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ORIGINAL ARTICLE

Brazilian meningococcal C conjugate vaccine: physicochemical, immunological, and thermal stability characteristics

Renata Chagas Bastos[1] · Marilza Batista Corrêa[2] · Iaralice Medeiros de Souza[2] · Milton Neto da Silva[2] · Denise da Silva Gomes Pereira[2] · Fernanda Otaviano Martins[2] · Camila da Silva Faria[2] · Ana Paula Dinis Ano Bom[1] · Maria de Lourdes Leal[2] · Ellen Jessouroun[3] · José Godinho da Silva Jr.[1] · Ricardo de Andrade Medronho[4] · Ivna Alana Freitas Brasileiro da Silveira[2]

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Abstract High temperature is known to cause some instability in polysaccharide-protein conjugated vaccines and studies under stress conditions may be useful in determining whether short-term accidental exposure to undesired conditions can compromise product quality. In this study, we examined the structural stability of three industrial batches of Brazilian Meningococcal C conjugate bulk (MPCT) incubated at 4, 37, and 55 °C for 5 weeks. The effect of exposure to the storage temperatures was monitored by HPLC-SEC, CZE, CD and NMR techniques. The immunological significance of any physicochemical changes observed in MPCT was determined by SBA and ELISA assays of serum from immunized mice. Fluorescence emission spectra at 4 and 37 °C were similar among all samples and compatible with the native fold of the carrier protein. Fluorescence spectra of MPCT stored at 55 °C decreased in intensity and had a significant red-shift, indicating conformational changes. Far-UV CD spectra revealed a trend toward loss of structural conformation as storage temperature was increased to 55 °C. The NMR data showed modified signal intensity of the aromatic and aliphatic residues, mainly for samples incubated at 55 °C, suggesting a partial loss of tertiary structure. About 50% free saccharide content was found in bulks stored at 55 °C, but no difference was observed in the IgG or SBA titers. The present study showed physicochemical methods alone are insufficient to predict the biological activity of a MPCT conjugate vaccine without extensive validation against immunological data. However, they provide a sensitive means of detecting changes induced in a vaccine exposed to adverse environmental condition.

Keywords Brazilian meningococcal C conjugate vaccine · Thermo stability · Physicochemical analysis · Immunological evaluation

✉ Renata Chagas Bastos
renata@bio.fiocruz.br

[1] Laboratório de Macromoléculas, Bio-Manguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

[2] Laboratório de Tecnologia Bacteriana, Bio-Manguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

[3] Programa de Vacinas Bacterianas, Bio-Manguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

[4] Escola de Química, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

Introduction

Vaccination programs worldwide are undoubtedly one of the greatest public health achievements of modern times. Most vaccines are large and complex molecular assemblies highly susceptible to environmental factors that can significantly affect their activity. Among these factors, the principal factor that affects the characteristics of all vaccines over time is temperature [1].

The stability of pharmaceutical molecules is a matter of great concern, as it affects the safety and efficacy of the drug product (*i.e.,* final container product). The maintenance of molecular conformation and hence of biological activity is dependent on covalent and non-covalent forces in biotechnological/biological products in which active components are typically proteins and/or polypeptides. These products are particularly sensitive to environmental factors, such as temperature changes, oxidation, light, ionic strength, and shear stress. Therefore, to avoid degradation and ensure

maintenance of biological activity, rigorous conditions for storage are usually necessary [2]. Inactivated and subunit vaccines are generally more stable over the long term than live attenuated vaccines but it is always necessary to conduct extensive stability studies, including both accelerated and long-term studies under different conditions (*e.g.*, pH, temperature, ionic strength, etc.) as part of vaccine formulation development. In addition, studies under actual storage conditions are important to establish shelf life and the expiration date at the recommended storage temperature [3].

Polysaccharide-protein conjugates are relatively more stable compared to live attenuated bacterial vaccines but the polysaccharide (PS) chains of such conjugates tend to degrade by hydrolysis [4]. The potency of these vaccines depends on effective conjugation of the saccharide to a protein carrier and preservation of the integrity of the conjugate molecule during shelf life. Thereby, factors adversely affecting conjugate stability may diminish vaccine potency by reducing the amount, accessibility, and solubility of the conjugated saccharide and carrier protein epitopes, limiting their protective efficacy [5, 6]. High temperature is known to cause physical instability, such as altered tertiary structure and protein aggregation, as well as chemical instability, such as dissociation of polysaccharides from the protein carrier, in this kind of vaccine. These changes can be assessed under thermal stress, the most widespread adverse condition used in the industry [7, 8]. Data on retained vaccine potency at temperatures other than recommended refrigerated storage are clinically useful because cold chain conditions might not always be maintained. Furthermore, studies under stress conditions may be useful in determining whether short-term accidental exposure to undesired conditions, such as improper transportation, can compromise product quality [4]. Therefore, effective methods are essential for monitoring quality and detecting changes that could potentially affect the efficacy of these vaccines. Establishing stability indicators for conjugate vaccine molecules is a key step to ensure their quality. Physicochemical analysis methods are widely used in pre- and post-licensing quality control of these vaccines to ensure compliance with manufacturing specifications and batch-to-batch product consistency [5, 6].

Understanding stability of the molecule helps in the selection of a proper formulation and package as well as providing proper storage conditions and shelf life, which is essential for regulatory documentation. Thus, the Food and Drug Administration and International Conference on Harmonization guidelines state the requirement of stability testing data to understand how the quality of a drug substance (*i.e.,* bulk material) and drug product changes with time under the influence of various environmental factors [9]. There is no single stability-indicating assay or parameter that provides the stability characteristics of a biotechnological/biological product. Consequently, a stability profile should be proposed ensuring that changes in product identity, purity, and potency will be detected [2].

