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Development and pre-clinical evaluation of a synthetic oligosaccharide-protein conjugate vaccine against *Neisseria meningitidis* serogroup C

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ABSTRACT

Significant improvement has been made in the development of vaccines against *Neisseria meningitidis* infections since the introduction of polysaccharide-protein conjugate vaccines. Conventional bacterial capsular polysaccharide (PS) based conjugate vaccines require unique and expensive manufacturing facilities, complex production processes and extensive quality testing. Synthetic oligosaccharide (OS) based approach is one of the novel technologies that is being developed to simplify production of conjugate vaccines. OSs can be chemically synthesized to a desired length long enough to represent the antigenic epitopes which often present as a homogenous mixture. We prepared OSs corresponding to tetramer and octamer of *N. meningitidis* serogroup C (MenC) PS by organic synthesis. The MenC tetramer and octamer were further conjugated with tetanus toxoid to produce respective monovalent conjugates having the desired physico-chemical characteristics. The conjugates were evaluated in a mouse model for immunogenicity and compared with a licensed PS conjugate vaccine. Synthetic conjugates could induce anti-MenC PS IgG as well as serum bactericidal titers at levels comparable to those elicited by the licensed vaccine. The increase in length of synthetic oligomers from tetramer to octamer did not appear to increase immunogenicity. The results establish the pre-clinical proof of concept for a synthetic MenC oligosaccharide conjugate vaccine candidate.

1. Introduction

Neisseria meningitidis infection is a significant cause of bacterial meningitis and septicemia and can be fatal in up to 50% of cases if left untreated. Six of *N. meningitidis* (Men) serogroups (A, B, C, W, X and Y) cause majority of epidemics globally and their distribution varies with geography. The epidemic potential also differs according to serogroup and the specific circulating meningococcal strain. Meningococcal infections are vaccine preventable and bacterial capsular polysaccharide (PS) is the key antigen used in meningococcal vaccine development. However, PS based meningococcal vaccines are poorly immunogenic in infants, and in addition MenC PS vaccine induces immunologic hyporesponsiveness in adults [1–4]. On the contrary, PS-protein conjugate vaccines are immunogenic in infants and induce long-term protection [1,5–7]. Recent meningitis outbreaks in Africa and an acute shortage of meningococcal C vaccine suggests the need for development of simple, effective and affordable MenC conjugate vaccine [8].

Bacterial PS or corresponding oligosaccharides (OSs) exist as heterogeneous mixtures with varying degree of polymerization. Immunogenic epitopes involved in antigen-antibody interactions are short glycan structures, often no longer than six to eight sugar residues [9]. Studies have been conducted for the production of defined length of OSs by chemical synthesis which results in highly pure homogenous products having minimal batch-to-batch variation. Such chemically defined oligomers can be tailor- made with a spacer arm at their reducing end to selectively conjugate it with the desired carrier protein. In some cases, very short oligomers like di- or tetra-saccharides have been found to be immunogenic when conjugated with a carrier protein [10,11]. Synthetic polyribosyl ribitol phosphate fragments having 6–9 repeating units when conjugated with several carrier protein, displayed antigenicity and immunogenicity in animal models [12,13]. The minimum length of synthetic MenW OS needed to generate effective antibody response is a tetrasaccharide, when conjugated with genetically detoxified diphtheria toxoid referred to as cross reactive material (CRM) [14]. Finding from Laura Morelli et al. suggests that synthetic oligomers of MenX longer than three repeating units might be needed to fully mimic the natural PS upon conjugation with

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CRM [15]. In a study by Silvia Fallarini et al. involving conjugation of synthetic oligomer analogues of MenA PS, immune response did not appear to be dependent on the oligomer length, as the conjugated monomer elicited similar IgG titers compared to conjugated dimer or trimer [16].

In this study our aim was to develop a vaccine candidate against MenC using synthetically prepared MenC tetramer (CTM) and octamer (COM) and their corresponding tetanus toxoid (TT) conjugates, which could generate immune response comparable to the licensed conjugate vaccine produced using bacterial PS. The work also aimed at evaluating the effect of oligomer chain length on immunogenicity of the conjugates in mouse immunogenicity studies.

2. Materials and methods

2.1. Reagents and chemicals

All the organic synthesis reactions to generate the Men C tetramer and octamer were performed using distilled or analytically pure HPLC grade solvents. All the reagents used were purchased from different reputed vendors with minimum 95% purity. The progress of the reaction was monitored by Thin layer chromatography (TLC). The TLC was checked on pre-coated Silica-gel 60 F_{254} on aluminum plates (Merck). TLCs of ultra-violet (UV)-inactive compounds were stained in 10% H_2SO_4 in methanol solution. Unless otherwise mentioned, all the column chromatography experiments during oligomer synthesis were performed on silica-gel (230–400 mesh). For conjugation experiments and analytical testing, S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA), N-(beta-Maleimidopropyloxy) succinimide ester (BMPS), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), sodium phosphate monobasic (NaH_2PO_4), 1-methyl-2-pyrrolidinone (NMP), dimethylsulfoxide (DMSO), N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesufonicacid) (HEPES), 2-mercaptoethanol, hydroxylamine hydrochloride, 2,4,6-trinitrobenzene sulfonic acid solution (5% w/v in water) (TNBS), 5.5′-dithio-bis(2-nitrobenzoic acid) (DTNB), N-acetylneuraminic acid, resorcinol, sodium dodecyl sulfate, sodium deoxycholate, sodium nitrate, bovine serum albumin (BSA) protein standards, β-mercaptoethanol were purchased from Sigma Aldrich. Sodium hydroxide (NaOH) pellets, hydrochloric acid (HCl), Folin Ciocalteu's phenol reagent, disodium tartarate dihydrate were from Merck Inc; copper sulphate ($CuSO_4{\cdot}5H_2O$) was from Sisco Research Laboratories and methylated human serum albumin (mHSA) for enzyme linked immunosorbent assay (ELISA) analysis was procured from National Institute for Biological Standards and Control (NIBSC). TT was purchased from Fabtech technologies Ltd., India.

