

## Procedure for the conjugation of the *Streptococcus pneumoniae* serotype 6B capsular polysaccharide to the tetanus toxoid

✉ Jean P Soubal<sup>1</sup>, Luis Peña<sup>1</sup>, Darielys Santana<sup>1</sup>, Yury Valdés<sup>1</sup>, Dagmar García<sup>2</sup>, Jessy Pedroso<sup>3</sup>, Félix Cardoso<sup>3</sup>, Humberto González<sup>4</sup>, Violeta Fernández<sup>5†</sup>, Vicente Vérez<sup>6</sup>

<sup>1</sup> Departamentos de Glicoconjugación, <sup>2</sup> Inmunología y <sup>3</sup> Análisis Centro de Química Biomolecular, CQB Ave. 21 y Calle 200, Reparto Atabey, Apdo. 16042, Municipio Playa, CP 11600, La Habana, Cuba

<sup>4</sup> Departamento de Desarrollo y Escalado, Instituto Finlay Calle 27, No. 19805, La Lisa, AP 16017, CP 11600, La Habana, Cuba

<sup>5</sup> Dirección de Investigaciones y <sup>6</sup> Dirección General, Centro de Química Biomolecular, CQB ✉ jean.pierre@cqb.cu

### ABSTRACT

*Streptococcus pneumoniae* causes annually 826 000 deaths in children under five years. The serotype 6B, one of higher incidence, is targeted by the Cuban research and development project to develop a conjugate vaccine. There is limited data on how modifications caused by conjugation affect the physicochemical and antigenic characteristics of polysaccharides, particularly for serotype 6B capsular polysaccharide (PS6B), despite being the least immunogenic among *S. pneumoniae* polysaccharides. In this work, a conjugation procedure was established for PS6B comprising: fragmentation by acid hydrolysis, activation by periodate oxidation, and conjugation to tetanus toxoid (TT) by reductive amination to increase its immunogenicity. Reaction conditions were set to obtain the polysaccharide in three molecular size ranges (1-10, 10-30, 30-100 kDa) and levels of oxidation. PS6B fragmentation below 10 kDa and oxidation above 24 % of the repetitive units implied the loss of antigenicity. Polysaccharide length but not oxidation level had an impact on the physicochemical characteristics of the conjugates in the tested conditions. Unlike the native polysaccharide, conjugated 10-30 kDa and 30-100 kDa PS6B were immunogenic in rabbits, with evidence of thymus-dependent response. The procedure described supports obtaining PS6B-TT conjugates reproducibly in the 30-100 kDa and 10-30 kDa molecular size ranges and with 8-18 % oxidized repeat units, which are immunogenic.

**Keywords:** polysaccharide, conjugate, *Streptococcus pneumoniae*, 6B serotype

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### RESUMEN

**Procedimiento de conjugación del polisacárido capsular de *Streptococcus pneumoniae* serotipo 6B a toxoide tetánico.** Anualmente *Streptococcus pneumoniae* provoca 826 mil muertes de niños menores de cinco años. El estudio del serotipo 6B, uno de los de mayor incidencia, es uno de los objetivos del proyecto de investigación-desarrollo para obtener una vacuna conjugada en Cuba. La estrategia de conjugación del polisacárido capsular del serotipo 6B (PS6B) fue fragmentarlo mediante hidrólisis ácida, activarlo mediante oxidación con periodato y conjugarlo a toxoide tetánico (TT) mediante aminación-reductiva. Existe poca información sobre cómo estas modificaciones afectan las características físico-químicas y antigénicas del polisacárido, en particular para PS6B, a pesar de ser el menos inmunogénico de los polisacáridos de *S. pneumoniae*. En tal sentido, se estableció un procedimiento para obtener conjugados inmunogénicos de PS6B a TT. Se crearon condiciones de reacción para obtener el polisacárido en tres rangos de tallas y niveles de oxidación. Se determinó que la fragmentación del polisacárido por debajo de 10 kDa y la oxidación de más del 24 % de las unidades repetitivas implican pérdida de antigenicidad. La talla del polisacárido tuvo impacto en las características físico-químicas de los conjugados en las condiciones evaluadas; no así el nivel de oxidación. A diferencia del polisacárido nativo, conjugados de PS6B de 10 a 30 kDa y 30 a 100 kDa fueron inmunogénicos en conejos, con evidencias de respuesta timo-dependiente. Los procedimientos que incluyen la obtención del PS6B de 10 a 30 kDa y 30 a 100 kDa con niveles de oxidación entre 8 y 18 % de las unidades repetitivas oxidadas, permitieron obtener conjugados a TT reproducibles e inmunogénicos.

**Palabras claves:** polisacárido, conjugado, *Streptococcus pneumoniae*, serotipo 6B

### Introduction

Yearly, *Streptococcus pneumoniae* (pneumococcus) causes 14.5 millions of pneumonia, bacteriemia and meningitis episodes worldwide, with 826 000 deaths among infants younger than five years [1]. Vaccines against this bacterium have been available for the last 30 years, mostly based on the envelope capsular polysaccharides (PS), and from up to 23 different circulating serotypes. However, those vaccines are not immunogenic in children younger than 2 years, the most susceptible population. This happens because their immune

system is unable to generate the adequate immune response against T-independent antigens, as the bacterial polysaccharides [2]. This problem has been addressed by developing conjugate vaccines, capable of inducing the required T-dependent immune responses against PS [3]. The first of these vaccines was licensed in 2000 (Prevenar®, against seven serotypes), and more recently other two (Synflorix® and Prevenar 13®, against 10 and 13 serotypes, respectively), but at very high prices in the market. In Cuba, there is a project currently

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at the research-development phase to develop a pneumococcal conjugate vaccine covering seven serotypes on its first stage: 1, 5, 6B, 14, 18C, 19F and 23F.

