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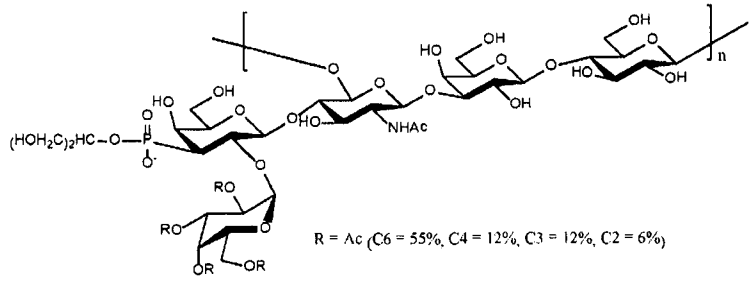
[Continued on next page]

(54) Title: STREPTOCOCCUS PNEUMONIAE CAPSULAR POLYSACCHARIDES AND CONJUGATES THEREOF

Figure 1



→6)-D-Glc*p*NAc-β-(1→3)-D-Gal*p*-β-(1→4)-D-Glc*p*-β-(1→

D-Gal*p*-α-(1→2)-D-Gal*p*(3-OPO$_3$-R^1)-β-(1→4

R^1 = CH(CH$_2$OH)$_2$

$C_{37}H_{61}NNaO_{31}P$, MW=1069.8

Ac= Acetyl

(57) **Abstract**: The invention relates to isolated Streptococcus pneumoniae serotype 15B capsular polysaccharide and processes for their preparation. The invention also relates to immunogenic conjugates comprising Streptococcus pneumoniae serotype 15B capsular polysaccharide covalently linked to a carrier protein, processes for their preparation and immunogenic compositions comprising them.

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Streptococcus pneumoniae capsular polysaccharides and conjugates thereof

Field of the invention

The invention relates to isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide and processes for their preparation. The invention also relates to immunogenic conjugates comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to a carrier protein, processes for their preparation and immunogenic compositions and vaccines comprising them.

Background

Streptococcus pneumoniae are Gram-positive, lancet shaped cocci that are usually seen in pairs (diplococci), but also in short chains or as single cells. They grow readily on blood agar plates with glistening colonies and display alpha hemolysis unless grown anaerobically where they show beta hemolysis. The cells of most pneumococcal serotypes have a capsule which is a polysaccharide coating surrounding each cell. This capsule is a determinant of virulence in humans, as it interferes with phagocytosis by preventing antibodies from attaching to the bacterial cells. Currently there are more than 90 known pneumococcal capsular serotypes identified, with the 23 most common serotypes accounting for approximately 90% of invasive disease worldwide. As a vaccine, the pneumococcal polysaccharide coat can confer a reasonable degree of immunity to *Streptococcus pneumoniae* in individuals with developed or unimpaired immune systems, but the capsular polysaccharide conjugated to a suitable carrier protein allows for an immune response in infants and elderly who are also at most risk for pneumococcal infections.

Since the introduction of the first 7-valent pneumococcal conjugate vaccine (PCV7 or Prevnar) in 2000, invasive disease from those seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) has nearly disappeared. The addition of serotypes 1, 3, 5, 6A, 7F and 19A in Prevnar 13 further decreased the numbers of invasive pneumococcal disease.

However, the incidence of invasive pneumococcal diseases caused by non-vaccine serotypes such as *Streptococcus pneumoniae* serotypes 15A, 15B and 15C has recently increased (see for example Beall B. et al, Journal of Clinical Microbiology. 44(3):999-1017, 2006, or Jacobs et Al, Clin Infect Dis. (2008) 47 (11): 1388-1395). None of the currently marketed pneumococcal vaccine provides an appropriate protection against serotype 15B *Streptococcus pneumoniae* in human and in particular in children less than 2 years old. Therefore, there is a need for immunogenic compositions that can be used to induce an

immune response against serotype 15B *Streptococcus pneumoniae*. It would also be an additional benefit if such immunogenic composition could be used to protect subjects against serotype 15C and/or 15A *Streptococcus pneumoniae*.

Summary of the invention

In one aspect the present disclosure provides an isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide having a molecular weight between 5kDa and 500kDa.

In a further aspect, the present disclosure provides an isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide comprising at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8, preferably at least 0.6 mM, acetate per mM of said *Streptococcus pneumoniae* serotype 15B capsular polysaccharide

In a further aspect, the present disclosure provides an isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide comprising at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8, preferably at least 0.6 mM glycerol per mM of said *Streptococcus pneumoniae* serotype 15B capsular polysaccharide.

In a further aspect, the present disclosure provides an immunogenic conjugate comprising an isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide disclosed herein covalently linked to a carrier protein. In one aspect, said carrier protein is CRM_{197}.

In a further aspect, the present disclosure provides an immunogenic composition comprising an immunogenic conjugate disclosed herein and a physiologically acceptable vehicle. In one aspect, said immunogenic composition further comprises at least one additional antigen. In one aspect, said immunogenic composition further comprises an adjuvant.

In a further aspect, the present disclosure provides a vaccine comprising an immunogenic composition as disclosed herein.

In a further aspect, the present disclosure provides a process for producing an isolated serotype 15B polysaccharide as disclosed herein, the process comprising the steps of:

(a) preparing a fermentation culture of *Streptococcus pneumonia* serotype 15B bacterial cells;

(b) lysing the bacterial cells in said fermentation culture;

(c) purifying *Streptococcus pneumoniae* serotype 15B capsular polysaccharide from the fermentation culture; and,

(d) sizing the purified *Streptococcus pneumoniae* serotype 15B capsular polysaccharide by high pressure homogenization.

In a further aspect, the present disclosure provides a process for producing an activated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide, said process comprising the step of reacting an isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide as disclosed herein with an oxidizing agent. In one aspect, the present disclosure provides an activated serotype 15B capsular polysaccharide obtained or obtainable by the above process.

In a further aspect, the present disclosure provides a process for the preparation of an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to a carrier protein, the process comprising the steps of:

(a) compounding an activated polysaccharide as disclosed herein with a carrier protein;

(b) reacting the compounded, activated polysaccharide and carrier protein with a reducing agent to form a serotype 15B capsular polysaccharide-carrier protein conjugate. In one aspect, the present disclosure provides an immunogenic conjugate obtained or obtainable by the above process.

In a further aspect, the present disclosure provides a method of protecting a subject against an infection with serotype 15B *Streptococcus pneumoniae,* the method comprising administering to a subject an immunogenic amount of the immunogenic composition or the vaccine disclosed herein.

In a further aspect, the present disclosure provides a method of treating or preventing a *Streptococcus pneumoniae* infection, disease or condition associated with serotype 15A, 15B and/or 15C *Streptococcus pneumoniae* in a subject, the method comprising the step of administering a therapeutically or prophylactically effective amount of an immunogenic composition or a vaccine disclosed herein.

Brief description of the drawings

Figure 1 - Structure of Pneumococcal Capsular polysaccharide Serotype 15B Repeat Unit

Detailed description of the invention

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included herein. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, certain preferred

methods and materials are described herein. In describing the embodiments and claiming the invention, certain terminology will be used in accordance with the definitions set out below.

Definitions

As used herein, the "molecular weight" of a polysaccharide or of a polysaccharide-carrier protein conjugate refers to molecular weight calculated by size exclusion chromatography (SEC) combined with multiangle laser light scattering detector (MALLS).

As used herein, the term "free polysaccharide" means a serotype 15B capsular polysaccharide that is not covalently conjugated to the carrier protein, but is nevertheless present in the serotype 15B capsular polysaccharide-carrier protein conjugate composition. The free polysaccharide may be non-covalently associated with (i.e., non-covalently bound to, adsorbed to, or entrapped in or with) the polysaccharide-carrier protein conjugate. The percentage of free polysaccharide is measured after the final purification of the serotype 15B capsular polysaccharide-carrier protein conjugate. Preferably it is measured within 4 weeks after the final purification. It is expressed as a percentage of the total polysaccharide in the sample.

As used herein, the term "serotype 15B polysaccharide" or "serotype 15B capsular polysaccharide" refers to a *Streptococcus pneumoniae* serotype 15B capsular polysaccharide.

As used herein, the term "serotype 15B glycoconjugate" or "serotype 15B conjugate" refers to an isolated serotype 15B polysaccharide covalently conjugated to a carrier protein.

As used herein, the term "degree of oxidation" (DO) refers to the number of sugar repeat units per aldehyde group generated when the isolated polysaccharide is activated with an oxidizing agent. The degree of oxidation of a polysaccharide can be determined using routine methods known to the man skilled in the art.

As used herein, the term "subject" refers to a mammal, including a human, or to a bird, fish, reptile, amphibian or any other animal. The term "subject" also includes household pets or research animals. Non-limiting examples of household pets and research animals include: dogs, cats, pigs, rabbits, rats, mice, gerbils, hamsters, guinea pigs, ferrets, monkeys, birds, snakes, lizards, fish, turtles, and frogs. The term "subject" also includes livestock animals.

Non-limiting examples of livestock animals include: alpaca, bison, camel, cattle, deer, pigs, horses, llamas, mules, donkeys, sheep, goats, rabbits, reindeer, yak, chickens, geese, and turkeys.

Isolated serotype 15B capsular polysaccharide

As shown in figure 1, the polysaccharide repeating unit of serotype 15B consists of a branched trisaccharide backbone (one N-acetylglucosamine (Glc_pNAc), one galactopyranose (Gal_p) and one glucopyranose (Glc_p)) with an αGal_p-βGal_p disaccharide branch linked to the C4 hydroxyl group of Glc_pNAc. The phosphoglycerol is linked to the C3 hydroxyl group of the βGal_p residue in the disaccharide branch. Serotype 15B capsular polysaccharide is O-acetylated and the total amount of O-acetylation is approximately 0.8 to 0.9 O-acetyl groups per polysaccharide repeating unit (see for example C. Jones et Al, *Carbohydrate Research*, 340 (2005) 403-409). Capsular polysaccharide from serotype 15C serotype has the identical backbone structure as serotype 15B but lacks the O-acetylation.

The isolated serotype 15B polysaccharide of the invention can be obtained by a process comprising the steps of:

(a) preparing a fermentation culture of serotype 15B *Streptococcus pneumonia* bacterial cells;

(b) lysing the bacterial cells in said fermentation culture;

(c) purifying serotype 15B polysaccharide from the fermentation culture; and,

(d) sizing the purified serotype 15B polysaccharide by high pressure homogenization.

Serotype 15B polysaccharides can be obtained directly from bacteria using isolation procedures known to one of ordinary skill in the art (see for example methods disclosed U.S. Patent App. Pub. Nos. 20060228380, 20060228381, 20070184071, 20070184072, 20070231340, and 20080102498 or WO2008118752). In addition, they can be produced using synthetic protocols.

Serotype 15B *Streptococcus pneumoniae* strains may be obtained from established culture collections (such as for example ATCC deposit strain No ATCC10354 or strain available from the Streptococcal Reference Laboratory of the Center for disease control and prevention, Atlanta, GA)) or clinical specimens.

The bacterial cells are preferably grown in a soy based medium. Following fermentation of bacterial cells that produce *Streptococcus pneumoniae* serotype 15B capsular polysaccharides, the bacterial cells are lysed to produce a cell lysate. The bacterial cells may be lysed using any lytic agent. A "lytic agent" is any agent that aids in cell wall breakdown and release of autolysin which causes cellular lysis including, for example, detergents. As used herein, the term "detergent" refers to any anionic or cationic detergent capable of inducing lysis of bacterial cells. Representative examples of such detergents for use within the methods of the present invention include deoxycholate sodium (DOC), N-lauroyl sarcosine, chenodeoxycholic acid sodium, and saponins.

In one embodiment of the present invention, the lytic agent used for lysing bacterial cells is DOC. DOC is the sodium salt of the bile acid deoxycholic acid, which is commonly derived from biological sources such as cows or oxen. DOC activates the LytA protein, which is an autolysin that is involved in cell wall growth and division in *Streptococcus pneumoniae*. The LytA protein has choline binding domains in its C-terminal portion, and mutations of the lytA gene are known to produce LytA mutants that are resistant to lysis with DOC.

In one embodiment of the present invention, the lytic agent used for lysing bacterial cells is a non-animal derived lytic agent. Non-animal derived lytic agents for use within the methods of the present invention include agents from non-animal sources with modes of action similar to that of DOC (i. e., that affect LytA function and result in lysis of *Streptococcus pneumoniae* cells). Such non-animal derived lytic agents include, but are not limited to, analogs of DOC, surfactants, detergents, and structural analogs of choline. In one embodiment, the non-animal derived lytic agent is selected from the group consisting of decanesulfonic acid, tert-octylphenoxy poly(oxyethylene)ethanols (e.g. Igepal® CA-630, CAS #: 9002-93-1, available from Sigma Aldrich, St. Louis, MO), octylphenol ethylene oxide condensates (e.g. Triton® X-100, available from Sigma Aldrich, St. Louis, MO), N-lauroyl sarcosine, N-lauroyl sarcosine sodium, lauryl iminodipropionate, sodium dodecyl sulfate, chenodeoxycholate, hyodeoxycholate, glycodeoxycholate, taurodeoxycholate, taurochenodeoxycholate, and cholate. In another embodiment, the non-animal derived lytic agent is N-lauroyl sarcosine. In another embodiment, the lytic agent is N-lauroyl sarcosine sodium.

The serotype 15B polysaccharide may then be isolated from the cell lysate using purification techniques known in the art, including the use of centrifugation, depth filtration, precipitation, ultra-filtration, treatment with activate carbon, diafiltration and/or column chromatography (See, for example, U.S. Patent App. Pub. Nos. 20060228380, 20060228381, 20070184071, 20070184072, 20070231340, and 20080102498 or WO2008118752). The purified serotype 15B capsular polysaccharide can then be used for the preparation of immunogenic conjugates.

Preferably, in order to generate conjugates with advantageous filterability characteristics and/or yields, sizing of the polysaccharide to a lower molecular weight (MW) range is performed prior to the conjugation to a carrier protein. Advantageously, the size of the purified serotype 15B polysaccharide is reduced while preserving critical features of the structure of the polysaccharide such as for example the presence of O-acetyl groups. Preferably, the size of the purified serotype 15B polysaccharide is reduced by mechanical homogenization.

In a preferred embodiment, the size of the purified serotype 15B polysaccharide is reduced by high pressure homogenization. High pressure homogenization achieves high shear rates by pumping the process stream through a flow path with sufficiently small dimensions. The shear rate is increased by using a larger applied homogenization pressure and exposure time can be increased by recirculating the feed stream through the homogenizer.

The high pressure homogenization process is particularly appropriate for reducing the size of the purified serotype 15B polysaccharide while preserving the structural features of the polysaccharide such as the presence of O-acetyl groups.

The isolated serotype 15B capsular polysaccharide obtained by purification of serotype 15B polysaccharide from the *Streptococcus pneumoniae* lysate and optionally sizing of the purified polysaccharide can be characterized by different parameters including for example the molecular weight, the mM of glycerol per mM of said serotype 15B capsular polysaccharide or the mM of acetate per mM of said serotype 15B capsular polysaccharide.

The degree of O-acetylation of the polysaccharide can be determined by any method known in the art, for example, by proton NMR (see for example Lemercinier and Jones (1996) Carbohydrate Research 296; 83-96, Jones and Lemercinier (2002) J. Pharmaceutical and Biomedical Analysis 30; 1233-1247, WO 05/033148 or WO00/56357). Another commonly used method is described in Hestrin (1949) J. Biol. Chem. 180; 249-261. Preferably, the presence of O-acetyl groups is determined by ion-HPLC analysis.

