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Development of meningococcal polysaccharide conjugate vaccine that can elicit long-lasting and strong cellular immune response with hepatitis B core antigen virus-like particles as a novel carrier protein

Lingling Xu [a,b], Zhengjun Li [b,c], Zhiguo Su [b], Yanli Yang [b], Guanghui Ma [b], Rong Yu [a,*], Songping Zhang [b,*]

[a] Key Laboratory of Drug Targeting and Drug Delivery System, Ministry of Education, West China School of Pharmacy, Sichuan University, Chengdu 610041, China
[b] State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China
[c] University of Chinese Academy of Sciences, Beijing 100049, PR China

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ABSTRACT

Neisseria meningitidis caused meningitis is life-threatening acute infection with high fatality and high frequency of severe sequelae. Meningococcal capsular polysaccharides can be used to prevent meningococcal disease; while conjugating the polysaccharides to carrier protein was found necessary to improve the immunogenicity and induce memory responses in infants and young children. Nevertheless, repeated administration of glycoconjugate vaccines might lead to carrier-induced epitope suppression due to limited number of carrier proteins. Here in this study, full-length hepatitis B core antigen virus-like particles (HBc VLPs) was used as a novel potential carrier protein for conjugation of meningococcal group C polysaccharides (CPS) with heterobifunctional polyethylene glycol (PEG) of different length (2, 5 and 10 kDa) as linkers. The physiochemical properties of the CPS-PEG-HBc conjugate vaccines were fully characterized. The TEM, DLS, native agarose gel electrophoresis, and HPLC analyses all confirmed the successful conjugation. As compared to plain CPS and the physical mixture of CPS and HBc, the immunization with the conjugate vaccines can generate about 10 times increase in CPS specific IgG titers with a significant boosting effect. HBc conjugation induced a shift to a Th1 cellular immune type response, as assessed by the increased IgG2a subclass production. In addition, vaccination of the conjugate vaccines elicited much enhanced avidity functional antibody and long-lasting immunological memory. IgG titers elicited by CPS-P2k-HBc, CPS-P5k-HBc and CPS-P10k-HBc at week 18 maintained 38.1%, 17.9% and 33.3% of their peak values. All these results demonstrated that HBc VLPs can be used as potential carrier protein to develop polysaccharide conjugate vaccines effective in eliciting long-lasting and strong cellular immune response.

1. Introduction

Neisseria meningitidis bacteria caused meningitis is life-threatening acute infection with high fatality (up to 50% when untreated) and high frequency (more than 10%) of severe sequelae. The risk of getting sick will be even higher for infants, adolescent and those with asplenia or complement deficiencies [1,2]. Based on the composition of capsular polysaccharide, there are 12 meningococcal serogroups, six of which (A, B, C, W, X and Y) responsible for the majority of invasive [3]. Though the polysaccharide meningococcal vaccines have been available over 40 years ago, their efficacy was largely limited by their inability to produce T-cell dependent immunologic memory responses and poor immunogenicity in infants and children less than 2 years old. For this reason, the first meningococcal conjugate vaccines were introduced in 1999 and have been successful in overcoming many of the shortcomings of polysaccharide vaccines [2]. In conjunction with a carrier protein, the hydrophilic carbohydrate are allowed to be presented on the antigen-presenting cell (APC) surface through major histocompatibility class II (MHC II), which in turn induces activation of T cells and maturation of the cognate B cell to become a memory B cell and subsequent production of carbohydrate-specific IgG antibodies [4]. Accordingly, the carrier protein acts role to anchor the polysaccharide to the B cell, which was the initial and the most important step for activation of the adaptive immune system. Therefore, an ideal protein carrier should be capable of inducing high level of B cell and T cell immunogenicity and affording high-density presentation of carbohydrate epitopes.

* Corresponding authors.
E-mail addresses: yurong@scu.edu.cn (R. Yu), spzhang@ipe.ac.cn (S. Zhang).

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To date, there are a limited number of carrier proteins included in licensed glycoconjugate vaccines: tetanus toxoid (TT), diphtheria toxoid (DT) [5], the non-toxic CRM197 variant of diphtheria toxin [6], a complex outer-membrane protein (OMP) mixture from N. meningitides [7], and non-typeable H. influenza-derived protein D [8]. With repeated or multivalent administrations of glycoconjugate vaccines, however, carrier associated immune interference including carrier-induced epitope suppression (CIES), carrier priming or bystander interference often arise [9–11]. Suppression of antibody response to the polysaccharides were often observed due to competition between peptides or capsular polysaccharides bound to homologous carrier proteins for a limited number of carrier specific primed helper T cells. In this regard, developing glycoconjugate vaccines based on some other novel carrier proteins might provide solution to this problem.

Virus-like particles (VLPs) containing repetitive, high density displays of viral proteins that can elicit both T cell and B cell immune responses, have made giant strides in the field of vaccinology over the last three decades [12,13]. As one kind of VLPs, full-length hepatitis B core (HBc) protein is the most fascinating one as a vaccine platform due to several of unique features [14–17]. HBc VLPS has high flexibility allowing insertion of over than 100 amino acids without affecting its ability to self-assemble into VLPs structure. The particulate nature affords the HBc VLPs inherent adjuvant properties by acting both as a T-helper (Th) cell-dependent and cell-independent antigen. Finally, the HBc VLPs are relative easy to produce and purify. Since the initial study by Clarke et al. in 1987 [18], a large number of antigens and epitopes have been genetic fused or chemically linked to HBc as vaccine development strategies [19–21], and several of which are in clinical and preclinical studies [15,16,18].

Considering the numerous advantages of HBc VLPs as vaccine carrier platform, developing a HBc-based polysaccharide conjugate vaccine is expected to break limitations in developing of glycoprotein vaccines with high efficacy and avoiding carrier-induced epitope suppression interference. Nevertheless, there were no relevant studies reported to the best of our knowledge. To this end, we aimed to use full-length HBc VLPs as carrier protein to develop a polysaccharide conjugate and to test its immunogenicity. Compared with the currently used carrier protein, the large particle size would also ensure a higher density presentation of epitopes for carbohydrate, which has been proven important for its immunogenicity [22].

