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(54) Title: POLYSACCHARIDE-PROTEIN CONJUGATES WITH ENHANCED IMMUNOGENICITY AND RAPID HIGH YIELDING PROCESS THEREOF

(57) Abstract: The present invention relates to polysaccharide – protein conjugates with enhanced immunogenicity displaying significantly high antibody titres. The carrier protein is obtained from group of gram positive bacteria, polysaccharide fragment is obtained from group of gram negative bacteria, preferably from *Haemophilus influenzae* serotype b (Hib), *Neisseria meningitidis* serogroup A and C (MenA and MenC). The present invention also relates to a rapid and high yielding process of preparing the polysaccharide–protein conjugates in which derivatized carrier protein reacts with cleaved and depolymerized polysaccharide fragments of optimum length to obtain polysaccharide – protein conjugate employing reductive amination chemistry. The present invention further relates to a chemical process of polysaccharide fragmentation to optimum length for use in conjugation.

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TITLE OF THE INVENTION

Polysaccharide – Protein Conjugates With Enhanced Immunogenicity And Rapid High Yielding Process Thereof

FIELD OF THE INVENTION

The present invention relates to polysaccharide – protein conjugates with enhanced immunogenicity and rapid high yielding conjugation process to obtain the same. More particularly, the present invention provides polysaccharide protein conjugate vaccines which are developed using optimized polysaccharide chain length to give enhanced immunogenicity. The present invention also relates to a rapid process of reductive amination to conjugate a polysaccharide to carrier protein with improved yields and higher immunogenicity.

BACKGROUND OF THE INVENTION

Vaccination (immunization) is a way to trigger the immune response. The process of vaccination involves the administration of vaccine to live entity which in turn activates the body's natural immune system. Vaccines comprise of small doses of an antigen which are preparations of weakened or killed pathogen, such as a bacterium or virus, or of a portion of the pathogen's structure that upon administration stimulates antibody production or cellular immunity against the pathogen.

Immunologically, antigens can be classified as either T-cell dependent (TD) or T-cell independent (TI) antigens. Proteins and peptides are usually TD antigens and require stimulation from helper T lymphocytes in order to elicit an immune response and thereby induce an immune response that is long lasting due to the formation of memory B and T

lymphocytes. In contrast, the TI antigens stimulate B cells to proliferate and differentiate into antibody-secreting effector cells without help from T cells and without formation of memory B and T lymphocytes. Most of these T-cell-independent antigens are microbial polysaccharides that stimulate the production of low-affinity antibodies.

In Gram-negative bacteria the polysaccharides present in bacterial capsules are important virulent factors showing poor immunogenicity. Conjugate vaccine is a product of coupling the microbial polysaccharide (PS) antigens with a carrier protein (CP), thereby converting the T-cell independent immune response into T-cell dependent immune response. The conjugate vaccines are used to immunize infants and children against invasive disease caused by bacteria that contain the capsular polysaccharide. The antigenic capsular polysaccharide is coupled with carrier proteins resulting in highly immunogenic conjugates.

Carbohydrate-protein conjugates are utilized extensively in basic research and as immunogens in a variety of bacterial vaccines. There have been significant efforts to develop simple and reliable methods for the construction of these conjugates. While direct coupling via reductive amination is an appealing approach, the same suffers many drawbacks. The existing conjugation processes via reductive amination are time consuming and low yielding processes while the conjugates thus obtained show less immunogenicity.

It is also a well-established fact that the immunologic performance of polysaccharide-protein conjugates is length-dependent, probably requiring multiple epitopes for optimization (Costantino et al., 1999). It is also known that smaller polysaccharide fragments with selective end group activation produces well defined conjugate vaccines with consistent

reproducibility. The higher sized polysaccharide molecule may have sterically hidden epitopes whereas the smaller fragments will have maximum epitopes exposed to the immune system after conjugation.

Several methods have been deployed for preparing smaller polysaccharide fragments including depolymerization of polysaccharides. Depolymerization of polysaccharides have been well described in the prior art. For instance, the article "Experimental design to optimize an *Haemophilus influenzae* type b conjugate vaccine made with hydrazide - derivatized tetanus toxoid" by Laferriere *et al.*, 2011 describes it in detail. One of the main drawbacks is that the process takes a long time which ranges from 32 hrs and above and have conjugate yields around 15%. Anderson *et al.*, 1986 also describe use of lower size of polysaccharide for conjugation with Diphtheria toxoid and found out that conjugates made from 20 repeat units of Hib-PRP were more immunogenic than those made with 8 repeat units. Their process also takes a very long time i.e. more than 5 days.

The present invention overcomes the drawbacks of the prior art by providing a rapid process of reductive amination to conjugate a polysaccharide to carrier protein with improved yields and higher immunogenicity. The process of the present invention requires a shorter conjugation time. The invention also provides small sized polysaccharide with enhanced immunogenicity through better exposure of the antigenic epitopes to the immune system.

OBJECT OF THE INVENTION

Thus the main object of present invention is to provide polysaccharide – protein conjugates with enhanced immunogenicity.

Another object of the present invention is to provide a chemical process for polysaccharide fragmentation.

Yet another object of the present invention is to provide Lower Molecular Weight (LMW) polysaccharide protein conjugate vaccine for *Haemophilus influenza*e type b (Hib) with enhanced immunogenicity.

Yet another object of the present invention is to provide Higher Molecular Weight (HMW) polysaccharide protein conjugate vaccine for *Neisseria meningitidis* serogroup A and C (MenA and MenC) with enhanced immunogenicity.

Yet another object of the present invention is to provide a rapid process of obtaining the polysaccharide-protein conjugate with shorter conjugation time and better yield of conjugate.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides polysaccharide – protein conjugates with enhanced immunogenicity and rapid high yielding conjugation process to obtain said polysaccharide – protein conjugates and their vaccine.

The present invention provides an optimized molecular weight polysaccharide – protein conjugate wherein polysaccharide is fragmented into a molecular weight in the range of 100 ± 40 kD, more preferably having an average molecular weight approximately 100 kD.

The present invention also provides an optimized low molecular weight polysaccharide to prepare Hib polysaccharide – protein conjugate wherein polysaccharide fragment is fragmented into a molecular weight in the range of 12 ± 6 kD, more preferably having an average molecular weight approximately 10 kd.

The present invention also discloses a process for chemical break down of Hib capsular polysaccharide i.e. PRP (Polyribosyl-Ribitol-Phosphate) to the size of 12 ± 6 kD with less polydispersity and reproducible results.

The present invention also discloses a process for chemical break down of native capsular polysaccharide, such as but not limited to, MenA and MenC to the size of 100 ± 40 kD which shows less polydispersity with reproducible results.

In one of the preferred embodiments, the present invention provides chemical degradation of polysaccharide, in which the polysaccharide sample is kept with sodium-metaperiodate for predetermined period and desalted directly on gel filtration column.

The said polysaccharide sample is purified and analytical assays of purified polysaccharide are carried out for assessing physicochemical properties including PS content, sialic acid content, phosphorus content, protein impurity, nucleic acid impurity, endotoxin, identity and moisture content. The purified polysaccharide is analysed for total content and is derivatized by generating aldehyde groups.

The invention further provides a polysaccharide – protein conjugate wherein selective end-group activation is possible on smaller polysaccharide fragments. The classical polysaccharide is fragmented to

lower mass by oxidizing agent such as metaperiodate to approximately 10 and 100 kD for Hib polysaccharide and Meningococcal serogroups, respectively and conjugated to derivatized carrier protein using reductive amination chemistry.

The carrier protein is the tetanus toxoid, CRM197 or other suitable carrier protein which is activated and analysed for protein content and degree of activation. The hydrazine monohydride is used to attach hydrazine groups to the carrier protein as linker in presence of a catalytic reagent. A non-limiting example of said catalytic reagent is EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride).

The conjugation step comprises the conjugation of derivatized polysaccharide and derivatized carrier protein such as tetanus toxoid. The polysaccharide-protein conjugate is purified further and analysed for ratio of protein content and polysaccharide content, free polysaccharide, size distribution and potency.

The present invention also carries out the analytics for physicochemical analysis of polysaccharide-protein conjugates. The analytics is carried out at every stage of the experiment, i.e. for initial polysaccharide sample, activated saccharide, carrier protein, bulk conjugate and final vaccine.

The conjugates produced are immunized into rat for Hib and into mice for MenA/MenC and titrated for serum antibodies to assess immunological potential. The antibody titers as determined by indirect IgG-ELISA for Hib and MenA/MenC and serum bactericidal assay for MenA and MenC conjugates are equivalent or higher than those obtained with the reference vaccines. Thus the process is cost effective and less time consuming.

The most important outcome of the invention is to obtain better immunogenicity with the conjugate produced using optimized molecular weight of each polysaccharide in a much shorter time and better conjugation yields.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts diagrammatic representation of the reductive amination process.

Fig. 2 depicts the Size Exclusion Chromatography using High performance liquid chromatographic system (SEC-HPLC) profiles of depolymerized and activated Hib PRP obtained on TSK 4000 – 5000 PWXL columns as monitored by RI detector.