The presence of O-acetyl in a purified, isolated or activated serotype 15B capsular polysaccharide or in a serotype 15B polysaccharide-carrier protein conjugate is expressed as the number of mM of acetate per mM of said polysaccharide or as the number of O-acetyl group per polysaccharide repeating unit.

The presence of glycerolphosphate side chains can be determined by measurement of glycerol using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) after its release by treatment of the polysaccharide with hydrofluoric acid (HF). The presence of glycerol in a purified, isolated or activated serotype 15B polysaccharide or in a serotype 15B polysaccharide-carrier protein conjugate is expressed as the number of mM of glycerol per mM of serotype 15B polysaccharide.

The isolated serotype 15B capsular polysaccharide can also be produced synthetically using methods known to the man skilled in the art.

In a preferred embodiment, the isolated serotype 15B capsular polysaccharide has a molecular weight between 5 and 500kDa, 50 and 500 kDa, 50 and 450kDa, 100 and 400kDa, 100 and 350 kDa. In a preferred embodiment, the isolated serotype 15B capsular polysaccharide has a molecular weight between 100 and 350kDa. In a preferred embodiment, the isolated serotype 15B capsular polysaccharide has a molecular weight between 100 and 300kDa. In a preferred embodiment, the isolated serotype 15B capsular polysaccharide has a molecular weight between 150 and 300kDa.

In a preferred embodiment, the isolated serotype 15B capsular polysaccharide comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM acetate per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the isolated serotype 15B capsular polysaccharide comprises at least 0.5, 0.6 or 0.7 mM acetate per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the isolated serotype 15B capsular polysaccharide comprises at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the isolated serotype 15B capsular polysaccharide comprises at least 0.7 mM acetate per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the presence of O-acetyl groups is determined by ion-HPLC analysis.

In a preferred embodiment, the isolated serotype 15B capsular polysaccharide comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM glycerol per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the isolated serotype 15B capsular polysaccharide comprises at least 0.5, 0.6 or 0.7 mM glycerol per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the isolated serotype 15B capsular polysaccharide comprises at least 0.6 mM glycerol per mM of said serotype 15B capsular

polysaccharide. In a preferred embodiment, the isolated serotype 15B capsular polysaccharide comprises at least 0.7 mM glycerol per mM of said serotype 15B capsular polysaccharide.

In a preferred embodiment, the isolated serotype 15B capsular polysaccharide has a molecular weight between 100 and 350 kDa, preferably 150 and 350kDa, and comprises at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide.

In a preferred embodiment, the isolated serotype 15B capsular polysaccharide has a molecular weight between 100 and 350 kDa, preferably 150 and 350kDa, and comprises at least 0.6 mM glycerol per mM of said serotype 15B capsular polysaccharide.

In a preferred embodiment, the isolated serotype 15B capsular polysaccharide comprises at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide and at least 0.6 mM glycerol per mM of said serotype 15B capsular polysaccharide.

In a preferred embodiment, the isolated serotype 15B capsular polysaccharide has a molecular weight between 100 and 350 kDa, preferably 150 and 350kDa, and comprises at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide and at least 0.6 mM glycerol per mM of said serotype 15B capsular polysaccharide.

Serotype 15B capsular polysaccharide-carrier protein conjugate

The isolated serotype 15B capsular polysaccharide may be conjugated to a carrier protein to obtain an immunogenic conjugate. The isolated polysaccharide can be conjugated to the carrier protein by methods known to the skilled person (See, for example, U.S. Patent App. Pub. Nos. 20060228380, 20070184071, 20070184072, 20070231340 or WO2011/100151).

In an embodiment, the polysaccharide may be activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide may be coupled directly or via a spacer (linker) group to an amino group on the carrier protein. For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using GMBS) or a haloacetylated carrier protein (for example using iodoacetimide or N-succinimidyl bromoacetate or SIAB, or SIA, or SBAP). Preferably, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the

amino-derivatised saccharide is conjugated to the carrier protein using carbodiimide (e.g. EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO93/15760, WO 95/08348 and WO 96129094.

Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with CDI (See Bethell *et al.,* 1979,1. Biol. Chern. 254:2572-4; Hearn *et al.,* 1981, J. Chromatogr.218:509-18) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

In a preferred embodiment, the isolated serotype 15B capsular polysaccharide is conjugated to the carrier protein by reductive amination. Reductive amination involves activation of the polysaccharide by oxidation and conjugation of the activated polysaccharide to a protein carrier by reduction.

Activation of serotype 15B capsular polysaccharide

An activated serotype 15B capsular polysaccharide is obtained by reacting an isolated serotype 15B capsular polysaccharide with an oxidizing agent. For example, said activated serotype 15B capsular polysaccharide can be obtained by a process comprising the following steps:

(a) preparing a fermentation culture of serotype 15B *Streptococcus pneumonia* bacterial cells;

(b) lysing the bacterial cells in said fermentation culture;

(c) purifying serotype 15B polysaccharide from the fermentation culture;

(d) sizing the purified serotype 15B polysaccharide by high pressure homogenization.

(e) reacting the sized serotype 15B polysaccharide with an oxidizing agent.

In a preferred embodiment, the concentration of isolated serotype 15B capsular polysaccharide which is reacted with an oxidizing agent is between 0.1 and 10 mg/mL, 0.5 and 5 mg/mL, 1 and 3 mg/mL, or about 2 mg/mL.

In a preferred embodiment, the oxidizing agent is periodate. The periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. The term 'periodate' includes both periodate and periodic acid. This term also includes both metaperiodate (IO_4^-) and orthoperiodate (IO_6^{5-}). The term 'periodate' also includes the various salts of periodate including sodium periodate and potassium periodate. In a preferred embodiment, the oxidizing agent is sodium periodate. In a preferred embodiment the periodate used for the oxidation of serotype 15B capsular polysaccharide is metaperiodate. In a preferred embodiment the periodate used for the oxidation of serotype 15B capsular polysaccharide is sodium metaperiodate.

In a preferred embodiment, the polysaccharide is reacted with 0.01 to 10, 0.05 to 5, 0.1 to 1, 0.5 to 1, 0.7 to 0.8, 0.05 to 0.5, 0.1 to 0.3 molar equivalent of oxidizing agent. In a preferred embodiment, the polysaccharide is reacted with about 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95 molar equivalent of oxidizing agent. In a preferred embodiment, the polysaccharide is reacted with about 0.15 molar equivalent of oxidizing agent. In a preferred embodiment, the polysaccharide is reacted with about 0.25 molar equivalent of oxidizing agent. In a preferred embodiment, the polysaccharide is reacted with about 0.5 molar equivalent of oxidizing agent. In a preferred embodiment, the polysaccharide is reacted with about 0.6 molar equivalent of oxidizing agent. In a preferred embodiment, the polysaccharide is reacted with about 0.7 molar equivalent of oxidizing agent.

In a preferred embodiment, the duration of the reaction is between 1 and 50, 10 and 30, 15 and 20, 15 and 17 hours or about 16 hours.

In a preferred embodiment, the temperature of the reaction is maintained between 15 and 45°C, 15 and 30°C, 20 and 25°C. In a preferred embodiment, the temperature of the reaction is maintained at about 23°C.

In a preferred embodiment, the oxidation reaction is carried out in a buffer selected from sodium phosphate, potassium phosphate, 2-(N-morpholino)ethanesulfonic acid (MES) or Bis-Tris. In a preferred embodiment, the buffer is potassium phosphate.

In a preferred embodiment, the buffer has a concentration of between 1 and 500 mM, 1 and 300mM, 50 and 200mM. In a preferred embodiment the buffer has a concentration of about 100mM.

In a preferred embodiment, the oxidation reaction is carried out at a pH between 4 and 8, 5 and 7, 5.5 and 6.5. In a preferred embodiment, the pH is about 6.

In preferred embodiment, the activated serotype 15B capsular polysaccharide is obtained by reacting 0.5 to 5 mg/mL of isolated serotype 15B capsular polysaccharide with 0.2 to 0.3 molar equivalent of periodate at a temperature between 20 and 25°C.

In a preferred embodiment, the activated serotype 15B capsular polysaccharide is purified. The activated serotype 15B capsular polysaccharide is purified according to methods known to the man skilled in the art such as gel permeation chromatography (GPC), dialysis or ultrafiltration/diafiltration. For example, the activated capsular polysaccharide is purified by concentration and diafiltration using an ultrafiltration device.

In a preferred embodiment, the invention relates to an activated serotype 15B capsular polysaccharide obtained or obtainable by the above disclosed process.

In a preferred embodiment, the degree of oxidation of the activated serotype 15B capsular polysaccharide is between 2 and 20, 2 and 15, 2 and 10, 2 and 5, 5 and 20, 5 and 15, 5 and 10, 10 and 20, 10 and 15, 15 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 15B capsular polysaccharide is between 2 and 10, 4 and 8, 4 and 6, 6 and 8, 6 and 12, 8 and 12, 9 and 11, 10 and 16, 12 and 16, 14 and 18, 16 and 20, 16 and 18, or 18 and 20.

In a preferred embodiment, the activated serotype 15B capsular polysaccharide has a molecular weight between 5 and 500kDa, 50 and 500 kDa, 50 and 450kDa, 100 and 400kDa, 100 and 350 kDa. In a preferred embodiment, the activated serotype 15B capsular polysaccharide has a molecular weight between 100 and 350kDa. In a preferred embodiment, the activated serotype 15B capsular polysaccharide has a molecular weight between 100 and 300kDa. In a preferred embodiment, the activated serotype 15B capsular polysaccharide has a molecular weight between 150 and 300kDa. In a preferred embodiment, the activated serotype 15B capsular polysaccharide has a molecular weight between 100 and 250kDa.

In a preferred embodiment, the activated serotype 15B capsular polysaccharide comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM acetate per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the activated serotype 15B capsular polysaccharide comprises at least 0.5, 0.6 or 0.7 mM acetate per mM of said serotype 15B

capsular polysaccharide. In a preferred embodiment, the activated serotype 15B capsular polysaccharide comprises at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the activated serotype 15B capsular polysaccharide comprises at least 0.7 mM acetate per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the presence of O-acetyl groups is determined by ion-HPLC analysis.

In a preferred embodiment, the activated serotype 15B capsular polysaccharide comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM glycerol per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the activated serotype 15B capsular polysaccharide comprises at least 0.5, 0.6 or 0.7 mM glycerol per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the activated serotype 15B capsular polysaccharide comprises at least 0.6 mM glycerol per mM of said serotype 15B capsular polysaccharide. In a preferred embodiment, the activated serotype 15B capsular polysaccharide comprises at least 0.7 mM glycerol per mM of said serotype 15B capsular polysaccharide.

In a preferred embodiment, the activated serotype 15B capsular polysaccharide has a molecular weight between 100 and 250 kDa and comprises at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide.

In a preferred embodiment, the activated serotype 15B capsular polysaccharide has a molecular weight between 100 and 250 kDa and comprises at least 0.6 mM glycerol per mM of said serotype 15B capsular polysaccharide.

In a preferred embodiment, the activated serotype 15B capsular polysaccharide comprises at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide and at least 0.6 mM glycerol per mM of said serotype 15B capsular polysaccharide.

In a preferred embodiment, the activated serotype 15B capsular polysaccharide has a molecular weight between 100 and 250 kDa and comprises at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide and at least 0.6 mM glycerol per mM of said serotype 15B capsular polysaccharide.

In an embodiment, the activated serotype 15B capsular polysaccharide is lyophilized, optionally in the presence of cryoprotectant/lyoprotectant. In an embodiment, said cryoprotectant/lyoprotectant is a saccharide. In a preferred embodiment, the saccharide is

selected from sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit. In a preferred embodiment, the saccharide is sucrose. The lyophilized activated capsular polysaccharide can then be compounded with a solution comprising the carrier protein.

In another embodiment, the activated serotype 15B capsular polysaccharide is compounded with the carrier protein and lyophilized optionally in the presence of cryoprotectant/lyoprotectant. In an embodiment, said cryoprotectant/lyoprotectant is a saccharide. In a preferred embodiment, the saccharide is selected from sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit. In a preferred embodiment, the saccharide is sucrose. The co-lyophilized polysaccharide and carrier protein can then be resuspended in solution and reacted with a reducing agent.

In an embodiment, the invention relates to a lyophilized activated serotype 15B capsular polysaccharide.

In an embodiment the invention relates to the co-lyophilized activated serotype 15B capsular polysaccharide and protein carrier. In a preferred embodiment, the protein carrier is CRM_{197}.

Conjugation of activated serotype 15B capsular polysaccharide with a carrier protein

The activated serotype 15B capsular polysaccharide can be conjugated to a carrier protein by a process comprising the step of:
(a) compounding the activated serotype 15B capsular polysaccharide with a carrier protein, and,
(b) reacting the compounded activated serotype 15B capsular polysaccharide and carrier protein with a reducing agent to form a serotype 15B capsular polysaccharide-carrier protein conjugate.

The conjugation of activated serotype 15B capsular polysaccharide with a protein carrier by reductive amination in dimethylsulfoxide (DMSO) is suitable to preserve the O-acetyl content of the polysaccharide as compared for example to reductive amination in aqueous solution where the level of O-acetylation of the polysaccharide is significantly reduced. In a preferred embodiment, step (a) and step (b) are carried out in DMSO.

In a preferred embodiment, step (a) comprises dissolving lyophilized serotype 15B capsular polysaccharide in a solution comprising a carrier protein and DMSO. In a preferred

embodiment, step (a) comprises dissolving co-lyophilized serotype 15B capsular polysaccharide and carrier protein in DMSO.

When steps (a) and (b) are carried out in aqueous solution, steps (a) and (b) are carried out in a buffer, preferably selected from PBS, MES, HEPES, Bis-tris, ADA, PIPES. MOPSO, BES, MOPS, DIPSO, MOBS, HEPPSO, POPSO, TEA, EPPS, Bicine or HEPB, at a pH between 6.0 and 8.5, 7 and 8 or 7 and 7.5. In a preferred embodiment the buffer is PBS. In a preferred embodiment the pH is about 7.3.

In a preferred embodiment, the concentration of activated serotype 15B capsular polysaccharide in step (b) is between 0.1 and 10 mg/mL, 0.5 and 5 mg/mL, 0.5 and 2 mg/mL. In a preferred embodiment, the concentration of activated serotype 15B capsular polysaccharide in step (b) is about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or 3 mg/mL.

In a preferred embodiment the initial input ratio (weight by weight) of activated serotype 15B capsular polysaccharide to carrier protein is between 5:1 and 0.1:1, 2:1 and 0.1:1, 2:1 and 1:1, 1.5:1 and 1:1, 0.1:1 and 1:1, 0.3:1 and 1:1, 0.6:1 and 1:1.

In a preferred embodiment the initial input ratio of activated serotype 15B capsular polysaccharide to carrier protein is about 0.6:1 to 1.5:1, preferably 0.6:1 to 1:1. Such initial input ratio is particularly suitable to obtain low levels of free polysaccharide in the immunogenic conjugate.

In a preferred embodiment the initial input ratio of activated serotype 15B capsular polysaccharide to carrier protein is about 0.4:1, 0.5:1, 0.6:1, 0.7:1, 0.8:1, 0.9:1, 1:1, 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, 1.9:1 or 2:1.

In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxyborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMe^{i}PrN-BH_3, benzylamine-BH_3 or 5-ethyl-2-methylpyridine borane (PEMB). In a preferred embodiment, the reducing agent is sodium cyanoborohydride. In a preferred embodiment, the reducing agent is sodium 2-Picoline Borane.