Outbreaks of meningococcal disease have been frequently associated with serogroup C to date [2,23]. In this study, meningococcal group C polysaccharide (CPS) we used as a representative vaccine antigen and HBc-VLPs as the carrier protein to develop a novel glycoconjugate vaccine. In order to avoid the self-crosslinking of CPS and realize high density presentation of CPS on the HBc particles, a heterobifunctional polyethylene glycol (PEG) was used as linker [24]. The physiochemistry properties, immunogenicity, immune persistence and antibody avidity of the CPS-PEG-HBc conjugate vaccines were fully investigated.

2. Materials and methods

2.1. Materials

Meningococcal serogroup C capsular polysaccharide (CPS) was kindly provided by Hualan Biologicals Engineering, Inc. (Xinxiang, China). Full-length hepatitis B core antigen virus like particle (HBc VLPs) was expressed in *E.* coli strain BL21 (DE3) and purified by ultra-centrifugation according to the method described in our previous study [25]. 2-iminothiolane (IT) was purchased from Sigma. 3,3′, 5,5′-tetramethylbenzidine (TMB) was purchased from Thermo, Horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG Fc antibody (HRP-IgG), HRP-conjugated goat anti-mouse IgG1 Fc antibody (HRP-IgG1) and IgG2a Fc antibody (HRP-IgG2a) were all purchased from Abcam (USA).

Heterobifunctional PEG linker of amine-PEG-maleimide with PEG chain of 2 kDa (P2k), 5 kDa (P5k) and 10 kDa, (P10k) were ordered from Nanocs Inc. (Boston, USA). Their polydispersity index (PDI), defined as ratio of weight-average molecular weight (Mw) and number-average molecular weight (Mn), were measured by multi-angle laser light scattering (MALLS) detector (DAWN EOS, λ = 690 nm, Wyatt Technology Corp., USA) and a refractive index (RI) detector (OPTILAB DSP, Wyatt Technology Corp., USA). PDI values of 1.18, 1.17 and 1.12 were determined for P2k, P5k and P10k, respectively.

2.2. Preparation of the CPS-PEG-HBc conjugate vaccines

The reaction route for preparing the CPS-PEG-HBc conjugate vaccines was schematically presented in Fig. 1. Briefly, HBc-VLPs (0.4 mg/ml) was firstly thiolated by incubating with 50-fold molar excess of IT in 20 mM phosphate buffer (pH 7.2) at 25 °C for 3 h. Followed by removal of the free IT using G25 desalting column (GE Healthcare), 0.1 mM of a heterobifunctional linker of amine-PEG-maleimide with PEG chain of 2 (P2k), 5 (P5k), and 10 kDa (P10k) was added to the thiolated HBc. The reaction was lasted overnight at 4 °C, and the unreacted PEG reagent was removed by ultrafiltration centrifugation using 15 ml Centricon with 50 kDa cutoff membrane (Millipore, USA) for five times. The thiolation extent of HBc was determined by measuring the thiol groups of the thiolated HBc using 5,5′-dithio-bis- (2-nitrobenzoic acid) (Ellman's reagent), and the subsequent derivatization degree of PEG was obtained by measuring the decrement of thiol groups after PEG linking.

CPS was activated by adding 10 μl of CNBr (50% (w/v) in chloroform) to 3 ml CPS solution (5 mg/ml) in 0.9% (w/v) NaCl. During 30 min incubating at room temperature, NaOH (0.5 M) was occasionally added to keep the solution at pH 10.5. Thereafter, 0.5 M HCl was added dropwise to adjust the solution to pH 8.5. Finally, the conjugate vaccines were obtained by incubation of the derived HBc with activated CPS, the final concentration of HBc and CPS in the reaction mixture were adjusted to the same value of 0.5 mg/ml. The incubation was in 20 mM phosphate buffer (pH 7.2) at 4 °C for 48 h. The conjugate vaccines prepared were referred as CPS-P2k-HBc, CPS-P5k-HBc, and CPS-P10k-HBc, respectively, in the following text.

Size exclusion chromatography (SEC) based on a Sephacryl S500HR column (1.6 cm × 70 cm, GE Healthcare, USA) was used to purify the CPS-PEG-HBc conjugate vaccines. PBS buffer (20 mM, pH 7.2) was used for column equilibration and sample elution at a flow rate of 2.0 ml/min. The conjugates were collected and concentrated using Amicon (Millipore) with 100 kDa cutoff membrane at 4 °C.

2.3. Quantitative assay of CPS

The total CPS contents in the intermediate products and the final conjugates were determined by resorcinol method [26]. The level of unconjugated polysaccharide in the conjugate vaccines was measured based on deoxycholic acid (DOC) precipitate of HBc [27]. Briefly, 100 μl 1% (w/v) deoxycholic sodium salt solution (pH 8.0) was added to 500 μl CPS-PEG-HBc conjugates samples with the concentration at about 100 μg/ml of HBc. The mixed solutions were kept in an ice bath for 30 min followed by adding 100 μl of 0.1 M HCl, then the conjugate participation was collected by centrifugation at 4 °C for 30 min leaving the unconjugated CPS (S) in the supernatant. While the conjugated CPS (P) in participation was then dissolved in 0.1 M NaOH. The unconjugated CPS

