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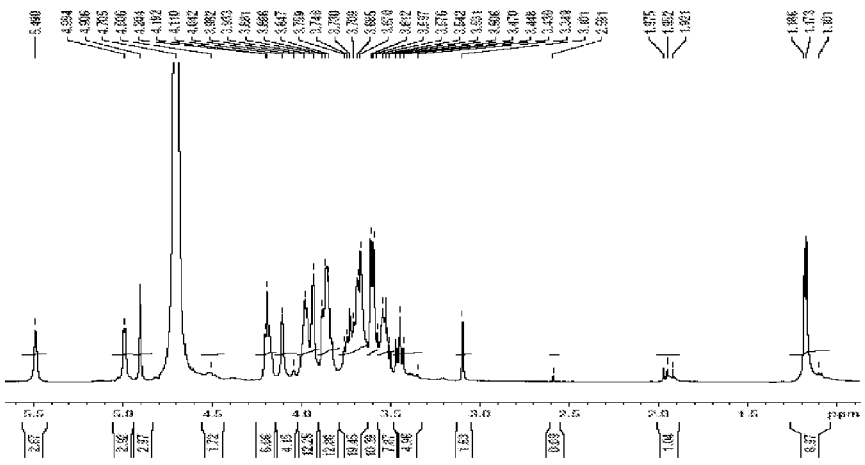
(54) Title: MULTIVALENT CONJUGATE VACCINES WITH BIVALENT OR MULTIVALENT CONJUGATE POLYSACCHARIDES THAT PROVIDE IMPROVED IMMUNOGENICITY AND AVIDITY

Figure 1A

6A PS (^{1}H NMR-30KD)



(57) Abstract: The disclosure describes compositions containing conjugates using novel linkers, bivalent polysaccharide conjugates, and methods of bivalent polysaccharide conjugation in the development of multivalent conjugate vaccines. Conjugation of capsular polysaccharides to carrier proteins is carried out using homo-bifunctional and/or hetero-bifunctional linkers of specific lengths. Incorporation of the linkers and their use in bifunctional linkers induces higher titers of functional antibodies with high avidity, eliciting higher immunologic memory, and reduced carrier protein effect. This provides immunochemically cross-reactive capsular polysaccharides wherein one or more cross-reactive capsular polysaccharides are conjugated sequentially or concurrently to carrier protein using bifunctional linkers bearing the same or different functional groups. Such a linker and the size of the capsular polysaccharides provides

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an effective multivalent conjugate vaccine with high antibody titers and a reduced carrier effect and results in reduction in the content of the capsular polysaccharide and protein per dose of vaccine which reduces reactogenicity.

MULTIVALENT CONJUGATE VACCINES WITH BIVALENT OR MULTIVALENT CONJUGATE POLYSACCHARIDES THAT PROVIDE IMPROVED IMMUNOGENICITY AND AVIDITY

Reference to Related Applications

This application claims priority to U.S. Provisional Application No. 62/517,905 filed June 10, 2017, the entirety of which is specifically incorporated by reference.

Background

1. Field of the Invention

The present invention is directed to multivalent conjugates, immunogenic compositions, and vaccines comprising carrier protein conjugated bacterial capsular polysaccharides and uses thereof. In particular, compositions of the invention comprise monovalent and multivalent bacterial capsular polysaccharide-protein conjugates, wherein the bacterial capsular polysaccharides and oligosaccharides are derived from serotypes of *Streptococcus pneumoniae*. The carrier protein is conjugated to bacterial capsular polysaccharides through mono functional as well as bi-functional linkers, preferably of defined lengths and the mono-functional or bi-functional linkers may be homo-mono-functional, homo-bi-functional, hetero-mono-functional, or hetero-bifunctional.

2. Description of the Background

Streptococcus pneumoniae is a Gram-positive pathogen responsible for invasive pneumococcal diseases (IPDs) such as pneumonia, bacteremia, meningitis, and acute Otitis media. Pneumonia is the most common manifestation of invasive pneumococcal disease, whereas bacterial spread within the respiratory tract may result in middle-ear infection, sinusitis or recurrent bronchitis. Pneumococcus is encapsulated with a chemically linked polysaccharide which results in serotype specificity. At least 90 pneumococcal serotypes are known of which about 23 account for 90% of invasive diseases and capsular polysaccharide is a poor immunogen.

There are currently three PCV vaccines available on the global market: PREVNAR®, SYNFLORIX®, and PREVNAR-13®. There is a need to address remaining unmet medical need for coverage of pneumococcal disease due to serotypes not found in PREVNAR-13® and potential for serotype replacement over time. There is a need for immunogenic compositions that can be used to induce an immune response

against additional *Streptococcus pneumoniae* serotypes in humans and in children less than two years old.

A capsular polysaccharide (CPS) is a key virulence determinant and generally insufficiently immunogenic to induce a T cell-dependent immune response in infants and children. Conjugation of a carrier protein to CPS can induce an immune response that undergoes class switching. Accordingly, a 7-valent (PCV-7, Pfizer Inc., USA), a 10-valent (Synflorox-10, GSK Vaccines) and a 13-valent pneumococcal conjugate vaccine (PCV-13, Pfizer Inc., USA) have been developed to efficiently prevent the incidence of IPDs. Reductive amination chemistry and cyanylation chemistry has been widely used to prepare the conjugate vaccines.

In these conjugates, the short C-N linkage (2.1Å) between CPS and carrier protein leads to steric shielding of the CPS epitopes by the carrier protein and low CPS/protein ratio. Important parameters are needed to minimize disadvantages of the current vaccines.

U.S. Patent No. 9,492,559 discloses immunogenic compositions comprising conjugated capsular polysaccharide antigens and uses thereof. The immunogenic compositions disclosed include an 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20-valent pneumococcal conjugate composition. Also disclosed is a 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25-valent pneumococcal conjugate composition.

International Application Publication No. WO 2014/097099A2 discloses a glycoconjugation process directed to several serotypes in addition to Preevnar-13 valent conjugates. New polysaccharide conjugates are added to formulation to increase efficacy of the vaccine.

U.S. Patent Application Publication No. 2011/023526 discloses a 15-valent pneumococcal polysaccharide-protein conjugate vaccine composition. This patent is directed to 15-valent conjugate vaccines made by adding two or more serotypes with currently available 1-3 vaccines.

International Application Publication No. WO 2016/207905 discloses multivalent pneumococcal conjugate vaccine. This application is directed to a 13 or greater valent conjugate vaccine and deletion of serotype 6A.

U.S. Patent Application Publication No. 2017/007713 discloses a linker containing ((2-oxoethyl) thio) with enhanced functionality.

International Application Publication No. WO 2014/092377 discloses a 13 valent composition wherein 12 serotypes were selected from the group consisting of serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F and one from 12 or 9N.

International Application Publication No. WO 2014/092378 discloses an immunogenic composition having 13 different polysaccharide-protein conjugates wherein each conjugate contained a capsular polysaccharide isolated from 12 serotypes selected from the group consisting of serotypes 1,3,4,5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F, and serotypes 22F or 33F.

Chinese Application Publication No. 101590224 discloses a 14-valent pneumococcal polysaccharide-protein conjugate vaccine containing serotypes 1, 2, 4, 5, 6A, 6B, 7F, 9N, 9V, 14, 18C, 19A, 19F and 23F.

Chinese Application Publication No. 104069488 discloses 14 valent polysaccharide protein conjugate wherein the 14 serotypes were 1,4,5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F.

International Application Publication No. WO 2016207905 discloses a multivalent Pneumococcal conjugate vaccine comprising conjugates of CRM 197 and at least 14 capsular polysaccharides selected from serotypes 1, 3, 4, 5, 6B, 7F, 9N, 9V, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.U.S. Patent 8,192,746 disclosed a 15 valent immunogenic composition comprising capsular polysaccharides from serotypes 1,3,4,5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F,22F, 23F, and 33F conjugated to CRM197.

International Application Publication No. WO 2013/191459 discloses a 15 valent composition comprising S. pneumoniae capsular polysaccharides form serotypes of 1,2,3,4,5, 6A, 6B, 7F, 9N, 9V, 14, 18C, 19A, 19F and 23F.

Chinese Application Publication No. 103656632 discloses multi valent pneumococcal capsular polysaccharide composition containing serotype 6A and at least one extra serotype selected from the group consisting of 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, l0A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F which provided protection against 24 different pneumococci serotypes.

Chinese Application Publication No. 103656631 discloses a multivalent pneumococcus capsular polysaccharide-protein conjugate composition comprising capsular

polysaccharides of pneumococcus of 24 different serotypes viz. 1, 2,3, 4, 5, 6A, 6B,7F, 8, 9N, 9V, l0A, llA, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

U.S. Patent Application Publication No. 2016/0324950 discloses immunogenic polysaccharide-protein conjugates comprising a capsular polysaccharide (CP) from Streptococcus agalactiae, also referred to as group B streptococcus (GBS), and a carrier protein, wherein the CP is selected from the group consisting of serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX. This was meant for treatment of chronic diabetes mellitus, cancer, heart failure, neurologic, and urologic conditions. The carrier protein capsular polysaccharide conjugates varied.

U.S. Patent No. 5,360,897 discloses immunogenic conjugate comprising reductive amination product of an intact capsular polymer of the bacterial pathogen S. pneumoniae having at least two carbonyl groups and a bacterial toxin or toxoid, said conjugate comprising a cross-linked conjugate in which there is a direct covalent linkage between the capsular polymer and the toxin or toxoid.

U.S. Patent No. 7,862,823 describes a multivalent conjugate vaccine composition with at least two different carrier proteins.

U.S. Patent No. 8,808,708 discloses a 13-valent immunogenic composition consisting of Polysaccharide-protein conjugates where serotypes consist of 1,3,4,5, 6A, 6B, 7F, 9V,14, 18C, 19A, 19F and 23F, and wherein the carrier protein is CRMI97.

U.S. Patent Application Publication No. 2009/0017059 discloses an immunogenic composition where serotypes 19A and 19F were conjugated to different bacterial toxoids.

International Application Publication No. WO 2011/110241 describes pneumococcal conjugate immunogenic compositions or vaccines wherein different conjugation chemistries were used for different components of the immunogenic composition or vaccine. Reductive amination was used for the conjugation of at least one serotype and a conjugation other than reductive amination was used for the conjugation of a different serotypes. The conjugation method selected for different serotypes allowed each serotype to be presented using a conjugation method that allowed the best presentation of the saccharide epitope. Some pneumococcal saccharides conjugated well using reductive amination, whereas other pneumococcal saccharides were conjugated differently to allow the ring structure to remain unbroken and provide better results.