In a preferred embodiment, the quantity of reducing agent used in step (b) is between about 0.1 and 10 molar equivalents, 0.5 and 5 molar equivalents, 1 and 2 molar equivalents. In a

preferred embodiment, the quantity of reducing agent used in step (b) is about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9 or 2 molar equivalents.

In a preferred embodiment, the duration of step (b) is between 1 and 60 hours, 10 and 50 hours, 40 and 50 hours; 42 and 46 hours. In a preferred embodiment, the duration of step (b) is about 44 hours.

In a preferred embodiment, the temperature of the reaction in step (b) is maintained between 10 and 40°C, 15 and 30°C or 20 and 26°C. In a preferred embodiment, the temperature of the reaction in step (b) is maintained at about 23°C.

In a preferred embodiment, the process for the preparation of an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to a carrier protein further comprises a step (step (c)) of capping unreacted aldehyde (quenching) by addition of $NaBH_4$.

In a preferred embodiment, the quantity of $NaBH_4$ used in step (c) is between 0.1 and 10 molar equivalents, 0.5 and 5 molar equivalent 1 and 3 molar equivalents. In a preferred embodiment, the quantity of $NaBH_4$ used in step (c) is about 2 molar equivalents.

In a preferred embodiment, the duration of step (c) is between 0.1 and 10 hours, 0.5 and 5 hours, 2 and 4 hours. In a preferred embodiment, the duration of step (c) is about 3 hours.

In a preferred embodiment, the temperature of the reaction in step (c) is maintained between 15 and 45°C, 15 and 30°C or 20 and 26°C. In a preferred embodiment, the temperature of the reaction in step (c) is maintained at about 23°C.

In a preferred embodiment the yield of the conjugation step (step b) is greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or 90%. In a preferred embodiment the yield of the conjugation step (step b) is greater than 60%. In a preferred embodiment the yield of the conjugation step (step b) is greater than 70%. The yield is the amount of serotype 15B polysaccharide in the conjugate x100 / amount of activated polysaccharide used in the conjugation step.

In a preferred embodiment, the process for the preparation of an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to a carrier protein comprises the steps of:

(a) preparing a fermentation culture of serotype 15B *Streptococcus pneumonia* bacterial cells;

(b) lysing the bacterial cells in said fermentation culture;

(c) purifying serotype 15B polysaccharide from the fermentation culture;

(d) sizing the purified serotype 15B polysaccharide by high pressure homogenization;

(e) reacting the sized serotype 15B polysaccharide with an oxidizing agent;

(f) compounding the activated serotype 15B polysaccharide with a carrier protein, and,

(g) reacting the compounded activated serotype 15B polysaccharide and carrier protein with a reducing agent to form a serotype 15B polysaccharide-carrier protein conjugate; and,

(h) capping unreacted aldehyde (quenching) by addition of $NaBH_4$.

In a preferred embodiment the yield of the conjugation step (step g) of the above process is greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or 90%. In a preferred embodiment the yield of the conjugation step (step g) is greater than 60%. In a preferred embodiment the yield of the conjugation step (step g) is greater than 70%. The yield is the amount of serotype 15B polysaccharide in the conjugate x100) / amount of activated polysaccharide used in the conjugation step.

After conjugation of the serotype 15B capsular polysaccharide to the carrier protein, the polysaccharide-protein conjugate can be purified (enriched with respect to the amount of polysaccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration, precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration.

In a preferred embodiment the carrier protein is non-toxic and non-reactogenic and obtainable in sufficient amount and purity. Carrier proteins should be amenable to standard conjugation procedures.

In a preferred embodiment, the activated serotype 15B capsular polysaccharide is conjugated to a carrier protein which is selected in the group consisiting of: DT (Diphtheria toxin), TT (tetanus toxid) or fragment C of TT, CRM_{197} (a nontoxic but antigenically identical variant of diphtheria toxin) other DT point mutants, such as CRM176, CRM228, CRM 45 (Uchida et al J. Biol. Chem. 218; 3838-3844, 1973); CRM 9, CRM102, CRM 103 and CRM107 and other mutations described by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc, 1992; deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala 158 to Gly and other mutations disclosed in US 4709017 or US 4950740;

mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in US 5917017 or US 6455673; or fragment disclosed in US 5843711, pneumococcal pneumolysin (Kuo et al (1995) Infect Immun 63; 2706-13) including ply detoxified in some fashion for example dPLY-GMBS (WO 04081515, PCT/EP2005/010258) or dPLY-formol, PhtX, including PhtA, PhtB, PhtD, PhtE (sequences of PhtA, PhtB, PhtD or PhtE are disclosed in WO 00/37105 or WO 00/39299) and fusions of Pht proteins for example PhtDE fusions, PhtBE fusions, Pht A-E (WO 01/98334, WO 03/54007, W02009/000826), OMPC (meningococcal outer membrane protein - usually extracted from N.meningitidis serogroup B - EP0372501), PorB (from N. meningitidis), PD (Haemophilus influenza protein D - see, e.g., EP 0 594 610 B), or immunologically functional equivalents thereof, synthetic peptides (EP0378881 , EP0427347), heat shock proteins (WO 93/17712, WO 94/03208), pertussis proteins (WO 98/58668, EP0471 177), cytokines, lymphokines, growth factors or hormones (WO10 91/01146), artificial proteins comprising multiple human CD4+ T cell epitopes from various pathogen derived antigens (Falugi et al (2001) Eur J Immunol 31 ; 3816-3824) such as N19 protein (Baraldoi et al (2004) Infect Immun 72; 4884-7) pneumococcal surface protein PspA (WO 02/091998), iron uptake proteins (WO 01/72337), toxin A or B of C. difficile (WO 00/61761). In an embodiment, the activated serotype 15B capsular polysaccharide is conjugated to DT (Diphtheria toxoid). In another embodiment, the activated serotype 15B capsular polysaccharide is conjugated to TT (tetanus toxid). In another embodiment, the activated serotype 15B capsular polysaccharide is conjugated to fragment C of TT. In another embodiment, the activated serotype 15B capsular polysaccharide is conjugated to PD (Haemophilus influenza protein D - see, e.g., EP 0 594 610 B).

In a preferred embodiment, the activated serotype 15B capsular polysaccharide of the invention is conjugated to CRM_{197} protein. The CRM_{197} protein is a nontoxic form of diphtheria toxin but is immunologically indistinguishable from the diphtheria toxin. CRM_{197} is produced by C. diphtheriae infected by the nontoxigenic phage $\beta197^{tox-}$ created by nitrosoguanidine mutagenesis of the toxigenic corynephage beta (Uchida, T. et al. 1971, Nature New Biology 233:8-11). CRM_{197} is purified through ultrafiltration, ammonium sulfate precipitation, and ion-exchange chromatography. The CRM_{197} protein has the same molecular weight as the diphtheria toxin but differs therefrom by a single base change (guanine to adenine) in the structural gene. This single base change causes an amino acid substitution glutamic acid for glycine) in the mature protein and eliminates the toxic properties of diphtheria toxin. The CRM_{197} protein is a safe and effective T-cell dependent

carrier for saccharides. Further details about CRM_{197} and production thereof can be found e.g. in US 5,614,382.

In an embodiment, the invention relate to an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to a carrier protein. In an embodiment, the invention relate to an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to a carrier protein by reductive amination. In an embodiment, the invention relate to an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to a carrier protein by reductive amination in DMSO. In a preferred embodiment, the carrier protein is CRM_{197}. In a preferred embodiment, the polysaccharide is an isolated serotype 15B capsular polysaccharide as defined herein. In a preferred embodiment, the polysaccharide is an isolated serotype 15B capsular polysaccharide as defined herein which has been sized by high pressure homogenization.

In a preferred embodiment, the immunogenic conjugate comprises less than about 50, 45, 40, 35, 30, 25, 20 or 15% of free serotype 15B capsular polysaccharide compared to the total amount of serotype 15B capsular polysaccharide. In a preferred embodiment the immunogenic conjugate comprises less than about 25% of free serotype 15B capsular polysaccharide compared to the total amount of serotype 15B capsular polysaccharide. In a preferred embodiment the immunogenic conjugate comprises less than about 20% of free serotype 15B capsular polysaccharide compared to the total amount of serotype 15B capsular polysaccharide. In a preferred embodiment the immunogenic conjugate comprises less than about 15% of free serotype 15B capsular polysaccharide compared to the total amount of serotype 15B capsular polysaccharide.

In a preferred embodiment, the immunogenic conjugate has a molecular weight between 3000 and 20000kDa; 5000 and 10000kDa; 5000 and 20000kDa; 8000 and 20000kDa; 8000 and 16000 KDa; or 10000 and 16000 KDa. The molecular weight of the immunogenic conjugate is measured by SEC-MALLS.

In a preferred embodiment, the ratio (weight by weight) of serotype 15B capsular polysaccharide to carrier protein in the conjugate is between 0.5 and 3. In a preferred embodiment, the ratio of serotype 15B capsular polysaccharide to carrier protein in the conjugate is between 0.4 and 2, 0.5 and 2, 0.5 and 1.5, 0.5 and 1, 1 and 1.5, 1 and 2. In a

preferred embodiment, the ratio of serotype 15B capsular polysaccharide to carrier protein in the conjugate is between 0.7 and 0.9.

Size exclusion chromatography media (CL-4B) can be used to determine the relative molecular size distribution of the conjugate. Size Exclusion Chromatography (SEC) is used in gravity fed columns to profile the molecular size distribution of conjugates. Large molecules excluded from the pores in the media elute more quickly than small molecules. Fraction collectors are used to collect the column eluate. The fractions are tested colorimetrically by saccharide assay. For the determination of Kd, columns are calibrated to establish the fraction at which molecules are fully excluded (V_0), (Kd=0), and the fraction representing the maximum retention (V_i), (Kd=1). The fraction at which a specified sample attribute is reached (V_e), is related to Kd by the expression, $Kd = (V_e - V_0)/ (V_i - V_0)$.

In a preferred embodiment, at least 20% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 30% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 40% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 60% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 70% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.
In a preferred embodiment, between 40% and 90% of the serotype 15B immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 90% of the serotype 15B immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 65% and 80% of the serotype 15B immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

In a preferred embodiment, the immunogenic conjugate comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM acetate per mM serotype 15B capsular polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.5, 0.6 or 0.7 mM acetate per mM serotype 15B capsular polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.6 mM acetate per mM serotype 15B capsular polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.7 mM acetate per mM serotype 15B capsular polysaccharide. In a preferred embodiment, the presence of O-acetyl groups is determined by ion-HPLC analysis.

In a preferred embodiment, the ratio of mM acetate per mM serotype 15B capsular polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 15B capsular polysaccharide in the isolated polysaccharide is at least 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, or 0.95. In a preferred embodiment, the ratio of mM acetate per mM serotype 15B capsular polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 15B capsular polysaccharide in the isolated polysaccharide is at least 0.7. In a preferred embodiment, the ratio of mM acetate per mM serotype 15B capsular polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 15B capsular polysaccharide in the isolated polysaccharide is at least 0.9. In a preferred embodiment, the presence of O-acetyl groups is determined by ion-HPLC analysis.

In a preferred embodiment, the ratio of mM acetate per mM serotype 15B capsular polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 15B capsular polysaccharide in the activated polysaccharide is at least 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, or 0.95. In a preferred embodiment, the ratio of mM acetate per mM serotype 15B capsular polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 15B capsular polysaccharide in the activated polysaccharide is at least 0.7. In a preferred embodiment, the ratio of mM acetate per mM serotype 15B capsular polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 15B capsular polysaccharide in the activated polysaccharide is at least 0.9. In a preferred embodiment, the presence of O-acetyl groups is determined by ion-HPLC analysis.

In a preferred embodiment, the immunogenic conjugate comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM glycerol per mM serotype 15B capsular polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.5, 0.6 or 0.7 mM glycerol per mM serotype 15B capsular polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.6 mM glycerol per mM serotype 15B capsular polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.7 mM glycerol per mM serotype 15B capsular polysaccharide.

The degree of conjugation is the number of lysine residues in the carrier protein that are conjugated to serotype 15B capsular polysaccharide. The evidence for lysine modification of the carrier protein, due to covalent linkages to the polysaccharides, is obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered, compared to the CRM_{197} protein starting material used to generate the conjugate materials.

In a preferred embodiment, the degree of conjugation of the immunogenic conjugate is between 2 and 15, 2 and 13, 2 and 10, 2 and 8, 2 and 6, 2 and 5, 2 and 4, 3 and 15, 3 and 13, 3 and 10, 3 and 8, 3 and 6, 3 and 5, 3 and 4, 5 and 15, 5 an 10, 8 and 15, 8 and 12, 10 and 15 or 10 and 12. In a preferred embodiment, the degree of conjugation of the immunogenic conjugate is between 2 and 5.

Immunogenic composition

The term "immunogenic composition" relates to any pharmaceutical composition containing an antigen, *e.g.*, a microorganism or a component thereof, which composition can be used to elicit an immune response in a subject.
As used herein, "immunogenic" means an ability of an antigen (or an epitope of the antigen), such as a bacterial capsular polysaccharide, or an immunogenic conjugate or immunogenic composition comprising an antigen, to elicit an immune response in a host such as a mammal, either humorally or cellularly mediated, or both.

In an embodiment, the disclosure relate to an immunogenic composition comprising an immunogenic serotype 15B capsular polysaccharide-carrier protein conjugate disclosed herein.

In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of binding to serotype 15B *Streptococcus pneumonia.* In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of binding to serotype 15B *Streptococcus pneumonia* as measured by a standard ELISA assay.

In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of binding to serotype 15B and 15A and/or 15C *Streptococcus pneumonia*. In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of binding to serotype 15B and 15A and/or 15C *Streptococcus pneumonia* as measured by a standard ELISA assay.

In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of binding to serotype 15B and 15C *Streptococcus pneumonia*. In an embodiment, the immunogenic composition disclosed

herein, when administered to a subject, induces the formation of antibodies capable of binding to serotype 15B and 15C *Streptococcus pneumonia* as measured by a standard ELISA assay.

In the ELISA (Enzyme-linked Immunosorbent Assay) method, antibodies from the sera of vaccinated subjects are incubated with polysaccharides which have been adsorbed to a solid support. The bound antibodies are detected using enzyme-conjugated secondary detection antibodies.

In an embodiment said ELISA assay is the standardized (WHO) ELISA assay as defined by the WHO in the 'Training manual for Enzyme linked immunosorbent assay for the quantitation of Streptococcus pneumoniae serotype specific IgG (Pn PS ELISA).' (accessible at http://www.vaccine.uab.edu/ELISA%20protocol.pdf ; accessed on March 31st, 2014).

The ELISA measures type specific IgG anti-*S. pneumoniae* capsular polysaccharide (PS) antibodies present in human serum. When dilutions of human sera are added to type-specific capsular PS-coated microtiter plates, antibodies specific for that capsular PS bind to the microtiter plates. The antibodies bound to the plates are detected using a goat anti-human IgG alkaline phosphatase-labeled antibody followed by a p-nitrophenyl phosphate substrate. The optical density of the colored end product is proportional to the amount of anticapsular PS antibody present in the serum.

In an embodiment, the immunogenic composition of the invention is able to elicit IgG antibodies in human which are capable of binding *S. pneumoniae* serotypes 15B polysaccharide at a concentration of at least 0.05, 0.1, 0.2, 0.3, 0.35, 0.4 or 0.5 µg/ml as determined by ELISA assay.

In an embodiment, the immunogenic composition of the invention is able to elicit IgG antibodies in human which are capable of binding *S. pneumoniae* serotypes 15C polysaccharide at a concentration of at least 0.05, 0.1, 0.2, 0.3, 0.35, 0.4 or 0.5 µg/ml as determined by ELISA assay.