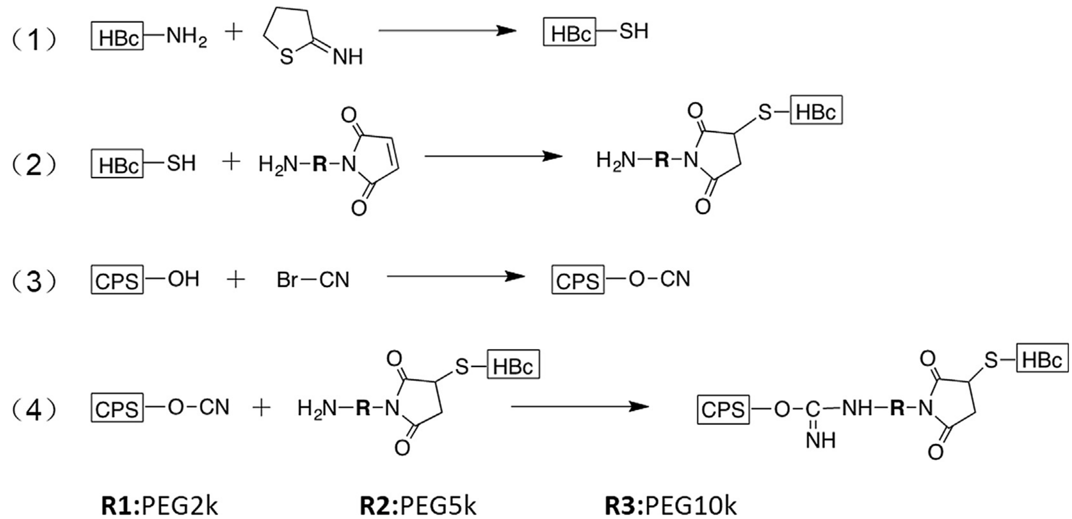

Fig. 1. Schematic representation of the preparation of CPS-P2k-HBc, CPS-P5k-HBc and CPS-P10k-HBc.

content (%) was according to the equation: CPS% = S/(S + P) × 100%. The CPS/HBc ratio (w/w) of the conjugate vaccines was calculated by measure the final content of CPS and HBc in the conjugates. Protein concentration was determined by Bradford method.

2.4. High performance size-exclusion chromatography (HPSEC) assay

Purity of the CPS-PEG-HBc conjugate vaccines was analyzed by HPSEC. The analysis was performed on an Agilent 1100 HPLC series system (Agilent, USA), equipped with a TSK G5000 PWXL column (75 mm × 30 cm, Tosoh Bioscience, Montgomeryville, PA). For each measurement, 100 µl of sample was injected and eluted with pH 6.8 phosphate-buffered saline (PBS) at 0.5 ml/min. Output was measured by absorbance at 280 nm and 214 nm. The retention time of the preparations was recorded.

To acquire the molecular weight (MW) of the HBc and the CPS-PEG-HBc conjugate vaccines, the column was also connected to a multi-angle laser light scattering (MALLS) detector (DAWN EOS, λ = 690 nm, Wyatt Technology Corp., USA) and a refractive index (RI) detector (OPTILAB DSP, Wyatt Technology Corp., USA). The buffer condition is the same as that for HPSEC analysis.

2.5. TEM, DLS and native agarose gel electrophoresis analysis

The morphology and structure of HBc-VLPs and the conjugate vaccines were observed by Hitachi HT7700 transmission electron microscopy (TEM, Hitachi Inc.,). A drop of sample with HBc concentration at about 0.2 mg/ml was applied to a 300 mesh copper grids carbon coated grid and negatively stained with 2% uranyl acetate. The specimens were observed at different magnification.

Dynamic light scattering (DLS) was performed to measure the size of HBc and the three vaccines, using a Malvern Zetasizer Nano ZS (Malvern Instruments, Southborough, Massachusetts) at 25 °C. The samples were at an HBc concentration of 0.2 mg/ml in PBS buffer (pH 7.2).

The HBc and the conjugate vaccines was also characterized by native agarose gel electrophoresis, which was performed on 0.8% gel for 40 min at 80 V. GelRed was use to staining the nucleic acid inside the HBc-VLPs and the conjugates.

2.6. Circular dichroism (CD) spectroscopy and fluorescence measurement

Changes in HBc secondary structure was evaluated by a Jasco-810 spectropolarimeter (Jasco, Japan), using a quartz cuvette with 0.1 cm pathlength. All the samples were at a protein concentration of 0.2 mg/ml in phosphate buffer (pH 7.2). The baseline of buffer was subtracted from the experimental spectra for corrections. The reported CD spectra are the average of three scans.

The intrinsic fluorescence of the HBc and the conjugate vaccines was analyzed using Hitachi F-4500 fluorescence spectrofluorometer (Hitachi, Japan) using a quartz cell of 1.0 cm path length. The emission spectra were excited at 280 nm with a slit width of 5.0 nm and recorded between 300 and 500 nm. The measurement was obtained at a protein concentration of 0.2 mg/ml in PBS buffer (pH 7.2) at room temperature.

2.7. Immunization schedule

Female BALB/c mice aged at 6–8 weeks (body weight about 18–22 g) were purchased from Beijing Vital Laboratory Animal Technology Company Ltd., (Beijing, China) and maintained with pathogen-free water and food. The animals were randomly divided into 6 groups for six animals per group. The six groups were CPS, HBc, physically mixed CPS and HBc, CPS-P2k-HBc, CPS-P5k-HBc, and CPS-P10k-HBc groups. For each group, the mice were immunized subcutaneously with 100 µl samples on days 0, 7, and 14. For HBc groups, the protein content was 5 µg for each injection. For other five groups, the CPS content was all 2.5 µg per dose (100 µl). Blood samples were taken from the mice at weeks 2, 3, 4, 6, 8, 10, 12, 14, 16, and 18, after injection of the samples. Sera were isolated and stored at −70 °C until use. All animals were treated according to the regulations of Chinese law and the local Ethical Committee.

2.8. ELISA assay

CPS specific antibodies (IgG, IgG2a and IgG1) were measured using 96-wells plates (Corning. USA). Experimental procedure was performed as described by Huang et al. [25]. The plates were coated with 100 µl of CPS (10 µg/ml) in 50 mM $NaHCO_3$, pH 9.6 at 4 °C overnight, respectively. Coating buffer were removed from the plates by washing three times with PBS buffer containing 0.05% v/v of Tween 20 (PBST). The plates were blocked with 200 µl of 1% BSA in PBST at 37 °C for 1 h and washed three times with PBST. Following an appropriate series of dilutions with PBST, 100 µl of diluted sera was incubated in the wells for 1 h at 37 °C. After washing the plates for five times with PBST, 100 µl secondary goat anti-mouse horseradish peroxidase conjugated IgG, IgG1, or IgG2a with 1:5000 dilution was added, and the plates were incubated for another 1 h at 37 °C. Thereafter, 100 μl TMB developing solution used as substrate was added and incubated at room temperature for 15 min, followed by quenching the reaction with 50 μl of 2 M H_2SO_4. The resultant solution was determined spectrometrically at 450 nm and 570 nm. The mean (X) and standard deviation (SD) for the PBS group was determined and positive-negative cutoffs calculated as X + 10SD [28].

