Optimization of the Process for Preparing Bivalent Polysaccharide Conjugates to Develop Multivalent Conjugate Vaccines against *Streptococcus pneumoniae* or *Neisseria meningitidis* and Comparison with the Corresponding Licensed Vaccines in Animal Models

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[Abstract] Objective: This study aimed to describe, optimize and evaluate a method for preparing multivalent conjugate vaccines by simultaneous conjugation of two different bacterial capsular polysaccharides (CPs) with tetanus toxoid (TT) as bivalent conjugates. Methods: Different molecular weights (MWs) of polysaccharides, activating agents and capsular polysaccharide/protein (CP/Pro) ratio that may influence conjugation and immunogenicity were investigated and optimized to prepare the bivalent conjugate bulk. Using the described method and optimized parameters, a 20-valent pneumococcal conjugate vaccine and a bivalent meningococcal vaccine were developed and their effectiveness was compared to that of corresponding licensed vaccines in rabbit or mouse models. Results: The immunogenicity test revealed that polysaccharides with lower MWs were better for Pn1-TT-Pn3 and MenA-TT-MenC, while higher MWs were superior for Pn4-TT-Pn14, Pn6A-TT-Pn6B, Pn7F-TT-Pn23F and Pn8-TT-Pn11A. For activating polysaccharides, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) was superior to cyanogen bromide (CNBr), but for Pn1, Pn3 and MenC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) was the most suitable option. For Pn6A-TT-Pn6B and Pn8-TT-Pn11A, rabbits immunized with bivalent conjugates with lower CP/Pro ratios showed significantly stronger CP-specific antibody responses, while for Pn4-TT-Pn14, higher CP/Pro ratio was better. Instead of interfering with the respective immunological activity, our bivalent conjugates usually induced higher IgG titers than their monovalent counterparts. Conclusion: The result indicated that the described conjugation technique was feasible and efficacious to prepare glycoconjugate vaccines, laying a solid foundation for developing extended-valent multivalent or combined conjugate vaccines without potentially decreased immune function.
Key words: multivalent conjugate vaccine; pneumococcal conjugate vaccine; meningococcal conjugate vaccine; bioconjugation; immunogenicity

During the last three decades, glycoconjugate vaccines against *Streptococcus pneumoniae* (Pn) and *Neisseria meningitidis* (Men) have been developed and proven effective in preventing infectious diseases caused by encapsulated bacteria[1]. Currently, a pneumococcal conjugate vaccine (PCV) containing 13 pneumococcal serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) and a meningococcal conjugate vaccine containing 4 meningococcal serogroups (MenA, MenC, MenY and MenW) are available on the market. However, the extensive use of conjugate vaccines is regularly followed by the so-called replacement phenomenon, i.e., the replacement of the serotype included in the current conjugate vaccine with "nonvaccine" serotypes[2, 3]. For example, in the cases of children under 5 years of age, 72% of the invasive pneumococcal diseases are caused by serotypes not included in any PCV[4]. Moreover, the introduction of meningococcal conjugate vaccines in Europe, the United States, Canada, and Africa also appears to be associated with declines in vaccine-type diseases and shifts in serogroup distribution in those geographic regions[5]. Given that more than 90 pneumococcal serotypes and 12 meningococcal serogroups have been described with distinct geographical and epidemiological distributions, there is a need to develop broader-spectrum conjugate vaccines containing nonvaccine pneumococcal serotypes and meningococcal serogroups. Furthermore, combination

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vaccines that protect against multiple pathogens are also preferred to realize an improved immunization rate and timely immunization.

Unfortunately, the preparation of multivalent or combination conjugate vaccines involves the manufacture of several separate bulk products, leading to time-consuming operations, multiple processes and risk control points. In addition, a reduced immune response to the target antigens was found when extending the valency of multivalent conjugate vaccines. Therefore, there is a need to improve the immunogenicity and avidity of conjugate vaccines to accommodate the increased number of serotypes without compromising the immune responses to all types included in the vaccine, which is not possible with conventional conjugation methods. In fact, there are good prospects for developing methods that enable the simultaneous incorporation of two or multiple polysaccharide antigens from different pathogens in a single bulk product. Other benefits of such a glycoconjugate may include a decrease of protein amount in the multivalent conjugate vaccine, avoidance of potential carrier-induced epitope suppression, convenience of developing extended-valent conjugates, and simplification of vaccine composition.

A few methods have been developed to manufacture similar multivalent conjugates[6–8]. However, the methods require either step-by-step synthesis or the utilization of specific groups of polysaccharides, which limit their practical application and universality. In addition, the safety and reactivity of the chemical groups incorporated in the final vaccine by the above methods have not been tested in any human trials.

In this paper, following the concept of a multicomponent construct, glycoconjugate vaccines against *pneumococci*, MenA, and MenC were developed by utilizing carbodiimide to mediate the formation of amide linkages between the hydrazine group of different derived polysaccharides and the carboxylate group of tetanus toxoid (TT). We focused on the simultaneous conjugation of two polysaccharides to the carrier protein as a bivalent conjugate, in which two polysaccharides are crosslinked to the carrier protein in a single construct with a net structure (fig. 1A). For the sake of readability, the net structure of the bivalent conjugate is simplified as CP Ⅰ-TT-CP Ⅱ, where CP Ⅰ and CP Ⅱ are different capsular polysaccharides (CPs). The steps from polysaccharide modification to conjugation are shown in fig. 1B–1D. Factors [e.g., different molecular weights (MWs) of polysaccharides, activating agents, and capsular polysaccharide/protein (CP/Pro) ratios] that may influence conjugation and immunogenicity were investigated and optimized. Five combinations of pneumococcal serotypes (Pn1 and Pn3, Pn4 and Pn14, Pn6A and Pn6B, Pn7F and Pn23F, Pn8 and Pn11A) as well as one combination of meningococcal serogroups (MenA and MenC) were used as representative cases. The immunogenicities of the six chemically conjugated bivalent vaccines and physically mixed monovalent combined conjugates were compared to determine whether there was immune interference in the multicomponent conjugates. To
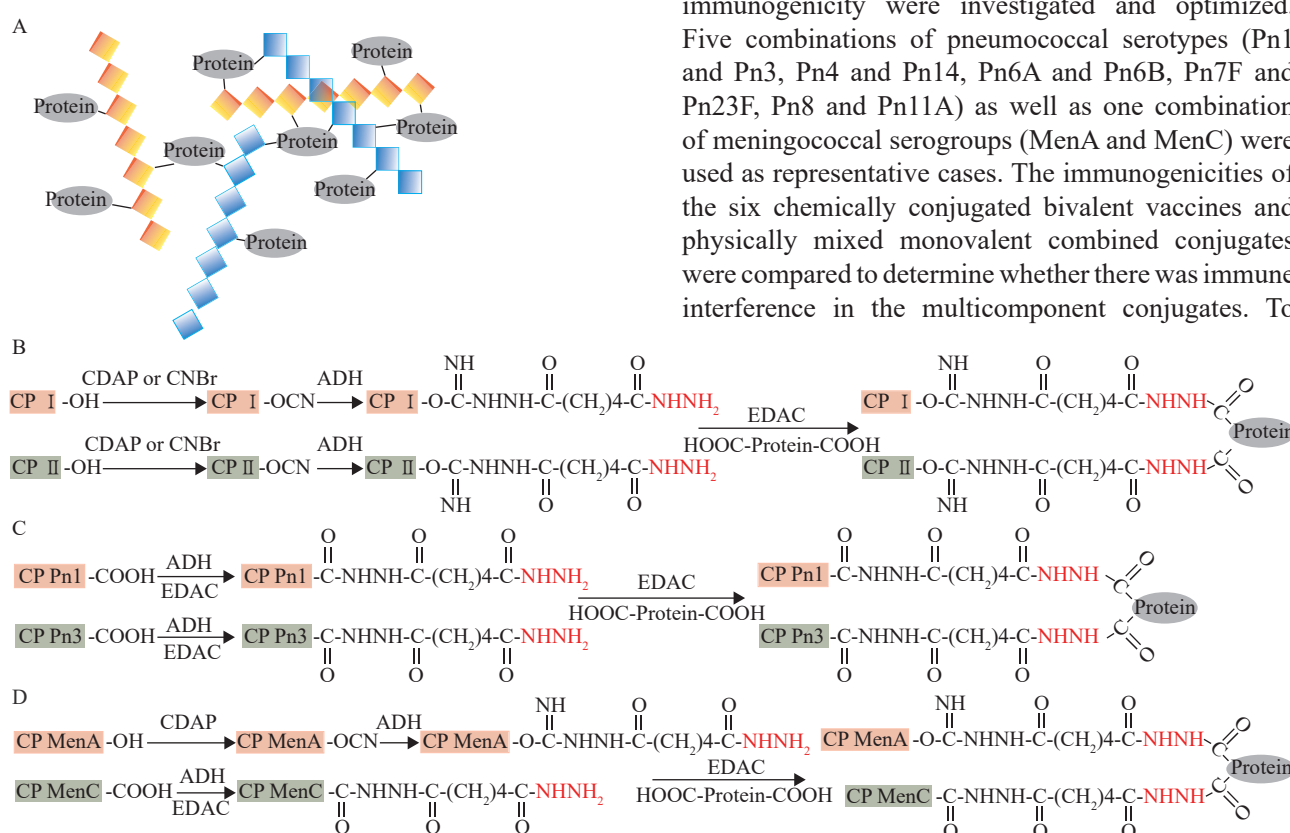