U.S. Patent No. 7,955,605 discloses a process of making carrier protein polysaccharide conjugate consisting serotype 19A where the activated serotype 19A polysaccharide and carrier protein are resuspended in dimethyl sulfoxide (DMSO) to form a conjugate.

U.S. Patent Application Publication No. 2010/0074922 discloses immunogenic composition containing 10 or more serotypes wherein 19F capsular saccharide was conjugated to diphtheria toxoid (DT), serotype 18C capsular saccharide is conjugated to tetanus toxoid and serotypes 1,4,5, 6B, 7F, 9V, 14 and 23F capsular saccharides are conjugated to Protein D from *Haemophilus influenza.*

U.S. Patent Application Publication No. 2010/0239604 discloses a composition comprising multivalent S. pneumoniae capsular saccharide conjugates wherein serotype 19A was conjugated to a first bacterial toxoid and 19F is conjugated to a second bacterial toxoid and 2-9 of the S. pneumoniae capsular saccharides are conjugated to protein D. Apart from increasing the scope of protection by developing vaccines which will offer protection against larger number of serotypes, efforts were focused on developing newer methods of synthesis.

U.S. Patent No. 7,709,001 describes a method of synthesis of carrier protein conjugate of capsular polysaccharide which consists of 1) reacting purified polysaccharide with a mild acid resulting in size reduction 2) reacting the polysaccharide of step 1 with an oxidizing agent in the presence of bivalent cations resulting in an activated polysaccharide; 3) compounding the activated polysaccharide with a carrier protein 4) reacting activated polysaccharide of step 3 and carrier protein with a reducing agent to form a polysaccharide - carrier protein conjugate; and 5) capping unreacted aldehydes in product of step 4 to yield an immunogenic polysaccharide - carrier protein conjugate.

International Application Publication No. WO 2014/097099 discloses a method of synthesizing a carrier protein conjugate, which involves a) reacting a saccharide with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and N-chlorosuccinimide (NCS) in an aqueous solvent to produce an activated saccharide; and b) reacting the activated saccharide with a carrier protein comprising one or more amine groups.

U.S. Patent Application Publication No. 2012/321658 discloses an immunogenic composition wherein serotypes 1,3, 19A and 19F linked to protein carriers either directly or

indirectly through a chemistry other than reductive amination, and one or more different saccharides is/are selected from a second group consisting of serotypes 4, 5, 6A, 6B,7F, 9V, 14, 18C and 23F which is/are linked to a protein carriers) by reductive amination.

Pneumococcal vaccines are based on 1) pneumococcal polysaccharide vaccine and 2) pneumococcal conjugate vaccines. PNEUMOVAX® marketed by Merck comprises of unconjugated polysaccharides belonging to serotypes 1,2,3,4,5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18e, 19F, 19A, 20, 22F, 23F and 33F. Infants and young children respond poorly to most pneumococcal polysaccharides. Immunogenicity of poor immunogens is enhanced by conjugating with carrier proteins. Polysaccharide protein conjugate vaccines are made using capsular polysaccharides linked to protein carriers. The conjugate induces T cell dependent enhanced immune response against the specific serotype.

Conjugates are synthesized using various reagents, such as homo bifunctional, hetero bifunctional linkers of varying lengths. Three pneumococcal conjugate vaccines are available in market, PREVNAR®, SYNFLORIX®, and PREVNAR-13®. PREVNAR® is a heptavalent vaccine that contains the capsular polysaccharides from serotypes 4, 6B, 9Y, 14, 18C, 19F and 23F, each conjugated to a carrier protein designated CRM197. SYNFLORIX® is a deca-valent vaccine from GSK Biologicals that incorporates ten capsular polysaccharides conjugated to protein D from NTHi offering coverage against three additional pneumococcal strains, serotypes 1, 5 and 7F. PREVNAR-13® is a tri-deca-valent vaccine containing 13 capsular polysaccharide prepared from thirteen serotype of Streptococcus pneumoniae (1, 3, 4, 5, 6A, 6B, 7F, 9Y, 14, 18C, 19 A, 19F, and 23F) conjugated to a carrier protein designated CRM197.

Increasing microbial resistance to antibiotics and the increasing number of immunocompromised persons have necessitated the development of pneumococcal vaccines with even broader protection, which leads to development of multivalent vaccines effective against increasing number of serotypes especially for coverage of pneumococcal disease due to serotypes not found in PREVNAR-13®. The need for a specific serotype depends on the region and antibiotic resistance developed. Thus, US patent 8192746 reports a multivalent immunogenic composition having 15 distinct polysaccharide-protein conjugates. Each conjugate consists of a capsular polysaccharide prepared from serotype of

Streptococcus pneumoniae (1, 3, 4, 5, 6A, 6B, 7F, 9\1, 14, 18C, 19A, 19F, 22F, 23F, or 33F) conjugated to a carrier protein CRM197. There is a need for vaccines that induce an immune response against serotype 15B, 15C, and 15A.

With increasing number of polysaccharide antigens in the multivalent conjugate vaccine formulations, the carrier protein content increases. This increase leads to an increase of immune response to the carrier protein which can cause a systemic overload.

Thus, there is a need to develop a pneumococcal vaccine that provides protection against increasing number of serotypes. Although a higher valent vaccine is highly desirable with a conjugation, preferably the immune response to the carrier protein is also reduced. In the development of multivalent vaccines that extend the scope of protection to additional serotypes, there is a need to improve immunogenicity and avidity of the conjugate vaccine to accommodate the increased number of serotypes without compromising the immune responses to all, which is not possible with conventional conjugation methods. In addition to protection against increasing number of serotypes, there is also a need for to develop new linkers for conjugation to improve the immune response even with the increasing number of serotypes as well as a decrease in the carrier protein response (and also avoiding steric hindrance).

Although many references recite efficacy of currently available vaccines, when adding multiple new serotypes, the immune responses decrease with increase in numbers to the original serotypes. Additional serotypes are needed to increase the efficacy of the immune response. In addition, the greater efficacy should preferably include a reduction of the immune response to carrier protein. Thus, there remains a great need for higher valency pneumococcal conjugate vaccines to provide a barrier against infections throughout the world.

Summary of the Invention

The present invention overcomes the problems and disadvantages associated with current strategies and designs and provides new immunogenic compositions comprising conjugated capsular saccharides and uses thereof.

One embodiment of the invention is directed to multivalent *S. Pneumoniae* conjugate vaccines comprising two groups of conjugates, wherein group one comprises monovalent bacterial capsular polysaccharide conjugates and group two comprises

multivalent conjugates containing conjugates of bivalent bacterial capsular polysaccharide having cross reactivity. Preferably, group one conjugates are composed of monovalent capsular polysaccharide conjugates of one or more *S. Pneumoniae* serotypes 1, 2, 3,4, 5, 7F, 8, 10A, 11A, 12F, 14, 17F, 18C, 20, 22F, 23F, 24F, 33F and 35B. Preferably, group two conjugates are composed of a bivalent or multivalent capsular polysaccharide conjugate of cross reactive serotypes of one, two or more of *S. Pneumoniae* serotypes 6A/6B/6C/6D, one, two or more of *S. Pneumoniae* serotypes 9V/9N/9A/9B, one, two or more of *S. Pneumoniae* serotypes 15B/15A/15C, or *S. Pneumoniae* serotypes 19A/19F; and carrier proteins. Preferably, the second group constituting the multivalent S. Pneumoniae conjugate vaccine comprises multivalent conjugates of S. Pneumoniae cross reactive serotypes wherein the conjugates are bivalent unimolecular and are derived from bacterial capsular polysaccharides. Preferably, the vaccine comprises capsular polysaccharide of two immunologically cross-reactive serotypes conjugated to the same carrier protein sequentially or concurrently. Preferably monovalent bacterial capsular polysaccharide conjugates of the first or second group are synthesized from native bacterial capsular polysaccharides with molecular weight ranges of 10 KDa to 50 KDa, 30 KDa-100 KDa, or 100 KDa-300 KDa.

Preferably, the bivalent capsular polysaccharide of two immunologically cross-reactive serotypes is represented by the formula PS1-Carrier Protein-PS2 and, also preferably, the conjugate comprises *6APS-CRM197-6BPS*. Preferably the carrier protein comprises Tetanus Toxoid, Diphtheria Toxoid, CRM197, Tetanus Toxoid fragments (TTHc), *N. meningitidis* protein PorB, RSV virus proteins, *B. Pertussis* proteins, Pertussis toxoid (PT), Adenylate cyclase Toxin (ACT), 69 KDa protein, Human Papilloma viral protein antigens, Human Papilloma virus VLP forms, Hepatitis *B* virus core antigen, Hepatitis *B* virus VLP forms, derivatives of HBsAg, or combinations thereof. Preferably a single dose of bivalent cross-reactive polysaccharide conjugates comprises less than 4 micrograms in comparison to monovalent conjugates of the same two polysaccharide vaccines which are 4 micrograms or more.

Preferably, total carrier protein quantity in the multivalent conjugate vaccine is significantly less than the quantity used in the mono conjugates of the individual polysaccharides of the same cross-reactive serotypes. Preferably, the vaccines of the present

invention, the carrier protein amount being conjugated to a bivalent cross-reactive polysaccharide has less protein per serotype in comparison to that of the monovalent conjugates of the same two polysaccharides thereby the carrier protein immune response generated by the vaccine is lower than the response to the carrier protein of vaccines made by others containing the mono conjugates of the individual polysaccharides. Preferably total carrier protein content in the multivalent conjugate vaccine is from 0.5 to 0.7 % by weight of the mono conjugates of the individual polysaccharides of the same cross-reactive serotypes (which is 1:1 ratio between PS: Carrier Protein). Preferably, the conjugate vaccine further comprises at least one adjuvant selected from the group consisting of aluminum or an aluminum salt, calcium phosphate, a liposome of monophosphoryl lipid A (MPLA), saponin QS-21, and/or a potent TLR7/8 agonist. Preferably the at least one adjuvant comprises an aluminum adjuvant selected from the group consisting of aluminum phosphate, aluminum sulfate and aluminum hydroxide. Preferably the bacterial polysaccharides are selected from the group consisting of cross reacting two or more serotypes from different bacterial capsular polysaccharides and/or the bacterial polysaccharides comprise: *S. pneumoniae* and *H. influenza* type a, b serotypes; *S. pneumoniae* and *Group B Streptococcus* serotypes, *H. influenza* type a, b serotypes, or *N. meningitis* serotypes. Preferably the capsular polysaccharides comprise polysaccharides derived from *Streptococcus pneumoniae, Haemophilus influenza, N. meningitis, Group B Streptococcus, or* Moraxella catarrhalis lipo-oligosaccharides (LOS). Also preferably, the S. pneumoniae capsular polysaccharide is immunochemically cross-reactive with serotypes selected from the group consisting of 6A/6B/6C/6D; 9V/9A/9B.9N; 15A/15B; 19A/19F and similar types of cross reactive polysaccharides. Preferably, the capsular polysaccharide is derived from *Haemophilus influenza* serotypes a/b/c/d/e/f, non-typeable Haemophilus influenza (NTHi) polysaccharides, or *Moraxella catarrhalis* Lipooligosaccharides(LOS), or *N. meningitis* serotypes A, B, C, Y, W-135 or X, or *Group B Streptococcus* serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII. IX and N, and N. meningitis serotypes A, C, Y, X, and W-135.