Sephadex G-10 resin (GE Healthcare) was used for desalting of derivatized OS samples. Amicon ultra centrifugal filters of 50 kDa MWCO (Millipore) were used for washing of derivatized TT and for removal of unconjugated OSs from MenC conjugates. Acrodisc 13 mm syringe filters, 0.2 μm PES (PALL life sciences) were used for filtration of purified conjugated MenC samples.

2.2. Synthesis of CTM and COM

The linear synthesis strategy was adopted for the CTM preparation as reported by Lin et al. [17] with minor optimizations. The retrosynthetic analysis for the synthesis is depicted in Fig. S1. Synthesis of MenC tetramer was based upon combining the propagation unit **3** with terminal unit compound **4** and then the iteration of same reaction. Both compounds **3** and **4** were prepared from sialic acid. The 6-carbon linker unit with terminal amine group was connected at the reducing end to facilitate the conjugation process. Combining propagation unit **3** with terminal unit **4** resulted in the formation of the dimer compound **2**. The dimer compound **2** was then subjected to chloroacetyl groups deprotection and then used for further elongation to form trimer. Iteration of same steps resulted in formation of protected tetramer, which upon deprotection followed by hydrogenation generated the MenC tetramer compound **1**.

COM was synthesized by a convergent approach by combining two tetrameric units (compound **7** & **8**) together as shown in retrosynthetic analysis (Fig. S2). The synthesis started with the protected sialic acid unit called as the propagation unit compound **3** and the terminal unit with linker compound **4** which were common for MenC tetramer synthesis also. Compound **8** was synthesized with terminal 6-carbon linker to ease the conjugation with proteins. The tetramer compound **8** was prepared by coupling two dimeric compounds termed as compound **11** and **12**. Another tetrameric compound **7** was prepared by coupling of dimer **9** having phosphate with the anomeric thiotolyl containing dimer compound **10**. Both dimeric compounds **9** and **10** were prepared from the sialic acid starting material after desired transformations. Chu et al. has reported the synthesis of α (2 → 9) oligosialic acids, from monomers to dodecamers [18]. We used the method reported by Lin et al. [17] for the phosphorylation of compound **9** and its coupling with compound **10** with minor modifications.

2.3. Characterization of oligomers

Total sialic acid was determined by resorcinol assay on dry content basis using a series of N-acetylneuraminic acid standards [19]. CTM and COM were characterized in detail by various techniques including high performance size exclusion chromatography (HPSEC) for estimation of molecular size distribution and, by one dimensional (^1H and ^{13}C) and two-dimensional (HSQC, DEPT) Nuclear Magnetic Resonance (NMR) spectroscopy for structure confirmation.

Purified OS (CTM and COM) were analyzed by HPSEC, on a TSKgel 3000 PWXL (7.8 × 300 mm, particle size 7 μm, TOSOH) with TSKgel PWXL guard column (6.0 × 40 mm, TOSOH). The mobile phase comprised of 0.1 M $NaNO_3$, pH 7.2 ± 0.1 and was used at the flow rate of 0.5 ml/min in isocratic mode for 30 min.

1D and 2D NMR experiments were recorded at room temperature on Bruker Avance 500 MHz using Deuterium oxide (D_2O) as solvent. Chemical shift value was expressed in ppm. Further, the OSs were analyzed with mass spectrometry for molecular mass.

2.4. Derivatization of oligomers

A schematic representation of the conjugation of synthetic MenC OS to TT via thioether linkage is depicted in Fig. 1. The solution of synthetic CTM (9.0 mg, 7.03 mM) in 0.1 M HEPES buffer containing 0.15 M NaCl, 10 mM EDTA, pH 7.5, was mixed with solution of SATA (4.08 mg, 17.5 mM) in dimethylsulfoxide. The solution was stirred for 1 h (h) at 25 ± 2 °C. The reaction mixture was applied to desalting over sephadex G-10 (GE Healthcare) column (23 ml bed volume), equilibrated with 0.1 M HEPES buffer containing 0.15 M NaCl, 10 mM EDTA, pH 7.5 ± 0.1. It was then collected in 4.0 ml eluent in isocratic mode using same buffer and subsequently concentrated to 500 μl on rotary evaporator (Buchi). The SATA-modified tetramer thus obtained was mixed with solution of hydroxylamine hydrochloride (8.4 mg) in 120 μl of 0.1 M HEPES buffer containing 0.15 M NaCl, 10 mM EDTA, pH 7.5 ± 0.1. After 2 h mixing at room temperature, the thiolated tetramer was stored at – 20 ± 2 °C till further use. The sugar content of thiolated tetra-saccharide was determined by resorcinol assay [19] and thiol (SH) content was determined by Ellman assay [20].