Fig. 3a and 3b depict SEC-HPLC elution profile of activated MenA and MenC polysaccharide on TSK 4000 – 5000 PWXL columns as monitored by RI detector.

Fig. 4 depicts the shift in SEC-HPLC profiles of Hib PRP-TT conjugates on TSK 4000 – 5000 PWXL columns as monitored by UV detector.

Fig. 5 depicts SDS-PAGE analysis of PRP-TT conjugates. The sample lanes contained following, lane 1 – protein standards for molecular weight, lane 2 – low molecular weight PRP-TT conjugate, lane 3 – high molecular weight PRP-TT conjugate, and lane 4 – native TT.

Fig. 6 depicts the shift in SEC-HPLC profiles of Men A – TT conjugate and Men C– TT conjugate in comparison with free tetanus toxoid on TSK 4000 – 5000 PWXL columns as monitored by UV detector.

Fig. 7 depicts the graphical representation of antibody inhibition percentage with Men C– TT conjugate in an inhibition ELISA.

Fig. 8 depicts Hib conjugate immunogenicity in rats by ELISA to determine serum IgG titres after three dosings on day 0, 28, and 42.The

antibody titers were evaluated on day 0, 28, 42, 49 and 70 at different dose levels and with different size of Hib PS.

Fig. 9 depicts Meningococcal serogroup A conjugate immunogenicity in mice by ELISA to determine serum anti-MenA IgG titres.

Fig. 10 depicts Meningococcal serogroup C conjugate immunogenicity in mice by ELISA to determine serum anti-MenC IgG titres.

Fig. 11 depicts Meningococcal serogroup C conjugate immunogenicity in mice by serum bactericidal assay to determine anti-MenC serum functional antibody titres.

DETAILED DESCRIPTION OF THE INVENTION WITH ILLUSTRATIONS AND EXAMPLES

Most proteins contain an abundance of carboxylic acid groups from C-terminal functionalities and aspartic and glutamic acid side chains. These groups are readily modified with nucleophilic compounds to yield stable imide product. While nucleophile functionalities easily react with aldehyde groups, they don't spontaneously react with carboxylate or carboxylic acid groups. The carboxylic acid group first must be activated with another compound that makes it reactive towards nucleophiles. In the present invention, the protein is treated with water-soluble crosslinker in aqueous solution for carboxyl to amine conjugation. One of the non-limiting examples of the said crosslinker is carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride). The crosslinker reacts with the available carboxylate groups to form a highly reactive intermediate ester. This active ester species further react with nucleophiles such as a hydrazide to yield stable imide product having bonds with extending terminal hydrazide groups (Fig. 1).

Several experiments are conducted to get sufficient labeling of TT with hydrazide molecules i.e. to achieve desirable degree of activation of the derivatized carrier protein e.g tetanus toxoid i.e. In this context, varying concentration of crosslinker is applied during activation reaction. Furthermore, it is observed that highly hydrazide activated TT (TT-H) tends to precipitate in reaction mixture which led to lower yield of activated TT. To counteract this, reaction mixture is observed during incubation and reaction is quenched if precipitation is observed. This is due to change in isoelectric point of TT, which is normally between 6.2 – 6.5. Isoelectric point increases to alkaline side due to loading of hydrazides on TT molecule which eventually leads to precipitation of protein at lower pH.

Activated TT is checked on SEC-HPLC to ensure removal of unreacted hydrazine and to know the profile of modified TT. Degree of activation (number of hydrazide per TT molecule) of more than 40 is achieved with this process, which is adequate for sufficient loading of activated polysaccharide on TT molecule.

Carbohydrates and other biological molecules that contain polysaccharides, such as glycoproteins, can be specifically modified at their sugar residues to produce reactive formyl functionalities. The formyl functionalities are relatively unreactive and oxidized to transform into amine reactive aldehydes. Periodate oxidation is the preferred route to transforming the hydroxyls of sugar residues into amine-reactive aldehydes. Periodate cleaves carbon-carbon bonds that possess adjacent hydroxyls, oxidizing the –OH groups to form highly reactive aldehydes. Terminal cis-glycols result in the loss of one carbon atom as formaldehyde and the creation of an aldehyde group on the former no. 2 carbon atom

(Fig. 1). The periodate oxidation is carried out by periodates. One of the non-limiting example of periodates is sodium metaperiodate. Varying the concentration of periodate during the oxidation reaction gives some specificity with regard to what sugar residues are modified. Oxidation of polysaccharide using greater concentrations of periodates results in the cleavage of adjacent hydroxyl-containing carbon-carbon bonds, which leads to sugar ring opening and generation of small sized polysaccharide fragments.

Reaction parameters are varied during depolymerization/activation reaction to obtain the desirable size of polysaccharide fragments and the desirable degree of activation. The varying reaction parameters comprise the concentration of periodate, different time period of exposure of periodate to polysaccharide. The concentration of periodate affects the molar ratio of polysaccharide to periodate in reaction mixture as illustrated in example 3 for Hib and in example 4 for MenA and MenC.

The polysaccharides from the group of gram negative bacteria including but not limited to *H. influenzae* b (Hib), *N. meningitidis* serogroup A and C (MenA and MenC) are cleaved to obtain lower molecular weight polysaccharide fragments. The lower molecular weight polysaccharide fragments are obtained in the range of 100 ± 40 kD for Men A and Men C, having an average molecular weight of 100kD, while in case of Hib the polysaccharide fragments are cleaved up to a molecular weight in the range of 12 ± 6 kD, having an average molecular weight of 10 kD.

During the conjugation step, aldehyde containing polysaccharide fragments react with hydrazine labelled proteins to form Schiff bases. A Schiff base is relatively labile bond that is readily reversed by hydrolysis

in aqueous solution. The formation of Schiff bases is enhanced at alkaline pH values, but they are still not completely stable unless reduced to secondary or tertiary amine linkages. A number of reducing agents can be used to convert the Schiff base into an alkylamine linkage. Once reduced, the bonds are highly stable. Reductive amination is best facilitated by the use of as reducing agent that has specificity towards the Schiff base structure and that will not affect the original aldehyde groups. A non-limiting example of such reducing reagents include sodium borohydride or sodium cyanoborohydride that has strong reducing power which rapidly converts unreacted aldehydes into non-reactive hydroxyls, effectively eliminating them from further participation in the conjugation process (Fig. 1). The course of conjugation is monitored by SEC-HPLC analysis with change in the retention time of the activated TT or polysaccharide in comparison to the conjugates.

Purified PS – TT conjugates are characterized for polysaccharide content, protein content and unconjugated free polysaccharide. Studies for testing antigenicity and immunogenicity of the polysaccharide – TT conjugates thus obtained are conducted.

Example 1: Derivatization of carrier protein (TT) with hydrazine for Hib-TT conjugate

100 mg of bulk TT is diafiltered against 0.1 M MES buffer containing 0.2 M NaCl, pH 6.5 using 50 kD MWCO amicon filter. 2.0 ml Hydrazine solution in the concentration of 0.4 M (from stock of 5M hydrazine in milli Q water (MQW) is mixed with 13.6 ml MES buffer. pH of the solution was adjusted to ~6.5 using 5N HCl. To this is added 0.48 ml EDC solution to make final concentration of EDC 30 mM (from stock of 1.5 M EDC in MQW). To this solution 2.0 ml diafiltered TT is added and the final volume of reaction

mixture is adjusted to 24.08 ml. The reaction mixture is stirred at room temperature for 4.0 hours. After incubation, the reaction is quenched by increasing the pH of the solution to ~9.0 by 5N NaOH. The solution is diafiltered against 30 mM NaCl, 3 mM Na_2CO_3, pH ~10.5 using 50 kD MWCO amicon filter. At least 6 washes of buffer is given to ensure the complete removal of unreacted hydrazine. Diafiltered activated TT solution is concentrated to 1.5 ml. The activated TT is analyzed on SEC-HPLC to ensure removal of unreacted hydrazine. Activated TT was analyzed for hydrazide labelling by TNBS assay. Protein content of activated TT was determined by Lowry's assay. Labelling of TT with hydrazide was expressed as degree of activation which was calculated by dividing the moles of hydazides by moles of protein present (Table 1).

Example 2: Derivatization of carrier protein (TT) with hydrazine for Mening-TT (MenA-TT and MenC-TT) conjugates

Tetanus toxoid is activated in the same way as for Hib-TT conjugate preparation in example 1. Activated TT is checked on SEC-HPLC to ensure removal of unreacted hydrazine and to know the profile of modified TT. Degree of activation (number of hydrazide per TT molecule) of 50 ± 5 (Table 1) is achieved with this process, which is adequate for sufficient loading of activated polysaccharide on TT molecule. The hydrazide activated TT (TT-H) is stored at pH ~10.5 at – 20 °C until further use.