In an embodiment, the immunogenic composition of the invention is able to elicit IgG antibodies in human which are capable of binding *S. pneumoniae* serotypes 15B and 15C polysaccharide at a concentration of at least 0.05, 0.1, 0.2, 0.3, 0.35, 0.4 or 0.5 µg/ml as determined by ELISA assay.

In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of killing serotype 15B *Streptococcus* pneumonia in an opsonophagocytosis assay (OPA) as disclosed herein. In an embodiment, the immunogenic composition disclosed herein, when tested in an OPA assay as disclosed herein, has an OPA titer greater than the OPA titer obtained with an unconjugated native *Streptococcus pneumonia* serotype 15B capsular polysaccharide.

In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of killing serotype 15C *Streptococcus* pneumonia in an opsonophagocytosis assay as disclosed herein. In an embodiment, the immunogenic composition disclosed herein, when tested in an OPA assay as disclosed herein, has an OPA titer greater than the OPA titer obtained with an unconjugated native *Streptococcus pneumonia* serotype 15C capsular polysaccharide.

In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of killing serotype 15B and 15C and/or 15A *Streptococcus pneumonia* in an opsonophagocytosis assay as disclosed herein.

In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of killing serotype 15B and 15C.

The pneumococcal opsonophagocytic assay (OPA), which measures killing of *S. pneumoniae* cells by phagocytic effector cells in the presence of functional antibody and complement, is considered to be an important surrogate for evaluating the effectiveness of pneumococcal vaccines.

Opsonophagocytic assay (OPA) can be conducted by incubating together a mixture of *Streptococcus pneumoniae* cells, a heat inactivated human serum to be tested, differentiated HL-60 cells (phagocytes) and an exogenous complement source (e.g. baby rabbit complement). Opsonophagocytosis proceeds during incubation and bacterial cells that are coated with antibody and complement are killed upon opsonophagocytosis. Colony forming units (cfu) of surviving bacteria that escape from opsonophagocytosis are determined by plating the assay mixture. The OPA titer is defined as the reciprocal dilution that results in a 50% reduction in bacterial count over control wells without test serum. The OPA titer is interpolated from the two dilutions that encompass this 50% killing cut-off.

An endpoint titer of 1:8 or greater is considered a positive result in these killing type OPA.

In an embodiment, the immunogenic composition of the invention is able to elicit a titer of at least 1:8 against *S. pneumoniae* serotype 15B in at least 50% of the subjects as determined by opsonophagocytic killing assay (OPA). In an embodiment, the immunogenic composition of the invention is able to elicit a titer of at least 1:8 against *S. pneumoniae* serotype 15B in at least 60%; 70%, 80%, 90%, or at least 93% of the subjects as determined by opsonophagocytic killing assay (OPA).

In an embodiment, the immunogenic composition of the invention is able to elicit a titer of at least 1:8 against *S. pneumoniae* serotype 15C in at least 50% of the subjects as determined by opsonophagocytic killing assay (OPA). In an embodiment, the immunogenic composition of the invention is able to elicit a titer of at least 1:8 against *S. pneumoniae* serotype 15C in at least 60%; 70%, 80%, 90%, or at least 95% of the subjects as determined by opsonophagocytic killing assay (OPA).

Formulation of the immunogenic composition of the present invention can be accomplished using art-recognized methods. For instance, the immunogenic conjugates of the invention can be formulated with a physiologically acceptable vehicle to prepare the composition. Examples of such vehicles include, but are not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions.

In a preferred embodiment, the immunogenic composition may comprise at least one additional antigen. In a preferred embodiments, the immunogenic composition may comprises at least one additional *Streptococcus pneumoniae* capsular polysaccharide.

In a preferred embodiment, the immunogenic composition may comprise at least one additional *Streptococcus pneumoniae* capsular polysaccharide conjugated to a carrier protein. In a preferred embodiment, said carrier protein is CRM_{197}.

In certain embodiments, the immunogenic composition comprises one or more adjuvants. As defined herein, an "adjuvant" is a substance that serves to enhance the immunogenicity of an immunogenic composition of this invention. Thus, adjuvants are often given to boost the immune response and are well known to the skilled artisan. Suitable adjuvants to enhance effectiveness of the composition include, but are not limited to:

(1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.;

(2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (defined below) or bacterial cell wall components), such as, for example,

(a) MF59 (PCT Pub. No. WO 90/14837), containing 5% Squalene, 0.5%5 Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below, although not required)) formulated into submicron particles using a microfluidizer such as Model 11OY microfluidizer (Microfluidics, Newton, MA),

(b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and

(c) Ribi™ adjuvant system (RAS), (Corixa, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of 3-O-deaylated monophosphorylipid A (MPL™) described in U.S. Patent No. 4,912,094 (Corixa), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™);

(3) saponin adjuvants, such as Quil A or STIMULON™ QS-21 (Antigenics, Framingham, MA) (U.S. Patent No. 5,057,540) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes);

(4) bacteriallipopolysaccharides, synthetic lipid A analogs such as aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa, and which are described in U.S. Patent No.6, 113,918; one such AGP is 2-[(R)-3-Tetradecanoyloxytetradecanoylamino]ethyl 2-Deoxy-4-Ophosphono-3-O-[(R)-3-tetradecanoyloxytetradecanoyl]-2-[(R)-3tetradecanoyloxytetradecanoylamino]-b-D-glucopyranoside, which is also know as 529 (formerly known as RC529), which is formulated as an aqueous form or as a stable emulsion, synthetic polynucleotides such as oligonucleotides containing CpG motif(s) (U.S. Patent No. 6,207,646);

(5) cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-15, IL-18, etc.), interferons (e.g., gamma interferon), granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), costimulatory molecules 87-1 and 87-2, etc.;

(6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT) either in a wild-type or mutant form, for example, where the glutamic acid at amino acid position 29 is replaced by another amino acid, preferably a histidine, in accordance with published international patent application number WO 00/18434 (see also WO 02/098368 and WO 02/098369), a pertussis toxin (PT), or an E. coli heat-labile toxin (LT), particularly LT-K63, LT-R72, CT-S109, PT-K9/G129 (see, e.g.,WO 93/13302 and WO 92/19265); and

(7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

In an embodiment of the present invention, the immunogenic compositions as disclosed herein comprise a CpG Oligonucleotide as adjuvant. A CpG oligonucleotide as used herein refers to an immunostimulatory CpG oligodeoxynucleotide (CpG ODN), and accordingly these terms are used interchangeably unless otherwise indicated. Immunostimulatory CpG oligodeoxynucleotides contain one or more immunostimulatory CpG motifs that are unmethylated cytosine-guanine dinucleotides, optionally within certain preferred base contexts. The methylation status of the CpG immunostimulatory motif generally refers to the cytosine residue in the dinucleotide. An immunostimulatory oligonucleotide containing at least one unmethylated CpG dinucleotide is an oligonucleotide which contains a 5' unmethylated cytosine linked by a phosphate bond to a 3' guanine, and which activates the immune system through binding to Toll-like receptor 9 (TLR-9). In another embodiment the immunostimulatory oligonucleotide may contain one or more methylated CpG dinucleotides, which will activate the immune system through TLR9 but not as strongly as if the CpG motif(s) was/were unmethylated. CpG immunostimulatory oligonucleotides may comprise one or more palindromes that in turn may encompass the CpG dinucleotide. CpG oligonucleotides have been described in a number of issued patents, published patent applications, and other publications, including U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068.

In an embodiment of the present invention, the immunogenic compositions as disclosed herein comprise any of the CpG Oligonucleotide described at pages 3 lines 22 to page 12 line 36 of WO2010/125480.

Different classes of CpG immunostimulatory oligonucleotides have been identified. These are referred to as A, B, C and P class, and are described in greater detail at pages 3 lines 22 to page 12 line 36 of WO2010/125480. Methods of the invention embrace the use of these different classes of CpG immunostimulatory oligonucleotides.

In an embodiment of the present invention, the immunogenic compositions as disclosed herein comprise an A class CpG Oligonucleotide. Preferably, the "A class" CpG oligonucleotide of the invention has the following nucleic acid sequence: 5' GGGGACGACGTCGTGGGGGGG 3' (SEQ ID NO: 1). Some non-limiting examples of A-Class oligonucleotides include: 5' G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G 3' (SEQ ID NO: 2) ; wherein * refers to a phosphorothioate bond and _ refers to a phosphodiester bond.

In an embodiment of the present invention, the immunogenic compositions as disclosed herein comprise a B class CpG Oligonucleotide. In one embodiment, the CpG oligonucleotide for use in the present invention is a B class CpG oligonucleotide represented by at least the formula:

5' $X_1X_2CGX_3X_4$ 3', wherein X1, X2, X3, and X4 are nucleotides. In one embodiment, X_2 is adenine, guanine, or thymine. In another embodiment, X_3 is cytosine, adenine, or thymine.

The B class CpG oligonucleotide sequences of the invention are those broadly described above in US patents Ps 6,194,388, 6,207,646, 6,214,806, 6,218,371, 6,239,116 and 6,339,068. Exemplary sequences include but are not limited to those disclosed in these latter applications and patents.

In an embodiment, the "B class" CpG oligonucleotide of the invention has the following nucleic acid sequence:

5' TCGTCGTTTTTCGGTGCTTTT 3' (SEQ ID NO: 3), or

5' TCGTCGTTTTTCGGTCGTTTT 3' (SEQ ID NO: 4), or

5' TCGTCGTTTTGTCGTTTTGTCGTT 3' (SEQ ID NO: 5), or

5' TCGTCGTTTCGTCGTTTTGTCGTT 3' (SEQ ID NO: 6), or

5' TCGTCGTTTTGTCGTTTTTTTCGA 3' (SEQ ID NO: 7).

In any of these sequences, all of the linkages may be all phosphorothioate bonds. In another embodiment, in any of these sequences, one or more of the linkages may be phosphodiester, preferably between the "C" and the "G" of the CpG motif making a semi-soft CpG oligonucleotide. In any of these sequences, an ethyl-uridine or a halogen may substitute for the 5' T; examples of halogen substitutions include but are not limited to bromo-uridine or iodo-uridine substitutions.

Some non-limiting examples of B-Class oligonucleotides include:

5' T*C*G*T*C*G*T*T*T*T*T*C*G*G*T*G*C*T*T*T*T 3' (SEQ ID NO: 8), or

5' T*C*G*T*C*G*T*T*T*T*T*C*G*G*T*C*G*T*T*T*T 3' (SEQ ID NO: 9), or

5' T*C*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T 3' (SEQ ID NO: 10), or

5' T*C*G*T*C*G*T*T*T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T 3' (SEQ ID NO: 11), or

5' T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*T*T*T*C*G*A 3' (SEQ ID NO: 12).

wherein * refers to a phosphorothioate bond.

In an embodiment of the present invention, the immunogenic compositions as disclosed herein comprise a C class CpG Oligonucleotide. In an embodiment, the "C class" CpG oligonucleotides of the invention has the following nucleic acid sequence:

5' TCGCGTCGTTCGGCGCGCGCCG 3' (SEQ ID NO: 13), or

5' TCGTCGACGTTCGGCGCGCGCCG 3' (SEQ ID NO: 14), or

5' TCGGACGTTCGGCGCGCGCCG 3' (SEQ ID NO: 15), or

5' TCGGACGTTCGGCGCGCCG 3' (SEQ ID NO: 16), or

5' TCGCGTCGTTCGGCGCGCCG 3' (SEQ ID NO: 17), or
5' TCGACGTTCGGCGCGCGCCG 3' (SEQ ID NO: 18), or
5' TCGACGTTCGGCGCGCCG 3' (SEQ ID NO: 19), or
5' TCGCGTCGTTCGGCGCCG 3' (SEQ ID NO: 20), or
5' TCGCGACGTTCGGCGCGCGCCG 3' (SEQ ID NO: 21), or
5' TCGTCGTTTTCGGCGCGCGCCG 3' (SEQ ID NO: 22), or
5' TCGTCGTTTTCGGCGGCCGCCG 3' (SEQ ID NO: 23), or
5' TCGTCGTTTTACGGCGCCGTGCCG 3' (SEQ ID NO: 24), or
5' TCGTCGTTTTCGGCGCGCGCCGT 3' (SEQ ID NO: 25).

In any of these sequences, all of the linkages may be all phosphorothioate bonds. In another embodiment, in any of these sequences, one or more of the linkages may be phosphodiester, preferably between the "C" and the "G" of the CpG motif making a semi-soft CpG oligonucleotide.

Some non-limiting examples of C-Class oligonucleotides include:
5' T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' (SEQ ID NO: 26), or
5' T*C_G*T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' (SEQ ID NO: 27), or
5' T*C_G*G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' (SEQ ID NO: 28), or
5' T*C_G*G*A*C_G*T*T*C_G*G*C*G*C*G*C*C*G 3' (SEQ ID NO: 29), or
5' T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C*G*C*C*G 3' (SEQ ID NO: 30), or
5' T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' (SEQ ID NO: 31), or
5' T*C_G*A*C_G*T*T*C_G*G*C*G*C*G*C*C*G 3' (SEQ ID NO: 32), or
5' T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C*C*G 3' (SEQ ID NO: 33), or
5' T*C_G*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3' (SEQ ID NO: 34), or
5' T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G 3' (SEQ ID NO: 35), or
5' T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*G*C*C*G*C*C*G 3' (SEQ ID NO: 36), or
5' T*C*G*T*C_G*T*T*T*T*A*C_G*G*C*G*C*C_G*T*G*C*C*G 3' (SEQ ID NO: 37), or
5' T*C_G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*G*C*C*G*T 3' (SEQ ID NO: 38)
wherein * refers to a phosphorothioate bond and _ refers to a phosphodiester bond.

In any of these sequences, an ethyl-uridine or a halogen may substitute for the 5' T; examples of halogen substitutions include but are not limited to bromo-uridine or iodo-uridine substitutions.

In an embodiment of the present invention, the immunogenic compositions as disclosed herein comprise a P class CpG Oligonucleotide. In an embodiment, the CpG oligonucleotide for use in the present invention is a P class CpG oligonucleotide containing a 5' TLR activation domain and at least two palindromic regions, one palindromic region being a 5' palindromic region of at least 6 nucleotides in length and connected to a 3' palindromic

region of at least 8 nucleotides in length either directly or through a spacer, wherein the oligonucleotide includes at least one YpR dinucleotide. In an embodiment, said oligoonucleotide is not T*C_G*T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G (SEQ ID NO: 27). In one embodiment the a P class CpG oligonucleotide includes at least one unmethylated CpG dinucleotide. In another embodiment the TLR activation domain is TCG, TTCG, TTTCG, TYpR, TTYpR, TTTYpR, UCG, UUCG, UUUCG, TTT, or TTTT. In yet another embodiment the TLR activation domain is within the 5' palindromic region. In another embodiment the TLR activation domain is immediately 5' to the 5' palindromic region.

In an embodiment, the "P class" CpG oligonucleotides of the invention has the following nucleic acid sequence: 5' TCGTCGACGATCGGCGCGCGCCG 3' (SEQ ID NO: 39).

In said sequences, all of the linkages may be all phosphorothioate bonds. In another embodiment, one or more of the linkages may be phosphodiester, preferably between the "C" and the "G" of the CpG motif making a semi-soft CpG oligonucleotide. In any of these sequences, an ethyl-uridine or a halogen may substitute for the 5' T; examples of halogen substitutions include but are not limited to bromo-uridine or iodo-uridine substitutions.