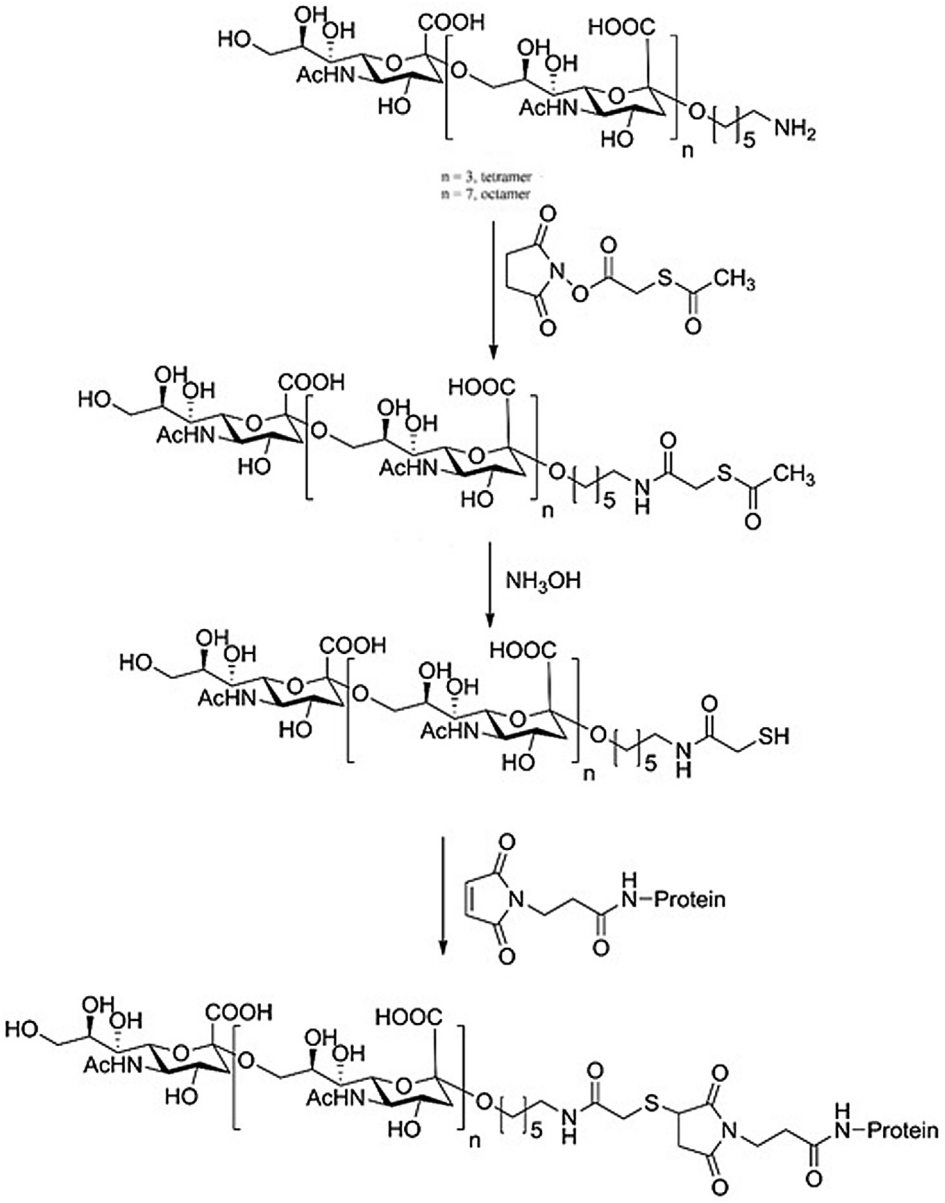

Fig. 1. Schematic diagram showing conjugation of synthetic MenC oligomers with tetanus toxoid via thio-ether linkage.

For the introduction of SH group to COM, the solution of oligomer (9.5 mg, 3.78 mM) in 0.1 M HEPES buffer containing 0.15 M NaCl, 10 mM EDTA, pH 7.5, was mixed with solution of SATA (2.6 mg, 11.4 mM) in dimethylsulfoxide. The rest of the procedure was followed as described for thiolation of tetramer.

2.5. Derivatization of TT

Solution of TT (20 mg/ml) in 0.1 M HEPES buffer, pH 7.6 ± 0.1 was reacted with solution of 3-(Maleimido) propionic acid N-hydroxysuccinimide ester (7.2 mg) in 1-Methyl-2-pyrrolidinone (135 μl). After 2 h mixing at room temperature, the reaction mixture was washed 6–7 times with 0.1 M PBS buffer containing 0.15 M NaCl, 5 mM EDTA, pH 6.8 ± 0.1 through 50 kDa cutoff centrifugal filters. Protein content of modified TT was determined by Lowry method [21] using Bovine Serum Albumin (BSA) as a standard. Maleimide labelling was estimated by Ellman assay [20], where maleimide labelled TT was first reacted with known amount of β-mercaptoethanol wherein unreacted β-mercaptoethanol was analyzed by reacting it with 5, 5′-Dithiobis (2-nitrobenzoic acid) (DTNB).

2.6. Preparation of CTM and COM conjugates

Solution of thiolated tetramer (5 mg/ml) in 0.1 M HEPES buffer containing 0.15 M NaCl, 10 mM EDTA, pH 7.5, was mixed with a solution of maleimide labeled TT (10 mg/ml) in 0.1 M PBS, 0.15 M NaCl, 5 mM EDTA, pH 6.8 ± 0.1; keeping a molar ratio of thiol groups to ester groups as 65:1. The reaction mixture was gently stirred overnight at 2 to 8 °C. The unconjugated OS from crude CTM-TT was removed by 8–10 washes with 0.1 M MES buffer containing, 0.2 M NaCl, pH 6.5 ± 0.1 through 50 kDa MWCO amicon ultra centrifugal filters. The purified conjugate was collected in the same buffer.

For the preparation of COM-TT, the procedure was similar to the one as described for CTM-TT, except for a minor change i.e. a molar ratio of SH groups to ester groups was kept at 80:1 while mixing thiolated COM with maleimide labeled TT.

2.7. Characterization of conjugates

The conjugates (CTM-TT and COM-TT) were analyzed by HPSEC, in comparison with modified TT used for conjugation, on a TSKgel 4000 PWXL (7.8 × 300 mm, particle size 7 μm, TOSOH) and TSKgel 3000 PWXL in series with TSKgel PWXL guard column. The mobile phase was 0.1 M $NaNO_3$, pH 7.2 ± 0.1, at the flow rate of 1.0 ml/min in isocratic mode for 30 min. Void and total column volume were determined using dextran, MW 50, 00,000–400, 00,000 (HIMEDIA) and deuterium oxide (D_2O, Merck), respectively. Saccharide peaks were detected with differential refractive index while protein and conjugate peaks were detected at 280 nm.

Total saccharide in purified conjugates was estimated by resorcinol assay [19], while the protein content was estimated by Lowry method [21]. The ratio of saccharide to protein was then calculated. The amount of free saccharide was determined after precipitation of conjugate sample with sodium deoxycholate [22]. The conjugate sample (900 μl containing approximately 100 μg saccharide content) was added to 80 μl of 1% w/v aqueous sodium deoxycholate solution, pH 6.8 ± 0.2. The reaction mixture was kept at 2–8 °C for 30 min followed by addition of 50 μl of 1 N HCl, and the sample was centrifuged at 6000*g* for 15 min. The supernatant was collected, and the free saccharide content was estimated by resorcinol assay [19].