Table 1: Derivatization of different batches of TT to generate active hydrazide groups

Batch no.	Scale of Expt. (amount of protein	Degree of Activation	Process recovery
TTH-01	25 mg	50.7	82%
TTH-02	50 mg	53.4	85%

TTH-03	50 mg	51.7	76%
TTH-04	100 mg	53.6	80%
TTH-05	100 mg	54.2	82%

Example 3: Depolymerization and activation of Hib PRP by oxidation

100 mg of Hib PRP is dissolved in 1000 µl of MQW. Moles of PRP monomer is calculated as ~136 mM considering 2000 µl volume of reaction mixture and 368.2 Dalton as molecular weight of PRP monomer. 200 mM stock of sodium periodate in MQW is prepared. 1 mole of PRP is reacted with 0.5 mole of sodium periodate. The reaction mixture is then incubated at 2-8°C by keeping in dark for 7-15 minutes. After incubation, reaction mixture is purified by sephadex G-25 column. Prior to this column is equilibrated with 1/5 x of 0.15 M MES, 0.2 M NaCl, pH 6.5. To this 2.5 ml sample is added for desalting and eluted with 1/5 x of 0.15 M NaCl, 0.2 M NaCl, pH 6.5.Or else the PS can be purified directly on PD10 columns. Eluted fraction of activated polysaccharide is concentrated to 500 µl using rotavac. Concentrated solution of activated polysaccharide is analyzed for size by SEC-HPLC (Fig. 2), for PRP content by orcinol assay and for aldehyde moles by BCA assay. Activation of polysaccharide is expressed as degree of activation which is calculated by dividing the moles of monomer present in polysaccharide by moles of aldehyde generated after oxidation with sodium metaperiodate (Table 2). The SEC-HPLC profiles of native and activated Hib PRP indicates that upon activation, polysaccharide fragments (≈10 kD) have low molecular weight than native PRP, suggesting degradation of PRP. The shift in the peak towards right in the figure 2 suggests depolymerisation of native polysaccharide.

Size of the native polysaccharide of higher kD are differentiated from lower kD activated polysaccharides or polysaccharide fragments by HPLC-SEC analysis using GPC columns in series (Fig. 2). Activated polysaccharide fragments of 6 – 18 kD molecular size with average degree of activation about 4 – 6 saccharide repeating units per aldehyde group is produced. Activated polysaccharides are stored at – 20 °C.

Table 2: Hib PRP activation

Batch no.	Scale of expt.	Size of Polysaccharide	Degree of Activation	Process Recovery
PRP-01	25 mg	≈10 kD	4.6	69.7%
PRP-02	25 mg	≈10 kD	7.4	66%
PRP-03	100 mg	≈10 kD	4.8	48.6%
PRP-04	10 mg	≈100 kD	80.2	71%
PRP-05	25 mg	≈100 kD	88.5	55%

Example 4: Depolymerization and activation of MenA and MenC polysaccharide by oxidation

For MenA, native polysaccharide is reacted in 0.2 molar excess of sodium metaperiodate ($NaIO_4$) at 25 °C for 4 hours. For MenC, native polysaccharide is reacted in 0.2 molar excess of $NaIO_4$ at 25°C for approx. 15 minutes. Depending upon the initial size of the polysaccharides, the time or the molar ratio may be changed from sample to sample. Excess of salt is desalted directly by PD10 columns from GE Amersham or on Sephadex G-25 column, equilibrated with 0.15 M MES buffer containing 0.2 M NaCl, pH 6.5. As the sample gets diluted in the process, it is concentrated on rotavapour to a desired final concentration of approx. 10 mg/ml. The concentration of the resulting activated polysaccharide is determined by phosphorous assay for MenA PS and by resorcinol-hydrochloric acid method for MenC PS. The aldehyde content of the

resulting polysaccharide is determined by BCA assay using glucose as a reference. Derivatization of polysaccharide is expressed as degree of activation, i.e. the number of saccharide repeats per aldehyde, which is calculated by dividing the moles of monomer present in polysaccharide by moles of aldehyde generated after oxidation with sodium metaperiodate. Activated polysaccharide is stored as dry powder after removing water using evaporation techniques which is selected from, but not limited to rotary evaporator or lyophilization. Rota vapour is preferred over lyophilisation because of being rapid process to concentrate and dry the product without any product disintegration.

Average degree of activation (DOA) of about 70 – 90 saccharide repeating units per aldehyde group are found for Men A polysaccharide (Table 3, Fig. 3a); while average degree of activation of 30 – 40 is found for activated MenC polysaccharide (Table 4, Fig. 3b). Fig. 3a and 3b displays the HPLC-SEC elution profiles of activated MenA and Men C polysaccharides, respectively when the samples are eluted in 0.1 M $NaNO_3$ at pH 7.2 and at the flow rate of 1.0 ml/min. on TSK gel G5000 PWXL + G4000 PWXL column series. The SEC-HPLC elution profiles of periodate oxidized Men A and Men C polysaccharides shows the shift in peak towards right suggesting depolymerisation of native polysaccharides.

Table 3: Derivatization of MenA Polysaccharide to generate aldehyde functional group

No.	Reaction Time with $NaIO_4$ @ RT	Content of Activated PS	Moles of CHO	Degree of Activation (DOA)
Lot 1	4 hrs	3.8 mg	0.259 mM	~88
Lot 2	4 hrs	3.5 mg	0.438 mM	~71
Lot 3	4 hrs	3.5 mg	0.293 mM	~90
Lot 4	4 hrs	4 mg	0.387 mM	~70

Table 4: Derivatization of MenC PS to generate aldehyde functional group

No.	Reaction Time with $NaIO_4$ @ RT	Content of Activated PS	Moles of CHO	Degree of Activation (DOA)
Lot 1	15 minutes	3.65 mg	0.89 mM	~32.9
Lot 2	12 minutes	3.4 mg	0.74 mM	~37.45
Lot 3	15 minutes	4.16 mg	0.92 mM	~36.65
Lot 4	11 minutes	7.0 mg	1.73 mM	~26.18
Lot 5	7 minutes	10 mg	1.47 mM	~44.44

Example 5: Conjugation reaction of activated Hib PRP and derivatized TT

Activated hydrazide-containing TT is diafiltered against 0.15 M MES buffer containing 0.2 M NaCl, pH 6.5. Activated aldehyde-containing Hib PRP is dissolved in 0.15 M MES buffer containing 0.2 M NaCl, pH 6.5.; Activated PRP and activated TT are mixed in 1:0.5 w/w to 1:0.75 w/w proportion for conjugation of polysaccharides. 1 to 1.5 equivalent of sodium cyanoborohydride to that of TT amount is added to the reaction mixture. The reaction mixture is incubated at 22±2 °C for 3 – 14 hours and treated with sodium borohydride for 1-2 hours. The sodium borohydride is taken in the ratio of at least 10 fold molar equivalent to the initial aldehyde content in the activated PS. PS-TT conjugates with an ≈10 kD molecular weight are purified by diafiltration against 0.15 M MES buffer containing 0.2 M NaCl, pH 6.5 (50 – 60 volumes) through 50 kD MWCO Amicon filter.

The course of conjugation is monitored by SEC-HPLC analysis with change in the retention time of the activated TT or PS in comparison to the conjugate. HPLC-SEC profile of the conjugate depicts that conjugation reaction is completed to maximum within three hours. Total conjugation time from the activation step to the final purified conjugate can be thus

achieved in 14-22 hrs. The SEC-HPLC profiles of native and activated TT and the PRP-TT conjugate (Fig 4) indicates that upon activation, the size of activated TT remains unchanged from the native TT, suggesting that little or no aggregation occurs. On the other hand, periodate activated Hib polysaccharide of 90 to 100 kD and polysaccharide fragment of ≈10 kD have lower molecular weight than the native PS, suggesting degradation of polysaccharide. After conjugation, high molecular weight peak appears, indicating the formation of Hib PRP-TT conjugates (Fig. 4).

SDS-PAGE analysis of PRP-TT conjugates also suggests that activated TT is successfully coupled to activated high molecular weight and low molecular weight PRP. SDS-PAGE for conjugates is performed using discontinuous gel/buffer system of Laemmli. The stacking layer contains 4% and separating layer contains 6% polyacrylamide. The electrophoretic runs are performed in Tris-glycine-SDS running buffer loading 5 µg of each protein sample, using the electrophoretic chamber with a voltage of 200V. After electrophoretic run, the gel is stained with comassie brilliant blue dye (Fig. 5).

Example 6: Conjugation reaction of activated MenA or MenC and derivatized TT

Activated hydrazide-containing TT is diafiltered against 0.15 M MES buffer containing 0.2 M NaCl, pH 6.5. Activated PS for both Men A and Men C are dissolved in 0.15 M MES buffer containing 0.2 M NaCl, pH 6.5. For conjugation of MenA PS, activated MenA polysaccharide and derivatized TT are mixed in 1:1.5 molar ratio, whereas for conjugation of MenC PS, activated MenC polysaccharide and derivatized TT are mixed in molar ratio of 1:2. 1.5 w/w equivalent of sodium cyanoborohydride to that of TT amount is added to the reaction mixture. The reaction mixture is

incubated from 3hrs to overnight at 25 °C, after which reaction is being quenched by addition of 10 molar excess of sodium borohydride to that of aldehyde moles and the reaction was incubated at 25 °C for 1-2 hours.