Fig. 1 Schematic diagram and synthesis workflow chart of the bivalent glycoconjugates

A: the crosslinking structure of the bivalent conjugate; B: None of the polysaccharides are rich in carboxyl groups. CP Ⅰ: capsular polysaccharide from the first serotype or serogroup bacteria; CP Ⅱ: capsular polysaccharide from the second serotype or serogroup bacteria. C: Both polysaccharides are rich in carboxyl groups. D: One polysaccharide is rich in carboxyl groups, whereas the other polysaccharide is not.

verify the effectiveness of the multivalent conjugate vaccines, a 20-valent pneumococcal conjugate vaccine (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F) and bivalent meningococcal vaccine (MenA and MenC) were developed, and their immunogenicities were compared to those of the corresponding licensed vaccines. Furthermore, a dose-ranging study of the developed 20-valent pneumococcal conjugate vaccine was also conducted in animal models.

1 MATERIALS AND METHODS

1.1 Materials

CPs of pneumococcus and meningococcus as well as TT were kindly provided by Chengdu Antigen Biotech Co., Ltd., China. 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), adipic acid dihydrazide (ADH), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC), bovine serum albumin (BSA), tetramarethylbenzidine (TMB), HRP-conju-gated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, and acetonitrile were purchased from Sigma-Aldrich, USA. Sodium hydroxide, hydrochloric acid, sodium chloride (NaCl), sodium bicarbonate, Tween-20, sulfuric acid, and triethylamine were purchased from Sinopharm Chemical Reagent Co., Ltd., China. Cyanogen bromide (CNBr) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd., China.

1.2 Preparation of Glycoconjugates as a Bivalent Construct

1.2.1 Depolymerization of Polysaccharides Native CPs were dissolved in 0.15 mol/L NaCl at 10 mg/mL. For depolymerization, 100 mL of the polysaccharide solution was transferred into a 250 mL-beaker, which was placed in an ice bath and put into the chamber of an ultrasonic cell disruption instrument (Ningbo Scientz, China). The ultrasonic treatment parameters and the MWs of the polysaccharides used for preparing the bivalent conjugates with different carbohydrate lengths are shown in table 1.

Table 1 Molecular weight (MW) of depolymerized polysaccharides obtained by ultrasonic treatment and used in a comparative study of the effect of different carbohydrate lengths on the immunogenicity of the conjugate

Conjugate	Polysaccharide	MW of a longer carbohydrate chain (ultrasonic treatment parameter)	MW of a shorter carbohydrate chain (ultrasonic treatment parameter)
Pn1-TT-Pn3	Pn1	389 kD (125 W, 30 s)	180 kD (200 W, 60 s)
	Pn3	403 kD (125 W, 30 s)	190 kD (200 W, 60 s)
Pn4-TT-Pn14	Pn4	425 kD (100 W, 40 s)	205 kD (250 W, 40 s)
	Pn14	384 kD (125 W, 30 s)	223 kD (250 W, 40 s)
Pn6A-TT-Pn6B	Pn6A	390 kD (150 W, 30 s)	178 kD (300 W, 30 s)
	Pn6B	396 kD (150 W, 30 s)	195 kD (300 W, 30 s)
Pn7F-TT-Pn23F	Pn7F	403 kD (150 W, 30 s)	228 kD (250 W, 60 s)
	Pn23F	373 kD (150 W, 30 s)	193 kD (250 W, 60 s)
Pn8-TT-Pn11A	Pn8	304 kD (125 W, 50 s)	117 kD (350 W, 60 s)
	Pn11A	334 kD (125 W, 50 s)	106 kD (350 W, 60 s)
MenA-TT-MenC	MenA	325 kD (not applicable)	325 kD (not applicable)
	MenC	343 kD (125 W, 30 s)	105 kD (400 W, 40 s)

MenA CP was not treated by ultrasound and remained as the native polysaccharide for preparing the bivalent conjugate.

1.2.2 Derivatization of Polysaccharides For CDAP cyanidation, the polysaccharides were activated and derivatized according to the general procedure described by Lees et al., with slight modifications[9]. Each polysaccharide was dissolved in 0.15 mol/L NaCl at 5 mg/mL. CDAP (0.5 mg of CDAP per mg of polysaccharide) was pipetted into the vortexed solution of polysaccharide, two volumes of aqueous 0.2 mol/L triethylamine were added to adjust and maintain the pH of the polysaccharide solution at 9.0 for 5 min, and then ADH equal to 3.5-fold weight of the polysaccharide was added. Subsequently, the pH was readjusted to 9.0 and maintained for 2 h, the solution was then adjusted to pH 7.0, allowed to stand overnight at 4°C , and further ultrafiltered with 0.15 mol/L NaCl using a Vivaflow 50 tangential flow ultrafiltration system (MW cutoff of 10 kD, Sartorius, Germany).

For CNBr cyanylation, polysaccharides were activated and derivatized according the method described by Chu et al, with some modifications[10]. Each polysaccharide was dissolved in 0.15 mol/L NaCl at 5 mg/mL, and the pH of the polysaccharide solution was increased to 10.8. Then, CNBr (0.5 mg of CNBr per mg of polysaccharide) was pipetted into the vortexed solution of polysaccharide, and the solution was maintained at pH 10.8 for 10 min. Next, the solution pH was decreased to 9.0, and ADH equal to 3.5-fold the weight of polysaccharide was added. Subsequently, the pH was readjusted to 9.0 and maintained for 15 min, and the solution was allowed to stand overnight at 4°C and further ultrafiltered with 0.15 mol/L NaCl using a Vivaflow 50 tangential flow ultrafiltration system (MW cutoff of 10 kD, Sartorius, Germany).