A non-limiting example of P-Class oligonucleotides include:

5' T*C_G*T*C_G*A*C_G*A*T*C_G*G*C*G*C_G*C*G*C*C*G 3' (SEQ ID NO: 40)

wherein * refers to a phosphorothioate bond and _ refers to a phosphodiester bond.

In one embodiment the oligonucleotide includes at least one phosphorothioate linkage. In another embodiment all internucleotide linkages of the oligonucleotide are phosphorothioate linkages. In another embodiment the oligonucleotide includes at least one phosphodiester-like linkage. In another embodiment the phosphodiester-like linkage is a phosphodiester linkage. In another embodiment a lipophilic group is conjugated to the oligonucleotide. In one embodiment the lipophilic group is cholesterol.

In an embodiment, all the internucleotide linkage of the CpG oligonucleotides disclosed herein are phosphodiester bonds ("soft" oligonucleotides, as described in the PCT application WO2007/026190). In another embodiment, CpG oligonucleotides of the invention are rendered resistant to degradation (e.g., are stabilized). A "stabilized oligonucleotide " refers to an oligonucleotide that is relatively resistant to *in vivo* degradation (e.g. via an exo- or endo-nuclease). Nucleic acid stabilization can be accomplished via backbone modifications. Oligonucleotides having phosphorothioate linkages provide maximal activity and protect the oligonucleotide from degradation by intracellular exo- and endo-nucleases.

The immunostimulatory oligonucleotides may have a chimeric backbone, which have combinations of phosphodiester and phosphorothioate linkages. For purposes of the instant invention, a chimeric backbone refers to a partially stabilized backbone, wherein at least one internucleotide linkage is phosphodiester or phosphodiester-like, and wherein at least one other internucleotide linkage is a stabilized internucleotide linkage, wherein the at least one

phosphodiester or phosphodiester-like linkage and the at least one stabilized linkage are different. When the phosphodiester linkage is preferentially located within the CpG motif such molecules are called "semi-soft" as described in the PCT application WO2007/026190.

The size of the CpG oligonucleotide (i.e., the number of nucleotide residues along the length of the oligonucleotide) also may contribute to the stimulatory activity of the oligonucleotide. For facilitating uptake into cells, CpG oligonucleotide of the invention preferably have a minimum length of 6 nucleotide residues. Oligonucleotides of any size greater than 6 nucleotides (even many kb long) are capable of inducing an immune response if sufficient immunostimulatory motifs are present, because larger oligonucleotides are degraded inside cells. In certain embodiments, the CpG oligonucleotides are 6 to 100 nucleotides long, preferentially 8 to 30 nucleotides long. In important embodiments, nucleic acids and oligonucleotides of the invention are not plasmids or expression vectors.

In an embodiment, the CpG oligonucleotides disclosed herein comprise substitutions or modifications, such as in the bases and/or sugars as described at paragraph 134 to 147 of WO2007/026190.

In an embodiment, the CpG oligonucleotide of the present invention is chemically modified. Examples of chemical modifications are known to the skilled person and are described, for example in Uhlmann E. et al. (1990), Chem. Rev. 90:543, S. Agrawal, Ed., Humana Press, Totowa, USA 1993; Crooke, S.T. et al. (1996) Annu. Rev. Pharmacol. Toxicol. 36:107-129; and Hunziker J. et al., (1995), Mod. Synth. Methods 7:331-417. An oligonucleotide according to the invention may have one or more modifications, wherein each modification is located at a particular phosphodiester internucleoside bridge and/or at a particular β-D-ribose unit and/or at a particular natural nucleoside base position in comparison to an oligonucleotide of the same sequence which is composed of natural DNA or RNA.

In some embodiments of the invention, CpG-containing nucleic acids might be simply mixed with immunogenic carriers according to methods known to those skilled in the art (see, e.g. WO03/024480).

In a particular embodiment of the present invention, any of the immunogenic composition disclosed herein comprises from 2μg to 100mg of CpG oligonucleotide, preferably from 0.1mg to 50 mg CpG oligonucleotide, preferably from 0.2mg to 10 mg CpG oligonucleotide, preferably from 0.3 mg to 5 mg CpG oligonucleotide, even preferably from 0.5 to 2 mg CpG oligonucleotide, even preferably from 0.75 to 1.5 mg CpG oligonucleotide. In a preferred embodiment, the immunogenic composition disclosed herein comprises approximately 1mg CpG oligonucleotide.

In a preferred embodiment, the adjuvant is an aluminum-based adjuvant selected from the group consisting of aluminum phosphate, aluminum sulfate and aluminum hydroxide. In one

embodiment, the immunogenic compositions described herein comprise the adjuvant aluminum phosphate.

In a preferred embodiments, the immunogenic compositions of the invention further comprise at least one of a buffer, a cryoprotectant, a salt, a divalent cation, a non-ionic detergent, an inhibitor of free radical oxidation, a diluent or a carrier.

The immunogenic composition optionally can comprise one or more physiologically acceptable buffers selected from, but not limited to Tris (trimethamine), phosphate, acetate, borate, citrate, glycine, histidine and succinate. In certain embodiments, the formulation is buffered to within a pH range of about 5.0 to about 7.0, preferably from about 5.5 to about 6.5.

The immunogenic composition optionally can comprise one or more non-ionic surfactants, including but not limited to polyoxyethylene sorbitan fatty acid esters, Polysorbate-80 (Tween 80), Polysorbate-60 (Tween 60), Polysorbate-40 (Tween 40) and Polysorbate-20 (Tween 20), polyoxyethylene alkyl ethers, including but not limited to Brij 58, Brij 35, as well as others such as Triton X-100; Triton X- 114, NP40, Span 85 and the Pluronic series of non-ionic surfactants (e. g. , Pluronic 121). In a preferred embodiment, the immunogenic composition comprises Polysorbate-80 or Polysorbate-40, preferably Polysorbate-80. In a preferred embodiment, the immunogenic composition comprises Polysorbate-80 at a concentration from about 0.001% to about 2% (with up to about 0.25% being preferred) or Polysorbate-40 at a concentration from about 0.001% to 1% (with up to about 0.5% being preferred).

The invention further relates to vaccines comprising the immunogenic composition of the invention.

Methods for inducing an immune response and protecting against infection

The present disclosure also includes methods of use for immunogenic compositions described herein. For example, one embodiment of the disclosure provides a method of inducing an immune response against *Streptococcus pneumoniae*, comprising administering to a subject an immunogenic amount of any of the immunogenic compositions described herein.

One embodiment of the disclosure provides a method of protecting a subject against an infection with *Streptococcus pneumoniae,* or a method of preventing infection with

Streptococcus pneumoniae, or a method of reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by *Streptococcus pneumoniae,* the methods comprising administering to a subject an immunogenic amount of any of the immunogenic compositions described herein.

One embodiment of the disclosure provides a method of protecting a subject against an infection with serotype 15B *Streptococcus pneumoniae,* or a method of preventing infection with serotype 15B *Streptococcus pneumoniae*, or a method of reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by serotype 15B *Streptococcus pneumoniae,* the methods comprising administering to a subject an immunogenic amount of any of the immunogenic compositions described herein.

One embodiment of the disclosure provides a method of protecting a subject against an infection with serotype 15C *Streptococcus pneumoniae,* or a method of preventing infection with serotype 15C *Streptococcus pneumoniae*, or a method of reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by serotype 15C *Streptococcus pneumoniae,* the methods comprising administering to a subject an immunogenic amount of any of the immunogenic compositions described herein.

One embodiment of the disclosure provides a method of protecting a subject against an infection with serotype 15A *Streptococcus pneumoniae,* or a method of preventing infection with serotype 15A *Streptococcus pneumoniae*, or a method of reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by serotype 15A *Streptococcus pneumoniae,* the methods comprising administering to a subject an immunogenic amount of any of the immunogenic compositions described herein.

One embodiment of the disclosure provides a method of treating or preventing a *Streptococcus pneumoniae* infection, disease or condition associated with serotype 15A, 15B and/or 15C (preferably 15B and/or 15C, more preferably 15B) *Streptococcus pneumoniae* in a subject, the method comprising the step of administering a therapeutically or prophylactically effective amount of an immunogenic composition described herein to the subject. Another embodiment provides a method of treating or preventing a *Streptococcus pneumoniae* infection, disease or condition associated with a serotype 15A, 15B and/or 15C (preferably 15B and/or 15C, more preferably 15B) *Streptococcus pneumoniae* in a subject, the method comprising generating a polyclonal or monoclonal antibody preparation from the immunogenic composition described herein, and using said antibody preparation to confer passive immunity to the subject.

In one embodiment, the disclosure relates to the use of the immunogenic conjugate or immunogenic composition disclosed herein for the manufacture of a medicament for protecting a subject against an infection with *Streptococcus pneumoniae,* and/or preventing infection with *Streptococcus pneumoniae*, and/or reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by *Streptococcus pneumoniae,* and/or protecting a subject against an infection with serotype 15A, 15B and/or 15C (preferably 15B and/or 15C, more preferably 15B) *Streptococcus pneumoniae* and/or preventing infection with serotype 15A, 15B and/or 15C (preferably 15B and/or 15C, more preferably 15B) *Streptococcus pneumoniae*, and/or reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by serotype 15A, 15B and/or 15C (preferably 15B and/or 15C, more preferably 15B) *Streptococcus pneumoniae.*

In one embodiment, the disclosure relates to the use of the immunogenic conjugate or immunogenic composition disclosed herein for protecting a subject against an infection with *Streptococcus pneumoniae,* and/or preventing infection with *Streptococcus pneumoniae*, and/or reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by *Streptococcus pneumoniae,* and/or protecting a subject against an infection with serotype 15A, 15B and/or 15C (preferably 15B and/or 15C, more preferably 15B) *Streptococcus pneumoniae* and/or preventing infection with serotype 15A, 15B and/or 15C (preferably 15B and/or 15C, more preferably 15B) *Streptococcus pneumoniae*, and/or reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by serotype 15A, 15B and/or 15C (preferably 15B and/or 15C, more preferably 15B) *Streptococcus pneumoniae.*

An "immune response" to an immunogenic composition is the development in a subject of a humoral and/or a cell-mediated immune response to molecules present in the immunogenic composition or vaccine composition of interest. For purposes of the present disclosure, a "humoral immune response" is an antibody-mediated immune response and involves the induction and generation of antibodies that recognize and bind with some affinity for the antigen in the immunogenic composition or vaccine of the disclosure, while a "cell-mediated immune response" is one mediated by T-cells and/or other white blood cells. A "cell-mediated immune response" is elicited by the presentation of antigenic epitopes in association with Class I or Class II molecules of the major histocompatibility complex (MHC), CD1 or other non-classical MHC-like molecules. This activates antigen-specific CD4+ T helper cells or CD8+ cytotoxic T lymphocyte cells ("CTLs"). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by classical or non-classical

MHCs and expressed on the surfaces of cells. CTLs help induce and promote the intracellular destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide or other antigens in association with classical or non-classical MHC molecules on their surface. A "cell-mediated immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells. The ability of a particular antigen or composition to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, by assaying for T-lymphocytes specific for the antigen in a sensitized subject, or by measurement of cytokine production by T cells in response to re-stimulation with antigen. Such assays are well known in the art. *See, e.g.*, Erickson *et al.* (1993) *J. Immunol.* 151:4189-4199; and Doe *et al.* (1994) *Eur. J. Immunol.* 24:2369-2376.

As used herein, "treatment" (including variations thereof, *e.g.*, "treat" or "treated") means any one or more of the following: (i) the prevention of infection or re-infection, as in a traditional vaccine, (ii) the reduction in the severity of, or, in the elimination of symptoms, and (iii) the substantial or complete elimination of the pathogen or disorder in question. Hence, treatment may be effected prophylactically (prior to infection) or therapeutically (following infection). In the present disclosure, prophylactic treatment is the preferred mode. According to a particular embodiment of the present disclosure, compositions and methods are provided that treat, including prophylactically and/or therapeutically immunize, a host animal against a serotype 15A, 15B and/or 15C (preferably 15B and/or 15C, more preferably 15B) *Streptococcus pneumoniae* infection. The methods of the present disclosure are useful for conferring prophylactic and/or therapeutic immunity to a subject. The methods of the present disclosure can also be practiced on subjects for biomedical research applications.

An "immunogenic amount", and "immunologically effective amount," both of which are used interchangeably herein, refers to the amount of antigen or immunogenic composition sufficient to elicit an immune response, either a cellular (T-cell) or humoral (B-cell or antibody) response, or both, as measured by standard assays known to one skilled in the art.

In a preferred embodiment, said subject is a human. In a most preferred embodiment, said subject is a newborn (i.e. under three months of age), an infant (from 3 months to one year of age) or a toddler (i.e. from one year to four years of age).

In an embodiment, the immunogenic compositions disclosed herein are for use as a vaccine.

In such embodiment, the subject to be vaccinated may be less than 1 year of age. For example, the subject to be vaccinated can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 months of age. In an embodiment, the subject to be vaccinated is about 2, 4 or 6 months of age. In another embodiment, the subject to be vaccinated is less than 2 years of age. For example the subject to be vaccinated can be about 12-15 months of age. In some cases, as little as one dose of the immunogenic composition according to the invention is needed, but under some circumstances, a second, third or fourth dose may be given (see regimen section).

In an embodiment of the present invention, the subject to be vaccinated is a human adult 50 years of age or older, more preferably a human adult 55 years of age or older. In an embodiment, the subject to be vaccinated is a human adult 65 years of age or older, 70 years of age or older, 75 years of age or older or 80 years of age or older.

In an embodiment the subject to be vaccinated is an immunocompromised individual, in particular a human. An immunocompromised individual is generally defined as a person who exhibits an attenuated or reduced ability to mount a normal humoral or cellular defense to challenge by infectious agents.

In an embodiment of the present invention, the immunocompromised subject to be vaccinated suffers from a disease or condition that impairs the immune system and results in an antibody response that is insufficient to protect against or treat pneumococcal disease.

In an embodiment, said disease is a primary immunodeficiency disorder. Preferably, said primary immunodeficiency disorder is selected from the group consisting of: combined T- and B-cell immunodeficiencies, antibody deficiencies, well-defined syndromes, immune dysregulation diseases, phagocyte disorders, innate immunity deficiencies, autoinflammatory disorders, and complement deficiencies. In an embodiment, said primary immunodeficiency disorder is selected from the one disclosed on page 24 line 11 to page 25 line 19 of the PCT application WO2010/125480.

In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated suffers from a disease selected from the groups consisting of: HIV-infection, acquired immunodeficiency syndrome (AIDS), cancer, chronic heart or lung disorders,

congestive heart failure, diabetes mellitus, chronic liver disease, alcoholism, cirrhosis, spinal fluid leaks, cardiomyopathy, chronic bronchitis, emphysema, Chronic obstructive pulmonary disease (COPD), spleen dysfunction (such as sickle cell disease), lack of spleen function (asplenia), blood malignancy, leukemia, multiple myeloma, Hodgkin's disease, lymphoma, kidney failure, nephrotic syndrome and asthma.

In an embodiment of the present invention, the immunocompromised subject to be vaccinated suffers from malnutrition.

In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated is taking a drug or treatment that lowers the body's resistance to infection. In an embodiment, said drug is selected from the one disclosed on page 26 line 33 to page 26 line 40 of the PCT application WO2010/125480.

In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated is a smoker.

In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated has a white blood cell count (leukocyte count) below 5 x 10^9 cells per liter, or below 4 x 10^9 cells per liter, or below 3 x 10^9 cells per liter, or below 2 x 10^9 cells per liter, or below 1 x 10^9 cells per liter, or below 0.5 x 10^9 cells per liter, or below 0.3 x 10^9 cells per liter, or below 0.1 x 10^9 cells per liter.