HPLC-SEC profile of the conjugate depicts that conjugation reaction is completed to maximum within three hours. Total conjugation time from the actvation step to the final purified conjugate can be achieved in 14-22 hrs. MenA PS – TT and MenC PS – TT conjugates are purified by 40-60% ammonium sulfate precipitation to remove unconjugated PS, and further diafiltered and stored in 0.15 M MES, 0.2 M NaCl, pH 6.5. MenA – TT and MenC – TT conjugates profiles are compared with TT by SEC-HPLC. Mixing ratio of activated PRP to activated TT is optimized to get the desirable loading of PRP to carrier protein. Conjugates are checked on SEC-HPLC during the course of reaction. Conjugates are observed to show shift in peak as compared to native TT, which suggests the loading of polysaccharides on TT molecule. (Fig. 6).

Example 7: Characterization of Hib PRP – TT conjugate:

Purified conjugates are analyzed by Lowry assay for protein content and orcinol assay for PRP content. Sodium deoxycholate precipitation is used for estimation of free polysaccharides in conjugate. 1 % w/v sodium deoxycholate solution is prepared in MQW. pH of the solution is adjusted to ~6.8 with 1N HCl. 80 µl of 1 % w/v sodium deoxycholate solution is added to 900 µl of conjugate sample. Reaction mixture is kept at 2 – 8 °C for 30 minutes. 50 µl of 1N HCl is added to this, and sample is centrifuged at 6000 x g for 15 minutes. Supernatant is collected and estimated by orcinol assay for free polysaccharide content.

The method of present invention gives reproducible results and optimal yields. After purification, these conjugates are analyzed for total and free

PRP content as well as protein content. The PRP to protein ratio is obtained in the range of 0.26 to 0.36. Percentage of free polysaccharides is also found below 10% (Table 5).

Table 5: PRP to protein ratio and % conjugation yield of various lots of PRP-TT conjugates

PRP-TT Conjugate Lot No	Size of activated PRP	PRP : Protein ratio	Free PRP	% yield of process
CB01	≈ 8 kD	0.31	1.3 %	15 %
CB02	≈8 kD	0.26	1.0 %	16 %
CB03	≈10 kD	0.36	1.1 %	16 %
CB04	≈8 kD	0.29	2.6 %	19 %

Example 8: Characterization of MenA/ MenC – TT conjugate

Purified MenA PS – TT and MenC – TT conjugates are characterized for their polysaccharide content, protein content and unconjugated free polysaccharide. Unconjugated polysaccharide in purified conjugates is determined by sodium deoxycholate precipitation method. 80 µl of 1 % w/v sodium deoxycholate solution of pH 6.8, is added with 900 µl of conjugate sample and reaction mixture is kept at 2 – 8 °C for 30 minutes. 50 µl of 1 N HCl is added to the reaction mixture andthen centrifuged at 6000 g for 15 minutes. Supernatant is collected and analyzed for total polysaccharide content by phosphorous assay for MenA – TT conjugates, and by resorcinol-hydrochloric acid method for MenC – TT conjugates. The percentage of unconjugated polysaccharide is calculated by dividing the amount of free polysaccharide detected by deoxycholate precipitation by the total amount of polysaccharide quantified in the conjugates. Protein content of the conjugates is determined by Lowry assay and the ratio of saccharide to protein is calculated mathematically.

Table 6 summarizes the characterization data of the purified conjugates. PS to protein ratio of these conjugates varied from 0.34 to 0.51 (wt/wt), and highest free PS remained close to 10 % (wt/wt).

Table 6. Polysaccharide to protein ratio and % conjugation yield of various lots of MenA – TT and MenC – TT conjugates

	Conjugate	PS/Protein ratio (wt/wt)	Unconjugated free PS	Conjugation % Yield
MenA PS – TT	MAC01	0.37	10 %	28 %
	MAC02	0.51	10 %	21 %
	MAC03	0.50	4 %	30 %
MenC PS – TT	MCC01	0.40	7 %	48 %
	MCC02	0.34	Not detectable	33 %
	MCC03	0.35	Not detectable	36 %

The Conjugation yield is calculated from the total active polysaccharide taken for conjugation, to the final content of the polysaccharide in the purified conjugate. Very high yield is achieved in both type of conjugates, i.e. MenA and MenC conjugates. The conjugation yield for Men A is achieved in the range above 25 % or above and that for MenC is achieved in the range of 30 % or above.

Example 9: Immunogenicity of Hib PRP - TT Conjugates

Group of animals are immunized with all the test samples at different dose levels to induce the significant immune response as compared to negative control. Maximum IgG response is seen on day 49, after two boosters given on day 28 and day 42.

The Hib PRP-TT conjugates prepared are immunized into Sprague dawley rats (5 – 8 weeks old) at different dose levels (1 and 0.5 µg) along with the licensed Hib conjugate vaccine available commercially at 1µg dose level. A group of 10 animals each were randomized on the basis of their body

weights. 200 µl of each Hib-PRP Conjugate is administered subcutaneously by single injection per animal on Day 0, 28 and 42 of the experiment. Approximately 300 to 800 µL blood from each animal is withdrawn from the retro-orbital plexus before the administration of test dose on Day 0 (pre bleed), 28, 42 and on the day of terminal collection (Day 49) maximum possible blood is withdrawn. The blood sera is collected and stored at -20°C or below until the samples are analyzed by ELISA. All the animals are sacrificed after final blood collection.

A quality controlled sera prepared by pooling sera is obtained from rats immunized with licensed Hib Conjugate Vaccine and in-house conjugates. The quality control serum is designated as containing an arbitrary anti-Hib IgG concentration of 5000 EU/ml which is used to generate standard ELISA curve to extrapolate values of IgG (EU/ml) in the test animal sera. IgG titer value for each animal against the standard is calculated using Combistat software. Then the Geometric mean IgG titer is calculated for each of the formulation (Table 7). In one of the study the IgG titters were evaluated till day 70 and at different dose levels and with different size of the Hib PS (Fig. 8).

Table 7 : Geometric mean for IgG titer on day 49 (+,- 95% Confidence Interval)

	Geometric mean for IgG titer on day49			
	Vehicle control	Licensed vaccine 1µg	Test vaccine 1µg	Test vaccine 0.5µg
Study 1	28 (52,25)	233(363,150)	451(594,343)	649(772,545)
Study 2	39 (46,33)	1200(2489,579)	2007(5120,787)	1956(4472,856)
Study 3	230 (362,146)	661 (1077,406)	2607 (6029,1128)	702 (1437,343)
Study 4	59 (134,26)	2385(3851,1477)	3546(6020,2088)	989(1840,531)

The immunogenicity study by ELISA reveals that said Hib PRP-TT conjugates under test showed significant fold rise in antibody titre as compared to vehicle control. Also the PS-TT conjugates give rise to equivalent or better antibody titres as compared to higher molecular weight reference vaccine. Also in three out of four studies there is equivalent or better response at lower dose level of 0.5 µg as compared to licensed vaccine (Table 7). Therefore, lower kD Hib conjugate vaccine of the present invention is equally to more immunogenic than the licensed comparator.

Example 10: Antigenicity of MenC -TT Conjugates

The antigenicity of MenC polysaccharide – TT conjugate are confirmed by in-vitro analysis by neutralizing the MenC antigens, with the sera against each type from a reference source. A control which contained only the reference antisera and no antigen is compared to the test sample. The extent of inhibition of sera by each antigen is compared with the no antigen control by ELISA.

Eight thousand fold diluted rabbit antiserum against *Neisseria meningitidis* serogroup C (Bacton Dickinson; 222281) is incubated for 1 hour at 37 °C with different antigens (Unconjugated Men C polysaccharide and MenC Polysaccharide – TT conjugate) at 10 µg/ml diluted in phosphate-buffered saline containing 0.1 % v/v Brij 35 and 5% FBS; in 96 well micro titer plate (Plate A). A separate plate (plate B) is coated with a mixture of MenC polysaccharide and methylated Human Serum Albumin (m-HSA) and subsequently blocked with 5% FBS after overnight incubation at 2 – 8 °C. To this plate B, serially diluted antiserum-antigen mix from plate A is added and incubated for 1 hour at 37 °C. The plate is washed with phosphate-buffered saline, pH 7.4 containing 0.1% Brij 35. The plate then

incubated for 60 minutes at 25 °C with peroxidase labelled anti-rabbit IgG antibodies in PBS, 0.1% Brij 35 and 5% FBS. The plate is washed again and incubated for 10 minutes at 25 °C with 100 µl peroxidase substrate, 3, 3′, 5, 5′ – tetramethylbenzidine-H_2O_2 in sodium acetate buffer. The reaction is stopped by adding 50 µl of 2M H_2SO4. The A450 is recorded on Tecan micro plate reader. MenC PS – TT conjugates neutralized the anti-PS antibodies significantly (Fig. 7). Unconjugated free MenC PS display lower inhibition as compared to conjugated PS.

For serum inhibition ELISA of Hib and MenA polysaccharide – TT conjugates, similar procedure is used with rabbit antiserum against *Haemophilus influenza* type b and *Neisseria meningitidis* serogroup A (BD; 222301) and unconjugated PS and PS – TT conjugates, as antigens.