The three polysaccharides (Pn1, Pn3, and MenC) are rich in carboxyl groups, which can bind to the hydrazine group of polysaccharides activated by CNBr

and CDAP in the conjugation reaction, leading to the crosslinking of different polysaccharide molecules. Therefore, the three polysaccharides were activated by EDAC and linked to ADH to block the carboxyl group and prevent crosslinking between polysaccharides. For EDAC activation, ADH equal to 3.5-fold weight of the polysaccharide was added to each polysaccharide solution (5 mg/mL in 0.15 mol/L NaCl). Then, the pH of the polysaccharide solution was adjusted to 5.7 at 5±3°C, and 0.6 mg of EDAC per mg of polysaccharide was added. The solution was maintained at pH 5.7 for 2 h, and the reaction was terminated by adjusting the pH to 7.0. Subsequent ultrafiltration was performed as in the CDAP cyanidation process to obtain the derived polysaccharide.

1.2.3 Simultaneous Conjugation of Two Different Polysaccharides with TT for Bivalent Conjugate Manufacturing   For the preparation of bivalent conjugates, two polysaccharide derivatives were first mixed together (total concentration=2 mg/mL) at appropriate proportions (data not shown) to ensure that the two polysaccharides in the resultant glycoconjugate would be balanced in terms of content (at a weight ratio close to 1, except 6A:6B=0.5). Then, a monomer of TT whose weight was half or equal to that of the polysaccharide was added to the mixture. Subsequently, the pH of the mixed solution was adjusted to 5.7, and EDAC double to the weight of polysaccharide was added. Following the reaction at pH 5.7 for 4 h, the bivalent conjugate was purified by size exclusion chromatography. Similar procedures except for the mixing of the two different polysaccharide derivatives were used to prepare the monovalent conjugates.

1.3 Analysis of Polysaccharides, Derivatives, and Bivalent or Monovalent Conjugates

For the depolymerized polysaccharides and subsequent derivatives, the MW and MW distribution were determined by high-performance size exclusion chromatography combined with a multiangle laser light scattering-ultraviolet-refractive index detector (HPSEC/MALLS-UV-RI, Wyatt, USA) and Astra 7.0.0.69 software (Wyatt, USA). The extent of derivatization with ADH was determined using the TNBS assay.

The pneumococcal polysaccharide content was determined by the rate nephelometry method based on a highly specific antigen–antibody reaction which was carried out using an IMMAGE 800 automatic biochemical analyzer (Beckman Coulter, USA). The MenA and MenC contents were determined by the acidic ammonium molybdate method and resorcinol method, respectively. The protein content was determined by the Lowry method. The free polysaccharide content was determined by cold-phenol precipitation. The free protein content was analyzed by high-performance liquid chromatography with a TSKgel G5000PWXL column (TOSOH, Japan) from the conjugated protein

and calculated by the area normalization method.

1.4 Animal Studies

The bivalent conjugates containing polysaccharides with different MWs, which were prepared by different activating agents and had different CP/Pro ratios, were separately and sequentially compared in animal models. For each comparative experiment, 50 female New Zealand white rabbits were equally randomized into ten groups for the pneumococcal conjugates, while 20 female BALB/c mice were equally randomized into two groups for the meningococcal conjugates. To determine whether there was immune interference among the multicomponent conjugates, the 5 bivalent pneumococcal conjugates were formulated together and compared with the formulations of the 10 corresponding monovalent conjugates, while the optimized bivalent meningococcal conjugate was compared with the mixture of MenA and MenC monovalent conjugates. For this comparative experiment, 10 rabbits divided into two groups (five each) and 20 mice divided into two groups (ten each) were used for the pneumococcal conjugates and meningococcal conjugates, respectively. To verify the effectiveness of the multivalent conjugate vaccines consisting of multicomponent constructs, the immunogenicity of a 20-valent pneumococcal conjugate vaccine composed of 10 bivalent conjugates, i.e., Pn1-TT-Pn3, Pn4-TT-Pn14, Pn5-TT-Pn9V, Pn6A-TT-Pn6B, Pn7F-TT-Pn23F, Pn8-TT-Pn11A, Pn10A-TT-Pn12F, Pn15B-TT-Pn18C, Pn19A-TT-Pn19F, and Pn22F-TT-Pn33F, which were formulated with aluminum phosphate adjuvant, was compared with that of the corresponding licensed vaccine in rabbits. Furthermore, a lyophilized bivalent meningococcal conjugate vaccine, MenA-TT-MenC, was compared to the corresponding licensed vaccine in a mouse model. In addition, a dose-ranging study of our 20-valent pneumococcal conjugate vaccine in a rabbit model was conducted in parallel with the comparative experiment. To ensure that the result was reliable, 10 rabbits were used for each group for the four pneumococcal vaccines. However, for the two meningococcal vaccines, 10 mice were again used for each group.

All animals were kept in a pathogen-free environment and fed *ad libitum*. The procedures for the care and use of the animals were approved by the ethics committee, and all applicable institutional and governmental regulations concerning the ethical use of animals were followed. Generally, each rabbit was injected intramuscularly with conjugates containing approximately 2.0 μg of each pneumococcal polysaccharide, with the exception of Pn6B, of which 4.0 μg was injected, while each mouse was injected subcutaneously with conjugates containing approximately 2.5 μg of each meningococcal polysaccharide. For the dose-ranging study, two other

dose levels, i.e., 1 µg and 5 µg, of each pneumococcal polysaccharide were adopted except that double the amount of Pn6B was used. Animals immunized with PBS were used as negative controls in all of the studies. Three doses of each vaccine were administered 2 weeks apart. Bleedings were performed 7 days after the last immunization.

1.5 ELISA Quantification

Serum samples from immunized rabbits and mice were analyzed for anti-CP IgG by ELISA. Microtiter plates were coated overnight at 2–8°C with 100 µL of pneumococcal or meningococcal polysaccharides at 5 µg/mL in PBS. Then, the wells were washed extensively and blocked with BSA. After another wash, 100 µL of serial dilutions of individual serum samples from animals were added and incubated at 37°C for 1 h. HRP-conjugated goat anti-rabbit IgG was used as the secondary antibody (for the meningococcal vaccine studies, the secondary antibody was substituted with a rabbit anti-mouse antibody), and TMB was used as the chromogenic substrate. The chromogenic reaction was stopped with 2 mol/L H_2SO_4, and the results were read at absorbance (A) of 450 nm and 630 nm (A_{450} and A_{630}) by an ELISA reader.

1.6 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 19.0 software for Windows (SPSS Inc., USA). The anti-CP IgG geometric mean titer (GMT) and 95% confidence interval (95% CI) of each animal group were calculated. Then, the anti-CP IgG GMT was logarithmically transformed to conform to or approximate a normal distribution, and an independent

samples t-test ($P<0.05$) and analysis of variance followed by a post hoc Student–Newman–Keuls test ($P<0.05$) were used to compare the anti-CP IgG GMT of two and more than two groups of animals, respectively.