2.8. Immunogenicity studies

A number of animal studies (3 representative studies included here) were performed at contract research organizations (CROs) to assess the ability of the synthetic conjugates to elicit anti-MenC IgG antibodies. All animal study protocols were approved by the institutional animal ethical committee and by the Indian Ministry of Environment and Forest. Studies were conducted as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

In study 1 the effect of antigen dosing was examined for CTM-TT conjugates, where groups of 8 female Swiss albino mice (5–9 weeks old) were immunized subcutaneously on day 0, 14 and 28 by injecting 200 μl volume containing 0.1, 0.5 and 1.0 μg of conjugated synthetic saccharide, and 1.0 μg of unconjugated saccharide (CTM). In study 2, groups of mice were immunized with COM-TT conjugates at 0.1, 0.5 and 1.0 μg dose of conjugated saccharide, 1.0 μg of unconjugated saccharide (COM only) and 1.0 μg of conjugated MenC polysaccharide. In study 3, group of animals were injected with 1 μg of either CTM-TT conjugates or COM-TT conjugates to compare their immunogenicity. In all three studies positive control group was injected with 1.0 μg conjugated MenC polysaccharide (licensed Men ACYW conjugate vaccine), while negative control group received normal saline.

For all studies, sera were collected at days 14 (post 1 dose serum), 28 (post 2 dose serum) and 35 (post 3 dose serum). Antibody (IgG) titers against specific saccharide and functional antibodies were determined by ELISA and SBA assay, respectively.

2.9. Detection of anti-MenC saccharide IgG by ELISA

Indirect ELISA method was performed as previously reported by Gheesling et al. [23] for the quantification of total anti-MenC PS IgGs. The microtiter plate (NUNC MAXISORP) wells were coated with 100 μl coating solution containing 5 μg/ml each of MenC PS and m-HSA in PBS buffer at pH 7.3 ± 0.1. The plates were incubated overnight at 2–8 °C. Next day, plates were washed with BPBS (0.1% Brij 35 in PBS pH 7.3) and blocked with 200 μl per well of 5% FBS solution in BPBS for 1 h at room temperature (RT as 25 ± 2 °C). Pre-diluted test sera samples (1:200) and quality control (QC) sera (1:400) in BPBS were added to coated plate wells (100 μl/well) followed by subsequent two-fold serial dilutions. The plates were incubated overnight at 2–8 °C. The plates were washed with BPBS and incubated with 100 μl per well of anti-mouse HRP conjugate (diluted 1:1000 in blocking buffer), followed by 1-h incubation at RT. The plates were washed with BPBS and incubated with 100 μl peroxidase substrate, 3, 3′, 5, 5′ – tetramethylbenzidine-H_2O_2 in sodium acetate buffer for 10 min at RT. The reaction was stopped by adding 50 μl of 2 M sulfuric acid. The well absorbance at 450/630 nm was measured using a Tecan multimode reader and the data transferred to an Excel file for analysis using the Combistat software. A QC serum (pool of hyper-immune sera from mice immunized with the licensed MenACYW conjugate vaccine) was given an arbitrary anti-MenC PS IgG concentration of 5000 ELISA units/ml (EU/ml) and used in every assay plate as standard. This was used to generate a standard ELISA curve for extrapolating optical density (OD) values in the test sera dilutions to determine the IgG concentration. The assay buffer blank comprised of antibody dilution buffer instead of serum. The geometric mean anti-MenC IgG concentration (EU/ml) was calculated for each of the animal group with 95% confidence intervals. Unpaired *t*-test was performed, and *p* values were calculated.

2.10. Serum bactericidal activity (SBA) assay

Serum bactericidal activity (SBA) against *N. meningitidis* strains was determined as previously reported by Maslanka et al. [24] with few modifications.

N. meningitidis serogroup C bacterial stock (ATCC® 13102™) was grown overnight on sheep blood agar plate at 37 ± 2 °C with 5% CO_2. Approximately 20 isolated colonies were picked and inoculated on the surface of another sheep blood agar plate and incubated at 37 ± 2 °C with 5% CO_2. After 4–5 h, one or two loopful of bacteria were suspended in assay buffer (Modified Hank's balanced salt solution fortified with bovine serum albumin) and diluted serially to get 250–1000 colony forming units per 10 μl. The QC and test sera samples were heat inactivated for 30 min at 56 ± 2 °C. In a 96 micro well plate, the diluted bacterial suspension was mixed with baby rabbit complement (Pel-Freeze) and serial two-fold dilutions of heat inactivated sera in a proportion of 1:1:2 (v/v/v). For negative controls, bacteria were incubated in separate wells, one with baby rabbit complement and without the test serum (Control-C). The other negative control had test serum and heat-inactivated baby rabbit complement (Control-S). The well contents were mixed, and the plates were incubated for 1 h at 37 ± 2 °C with 5% CO_2. Sample (10 μl) from each well was plated on blood agar plate by tilt plate method. The blood agar plates were incubated overnight at 37 ± 2 °C with 5% CO_2 and colonies were counted. The highest serum dilution showing ≥50% decrease in colony-forming units per ml after incubation of bacteria with reaction mixture, as compared to Control-C was considered as the SBA titer for a sample. The geometric mean anti-MenC SBA titer was calculated for each of the animal group with 95% confidence intervals. The different groups were compared by unpaired *t*-test and p values were calculated.