Example 11: Immunogenicity of MenA & MenC Conjugates

Groups of 6 female BALB/c mice of 5-9 weeks are immunized on days 0, 14 and 28 with 1 µg of MenA and MenC conjugated PS antigen formulated in normal saline individually and in combination (Table 9). All immunizations are performed by administering 200 µl of vaccine dilution via subcutaneous route. Normal saline alone is used for negative control group, and a licensed vaccine is used for positive control group. Sera is collected at days 14, 28 and 35. Specific anti-PS IgG antibody titers are estimated by ELISA.

Ninety six-well plates (Nunc Maxisorp) are coated with MenA PS (for testing of MenA conjugates) or MenC PS (for testing of MenC conjugates) by adding 100 µl per well mixture of a 5 µg/ml PS and m-HSA in PBS buffer, pH 7.4. Plates are incubated overnight at 4 °C and then washed three times with PBS buffer (0.1 % Brij 35 in PBS, pH 7.4) and blocked with 200 µl per well of 5% FBS solution in PBS buffer (0.1 % Brij 35 in PBS, pH

7.4) for 1 hour at 37 °C. Each incubation step is followed by three PBS buffer wash. Reference and test sera samples are diluted in PBS buffer (0.1% Brij 35, 5% FBS in PBS, pH 7.4), transferred into coated-blocked plates (200 µl), and serially twofold diluted followed by overnight incubation at 4 °C. Then 100 µl per well of 1:1000 diluted peroxidase conjugated anti-mouse IgG are added and left for 1 hour at 25 °C. The 100 µl per well of substrate, 3, 3′, 5, 5′ – tetramethylbenzidine-H_2O_2 is added for color development. After 10 minutes of development at 25 °C, reaction is stopped by adding 50 µl of 2 M H_2SO_4, and optical density is measured at 450 nm on Tecan micro plate reader.

The maximum IgG titers for MenA conjugates are achieved on day 35 after two boosters. It is observed that for MenA the increase in titer value in comparison to negative control is approximately 120 fold in licensed Men ACYW-DT conjugate vaccine, 330 fold for MenA-TT conjugate & 220 fold for MenA-TT conjugate in combination. (Table 8)

Table 8: Geometric mean for IgG titer by ELISA (+,- 95%Confidence Interval) for MenA formulations in mouse model, dosing on day 0, 14 and 28 (Fig 9).

	Vehicle Control	1 µg licensed vaccine	1 µg Men A – TT conjugate	(1+1) µg (Men A-TT) + (Men C-TT) conjugate
Day 14	1 (1,1)	3 (4,2)	498 (329,198)	229 (242,118)
Day 28	1 (1,1)	430 (2247,361)	11717 (4485,3243)	6392 (6306,3174)
Day 35	37 (53,22)	4444 (9937,3071)	12302 (3913,2969)	8088 (5760,3364)

The maximum IgG titers for MenC conjugates are achieved on day 35 after two boosters. It is observed that for MenC the increase in titer value in comparison to vehicle control is approximately 10 fold in licensed vaccine,

322 fold for MenC-TT conjugate & 250 fold for MenC-TT conjugate in combination (Table 9).

Table 9: Geometric mean for IgG titer by ELISA (+,- 95%Confidence Interval) for MenC formulations in mouse model, dosing on day 0, 14 and 28 (Fig 10)

	Vehicle control	1 µg licensed vaccine	1 µg Men C – TT conjugate	(1+1) µg (Men A-TT) + (Men C-TT) conjugate
Day 14	18 (15,08)	30 (18,11)	605 (329,213)	637 (519,286)
Day 28	15 (7,5)	78 (220,58)	6327 (8701,3663)	6063 (4354,2534)
Day 35	28 (44,17)	305 (595,202)	9039 (8059,4261)	7046 (3602,2384)

The overall trend for IgG titer values on day 14 and 28 for both MenA & MenC conjugates is found similar in trend to day 35 response, as determined by ELISA.

Example 12: Serum Bactericidal Assay (SBA) for MenC Conjugates

Equal volume from each of the sera sample belonging to a group of mice are pooled together to make group sera pools for testing by serum bactericidal assay. The assay is performed as follows:

Streak the *N. meningitidis* serogroup C target strain for single colony isolation and incubate overnight (16-24 hours) at 37^0C with 5% CO_2 on a Sheep blood agar plate. The strain is subcultured by spreading cells (~50 CFU) over the entire surface of another Sheep Blood agar plate and then incubated for 4 h at 37^0C with 5% CO_2. Bacteria are resuspended in ~ 5 ml of bactericidal buffer. 1 mL of suspension is removed and absorbance is measured at a wave length of 650nm. Suspension OD_{650} is being adjusted to 0.1, anddilution is completed at 1:2500 dilution. The sera are diluted serially 2-fold and assay buffer is added in control wells.

10µl of the working solution of bacteria is added to every well. 10 µl of heat inactivated (kept at 56^{0}C for 30 min.) complement is added to all inactive complement control wells and 10 µl of said inactivated complement is added to sera containing wells and active complement control wells. Shake the plates and incubate the plates for 1 hour at 37^{0}C without CO_2.

After incubation, spot 10µL from each wells on slanted blood agar plate. Incubate all agar plates for overnight at 37^{0}C with 5% CO_2. Count the number of colonies on each spot of the plates. The highest serum dilution showing ≥50% killing of bacteria as compared to complement control is considered as the SBA titre of that serum sample.

The SBA data shows a negative response from the vehicle immunizations after 3 doses on 2 week interval, whereas the licensed conjugate vaccine showed an increase of functional antibody titres (Fig 11). The vaccine formulations under test showed significantly high SBA titres as compared to the licensed vaccine control indicating the vaccine to be effective in-vivo in the mouse model.

Similar kind of protocol is used for the SBA testing of Men A by streaking the *N. meningitidis* serogroup A.

We claim

1. Polysaccharide – protein conjugate with enhanced immunogenicity comprising of carrier protein, polysaccharide fragment wherein
   - said carrier protein is obtained from gram positive bacteria, preferably but not limited to tetanus toxoid (TT) and CRM197.
   - said polysaccharide fragment is obtained from group of gram negative bacteria including but not limited to *Haemophilus influenzae* serotype b (Hib), *Neisseria meningitidis* serogroup A and C (MenA and MenC).
2. The polysaccharide – protein conjugate as claimed in claim 1 wherein said polysaccharide is Hib, MenA or MenC capsular polysaccharide.
3. The polysaccharide – protein conjugate as claimed in claim 1 wherein the percentage of free polysaccharide is below 10% and the ratio of activated polysaccharide to activated TT is
   - in the range of 0.2 to 0.5 (wt/wt), more preferably between 0.25 to 0.35 (wt/wt) for Hib PRP – TT conjugate
   - in the range of 0.2 to 0.8(wt/wt), more preferably between 0.3 to 0.7 (wt/wt) for MenA–TT conjugate and MenC–TT conjugate.
4. The polysaccharide – protein conjugate as claimed in claim 1 wherein said polysaccharide – protein conjugate displays significantly high antibody titre at the doses of 0.5 µg to 1 µg.
5. Process of preparing the polysaccharide–protein conjugate as claimed in claim 1, said process comprising the steps of:
   (a) derivatization of at least one carrier protein by reacting the carrier protein with at least one nucleophile in the presence of cross linker, wherein said carrier protein is obtained from gram positive bacteria preferably selected from tetanus toxoid and CRM 197;

(b) cleavage and depolymerisation of at least one high molecular weight polysaccharide by reacting said polysaccharide with at least one oxidizing agent resulting into cleaved and activated smaller sized polysaccharide fragments wherein said high molecular weight polysaccharide is obtained from group of gram negative bacteria including but not limited to *H. influenzae* type b (Hib PRP), *N. meningitidis* serogroup A (MenA) and *N. meningitidis* serogroup C (MenC);

(c) conjugation reaction of said derivatized carrier protein of step (a) with said activated polysaccharide fragment of step (b) to obtain polysaccharide – protein conjugate employing reductive amination chemistry;