A major problem when assessing vaccine stability is the fact that many vaccines possess a specific biological activity that cannot be fully characterized by physicochemical methods alone. Biological assays play an important role in the quality control of vaccines and are essential for verifying vaccine quality. Vaccine stability testing is based on determining a change in vaccine properties, which may be a direct or indirect indicator of vaccine immunogenicity or maintenance of efficacy [1]. The use of relevant physicochemical, biochemical, and immunochemical analytical methodologies permits a comprehensive characterization of the drug substance and/or drug product as well as accurate detection of degradation changes occurring during storage [2].

In this study, we examined the structural stability of three industrial batches of Brazilian Meningococcal C conjugate bulk (MPCT) which were incubated at 4, 37, and 55 °C for 5 weeks, based on the experimental conditions described by Ho *et al*., 2002. Sialic acid (Neu5*N*Ac), protein content, and pH of each sample were monitored during the incubation period. In addition, the effect of exposure to various storage temperatures on molecular integrity, free saccharide content, and carrier protein folding of treated conjugate vaccines was monitored by high performance liquid chromatography-size exclusion chromatography (HPLC-SEC), capillary zone electrophoresis (CZE), as well as fluorescence, circular dichroism (CD), and nuclear magnetic resonance (NMR) spectroscopic techniques. The immunological significance of any physicochemical changes observed in treated conjugate bulks was determined by a serum bactericidal antibody assay (SBA) and enzyme-linked immunosorbent assay (ELISA) of serum from immunized mice.

Materials and methods

Vaccine MPCT was obtained and controlled as described before [10–14]. Briefly, three consecutive conjugation reactions between periodate-oxidized meningococcal C polysaccharide (MPCO) and hydrazide-activated monomeric tetanus toxoid (MATT) were performed by reductive amination. For each reaction, MPCO was covalently linked to MATT in the presence of sodium cyanoborohydride (Sigma Aldrich., St. Louis, MO, USA), overnight. The reaction was stopped by adding adipic acid dihydrazide (ADH) (Sigma) to block unreacted aldehyde groups. The conjugates (called MPCT Industrial Batches #1, #2, and #3) were purified by tangential flow filtration. After sterile filtration, the purified batches were stored at 4 °C in 1.0 mM PBS, pH 7.2.

Study design Triplicate samples from each sterile conjugate batch were aseptically transferred to sterile vials (2.0 mL) maintained at 4 (default temperature), 37 and 55 °C for 5 weeks. At the end of the incubation period, all samples were

stored at 4 °C before performing the physicochemical and immunogenicity analyses. Methods used to measure the stability of the conjugate included, Neu5*N*Ac and protein contents, pH, proton NMR, CD and fluorescence spectroscopy, SEC, CZE, and immunological assays.

Measurements

Sialic acid and protein content Neu5*N*Ac and total protein contents in each sample were determined at the end of incubation time using the methods of Svnennerholm [15] and Lowry [16] respectively, and the results were used to calculate the polysaccharide-protein ratio (PS/Prot).

pH Bulk pH was determined at the end of each incubation week until the end of week 5 using a benchtop pH meter (Seven easy; Mettler Toledo, Columbus, Ohio) at room temperature. The pH meter was calibrated using pH 4.0, 7.0, and 10.0 standard buffer solutions (Merck Millipore, Billerica, Massachusetts, EUA). Samples were equilibrated to room temperature for approximately 15 min prior to measurements. The samples were prepared in 45 vials containing 2.0 mL of conjugate bulk for each MPCT batch. Triplicate pH measurements were taken of all three conjugate batches and the three temperatures in the study.

Physicochemical methods

Circular dichroism and fluorescence spectroscopy CD measurements were taken using a Jasco J-815 spectropolarimeter (Jasco Corp., Tokyo, Japan) with a 2.0 mm path-length quartz cuvette. Conjugates were diluted to a final protein concentration of 1 mg mL^{-1}. Far-UV spectra were measured at 20 ± 1°C from 200 to 260 nm, averaged over three scans at a speed of 50 nm min^{-1}, and collected in 1 nm steps. The alpha-helix structure was monitored by a maximum peak ellipticity at 220 nm. The buffer baselines were subtracted from their respective sample spectra.

Fluorescence emission spectra of the same bulk samples were recorded using a Jasco FP-6500 spectrofluorimeter. Tryptophan (Trp) fluorescence emission spectra were obtained by setting the excitation wavelength at 280 nm, and the emission spectrum was recorded from 295 to 415 nm. Trp fluorescence intensity was measured as spectral fluorescence area, and the spectral shifts were quantified as changes in the average energy of emission (spectral center of mass, $<\upsilon>$) according to the equation:

$$< \upsilon >= \sum \upsilon_i \cdot F_i / \sum F_i$$

where *Fi* is the fluorescence emission at wavenumber υ_i, and the summation was carried out over the range of measured values of *F*. Experiments followed by light scattering (LS) data had an excitation wavelength at 320 nm and emission wavelengths of 300–340 nm.

High performance liquid chromatography-size exclusion chromatography The chromatographic analysis was performed on a TSK-G® 4000 PWxL analytical column (7.8 mm × 30 cm) (Tosoh Bioscience, King of Prussia, PA, USA) with a fractionation range of Mr. 1×10^4–1.5×10^6 Da. Bulk conjugate samples containing 100 μg protein in 100 μl were loaded onto the SEC 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 and 254 nm. Void and bed volumes were calibrated with Blue Dextran (GE Healthcare, Uppsala, Sweden) and acetone (1%, v/v) in 0.2 M NaCl, respectively. The samples were analyzed by measuring the proportion of conjugate eluting in a specific column volume or distribution coefficient (Kd). The measurement was done according to $Kd = (V_e - V_o)/(V_t - V_o)$ where V_o is the void elution volume of the column determined with Blue Dextran, V_t is the peak elution volume determined with acetone, and V_e is the peak elution volume of the analyzed conjugate bulk. Results are expressed as elution volume (V_e; mL).