White blood cell count (leukocyte count): The number of white blood cells (WBCs) in the blood. The WBC is usually measured as part of the CBC (complete blood count). White blood cells are the infection-fighting cells in the blood and are distinct from the red (oxygen-carrying) blood cells known as erythrocytes. There are different types of white blood cells, including neutrophils (polymorphonuclear leukocytes; PMNs), band cells (slightly immature neutrophils), T-type lymphocytes (T cells), B-type lymphocytes (B cells), monocytes, eosinophils, and basophils. All the types of white blood cells are reflected in the white blood cell count. The normal range for the white blood cell count is usually between 4,300 and 10,800 cells per cubic millimeter of blood. This can also be referred to as the leukocyte count and can be expressed in international units as 4.3 - 10.8 x 10^9 cells per liter.

In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated suffers from neutropenia. In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated has a neutrophil count below 2 x 10^9 cells per liter, or below 1 x 10^9 cells per liter, or below 0.5 x 10^9 cells per liter, or below 0.1 x 10^9 cells per liter, or below 0.05 x 10^9 cells per liter.

A low white blood cell count or "neutropenia" is a condition characterized by abnormally low levels of neutrophils in the circulating blood. Neutrophils are a specific kind of white blood cell that help prevent and fight infections. The most common reason that cancer patients experience neutropenia is as a side effect of chemotherapy. Chemotherapy-induced neutropenia increases a patient's risk of infection and disrupts cancer treatment.

In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated has a CD4+ cell count below 500/mm^3, or CD4+ cell count below 300/mm^3, or CD4+ cell count below 200/mm^3, CD4+ cell count below 100/mm^3, CD4+ cell count below 75/mm^3, or CD4+ cell count below 50/mm^3.

CD4 cell tests are normally reported as the number of cells in mm^3. Normal CD4 counts are between 500 and 1600, and CD8 counts are between 375 and 1100. CD4 counts drop dramatically in people with HIV.

In an embodiment of the invention, any of the immunocompromised subject disclosed herein is a human male or a human female.

The amount of a conjugate in a composition is generally calculated based on total polysaccharide, conjugated and non-conjugated for that conjugate. For example, a conjugate with 20% free polysaccharide will have about 80 µg of conjugated polysaccharide and about 20 µg of non-conjugated polysaccharide in a 100 µg polysaccharide dose. The protein contribution to the conjugate is usually not considered when calculating the dose of a conjugate. Generally, each dose will comprise 0.1 to 100 µg of polysaccharide, particularly 0.1 to 10 µg, and more particularly 1 to 10 µg and more particularly 1 to 5 µg. Preferably each dose will comprise about 1.1, 2, 2.2, 3, 3.3, 4, 4.4 µg of polysaccharide.
Optimal amounts of components for a particular immunogenic composition or vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects can receive one or several booster immunizations adequately spaced.

The effectiveness of an antigen as an immunogen, can be measured either by proliferation assays, by cytolytic assays, such as chromium release assays to measure the ability of a T-cell to lyse its specific target cell, or by measuring the levels of B-cell activity by measuring the levels of circulating antibodies specific for the antigen in serum. An immune response may also be detected by measuring the serum levels of antigen specific antibody induced following administration of the antigen, and more specifically, by measuring the

ability of the antibodies so induced to enhance the opsonophagocytic ability of particular white blood cells, as described herein. The level of protection of the immune response may be measured by challenging the immunized host with the antigen that has been administered. For example, if the antigen to which an immune response is desired is a bacterium, the level of protection induced by the immunogenic amount of the antigen is measured by detecting the percent survival or the percent mortality after challenge of the animals with the bacterial cells. In one embodiment, the amount of protection may be measured by measuring at least one symptom associated with the bacterial infection, *e.g.*, a fever associated with the infection. The amount of each of the antigens in the multi-antigen or multi-component vaccine or immunogenic compositions will vary with respect to each of the other components and can be determined by methods known to the skilled artisan. Such methods would include procedures for measuring immunogenicity and/ or *in vivo* efficacy.

The disclosure further provides antibodies and antibody compositions which bind specifically and selectively to the capsular polysaccharides or immunogenic conjugates of the present disclosure. In some embodiments, antibodies are generated upon administration to a subject of the capsular polysaccharides or immunogenic conjugates of the present disclosure. In some embodiments, the disclosure provides purified or isolated antibodies directed against one or more of the capsular polysaccharides or immunogenic conjugates of the present disclosure. In some embodiments, the antibodies of the present disclosure are functional as measured by killing bacteria in either an animal efficacy model or via an opsonophagocytic killing assay. In some embodiments, the antibodies of the disclosure confer passive immunity to a subject. The present disclosure further provides polynucleotide molecules encoding an antibody or antibody fragment of the disclosure, and a cell, cell line (such as hybridoma cells or other engineered cell lines for recombinant production of antibodies) or a transgenic animal that produces an antibody or antibody composition of the disclosure, using techniques well-known to those of skill in the art.

Examples

Example 1: Preparation of isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide

1.1 Fermentation and Purification

Serotype 15B capsular polysaccharides can be obtained directly from bacteria using isolation procedures known to one of ordinary skill in the art (see for example methods disclosed U.S. Patent App. Pub. Nos. 20060228380, 20060228381, 20070184071, 20070184072,

20070231340, and 20080102498 or WO2008118752). The serotype 15B *Streptococcus pneumonia* were grown in a seed bottle and then transferred to a seed fermentor. Once the targeted optical density was reached, the cells were transferred to a production fermentor. The fermentation was broth was inactivated by the addition of N-lauroyl sarcosine and purified by ultrafiltration and diafiltration.

The purified *Streptococcus pneumoniae* serotype 15B polysaccharide was then sized by high pressure homogenization using a PANDA 2K homogenizer ® (GEA Niro Soavi) to produce the isolated *Streptococcus pneumoniae* serotype 15B polysaccharide.

Preferably, the isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide obtained by the above process comprises at least 0.6 mM acetate per mM of serotype 15B capsular polysaccharide and has a molecular weight between 50kDa and 500kDa, preferably 150 to 350kDa.

1.2 Oxidation of Isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide

Polysaccharide oxidation was carried out in 100 mM potassium phosphate buffer (pH 6.0 ± 0.2) by sequential addition of calculated amount of 500 mM potassium phosphate buffer (pH 6.0) and WFI to give final polysaccharide concentration of 2.0 g/L. If required, the reaction pH was adjusted to pH 6.0, approximately. After pH adjustment, the reaction temperature was adjusted to 23 ± 2 °C. Oxidation was initiated by the addition of approximately 0.25 molar equivalents of sodium periodate. The oxidation reaction was performed at 23 ± 2 °C during 16 hrs, approximately.

Concentration and diafiltration of the activated polysaccharide was carried out using 10K MWCO ultrafiltration cassettes. Diafiltration was performed against 20-fold diavolumes of WFI. The purified activated polysaccharide was then stored at 5 ± 3°C. The purified activated saccharide was characterized inter alia by (i) saccharide concentration by colorimetric assay; (ii) aldehyde concentration by colorimetric assay; (iii) Degree of Oxidation (iv) Molecular Weight by SEC-MALLS and (v) presence of O-acetyl and glycerol.

SEC-MALLS is used for the determination of the molecular weight of polysaccharides and polysaccharide-protein conjugates. SEC is used to separate the polysaccharides by hydrodynamic volume. Refractive index (RI) and multi-angle laser light scattering (MALLS) detectors are used for the determination of the molecular weight. When light interacts with matter, it scatters and the amount of scattered light is related to the concentration, the square

of the dn/dc (the specific refractive index increments), and the molar mass of the matter. The molecular weight measurement is calculated based on the readings from the scattered light signal from the MALLS detector and the concentration signal from the RI detector.

The degree of oxidation (DO = moles of sugar repeat unit / moles of aldehyde) of the activated polysaccharide was determined as follows:

The moles of sugar repeat unit is determined by various colorimetric methods, example by using Anthrone method. By the Anthrone mthod, the polysaccharide is first broken down to monosaccharides by the action of sulfuric acid and heat. The Anthrone reagent reacts with the hexoses to form a yellow-green colored complex whose absorbance is read spectrophotometrically at 625nm. Within the range of the assay, the absorbance is directly proportional to the amount of hexose present.

The moles of aldehyde also is determined simultaneously, using MBTH colorimetric method. The MBTH assay involves the formation of an azine compound by reacting aldehyde groups (from a given sample) with a 3-methyl-2-benzothiazolone hydrazone (MBTH assay reagent). The excess 3-methyl-2-benzothiazolone hydrazone oxidizes to form a reactive cation. The reactive cation and the azine react to form a blue chromophore. The formed chromophore is then read spectroscopically at 650 nm.

Preferably, the activated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide obtained by the above process comprises at least 0.6 mM acetate per mM of serotype 15B capsular polysaccharide and has a molecular weight between 50kDa and 500kDa, preferably 100 to 250kDa.

1.3 Conjugation of activated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide with CRM_{197}

The conjugation process consists of the following steps:

a) Compounding with sucrose excipient and lyophilization

b) Reconstitution of the lyophilized activated polysaccharide and CRM_{197}

c) Conjugation of activated polysaccharide to CRM_{197} and capping

d) Purification of the conjugate

a) Compounding with Sucrose excipient, and Lyophilization

The activated polysaccharide was compounded with sucrose to a ratio of 25 grams of sucrose per gram of activated polysaccharide. The bottle of compounded mixture was then lyophilized. Following lyophilization, bottles containing lyophilized activated polysaccharide

were stored at -20 ± 5°C. Calculated amount of CRM_{197} protein was shell-frozen and lyophilized separately. Lyophilized CRM_{197} was stored at -20 ± 5°C.

b) Reconstitution of Lyophilized Activated Polysaccharide and CRM_{197} Protein

Lyophilized activated polysaccharide was reconstituted in anhydrous dimethyl sulfoxide (DMSO). Upon complete dissolution of polysaccharide, an equal amount of anhydrous DMSO was added to lyophilized CRM_{197} for reconstitution.

c) Conjugation and Capping

Reconstituted activated polysaccharide was combined with reconstituted CRM_{197} in the reaction vessel (input ratio: 0.8:1), followed by mixing thoroughly to obtain a clear solution before initiating the conjugation with sodium cyanoborohydride. The final polysaccharide concentration in reaction solution is approximately 1 g/L. Conjugation was initiated by adding 1.0 – 1.5 MEq of sodium cyanoborohydride to the reaction mixture and was incubated at 23 ± 2 °C for 40-48 hrs. Conjugation reaction was terminated by adding 2 MEq of sodium borohydride ($NaBH_4$) to cap unreacted aldehydes. This capping reaction continued at 23 ± 2°C for 3 ± 1 hrs.

d) Purification of the conjugate

The conjugate solution was diluted 1:10 with chilled 5 mM succinate-0.9% saline (pH 6.0) in preparation for purification by tangential flow filtration using 100-300K MWCO membranes. The diluted conjugate solution was passed through a 5 µm filter and diafiltration was performed using 5 mM succinate-0.9% saline (pH 6.0) as the medium. After the diafiltration was completed, the conjugate retentate was transferred through a 0.22µm filter.

The conjugate was diluted further with 5 mM succinate / 0.9% saline (pH 6), to a target saccharide concentration of approximately 0.5 mg/mL. Final 0.22µm filtration step was completed to obtain the immunogenic conjugate.

Preferably, the conjugate obtained by the above process comprises at least 0.6 mM acetate per mM of serotype 15B capsular polysaccharide, has a molecular weight between 3000 and 20000kDa and has a degree of conjugation between 2 and 6.

Example 2: Characterization of immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to a CRM_{197}

Conjugate 1 was prepared by the process disclosed in example 1. Conjugates 2 and 3 were prepared by a similar process using different amount of oxidizing agent. Conjugate 4 was prepared by a similar process except that the purified serotype 15B capsular polysaccharide was not sized and was activated to a lower DO (higher oxidation level) and the conjugation was performed in aqueous medium. Conjugate 5 was prepared by a similar process except that the purified serotype 15B capsular polysaccharide was sized by chemical hydrolysis and the conjugation was performed in aqueous medium. Conjugates 6 and 7 were prepared by a similar process except that the purified serotype 15B capsular polysaccharide was not sized. The obtained conjugates were characterized and the results are summarized in Table 1.

Table 1: ***Streptococcus pneumoniae*** **serotype 15B capsular polysaccharide-CRM_{197} conjugates**

Conjugate	1	2	3	4	5	6	7
Polysaccharide	Sized	Sized	Sized	Native	Hydrolyzed	Native	Native
O-Acetylation; Polysaccharide (µmol acetate/µmol poly)	0.69	0.69	0.69	1.01	0.66	0.76	NA
Solvent medium	DMSO	DMSO	DMSO	Aqueous	Aqueous	DMSO	DMSO
Activated Polysaccharide DO	11.4	5.8	9.7	4.8	8.8	5	12
Activated Polysaccharide MW	196KDa	218KDa	235KDa	435 KDa	270KDa	431KDa	460KDa
Yield (%)	87.2	64	63.7	96.2	78.8	24.2	26.2
Saccharide Protein Ratio	0.68	0.65	0.71	1.22	1.29	0.9	1.5
Free Saccharide (%)	< 5	< 5	6.1	18.1	14.2	8.8	18
Conjugate MW, SEC-MALLS	6190KDa	7090KDa	7937KDa	1766KDa	1029KDa	6293KDa	4466KDa
O-Acetylation, Conjugate (µmol acetate/µmol poly)	0.68	0.7	0.68	0.61	0.44	0.85	NA
< 0.3 Kd (%), SEC	NA	73	NA	NA	62	NA	NA
Degree of Conj (AAA); Modified Lys	3.7	3.9	4.1	NA	3.4	NA	NA
% O-Acetyl Retained in Conjugate	99%	100%	99.5%	60%	67%	100%	NA

The percentage of free polysaccharide is measured by a procedure utilizing aluminium hydroxide gel to bind protein and covalently bound saccharide for removal by centrifugation. Samples are mixed with phosphate buffered aluminium hydroxide gel and centrifuged. Bound saccharide is pelleted with the gel and free saccharide remains in the supernatant. The resulting supernatant and controls samples are quantitated by appropriate colorimetric

assays to determine the percentage of free saccharide and to confirm sufficient removal of protein and recovery of saccharide.

For the Amino Acid analysis the polysaccharide-protein sample is first hydrolyzed into its individual components as free amino acids, using 6N hydrochloric acid (HCl) hydrolysis under vacuum and heat (160° C for 15 minutes). After hydrolysis, the samples are analyzed using Amino Acid Analyzer. The individual amino acids are separated through ion exchange chromatography using a step gradient of sodium citrate buffer with temperature and flow rate changes. After separation, the amount of each amino acid residual is quantitatively determined using a postcolumn ninhydrin coupling detection system. In this system, the ninhydrin is mixed with the column eluate in the postcolumn reactor system and the mixture passed into the photometer. The reaction of ninhydrin with eluated amino acids yields a purple compound that absorbs maximally at 570 nm. This absorbance is a linear response (function) of the amount of α-amino groups present and this reaction provides quantitative colorimetric assay for all organic compounds with α-amino groups. In the reaction with imino acids such as proline and hydroxylproline, which do not have free amino group, a bright yellow compound is generated and monitored at 440 nm. The peak areas for each amino acid are calculated using both 570 and 440 nm wavelength outputs.

The yield is calculated as follows: (amount of polysaccharide in the conjugate x100) / amount of activated polysaccharide.