2 RESULTS

2.1 Depolymerization of Polysaccharides and Effect of Different Polysaccharide Lengths on Bivalent Conjugates

For the 6 studied groups of bivalent conjugates, the polysaccharide of MenA tended to hydrolyze significantly in aqueous solution due to the presence of a phosphodiester linkage between the anomeric position and the N-acetyl group at position 2 of mannosamine, which can assist the leaving of a phosphomonoester group[11]. Therefore, the polysaccharide of MenA remained native for bioconjugation, while one of the MenC polysaccharides was depolymerized to obtain a similar MW as that of MenA, and another was depolymerized to obtain a MW of approximately 100 kD. The MWs of the depolymerized polysaccharides obtained by ultrasonic treatment and used in the comparative study of the effect of different polysaccharide MWs on the immunogenicity of the conjugates are listed in table 1. In general, the lower MW polysaccharides were approximately 200 kD smaller than the higher MW polysaccharides. In fig. 2, it can be seen that after ultrasonic processing, the value of polydispersity [MW/number average molecular weight (Mn)] decreased, indicating a uniform and slightly narrow MW distribution of the polysaccharide
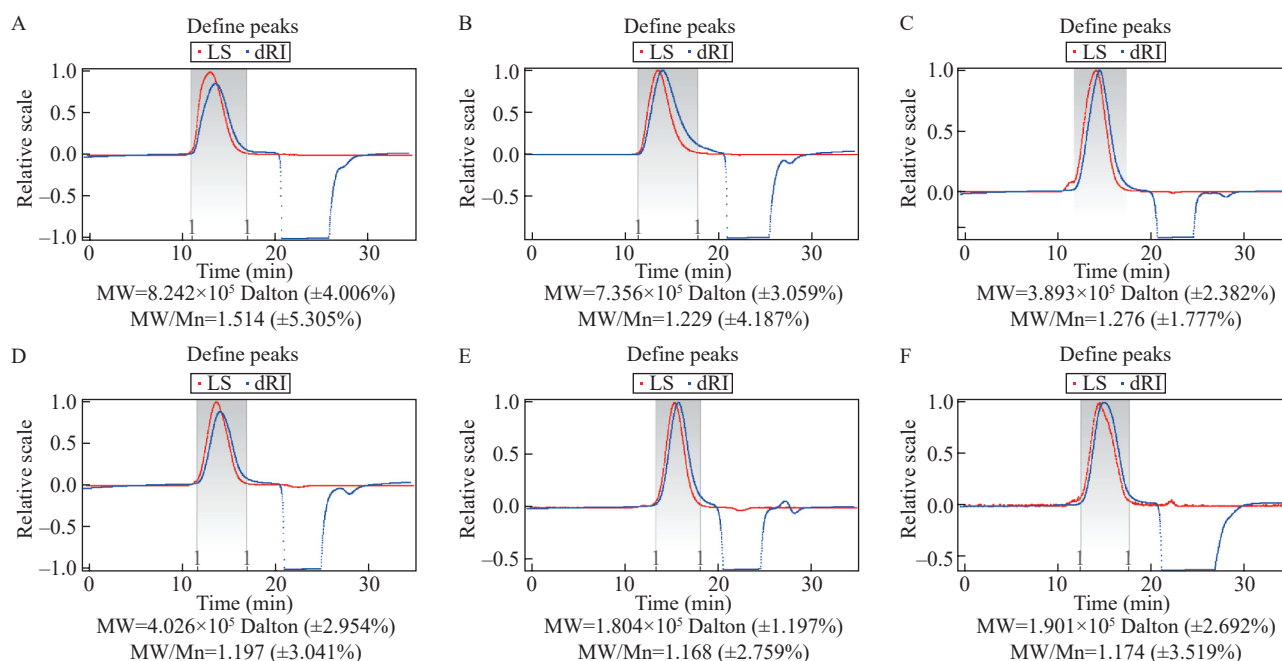

Fig. 2 HPSEC/MALLS-UV-RI collection of native and depolymerized polysaccharides

The red line represents the laser signal. The blue line represents the refractive index signal. The percentage value in the brackets is the relative standard deviation (RSD). A: native Pn1 CP; B: native Pn3 CP; C: depolymerized Pn1 CP with longer carbohydrate chains; D: depolymerized Pn3 CP with longer carbohydrate chains; E: depolymerized Pn1 CP with shorter carbohydrate chains; F: depolymerized Pn3 CP with shorter carbohydrate chains

obtained after depolymerization. Other polysaccharides also showed similar phenomena (data not shown).

The physiochemical properties of the bivalent conjugates prepared with different polysaccharides with different MWs are shown in table 2. In all of the bivalent conjugates, the CP/Pro ratio was approximately equal to 1, and the free polysaccharide and protein contents were below 15% and 2%, respectively.

Table 2 Partial characteristics of the bivalent conjugates prepared by polysaccharides with different molecular weights (MWs)

Conjugate	MWs of the two CPs	Ps content (µg/mL)	Pro content (µg/mL)	Free Ps	Free Pro
Pn1-TT-Pn3	389 kD+403 kD	45.89/40.44	88.32	11.78%/10.59%	0.18%
	180 kD+190 kD	30.39/35.30	83.93	10.08%/7.67%	0.48%
Pn4-TT-Pn14	425 kD+384 kD	47.72/42.46	85.48	7.90%/8.76%	0.34%
	205 kD+223 kD	36.94/33.75	70.37	5.88%/8.54%	0.50%
Pn6A-TT-Pn6B	390 kD+396 kD	42.63/77.83	103.30	8.53%/7.60%	0.42%
	178 kD+195 kD	31.64/70.25	82.83	8.34%/9.86%	0.40%
Pn7F-TT-Pn23F	403 kD+373 kD	47.74/53.56	112.37	8.52%/10.18%	0.41%
	228 kD+193 kD	35.88/33.49	80.48	9.09%/8.33%	0.62%
Pn8-TT-Pn11A	304 kD+334 kD	56.14/50.18	99.50	10.39%/7.49%	0.28%
	117 kD+106 kD	44.05/38.86	90.08	6.88%/8.22%	0.49%
MenA-TT-MenC	325 kD+343 kD	70.82/75.54	135.54	8.44%/10.75%	0.53%
	325 kD+105 kD	68.16/74.81	115.32	9.73%/9.26%	0.44%

CP: capsular polysaccharide; Ps: polysaccharide; Pro: protein

To determine the effect of the polysaccharide MW on immunogenicity, the serum responses to bivalent conjugates prepared from depolymerized polysaccharides with different MWs were compared in animal models. As shown in fig. 3, Pn1-TT-Pn3 and MenA-TT-MenC with lower polysaccharide MWs induced higher anti-CP IgG GMTs, whereas Pn4-TT-Pn14, Pn6A-TT-Pn6B, Pn7F-TT-Pn23F, and Pn8-TT-Pn11A with higher polysaccharide MWs induced higher anti-CP IgG GMTs. The differences in Pn1, Pn6B, and Pn14 between depolymerized polysaccharides with higher and lower MWs were statistically significant at the level of 0.05, suggesting the influence of depolymerization on the immunogenicity of polysaccharides. Based on this result, Pn1, Pn3, and MenC polysaccharides with lower MWs were used for the subsequent experiments, while for the nine other polysaccharides except MenA, the higher MW polysaccharides were chosen.

2.2 Derivatization of Polysaccharides and Effect of Different Activating Agents on Bivalent Conjugates

CDAP, CNBr, and EDAC were used to activate the polysaccharides. The MW of each polysaccharide before and after derivatization as well as the degree of derivatization (ADH/CPs, w/w, %) are listed in table 3. The MWs of the polysaccharide derivatives activated by CNBr were usually larger than those of the polysaccharide derivatives activated by CDAP.

The physiochemical properties of the bivalent conjugates prepared by different activating agents are shown in table 4. The immunogenicities of the six bivalent conjugates prepared by CDAP-, CNBr-, and/or EDAC-activated polysaccharides are shown in fig. 4. There was a significant difference in the anti-CP IgG GMTs of Pn4, Pn6B, and Pn7F between the two cyanylating agents, showing a distinctly higher value for the CDAP group, while Pn3 and Pn23F showed
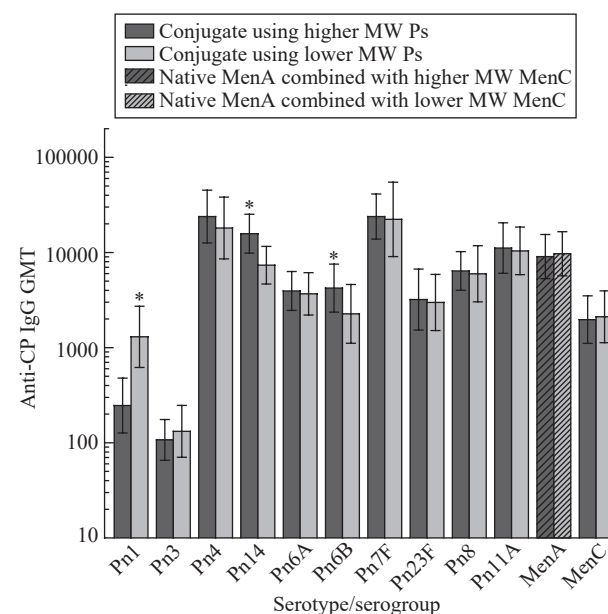