wherein

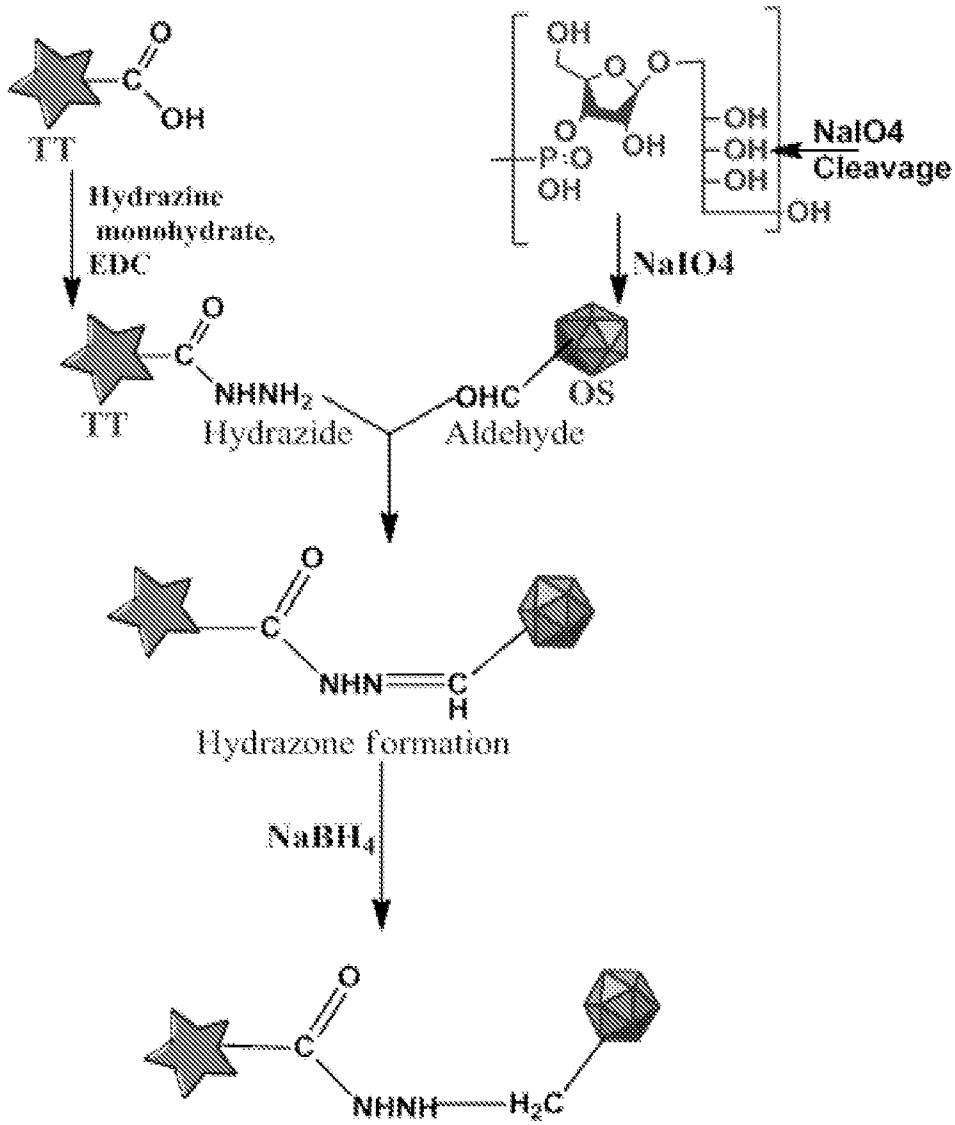
- said conjugation reaction takes short conjugation time of 14 to 22 hrs from stage of activation of polysaccharide till final purification of the conjugate,
- said process results into higher yields of polysaccharide – protein conjugate,
- said polysaccharide – protein conjugate displays significantly high antibody titre including functional antibodies.

6. The process of preparing polysaccharide – protein conjugate as claimed in claim 5 wherein said crosslinker is a chemical crosslinking reagent to facilitate carboxyl-to-amine crosslinking, said crosslinker preferably being Hydrazine monohydrate in presence of EDC.
7. The process of preparing polysaccharide – protein conjugate as claimed in claim 5 wherein said nucleophile is selected from a group of reducing agents, preferably being hydrazine.

8. The process of preparing polysaccharide – protein conjugate as claimed in claim 5 wherein the degree of activation of said derivatized tetanus toxoid is 50 ± 5.
9. The process of preparing polysaccharide – protein conjugate as claimed in claim 5 wherein said oxidizing agent is selected from group of periodates, preferably being sodium periodate or sodium metaperiodate.
10. The process of preparing polysaccharide – protein conjugate as claimed in claim 5 wherein
    - said cleaved and activated Hib PRP fragment has a molecular weight in the range of 12 ± 6 kD with average degree of activation having one aldehyde group per 3 - 15 saccharide repeating units,
    - said cleaved and activated Men A polysaccharide has a molecular weight of 100 ± 40 kD with average degree of activation having one aldehyde group per 40-120 saccharide repeating units, and
    - said cleaved and activated Men C polysaccharide has a molecular weight of 100 ± 40 kD with average degree of activation having one aldehyde group per 20-80 saccharide repeating units.
11. The process of preparing polysaccharide – protein conjugate as claimed in claim 5 wherein said derivatized carrier protein and said activated polysaccharide fragment are conjugated in the presence of at least one reducing agent to obtain highly stable lower molecular weight PS-TT conjugate.
12. The process of preparing polysaccharide – protein conjugate as claimed in claim 11 wherein said reducing agent is selected from a group having specificity towards Schiff base structure, such as sodium borohydride or sodium cyanoborohydride.

13. The process of preparing polysaccharide – protein conjugate as claimed in claim 5 wherein ratio of activated polysaccharide to derivatized TT in purified conjugate is in the range of
    - between 0.2 to 0.5 (wt/wt) and preferably between 0.25 to 0.35 (wt/wt) for Hib PRP–TT conjugate ,
    - between 0.2 to 0.8 (wt/wt) and preferably between 0.3 to 0.7 (wt/wt) for MenA–TT conjugate and MenC–TT conjugate.
14. The process of preparing polysaccharide – protein conjugate as claimed in claim 5 wherein said conjugation percent yield using reductive amination chemistry is
    - 16% to 25% for Hib PRP - TT conjugate,
    - 30% to 50% for Men C – TT conjugate, and
    - 20% to 30% for Men A – TT conjugate.
15. The polysaccharide – protein conjugate as claimed in claim 1 wherein dose of Hib PRP-TT conjugate is in the range of 0.5 µg to 1 µg in rat model.
16. The polysaccharide – protein conjugate as claimed in claim 1 wherein dose of MenA and MenC PS-TT conjugate is in the range of 0.5 µg to 1 µg in mouse model.
17. The polysaccharide – protein conjugate as claimed in claim 1 wherein the antibody titre for Hib PRP polysaccharide – protein conjugate is upto 60 fold higher for 1µg dose and 50 fold higher for 0.5 µg dose as compared to the vehicle control antibody titres.
18. The polysaccharide – protein conjugate as claimed in claim 1 wherein the increase in IgG titer value as compared to vehicle control is
    - 330 fold for MenA-TT conjugate, and
    - 220 fold for MenA-TT+MenC-TT conjugate in combination.
    - 322 fold for Men C-TT conjugate and
    - 250 fold for MenA-TT+MenC-TT conjugate in combination.

19. The polysaccharide – protein conjugate as claimed in claim 1 wherein : Serum Bactericidal Assay (SBA) titres for MenC are significantly high with the test MenC-TT or MenC-TT+MenA-TT conjugate vaccine as compared to the vehicle control or the licensed comparator vaccine.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Cleavage
NaIO4
TT
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Hydrazide
OHC
OS
Aldehyde
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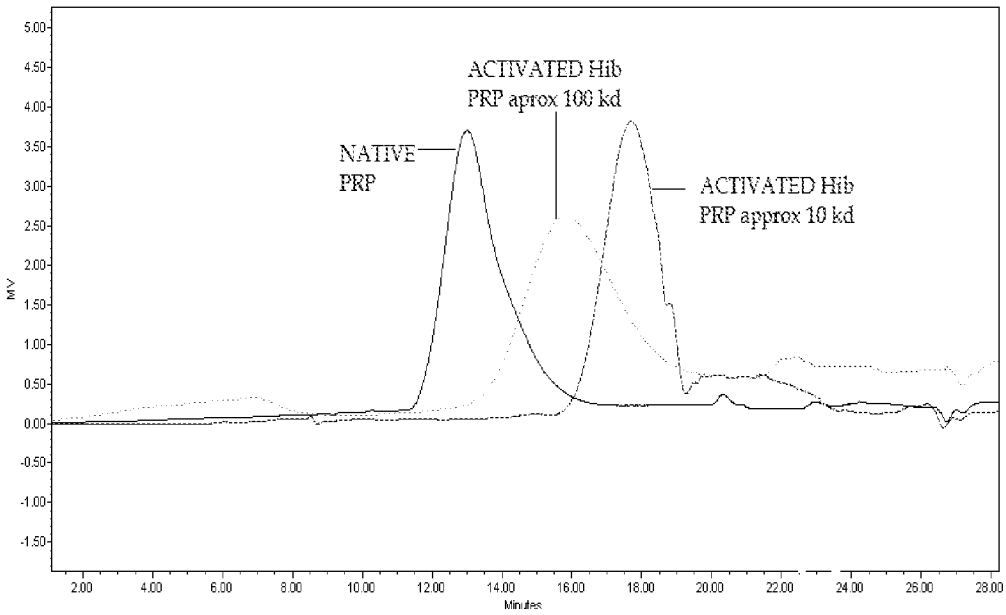
C
H
Hydrazone formation
NaBH4
NHNH
H2C

Fig. 1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NATIVE
PRP
ACTIVATED Hib
PRP approx 10 kd
MV
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4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
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2.00
4.00
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Fig. 2