Among them, serotype 6B is one of the most frequently causing invasive diseases in children younger than five years in countries either developed or underdeveloped [4], like in Cuba and its geographic area [5]. It is also one of the most common causes of otitis media worldwide [6]. The three commercially available vaccine formulations contain the serotype 6B PS (PS6B), a component of most of the vaccine candidates assayed in clinical trials. Most of the studies describe the conjugate of this serotype as the less immunogenic [7, 8]. Its repetitive unit (RU) comprises the sequence  $\rightarrow 2$ - $\alpha$ -D-Galp-(1-3)- $\alpha$ -D-Glcp-(1-3)- $\alpha$ -L-Rhap-(1-4)-D-RibOH-(5-PO<sub>4</sub>) $\rightarrow$  [9]. This polymeric structure is linear, with phosphodiester bonds which confers it a periodic negative charge. Its low immunogenicity results from its relatively simple structure and its similarity to DNA [10].

The conjugation strategy proposed by our project comprises the fragmentation of PS by acid hydrolysis, followed by PS activation by periodate oxidation and conjugation to the tetanus toxoid (TT) by reductive amination. Although this conjugation method is frequently used, there are few studies elucidating how such modifications affect the physicochemical and antigenic properties of PS in general. Particularly for PS6B, it has not been previously studied, in spite of its low immunogenicity. PS modifications during the conjugation process could affect the natural epitope conformation and promote the appearance of neoantigenic structures that subsequently affect the specificity of the immune response attained [11]. Therefore, the present study established a procedure to conjugate the PS6B to TT. It required to establish reproducible reaction conditions on each step and to determine the size and activation levels required for PS in order to preserve its epitopes and to efficiently generate its immunogenic conjugates.

## Materials and methods

PS from serotypes 6B (batch 806), 18C (batch 801) and 19F (batch 802), and TT protein (batch 6017), were produced by the Development and Scale up department, and the Production plant II, respectively of the Finlay Institute, Havana, Cuba.

### Fragmentation of native PS6B

The dissolution of 50 mg of PS6B was added with either a dissolution of acetic acid (Merck) or trifluoroacetic acid (TFA; Merck, Germany) to a final concentration of 0.25 M or 0.1 M, respectively. Each dissolution was further incubated at 70 °C and aliquots were collected at different times (Table 1). Aliquots were neutralized and molecular size fragmented by ultrafiltration (Amicon, Millipore Corp., Bedford, MA), with distilled water using regenerated cellulose membranes (Millipore, USA) with subsequent molecular cutoffs of 100, 30, 10 and 1 kDa. Each fraction was denominated according to the interval of cutoff values of the membrane retaining it and the one of the previous fragmentation step, and carbohydrate content was determined. The condition providing the highest yield for each fraction was reproduced, and the products

**Table 1. Study of hydrolysis conditions of the capsular polysaccharide of *Streptococcus pneumoniae* serotype 6B**

Conditions	Acid	† (min)	Yield (%)		
			1-10 kDa	10-30 kDa	30-100 kDa
AcOH 0.25 M		90	nd	0	65.1
		120	nd	3.2	82.9†
		180	0	38.3	60.8
TFA 0.1 M		30	nd	46.2	55.9
		60	2.0	86.3†	10.6
		90	37.9	59.7	0
		120	71.2†	30.1	nd

† Selected conditions.

AcOH: acetic acid; TFA: trifluoroacetic acid.

nd: not determined.

obtained were analyzed attending to carbohydrate content, by monodimensional proton nuclear magnetic resonance (NMR-<sup>1</sup>H), size exclusion-high performance liquid chromatography (SE-HPLC) and in antigenicity studies. Yields (Y) were determined according to the following formula:

$$Y = \frac{\text{final carbohydrate mass}}{\text{initial carbohydrate mass}} \times 100$$

### Activation of fragmented PS6B

A dissolution of 20 mg of fragmented PS6B (fractions 10-30 kDa or 30-100 kDa) was added with a sodium periodate dissolution (NaIO<sub>4</sub>; Riedel-de Haen) to final concentrations of 2.5, 5 or 10 mM in PBS, pH 7.0, and were further incubated for 3 h in the darkness. The reaction was stopped by adding 5  $\mu$ L of glycerol (Plu-sone). The reaction mix was diafiltered against 5 volumes of distilled water, using a membrane with cutoff values of 10 kDa for the 10-30 kDa fraction or 30 kDa for the 30-100 kDa fraction. The products obtained were analyzed for carbohydrate and aldehyde groups content, by NMR-<sup>1</sup>H, SE-HPLC and antigenicity. The activation level was expressed as the percentage of oxidized repetitive units (ORU), calculated by the following formula:

$$\text{ORU} = \frac{\text{moles of aldehyde groups}}{\text{moles of RU}} \times 100$$

where:

RU: repetitive units

Yield was determined ad in the fragmentation study.

### Conjugation of activated PS6B

Polysaccharides from 10-30 kDa and 30-100 kDa fractions were used, with ORU percentage ranges 8-12 and 13-18%. PS (20 mg) were added with 1 mL of 10 mg/mL TT dissolution. Subsequently, 4 mg of sodium borohydride (Merck, Germany) were added and incubated for approximately 18 h at room temperature. Two milligrams of sodium borohydride were further added and incubated for 2 h to eliminate the remaining aldehyde groups. The reaction mix was diafiltered against 10 volumes of distilled water with a 100 kDa cutoff membrane. The products obtained were analyzed for carbohydrate and protein content, by NMR-<sup>1</sup>H, SE-HPLC and antigenicity.

### Analytical methods

Carbohydrate content was determined by the orcinol-sulfuric acid method [12] using a glucose standard

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curve, and the carbonyl group by the modified Park-Johnson's method [13], also against a glucose standard curve. Protein content of the conjugates was assessed by the Lowry's method [14], with a bovine serum albumin (BSA) standard curve.