Capillary zone electrophoresis The quantity of free polysaccharide was evaluated by CZE with a capillary electrophoresis (CE) system (HP 3D CE), containing a photodiode array detector and equipped with forced air temperature control, platinum electrodes, and ChemStation Software G1601A for instrument control and data analysis. A fused silica capillary coated with polyimide (112.5 cm in length, effective size of 104 cm, and 50 μm in inner diameter; Agilent Technologies, Palo Alto, CA, USA) was used. The analysis was performed using sodium tetraborate (TBNa) buffer 50 mM, pH 10.0, 30 kV, 40 °C, monitored at a wavelength of 200 nm, as described previously [13]. Sugar-free content of the conjugate bulks was determined from the slope of the calibration line using the bulk as the matrix.

Proton nuclear magnetic resonance Analysis of dry samples (10 mg) dissolved in deuterium oxide (D_2O) (D, 99.9% + 0.01% DMSO-D_6 (*w*/w) + 0.01% DSS-D_6, Cambridge Isotope Laboratories Inc., Tewksbury, Massachusetts, EUA) were recorded at 400 MHz and 25 °C using a Bruker Avance III HD 400 spectrometer with 5 mm probes and a Topspin 3.2 software, as described previously [11, 14].

Immunological assays

Animal immunization Conjugate bulks were diluted under aseptic conditions with sterile diluent: Alhydrogel (Brenntag; 350 μg Al^{3+}/dose) in saline, pH ~ 5.6) prior to injection. All animal protocols were approved by the Oswaldo Cruz Foundation Ethical Committee (CEUA/FIOCRUZ number

LW65/14). Groups of five Swiss mice per sample (12–17 g; 20 animals per conjugate batch) were injected intramuscularly on day 0 with the equivalent of 1/10 of a single human dose (1 μg saccharide/200 μL) stored at 4, 37, and 55 °C. The animals received two additional doses on days 15 and 30 with the same amount of material. Sera collected on day 45 were pooled and tested for anti-meningococcal C polysaccharide (MenCPS) antibodies by ELISA and for serum bactericidal activity. Sera were isolated and stored at −20 °C until use.

Enzyme-linked immunosorbent assay Antibody levels (IgG titers) against MenCPS in sera were determined by ELISA as described by Gheesling *et al*., 1994 [17]. Briefly, plates (Immulux REF 1010; Dynex, Chantilly, VA, USA) were coated overnight at 4 °C with 100 μL MenCPS (5 μg mL^{-1}) in 10 mM PBS, pH 7.4, co-mixed with methylated human serum albumin (5 μg mL^{-1}). Tris-buffered saline (TBS) (20 mM Tris-HCl, 0.5 M NaCl, 0.02% Tween 20) with 5% fetal calf serum was used as a blocking reagent, and the plates were incubated for 60 min at room temperature (RT). After washing four times with 200 μL washing buffer (TBS with 0.05% Tween 20), antiserum samples and in-house standard serum at serial two-fold dilution starting from 1:1000 (*v*/v) were added to each well. The plates were incubated with anti-mouse IgG conjugated with alkaline phosphatase (A-3688; Sigma-Aldrich) diluted 1:3000 (*v*/v) for 120 min at RT. All sera were titrated in duplicate, and titers were determined by reading absorbance at 405 nm in VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale. CA, USA). ELISA titers were calculated using arbitrary ELISA units in reference to a standard serum (1000 EU mL^{-1}) and expressed as Ln transformed values (Ln U mL^{-1}).

Serum bactericidal antibody assay *N. meningitidis* serogroup C strain C11 (phenotype C:16:P1.7–1,1) was streaked on a Columbia blood agar plate and incubated overnight at 37 °C in 5% CO_2. The next day, 10–20 colonies were subcultured on another Columbia blood agar plate and incubated for 4 h at 37 °C in 5% CO_2. The cells were suspended in bactericidal buffer (Hanks balanced salt solution) and adjusted to 1.5×10^4 CFU. The bactericidal assay was performed by the tilt method as follows: 20 μL of heat inactivated (56 °C for 30 min) sera from mouse immunized with MPCT was 10-fold serially diluted in bactericidal buffer in a U-bottom, 96-well plate. A 10 μL aliquot per well of the bacterial suspension and 10 μL of human complement were added and previously shown to lack detectable intrinsic bactericidal activity. The plates were incubated for 60 min at 37 °C and a 10 μL drop of the mixture from each well was plated out on Columbia blood agar plates at the end of the incubation period and incubated overnight at 37 °C in 5% CO_2. SBA titers are expressed as the reciprocal serum dilution at which ≥50% of cells lysed, compared to the number of cells present prior to incubation with sample serum and complement [18]. The original surrogate of natural protection for meningococcal serogroup C (MenC) disease was established by Goldschneider *et al*. as an SBA titer with human complement (hSBA) ≥ 4 [19]. Positive and negative serum controls were included in every assay.

Statistical analysis

The data in this study were not normally distributed. IgG titers were logarithmically transformed (Ln) and expressed as the geometric mean plus standard deviation. SBA titers were log-transformed and described as the mid-value antibody titer (t_{mv}) according to the formula:

$$logt_{mv} = (logt_s + logr_n)/2$$

where: log t_s is the standard titer and logr_n is the reciprocal of the next dilution [20]. SBA data are expressed as the geometric mean. Differences in antibody levels and SBA titers among sera from immunized mice were assessed with the non-parametric Mann–Whitney test in the high temperature and control temperature groups. 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).

Results

This study was undertaken to evaluate thermal stability of the conjugate molecule obtained by modified reductive amination conjugation method employed to produce a Brazilian conjugate vaccine. This evaluation was made using spectroscopic and chromatographic techniques as well as immunogenicity assays in animal models.

The results showed that the PS/Prot ratio (data not shown) and pH values (Table 1) did not change after 5 weeks of storage at any of the temperatures studied.