Another embodiment of the invention is directed to conjugate vaccines for the treatment or prevention of infection by Gram-positive and Gram-negative pathogens comprising a therapeutically effective amount of the conjugate vaccine of the invention and, optionally, a pharmacologically acceptable carrier. Preferably the capsular polysaccharides

are derived from *Haemophilus influenza, N. meningitis, Group B Streptococcus, N. meningitis, H. influenza,* Moraxella catarrhalis lipo-oligosaccharides (LOS), and combination thereof.

Another embodiment of the invention is directed to methods for coupling polysaccharides with carrier protein comprising: activating the polysaccharide; attaching a define length spacer arm of about 2.0-40Å to the activated polysaccharide; and attaching the activated polysaccharide attached spacer arm to a carrier protein.

Another embodiment of the invention is directed to methods coupling a carrier protein with polysaccharides comprising: activating the said carrier protein, reducing the carrier proteins disulfide to create sulfhydryl groups, preferably creating a sulfhydryl group using 2-iminothiolane (2-IT), SMPH like bi-functional linker; attaching a defined length spacer arm of 4-40Å to the activated carrier protein; and attaching the polysaccharide to a spacer arm attached to activated carrier protein. Preferably the activated carrier protein is selected from the group consisting of cross-reactive material (CRM197) obtained or derived from *C. diphtheria*, or recombinant CRM197 obtained or derived from *P. fluorescens* or *E. coli*.

Another embodiment of the invention is directed to bifunctional linkers that are is homo-bifunctional or hetero-bifunctional.

Another embodiment of the invention is directed to multivalent S. Pneumoniae conjugate vaccine wherein carrier protein is cross-reactive material (CRM197) obtained from C. diphtheria, recombinant CRM197 obtained from *P. fluorescens*, or recombinant CRM197 obtained from E. coli.

Other embodiments and advantages of the invention are set forth in part in the description, which follows, and in part, may be obvious from this description, or may be learned from the practice of the invention.

Description of the Figures

Figure 1A Size reduced capsular polysaccharide of serotype 6A 1H-NMR spectra (500MHz)-NMR data shows no loss of structural integrity compared to native PS.

Figure 1B Size reduced capsular polysaccharide of serotype 6B 1H-NMR spectra (500MHz)-NMR data shows no loss of structural integrity compared to native PS.

Figure 2A Capsular polysaccharide specific antibodies (total IgG) using multiplex bead based assay procedure (Polysaccharides used for these conjugates are in the range of 10-50KDa).

Figure 2B Capsular polysaccharide specific antibodies (total IgG) using multiplex bead based assay procedure wherein polysaccharides are in the range of 200-300 KDa or more.

Figure 2C Bi-valent conjugates of 6A and 6B capsular polysaccharide specific antibodies (total IgG) using multiplex bead based assay procedure wherein polysaccharides are in the range of 10-50 KDa and 200-400KDa.

Figure 3A Monovalent conjugates synthesis work flow chart.

Figure 3B Flow chart of PS1 and PS2 activation with linkers.

Figure 4A Bivalent unimolecular conjugates and bi-valent conjugates synthesis workflow chart.

Figure 4B CRM chemical couplings.

Figure 5 CDAP (1-cyano-4-dimethylaminopyridinium tetrafluoroborate, Cyanuric chloride (2,4,6-Trichloro-1,3,5-triazine), cyanogen bromide (CNBr).

Figure 6 Thiolation of CRM 197 with iminotiolene.

Description of the Invention

Streptococcus pneumoniae is a Gram-positive bacterium which can cause diseases such as pneumonia, bacteraemia, meningitis, and acute Otitis media. Pneumococcus is encapsulated with a chemically linked polysaccharide which results in serotype specificity. At least 90 pneumococcal serotypes are known of which about 23 account for 90% of invasive diseases. The protection against invasive pneumococci disease is related to the antibody specific to the capsular polysaccharide, the protection is therefore serotype specific.

It was surprisingly discovered that multivalent S. Pneumoniae conjugate vaccine comprising of a linker between the carrier protein and the polysaccharide to form two groups of conjugates, wherein group one comprises monovalent bacterial capsular polysaccharide conjugates and the other group comprises multivalent carrier protein conjugates provides substantially improved results. Specifically, the bivalent conjugates and bivalent uni-molecular conjugates are preferably synthesized by the reaction between carrier protein and bifunctional linkers attached to cross reactive S. Pneumoniae serotypes. Results achieved

are substantially improved compared to vaccines containing multivalent S. Pneumoniae conjugate vaccine containing monovalent bacterial capsular polysaccharide conjugates with the same number of serotypes with a direct conjugation between the two instead of a linker.

One embodiment of the invention is directed to multivalent conjugate vaccines comprised of bivalent-polysaccharide protein conjugates with enhanced immunogenicity. Bivalent conjugates with general structure PS1-carrier protein-PS2 have higher immunogenicity compared to similar monovalent conjugates wherein PS1 and PS2 are two different serotype polysaccharides from gram-negative and gram-positive bacterial pathogens. By developing a bi-valent conjugate vaccine, the efficacy of the vaccine increases and carrier immunogenicity is reduced. The chemistry disclosed herein substantially increases the conjugates immunogenicity, at the same time reduces carrier protein load.

Another embodiment of the invention is directed to vaccines with lower molecular weight polysaccharides and longer arm bifunctional linkers preferably with enhanced immunogenicity. Another embodiment of the invention is directed to providing higher immunogenicity and avidity of bivalent conjugates as well as lower carrier protein immunogenicity. Another embodiment of the invention is directed to reducing conjugate vaccine dose with higher immunogenicity.

As disclosed herein, four parameters have been introduced to minimize the disadvantages of conventional vaccines:

- Polysaccharide size is preferably 10-50KDa.
- Cross-reactive polysaccharides concurrent conjugation to carrier protein.
- Two or more cross reactive serotypes are conjugated concurrently with carrier proteins.
- A long hetero- or homo-bifunctional spacer arm preferably of from 2-40Å (also 2-40Å, 4-40Å, 10-40Å, 20-40Å, 9-20Å, 5-20Å, 5-30Å).

These four parameters taken together are profoundly effective to increase the conjugates polysaccharide/protein ratio, to reduce carrier protein load, and to provide several folds of increase in immunogenicity and avidity.

The present invention is directed to polysaccharide–protein conjugates with enhanced immunogenicity displaying significantly high antibody titers. The carrier protein is obtained from, for example, tetanus toxoid, diphtheria toxoid, CRM197, tetanus toxoid

fragments (TTHc), *N. meningitidis* protein PorB, RSV virus proteins, *B. Pertussis* proteins like pertussis toxoid (PT), adenylate cyclase toxin (ACT), 69KDa protein and Human Papilloma viral protein antigens or its VLP form, Hepatitis B core antigen or its VLP form or derivatives of HBsAg, and other conventional carriers. Polysaccharide fragment is obtained from group of group of gram positive bacteria and gram-negative bacteria, preferably from immunochemically cross-reactive polysaccharides of *S. Pneumoniae*. The present invention is also directed to a process of preparing the polysaccharide–protein conjugates in which carrier protein reacts with cleaved and depolymerized polysaccharide fragments of optimum chain length.

Immunogenic compositions of the present invention provide for appropriate level and improved protection against S. *pneumoniae* serotypes not found in PREVNAR-13®, and SYNFLORIX-10®.

Bivalent conjugates with cross-reactive polysaccharides of *S. Pneumoniae* serotypes (6A/6B, 9V/9N, 15A/15B and 19A/19F and similar cross-reactive serotypes) with short chain molecular size (10-50KDa) was used to prepare 16-26-valent pneumococcal CPS conjugate vaccine in the present study. Pneumococcus type 6A and 6B polysaccharide was used as the model cross-reactive CPSs. CRM197 was used as the carrier protein for its clinical acceptance.

Multivalent monoconjugates have also been prepared using shorter PS chain length (0-50KDa), long spacer arm (9-40Å) with homo or hetero-bifunctional PEG or non-PEG linker with carrier protein CRM197.

CPS was activated either by oxidation or by cyanylation chemistry and oxidized by sodium periodate and introduced with either -reactive aldehyde or isothiocyanate (-OCN) groups in CPS.

Two strategies (short and long linker, short and long CPSs) were used to introduce, respectively. Physicochemical and immunological characteristics of the bivalent conjugates vaccines were then investigated independently or combining with multivalent conjugate formulation.

The following examples illustrate embodiments of the invention, but should not be viewed as limiting the scope of the invention.

Examples

Example 1 Polysaccharide size reduction, activation and conjugation process for multiple S. pneumoniae serotypes -1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 10A, 11A, 12F, 14, 15A, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F and 35B

6A and 6B Polysaccharide

100 mg each of capsular polysaccharides of *S. pneumoniae* 6A and 6B is dissolved in 10ml of aqueous solution containing 10mM of Acetic acid or 0.1 M HCl at pH 2.5-3.0 and hydrolysis is carried out by maintaining the solution at a temperature of 60-85°C for a period of 60-120 mins. The so-obtained oligosaccharides after neutralization, diafiltered using 3-10KDa TFF Centricon filters. Upon ^{1}H NMR analysis (**Figures 1A and 1B**), the oligosaccharides formed show no loss of structural integrity or loss of epitope or repeat unit structure. Polysaccharides were measured using Anthrone assay and molecular size distributions (KDa) obtained are in the range of 10-50KDa, 30-100KDa, and 100-300KDa.

CPS (50 mg) moiety (native polysaccharides of size between ≥200-500KDa or size-reduced polysaccharides of size between 10-50KDa) were activated cyanylation reagents commonly used in activation process (**Table 1**). Polysaccharide molecular size distributions were determined using SEC-HPLC (Shodex SB-405 and SB-406 SEC columns) with analysis using (10-1000KDa) Pollulan mixture as reference standard (Pollulan standards from Shodex, USA).

Short spacer arm was introduced to PS by reaction with 5-8-fold molar excess of ADH (Sigma) at pH 5.6-6.0 for 3-5 hr. Long spacer arm (bifunctional linker or long 4-arm linker) was introduced into PS by reaction with 5-10-fold molar excess of at pH 5.6-6.0 for 3-5 h.