RU structure was followed by NMR-<sup>1</sup>H. The samples were prepared in D<sub>2</sub>O. Spectra were obtained at 25 °C using a 250 MHz equipment (Bruker AC-250F). Signals were initially assigned according to van Dam *et al.* [15]. Chromatography analyses were done by SE-HPLC (Knauer Smartline, Germany) using a TSK 5000PW (TOSOH) column with refractive index detector. The distribution constant ( $K_D$ ) was calculated as follows:

$$K_D = \frac{(T_r - T_0)}{(T_t - T_0)} \times 100$$

where:

$T_r$ : sample retention time;

$T_0$ : retention time of a 2000 kDa dextran standard (Blue Dextran, Sigma);

$T_t$ : NaN<sub>3</sub> retention time.

The relative molecular weight (M<sub>W</sub>r) was determined by using a calibration curve of dextran standards (American Polymer Standard Corp.). The width at half peak height was determined with the program ClarityChrom 2.4.4.80 (Knauer, Germany).

The unbound protein content was estimated by the same SE-HPLC system using a Superose 12 column (Pharmacia, USA) with ultraviolet detector. The area under the curve for each peak was integrated with the ClarityChrom 2.4.4.80 program.

#### Antigenicity determination

The antigenicity of polysaccharide derivatives was determined by an inhibition immunoenzymatic assay (ELISA). A standard serum against SP6B (factor 6c, Statem Serum Institute, Copenhagen, Denmark) diluted 1/3200 was used, followed by incubation with each inhibitor at concentrations ranging 0.005-500 µg/mL, overnight at 4 °C. The fragmented polysaccharides were used as inhibitors, either active or conjugated accordingly. Additionally, serotypes 18C and 19F PS were included as negative controls (non-related polysaccharides). For ELISA assays, polystyrene 96-well microtiter plates (Maxisorp, Nunc, Denmark) were coated with 10 µg/mL PS6B in phosphate buffer saline (PBS), pH 7.2, and incubated overnight at 37 °C. Plates were subsequently blocked with 1 % BSA for 30 min at 37 °C, and then, the anti-SP6B standard serum preincubated with the different inhibitors was added. Afterwards, the anti-rabbit IgG-horseradish peroxidase conjugate (HRP; Sigma, Germany) was added at a 1:10 000 dilution (in PBS, 0.3 % Tween-20, 0.01 M EDTA, 1 % BSA) and further incubated for 90 min at room temperature. Microplates were washed thrice after each step with washing solution (PBS, 0.05 % Tween-20). Finally, the HRP substrate solution was added (0.5 mg/mL ortho-phenylenediamine and 0.1 % (v/v) H<sub>2</sub>O<sub>2</sub>) in citrate buffer solution, pH 5.6, and the reaction was developed for 20 min in the darkness. The reaction was stopped by adding 3 M HCl. Absorbance was determined at 492 nm and the percent inhibition (PI) was calculated as follows:

$$PI = 100 - \left( \frac{\text{absorbance of serum plus inhibitor}}{\text{absorbance of serum}} \times 100 \right)$$

#### Rabbit immunization

Two conjugates obtained from PS activated at ORU percentages ranging 13-18 % from fractions 10-30 kDa (Cj10-30) and 30-100 kDa (Cj30-100) were administered. Three groups of New Zealand White rabbits (Cenpalab, Havana, Cuba), five animals each, were immunized with 4 µg of conjugated PS, 25 µg of non-conjugated PS or placebo, in aluminum phosphate. Three doses of each immunogen were administered on days 0 (start of the study), 14 and 28 respectively. Blood was extracted from all the animals on days 7, 21 and 35. Sera were stored at -20 °C until use. The study was conducted in compliance with international guidelines for ethical conduct in the care and use of laboratory animals for scientific purposes. The Ethics Committee and the Quality Control Department of the Center of Biomolecular Chemistry (CQB, Havana, Cuba) approved all the experimental procedures.

#### Evaluation of the immune response induced by the conjugates

The IgG antibody response against PS was determined by an indirect ELISA, using the native PS6B for coating [16]. The antibody titer was calculated by a regression analysis between optical density (OD) values and the base ten log of the reciprocal of the serum dilution. Antibody titer was considered as the reciprocal of the serum dilution at which the absorbance reached twice the value of the 1/100 pre-immune serum. Titers above 50 were regarded as positive.

The IgG response against the TT carrier protein was determined as previously described to assess the response against the polysaccharide. In this case, plates were coated with 1 µg/mL TT in PBS, pH 7.2.

IgG antibody avidity against PS6B was determined by an ELISA similar to that used for characterizing the response against the polysaccharide, with some modifications. After serum incubation, a step of incubation with 0.5 M ammonium thiocyanate (NH<sub>4</sub>SCN, Merck) for 15 min was applied to half of the replicates. The resulting two dilution curves for each sample, with and without NH<sub>4</sub>SCN allowed the calculation of the avidity index (AI) as follows [17]:

$$AI = 100 \times \frac{\text{titer with NH}_4\text{SCN}}{\text{titer without NH}_4\text{SCN}}$$

The serum dilution showing an absorbance value of half the value of the serum diluted 1/100 without NH<sub>4</sub>SCN was considered as titer.

#### Statistical analyses

Results of the physicochemical evaluations were expressed as arithmetic means ± standard deviation of five independent experiments (three in the case of conjugates); the variation coefficient was also calculated for fragmentation yields and PS oxidation levels. Immunological evaluations were expressed as arithmetic means ± standard deviation of five animals. The statistically significant differences between groups were determined by the non-parametric Kruskal-Wallis test, and when present, a Dunn's test was used *a posteriori*. Means were compared against reference

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values by the Wilcoxon test. Probability values (p) lower than 0.05 were considered as statistically significant. All the analyses were done with the Graph Pad Prism 4.03 program.