2.11. Inhibition ELISA

The antigenicity of synthetic tetramer, octamer and their respective conjugates was compared with no-antigen control (NAC) by inhibition ELISA. Inhibition studies were performed using the anti-MenC PS IgG ELISA procedure as described in Section 2.9 with following modifications. The 1:250-fold diluted rabbit anti MenC-polysaccharide serum (in-house) was incubated for 1 h at 37 ± 2 °C with 50, 100 and 200 μg/ml of conjugated or unconjugated CTM or COM. This mixture was used as sample in the indirect ELISA procedure with 1:1000 dilution of the secondary antibody

(anti-rabbit IgG HRP, Sigma). The percentage inhibition of optical density with different concentrations of antigens was calculated as compared to optical density obtained with the NAC.

$$Percent\ inhibition = (NAC\ OD - - Test\ sample\ OD)/NAC\ OD \times 100.$$

3. Results

3.1. NMR and HPSEC analysis of synthetic oligomers

CTM and COM were fully characterized using 1D and 2D NMR spectroscopy and mass analysis (Fig. S3A–D and S4 A–D). The ^{1}H NMR of both CTM and COM clearly showed the peaks corresponding to the ring protons. Further, the presence of linker was confirmed from peaks at δ 3.5 and 2.9 ppm. The remaining protons of the linker part appeared below δ 2 ppm in the form of multiplet. All alpha linkages in CTM and COM were confirmed by the 2D NMR data i.e. correlation spectroscopy (COSY) and heteronuclear single quantum coherence (HSQC) spectroscopy.

All CTM (four repeating units of N-acetyl neuraminic acid) and COM (eight repeating units of N-acetyl neuraminic acid) samples had >90% of sialic acid (wt/wt) as expected. As analysed by HPSEC, both CTM and COM preparations showed single peak on TSK gel 3000 PWXL column (Fig. 2A and 2B).

3.2. Preparation of conjugates of synthetic oligomers

CTM-TT and COM-TT were prepared using thio-ether linkage. The amine group at the end of saccharide sample was reacted with SATA followed by de-acetylation with hydroxylamine hydrochloride to introduce SH groups. The derivatization including purification of thiolated saccharide was completed in 4–5 h, with 50–80% derivatization (0.5–0.8 –SH mole/saccharide mole), as quantified by Ellman assay. Purification of thiolated saccharide resulted in 60–80% saccharide recovery. The maleimido groups were incorporated in TT by reaction with BMPS. The derivatized TT was found to have 30–40 maleimide/TT (mol/mol) with 80–95% protein recovery. The derivatization results for CTM, COM and TT have been summarized in Table 1. The analysis of conjugation mixtures on HPSEC confirmed conjugate formation without residual free protein (Fig. 2C). Purified CTM-TT and COM-TT conjugates were analyzed for saccharide/protein ratio and unconjugated saccharide. The characterization data has been summarized in Table 2, suggesting achievement of saccharide to protein ratio from 0.25 to 0.40 w/w and free saccharide less than 10% for different conjugate lots. The conjugation reaction yields ranged from 20 to 45% based on saccharide content.

3.3. Antigenicity and immunogenicity of conjugates

The antigenicity of unconjugated oligomers and their respective conjugates were compared with "no antigen control" by inhibition ELISA. As shown in Fig. 2D, unconjugated saccharides showed very low (10–25% at 200 μg/ml) inhibition of anti-MenC PS antibodies. However, antigenicity of synthetic saccharides remarkably increased upon conjugation with carrier protein. The saccharide-TT conjugates at 200 μg/ml antigen concentration were found to inhibit 50% of the ELISA response as compared to NAC.

The ability of CTM-TT and COM-TT conjugates to induce anti-MenC antibodies in mice was assessed in a number of animal studies with the objectives of comparing the effect of saccharide chain length on immunogenicity and to evaluate immune response with different doses of conjugated saccharide. In all studies, the licensed MenACYW polysaccharide conjugate vaccine was used as a comparator.

CTM-TT conjugates induced significantly high anti-MenC PS IgG concentration as compared to negative control group (p value-0.007), while unconjugated CTM did not show appreciable anti-MenC PS IgG response (Fig. 3A). The anti MenC PS IgG response from CTM-TT conjugates decreased with decreasing antigen dose from 1.0 μg to 0.1 μg. As depicted in Fig. 3B, COM-TT conjugates elicited higher absolute anti-MenC PS IgG antibody concentration as compared to the negative control group (p value – 0.139) (Note: The original p value 0.139 has been mentioned considering inherent variations in the population, however, p value came out to be 0.00257 after removal of a high titred COM-TT serum value from the calculation as an outlier). Mice immunized with 1 μg of unconjugated COM did not show any significant anti-MenC PS IgG response. The IgG response to COM-TT conjugates also showed dose dependency, as IgG concentrations decreased with decreasing antigen amount from 1.0 μg to 0.1 μg.

In study 3, CTM-TT and COM-TT conjugates produced higher post 3-dose anti-MenC PS IgG levels as compared to those from licensed vaccine at 1 μg antigen dose level (p value – 0.006 and 0.021, respectively) (Fig. 3C). However, antibody titers between CTM-TT and COM-TT conjugates were found to be statistically similar (p value – 0.142).

SBA assays were performed with sera against *N. meningitidis* serogroup C bacterial stock and the results indicate that immunization with CTM-TT and COM-TT conjugates could induce anti-meningococcal functional antibodies in mice. Highest bactericidal titers were observed post 3 doses with 1 μg conjugates, which also correlates with respective groups' IgG concentrations (Fig. 3A and 3B). Absolute mean bactericidal titers generated by both CTM-TT (p value – 0.729) and COM-TT (p value – 0.266) conjugates were higher as compared to the licensed vaccine (Fig. 2A and 2B), however the difference was not statistically significant. Also, there was no significant difference between titers of CTM-TT and COM-TT conjugates (p value – 0.906) (Fig. 3C). The post 3 dose mean anti-MenC PS IgG concentrations against CTM-TT and COM-TT conjugates were higher than those with respective post 2 dose in all of the animal studies (Fig. 3C). However, few groups, generated higher post 2 dose bactericidal titers than post 3 doses (day 35).