The specificity of CPS-specific IgG antibody was evaluated by competitive-inhibition ELISA [24]. The plate was coated with CPS and blocked by adding blocking buffer. 0–15 μg free CPS solutions were prepared in 50 μl of blocking buffer. The analysis was carried out by adding 50 μl of 0–15 μg CPS solution to 100 μl mice serum with 100-fold dilution. The assays were carried out as mentioned above. Absorbance at 450 nm was read.

The avidity of the CPS-specific antibodies was investigated employing the ammonium thiocyanate elution ELISA method [29]. Briefly, the plate pre-coated with CPS conjugate was blocked with blocking buffer, followed by incubation of the diluted mice sera at 37 °C for 1 h to give an absorbance between 0.8 and 1.0 at 450 nm. Then, 100 μl sodium thiocyanate with different dilutions (0–0.75 M) was added for 15 min at room temperature. Then, the assay continued as described above. The IgG avidity index (AI) corresponding to ammonium thiocyanate concentration needed to reduce the absorbance by 50% and was calculated as described below according to [29]:

$$AI = \frac{(\log 50 - \log A) \times (B - A)}{\log B - \log A} + A \quad (1)$$

where AI corresponding to the avidity index, log50 = 1.70; NaSCN concentration able to reduce the absorbance by 50% in the initial IgG level was calculated; A and B corresponding to the lowest and the highest NaSCN concentration that gives reduction of the absorbance lower than 50% and higher than 50%, respectively[30].

2.9. Statistical analysis

Results were analyzed using GraphPadPrism 6 software (GraphPad Software, San Diego, CA, USA). The values of $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered statistically significant between the experimental groups, respectively.

3. Results and discussions

3.1. Preparation and purification of the CPS-PEG-HBc conjugate vaccines

In principle, HBc could be possibly conjugated with CNBr activated CPS directly through the reaction between -O-CN in CNBr activated CPS and the primary amine in HBc. However, in our preliminary experiment, we were not able to obtain conjugation of HBc-CPS without PEG spacer. Steric hindrance could be one of the major reason, since both HBc and CPS has large molecular weight. Low reactivity of primary amine in HBc with -O-CN in CNBr activated CPS could also be possible reason.

To realized effective conjugation of CPS with HBc, a heterobifunctional linker amine-PEG-maleimide was used. As illustrated in Fig. 1, HBc was firstly thiolated by IT reagent to introduce thiols groups, which were reacted with the maleimide groups in heterobifunctional amine-PEG-maleimide linker. The amine groups left on the other side in the PEG linkers were further coupled with the -O-CN groups in CNBr activated CPS.

By measuring the thiol groups of the thiolated HBc using 5,5′-dithio-bis- (2-nitrobenzoic acid) (Ellman's reagent), the thiolation extent of HBc was estimated to be about 0.094 μmol thiol group per mg HBc. The derivatization degree of P2k, P5k, and P10k to the thiolated HBc were 0.012, 0.01, and 0.012 μmol PEG per mg HBc, respectively, calculated based one the decrement of thiol groups after PEG modification.

HPSEC equipped with a TSK 5000 PWXL column was used to analyze the HBc before and after modification. As shown in Fig. 2a, the pure HBc was eluted as a single peak at about 15 min. The retention time of the thiolated HBc advanced slightly, but no aggregation was observed through disulfide formation. The further PEGylation of HBc lead to more remarkable advance in retention time and broader peak due to the conjugation and polydispersity of PEG.

Fig. 2b shows the chromatograms for purification of the CPS-PEG-HBc conjugate vaccines reaction mixtures on a Sephacryl S500HR SEC column (1.6 cm × 70 cm). Since there are overlaps in retention time of un-reacted HBc and CPS, as well as the conjugate vaccines, the elution peaks between 80 and 110 ml were collected for the conjugate vaccines to obtain product with higher purity. The purified vaccines were then analyzed by TSK 5000 PWXL HPSEC connected with MALLS, the chromatograms are presented in Fig. 2c. Assuming the dn/dc value for HBc of 0.185 ml/g and the value for CPS of 0.171 ml/g [31], the molecular weight of unconjugated HBc and CPS were calculated to be 6.64×10^3 kDa and 1.08×10^2 kDa using the ASTRA software (Wyatt Technology, USA). Compared to the pure HBc and CPS, significant advancement in retention time was observed for all these three conjugate vaccines, indicating a successful conjugation of HBc and CPS through PEG linker. To obtain the *M*w of the conjugated vaccine, $dn/dc_{(conjugate)}$ was calculated according to the following equation:

$$dn/dc(Conjugate) = \frac{R \times dn/dc(CPS) + dn/dc(HBc)}{R + 1} \quad (2)$$

where the *R* represent the CPS/HBc ratio (w/w). By comparing the protein content and CPS content in the purified conjugate vaccines determined through Bradford assay and deoxycholic acid precipitation methods, the *R* values were determined to be 0.62, 0.43 and 0.54, for CPS-P2k-HBc, CPS-P5k-HBc, and CPS-P10k-HBc, respectively. Accordingly, the dn/dc value of each of these three vaccines was 0.180 ml/g (P2k), 0.181 ml/g (P5k), 0.180 ml/g (P10k). Using these $dn/dc_{(conjugate)}$ values and the ASTRA® software, the average *M*w of the conjugated vaccines were calculated to be 1.04×10^4 kDa (P2k), 9.11×10^3 kDa (P5k), and 1.01×10^4 kDa (P10k), respectively.

Based on the molecular structure and compositions of the conjugate vaccines, the CPS/HBc ratios (*R*, w/w) of the conjugates can also be approximately calculated from the molecular weight of each compound using the following equation:

$$R = (M - M_2 - M_3 \times n)/M_2 \quad (3)$$

where M, M_2 and M_3 were the molar mass of the conjugate vaccines, HBc, and PEG, which are determined by HPSEC-MALLS; n represents the numbers of PEG linked to each HBc VLP. The calculated *R* values for CPS-P2k-HBc, CPS-P2k-HBc and CPS-P2k-HBc were 0.56, 0.37, and 0.51, respectively, which are all close to their corresponding values determined from deoxycholic acid precipitate analysis.