The conformational state of the MPCT stored at 4, 37, and 55 °C was verified by fluorescence spectroscopy and CD. Fluorescence emission spectra at the control temperature (4 °C) and 37 °C were similar among all samples and exhibited fluorescence emission λ_{max} (F_{max}) of 324 nm when

Table 1 Average pH values for all Brazilian Meningococcal C conjugate bulk (MPCT) batches measured during the five weeks of incubation at each temperature

Temperature	MPCT batch #1	MPCT batch #2	MPCT batch #3
4 °C	6.98 ± 0.04	6.96 ± 0.04	6.95 ± 0.03
37 °C	6.90 ± 0.04	6.86 ± 0.02	6.95 ± 0.05
55 °C	6.97 ± 0.09	6.92 ± 0.06	6.74 ± 0.06

excited at 280 nm, indicating the native fold of the carrier protein. Fluorescence spectra of MPCT bulks stored at 55 °C decreased in intensity and a significant red-shift (λ_{max} 328 nm) was detected, indicating conformational changes in the carrier protein. The fluorescence results were summarized by spectral area values of conjugated protein, which were similar at 4 and 37 °C. A decrease in spectral area was observed for the bulks stored at 55 °C (Fig. 1). This finding agrees with data obtained for the center of mass and LS which varied only in the conjugate batches held at 55 °C (data not shown).

Far-UV CD spectra for the MPCT conjugates showed a trend toward loss of structural conformation as storage temperature was increased from 4 to 55 °C (Fig. 2). The MPCT stored at 55 °C revealed a secondary structure change due to a decrease in negative ellipticity (Fig. 2, Inset), indicating some unfolding of the carrier protein at this temperature. The alpha-helix structure was monitored by maximum peak ellipticity at 220 nm.

^{1}H NMR was used as a complementary approach to obtain preliminary information about the structural changes in MPCT samples incubated at different temperatures. The NMR profile of MPCT stored at 4 °C was used as the default.

The NMR data showed modified signal intensity of the aromatic and aliphatic residues, mainly for samples incubated at 55 °C.

Increased signal intensity was detected for aromatic (6.5–7.5 ppm) and aliphatic (0.3–1.6 ppm) signals, indicating a protein structural modification due to temperature and suggesting a partial loss of tertiary structure (Fig. 3).

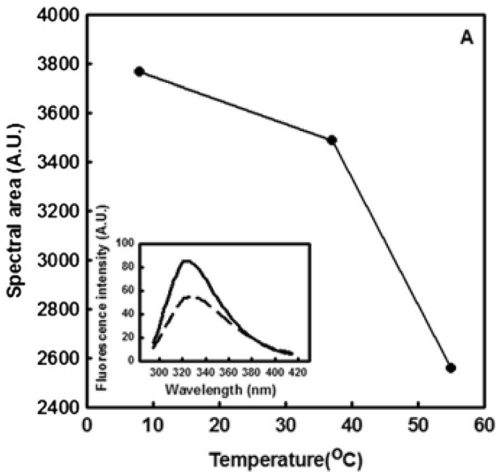

Fig. 1 Fluorescence spectral area of a representative Brazilian Meningococcal C conjugate bulk (MPCT) batch at 4, 37, and 55 °C. The lower spectral area is shown for the highest incubation temperature. Inset: fluorescence emission spectra at 4 °C (solid line) or 55 °C (dashed line). A red-shift from λ_{max} 324 nm to 328 nm and a decrease in fluorescence intensity were observed for samples incubated at 55 °C. The samples were excited at 280 nm and emissions were collected from 295 to 415 nm at 25 °C

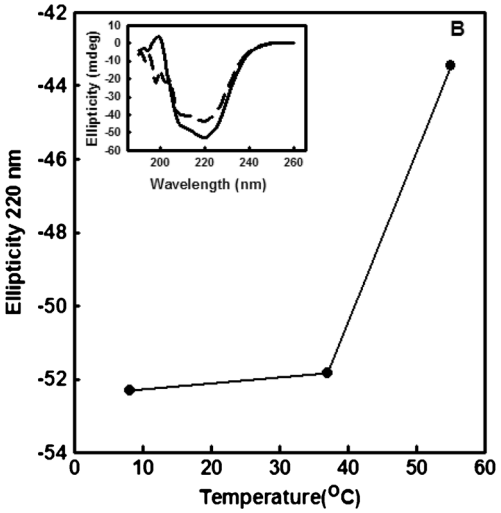

Fig. 2 Far-UV circular dichroism (CD) ellipticity (220 nm) of a representative Brazilian Meningococcal C conjugate bulk (MPCT) batch at 4, 37, and 55 °C. Inset: MPCT CD spectra at 4 °C (solid line) and 55 °C (dashed line). The decrease in secondary structural content at the highest temperature is shown as a decrease in negative ellipticity. Spectra were measured at 20 ± 1 °C

HPLC-SEC was used to monitor molecular integrity of the conjugate bulks. The MPCT control samples (stored at 4 °C), as well as those stored at 37 and 55 °C eluted as a single component, and all presented almost the same elution volume. Consequently, all samples were approved according to Kav range (0.2–0.35) [14]. On the other hand, the HPLC profile of the bulk conjugates stored at 55 °C for 5 weeks showed a slight peak base broadening (Fig. 4).

To determine if the change in the HPLC-SEC profile of samples stored at 55 °C was accompanied by hydrolysis of MenC polysialic acid, the quantity of free saccharide was measured by CZE. As a result, a progressive increase in the peak corresponding to free saccharide was observed as storage temperature increased. The average percentages of free saccharide were 10.4, 21.0, and 51.6% for samples stored at 4, 37, and 55 °C, respectively. These results show unconjugated sugar values above the specified limit (20%) for samples stored at 37 and 55 °C. Two peaks were observed at 9–10 min and 214 and 254 nm, suggesting the presence of degraded protein (data not shown).

The immunogenicity of the MPCT bulk was evaluated in mice to check the effect of structural interference triggered by temperature on the immune response. All MPCT bulks induced high total IgG measured by ELISA and SBA (≥4). According to our results, although chemical modifications were observed in the IgG and SBA titers after three doses, no differences were observed between the MPCT bulks stored at high temperatures (37 and 55 °C) when compared with the control temperature (Table 2).