4. Discussion

Conjugate vaccines have become an important part of ongoing immunization programs globally [25,26]. However, there is still a need for affordable and effective conjugate vaccines to prevent several infectious diseases. More specifically, an affordable and effective MenC conjugate vaccine is needed to counter the emergency during outbreaks [8]. The manufacturing of affordable MenC conjugate vaccine using conventional fermentation-based technology has several limitations. PS derived from bacterial fermentation is associated with multiple limitations like bio-safety compliant expensive manufacturing facility to handle pathogenic organisms, host cell impurities in purified PS and lot to lot variations etc. Various research studies have established that only a small portion of large PS molecule represents antigenic epitope, and hence conjugates made from these OSs are sufficient in eliciting the required immune response [27,28]. Chemical synthesis of desired length OSs yields a structurally well-defined carbohydrate with high purity. Moreover, chemical synthesis not only provides batch-to-batch consistency in vaccine manufacturing but also warrant less stringent manufacturing facility requirements as there is no handling of pathogenic organisms [29]. In this work, we aimed to compare the immune response from synthetic oligomer-based conjugates to that from conventional PS conjugate vaccine and to study the effect of saccharide chain length on immunogenicity of synthetic oligomers. CTM was prepared by linear chemical synthesis,

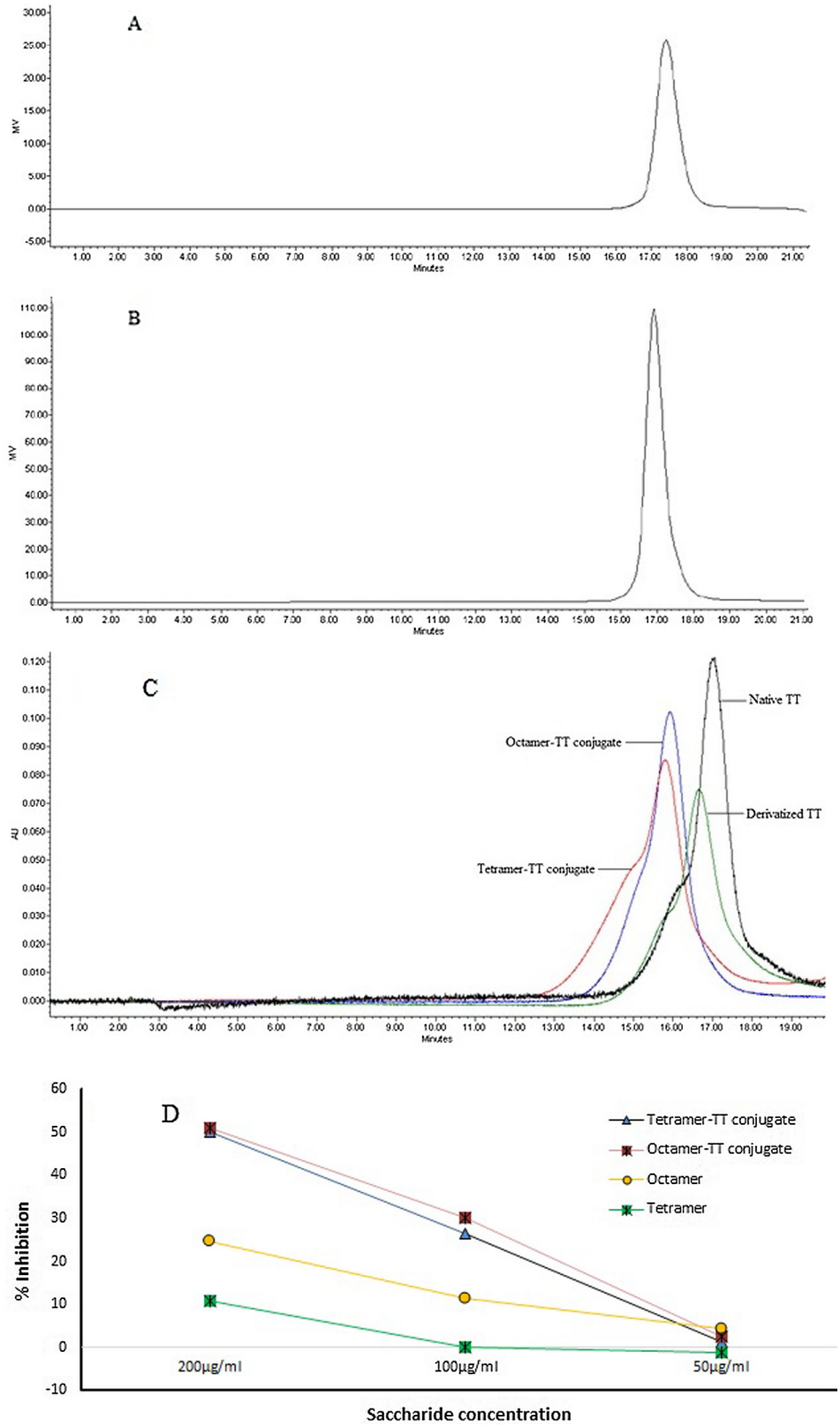

Fig. 2. HPSEC profiles of chemically synthesized **(A)** MenC tetramer, and **(B)** MenC octamer; samples analyzed on TSKgel 3000 PWXL and data recorded using RI detector. **(C)** Comparison of HPSEC profiles of MenC tetramer-TT and octamer-TT conjugates with native and derivatized tetanus toxoid; samples analyzed on TSKgel 4000–5000 PWXL columns in series and data recorded using PDA detector (**D**) Percent inhibition of ELISA response to rabbit sera containing anti-MenC PS antibodies with 50–200 µg/ml of different antigens (unconjugated synthetic MenC tetramer, MenC octamer and their conjugates with tetanus toxoid).

Table 1
Characterization of derivatized MenC tetramer, octamer and tetanus toxoid.