From Fig. 2(b), it is clear that the Sephacryl S500HR SEC column cannot make complete separation of the conjugates from the unconjugated CPS. As listed in Table 1, the content of unconjugated CPS in the three conjugate vaccines was around 10%. This value is close to many of reported polysaccharide conjugate vaccines using conventional tetanus toxoid as carrier proteins [24,30,32]. The WHO requirements have not established a maximal free polysaccharide content allowed for these vaccines, but this rate would be acceptable considering the established limits for Hib conjugate vaccines (maximal of 20% free PRP, [33]).

The preparation process of the HBc-PEG-CPS conjugated vaccines was also monitored by native agarose gel electrophoresis

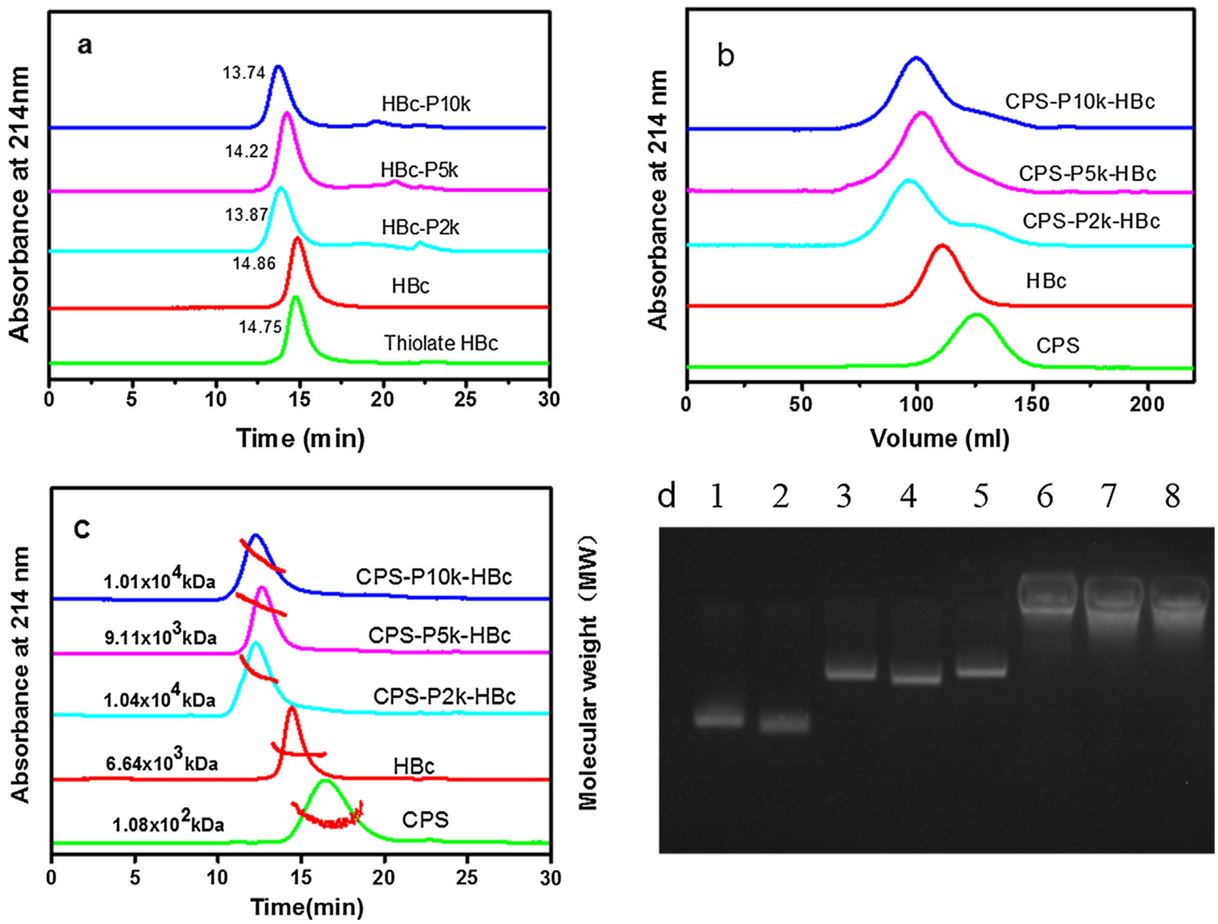

Fig. 2. Purification and analysis of HBc, CPS and the three CPS-PEG-HBc conjugate vaccines. (a) HPSEC analysis of HBc and the modified samples on a TSK G5000 PWXL column (300 × 7.8 mm, I.D.) eluted by 50 mM PB (pH 6.8) at a flow rate of 0.5 ml/min. (b) Purification of the three vaccines performed on a Sephacryl S500HR column (1.6 cm × 70 cm) at a flow rate of 2 ml/min. (c) HPSEC-MALLS assay of the CPS, HBc and the three purified vaccines on TSK G5000 PWXL column. (d) Native agarose gel electrophoresis analysis. Lanes: 1, original HBc; 2, thiolated HBc; 3, HBc-P2k; 4, HBc-P5k; 5, HBc-P10k; 6, HBc-P2k-CPS; 7, HBc-P5k-CPS; 8, HBc-P10k-CPS.

Table 1
Analysis the molar mass, free CPS and CPS/HBc ratio of the conjugate vaccines.

