological response against capsular polysaccharides and in prevention of diseases caused by encapsulated bacteria. Reductive amination was used in this process and has proven to be an effective technique for conjugation of bacterial polysaccharides to carrier proteins. Several monovalent meningococcal conjugate vaccines, using this technique mainly for groups A and C are also available but at the moment, non-monovalent vaccine was licensed against MenW [42–45].

The development of a MenW conjugate vaccine makes part of a project to develop an autochthonous multivalent conjugate vaccine against *N. meningitidis* to attend the Brazilian Immunization Program. The polysaccharide obtained for conjugation studies was naturally produced with non-*O*-acetyl groups. The O-acetylation status for group W seems to be unrelated to the production of functional antibodies [26, 43]. Due to the characteristics of MenW polysaccharide obtained in our laboratory, it was necessary to study parameters to develop a conjugate vaccine against this group. Since a modern purification process using Celite as filtrate assistant was used, the length of the obtained polysaccharide was higher than the one obtained with ethanol fractionation and phenol extraction [19, 20, 46]. The alteration in PSW length was confirmed by HPLC-SEC assays and after evaluation of three SEC columns with different fractionation ranges and showed that TSK® 5000 PW_XL was able to provide an adequate separation profile. The length of the native polysaccharide also affected the oxidation reaction. Different from what was observed in other studies, we obtained an oxidized polysaccharide reduced in length and able to be distinguished from native one using a short reaction time at room temperature [16, 17, 22].

After obtaining the oxidized polysaccharide, the conjugate reaction was studied. Several parameters such as reactant proportion and concentration, buffer composition, pH and temperature were evaluated to reach higher conjugation ratios. The ratio is a critical measure in vaccine potency because it allows the evaluation of the conjugation efficiency [23]. The determination of the best parameters to obtain the MenW conjugate was done by the production of fourteen batches. HPLC-SEC analysis confirmed the success of the conjugation reaction. Two pilot batches with a five-fold volume were produced to confirm the parameters established. As was observed by Gudlavalleti and coworkers, conjugate batches obtained presented K_D around 0.15 even with different conjugation ratio [27]. ^{1}H NMR spectroscopy, which has proven to be a structurally sensitive and reproducible technique to control the identity and integrity of bacterial polysaccharides used in vaccine manufacture is also used for controlling the success of this activation and conjugation steps through the aldehyde group characterization and protein signal's introduction, respectively. This approach was applied to monitor one of these batches, PSW-TTH 15 showing the success of the methodologies developed. This batch was also used to conduct immunological tests of the vaccine and dose–response studies.

The immunological assays used to assess the immunogenicity of the PSW-TTH vaccine were SBA using baby rabbit complement to measure functional activity against MenW and ELISA to measure total MenW IgG serum anti-capsular antibody concentrations. Both assays have been standardized to yield reproducible data. The PSW-TTH 15 conjugate vaccine produced with our methodology was immunogenic in mice. All concentrations of conjugate were able to generate a rise in IgG response. This IgG response clearly showed T-cell dependent immune response after coupling the plain polysaccharide to an immunogenic protein carrier. It also showed a proper functional activity with bactericidal titers much higher than 1:8 (between 1:256 and 1:1024) for all vaccinated animal groups (0.1, 0.5, 1.0 and 10 µg of polysaccharide/dose). These titers are greater than the ones published by Beresford and coworkers (2017) that showed SBA titers with GMT between 1:64 and 1:128 using mice immunized with tetravalent ACWY conjugate vaccine with tetanus toxoid as carrier protein [47]. Gudlavalleti and coworkers observed that rSBA titers for conjugate batches produced with non-O-acetylated polysaccharide and the same carrier protein were higher than the observed with de-*O*-acetylated PSW and *O*-acetylated PSW and are in agreement with the results found in the present work. However, our conjugate batches presented slightly higher SBA titers with a dose of 1.0 µg of PSW using the same complement source [27]. The choice of doses used in our experiments was made based on publications that used ten-fold fractions of plain human dose, in monovalent and tetravalent meningococcal vaccines [47, 48]. We also used a dose of 0.5 µg because in commercially multivalent vaccine the MenW polysaccharide per dose was frequently

around 5.0 µg [36–38]. ELISA showed significant differences between IgG titers of mice immunized with doses greater than or equal to ten-fold. However, no differences were observed among sera of mice immunized with the four conjugate concentrations by SBA. The assessment of immune responses for meningococcal conjugate vaccines has included a fourfold rise in antibody titer between pre- and post-immunizations sera. For this reason, the ELISA and SBA titers suggested that doses above 0.5 µg of PSW could be recommended for mice immunization. Although ELISA test has been standardized and could predict protection, intrinsic variation up to 30% was observed in literature [31]. In this way the results obtained with this test are not often correlated with the titers obtained in rSBA assay that is the WHO recommended test for release of meningococcal vaccines [49]. It happens because not all antibodies detected by ELISA have bactericidal activity, which characterizes their functionality [50].

We developed some methodologies to produce a Brazilian meningococcal C conjugate vaccine and have applied them to obtain a conjugate vaccine against meningococcal W [16, 17, 22]. However, there are some differences among meningococcal polysaccharides constitution that modify the characteristics of the oxidation and conjugation reactions and strength the need to standardize these procedures due to matrix differences. Our results suggested that the galactose moiety of MenW polysaccharide have influenced in oxidation reaction pattern since we obtained PSWoxi with a shorter time of reaction eluting after native PSW. In addition, we obtained MenW conjugates using different conditions of reactant proportion and concentration, and temperature, but with the same buffer used for MenC. These conditions resulted in MenW conjugates with a ratio around 0.5 within the ratio range obtained for MenC conjugates by Bastos and cowokers after optimization of conjugation reaction since at the beginning of the platform development lower ratios were obtained [16, 22]. These results showed a high level of carbohydrate loading that is uncommon using reductive amination but is desirable to induce a good immune response [26, 49]. Although MenW conjugate batches presented a conjugate ratio of 0.5 they induced rSBA titers lower than the observed for MenC conjugates even using different complement source [21]. However, we should consider that in general, rSBA titers obtained for MenW conjugates are lower than those observed for other meningococcal groups like MenC using tetanus toxoid as carrier [51].

In conclusion, the results obtained in the present paper, have established the best conditions to produce MenW conjugate vaccine and showed the efficacy of conjugate vaccine in inducing a good immune response in mice. Further experiments need to be done to scale up the conjugation reaction and then allow the use of this conjugate in clinical trials.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval All animal protocols were submitted and approved by the Oswaldo Cruz Foundation Ethical Committee with licenses numbers LW-65/14 and LW-3/20.

Consent to participate Not applicable.

Consent for publication All authors have consented to publish this material.

Conflicts of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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