Lot No of thiolated MenC tetramer	-SH/ saccharide (mol/mol)	% Recovery of saccharide	Lot No of thiolated MenC octamer	-SH/ saccharide (mol/mol)	% Recovery of saccharide	Lot No of maleimide labelled TT	Maleimide/ TT (mol/mol)	% Recovery of TT
Lot 1	0.66	62	Lot 1	0.74	70	Lot 1	38	74
Lot 2	0.55	70	Lot 2	0.72	68	Lot 2	41	95
Lot 3	0.60	86	Lot 3	0.88	64	Lot 3	35	98
Lot 4	0.60	70	Lot 4	0.64	70	Lot 4	38	91
Lot 5	0.60	70	Lot 5	0.57	73	Lot 5	35	88

Table 2
Characterization of various lots of MenC tetramer-TT and MenC octamer-TT conjugates.

MenC tetramer-TT conjugates				MenC octamer-TT conjugates			
Conjugate Lot No	saccharide/ protein ratio (w/w)	Unconjugated saccharide %	Conjugated saccharide yield %	Conjugate Lot No	saccharide/ protein ratio (w/w)	Unconjugated saccharide %	Conjugated saccharide yield %
Lot 1	0.25	Less than 1	32	Lot 1	0.30	Less than 1	28
Lot 2	0.28	Less than 1	47	Lot 2	0.29	Less than 1	21
Lot 3	0.28	5.9	38	Lot 3	0.34	Less than 1	37
Lot 4	0.25	9.9	35	Lot 4	0.36	2.0	22

whereas, COM was synthesized by convergent approach. The 6-carbon linker unit with terminal amine group was attached during synthesis at the reducing end of the oligomers to facilitate conjugation. The MenC PS is a homopolymer of α 2 → 9 linked N-acetyl neuraminic acid with O-acetyl groups at C-7 or C-8 of its sialic acid residues. Michon et al. [30] demonstrated that mouse immune response against MenC PS was highly dependent on the degree of O-acetylation and that lower degree of O-acetylation induced higher SBA titers. North American Vaccine Inc. developed and licensed MenC-TT conjugate vaccine using O-acetyl negative polysaccharide. Based on these observations, we synthesized non-O-acetylated MenC tetramer (CTM) and octamer (COM) for the evaluation of immunogenicity.

Both CTM and COM were coupled independently to TT using a thio-ether chemistry approach. The active ester end of SATA was made to react with terminal amine group of OSs to obtain modified OSs that contained a protected sulfhydryl and can be stored at -20 ± 2 °C without degradation, and subsequently deprotected with an excess of hydroxylamine to generate nucleophilic thiol groups. We have optimized the reaction conditions to obtain at least 60–70% derivatization of oligomers. We achieved 60–80% recovery of thiolated OSs after purification for both CTM and COM. During derivatization process, all buffers contained EDTA to prevent metal-catalyzed oxidation of sulfhydryl groups. Although sulfhydryl groups are highly susceptible to oxidation and formation of disulfide cross linkers, we found that more than 90% reactive –SH groups on derivatized CTM and COM were intact up to one week when stored at −20 °C. TT was reacted with BMPS to introduce an electrophilic maleimide group at one end. The chosen reaction conditions resulted in derivatized tetanus toxoid which contained 30–40 mol/mol maleimide/TT with 70–90% protein recovery. The electrophilic maleimido group from tetanus toxoid facilitated conjugation to nucleophilic sulfhydryl groups of thiolated OS. Several lots of CTM-TT and COM-TT conjugates were found to give good conjugation yields with very low residual unconjugated saccharide in purified conjugates.

To determine the antigenicity of synthetic oligomers and synthetic oligomer-conjugates, inhibition ELISA was performed where serum containing anti-MenC PS IgG against licensed MenC PS conjugate vaccine was neutralized with different concentrations of CTM, COM and their respective conjugates. A moderate inhibition was observed with unconjugated OSs; however, OS-TT conjugates were able to neutralize anti-MenC PS antibodies significantly higher than unconjugated OSs. The findings suggest that synthetic oligomers mimic the epitopes recognized by anti-MenC antibodies, however this binding is higher for their respective conjugates, possibly due to proper orientation or more availability of antigenic epitopes to bind with antibodies. This phenomenon of lesser inhibition with smaller OSs as compared to their conjugates has been reported earlier as well [31–33]. Increased inhibition is solely the effect of sera neutralization by saccharide and not the carrier protein as during testing, ELISA plates were coated with mixture of MenC PS and m-HSA which binds with un-neutralized anti-MenC PS antibodies only.

In several immunogenicity evaluations conducted in mice, both CTM-TT and COM-TT were found to be immunogenic as they elicited much higher absolute anti-MenC PS IgG and SBA titers as compared to negative control group. These conjugates have shown dose dependent response as both IgG and SBA titers decreased with decrease in the OS dose from 1.0 to 0.1 μg. Maximum response was observed when mice were injected with 1 μg conjugated saccharide. In comparison to licensed vaccine (MenACYW conjugate vaccine was used due to the lack of any licensed monovalent MenC conjugate vaccine in India), both CTM-TT and COM-TT conjugates showed better or comparable immune response in terms of absolute antibody titers. Unconjugated OSs elicited only minimal immune response. Initially, CTM-TT and COM-TT conjugates were evaluated in two separate animal studies (study 1 and 2), and hence it was not appropriate to draw any comparison between their immunological behavior. Therefore, one independent study (study 3) was conducted to check the effect of saccharide chain length on immunogenicity of these synthetic OS conjugates. We found that CTM-TT conjugates at 1 μg dose elicited comparable immune response with licensed vaccine. However, COM-TT conjugates did not seem to further improve the IgG and SBA titers at the same dose, which indicates that there is no effect of increasing saccharide chain length from tetramer to octamer on enhancing immune response. One limitation of the current immunogenicity studies is that the monovalent CTM-TT and COM-TT conjugate vaccine formulations have been compared