Conjugates	Calculated Mw (kDa)	Unconjugated CPS (%)	CPS/HBc Ratio (w/w)[a]	CPS/HBc Ratio (w/w)[b]
CPS-P2k-HBc	10400 ± 100.2	11.78 ± 2.9	0.62	0.56
CPS-P5k-HBc	9110 ± 82	12.36 ± 3.7	0.43	0.37
CPS-P10k-HBc	10070 ± 103	9.54 ± 2.6	0.54	0.51

[a] The values represent was calculated by deoxycholic acid precipitate.
[b] The values represent was calculated by multi-angle light scattering analysis.

analysis. The nucleic acids inside the HBc-VLPs were visualized by GelRed staining, so that the mobility of the HBc before and after conjugation with CPS can be visualized. Compared with the original HBc-VLPs, PEG modification lead to significant lower mobility. After conjugation with CPS, the HBc-PEG-CPS conjugated vaccines even cannot migrate in agarose gel due to significant increase in the molecular weight. Both agarose gel electrophoresis analysis and HPSEC indicate that there was no free HBc-VLPs in the prepared conjugate vaccines.

3.2. Structure analysis of the CPS-PEG-HBc conjugate vaccines

The secondary structure of the HBc-VLPs and the CPS-PEG-HBc conjugate vaccines were characterized by dichroism spectroscopy (CD) analysis, and the profiles were shown in Fig. 3a. Compared with the HBc-VLPs, the characteristics negative peaks at around 208 and 222 nm, corresponding to α-helix, slightly changed after conjugation with CPS. Particularly, there was a marginal decrease in the ellipticity values at 222 nm for the three vaccines, indicating a slight decrease in α-helix content compared to the original HBc. It was speculated that the conjugation of CPS on the HBc particle surface lead to stretching of peptide chain due to increased hydrophilicity. The same fluorescence maximum emission wavelength (Fig. 3b) indicated that the conjugation of CPS to HBc did not lead to noteworthy conformational change.

The integrity of the unconjugated HBc-VLPs and conjugated vaccines was characterized by DLS and TEM. The particle size distributions based on volume distribution determined by DLS show that the unconjugated HBc-VLPs has diameter about 28.2 nm (Fig. 3c). After CPS conjugation, significantly increased particle size were the hydrodynamic diameters of the three conjugate vaccines increased significantly to 122.4 nm (CPS-P2k-HBc),

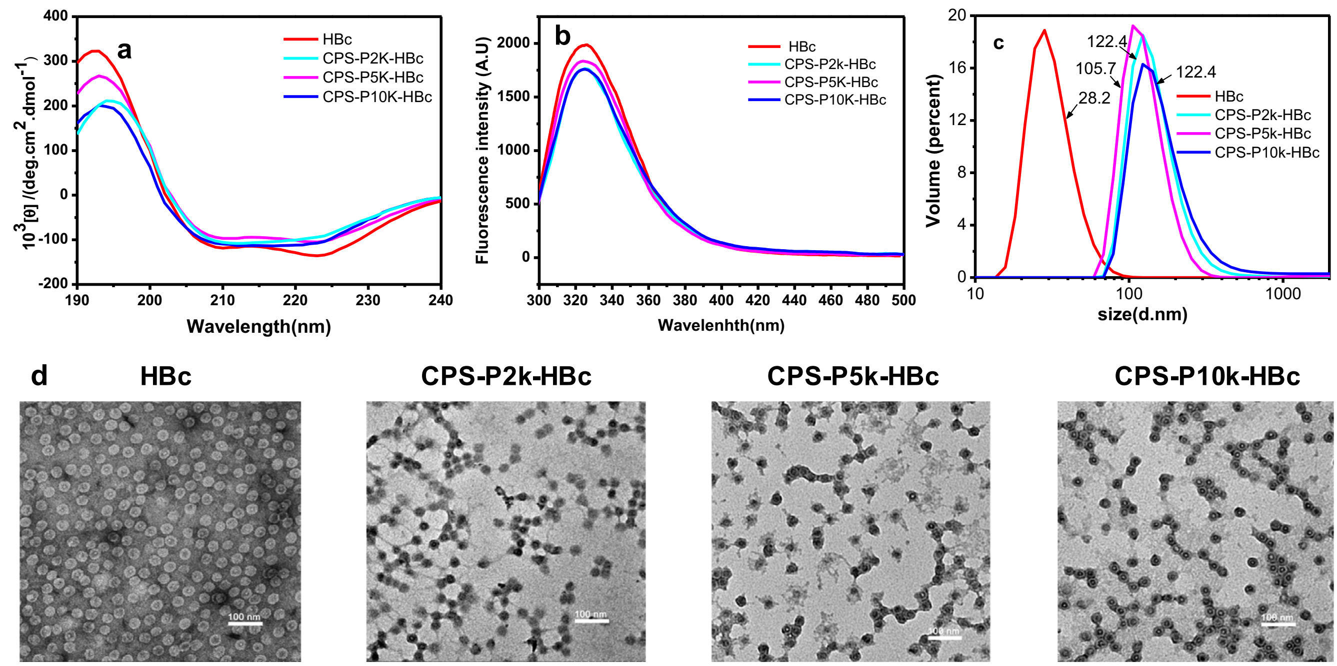

Fig. 3. (a) Circular dichroism spectra, (b) Intrinsic fluorescence, (c) DLS, and (d) TEM analyses of HBc and the three conjugate vaccines.

105.7 (CPS-P5k-HBc), and 122.4 nm (CPS-P10k-HBc), respectively. While the TEM images of the HBc-VLPs and the conjugates indicate the particle structure of the VLPs were well preserved after conjugated with CPS, though the surfaces of the three vaccines become diffuse and some adhesion between particles were observed due to surrounding of capsular polysaccharide. It was considered both the hydrated layer of PEG linker and the CPS contributed to such significant increase in molecular volume and the inconsistence in DLS and TEM characterizations.