Fig. 3 Comparison of the anti-CP IgG GMTs induced by glyco-conjugates using depolymerized polysaccharides with higher and lower molecular weights (MWs)
The error bar represents the 95% confidence interval of the geometric mean titer (GMT). An asterisk above the bar indicates that the IgG GMT is significantly greater than that of the same serotype/serogroup glycoconjugates ($P<0.05$).

a nonsignificantly higher value for the CNBr group. Due to its high immunogenicity, low toxicity, and convenient handling, CDAP was adopted to activate the polysaccharides not rich in carboxyl groups. For the carboxyl group-rich polysaccharides of Pn1, Pn3, and MenC, EDAC was used as an activating agent to prepare the derivatives. As expected, the anti-CP IgG GMTs of the polysaccharides derived from EDAC treatment were greater than those of the polysaccharides derived from the CDAP and CNBr methods, among which MenC showed a significant difference between

Table 3 Molecular weight (MW) and derivatization degree of the studied polysaccharides activated by different activation agents

Polysaccharide	MW before activation	Activation by CDAP		Activation by CNBr		Activation by EDAC	
		MW	ADH/CPs	MW	ADH/CPs	MW	ADH/CPs
Pn1	180 kD	210 kD	1.18%	228 kD	0.21%	223 kD	3.55%
Pn3	190 kD	235 kD	3.44%	401 kD	2.24%	219 kD	8.13%
Pn4	425 kD	450 kD	1.49%	455 kD	0.75%	/	/
Pn14	384 kD	442 kD	2.03%	615 kD	1.09%	/	/
Pn6A	390 kD	450 kD	1.47%	585 kD	0.98%	/	/
Pn6B	396 kD	447 kD	1.50%	557 kD	0.92%	/	/
Pn7F	403 kD	520 kD	1.78%	570 kD	0.98%	/	/
Pn23F	373 kD	439 kD	2.36%	523 kD	1.35%	/	/
Pn8	304 kD	339 kD	2.08%	446 kD	0.94%	/	/
Pn11A	334 kD	380 kD	1.48%	508 kD	0.81%	/	/
MenA	325 kD	345 kD	2.50%	354 kD	1.26%	/	/
MenC	105 kD	115 kD	0.98%	111 kD	0.53%	124 kD	5.87%

CDAP: 1-cyano-4-dimethylaminopyridinium tetrafluoroborate; CNBr: cyanogen bromide; EDAC: *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; ADH: adipic acid dihydrazide; CPs: capsular polysaccharides. /: not applicable

Table 4 Partial characteristics of the bivalent conjugates prepared by different activating agents

Conjugate	Activation agent	Ps content (μg/mL)	Pro content (μg/mL)	Free Ps	Free Pro
Pn1-TT-Pn3	CDAP	30.90/35.78	83.27	10.35%/8.62%	0.44%
	CNBr	27.82/31.54	78.84	9.22%/9.09%	0.37%
	EDAC	33.26/29.97	77.78	10.42%/10.23%	0.41%
Pn4-TT-Pn14	CDAP	48.81/49.20	98.68	7.23%/10.29%	0.35%
	CNBr	48.25/42.46	90.83	9.34%/6.89%	0.47%
Pn6A-TT-Pn6B	CDAP	40.84/76.35	104.55	8.77%/12.82%	0.50%
	CNBr	41.65/75.03	96.73	10.57%/11.34%	0.56%
Pn7F-TT-Pn23F	CDAP	44.46/49.71	102.66	8.45%/9.27%	0.25%
	CNBr	46.32/51.99	103.47	7.09%/9.07%	0.37%
Pn8-TT-Pn11A	CDAP	56.51/51.66	92.65	10.56%/8.33%	0.41%
	CNBr	47.28/55.04	100.92	7.04%/9.36%	0.31%
MenA-TT-MenC	CDAP	68.76/62.44	130.11	9.19%/5.06%	0.55%
	CNBr	71.80/77.52	122.63	10.87%/8.58%	0.38%
	EDAC	69.65/75.27	137.31	9.34%/9.16%	0.44%

Ps: polysaccharide; Pro: protein; CDAP: 1-cyano-4-dimethylaminopyridinium tetrafluoroborate; CNBr: cyanogen bromide; EDAC: *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride; ADH: adipic acid dihydrazide

the EDAC method and the CNBr method.

2.3 Effect of Different CP/Pro Ratios on Bivalent Conjugates

The bivalent conjugates with lower CP/Pro ratios (ranging from 0.47 to 0.57) and higher CP/Pro ratios (ranging from 0.86 to 1.17) are shown in table 5. Generally, lower free polysaccharide and higher free protein contents were found in the bivalent conjugates bearing lower CP/Pro ratios.

The comparison of the anti-CP IgG GMTs induced by the bivalent conjugates with different CP/Pro ratios is shown in fig. 5. For Pn6A, Pn6B, Pn8, and Pn11A, the rabbits immunized with pneumococcal conjugates with lower CP/Pro ratios showed significantly stronger CP-specific antibody responses. For Pn4 and Pn14, significantly higher anti-CP IgG GMTs were obtained with higher CP/Pro ratios. For the other serotypes, no significant difference was found. Interestingly, for the bivalent conjugates of Pn7F-TT-Pn23F, one serotype showed a nonsignificantly higher anti-CP IgG GMT for glycoconjugates with a higher CP/Pro ratio, while the other serotype showed a higher anti-CP IgG GMT for glycoconjugates with a lower CP/Pro ratio. A similar phenomenon was also found in mice immunized with the MenA-TT-MenC bivalent conjugates with different CP/Pro ratios (fig. 5).

2.4 Monovalent and Bivalent Conjugates: An Immune Interference Study

All of the polysaccharide derivatives prepared with optimized MWs and activating agents, either a mixture of two CPs-ADH or a single CP-ADH, were conjugated to TT. The partial characteristics of the bivalent and monovalent conjugates used in the immune interference study are listed in table 6.