## Results

### Fragmentation of the capsular PS6B

PS was fragmented by acid hydrolysis. The study was carried out by two acids dissolutions, assayed at different times. The conditions yielding the highest PS amounts were: 2 h in 0.25 M acetic acid for fraction 30-100 kDa, 1 h in 0.1 M TFA for fraction 10-30 kDa, and 2 h in 0.1 M TFA for fraction 1-10 kDa (Table 1). These conditions reproducibly yielded more than 50 % PS (Table 2). The three processes rendered variation coefficients (VC) below 6 %.

NMR-<sup>1</sup>H spectra recorded for the fragmented PS were very similar to that of the native PS, indicating that the RU structure was preserved. Low intensity signals at 3.22 ppm and around 2.1 ppm in the native PS spectrum corresponded to polysaccharide C [18]. This contaminant, common among pneumococcal PS, was found at very low amounts in the starting material and decreased even more after fragmentation. There were no signals of end-product monosaccharides resulting from size reduction, not even in the 1-10 kDa PS fraction (Figure 1).

$K_D$  increased conversely with size reduction.  $W_{0.5}$  values for fragmented PS were lower than those of the native PS, indicating a decrease in polydispersion. The rMW for each fraction was higher than the cut-off values of the flat ultrafiltration membranes used (Table 2).

Antigenicity, evaluated by an inhibition ELISA and using a polyclonal serum specific for PS6B, was only similar to that of the native PS for fragmented PS fractions 30-100 kDa and 10-30 kDa. In fact, the concentrations of these two fractions required to achieve 50 % inhibition were in the same order of that of the native PS, while that of the PS fraction 1-10 kDa was two orders higher (Figure 2A). For this reason, fractions 30-100 kDa and 10-30 kDa were selected for further experimentation.

### Activation of fragmented PS6B

Fragmented PS were activated by periodate oxidation. A study was conducted for this reaction in fractions 10-30 kDa and 30-100 kDa, varying the  $\text{NaIO}_4$ /PS ratio (Table 3). A linear relationship was achieved for the equivalent amounts of  $\text{NaIO}_4$  and the number of aldehyde groups generated ( $R^2 = 1.0$  in the 10-30 kDa fraction and  $R^2 = 0.99$  in the 30-100 kDa fraction). ORU VC obtained at the different conditions ranged 11-20 %. There were no differences between the results attained for both working fractions. More than 85 % of the starting amount of PS was recovered in all the experiments, indicating that the oxidation reaction conditions did not generate fragmentation.

NMR-<sup>1</sup>H spectra of activated PS showed the chemical shifts and relative intensities of signals typical of the non-inactivated PS fragments (Figure 3A). There were no signals corresponding to the carbonyl proton at lower fields. Noteworthy, two new signals appeared at 5.10 and 5.09 ppm following those of the anomeric

**Table 2. SE-HPLC evaluation (TSK 5000PW) of *Streptococcus pneumoniae* serotype 6B capsular polysaccharide fragmented under the conditions selected†**

Fraction	Yield (%)	$K_D$	$W_{0.5}$ (min)	rMW (kDa)
30-100 kDa	84.0 ± 3.8	0.44 ± 0.02	1.47 ± 0.06*	134 ± 6
10-30 kDa	83.2 ± 3.0	0.57 ± 0.02	1.27 ± 0.11*	55 ± 8
1-10 kDa	69.2 ± 3.7	0.67 ± 0.02	1.04 ± 0.10*	20 ± 3
PsN	-	0.2	1.70	643

† Results reported as average ± standard deviation (n = 5).

\* p vs. 1.70 (0.031).

$K_D$ : distribution constant.

$W_{0.5}$ : peak width at half peak height.

rMW: relative molecular weight.

PsN: native polysaccharide.

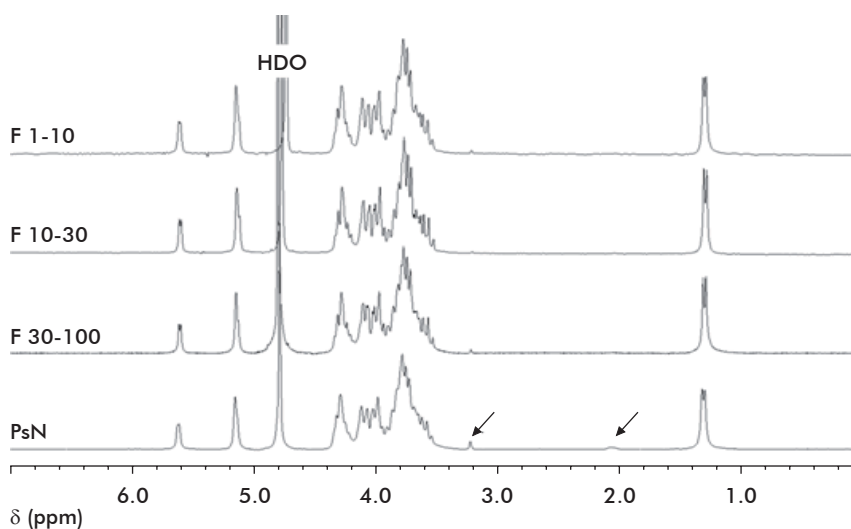
protons of ramnose and glucose superimposed at 5.15 ppm. The relative intensities of those two new signals were higher for PS showing the highest oxidation level (Figure 3B). These correspond to the signal of the anomeric proton of ramnose produced by the shortage in ORU of ribitol.

$K_D$  values were the same as for the inactivated PS for the given fractions (Tables 2 and 3). These results confirmed that none of the oxidation conditions used seems to generate PS fragmentation.