Table 1

Polysaccharide size distribution (KDa) used for conjugation

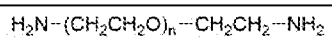
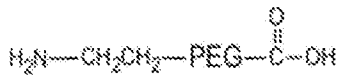
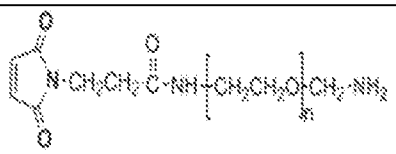
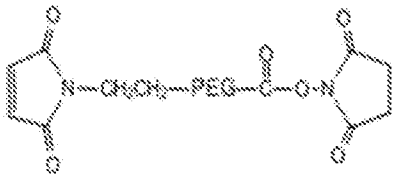
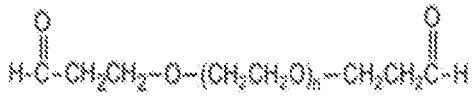
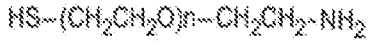
PS	Polysaccharide KDa
6A	10-30KDa
6B	20-50KDa
15B	20-40KDa
18C	20-50KDa
22F	10-30KDa

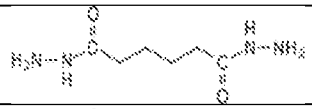
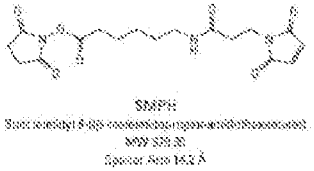
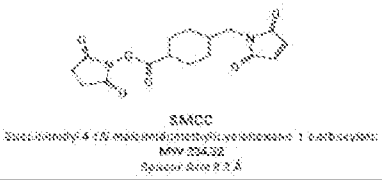
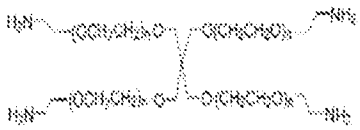
Activated PS is further derivatized with short arm linker (adipic acid di-hydrazide, ADH, 174.2g/mole), one more spacer arm linkers with varying size from 2-4Å to 8-20Å (600g/mol-3.5g/mole).

Homo or hetero-bifunctional PEG linkers with diamine functional groups attached, e.g. NH_2-PEG0.6K-NH_2, NH_2-PEG3.5K-COOH (**Table 2**).

Table 2

Short and long chain linker used for polysaccharide or carrier protein derivatization used (several other linkers either in pegylated form or non-pegylated form have also been used)

Linker No.	Linker Structure	Chemical Structures/KDa or Å used
1	NH_2-PEG- NH_2/NHS	 1K and 3.5K
2	NHS/NH_2-PEG--COOH	 1K and 3.5K
3	Mal-PEG- NH_2	 1K and 3.5K
4	Mal-PEG-NHS	 1K and 3.5K
5	CHO-PEG-CHO	 1K and 3.5K
6	SH-PEG-NH_2	 1K and 3.5K

7	ADH	
8	HZ-PEG-HZ	
9	SMPH	
11	SMCC	
12	4-Arm-PEG-NH_2 or NHS	

Mal-Maleimide, **NHS-**Succinimide, **PEG**-Polyethylene glycol derivatives, **ADH**-Adipic acid di-hydrazide.

Two aliquots of 2 ml each of the derivatized CPS (10 mg/ml) were mixed with 1 ml aliquot of the two CRM197 protein samples (10 mg/ml) at 4°C for 8-12hrs. The conjugates with long and short spacer arm were purified by a 100-300 KDa Centricon filters (EMD Millipore) (**Table 3**).

Table 3

Physicochemical Characterization of mono-valent Conjugates

PS	Activated PS KDa by SEC-HPLC	Conjugate KDa by SEC-HPLC	PS: Protein ratio	Free PS%
6A	10-30KDa, 200-300KDa	>200-300, >2500	0.5-2, 1-2	<2
6B	20-50KDa,200-400KDa	>300-500, >2500	0.5-2, 1-2	<1
15B	20-40KDa	>300-500	0.5-2, 1:1	<1
18C	20-50KDa	>300-500	0.5-2, 1:1	<2
22F	10-30KDa	>200-300	0.5-2, 1:1	<1

Note: Internal std. for KDa determination of PS for SEC-HPLC:Pullulan std. mixture (2 KDa-2500 KDa).

Example 2 Activation of size reduced polysaccharides of different molecular weights

Oligosaccharides of different molecular weights synthesized as described in example 1 were activated. Cyanylation reagents commonly used in activation process.

CDAP (1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (Sigma Aldrich, USA)) cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) or cyanogen bromide (CNBr) and coupling carrier protein (see **Figures 5 and 6**).

Polysaccharide solution (10 mg/ml) was incubated with 10mg/ml CDAP (100mg/ml in acetonitrile) in 2M NaCl or 200-300mM bicarbonate buffer at RT for 4-6 minutes. pH was maintained at 10-10.5 using either 1N NaOH or 1N HCl. Then, pH was adjusted to 8.1-8.2, pegylated linkers (Hz-PEG-HZ) were allowed to react with CDAP treated PS. For 8-12 hrs at RT. The reaction mixtures were depth filtered followed by 100-300KDa cutoff centricon fiters 5-8 times using 150 mM NaCl.

Derivatization of activated size reduced Polysaccharides

Activated oligosaccharides were further derivatized with short chain homo-bifunctional hydrazide linker. Typical reagent was adipic Acid di-hydrazide, ADH, Molecular weight 174.2 g/mole). Homo or hetero-bifunctional PEG linkers bearing di-amine, di-hydrazide, or amine or hydrazide-carboxylic acid/aldehyde functional groups, e.g. NH2-PEG(1K-3.5K)-NH2, HZ-PEG(1-3.5K)-HZ, NH2-PEG3.5K-COOH were used. (**Table** 2). Several other homo-or hetero-bifunctional spacer arms can also be used for derivatization (**Table** 2). Short spacer arm was introduced to oligosaccharide by reaction with 5-8fold molar excess of adipic acid di-hydrazide (Sigma) at pH 5.8-6.0 for 3-5 hr. long chain linker (bifunctional linker or long tetra functional linker (**Table 2**), No. 12 four arm linker) was introduced into Polysaccharide by reaction with 5-10-fold molar excess of the linker to the oligosaccharide at pH 5.8-6.0 for 3-5 hrs. at RT.

Derivatization of carrier protein with short or long-linkers

Carrier protein CRM197 was further derivatized with short chain homo-bifunctional hydrazide linker. Typical reagent was adipic Acid di-hydrazide, ADH, molecular weight 174.2 g/mole). Homo or hetero-bifunctional PEG linkers bearing di-amine, di-hydrazide, or amine or hydrazide-carboxylic acid/aldehyde functional groups, e.g., NH2-PEG(1K-3.5K)-NH2, HZ-PEG(1-3.5K)-HZ, NH2-PEG3.5K-COOH were used. (**Table 2**). Several other homo-or hetero-bifunctional spacer arms can also be used for derivatization as listed in

Table 2). Short spacer arm was introduced to carrier protein CRM197 by reaction with 5-8 fold molar excess of adipic Acid di-hydrazide (Sigma) at pH 5.8-6.2 in 300-600 mM MES buffer for 3-5 hr at RT. Long chain linker (bifunctional linker or long tetra functional linker (**Table 2**, No. 12 four arm linker) was introduced into carrier protein by reaction with 5-10-fold molar excess of the linker to the oligosaccharide at pH 5.8-6.2 in 300-600mM MES buffer for 3-5 hr at RT (room temperature).

Example 3 Cross-reactive Polysaccharide serotypes activation and attachment of short or long-spacer arm linkers (serotypes of interest are 6A/6B, 9V/9N, 15A/15B and 19A/19F or any other cross-reactive serotypes).

Activation of the oligosaccharide derived from the capsular polysaccharide of *S. Pneumoniae* Type 6A and 6B conjugation with CRM197 and introduction of the primary amino groups to the oligosaccharides concurrently.

Native or size reduced polysaccharide of serotype 6A and 6B (≥200-400KDa) were conjugated using the same procedure as described in Examples 1 and 2.

The oligosaccharides mixture thus obtained as reported in Example 1 are dissolved in WFI, to an end concentration of 10 mg/ml. At the end of the reaction, the Oligosaccharide are purified by diafiltration using 3-10 KDa Centricon filters.

The Oligosaccharides into which the amino groups have been introduced are diluted to a concentration of 10 mg/ml in an aqueous solution of DMSO (at 20-30% v/v) to DMSO containing ADH short linker or long spacer arm linkers in molar excess relatively to the amino groups introduced into the oligosaccharide (usually 5-10:1). The reaction was carried out by keeping the solutions at RT for a time of 4-12 hours. At the end of the period, oligosaccharide was again purified using 3-10 KDa Centricon filters.

Example 4 Synthesis of Pneumococcal polysaccharide monovalent conjugates

Two separate aliquots of same or differently size reduced and derivatized size reduced Polysaccharides (with short spacer arm ADH and Long spacer arm HZ-PEG-HZ) as synthesized in example 3 (10 mg/ml) were mixed with 1 ml aliquot of the CRM197 protein sample (10 mg/ml) at 4 °C for 8-12hrs. The conjugates containing both long and short chain linkers were purified using 100-300KDa centricon filters (EMD Millipore). Each monovalent conjugates were assayed for total polysaccharide content by either anthrone or uronic acid assay, total protein content by BCA or Lowry assay (**Table 4**).

All other cross-reactive Polysaccharide conjugates are made using the same procedure as above.