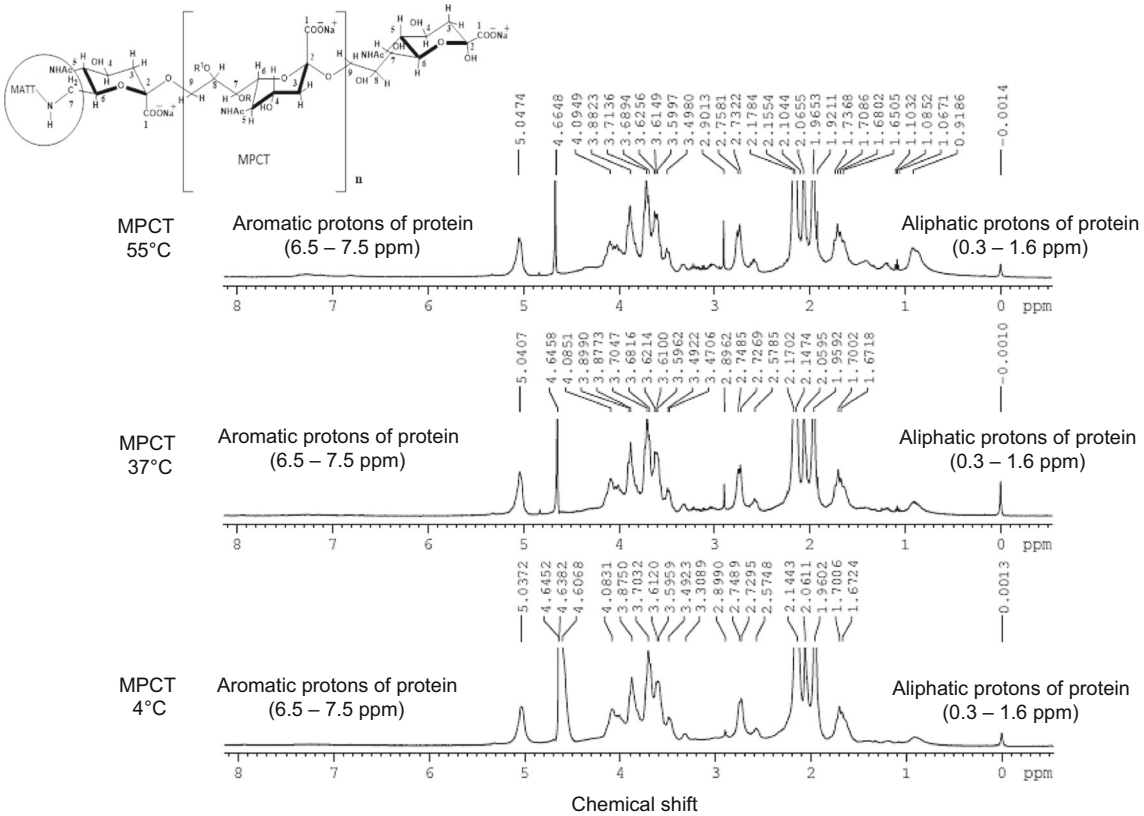

Fig. 3 400 MHz ^{1}H Nuclear magnetic resonance (NMR) spectra of a representative Brazilian Meningococcal C conjugate bulk (MPCT) conjugate bulk stored at the stated temperatures for 5 weeks. Signal intensity increased for aromatic (6.5–7.5 ppm) and aliphatic (0.3–1.6 ppm) signals as temperature increased from 4 to 55 °C

Discussion

A major concern during vaccine formulation is the ability to preserve biological activity during storage and handling until use. Careful attention to storage and handling is vital to ensure optimal potency of vaccines at the time of immunization [4]. According to the World Health Organization (WHO) recommendations for the production and control of meningococcal C conjugate vaccines, adequate stability studies are an essential part of vaccine development [21]. Vaccines against the same disease from different manufacturers should be considered individually because they may display different stability

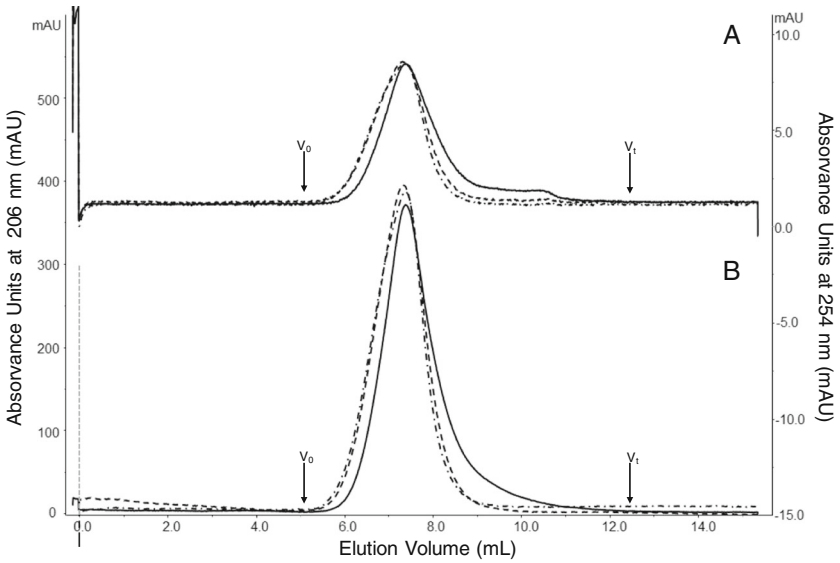

Fig. 4 High performance liquid chromatography-size exclusion chromatography chromatogram of a representative Brazilian Meningococcal C conjugate bulk (MPCT) using a TSK-G® 4000 PWxL analytical column. Peak base broadening is shown for bulk stored at 55 °C (solid line) compared to the profiles obtained at 4 °C (dashed line) and 37 °C (dashed and dotted line). The MPCT bulk was stored at the indicated temperatures for 5 weeks and monitored at 254 nm (A) and 206 nm (B)