While evaluating antigenicity, PS with a similar activation level up to 17.2 % ORU behaved similar to the fragmented PS of the same size. For these PS, concentrations required for a 50 % inhibition of serum were 1 and 2 times that of the native PS, respectively. On the contrary, the PS activated at 24.4 % ORU showed a drop in the inhibition capacity depending on its concentration, requiring a concentration 25-fold than that of the native PS to achieve a 50 % inhibition. Activation at 62.5 % ORU caused a drastic decrease in the inhibition capacity (Figure 3B). These results indicate that the activated PS antigenicity becomes affected when ORU is higher than 24.4 %.

### Conjugation of activated PS6B

Activated PS of the working fractions 10-30 kDa and 30-100 kDa were conjugated at two ranges of



**Figure 1. Evaluation of the preservation of repetitive unit structure in fragmented *Streptococcus pneumoniae* serotype 6B capsular polysaccharide (PS). Arrows indicate the signals corresponding to contaminant polysaccharide C. F1-10, F10-30 and F30-100: PS of the fragmented working fractions of 1-10 kDa, 10-30 kDa and 30-100 kDa, respectively. HDO: semiheavy water. PsN: Native PS.**

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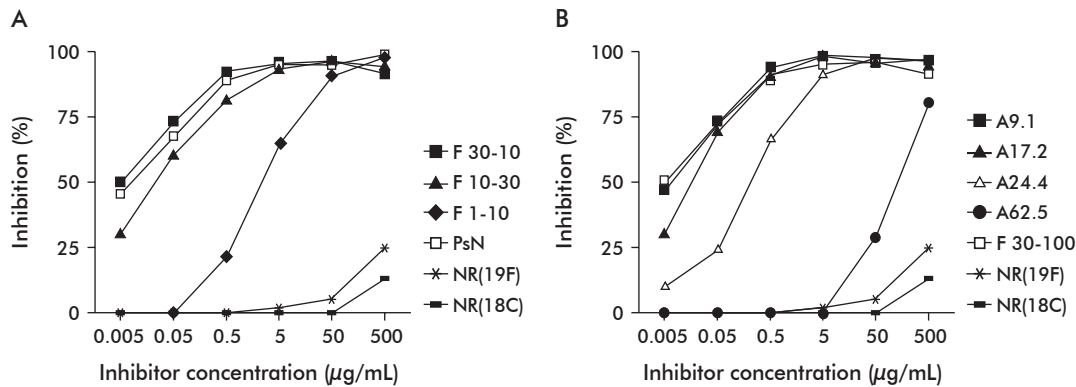


Figure 2. Antigenicity evaluations by inhibition ELISA of a polyclonal serum specific against the capsular polysaccharide (PS) of *Streptococcus pneumoniae* serotype 6B. A) Evaluation of PS fragmented working fractions. B) Evaluation of PS with different levels of activation. PsN: native PS; F1-10, F10-30 and F30-100: PS of the fragmented working fractions of 1-10 kDa, 10-30 kDa and 30-100 kDa, respectively. A9.1, A17.2, A24.4 and A62.5: PS activated with 9.1, 17.2, 24.4 and 62.5 % of oxidized repetitive units, respectively. NR (19F) and NR (18C): Non-related PS from serotypes 19F and 18C.

activation levels to TT. There was no free protein detected in the conjugates obtained. Figure 4A shows the typical chromatograms. The differences detected in the PS activation levels had no influence on the properties evaluated. Otherwise, PS from the 10-30 kDa had higher  $K_D$  and lower PS/protein ratio than that obtained from the 30-100 kDa (Table 4).

NMR- $^1\text{H}$  of PS conjugates retained the typical chemical shifts and relative intensities. There were new signals of low intensities in the range 1.0-0.8 ppm, corresponding to the aliphatic amino acids of the protein (Figure 4B). On the other hand, the capacity to inhibit the reference serum remained almost the same. The concentration required to achieve 50 % inhibition were of the same order of magnitude compared to that of the activated PS prior to conjugation (Figure 4C). These results indicated that conjugation did not significantly affect either the structure or the exposure of PS.

#### Evaluation of the antibody immune response generated by the conjugates

The conjugates obtained from the fractions 10-30 kDa (Cj10-30) and 30-100 kDa (Cj30-100) were tested by subcutaneous immunization of New Zealand White rabbits in three doses. The non-conjugated PS was administered as control and did not induce positive anti-PS6B antibody titers at any of the time points evaluated, and the response remained at baseline level as placebo (Figure 5). On the contrary, both conjugates generated IgG antibody titers against PS, with logarithmic titers above 2 after the second dose. On day 21, the Cj30-100 conjugate antibody titer was very highly statistically significant compared to that of the Cj10-30 ( $p < 0.001$ ). This difference did not remain after the third immunization, due to an increase in the immune response generated by Cj10-30 ( $p < 0.001$ ); but not by Cj30-100 (Figure 5A).

The quality of the response was evaluated by determining the AI for antibodies against PS on days 21 and 35. After the second dose, sera from the group receiving Cj10-30 overcame the 50 % of inhibitory activity (IA), in contrast to those of the group immunized with Cj30-100, which were lower ( $p < 0.001$ ).

Table 3. Results of the periodate oxidation reaction for the *Streptococcus pneumoniae* serotype 6B capsular polysaccharide fractions 10-30 kDa and 30-100 kDa<sup>†</sup>

Fraction	mol $\text{NaIO}_4$ / mol RU	ORU (%)	Yield (%)	$K_D$
10-30 kDa	0.18	$9.9 \pm 1.6$	$92.7 \pm 4.5$	$0.57 \pm 0.05$
	0.35	$15.7 \pm 2.1$	$92.6 \pm 6.1$	$0.56 \pm 0.05$
	0.71	$27.6 \pm 6.4$	$95.4 \pm 3.2$	$0.56 \pm 0.05$
30-100 kDa	0.18	$9.0 \pm 1.0$	$92.5 \pm 4.0$	$0.43 \pm 0.02$
	0.35	$15.1 \pm 2.5$	$95.8 \pm 4.2$	$0.43 \pm 0.02$
	0.71	$27.9 \pm 5.4$	$93.7 \pm 4.9$	$0.44 \pm 0.02$

<sup>†</sup> Results reported as average  $\pm$  standard deviation ( $n = 5$ ).