The anti-CP IgG GMTs of animals immunized with the bivalent chemical conjugates and physically mixed monovalent conjugates are depicted in fig. 6. All of the bivalent conjugates induced better immunogenicity, among which the Pn4, Pn14, Pn6A, Pn6B, Pn7F, Pn23F, Pn11A, and MenC in bivalent conjugates showed significantly higher anti-CP IgG GMTs than those in the monovalent conjugates, except that anti-MenA IgG GMT in a bivalent conjugate was slightly lower than that in the monovalent conjugate. Therefore, it was concluded that
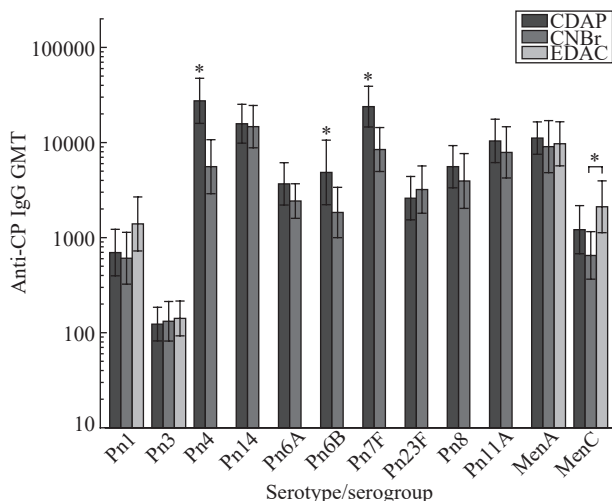
Fig. 4 Comparison of the anti-CP IgG geometric mean titers (GMTs) induced by glycoconjugates prepared by CDAP-, CNBr-, and/or EDAC-activated polysaccharides
The error bar represents the 95% confidence interval of the GMT. An asterisk above the bar indicates that the IgG GMT is significantly greater than that of the same serotype/serogroup glycoconjugates ($P<0.05$).
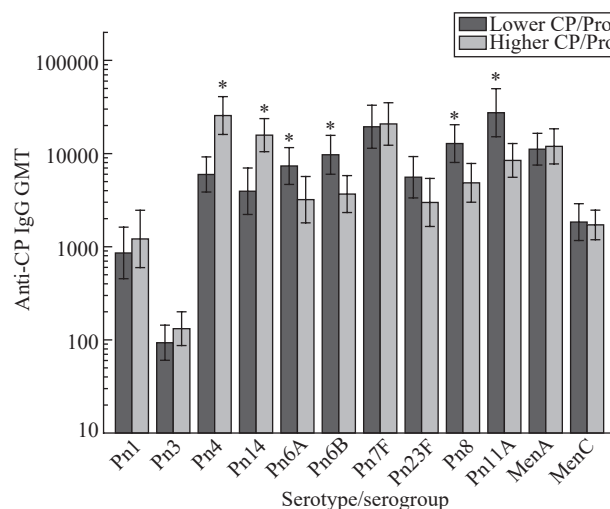
Fig. 5 Comparison of the anti-CP IgG geometric mean titers (GMTs) induced by bivalent glycoconjugates with lower and higher CP/Pro ratios
The error bar represents the 95% confidence interval of the GMT. An asterisk above the bar indicates that the IgG GMT is significantly greater than that of the same serotype or serogroup glycoconjugates ($P<0.05$).

Table 5 Capsular polysaccharide/protein (CP/Pro) was added to the reaction and the partial characteristics of the bivalent conjugates used in the lower and higher CP/Pro studies

Conjugate	CP/Pro ratio	Ps content (μg/mL)	Pro content (μg/mL)	Free Ps	Free Pro
Pn1-TT-Pn3	0.57	44.47/41.88	151.49	6.79%/5.09%	0.58%
	0.88	32.82/37.90	80.55	9.44%/8.23%	0.38%
Pn4-TT-Pn14	0.47	48.24/41.20	190.92	4.52%/4.89%	0.73%
	0.95	41.22/46.91	92.77	9.55%/10.87%	0.52%
Pn6A-TT-Pn6B	0.55	36.28/67.97	189.54	5.24%/6.34%	0.98%
	1.15	38.94/73.63	97.89	10.77%/9.66%	0.35%
Pn7F-TT-Pn23F	0.48	53.25/48.48	210.33	5.06%/4.82%	1.07%
	0.86	50.74/43.66	110.06	9.45%/9.20%	0.33%
Pn8-TT-Pn11A	0.52	52.78/47.69	193.21	5.33%/7.54%	1.42%
	1.01	48.61/51.26	98.88	8.46%/12.43%	0.24%
MenA-TT-MenC	0.53	78.23/71.74	280.71	7.23%/7.90%	0.57%
	1.17	79.69/70.26	128.16	7.77%/9.61%	0.51%

Ps: polysaccharide; Pro: protein

there is probably some enhancement rather than immune interference between the two carbohydrate antigens in a single bivalent conjugate bulk product.

2.5 Comparison to Licensed Vaccines and Dosage Experiment

Based on the five studied combinations of bivalent pneumococcal conjugates, a 20-valent pneumococcal conjugate vaccine (20vPnC) including 10 bivalent conjugates (Pn1-TT-Pn3, Pn4-TT-Pn14, Pn5-TT-Pn9V, Pn6A-TT-Pn6B, Pn7F-TT-Pn23F, Pn8-TT-Pn11A, Pn10A-TT-Pn12F, Pn15B-TT-Pn18C, Pn19A-TT-Pn19F, and Pn22F-TT-Pn33F) was prepared and compared with the corresponding licensed vaccine (13vPnC) in rabbits. Moreover, a bivalent meningococcal conjugate vaccine (MenA and MenC) was also formulated and compared with a licensed vaccine in mice. The serologic immune responses (anti-CP IgG titers) are depicted in fig. 7 and fig. 8, respectively. Under the same dosage of 2 μg, except

that pneumococcal serotype 19A induced slightly higher anti-19A IgG titers in the licensed 13vPnC vaccine, all other common serotypes induced higher anti-CP IgG titers in the 20vPnC formulation. Notably, the anti-CP IgG GMT induced by the polysaccharides of Pn4, Pn5, Pn6A, Pn6B, Pn7F, Pn9V, and Pn14 in 20vPnC was significantly greater than that in the licensed 13vPnC vaccine. However, it should be noted that the lowest anti-Pn3 IgG GMT was shown in both the 20vPnC and licensed 13vPnC vaccines. This finding was in accordance with previous studies that also found a low immunogenicity value (IgG geometric mean concentrations and/or OPA GMTs) of Pn3 in human trials performed by other researchers[12, 13]. For the three different dosages of 20vPnC, the immunogenicity did not always show an intrinsic dose correlation, as reflected for Pn1, Pn5, Pn6B, Pn8, Pn10A, Pn14, Pn19A, and Pn19F (fig. 7). For the meningococcal vaccine, there was a significant difference in the anti-

Table 6 Partial characteristics of the monovalent and bivalent conjugates used in the immune interference study

Conjugate	Ps content (µg/mL)	Pro content (µg/mL)	Free Ps	Free Pro
Pn1-TT-Pn3	33.53/36.24	86.22	8.50%/6.88%	0.23%
Pn1-TT	59.65	101.32	7.75%	0.68%
Pn3-TT	62.50	113.67	8.31%	0.45%
Pn4-TT-Pn14	48.25/42.18	98.60	10.34%/11.78%	0.44%
Pn4-TT	101.33	95.61	8.57%	0.58%
Pn14-TT	85.44	90.32	7.90%	0.41%
Pn6A-TT-Pn6B	38.24/82.11	170.44	9.54%/8.76%	0.99%
Pn6A-TT	80.23	155.90	8.66%	1.33%
Pn6B-TT	87.31	163.88	9.18%	0.86%
Pn7F-TT-Pn23F	51.38/58.57	204.56	4.99%/6.84%	0.77%
Pn7F-TT	113.68	198.73	7.15%	0.92%
Pn23F-TT	105.92	196.19	6.30%	0.87%
Pn8-TT-Pn11A	55.27/63.77	210.84	6.83%/5.35%	1.05%
Pn8-TT	107.45	228.98	4.67%	0.85%
Pn11A-TT	111.69	202.27	6.16%	0.94%
MenA-TT-MenC	85.12/80.24	150.34	5.22%/7.68%	0.34%
MenA-TT	150.75	226.90	8.77%	0.58%
MenC-TT	148.33	205.24	5.58%	0.34%

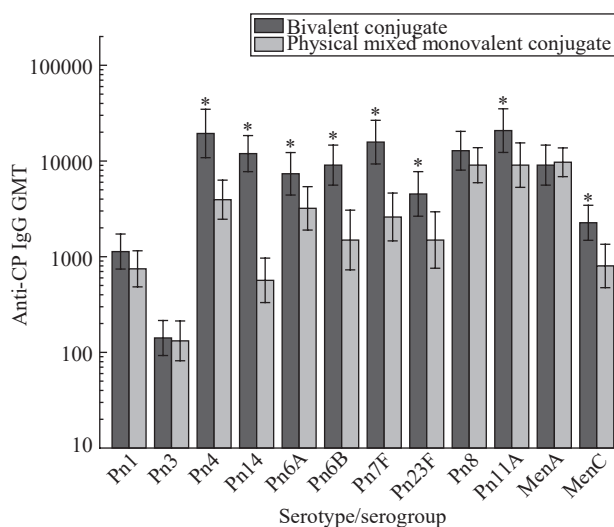Ps: polysaccharide; Pro: protein

Fig. 6 Comparison of the anti-CP IgG geometric mean titers (GMTs) induced by bivalent glycoconjugates and physically mixed monovalent conjugates
The error bar represents the 95% confidence interval of the GMT. An asterisk above the bar indicates that the IgG GMT is significantly greater than that of the same serotype or serogroup glycoconjugates ($P<0.05$).