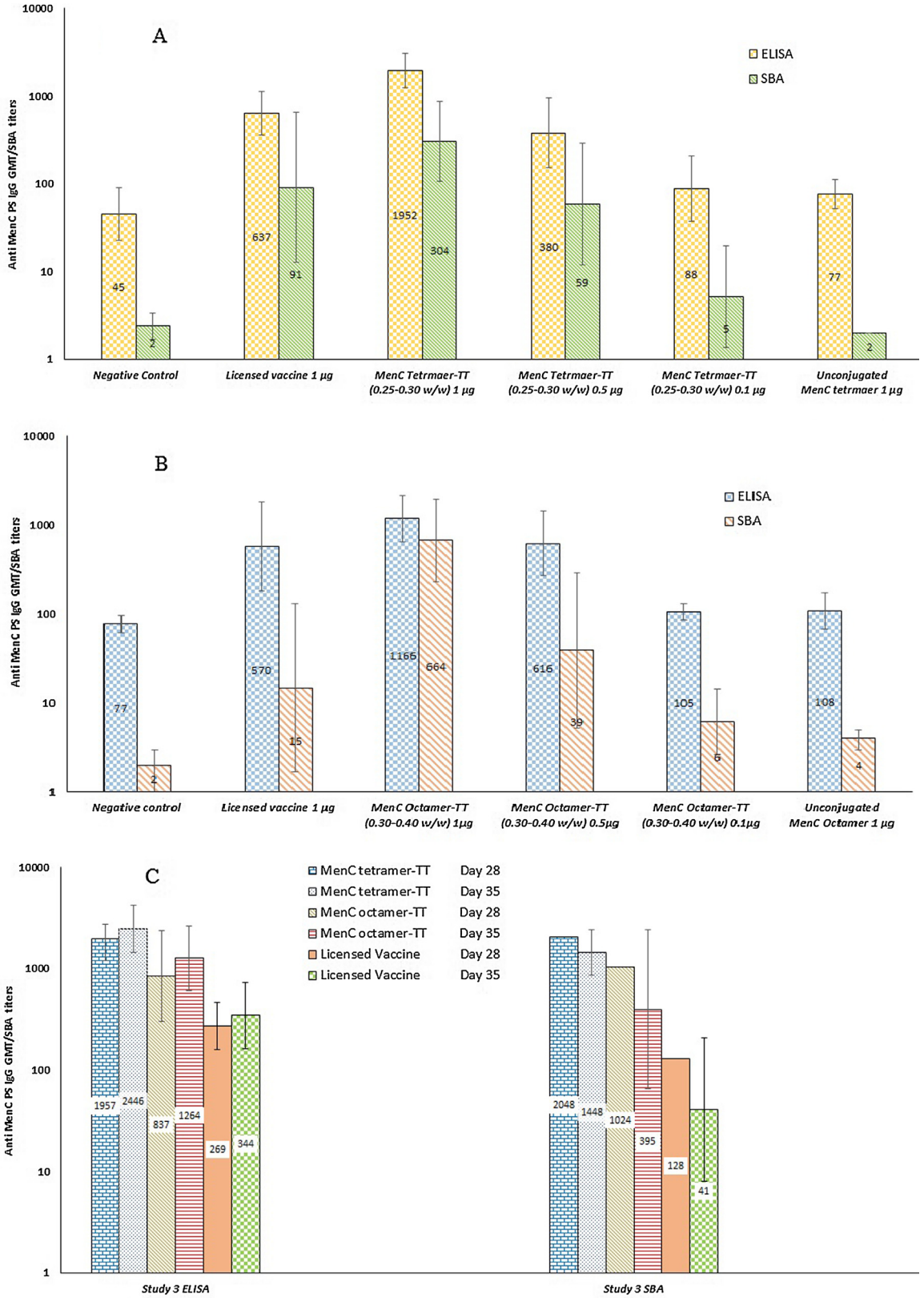

Fig. 3. Immunogenicity of synthetic MenC tetramer-TT and MenC-octamer TT conjugates. BALB/c mice were immunized subcutaneously on day 0, 14 and 28 and the blood sera tested for IgG and functional antibodies on day 28 (post 2) and day 35 (post 3) as required. **(A)** Study 1 – post 3 dose IgG and SBA titers of different doses of MenC tetramer-TT conjugate compared to the vehicle control, licensed vaccine and unconjugated saccharide **(B)** Study 2 – post 3 dose IgG and SBA titers of different doses of MenC octamer-TT conjugate compared to the vehicle control, licensed vaccine and unconjugated saccharide **(C)** Study 3– post 2 and post 3 dose IgG and SBA titers of 1 μg dose each of MenC tetramer-TT and octamer-TT conjugates compared to 1 μg dose of licensed vaccine; Geometric Mean of anti-MenC IgG concentrations(post 2 and 3 dose) and SBA titers (post 3 dose) are presented with 95% confidence interval, post 2 dose SBA titers are for pooled sera.

against a tetravalent MenACYW licensed conjugate vaccine comparator due to non-availability of a licensed monovalent MenC PS conjugate vaccine in India.

In the last three decades, PS conjugate vaccines have made great impact in the prevention of bacterial infections. As immunogenic epitope of glycoconjugate usually comprises small glycan structure, sometimes as short as di- to tetra-saccharide, significant efforts recently have been put on the development of well-defined OSs via chemical synthesis. Synthetic OSs offer numerous advantages as mentioned earlier. If a glycoconjugate with chemically synthesized short saccharides could give protective functional antibodies, it would be good as an alternative to the conventional PS conjugate vaccine. In conclusion, we report successful organic synthesis of well characterized CTM and COM, and their TT conjugates. The conjugation efficiency and homogeneity in different conjugate lots was good. The conjugates showed antigenicity *in vitro* and induced immunological response when injected subcutaneously in mice. However, increasing the length of saccharide beyond tetramer was not found to improve immunogenicity in a mouse model.

The hallmark of the study is the presentation of MenC synthetic tetramer conjugate as a pre-clinical proof of concept of an effective meningococcal conjugate vaccine candidate. Moreover, the development of synthetic meningococcal vaccines may serve as a plausible platform to answer the inequitable access of PS conjugate vaccines in the developing world.

Contributors

MKC lead the overall project. KH, RR, JD, SH, NK, DS conducted the experiments. JD drafted the manuscript. MKC reviewed the manuscript.

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Declaration of Competing Interest

None of the authors has conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.07.053.

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