Conjugates (4 and 5) generated using in aqueous medium demonstrated significant loss in O-acetyl levels. Conjugates generated in DMSO solvent, using native polysaccharide without MW sizing (6 and 7) did not demonstrate loss in O-acetyl levels. However, the conjugate yields were very poor in addition to poor filterability characteristics. Conjugates generated in DMSO using polysaccharides that were sized by high pressure homogenization (1, 2 and 3) had high yield and better filterability characteristics with significant preservation of O-acetyl levels. These conjugates also had very low levels of free polysaccharides.

Example 3: Opsonophagocytic activity (OPA) assay

The immunogenicity of the conjugates of the invention can be assessed using the opsonophagocytic assay (OPA) described below.

Groups of 30 6-7 week old female Swiss Webster mice were immunized with 0.001 µg, 0.01 µg, or 0.1 µg of test conjugates via the subcutaneous route on week 0. The mice were boosted with the same dose of conjugate on week 3 and then bled at week 4. Serotype-specific OPAs were performed on week 4 sera samples.

OPAs are used to measure functional antibodies in murine sera specific for *S. pneumoniae* serotype 15B. Test serum is set up in assay reactions that measure the ability of capsular polysaccharide specific immunoglobulin to opsonize bacteria, trigger complement deposition, thereby facilitating phagocytosis and killing of bacteria by phagocytes. The OPA titer is defined as the reciprocal dilution that results in a 50% reduction in bacterial count over control wells without test serum. The OPA titer is interpolated from the two dilutions that encompass this 50% killing cut-off.

OPA procedures were based on methods described in Hu et al., Clin Diagn Lab Immunol 2005;12(February (2)):287–95 with the following modifications. Test serum was serially diluted 2.5-fold and added to microtiter assay plates. Live serotype 15B target bacteria were added to the wells and the plates were shaken at 37°C for 30 minutes. Differentiated HL-60 cells (phagocytes) and baby rabbit serum (3- to 4-week old, Pel-Freez®, 6.25% final concentration) were added to the wells, and the plates were shaken at 37°C for 45 minutes. To terminate the reaction, 80 µL of 0.9% NaCl was added to all wells, mixed, and a 10µL aliquot were transferred to the wells of MultiScreen HTS HV filter plates (Millipore®) containing 200 µL of water. Liquid was filtered through the plates under vacuum, and 150 µL of HySoy medium was added to each well and filtered through. The filter plates were then incubated at 37°C, 5% CO_2 overnight and were then fixed with Destain Solution (Bio-Rad). The plates were then stained with Coomassie Blue and destained once. Colonies were imaged and enumerated on a Cellular Technology Limited (CTL) ImmunoSpot Analyzer®. Raw colony counts were used to plot kill curves and calculate OPA titers.

The immunogenicity of conjugates 1 and 2 has been tested according to the above mentioned assay. One additional conjugate and an unconjugated native *S. pneumoniae* serotype 15B capsular polysaccharide (unconjugated PS) were also tested in the same assay:

Conjugate 9 was prepared by conjugation of native (i.e not sized) serotype 15B capsular polysaccharide to CRM_{197} by reductive amination in aqueous solution.

The results are shown in table 2.

Table 2: OPA Titers of Animal Testing

OPA GMT (Geometric Mean antibody Titer) (95% CI)			
	0.001 µg	0.01 µg	0.1 µg
Conjugate 1	485 (413, 569)	804 (565, 1145)	1563 (1048, 2330)
Conjugate 2	556 (438, 707)	871 (609, 1247)	1672 (1054, 2651)
Conjugate 9	395 (329, 475)	856 (627, 1168)	1802 (1108, 2930)
Unconjugated PS	-	-	698 (466, 1045)

As shown in the above table, conjugates 1 and 2, when administered to mice, generate antibodies capable of opsonizing serotype 15B *S. pneumoniae*, triggering complement deposition, thereby facilitating phagocytosis and killing of bacteria by phagocytes. In addition, despite their lower molecular weight, they also exhibited similar level of immunogenicity as compared to conjugate 9 which has not been sized.

Example 4: Cross-functional OPA responses between serotype 15B and serotype 15C

Pneumococcal serogroup 15 includes four structurally-related serotypes: 15A, 15B, 15C, and 15F. Serotypes 15B and 15C are undistinguishable by genetic typing techniques and have similar capsular polysaccharide (PS) composition, except that the 15B-PS is the O-acetylated variant of 15C-PS. To understand whether anti-capsular PS antibodies for serotype 15B are functionally cross-reacting with serotype 15C, 10 rabbits were immunized with PCV16v and PCV20v vaccines both containing an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to CRM_{197} as disclosed herein as part of their formulation. Sera from pre- and post-vaccination were tested in OPA assays against serotypes 15B and 15C target pneumococcal strains.

Of the 10 rabbits from each group, 100% had OPA response to serotype 15B following immunization with a serotype 15B conjugate. Of these same samples, 100% had OPA response to serotype 15C as well (Table 1 and Table 2). Low OPA titers were observed in prevaccination sera in 15C OPA. However, over 10-fold GMT OPA titer increase with post vaccination sera compared to pre vaccination demonstrated that the immunogenic conjugates of the invention induces the formation of antibodies capable of killing serotype 15B and 15C *Streptococcus* pneumonia in an OPA.

PCV16v is a 16-valent conjugates composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F (16vPnC) all individualy conjugated to CRM_{197}.

PCV20v is a 20 valent conjugates composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F (20vPnC) all individualy conjugated to CRM_{197}.

Table 1. OPA Titers Against serotypes 15B and 15C strains in Rabbit Sera Pre and Post vaccination with PCV16v

	15B OPA		15C OPA	
Animal	wk0	wk4	wk0	wk4
1	4	4129	50	2524
2	4	1645	182	472
3	4	1131	126	818
4	4	3199	50	1189
5	4	2664	36	727
6	4	4589	68	2492
7	11	3601	169	1137
8	4	1838	165	672
9	4	1334	98	528
10	4	1108	204	2425
GMT	**4**	**2222**	**98**	**1075**

Table 2. OPA Titers Against serotypes 15B and 15C strains in Rabbit Sera Pre and Post vaccination with PCV20v

	15B OPA		15C OPA	
Animal	wk0	wk4	wk0	wk4
1	4	3784	indeterminable*	2353
2	4	862	480	938
3	4	3056	69	1497
4	4	1948	indeterminable*	1316
5	4	2360	4	4665
6	4	1594	indeterminable*	1835
7	4	4943	172	4085
8	4	2419	117	1458
9	4	1245	indeterminable*	527
10	4	616	indeterminable*	545
GMT	**4**	**1917**	**77**	**1515**

* Titer cannot be determined due to bad killing curves

Claims

1. An isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide comprising at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM acetate per mM of said serotype 15B capsular polysaccharide.

2. The isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide having a molecular weight between 5kDa and 500kDa.

3. The polysaccharide according to claim 2 having a molecular weight between 100kDa and 350kDa.

4. The isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide comprising at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM glycerol per mM of said serotype 15B capsular polysaccharide.

5. The polysaccharide according to any one of claims 1 to 4 comprising at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide.

6. The polysaccharide according to any one of claims 1 to 5 comprising at least 0.6 mM glycerol per mM of said serotype 15B capsular polysaccharide.

7. An immunogenic conjugate comprising an isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide according to any one of claims 1 to 6 covalently linked to a carrier protein.

8. The immunogenic conjugate according to claim 7 where the carrier protein is CRM_{197}.

9. The immunogenic conjugate according to claim 7 or 8 where the immunogenic conjugate comprises less than about 50, 45, 40, 35, 30, 25, 20 or 15% of free serotype 15B capsular polysaccharide compared to the total amount of serotype 15B capsular polysaccharide.

10. The immunogenic conjugate according to any one of claims 7 to 9 where the immunogenic conjugate comprises less than about 40% of free serotype 15B capsular polysaccharide compared to the total amount of serotype 15B capsular polysaccharide.

11. The immunogenic conjugate according to any one of claims 7 to 10 where the immunogenic conjugate comprises less than about 20% of free serotype 15B capsular polysaccharide compared to the total amount of serotype 15B capsular polysaccharide.

12. The immunogenic conjugate according to any one of claims 7 to 11 where the immunogenic conjugate has a molecular weight between: 3000 and 20000kDa; 8000 and 20000kDa; 8000 and 16000 KDa; or 10000 and 16000 KDa.

13. The immunogenic conjugate according to any one of claims 7 to 12 where the immunogenic conjugate has a molecular weight between 10000 and 16000 KDa.

14. The immunogenic conjugate according to any one of claims 7 to 13 where the ratio of serotype 15B capsular polysaccharide to carrier protein in the conjugate is between 0.4 and 2.

15. The immunogenic conjugate according to any one of claims 7 to 13 where the ratio of serotype 15B capsular polysaccharide to carrier protein in the conjugate is between 0.7 and 0.9.

16. The immunogenic conjugate according to any one of claims 7 to 15, wherein at least 40% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

17. The immunogenic conjugate according to any one of claims 7 to 15, wherein at least 50% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

18. The immunogenic conjugate according to any one of claims 7 to 15, wherein at least 60% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

19. The immunogenic conjugate according to any one of claims 7 to 17 wherein the degree of conjugation is between: 2 and 15; 2 and 13; 2 and 10; 2 and 8; 2 and 6; 2 and 5; 2 and 4; 3 and 15; 3 and 13; 3 and 10; 3 and 8; 3 and 6; 3 and 5; 3 and 4; 5 and 15; 5 an 10; 8 and 15; 8 and 12; 10 and 15; or 10 and 12.

20. The immunogenic conjugate according to any one of claims 7 to 17 wherein the degree of conjugation is between 2 and 6.

21. The immunogenic conjugate according to any one of claims 7 to 17 wherein the degree of conjugation is between 3 and 5.

22. An immunogenic composition comprising an immunogenic conjugate according to any one of claims 7 to 21 and a physiologically acceptable vehicle.

23. The immunogenic composition according to claim 22 further comprising at least one additional antigen.

24. The immunogenic composition according to claim 22 or 23 further comprising an adjuvant.

25. The immunogenic composition according to claim 24 where the adjuvant is aluminium phosphate.

26. A vaccine comprising an immunogenic composition according to any one of claims 22 to 25.

27. A process for producing a polysaccharide according to any one of claims 1 to 6, said process comprising the steps of:
(a) preparing a fermentation culture of serotype 15B *Streptococcus pneumonia* bacterial cells;
(b) lysing the bacterial cells in said fermentation culture;
(c) purifying *Streptococcus pneumoniae* serotype 15B capsular polysaccharide from the fermentation culture;
(d) sizing the purified *Streptococcus pneumoniae* serotype 15B capsular polysaccharide by high pressure homogenization.

28. A process for producing an activated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide, said process comprising the step of reacting the isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide of any one of claims 1 to 6 with an oxidizing agent.

29. The process according to claim 28 where said process comprises the step of reacting said isolated *Streptococcus pneumoniae* serotype 15B capsular polysaccharide with 0.1 to 0.3 molar equivalent of sodium periodate during 15 to 20 hours at a temperature between 20 and 25°C.

30. An activated serotype 15B capsular polysaccharide obtained or obtainable by the process of claim 28 or 29.

31. An activated serotype 15B capsular polysaccharide having a molecular weight between about 5 and 500 kDa, about 50 and 450kDa, about 100 and 400kDa, about 100 and 350 kDa, about 100 and 300kDa.

32. The activated polysaccharide according to claim 31 comprising at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM acetate per mM of said serotype 15B capsular polysaccharide.

33. The activated polysaccharide according to claim 31 comprising at least 0.6 mM acetate per mM of said serotype 15B capsular polysaccharide.

34. The activated polysaccharide according to any one of claims 31 to 33 comprising at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 mM glycerol per mM of said serotype 15B capsular polysaccharide.

35. The activated polysaccharide according to any one of claims 31 to 34 comprising at least 0.6 mM glycerol per mM of said serotype 15B capsular polysaccharide.

36. The activated polysaccharide according to any one of claims 31 to 35, characterized by a degree of oxidation between 2 and 20, 2 and 15, 2 and 10, 2 and 5, 5 and 20, 5 and 15, 5 and 10, 10 and 20, 10 and 15, or 15 and 20.

37. The activated polysaccharide according to any one of claims 31 to 36, characterized by a degree of oxidation between 5 and 15.

38. The activated polysaccharide according to any one of claims 31 to 36, characterized by a degree of oxidation between 10 and 20.

39. The activated polysaccharide according to any one of claims 31 to 36, characterized by a degree of oxidation between 15 and 20.

40. The activated polysaccharide according to any one of claims 31 to 36, characterized by a degree of oxidation between 10 and 15.

41. A process for the preparation of an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 15B capsular polysaccharide covalently linked to a carrier protein, the process comprising the steps of:
(a) compounding the activated polysaccharide of anyone of claims 30 to 40 with a carrier protein; and
(b) reacting the compounded, activated polysaccharide and carrier protein with a reducing agent to form a serotype 15B capsular polysaccharide-carrier protein conjugate.

42. The process according to claim 41 wherein the carrier protein is CRM_{197}.

43. The process according to claim 41 or 42 wherein step (a) and step (b) are carried out in DMSO.

44. The process according to claim 41 or 42 wherein step (a) and step (b) are carried out in aqueous solution.

45. The process according to any one of claims 41 to 44 wherein the concentration of activated serotype 15B capsular polysaccharide in step (b) is between 0.1 and 10 mg/mL, 0.5 and 5 mg/mL or 0.5 and 2 mg/mL.

46. The process according to any one of claims 41 to 44 wherein the concentration of activated serotype 15B capsular polysaccharide in step (b) is about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or 3mg/mL.

47. The process according to any one of claims 41 to 44 wherein the concentration of activated serotype 15B capsular polysaccharide in step (b) is about 0.5 mg/mL.

48. The process according to any one of claims 41 to 44 wherein the concentration of activated serotype 15B capsular polysaccharide in step (b) is about 1 mg/mL.

49. The process according to any one of claims 41 to 44 wherein the concentration of activated serotype 15B capsular polysaccharide in step (b) is about 1.5 mg/mL.

50. The process according to any one of claims 41 to 44 wherein the concentration of activated serotype 15B capsular polysaccharide in step (b) is about 2 mg/mL.

51. The process according to any one of claims 41 to 50 wherein the initial input ratio of activated serotype 15B capsular polysaccharide to carrier protein is between 5:1 and 0.1:1, 2:1 and 0.1:1, 2:1 and 1:1, 1.5:1 and 1:1, 0.1:1 and 1:1, 0.3:1 and 1:1, 0.6:1 and 1:1, or 0.6:1 and 1.5:1.

52. The process according to any one of claims 41 to 50 wherein the initial input ratio of activated serotype 15B capsular polysaccharide to carrier protein is about 0.4:1, 0.5:1, 0.6:1, 0.7:1, 0.8:1, 0.9:1, 1:1, 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, 1.9:1 or 2:1.

53. The process according to any one of claims 41 to 50 wherein the initial input ratio of activated serotype 15B capsular polysaccharide to carrier protein is about 0.6:1.

54. The process according to any one of claims 41 to 50 wherein the initial input ratio of activated serotype 15B capsular polysaccharide to carrier protein is about 0.8:1.

55. The process according to any one of claims 41 to 50 wherein the initial input ratio of activated serotype 15B capsular polysaccharide to carrier protein is about 1:1.

56. The process according to any one of claims 41 to 50 wherein the initial input ratio of activated serotype 15B capsular polysaccharide to carrier protein is about 1.5:1.

57. The process according to any one of claims 41 to 50 wherein the initial input ratio of activated serotype 15B capsular polysaccharide to carrier protein is about 2:1.