RU: repetitive units.

ORU: oxidized repetitive units.

$K_D$ : distribution constant.

Following the third dose, IA increased in both groups ( $p < 0.001$ ) to near 90 %, reaching a level similar to that of the reference serum (Figure 5B).

Regarding the response against the carrier protein, there were no differences after three doses among the groups immunized with both conjugates ( $p > 0.05$ ) (Figure 5C); in spite of the TT dose being 70 % higher in the Cj10-30.

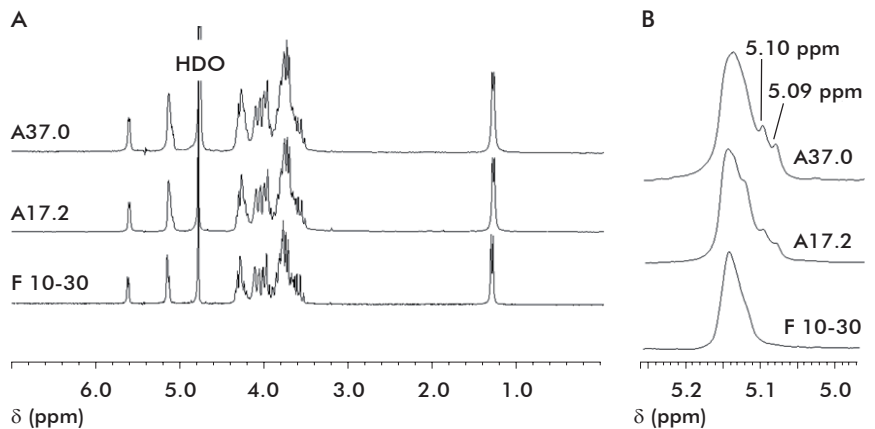


Figure 3. Preservation of the repetitive unit of the activated capsular polysaccharide (PS) fractions of *Streptococcus pneumoniae* serotype 6B. A) Superposition of NMR- $^1\text{H}$  spectra from PS at different activation levels and the respective fragmented PS. B) Detail of the spectral area from 5.0 to 5.25 ppm showing the new signals corresponding to the activated PS. A37.0 and A17.2: PS activated with 37.0 and 17.2 %, respectively of oxidized RU; F10-30: Fragmented PS.

**Table 4.** Physicochemical properties of the *Streptococcus pneumoniae* serotype 6B capsular polysaccharide (PS) conjugates to tetanus toxoid †

PS fraction	ORU (%)	PS/Prot.	$K_D$
10-30 kDa	13-18	$0.7 \pm 0.3$	$0.42 \pm 0.02$
	8-12	$0.8 \pm 0.3$	$0.41 \pm 0.04$
30-100 kDa	13-18	$1.7 \pm 0.4$	$0.28 \pm 0.02$
	8-12	$1.6 \pm 0.4$	$0.31 \pm 0.05$

† Results reported as average  $\pm$  standard deviation ( $n = 3$ ). None of the fractions contained unbound protein.

ORU: oxidized repetitive units.

PS/prot.: Carbohydrate/protein ratio (w/w).

$K_D$ : distribution constant.

## Discussion

The methods available to obtain PS-protein conjugates are quite varied [19]; but few are suitable for vaccine development. The controlled periodate oxidation of PS was selected, followed by reductive amination of the produced aldehyde groups by the amino groups of the protein. This is a simple and scalable method, applicable to PS from pneumococcal serotypes included in the Cuban vaccine project. It is also the method used to produce the Prevenar® and Prevenar13® vaccines [20]. In contrast to procedures used to generate commercial vaccines against pneumococci, PS was fragmented and the desired size range selected. By these means, a more efficient and consistent process is guaranteed, also favoring the quality of the immune response generated while administering the conjugate [21].

Moreover, the difference in the rMW distribution of the conjugate supported the exclusion of part of the free PS, as indicated by the change in the PS/protein ratio of the final conjugates compared to the reaction mix. A significant amount of free PS for this serotype could decrease the conjugate immunogenicity, more significantly for PS at higher rMW [22]. Acid hydrolysis was selected among the PS fragmentation methods available, due to its applicability to most PS molecules of the vaccine candidate and the easiness for scale up.

A methodology was followed considering all the factors needed, even those counteracting, to find the most adequate reaction conditions. Particularly relevant were the preservation of PS natural epitopes and the enhancement of the thymus-dependent character of the antigen once conjugated, reaction times no longer than 3 h a day, highly reproducible and potentially robust processes, rendering PS yields above 50 %.

The fragmentation study involved two acids of different strengths. Acetic acid supported to obtain PS in the 30-100 kDa fraction and TFA in the fractions 10-30 kDa and 1-10 kDa. Yields were higher than predicted and the reproducibility was regarded as adequate. These conditions would require further adjustment when working with native PS batches of other rMW distributions.