Table 4

Physicochemical Characterization of Bi-valent Conjugate of general structure 6A-CRM197-6B

PS	Activated Oligosaccharide KDa	Conjugate KDa	Oligosaccharide: Protein ratio (Weight ratio)	Free Oligosaccharide % by weight
6A	≥100-300KDa	>200-300KDa, >2500KDa	0.5-2, 1-2	<2
6B	≥200-400KDa	>300-500KDa,>2500KDa	0.5-2, 1-2	<1
6C	≥200-400KDa	>300-500KDa,>2500KDa	0.5-2, 1-2	<1
15B	≥100-300KDa	>300-500KDa, >1500KDa	0.5-2, 1:1	<1
15A	≥100-300KDa	>300-500KDa, >1500KDa	0.5-2, 1:1	<1
18C	≥100-300KDa	>300-500KDa, >1500KDa	0.5-2, 1:1	<2
22F	≥100-300KDa	>200-300KDa, >1000KDa	0.5-2, 1:1	<1
1	≥100-300KDa	>200-300KDa, >2500KDa	0.5-2, 1-2	<2
3	≥200-400KDa	>300-500KDa,>2500KDa	0.5-2, 1-2	<1
4	≥100-300KDa	>300-500KDa, >1500KDa	0.5-2, 1:1	<1
7F	≥100-300KDa	>300-500KDa, >1500KDa	0.5-2, 1:1	<2
9V	≥100-300KDa	>200-300KDa, >1000KDa	0.5-2, 1:1	<1
9N	≥100-300KDa	>200-300KDa, >1000KDa	0.5-2, 1:1	<1
14	>100-300KDa	>200-300KDa, >2500KDa	0.5-2, 1-2	<2
18C	≥200-400KDa	>800KDa,>2500KDa	0.5-2, 1-2	<1
19A	≥100-300KDa	>300-500KDa, >1500KDa	0.5-2, 1:1	<1
19F	≥100-300KDa	>300-500KDa, >1500KDa	0.5-2, 1:1	<2
23F	≥100-300KDa	>200-300KDa, >1000KDa	0.5-2, 1:1	<1
33F	≥100-300KDa	>200-300KDa, >2500KDa	0.5-2, 1-2	<2

Note: Internal std. for SEC-HPLC (KDa): Pollulan std mixture (2KDa-1200KDa)

Example 4 Investigational Formulation of 16V-or higher valent Pneumococcal Conjugate vaccine

Pneumo Polysaccharide -CRM197 conjugates for serotypes containing 1, 3, 5, 7F, 14, 15B, 18C, 22F, 23F, 33F, 35B and cross-reactive polysaccharide conjugates 6A, 6B, 9V, 9N, 15A, 15B, 19A, and 19F were combined to yield final antigen concentration of 4.0 µg PS/mL. Sodium chloride (150 mM) solution, 10-20 mM Histidine, Succinic acid and 0.001% Tween-20 was also used during the formulation process as diluent, and aluminum phosphate (Adju-Phos, Brenntag, USA) was used as investigational adjuvant. 16-V conjugate was aseptically filled in 2mL sterile vials. PNEUMOVAX® (Merck, USA) or PREVNAR-13® (Pfizer, USA) was used as two control commercial vaccine formulation.

Example 5 Immunogenicity studies of Conjugates

A New Zealand white rabbit model (NZW) was selected in this work to compare the immunogenicity of the Pneumo PS-CRM197 conjugates. Rabbits from all groups (16-V {valent}, PREVNAR-13®, and PNEUMOVAX®) were examined for clinical signs before and after immunization periods. For all groups, pre-immunization, booster dose (7 and 14-days) and terminal bleed (28 days) were collected and aliquoted and store at minus 80°C until use. Multiplexed Immunogenicity assay for the determination of Total IgG were performed according to the standard protocol using reference standard serum 007 (CBER, FDA, USA). Reference serum and rabbit serum were diluted and pre-adsorbed for cross-reacting antibodies by treatment with pneumococcal CWPS and either 22F PS or 25PS. Human monoclonal anti-polysaccharide antibodies (Pamlico Biopharma, USA) were used for total IgG estimation. Bio-Plex 200 (Bio-Rad). Multiplex reader was used as per manufacturer's instructions (see **Figures 2A, 2B and 2C**).

Example 5 *S. Pneumoniae* Cross-reactive capsular Polysaccharide serotypes activation and attachment of short and long-spacer linkers

Serotypes of 6A/6B, 9V/9N, 15A/15B and 19A/19F which are cross-reactive serotypes are used for the synthesis of bi-valent conjugates containing capsular poly saccharides and carrier protein. Bivalent conjugates by definition contain two capsular polysaccharide attached to CRM 197 simultaneously or concurrently.

Activation of the size reduced polysaccharide derived from the capsular polysaccharide of *S. Pneumoniae* Type 6A and 6B, conjugation with CRM197 and

introduction of the primary amino or hydrazide groups to the oligosaccharides carried out concurrently.

Native polysaccharides or size reduced oligosaccharide of serotype 6A and 6B (≥200-500KDa) were conjugated using the same procedure as described in Example 1 -4.

The size reduced polysaccharides mixtures thus obtained were dissolved in water for injection, so that the final concentration was 10 mg/ml. The size reduced polysaccharides into which the amino or hydrazide groups were introduced were diluted to a concentration of 10 mg/ml in an aqueous solution of dimethyl sulfoxide (DMSO) so the percentage of DMSO was in the range of 20-30% (v/v). This was added to DMSO containing short chain linker such as ADH or long chain linkers as described in **Table 2** in molar excess relatively to the amino/hydrazide groups introduced into the size reduced polysaccharides (usually 5:1 or 10:1), more specifically 8:1.

The reaction was carried out at room temperature for a duration of 4-12 hours. At the end of the reaction period, the reaction product was again purified using 3-10KDa Centricon filters.

Example 6 Simultaneous or concurrent Conjugation of *S. pneumoniae* oligosaccharides of Type 6A and Type 6B with CRM197 carrier Protein as bivalent conjugates manufacturing.

The aqueous solution containing 15 mg/ml of CRM197, was added to DMSO containing the linker attached oligosaccharide (20-30% in water) derived from the capsular polysaccharide of *S. pneumoniae* Type 6A. The ratio of linker attached oligosaccharide to CRM197 was selected from 1:1,2:1,1:2. The mixture so obtained was kept, under mild stirring, at room temperature for 8-12hrs. At the end of said time, the solution containing the derivatized oligosaccharide derived from the capsular polysaccharide of *S. Pneumoniae* 6B was added. The molar ratio of capsular polysaccharide of *S. Pneumoniae* 6B to the CRM197, was selected from 1:1,2:1,1:2). The resulting mixture was kept for 8-12hrs at room temperature (**Table 5**). The conjugation reaction can also be carried out by adding, at the same time (concurrently), to the CRM197-containing solution, the two-activated oligosaccharide respectively derived from the capsular polysaccharide of *S. pneumoniae* Type 6A and from the capsular polysaccharide of *S. pneumoniae* Type 6B. The oligosaccharide-protein conjugates so obtained were dialyzed using 100-300KDa dialysis

membrane (Spectrum lab, USA), conditioned in 0.01 M phosphate buffer containing 0.2M NaCl (pH=6.6-7.0) and finally filtered through a 0.22 µm filter.

All other cross-reactive Polysaccharide conjugates were made using the same procedure as used above. Reaction sequences are depicted in **Figures 3A, 3B, 4A, and 4B**.

Table 5

Comparisons of PS Contents

Bivalent Oligosaccharide	Activated oligosaccharide KDa	Conjugate KDa	Total Polysaccharide Protein ratio by weight	Free oligosaccharide % by weight
6A-6B	≥100-300	2.0:1.5	2-1.5 (1:0.75)	<2
6A-6B	≥100-300, ≥300	>1200-2500KDa	2-1.4 (1: 0.7)	<3
19A-19F	≥100-300	>500-800KDa	2-1.6 (1:0.8)	<2
15A-15B	≥100-300, ≥300	>500-1000KDa	2-1.3 (1: 0.65)	<3
9V-9N	≥100-300, ≥300	>500-1000KDa	2-1.3 (1: 0.65)	<3

Example 7 Investigational Formulation of 18-Valent or higher valent Pneumococcal Conjugate vaccine.

Pneumococcal Polysaccharide-CRM197 conjugates for serotypes containing 1, 3, 5, 7F, 14, 18C, 22F, 23F, 33F, 35B (10 serotypes Polysaccharides) and cross-reactive polysaccharide conjugates of (6A, 6B), (9V, 9N), (15A, 15B) and (19A, 19F) (8 serotypes) were combined to yield final polysaccharide concentration of 2.2-4.4 µg PS/mL (1.1-2.2 µg/human dose, 0.5 mL). Sodium chloride (150mM) solution, 10-20 mM histidine, 20 mM HEPES or MOPS buffer and 0.001% Tween-20 was also used during the formulation process as diluent, and aluminum phosphate (Adju-Phos, Brenntag, USA) was used as investigational adjuvant.

18-valent or higher valent (>20V-24V) conjugate was aseptically filled in 2mL sterile vials. PNEUMOVAX® (Merck, USA) and/or PREVNAR-13® (Pfizer, USA) were used as controls.

Example 9 Immunogenicity studies of the Conjugates.

A New Zealand white rabbit model (NZW) was selected in this work to compare the immunogenicity of the Pneumococcal PS-CRM197 conjugates. Rabbits from all groups (18

or higher-Valent conjugates, PREVNAR-13®, Pfizer and PNEUMOVAX®-23 (Merck USA) were examined for serological titers before and after immunization periods. For all groups, pre-immunization, booster dose (7 and 14-days) and terminal bleed (28 days) were collected and aliquoted and store at -80°C until use. Immunogenicity assay for the determination of Total IgG were performed according to the standard protocol using reference standard serum 007 (CBER, FDA, USA). Reference serum and Rabbit serum were diluted and pre-adsorbed for cross-reacting antibodies by treatment with Pneumococcal CWPS and non-vaccine serotype 25PS. Human/rabbit/mouse monoclonal anti-polysaccharide antibodies were used for total IgG estimation. Bio-Plex 200 (Bio-Rad) reader were used as per the manufacturer's instructions.

Immunogenicity of the conjugates, i.e. capsular polysaccharide specific antibodies (total IgG) were measured using bead-based ELISA assay method were given in **Table 6**. Total IgG values were compared head to head with PREVNAR-13® in rabbit immunogenicity data. 14- day data shows significant increase in titer in IVT-18V-1 vaccine compared to PREVNAR-13® vaccine. Similarly, IVT-18V-1 data has significant booster on IgG values as compared to PREVNAR-13® (**Table 6**).