MenC IgG GMT between our bivalent conjugate vaccine and the licensed vaccine in mouse models, as shown in fig. 8, while the MenA polysaccharides induced comparable anti-MenA IgG GMTs. Therefore, both our multivalent pneumococcal and bivalent meningococcal conjugate vaccines are obviously better than commercially available vaccines.

3 DISCUSSION

Since the first semisynthetic chemical conjugate vaccine was licensed in the 1980s to protect humans against *H. influenzae* type b infection, conjugate vaccines against *N. meningitidis* and *S. pneumoniae* have been developed and registered using the same approach (i.e., bacterial growth to produce CP antigens and chemical coupling to carrier proteins)[14]. Although the carrier protein is of utmost importance for converting the T cell-independent polysaccharide antigen into a T cell-dependent antigen, other parameters, such as the polysaccharide MW, exposure of the charged functional groups, activating agents, CP/Pro ratio, linker used for conjugation, and conjugation biochemistry, can have impacts on the immunogenicity of conjugate vaccines[15–17].

From a manufacturing perspective, the use of shorter depolymerized CPs facilitates consistency in the production, sterile filtration, and purification of the resulting conjugates (particularly from unreacted polysaccharides) and aids in their characterization[14, 18]. In regard to dose control, the incorporation of two polysaccharides in the bivalent conjugate at an equal amount was required. Due to the polyfunctional and polydisperse nature of native biomolecules, the benefit of using depolymerized polysaccharides with a more homogeneous MW may contribute to achieving a ratio of different polysaccharides close to 1[8]. Therefore, native polysaccharides were excluded, and two depolymerizing polysaccharides with different MWs were used for the conjugation reaction in this study, except for MenA due to its low hydrolysis resistance. However, which polysaccharide MW is best for conjugate vaccine immunogenicity is disputed. In an immunological activity study of a Hib conjugate vaccine prepared using different polysaccharide chain lengths, low-MW 10-kD PRP-TT conjugates were found to be more immunogenic than their high-MW counterparts (50- and 100-kD PRP-TT)[19]. Lockyer *et al* found
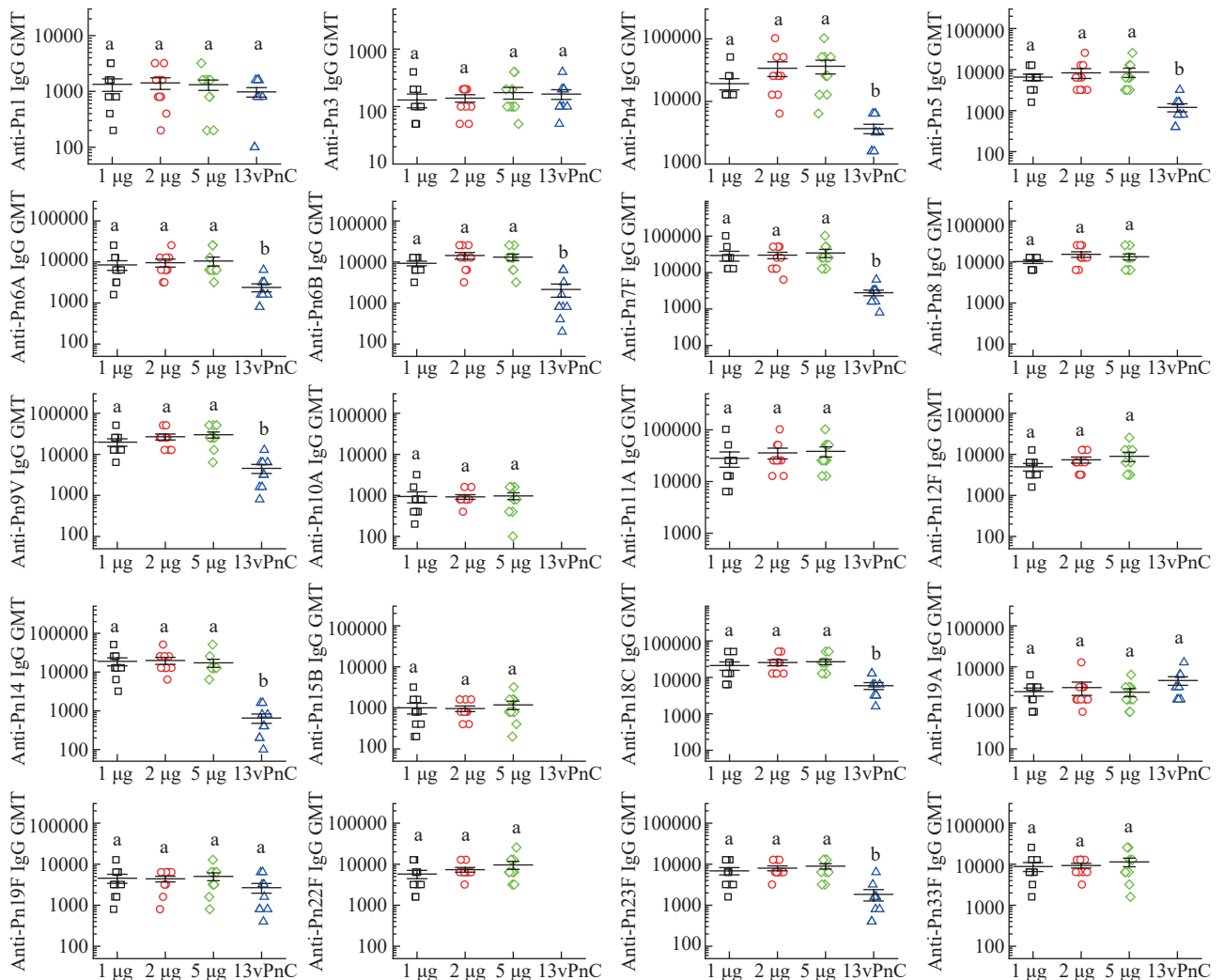
Fig. 7 Comparison of the anti-CP IgG titers induced by different doses of the developed 20-valent pneumococcal glycoconjugate vaccine and the licensed 13-valent pneumococcal conjugate vaccine

The long horizontal line in the middle of the scatter plot represents the mean value of ten IgG titers. The error bars represent the standard error. Different letters above the scatters in each pneumococcal serotype indicate significantly different IgG geometric mean titers (GMTs) according to the Student–Newman–Keuls test ($P<0.05$).
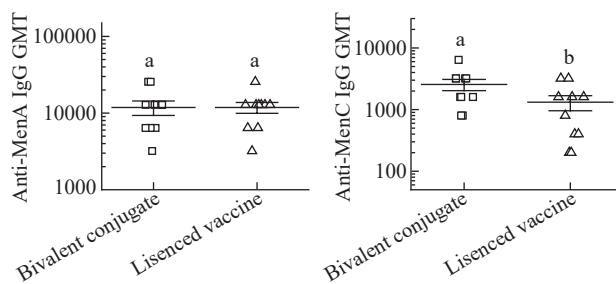

Fig. 8 Comparison of the anti-CP IgG titers induced by the developed bivalent meningococcal glycoconjugate vaccine and the corresponding licensed vaccines