3.3. CPS-specific IgG and its subclasses

CPS-specific IgG and its subclass in the mice sera were determined in BALB/c mice immunized subcutaneously with CPS, the physical mixture of CPS and HBc, CPS-P2k–HBc, CPS–P5k–HBc and CPS-P10k-HBc. The IgG titer elicited by free CPS was rather low and showed no apparent increase with two-boosted vaccination (Fig. 4a). While the mice treated by the three conjugate vaccines exhibited high and increasing CPS-specific IgG titers following

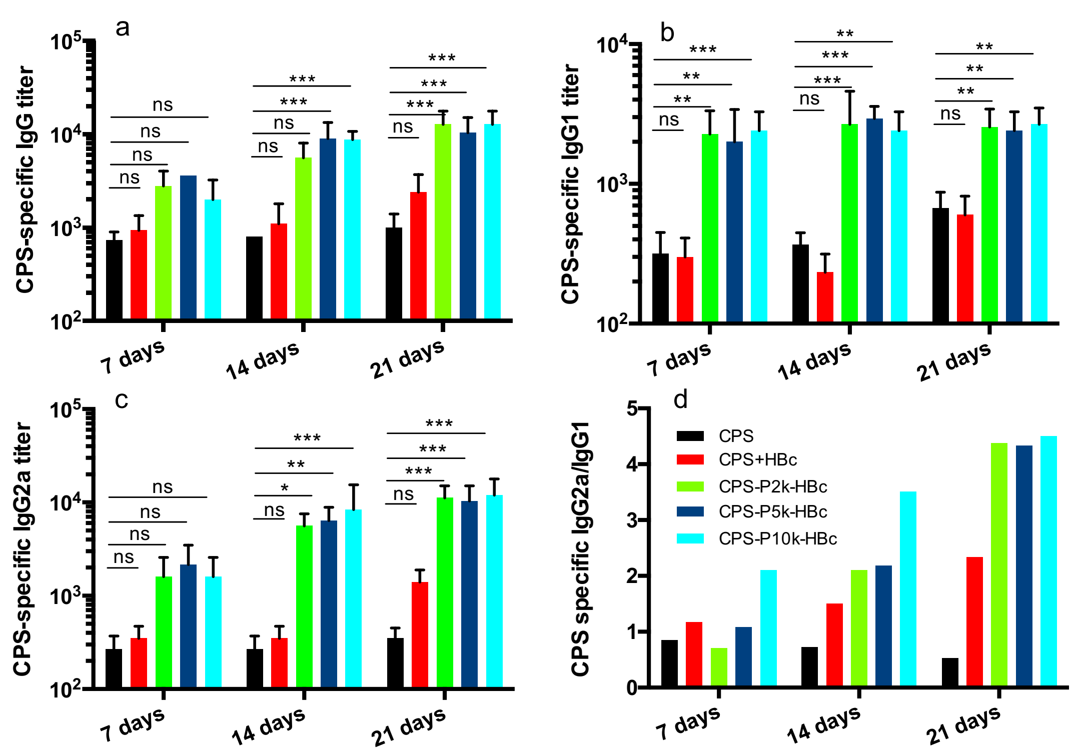

Fig. 4. Production of (a) CPS specific antibodies, (b) IgG1, (c) IgG2a and (d) ration of IgG2a to IgG1 in the sera on days 7, 14, and 21. Bars represent mean ± S.D. (n = 6) (* P < 0.05; ** P < 0.01; *** P < 0.001; ns, not significant).

a booster vaccination. The second injections of the three conjugate vaccines elicited boosting effects (about two- to six-fold rise) as compared to the first injection. On days 21, the anti-CPS titers for the three conjugate vaccines were about 10 times higher than CPS alone ($P < 0.001$), injection. This IgG response clearly showed T-cell dependent immune response after coupling the plain polysaccharide to HBc carrier [30]. Moreover, the antibody titers were also significantly higher ($P < 0.001$) than that elicited by the physical mixture of two components, indicating the conjugation of CPS with HBc was more efficient than simple mixing to induce stronger humoral response. The total specific IgG titer elicited by three conjugate vaccines with 2 kDa PEG, 5 kDa PEG, and 10 kDa PEG have no significant difference, indicating that different length of PEG did not affect the significantly effect on immunogenicity of the conjugate vaccines during 21 days' measurements.

The IgG1 and IgG2a subclasses on days 7, 14, and 21 were further assessed to characterize the antigen specific antibody responses induced by CPS and the conjugated CPS (Fig. 4b, c).

For all of the three conjugation vaccines, the enhancement in IgG2a titers was more noteworthy than in IgG1 titers. On day 21, CPS–P2k–HBc, CPS–P5k–HBc and CPS-P10k-HBc elicited 36.6-fold ($P < 0.001$), 29.7-fold ($P < 0.01$) and 34.3-fold ($P < 0.001$) rise in IgG2a titers respectively, as compared with that of CPS (Fig. 4c). The IgG2a/IgG1 ratio was calculated to further analyze Th1/Th2 type of the induced immune response of CPS and the conjugated ones. On day 21, the IgG2a/IgG1 ratio for free CPS was 0.525, indicating a predominate Th2-type immune response, which was in consistent with most of previous report [24,34]. While the CPS-specific IgG2a/IgG1 ratios of the three conjugate vaccines all increased gradually to above 4.0 (Fig. 4d), indicating a Th1-type cellular immune response was predominantly activated by conjugating the CPS to HBc-VLPs. Increase in IgG2a/IgG1 ratios was usually found in polysaccharide conjugate vaccine with other protein like TT as carrier protein [24,34], nevertheless, such shifting of Th1 type immune from Th 2 type immune response found in CPS conjugation vaccine using HBc as carrier protein was rarely reported, unless TLR7 agonist and Alum-OH adjuvant were applied [35]. The inherent adjuvant properties of HBc-VLPs were considered to play major roles in the enhancement in the cellular immune response found in this work due to their particulate nature [15]. VLPs can enter both MHC class I and class II antigen processing pathways in antigen presenting cells, eliciting both humoral and cellular immune responses [36]. Specifically, the protamine-like domain in the full-length HBc VLPs would enable the VLPs and the linked CPS a high potential to bind to the cell surface heparan sulfate on a variety of cell type to increase the antigen presentation [37].

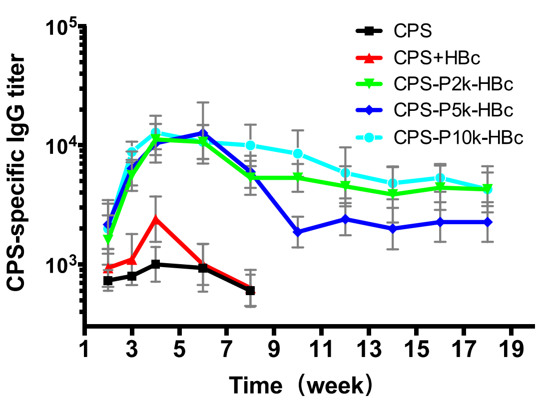

Fig. 5. Persistence of the CPS-specific IgG during 18 weeks period after injections of the vaccines. Bar represents mean ± S.D. from 6 mice per group.