Table 6

Capsular Polysaccharides specific antibodies (Total IgG in µg/ml) using Multiplex bead-based ELISA assay for 18V-monovalent conjugate vaccines

PREVNAR-13® 2.2µg/dose	(IgG) 14day/zero day	(IgG) 28day /Zero day	IVT-18V-1 2.2µg/dose	(IgG) 14day /Zero day	(IgG) 28day /Zero day
1	45	350	1	375	1500
3	47	200	3	48	480
6A	188	560	6A	775	3775
6B	165	780	6B	662	3662
18C	50	280	18C	306	3560
19A	45	235	19A	233	2500
19F	29	290	19F	72	720
4	49	230	4	150	750
5	186	700	5	550	3550

7F	180	680	7F	332	3860
9V	52	520	9V	212	2400
9N	-	-	9N	200	2200
14	85	400	14	272	2890
15A	-	-	15A	672	3900
15B	-	-	15B	750	4000
18C	175	800	18C	550	5500
22F	-	-	22F	1000	8000
23F	53	450	23F	212	2420

Note: IVT-18V == 18-V conjugate vaccine (monovalent conjugates mixed together); 9N, 15A, 15B, 22F and 23F serotype are not present in PREVNAR-13®, so IgG values not measured; 18-V formulation as monovalent conjugates were prepared using 2.2 µg for each serotype except 4.4 µg of 6B conjugate. Sodium chloride (150mM) solution, 10-20 mM histidine, 20 mM HEPES or MOPS buffer and 0.001% Tween-20 was also used during the formulation process as diluent, and aluminum phosphate (Adju-Phos, Brenntag, USA) was used as investigational adjuvant; capsular polysaccharides antibodies (total IgG) using bead-based ELISA: 18-V conjugate vaccine formulation-2 (IVT-18V-2): 10-V formulation as monovalent conjugates and remaining 8-V added as bivalent-conjugates which includes 6A/6B, 9V/9N, 15A/15B and 19A/19F. (vaccine dose used as 2.2 µg for each serotype except 4.4 µg of 6B) 10-V formulation as monovalent conjugates and remaining 8-V added as bivalent-conjugates which includes 6A/6B, 9V/9N, 15A/15B and 19A/19F. 6A-6B bivalent unimolecular conjugates are used as 2.2 µg/dose, remaining bivalent conjugates are used as 2.2 µg/dose. Sodium chloride (150mM) solution, 10-20 mM histidine, 20 mM HEPES or MOPS buffer and 0.001% Tween-20 was also used during the formulation process as diluent, and aluminum phosphate (Adju-Phos, Brenntag, USA) was used as investigational adjuvant.

Immunogenicity of the conjugates, capsular polysaccharide specific antibodies (total IgG) were measured using bead-based ELISA assay method were given in **Table 7**. Total IgG values were compared head to head with PREVNAR-13® in rabbit immunogenicity data. 14-day data shows significant increase in titer in IVT-18V-2 vaccine compared to PREVNAR-13® vaccine. Interestingly, IVT-18V-2 total IgG data for bivalent conjugates

serotypes (for example. 6A/6B, 9V/9N, 15A/15B, and 19A/19F) has significant booster on IgG values as compared to IVT-18V-1 formulation with monovalent conjugates. Therefore, it can be concluded that Bivalent conjugates has better immunogenicity in comparison to monovalent conjugates (**Table 7**). Therefore, IVT-18V-2 conjugate vaccine formulation has superior immunogenicity not only against PREVNAR-13® but also against IVT-18V-1 formulation. Polysaccharide conjugated with either 1-3.5K linker (HZ-PEG-HZ) elicits much higher immunogenicity in compared to short linker (ADH) or no linker conjugates as in PREVNAR-13®.

Table 7

Capsular Polysaccharides antibodies (total IgG) using Multiplex bead-based ELISA

PREVNAR-13® 2.2µg/dose	Ratio 14day/0day	Ratio 28day/0 day	IVT-18V-2 2.2µg/dose	Ratio 14day/0day	Ratio 28day/0 day
1	45	350	1	375	1500
3	47	200	3	50	530
6A	188	560	6A/6B	875/762	4375/4662
6B	165	780			
18C	50	280	18C	316	3600
19A	45	235	19A/19F	300/198	3500/2700
19F	29	290			
4	49	230	4	180	1000
5	186	700	5	550	3600
7F	180	680	7F	360	4100
9V	52	520	9V/9N	350/300	3400/3200
9N	-	-			
14	85	400	14	310	32000
15A	-	-	15A/15B	872/850	5900/5600
15B	-	-			
18C	175	800	18C	600	6800
22F	-	-	22F	1020	8150
23F	53	450	23F	300	3200

Note: 1VI-18V-2 = 10-monovalent conjugates and 4 bivalent conjugates mixed together; 18-V conjugate vaccine formulation (IVT-18V-3): 10-V formulation as monovalent

conjugates used as 2.2 µg/dose and remaining 8-V added as bivalent-conjugates which includes 6A/6B, 9V/9N, 15A/15B and 19A/19F used as 1.1 µg/dose, except 6B 2.2 µg/dose.

Immunogenicity of the conjugates, i.e. capsular polysaccharide specific antibodies (total IgG) were measured using Multiplex bead-based ELISA assay method were given in **Table 8**. Total IgG values were compared head to head with PREVNAR-13® in rabbit immunogenicity data. 14-day data shows significant increase in titer in IVT-18V-3 vaccine compared to PREVNAR-13® vaccine. Interestingly, IVT-18V-3 formulations with lower dose (2.2 vs 1.1ug dose), total IgG data for bivalent conjugates serotypes (for example. 6A/6B, 9V/9N, 15A/15B, and 19A/19F) has comparable IgG values as compared to IVT-18V-2 formulations for bivalent conjugate serotypes. Therefore, it can be concluded that bivalent conjugates has better immunogenicity in comparison to monovalent conjugates with lower dose. Therefore, IVT-18V-2 conjugate vaccine formulation has superior immunogenicity not only against PREVNAR-13® but also against IVT-18V-1 formulation. Polysaccharide conjugated with either 1-3.5K linker (HZ-PEG-HZ) elicits much higher immunogenicity in compared to short linker (ADH) or no linker conjugates as in PREVNAR-13® (**Table 8**).

Table 8

Total IgG data for bivalent conjugates serotypes

PREVNAR-13® 2.2µg/dose	Ratio 14day/0day	Ratio 28day/0day	IVT-18V-2 2.2µg/dose	Ratio 14day/0	Ratio 28day/0day
1	45	350	1	375	1500
3	47	200	3	50	530
6A	188	560	6A/6B	825/860	4275/4900
6B	165	780			
18C	50	280	18C	316	3600
19A	45	235	19A/19F	275/250	3400/3000
19F	29	290			
4	49	230	4	180	1000
5	186	700	5	550	3600
7F	180	680	7F	360	4100

9V	52	520	9V/9N	320/380	3300/3800
9N	-	-			
14	85	400	14	310	32000
15A	-	-	15A/15B	790/900	5800/6200
15B	-	-			
18C	175	800	18C	600	6800
22F	-	-	22F	1020	8150
23F	53	450	23F	300	3200

Note: 1VI-18V-3 = 10-monovalent conjugates and 4 bivalent conjugates mixed together.

Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. All references cited herein, including all publications, U.S. and foreign patents and patent applications, are specifically and entirely incorporated by reference. It is intended that the specification and examples be considered exemplary only with the true scope and spirit of the invention indicated by the following claims. Furthermore, the term "comprising of" includes the terms "consisting of" and "consisting essentially of."

Claims

1. A multivalent conjugate comprising:

first and second group conjugates, wherein:

first group conjugates comprise a monovalent capsular polysaccharide conjugate of *S. Penumoniae* serotypes 1, 2, 3,4, 5, 7F, 8, 10A, 11A, 12F, 14, 17F, 18C, 20, 22F, 23F, 24F, 33F and 35B; and

second group conjugates comprising a bivalent or multivalent capsular polysaccharide conjugate of cross reactive serotypes of *S. Penumoniae* serotypes 6A/6B/6C/6D, *S. Penumoniae* serotypes 9V/9N/9A/9B, *S. Penumoniae* serotypes 15B/15A/15C, or *S. Penumoniae* serotypes 19A/19F with a bifunctional linker; and

a carrier protein.

2. The multivalent conjugate of claim 1, wherein the first and/or second group conjugates are bivalent and are derived from bacterial capsular polysaccharides.
3. The multivalent conjugate of claim 2, wherein the bivalent capsular polysaccharide comprises two immunologically cross-reactive serotypes is represented by the formula PS1-Carrier Protein-PS2.
4. The multivalent conjugate of claim 1, wherein the bivalent capsular polysaccharide comprises two immunologically cross-reactive serotypes conjugated to the same carrier protein sequentially or concurrently.
5. The multivalent conjugate of claim 1, wherein the first and/or second group conjugates comprise bivalent capsular polysaccharides from 10 kDa to 50 kDa, from 30 KDa-100KDa, and/or from 100KDa-300KDa.
6. The multivalent conjugate of claim 1, wherein the conjugate comprises *6APS-CRM197-6BPS*.
7. The multivalent conjugate of claim 1, wherein carrier protein comprises tetanus toxoid, diphtheria toxoid, CRM197, tetanus toxoid fragments (TTHc), *N. meningitidis* protein PorB, RSV virus proteins, *B. Pertussis* proteins, Pertussis toxoid (PT), adenylate cyclase toxin (ACT), 69 KDa protein, Human Papilloma viral protein antigens, Human Papilloma virus VLP forms, Hepatitis *B* virus core antigen, Hepatitis *B* virus VLP forms, derivatives of HBsAg, and/or combinations thereof.

8. The multivalent conjugate of claim 1, wherein a single dose of bivalent cross-reactive polysaccharide conjugates comprises less than 4 micrograms in comparison to monovalent conjugates of the same two polysaccharide vaccines which are 4 micrograms or more.
9. The multivalent conjugate of claim 1, wherein the quantity of carrier protein is from 0.5% to .7%, by weight, of the conjugates of the capsular polysaccharides and of the same cross-reactive serotype as the capsular polysaccharides.
10. The multivalent conjugate of claim 1, which has a ratio of about 1:1 of capsular polysaccharide to carrier protein.
11. The multivalent conjugate of claim 1, wherein carrier protein quantity is significantly less than the quantity used in making conjugates of the capsular polysaccharides of the same cross-reactive serotypes.
12. The multivalent conjugate of claim 1, further comprising at least one adjuvant selected from the group consisting of aluminum salt, calcium phosphate, a liposome of monophosphoryl lipid A (MPLA), saponin QS-21, and/or a potent TLR7/8 agonist.
13. The multivalent conjugate of claim 12, wherein the aluminum salt is selected from the group consisting of aluminum phosphate, aluminum sulfate and/or aluminum hydroxide.
14. The multivalent conjugate of claim 1, wherein the capsular polysaccharides are selected from the group consisting of cross reacting serotypes of bacterial capsular polysaccharides.
15. The multivalent conjugate of claim 14, wherein the bacterial capsular polysaccharides comprise one or more serotypes of *S. pneumoniae, H. influenza* type a and/or b; *S. pneumoniae, Group B Streptococcus*, and/or *N. meningitis*.
16. The multivalent conjugate of claim 1, wherein the capsular polysaccharides comprise polysaccharides derived from *Streptococcus pneumoniae, Haemophilus influenzae, N. meningitis, Group B Streptococcus, or* Moraxella catarrhalis lipo-oligosaccharides (LOS).
17. The multivalent conjugate of claim 16, wherein the S. pneumoniae capsular polysaccharide is immunochemically cross-reactive with serotypes selected from the

group consisting of 6A/6B/6C/6D; 9V/9A/9B.9N; 15A/15B; 19A/19F and similar types of cross reactive polysaccharides.