The long horizontal line in the middle of the scatter plot represents the mean value of ten IgG titers. The error bars represent the standard error. The error bars represent standard error. Different letters above the scatters in each meningococcal serogroup indicate significantly different IgG geometric mean titers (GMTs) according to the Student–Newman–Keuls test ($P<0.05$).

that vaccine conjugates prepared from longer MenC polysaccharides produced higher anti-MenC IgG1 and

IgG2b titers after the second dose in a mouse study[20]. In addition, Laferriere et al have reported that there was an increase in the immunogenicity of Pn14-TT conjugates with an increasing polysaccharide chain length[21], while little variation in antibody titers with increasing length was found in pneumococcal polysaccharides of serotypes 3, 6A, 18C, 19F, and 23F[22]. Moreover, a pentavalent semisynthetic oligosaccharide-based glycoconjugate vaccine containing 5 serotype antigens as well as a coformulation of existing vaccines proved highly efficacious in a rabbit model considering the three most important indicators of vaccine efficacy[23], indicating that glycoconjugates containing minimal protective glycan epitopes (only one or a few repeating units of bacterial CPs) were as effective as and even more efficient than those containing much longer saccharide chains, such as an efficacious, semisynthetic glycoconjugate vaccine candidate against *S. pneumoniae* serotype 1 using a synthetic ST1 trisaccharide with a very low MW carrying D-AAT

at the nonreducing end developed by Schumann *et al*[24] and multiple glycoconjugate vaccines prepared by chemically synthesized *S. pneumoniae*-related oligosaccharides[25]. In this study, there was no significant difference in the immunogenicity between bivalent conjugates using high- and low-MW polysaccharides of pneumococcal serotypes 3, 4, 6A, 7F, 8, 11A, and 23F and serogroup MenC. However, a significant decrease in IgG GMTs of anti-Pn14 and anti-Pn6B as well as a significant increase in the IgG GMT of anti-Pn1 with a decreasing polysaccharide MW were found. Reasonable explanations of these results should be further explored.

Cyanylation chemistry initially utilized CNBr to create reactive cyanoester groups on polysaccharides, as used in the first Hib conjugate reported in 1980 by Schneerson *et al*[26, 27]. While CNBr activation has proven to be a very useful method, CNBr is toxic, is difficult to work with in small quantities, and has a number of other limitations, such as low yields, multiple side reactions, batch-to-batch variation, and a risk of destroying the carbohydrate structure and, hence, the naturally occurring epitopes[9]. The CNBr methodology has been largely replaced by techniques using the superior cyanylating agent CDAP[27]. These two cyanylating agents were comparatively studied in this paper. For Pn4, Pn6B, and Pn7F, CDAP activation was significantly superior to CNBr treatment, while comparable immunogenicities were observed for Pn1, Pn3, Pn6A, Pn8, Pn11A, Pn14, Pn23F, MenA, and MenC. Furthermore, Jin *et al* observed that the immunogenicity of GAMP$_{CDAP}$*AH*-BSA was significantly superior to that of GAMP$_{CNBr}$*AH*-BSA, but a higher geometric mean concentration with GAMP$_{CNBr}$*AH*-BSA was induced than that with GAMP$_{CDAP}$*AH*-BSA in mice[28]. The explanation for these conflicting results needs to be further investigated. When the carboxyl groups of Pn1, Pn3, and MenC were blocked by ADH through EDAC activation and thus prevented the binding to the hydrazine group of derived polysaccharides and consequent crosslinking between polysaccharides in the preparation of bivalent conjugates, the two polysaccharides were effectively conjugated to the carrier protein, which is essential for the generation of glycan$_p$-peptides in antigen processing and for presentation to carbohydrate-specific CD4$^+$ T helper cells, with the consequent production of carbohydrate-specific IgG antibodies[29]; this conclusion was supported by the higher GMTs than those derived from the CDAP and CNBr methods.

It has become evident that as the number of glycoconjugates and the dosage of carrier protein included in conjugate vaccines increase, so does the likelihood of interference with the immune response to conjugated antigens[30]. Moreover, high concentrations of the same protein carrier at the site of injection lead to competition for a limited number of specific carrier protein-primed T cells[31]. This effect results in one or more components being unable to elicit a sufficient immune response[8, 32]. Therefore, an excessive carrier protein content should be avoided in the conjugate vaccine. However, an inadequate protein content in the glycoconjugate also had a negative impact on the vaccine effectiveness. This finding is supported by the theory that glycan$_p$-peptides bind to MHC Ⅱ molecules and that the glycan portion is presented to T$_{carb}$ cells[29], which has been discussed above. In other words, excessive or inadequate carrier protein content in the glycoconjugate may lead to a decreased immune response, and the suitable CP/Pro ratio of each polysaccharide is markedly different, as demonstrated in this study.

An important aspect that should be noted is the immunological activity of multiple pathogen polysaccharides incorporated in the same multivalent conjugate. No immune interference has been found in many studies that investigated similar bivalent conjugates. Porro *et al* have reported the immunological activity of a hybrid artificial molecule (polysaccharides from pneumococcal serotype 6A and meningococcal serogroup C, CRM197) tested in two animal models and provided evidence for anamnestic induction of the production of serum antibodies specifically directed against the three distinct native molecules. However, the immunological responses were compared to those against unconjugated oligosaccharides rather than the corresponding monovalent conjugates[33]. Additionally, Adamo *et al* prepared and evaluated a bivalent unimolecular conjugate vaccine against serogroup A and C meningococcal disease, which showed good immunogenicity in comparison with that for monovalent MenA and MenC vaccines and their combinations, thus indicating that there was no immune interference among the carbohydrates in the bivalent unimolecular construct after the third immunization[6]. Based on Ugi multicomponent bioconjugation, Méndez *et al* and Humpierre *et al* found that the bivalent Pn14-TT-Pn7F, Pn18C-Pn1-TT, Pn18C-Pn14-TT, and Pn6B-Pn1-TT conjugates showed immunogenic behavior equivalent to that of their monovalent analogs[7, 8]. For our bivalent conjugate, there was also no immune interference but probably some enhancement between the two carbohydrate antigens loaded onto the same carrier protein. Therefore, in addition to the easier production processes, there is a potential advantage for our conjugation technique to alleviate the immune interference from additional valency to existing conjugate vaccines, which has been proven extremely difficult and functionally challenging in conventional conjugation technologies. A clinical trial that compared PCV13 with PCV7 revealed that immune responses to the seven common serotypes in PCV7 were lower

when PCV13 was applied[34]. Similar results also have been presented for an investigational 20-valent PCV[35]. However, for our developed 20-valent PCV, comparable and even better immunogenicity was obtained than that of a licensed 13-valent PCV.

In a mouse model evaluating the immunogenicity differences of a 15-valent pneumococcal conjugate vaccine (PCV15) based on a vaccine dose, statistical significance could not be determined from pooled sera of BALB/c mice immunized three times with PCV15 at 2, 0.4, 0.08, or 0.016 µg per polysaccharide (with the concentration of 6B doubled in each case) despite achieving the highest absolute antibody titers for eleven of the fifteen serotypes contained in the vaccine (4, 6B, 9V, 14, 18C, 19F, 1, 3, 6A, 19A, and 22F) in the 0.4-µg polysaccharide vaccine formulation[36]. Higher anti-CP IgG GMTs did not always appear in the higher dose formulations, which may suggest that this higher dose saturated the immune system, an observation referred to as a prozone effect[36]. This phenomenon was also observed in our 20-valent pneumococcal conjugate vaccine study using a rabbit model.

In summary, our results indicated that the described conjugation technique was feasible and efficacious to prepare glycoconjugate vaccines, laying a solid foundation for developing extended-valency multivalent or combined conjugate vaccines without potentially decreased immune function.

Acknowledgments

Conflict of Interest Statement

The authors have no conflict of interest.

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