3.4. Immune persistence of antibody avidity analyses of the conjugate vaccines

Fig. 5 presents the CPS-specific IgG titers during 18 weeks after three times immunization with each of vaccine. CPS can induce low antibody titers that gradually vanish. The titers of the physical mixtures of CPS and HBc increased temporarily but dropped down rapidly at the sixth week. It may be due to the transient adjuvant effect of co-administration of HBc and CPS. The CPS-specific IgG titers elicited by the three conjugate vaccines increased gradually after each of injection and a peak plateau was observed in weeks 4–6, and there was no significant difference in antibody titers in these three vaccines with different PEG chain length. After week 6, however, the IgG titers elicited by CPS-P5k-HBc showed a faster decrease than those by CPS-P2k-HBc and CPS-P10k-HBc, probably due to the relative low CPS/HBc ratio of CPS-P5k-HBc compared with the other two vaccines (Table 1). The CPS-P10k-HBc shows the best immune persistence, such that 38.1% of the peak IgG titers was remained at week 18, followed by CPS-P2k-HBc (33.3% of peak value) and CPS-P5k-HBc (17.9% of the peak value). Huang et al. [24] prepared meningococcal group Y polysaccharide conjugate vaccine using TT as carrier protein and PEG of different chain length as linkers. It was found the conjugate vaccine prepared with 2 kDa PEG had the best immune persistence that about 23.0% of its peak value was remained at week 18. This revealed that conjugate vaccine prepared with HBc as carrier protein can prolong the

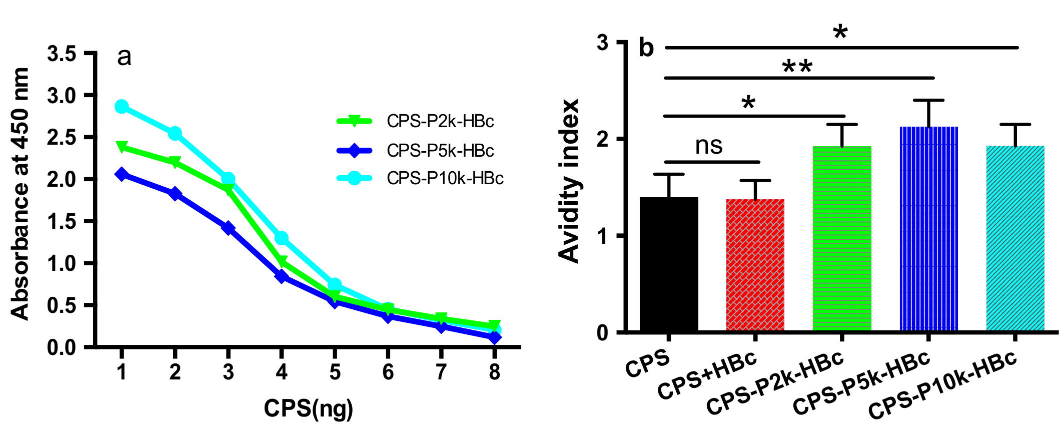

Fig. 6. Competitive-inhibition ELISA analysis of CPS-specific IgG elicited by the three vaccines (a) and avidity measurement of CPS-specific IgG (b). Avidity Index (AI) calculated by the NaSCN concentration needed to reduce the absorbance by 50%. Bar represents mean ± S.D. from 6 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, ns – no significant.

persistence of CPS-specific antibody titer and induced immunological memory in the mice more efficiently.

Fig. 6a shows the results of competitive-inhibition ELISA analysis of CPS-specific IgG elicited by the three vaccines. Obviously, the free CPS added in the serum will competitively bind to the CPS-specific IgG in the serum, thus inhibit the IgG binding to the immobilized CPS during ELISA assay. Even though, the serum IgG showed high affinity to bind the immobilized CPS in the absence of free CPS, until the free CPS amount reach to 15 μg. This indicated that the IgG elicited by all these three conjugate vaccines are CPS specific.

Avidity maturation is an important marker of induction of immunologic memory and it has been shown that higher avidity anticapsular antibodies are more active than lower avidity antibodies in eliciting complement-mediated bacteriolysis [30]. According to the avidity index values presented in Fig. 6b, the CPS-specific IgG elicited by all these three conjugate vaccines showed higher avidity than that of free CPS and the physical mixture of CPS and HBc VLPs ($P < 0.05$). Therefore, we can conclude that the conjugation of CPS with HBc can significantly increase antigen–antibody binding affinity. Moreover, the three conjugates showed no significant difference in the IgG avidity (P greater than 0.05).

4. Conclusion

In the present study, a full-length HBc VLPs was explored for the first as carrier protein to prepare a CPS conjugate vaccine using heterobifunctional PEG of different chain length as linker. The physiochemical properties of the synthesized conjugate vaccines were fully characterized by CD, fluorescence analysis, DLS, TEM, native agarose electrophoresis, et al. Immunization tests on mice show that compared with free CPS and the physical mixture of CPS and HBc VLPs, the CPS-PEG-HBc conjugate vaccines can elicit significantly higher CPS-specific antibody, much prolonged immune persistence, as well as enhanced CPS-specific antibody avidity. Moreover, benefited from the inherit adjuvant properties of HBc VLPs, both strong humoral and cellular immune responses were efficiently elicited. In summary, we have demonstrated that the HBc VLPs can potentially be an excellent carrier protein alternative to currently used TT and DT for development of a groups of polysaccharide conjugate vaccines. The next work will be focused on immunology mechanism analysis to confirm these generic advantages of employing HBc VLPs as carrier protein and delivery system for the polysaccharide conjugate vaccines. It is very likely that new findings and surprises will be reported in the future on the use of HBc-based VLPs in development of novel vaccines for fundamental and applied purposes.

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Conflict of interest statement

The authors declare no conflicts of interest, and all authors attest they meet the ICMJE criteria for authorship.

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