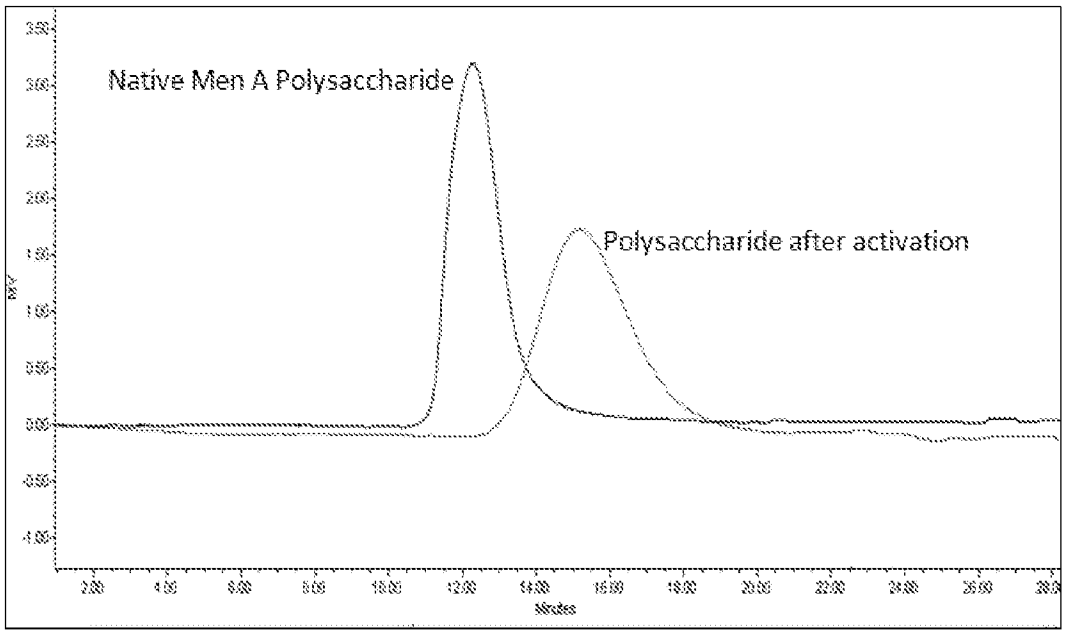

Native Men A Polysaccharide
Polysaccharide after activation

Fig. 3a

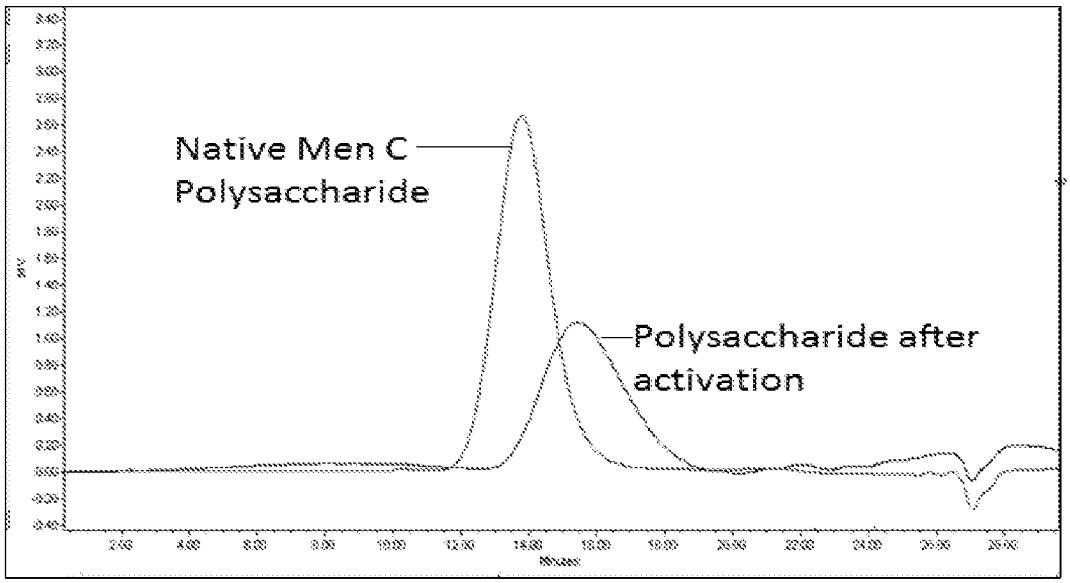
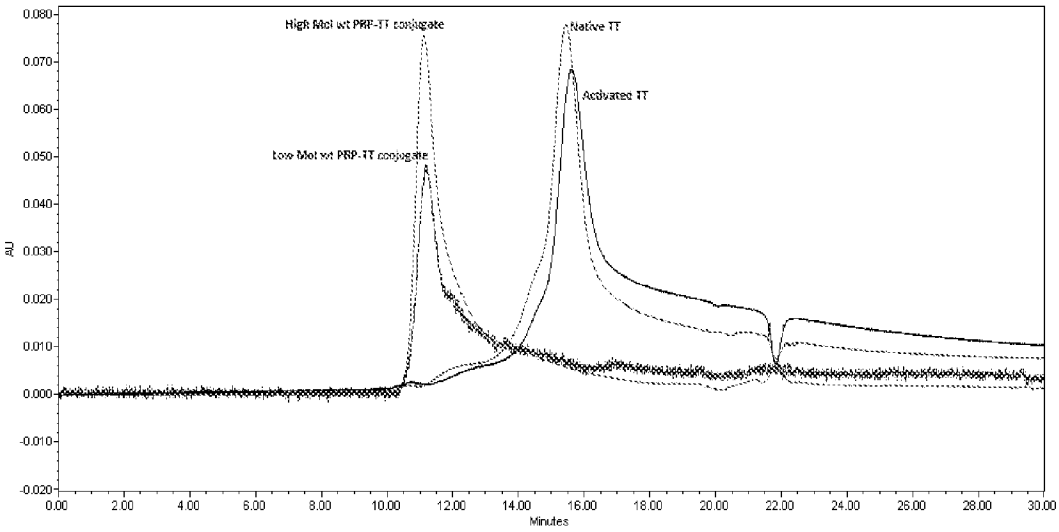

Native Men C
Polysaccharide
Polysaccharide after
activation

Fig. 3b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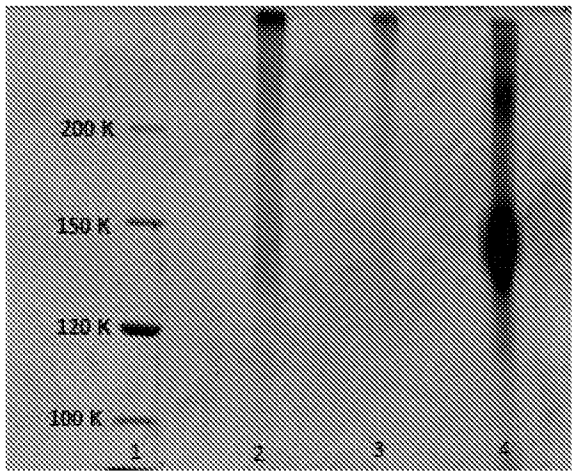
Native TT
Activated TT
Low Mol wt PRP-TT conjugate
AU
Minutes

Fig. 4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2
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4

Fig. 5

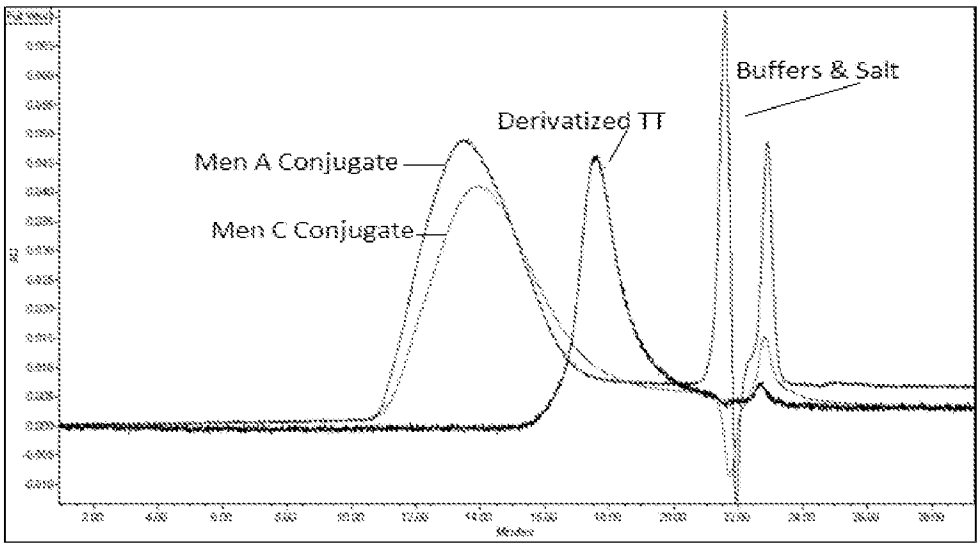
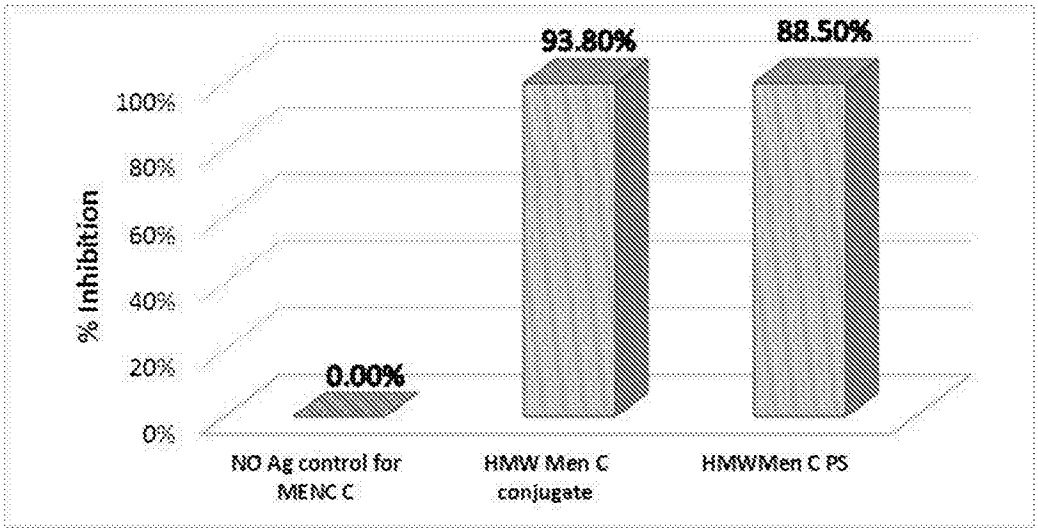