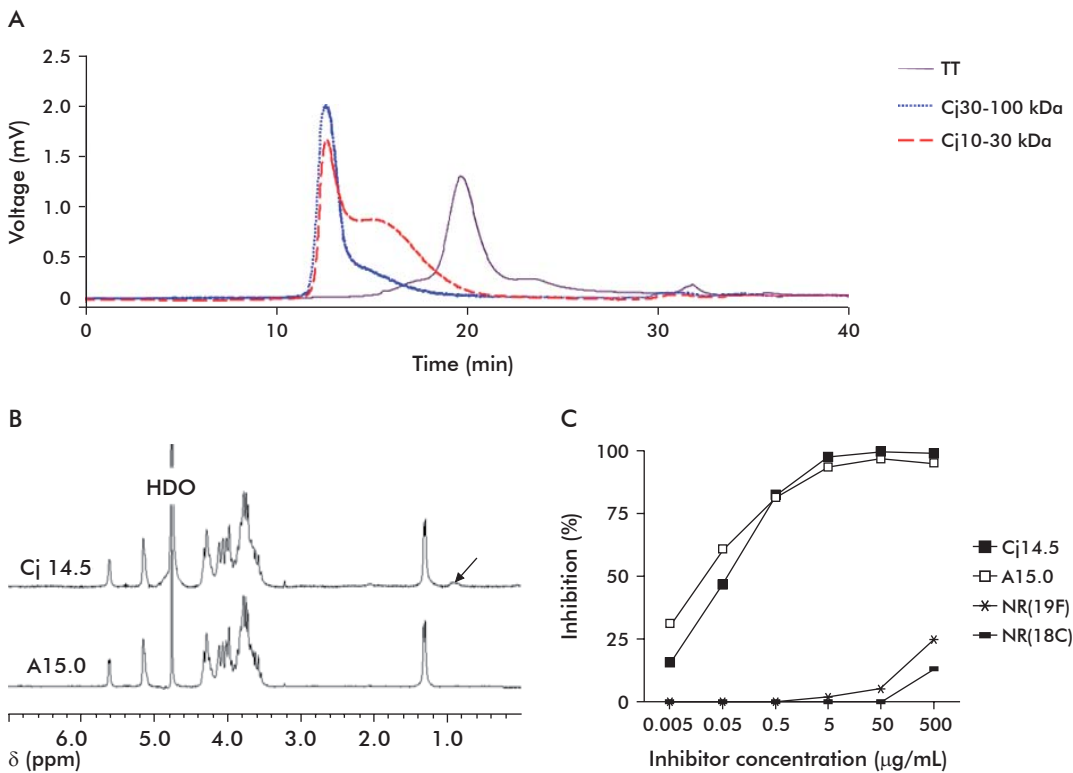
The fragments retained the RU chemical structure in the three fractions, as expected, regarding the absence of PS6B side chains susceptible to hydrolysis. Significantly, antigenicity was preserved at the higher

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**Figure 4.** Physicochemical and immunochemical characterization of the capsular polysaccharide (PS) of *Streptococcus pneumoniae*, serotype 6B conjugates. A) Superposition of chromatograms of the conjugates and the tetanus toxoid (TT) carrier protein. B) Superposition of PS NMR- $^1\text{H}$  spectra. The arrow indicates the signal corresponding to the aliphatic amino acids of the carrier protein. C) Chart of inhibition vs. inhibitor concentration. Cj10-30 kDa and Cj30-100 kDa: conjugates obtained from PS fractions 10-30 kDa and 30-100 kDa, respectively. Cj14.5: conjugate obtained from PS with 14.5 % of oxidized repetitive units (ORU). A15.0: PS activated with 15.0 % ORU. NR (19F) and NR (18C): Non-related PS from serotypes 19F and 18C.