18. The multivalent conjugate of claim 1, wherein the capsular polysaccharide is derived from *Haemophilus influenzae* serotypes a/b/c/d/e/f, non-typeable Haemophilus influenzae (NTHi) polysaccharides, and/or *Moraxella catarrhalis* Lipooligosaccharides(LOS).
19. The multivalent conjugate of claim 1, wherein one or more of the capsular polysaccharides are derived from *N. meningitis* serotypes A, B, C, Y, W-135 or X.
20. The multivalent conjugate of claim 1, wherein one or more of the capsular polysaccharides are derived from *Group B Streptococcus* serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII, IX, or N.
21. The multivalent conjugate of claim 1, wherein the quantity of a single dose of bivalent cross-reactive polysaccharide conjugates is equivalent to the quantity of individual monovalent conjugates of the same two polysaccharides.
22. The multivalent conjugate of claim 1, wherein a single dose of bivalent cross-reactive polysaccharide conjugates generates a lower immune response to the carrier protein in comparison to monovalent conjugates of the same two polysaccharides.
23. An immunogenic composition for the treatment or prevention of infection by Gram-positive and Gram-negative pathogens comprising a therapeutically effective amount of the multivalent conjugate of claim 1 and a pharmacologically acceptable carrier.
24. The immunogenic composition of claim 23, comprising capsular polysaccharides derived from *Haemophilus influenza, N. meningitis*, *Group B Streptococcus*, *N. meningitis, H. influenza,* and combination thereof.
25. A method for coupling polysaccharides with carrier protein comprising:

    activating the polysaccharide;

    attaching a spacer arm of about 2.0-40Å to the activated polysaccharide; and

    attaching the activated polysaccharide attached to spacer arm to a carrier protein.
26. A method for coupling a carrier protein containing a disulfide with polysaccharides comprising:

    activating the carrier protein to form an activated carrier protein;

    reducing the disulfide of the carrier protein to create a sulfhydryl group;

attaching a spacer arm of 2.0-40Å to the activated carrier protein; and

attaching the polysaccharides to the spacer arm attached to the activated carrier protein.

27. The method of claim 26, wherein the activated carrier protein is selected from the group consisting of cross-reactive material (CRM197) obtained or derived from *C. diptheriae*, or recombinant CRM197 obtained or derived from *P. fluorescens* or *E. coli*.

Figure 1A

6A PS (^{1}H NMR-30KD)

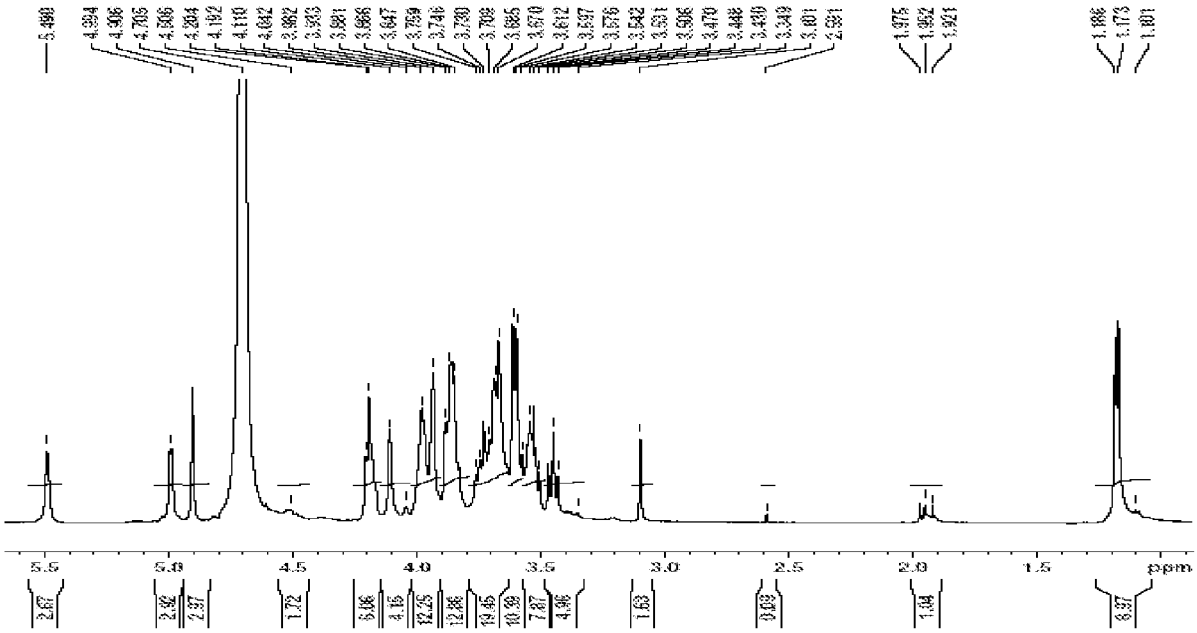


Figure 1B

6B PS (^{1}H NMR-30KD)