Table 2 Total IgG titers measured by enzyme-linked immunosorbent (ELISA) and serum bactericidal antibody assays in immunized mice. Antibody levels in animal serum obtained after immunization with bulk conjugate stored at control temperature were compared with that immunized with the Brazilian Meningococcal C conjugate bulk (MPCT) stored at extreme temperatures

Temperature		4 °C		37 °C		55 °C	
Immunologic test		ELISA (Ln U mL^{-1})	SBA (GMT)[a]	ELISA (Ln U mL^{-1})	SBA (GMT)	ELISA (Ln U mL^{-1})	SBA (GMT)
Conjugate batch	# 1	6.95 ± 0.13	5787.6	6.49 ± 0.06	5790.3	6.06 ± 0.13	3249.1
	# 2	7.03 ± 0.04	5787.6	6.28 ± 0.10	5790.3	6.83 ± 0.35	8685.0
	# 3	5.92 ± 0.14	1447.1	5.94 ± 0.09	5790.3	7.41 ± 0.13	2895.3

[a] GMT (geometric mean titer); Statistical analysis was done by a non-parametric Mann–Whitney method with 95% confidence interval. No differences were observed in the ELISA titers obtained from MPCT stored at 4 °C compared with that stored at 37 °C ($p = 0.1082$) or 55 °C ($p = 0.1239$). Similarly, no differences were observed in the SBA titers between 4 and 37 °C ($p = 0.0646$), as well as 4 and 55 °C ($p = 0.9235$). The original natural protection surrogate for meningococcal serogroup C (MenC) disease was established as an SBA titer with human complement (hSBA) of ≥4 [19]

and reactogenicity profiles. Furthermore, studies under stressed conditions are useful in determining whether short-term accidental exposure to undesired conditions, such as during transport, can compromise product quality [4].

Physicochemical analysis methods have been widely used to determine the structural integrity and consistency of conjugate vaccines for controlling their quality. These methods provide a sensitive means of detecting changes induced in the vaccine by exposure to adverse environmental conditions that may influence the immunological properties of the conjugates. In addition, these procedures can also be used to monitor the need for subsequent biological testing [6, 23].

Despite that the MPCT was stored in a saline-based buffer, the possible effect of different temperatures on sample pH was investigated. The pH stability of a conjugate vaccine can be critically important, as changes in pH can trigger PS depolymerization. For example, decreases in pH can occur because of the de-*O*-acetylation process [6]. According to our results, after a 5 week incubation, no changes in pH of the bulks were observed in different storage temperatures (Table 1). Furthermore, the Neu5NAc and protein contents were not affected by temperature; therefore, the PS/Prot did not change, suggesting little or none alteration in conjugate structure.

Intrinsic fluorescence spectroscopy is a technique commonly used to detect conformational stability of protein molecules [7]. It has been used to characterize conjugated tetanus toxoid (TT) due to the spectral features of its aromatic amino acid side-chains [22, 23]. An excitation wavelength (λ_{ex}) of 280 nm gives an emission spectrum that is sensitive to solvent exposure of the Trp side chains and to a lesser extent tyrosine (Tyr), whereas λ_{ex} of 295 nm is used to obtain spectra almost exclusively due to Trp. The emission maximum (λ_{max}) of Trp is highly sensitive to the local environment surrounding its indole ring. Trp is a complex fluorophore with two nearby isoenergetic transitions that are affected differently by solvent polarity. In contrast, Tyr is rather insensitive to its local environment, so the λ_{max} appears to occur from a single electronic state. Trp is generally located within the core in the protein native folded state, whereas it is in a partially folded or unfolded state when exposed to solvent. In this sense, changes in conformation of a protein arising from Trp side chain solvent exposition can be tracked by red-shifts, *i.e.*, to longer λ_{max} upon protein unfolding and exposure of side chains; and by blue-shifts, *i.e.*, to shorter λ_{max} upon internalization of this residue. Trp displays high fluorescence intensity in a hydrophobic environment (buried within the core of the protein) whereas fluorescence intensity decreases in a hydrophilic environment (exposed to solvent) [23, 24].

According to our results, the observed red shift and F_{max} reduction for MPCT bulks incubated at 55 °C compared with samples stored at the control temperature (4 °C) could be due to partial protein unfolding (Fig. 1). A previous study showed some carrier protein denaturation after incubation at or above 37 °C of a Meningococcal C de-*O*-acetylated oligosaccharide-TT conjugate bulk, which may be considered indicative of higher thermal stability for the MPCT molecule [22].

Formaldehyde-mediated toxoid production does not involve extensive conformational changes [25, 26]. In the same way, some authors have reported little difference between the F_{max} of TT prior to and following conjugation, indicating that conjugation of the TT monomer to saccharide chains results in very little change in its conformation. This structural stability has been attributed to the formaldehyde-induced cross-linking that occurs during the detoxification stage of its manufacture [22, 23]. Indeed, our previous data show that TT and MATT are not different when evaluated by intrinsic fluorescence spectroscopy (data not shown).

Far-UV CD has been used to obtain information on changes in secondary structures of conjugated carrier proteins as a result of exposure to different temperatures [7, 22, 26, 27]. The far-UV CD analysis showed a maximum peak ellipticity at 220 nm for MPCT at all incubation temperatures studied and a substantial decrease in secondary structure when incubated at 55 °C (Fig. 2). This observation differs from that of Ho *et al*. (2002) who showed no apparent effect of high storage temperature on the conjugated toxoid secondary structure for MenC bulk stored in 150 mM NaCl, pH 5–6 [22]. An earlier study aimed at detecting structural modifications in

CRM197 following conjugation with the meningococcal C capsular oligosaccharide and incubation at various temperatures reported a relatively comparable result. In this case, no secondary structural change was detected in the carrier protein from vaccine bulk maintained in 0.9% (w/v) NaCl (approx. pH 5.6) compared to the CRM197 of a conjugate bulk stored in 10 mM sodium phosphate buffer (pH 7.2) [27]. The TT conformation is considered to be a characteristic of good vaccine quality, although there is no evidence suggesting that maintenance of the native-like conformation is a requirement for immunogenicity and protection [23]. The TT maintains a high degree of secondary and tertiary structure compared to that of the native toxin [28]. It also has remarkable thermal stability, retaining its conformational structure and polyclonal antibody binding properties after storage at 37 °C for at least 2 months [22]. This toxoid structural behavior could explain why there was no change in the MPCT conjugate secondary structure after the 37 °C incubation.