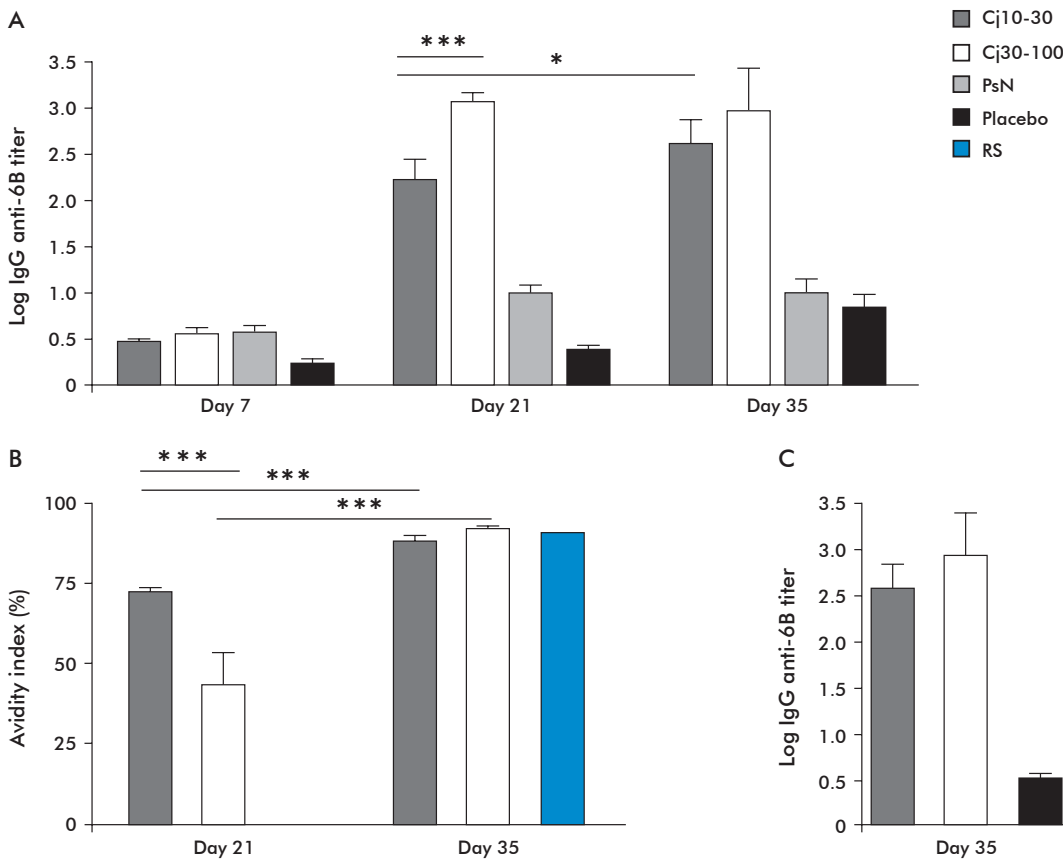


Figure 5. Immunological evaluation of the capsular polysaccharide of *Streptococcus pneumoniae*, serotype 6B (PS6B) conjugates. Groups of five New Zealand White rabbits were immunized with three doses of either 4  $\mu$ g of PS6B conjugated to tetanus toxoid (TT), 10  $\mu$ g of non-conjugated PS6B or placebo on days 0, 14 and 28. Bars indicate average values plus standard deviation. A) Titers of IgG antibodies against PS6B. B) Avidity index of IgG antibodies against PS6B. C) Titers of IgG antibodies against TT after the third dose. Cj10-30 and Cj30-100 are the conjugates obtained from PS6B fractions in the molecular weight ranges 10-30 kDa and 30-100 kDa, respectively; PsN: Native PS; SR: reference serum. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ .

size PS fractions, but was affected in the 1-10 kDa fraction. This evidenced the preservation of the conformational epitopes essential for recognizing the native PS which are absent at lower sizes. Although previous reports on this matter were not found in the literature for PS6B, there were previous studies with other PS, such as those from *Streptococcus B* type III [23, 24], *Neisseria meningitidis* serogroups Y and W<sub>135</sub> [25] and *S. pneumoniae* serotype 14 [26, 27]. In this last PS, conformational epitopes were also relevant for the generation of functional antibodies by the conjugates [28].

Reproducible conditions for PS oxidation were established while studying the PS6B activation. A linear relation was found between the equivalent amount of NaIO<sub>4</sub> and the number of aldehyde groups generated, which supported the generation of an activation level in a preselected narrow range.

The periodate oxidation reaction occurs between adjacent hydroxyl groups followed by the rupture of the bond between the involved carbons which become aldehydes. The PS6B RU bears three of such sites: between positions C1-C2 and C2-C3 in ribitol and C3-C4 in galactose. Following the reactivity order described by Kim *et al.* [29], the oxidation of this PS must occur completely in ribitol since it is a linear

chain. In support of this affirmation, it is known that under oxidation conditions of 0.87 moles of NaIO<sub>4</sub>/mol of UR, the *N. meningitidis* serogroup W<sub>135</sub> oxidation proceeds in the side chain of sialic acid at C7-C8 and C8-C9 positions, and not at the C2-C3 and C3-C4 of galactose [30].

In this work, the NMR-<sup>1</sup>H revealed signals congruent with the shielded anomeric proton of rhamnose, due to a shortage of the adjacent ribitol residue. On the contrary, there was no signal for the unshielded anomeric proton of galactose, present when it is oxidized. This implies that no monosaccharide ring was broken, what should minimize any perturbation in the PS conformation. Nevertheless, there was a decrease in antigenicity for RU above 24 % in spite of the fact that no PS fragmentation was detected. This indicates that more than 3/4 of ribitol residues were required intact for a proper antigenic recognition. Based on these results, the 0.71 moles of NaIO<sub>4</sub>/mol of RU oxidation conditions were avoided.

Conjugates obtained from PS of 8-12 % and 13-18 % ORU, and activated with 0.18 and 0.35 moles of NaIO<sub>4</sub>/mol of RU, respectively, showed no differences in any of the physicochemical parameters evaluated. Conjugate immunogenicity is not expected to vary when generated from PS of the same size without any

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other difference than in the activation level. It has been observed that PS conjugates of group B *Streptococcus type III* with higher 18-89 % ORU range, higher than the one evaluated by our group, showed increased immunogenicity due to a rise in conjugate cross-linking [31]. Besides, this result was not further reproduced, due to a slight decrease in immunogenicity at a higher ORU percentage [32].

Otherwise, the PS size did influence in the physico-chemical properties of the conjugates, the 30-100 kDa fraction generating the highest rMW and PS/protein ratio. The characterization of conjugates generated from a similar size PS was highly reproducible.

The immune response induced by the conjugates was quantitatively and qualitatively higher than that obtained by the non-conjugated PS. The raise of IgG antibody titers and avidity after each dose of conjugate indicated a thymus-dependent response, involving affinity maturation [33]. The increase in antibody avidity tends to correlate with the increase in protective capacity [34].

The Cj30-100 was more immunogenic than Cj10-30 although no statistically significant after the third dose. This result coincides with that obtained by Daum et al. [35] in children immunized with oligosaccharide or polysaccharide conjugate vaccines. These differences in the elicited immune responses could be influenced by the PS length, but also by the conjugate size [36], the PS/protein ratio [37] or a combination of both factors. In fact, several studies addressing the influence of

PS length on the immunogenicity of their respective conjugates achieved different results [32, 38]. At the same time, the response induced by the Cj10-30 conjugate seems to be more thymus-dependent, due to a faster increase in avidity. The native structure of the polysaccharide determines its thymus-independent type 2 nature, and part of this characteristic was preserved once conjugated to a protein [39]; while conjugates of lower rMW could favor better antigen recognition, a T-dependent one, therefore maximizing the effect of the carrier protein [11]. Nevertheless, those differences after three doses for both conjugates generated IgG antibody responses similar in titers and avidity.

In summary, the procedures described herein, which included the fragmentation of PS6B at molecular size cut-offs of 10-30 and 30-100 kDa, respectively, and their activation at levels from 8 to 18 % of oxidized repetitive units, allowed to obtain reproducibly PS6B conjugates to TT which demonstrated to be immunogenic. The methodology followed to find and establish each reaction conditions is also applicable for establishing polysaccharide conjugation procedures for other pneumococcal serotypes and bacteria.

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