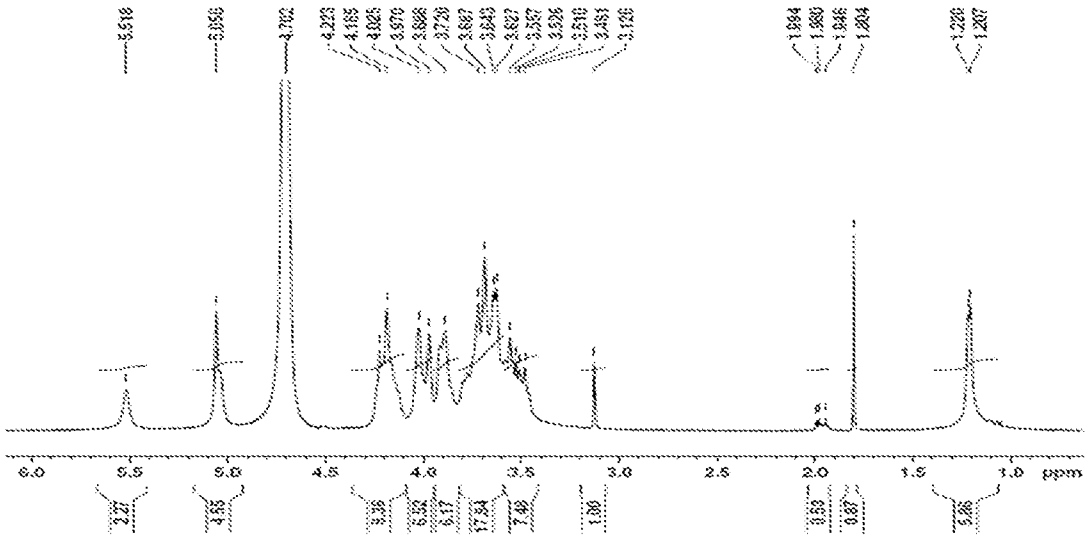


Figure 2A

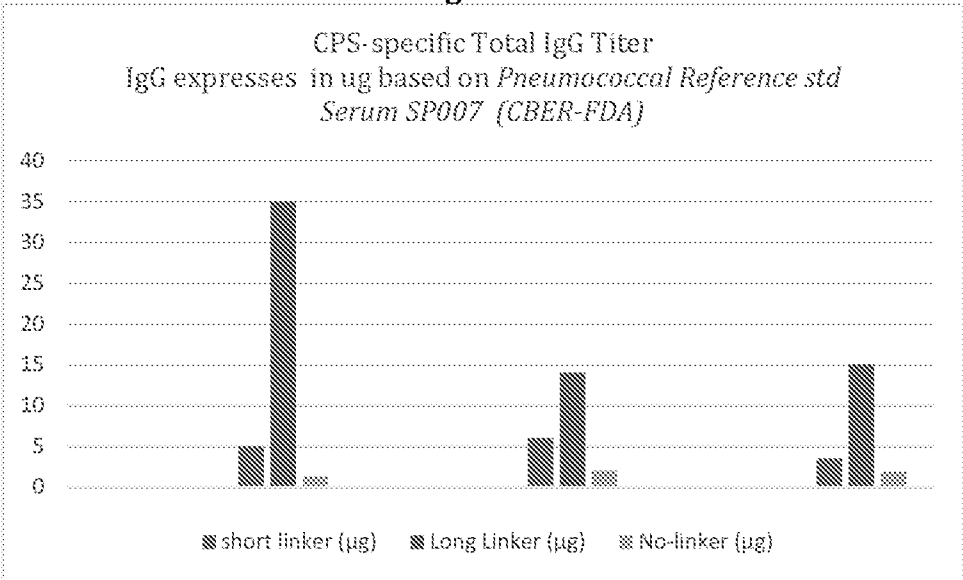


Figure 2B

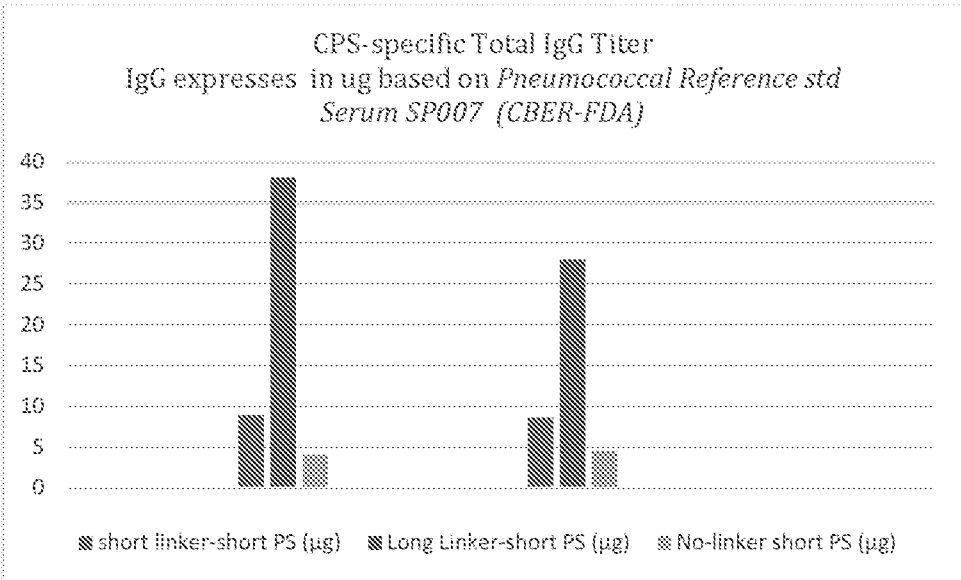


Figure 2C

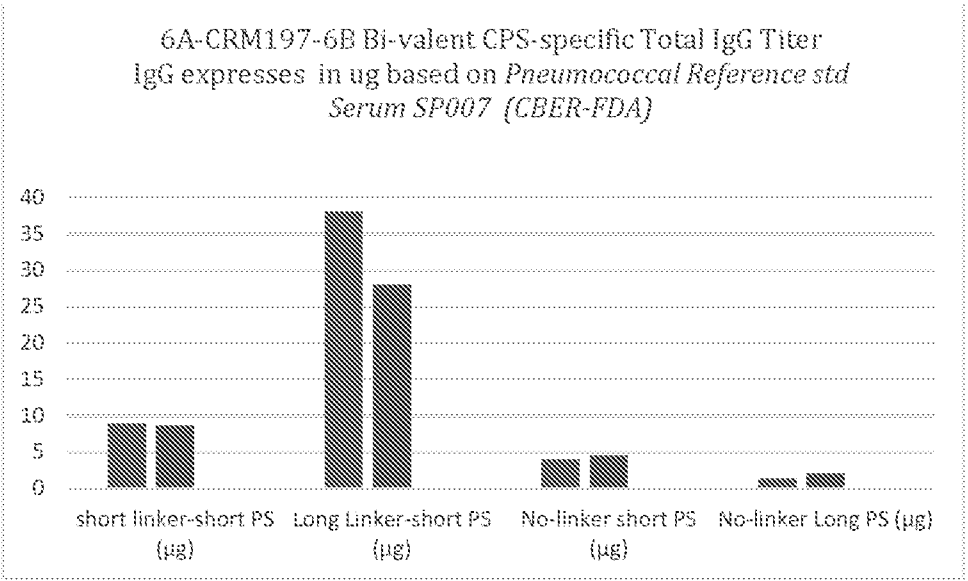

6A-CRM197-6B Bi-valent CPS-specific Total IgG Titer
IgG expresses in ug based on Pneumococcal Reference std Serum SP007 (CBER-FDA)
40
35
30
25
20
15
10
5
0
short linker-short PS (µg)
Long Linker-short PS (µg)
No-linker short PS (µg)
No-linker Long PS (µg)

Figure 3A

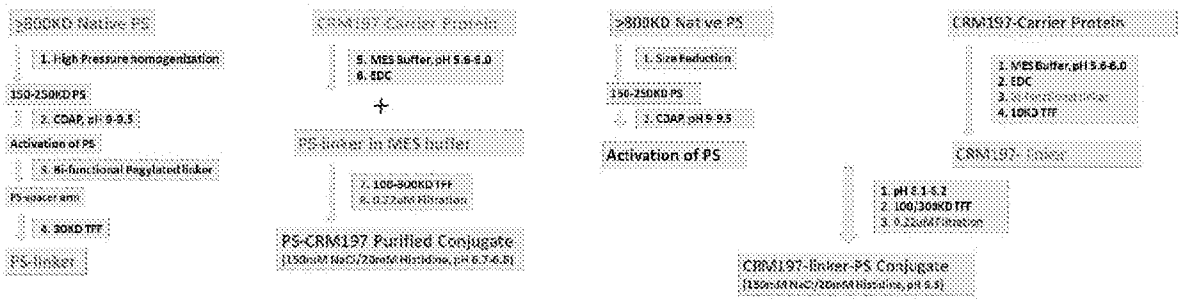


Figure 3B

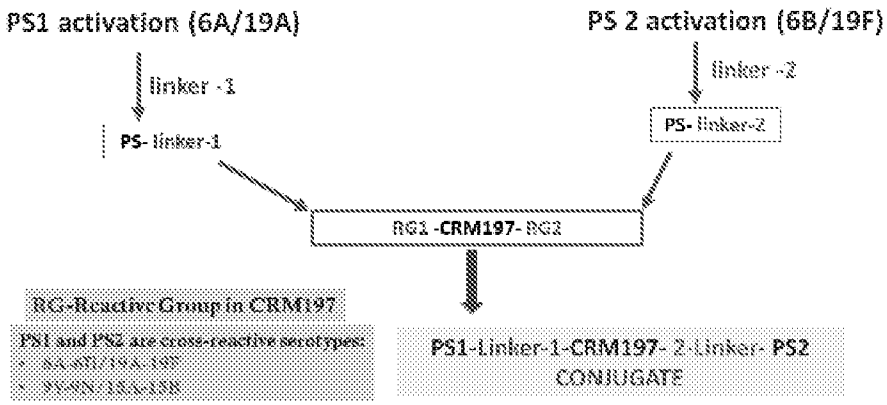


Figure 4A

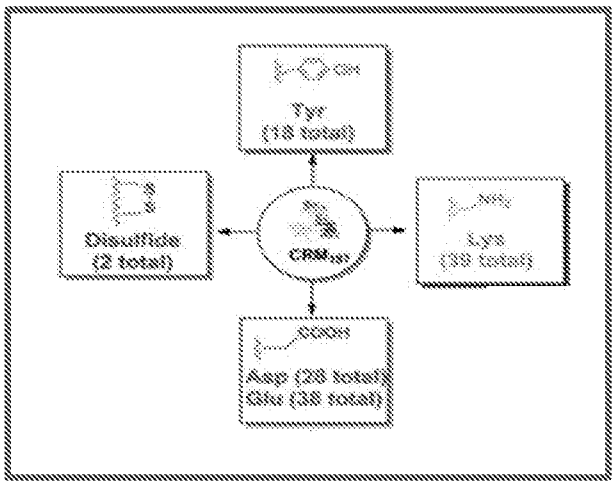


Figure 4B

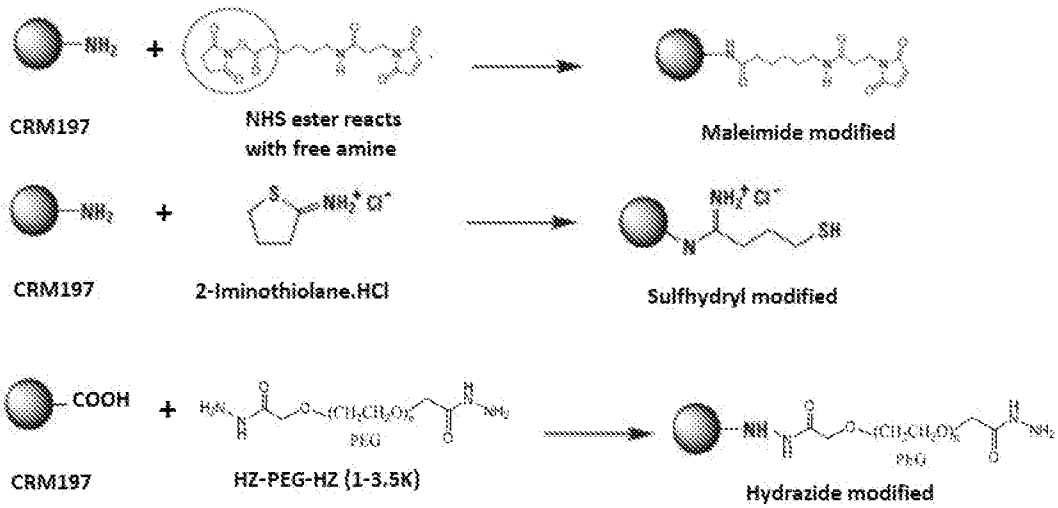


Figure 5

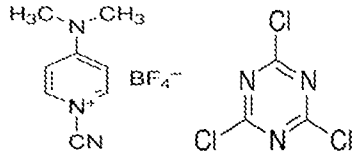
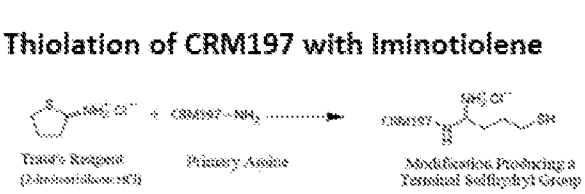


Figure 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/36868

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/09, A61K 47/48 (2018.01)
CPC - A61K 2039/6037, A61K 39/092, A61K 47/48261

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	US 2011/0195086 A1 (CAULFIELD et al.) 11 August 2011 (11.08.2011) Para [0014]; Para [0026];	25 ------- 26-27
A	US 2007/0231340 A1 (HAUSDORFF et al.) 04 October 2007 (04.10.2007) Para [0009]; Para [0010]; Para [0028];	1-24
A	US 2014/0105926 A1 (GLAXOSMITHKLINE BIOLOGICALS S.A.) 17 April 2014 (17.04.2014) Para [0108]; Para [0110]; Para [0382];	1-24
A	US 2004/0096461 A1 (MICHON et al.) 20 May 2004 (20.05.2004) Para [0049]; Para [0053];	1-24
A	WU et al. "Development of pneumococcal polysaccharide conjugate vaccine with long spacer arm", 2013, Vaccine, Vol. 31: pgs. 5623-5626 Pg. 5623, Col.1, Para [1];	25-26
A	US 2015/0216996 A1 (PFIZER INC.) 06 August 2015 (06.08.2015) Para [0054]; Para [0127]; Para [0134];	26-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 October 2018	25 OCT 2018

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/36868

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See Supplemental Box

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/36868

Continuation of Box III: Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-24 are directed to a multivalent conjugate.

Group II: Claims 25-27 are directed to a method for coupling polysaccharides with a carrier protein.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

special technical features

The invention of Group I includes the special technical feature of comprising: first and second group conjugates, wherein: first group conjugates comprise a monovalent capsular polysaccharide conjugate of S. Penumoniae serotypes 1, 2, 3,4, 5, 7F, 8, 10A, 11A, 12F, 14, 17F, 18C, 20, 22F, 23F, 24F, 33F and 35B; and second group conjugates comprising a bivalent or multivalent capsular polysaccharide conjugate of cross reactive serotypes of S. Penumoniae serotypes 6A/6B/6C/6D, S. Penumoniae serotypes 9V/9N/9A/9B, S. Penumoniae serotypes 15B/15A/15C, or S. Penumoniae serotypes 19A/19F with a bifunctional linker not required by the claims of Group II.

The invention of Group II includes the special technical feature of method for coupling polysaccharides with carrier protein comprising: activating the polysaccharide; attaching a spacer arm of about 2.0-40A to the activated polysaccharide; and attaching the activated polysaccharide attached to spacer arm to a carrier protein, not required by the claims of Group I.

common technical features

Groups I and II share the common technical feature of a polysaccharide and a carrier protein. However, this shared technical feature does not represent a contribution over prior art as being anticipated by US 2007/0231340 A1 to Hausdorff et al. (hereinafter 'Huasdorff') which discloses a polysaccharide conjugated to carrier protein (Para [0036]; Accordingly, the present invention provides a multivalent immunogenic composition comprising 13 distinct polysaccharide-protein conjugates, wherein each of the conjugates contains a different capsular polysaccharide conjugated to a carrier protein...).

As the common technical features were known in the art at the time of the invention, these cannot be considered special technical feature that would otherwise unify the groups.

Therefore, Groups I and II lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.