The conjugated MPCT protein was structurally stable when stored at temperatures up to 37 °C. Storage at 55 °C caused conformational changes in the conjugated toxoid structure as indicated by the decrease in secondary structural content (Fig. 2) and the red shift with the F_{max} reduction (Fig. 1), indicating higher mobility of the aromatic side chains and exposure to solvent following storage at high temperature. Taken together, the fluorescence and CD data revealed conformational changes in the protein at 55 °C. These findings contribute to define the stability parameters of the MCPT vaccine.

Proton NMR is commonly used to characterize, evaluate, and establish batch-to-batch consistency of conjugate vaccines, as recommended by the WHO [21]. ^{1}H NMR spectra of conjugate vaccines usually show a superimposition of relatively sharp signals originating from the saccharide component and broader peaks arising from the carrier protein. The MPCT peaks were previously assigned and detected between 1.5 and 5.4 ppm, indicating the presence of typical monomer signals [10, 11, 14, 29]. Peaks originating from the carrier protein have been observed between 0.3 and 1.6 ppm (aliphatic regions) and 6.5 and 7.5 ppm (aromatic regions), and are broader because the protein has less mobility than that of the saccharide moieties [22, 27]. The changes observed in the aromatic and aliphatic amino acid regions suggest structural modifications in the carrier protein with increasing incubation temperature (mainly 55 °C) (Fig. 3). This feature is similar to that described by Ho *et al*., 2001 and 2002 [22, 27] in which relative intensities of the peaks at 0.88 ppm and 6.6 and 7.4 ppm also increased in the spectrum of conjugate bulks stored at 55 °C. These results were associated with higher mobility of both aliphatic and aromatic regions of the conjugated protein, probably following partial loss of some of its tertiary structure [22].

Unlike globular proteins, conjugates have long branches of carbohydrate chains attached to the carrier protein [5, 22]. In fact, an elongated surface structure was proposed for TT, which would provide greater surface area for conjugation with polysaccharides than a more globular structure [30]. Consequently, conjugate elution volumes depend on hydrodynamic size, molecular weight, and possibly charge interactions between the molecules and the column matrix [5]. The TSK PWXL polymeric matrix was chosen to minimize these interactions in the chromatographic profiles. So, the HPLC profiles of MPCT bulks stored at 55 °C showed only a slight modification (peak base broadening) and no changes to hydrodynamic size or molecular weight were detected in the conjugate (Fig. 4). According to Bolgiano *et al*. (2001), the polysaccharide component of the conjugate vaccine depolymerizes at a rate that can vary with the type of conjugate under normal storage conditions and whether it is stored in an aqueous or lyophilized state [31]. Hence, the peak base broadening observed for the MPCT HPLC profile incubated at 55 °C suggests product degradation, suggesting greater exposure of the saccharide attached to the carrier protein due to structural changes in the conjugated toxoid under high temperature storage. Dissociation of these molecules and increasing size heterogeneity could also be a possible explanation. A similar behavior was observed by Ho *et al*. (2002) [22].

The quantification of free oxidized polysaccharide in MPCT by CZE [13] showed loss of saccharide following storage of bulk conjugates for 5 weeks at ≥37 °C). Free saccharide content was approximately 10% for MPCT bulk incubated at control temperature, whereas it was above the specified limit (20%) for samples incubated at 37 and 55 °C. A previous study reported a similar behavior for a meningococcal C de-*O*-acetylated oligosaccharide-TT conjugate vaccine after incubation under adverse conditions. Free saccharide content increased from 5 to 7% to about 20% when samples were stored for 5 weeks at 37 °C and to 70% during storage at 55 °C [22]. Another study using a combined conjugate vaccine against *Haemophilus influenzae* type b and meningococcal serogroup C (Hib/MenC), where both capsular polysaccharides were individually conjugated to TT, showed only a small increase in the percentage of free polysialic acid for the MenC component of the combined vaccine, from approximately 8% to 11–12%, when exposed to the highest temperatures [32]. In addition, another study assessed the stability and immunogenicity of meningococcal oligosaccharide C-CRM197 conjugate vaccines obtained by reductive amination. They showed 14% free saccharide content for a vaccine prepared by indirect coupling via a spacer to the carrier protein and about 42% free saccharide content in the bulk vaccine prepared by direct conjugation of oligosaccharide and stored at 55 °C [5]. Only the meningococcal polysaccharide that is covalently bound to the carrier protein is immunologically important for clinical protection, and excessive levels of unbound saccharide could potentially result in immunological hyporesponsiveness [4]. Taken together,

these results suggest that the structural stability of these conjugates may be influenced by the conjugation chemistry and the manufacturing process [22].

The immune response of murine serum was determined by the SBA titer and serogroup C specific IgG measured by ELISA. The SBA titer reflects functional antibodies, whereas the ELISA measures total or isotype-specific serum antibody response [6, 33, 34]. Protection from meningococcal infection depends not only on innate immunity, particularly a functional complement system, but also on the humoral antibody response. The "*in vitro*" SBA functional assay tests the ability of antibody-containing serum to kill meningococci in the presence of exogenous complement and is the only validated measure of protection against MenC disease [19, 35, 36]. An SBA titer ≥4 has been utilized to putatively indicate protection when using human complement serogroup C [19].