Buffers & Salt
Derivatized TT
Men A Conjugate
Men C Conjugate

Fig. 6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100%
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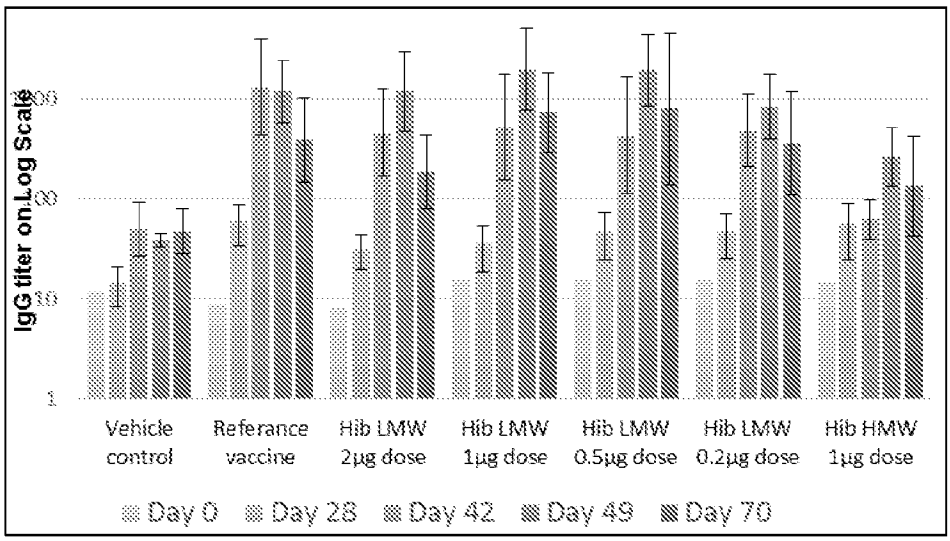
% Inhibition
NO Ag control for MENC C
HMW Men C conjugate
HMWMen C PS

Fig. 7


IgG titer on Log Scale
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100
1000
Vehicle control
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Hib LMW 1µg dose
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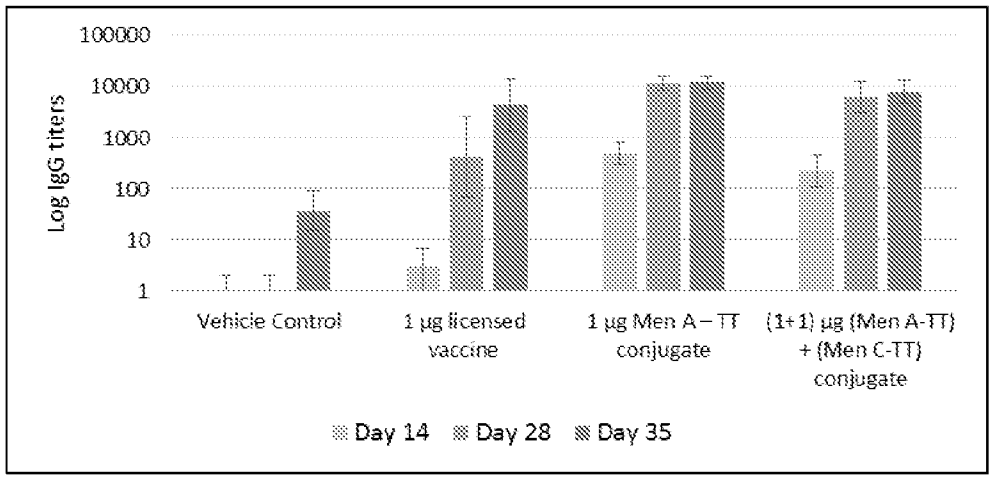
Hib LMW 0.2µg dose
Hib HMW 1µg dose
Day 0
Day 28
Day 42
Day 49
Day 70

Fig. 8


Log IgG titers
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10
100
1000
10000
100000
Vehicle Control
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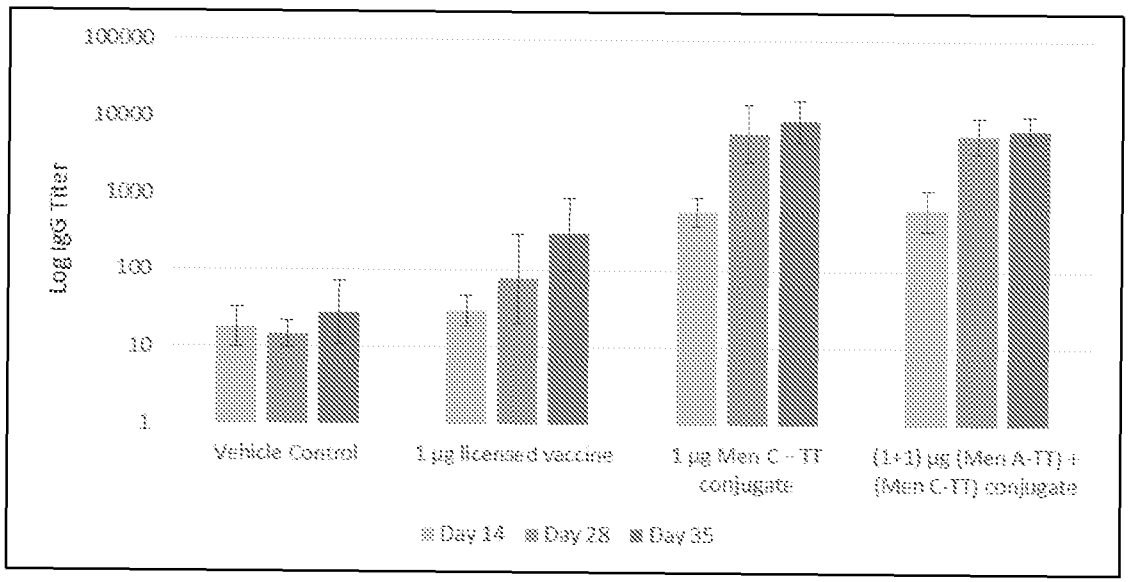
1 µg Men A – TT conjugate
(1+1) µg (Men A-TT) + (Men C-TT) conjugate
Day 14
Day 28
Day 35

Fig 9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10000
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1
Log IgG Titer
Vehicle Control
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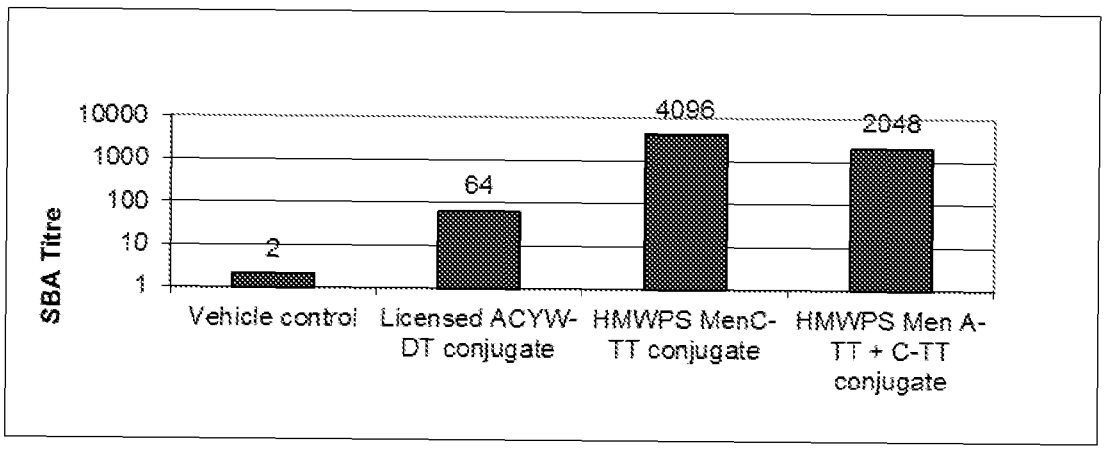
1 µg Men C – TT conjugate
(1+1) µg (Men A-TT) + (Men C-TT) conjugate
Day 14
Day 28
Day 35

Fig 10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2048
Vehicle control
Licensed ACYW-DT conjugate
HMWPS MenC-TT conjugate
HMWPS Men A-TT + C-TT conjugate

Fig 11