In our data, a storage temperature of 55 °C caused some loss (51.6%) of polysaccharide from the MPCT molecules. Unexpectedly, this did not result in a significant reduction in the mouse primary IgG response to the polysaccharide ($p = 0.9235$). This phenomenon might be related to the nature of TT as a good T cell immunogen, which provides a strong primary response, as well as the fact that this large 150 kDa protein enables many oligosaccharide chains to attach per mole carrier protein [27]. Some authors have studied the accessibility of epitopes on conjugated TT molecules using monoclonal IgG antibodies (mAbs), which are capable of recognizing not only linear but also conformational epitopes in both toxin and toxoid [23]. They observed that conjugates had significantly lower binding to the mAbs than did the carrier protein alone. On the other hand, mAbs recognizing linear epitopes also showed a preference for binding TT molecules with higher PS loading. This behavior was also observed in the study by Rana *et al*., 2015 [37] who proposed a relationship between polysaccharide size and conjugate immunogenicity. They showed comparable or better antibody titers for conjugates made of smaller sized saccharides compared with higher molecular weight saccharides, suggesting that saccharide fragments of shorter chain length, as opposed to high-molecular-weight, may be able to elicit a better T-cell dependent antibody response. According to the authors, this could be explained by the extent of glycosylation in the conjugates as a conjugate was made of smaller sized saccharides with a higher degree of glycosylation on a molar basis. However, the size of the saccharide must be sufficiently large to express epitopes representative of the native antigen, meaning that the presence of conformational epitopes may be an important determinant of the optimal size of the oligosaccharides used in conjugate vaccines [37].

The evaluation of physicochemical changes in immunogenicity of Hib oligosaccharide–CRM197 conjugate vaccines showed a parallel reduction in the levels of bactericidal activity and anti-Hib oligosaccharide IgG in the sera of animals immunized with Hib vaccines stored at 55 °C [31]. This was not seen in our results, given that both IgG and SBA titers were maintained in animals immunized with MPCT stored at 55 °C compared with that stored at the control temperature.

Our results show about 50% free saccharide content in MPCT stored at 55 °C, probably due to hydrolysis of glycosidic bonds in the oligosaccharide chains, but no difference was observed in the IgG or SBA titers (Table 2). This result suggests that there may still be sufficient conjugated saccharide to elicit an antibody response in this system, as considered previously [22]. It has been speculated that more stable conformational epitopes of PS species can result from the restriction of rotational movement of individual sugar residues and that these conformations may be preferred by antibodies. Although, the implications of these structural requirements are still poorly understood [37–39].

A polysaccharide vaccine usually elicits a very poor or undetectable IgG antibody response, whereas meningococcal C conjugate vaccines are very immunogenic, eliciting both IgG and IgM antibody responses directly against the polysaccharide in mice after one dose, although the serum IgG antibody response is comparatively greater [5]. In a previous study that assessed thermal stability of meningococcal C-TT (MenC-TT) and Hib-TT conjugate vaccines, a reduction in both IgG and IgM responses for MenC-TT was noted following storage at higher temperatures, but the decrease was not significant. On the other hand, an enhanced Hib polysaccharide response was detected when the Hib-TT vaccine was stored at 55 °C, again without significance [22]. Furthermore, no significant reduction in the primary IgG response was observed in mice immunized with a MenC bulk exposed to high temperature when compared to the bulk stored at control temperature. However, a significant decrease in the IgM titer was detected in mice injected with the same sample after storage at 55 °C [5]. Increasing the storage temperature to 37 or 56 °C reduced the IgG anti-MenC response slightly but not significantly by 31 and 19%, respectively [32]. These reductions in immunological responses were correlated with increased amounts of free saccharide in the treated samples and the lack of a statistical difference may be due to the inherently very high variation in the animal-to-animal responses as observed by other authors [37]. Although several studies have observed some changes in carrier protein conformation following storage under adverse conditions, only loss of bound saccharide has been associated with a decrease of immunogenicity in laboratory animals [22].

The presence of free PS or TT (100–400%) had little effect on the SBA titers of mice immunized with the serogroup A conjugate vaccine, which was produced using the same conjugation methodology used to produce the MPCT [40]. These results suggest that quite extensive degradation of oligosaccharide chains is necessary to reduce the anti-polysaccharide immune response, at least in animal

models, even though denaturation of the protein carrier is a sensitive indicator of MenC conjugate vaccine exposure to adverse conditions [5, 22].

The physicochemical changes observed in our study seem to have resulted essentially in conformational changes and modifications to the carrier protein. These changes are unlikely to affect cell T helper epitopes, as they are continuous and dependent on the primary amino acid sequence [31].

Polysaccharide-protein conjugate vaccines are regarded as well-defined, purified subunit vaccines but little had been reported on the accessibility of key protein epitopes that might serve as the glycopeptide epitopes presented to antigen-presenting cells in its role as a carrier protein, or even as protective toxin neutralising epitopes. Although the carrier protein is of utmost importance in converting the T-cell independent polysaccharide antigen to a Tcell dependent antigen, other parameters, for example saccharide chain length, the linker used for conjugation, and random or selective carbohydrate modification, can have an impact on the immunogenicity of conjugate vaccines. A study aming to analyze the conformation and structure of the TT used as carrier in a variety of glycoconjugates showed that the various TT molecules in these glycoconjugates had different accessibility to carrier-specific monoclonal antibodies, and concluded that the stimulation of B- and T-cells to glycoprotein conjugates and thus the effectiveness of the conjugates may be determined by different carrier structures [23, 41]."

In general, MenC conjugate vaccines are stable over a wide range of storage temperatures. A short-term stability evaluation of these vaccines over 5 weeks at a variety of temperatures demonstrated that the structural stability of the polysaccharide chains and of the carrier protein vary between conjugate vaccines [4]. In the same way, a long-term real-time study of a MenC conjugate vaccine demonstrated robust thermal stability at both refrigerated (42 months) and room temperatures (9 months), even though vaccine shelf life at refrigerated temperatures is generally about 2 years [4, 8].

In conclusion, the present study showed that physicochemical methods alone are insufficient to predict the biological activity of a MPCT conjugate vaccine without extensive validation against immunological data. However, the physicochemical examination provides a sensitive means of detecting changes induced in a vaccine exposed to adverse environmental conditions, which may influence the immunological properties of the conjugates.

Compliance with ethical standards

Conflict of interest None of the authors has conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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