58. The process according to any one of claims 41 to 57 wherein in step (b), the activated polysaccharide is reacted with between about 1 and 2 molar equivalent of sodium cyanoborohydride during about 40 to 50 hours at a temperature between about 20 to 26°C.

59. The process according to anyone of claims 41 to 58 wherein said process comprises the additional following step:
(c) capping unreacted aldehyde by addition of $NaBH_4$.

60. The process according to anyone of claims 41 to 59 wherein said process further comprises the step of formulating the conjugate in a multivalent vaccine.

61. The process according to any one of claims 41 to 60 wherein the yield of the conjugation step (b) is greater than 50%.

62. The process according to any one of claims 41 to 60 wherein the yield of the conjugation step (b) is greater than 60%.

63. An immunogenic conjugate obtained or obtainable by the process of any one of claims 41 to 62.

64. The immunogenic conjugate according to claim 63 where the immunogenic conjugate comprises less than about 40% of free serotype 15B capsular polysaccharide compared to the total amount of serotype 15B capsular polysaccharide.

65. The immunogenic conjugate according to any one of claims 63 to 64 where the immunogenic conjugate comprises less than about 20% of free serotype 15B capsular polysaccharide compared to the total amount of serotype 15B capsular polysaccharide.

66. The immunogenic conjugate according to any one of claims 63 to 65 where the immunogenic conjugate has a molecular weight between: 3000 and 20000kDa; 5000 and 10000kDa; 5000 and 20000kDa; 8000 and 20000kDa; 8000 and 16000 KDa; or 10000 and 16000 KDa.

67. The immunogenic conjugate according to any one of claims 61 to 66 where the ratio of serotype 15B capsular polysaccharide to carrier protein in the conjugate is between 0.4 and 2.

68. The immunogenic conjugate according to any one of claims 61 to 65 where the ratio of serotype 15B capsular polysaccharide to carrier protein in the conjugate is between 0.7 and 0.9.

69. The immunogenic conjugate according to any one of claims 61 to 68, wherein at least 40% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

70. The immunogenic conjugate according to any one of claims 61 to 68, wherein at least 60% of the immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

71. The immunogenic conjugate according to any one of claims 61 to 70 wherein the degree of conjugation is between: 2 and 15; 2 and 13; 2 and 10; 2 and 8; 2 and 6; 2 and 5, 2 and 4; 3 and 15; 3 and 13; 3 and 10; 3 and 8; 3 and 6; 3 and 5; 3 and 4; 5 and 15; 5 an 10; 8 and 15; 8 and 12; 10 and 15; or 10 and 12.

72. The immunogenic conjugate according to any one of claims 61 to 70 wherein the degree of conjugation is between 2 and 6.

73. The immunogenic conjugate according to any one of claims 61 to 72 wherein the ratio of mM acetate per mM serotype 15B capsular polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 15B capsular polysaccharide in the activated polysaccharide is at least 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, or 0.95, preferably at least 0.7 or at least 0.9.

74. The immunogenic conjugate according to any one of claims 61 to 72 wherein the ratio of mM acetate per mM serotype 15B capsular polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 15B capsular polysaccharide in the activated polysaccharide is at least 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, or 0.95, preferably at least 0.7 or at least 0.9.

75. An immunogenic composition comprising an immunogenic conjugate according to any one of claims 61 to 74 and a physiologically acceptable vehicle.

76. A vaccine comprising an immunogenic composition according to claim 75.

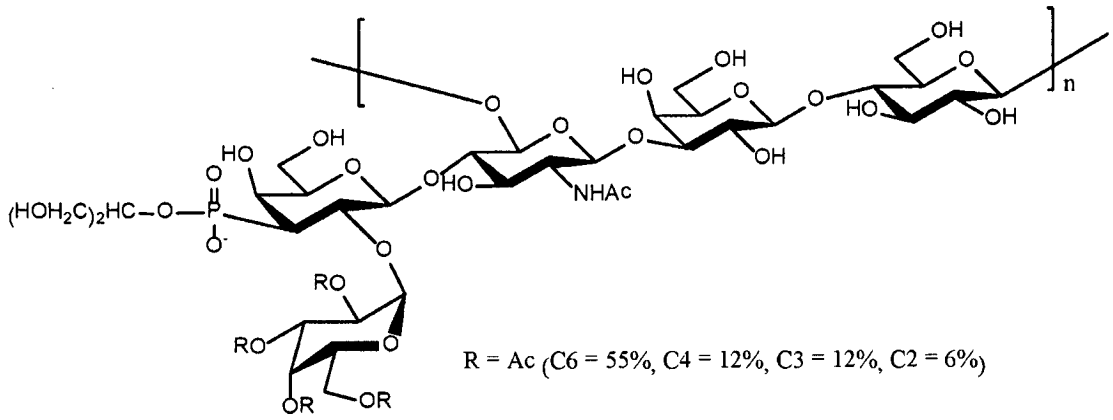
77. A method of protecting a subject against an infection with serotype 15B *Streptococcus pneumoniae*, the method comprising administering to a subject an immunogenic amount of the immunogenic composition of any one of claims 22 to 25 or 75, or the vaccine of claim 26 or 76.

78. A method of treating or preventing a *Streptococcus pneumoniae* infection, disease or condition associated with serotype 15A, 15B and/or 15C *Streptococcus pneumoniae* in a subject, the method comprising the step of administering a therapeutically or prophylactically effective amount of an immunogenic composition according to any one of claims 22 to 25 or 75, or the vaccine of claim 26 or 76.

79. A method of treating or preventing a *Streptococcus pneumoniae* infection, disease or condition associated with serotype 15B and/or 15C *Streptococcus pneumoniae* in a subject, the method comprising the step of administering a therapeutically or prophylactically effective amount of an immunogenic composition according to any one of 22 to 25 or 75, or the vaccine of claim 26 or 76.

80. A method of treating or preventing a *Streptococcus pneumoniae* infection, disease or condition associated with serotype 15B *Streptococcus pneumoniae* in a subject, the method comprising the step of administering a therapeutically or prophylactically effective amount of an immunogenic composition according to any one of claims 22 to 25 or 75, or the vaccine of claim 26 or 76.

Figure 1



→6)-D-Glc*p*NAc-β-(1→3)-D-Gal*p*-β-(1→4)-D-Glc*p*-β-(1→

D-Gal*p*-α-(1→2)-D-Gal*p*(3-OPO$_3$-R^1)-β-(1→4

R^1 = CH(CH$_2$OH)$_2$

$C_{37}H_{61}NNaO_{31}P$, MW=1069.8

Ac= Acetyl

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(74) Agent: **OLSON, A. Dean**; Pfizer Worldwide Research & Development, Eastern Point Road MS8260-2141, Groton, Connecticut 06340 (US).

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Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*

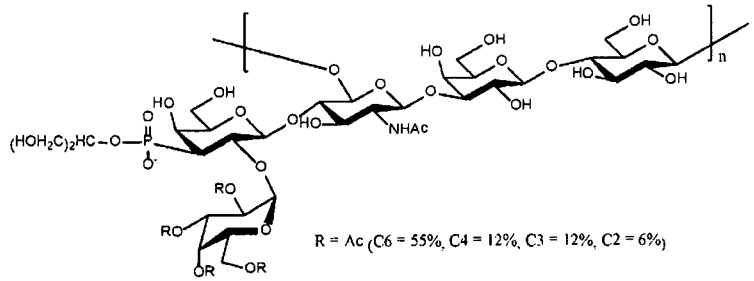
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

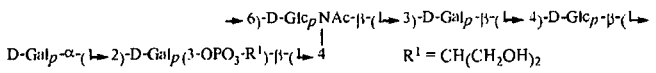
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

[Continued on next page]

(54) Title: STREPTOCOCCUS PNEUMONIAE CAPSULAR POLYSACCHARIDES AND CONJUGATES THEREOF

Figure 1





$C_{37}H_{61}NNaO_{31}P$, MW=1069.8

Ac= Acetyl

(57) Abstract: The invention relates to isolated Streptococcus pneumoniae serotype 15B capsular polysaccharide and processes for their preparation. The invention also relates to immunogenic conjugates comprising Streptococcus pneumoniae serotype 15B capsular polysaccharide covalently linked to a carrier protein, processes for their preparation and immunogenic compositions comprising them.

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

B. FIELDS SEARCHED

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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 157 899 A2 (AMERICAN CYANAMID CO [US]) 16 October 1985 (1985-10-16) example 6 -----	1-3,5 2,3
X	BUSSAT B ET AL: "Molecular size characterization of bacterial capsular polysaccharide vaccines by high performance liquid chromatography", BIOLOGICALS, ACADEMIC PRESS LTD., LONDON, GB, vol. 18, no. 2, 1 April 1990 (1990-04-01), pages 117-121, XP022954054, ISSN: 1045-1056, DOI: 10.1016/1045-1056(90)90021-Q [retrieved on 1990-04-01] See e.g. the abstract; page 117, right column, first and third paragraphs; and section bridging pages 117 and 118 ----- -/--	1,5

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
4 September 2015	25/09/2015
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Valcárcel, Rafael

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INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 714 354 A (ARNOLD FRANK JOSEF [US] ET AL) 3 February 1998 (1998-02-03) See e.g. the abstract; Flowsheet 1 on columns 3 and 4; column 6, lines 28-31	1-3,5
X Y A	WO 2008/157590 A1 (BAXTER INT [US]; BAXTER HEALTHCARE SA [CH]; MICHON FRANCIS [US]; SARKA) 24 December 2008 (2008-12-24) See e.g. the abstract; paragraph [0059]; claims 29, 33 and 34	7,77-80 9-21 1,5
X	PER-ERIK JANSSON ET AL: "Structural studies of the capsular polysaccharide from Streptococcus pneumoniae types 15B and 15C", CARBOHYDRATE RESEARCH, vol. 162, no. 1, 1 April 1987 (1987-04-01), pages 111-116, XP055210747, ISSN: 0008-6215, DOI: 10.1016/0008-6215(87)80205-7 See e.g. the abstract	2,3
Y	J G HOWARD ET AL: "Studies on Immunological Paralysis: V. THE INFLUENCE OF MOLECULAR WEIGHT ON THE IMMUNOGENICITY, TOLEROGENICITY AND ANTIBODY-NEUTRALIZING ACTIVITY OF TYPE III PNEUMOCOCCAL POLYSACCHARIDE", IMMUNOLOGY., vol. 21, no. 3, 1 January 1971 (1971-01-01), pages 535-546, XP55210763, GB ISSN: 0019-2805 See e.g. the abstract	2,3
X Y	EP 2 384 765 A2 (GLAXOSMITHKLINE BIOLOG SA [BE]) 9 November 2011 (2011-11-09) See e.g. paragraphs [0014], [0016], [0017], [0020], [0077], [0089], [0111] to [0123], [0125], [0137], [0140], [0141]; claim 11	7-26, 77-80 9-21
X	US 2013/273098 A1 (BLUE JEFFREY T [US] ET AL) 17 October 2013 (2013-10-17) See e.g, the abstract; paragraphs [0011], [0012], [0013], [0055] or [0111]	7,22-26
X	US 5 623 057 A (KNISKERN PETER J [US] ET AL) 22 April 1997 (1997-04-22) See e.g. claim 4	2,3,7
Y	WO 2011/110531 A2 (GLAXOSMITHKLINE BIOLOG SA [BE]; BIEMANS RALPH LEON [BE]; DUVIVIER PIER) 15 September 2011 (2011-09-15) See e.g. claims 9, 20 or 24	9-21

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2015/050316

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

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   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

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3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2015/050316

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0157899 A2	16-10-1985	AT 399655 B	26-06-1995
		AU 569946 B2	25-02-1988
		AU 4101685 A	17-10-1985
		CA 1216235 A1	06-01-1987
		DE 3485778 D1	23-07-1992
		DE 3485778 T2	14-01-1993
		EP 0157899 A2	16-10-1985
		ES 8602417 A1	16-03-1986
		JP H0641422 B2	01-06-1994
		JP S60218325 A	01-11-1985
		US 4686102 A	11-08-1987
		ZA 8502724 A	27-11-1985
US 5714354 A	03-02-1998	NONE	
WO 2008157590 A1	24-12-2008	AU 2008265767 A1	24-12-2008
		CA 2692069 A1	24-12-2008
		CN 101754773 A	23-06-2010
		EP 2170391 A1	07-04-2010
		JP 5555623 B2	23-07-2014
		JP 2010532793 A	14-10-2010
		US 2010189740 A1	29-07-2010
		US 2013197203 A1	01-08-2013
		WO 2008157590 A1	24-12-2008
EP 2384765 A2	09-11-2011	AR 058706 A1	20-02-2008
		AR 058707 A1	20-02-2008
		AT 520415 T	15-09-2011
		AU 2006327036 A1	28-06-2007
		AU 2006327040 A1	28-06-2007
		AU 2006327041 A1	28-06-2007
		AU 2010241281 A1	25-11-2010
		BR PI0620163 A2	01-11-2011
		BR PI0620193 A2	01-11-2011
		BR PI0620460 A2	16-11-2011
		CA 2633772 A1	28-06-2007
		CA 2634885 A1	28-06-2007
		CA 2634887 A1	28-06-2007
		CA 2808919 A1	28-06-2007
		CA 2816182 A1	28-06-2007
		CL 2013002106 A1	07-03-2014
		CN 103251940 A	21-08-2013
		CN 103585623 A	19-02-2014
		CR 10119 A	29-07-2008
		CR 10122 A	21-08-2008
		DK 1962899 T3	31-10-2011
		DK 1968631 T3	15-06-2015
		EA 200801344 A1	30-12-2008
		EA 200801367 A1	30-12-2008
		EA 200801374 A1	30-12-2008
		EP 1962899 A2	03-09-2008
		EP 1968631 A2	17-09-2008
		EP 1973564 A2	01-10-2008
		EP 2382986 A2	02-11-2011
		EP 2384765 A2	09-11-2011
		EP 2402025 A2	04-01-2012
		ES 2539795 T3	06-07-2015
		HK 1120230 A1	09-12-2011

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2015/050316

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		HR P20110685 T1	31-10-2011
		IL 191913 A	31-07-2012
		IL 192084 A	28-11-2013
		JO 2813 B	15-09-2014
		JP 5461838 B2	02-04-2014
		JP 5579387 B2	27-08-2014
		JP 5600337 B2	01-10-2014
		JP 5615323 B2	29-10-2014
		JP 5749689 B2	15-07-2015
		JP 2009520759 A	28-05-2009
		JP 2009520760 A	28-05-2009
		JP 2009520761 A	28-05-2009
		JP 2012211165 A	01-11-2012
		JP 2012229228 A	22-11-2012
		JP 2012232985 A	29-11-2012
		JP 2014205673 A	30-10-2014
		KR 20080081060 A	05-09-2008
		KR 20080081063 A	05-09-2008
		KR 20130103831 A	24-09-2013
		KR 20140036327 A	25-03-2014
		MA 30062 B1	01-12-2008
		MA 30065 B1	01-12-2008
		MA 30066 B1	01-12-2008
		MY 147495 A	14-12-2012
		MY 148141 A	15-03-2013
		MY 150719 A	28-02-2014
		NZ 569076 A	26-08-2011
		NZ 569077 A	26-08-2011
		NZ 569168 A	24-02-2012
		NZ 596500 A	31-05-2013
		PE 10582007 A1	27-11-2007
		PE 13052007 A1	14-12-2007
		PT 68631 E	20-07-2015
		PT 1962899 E	19-10-2011
		SG 168517 A1	28-02-2011
		SI 1962899 T1	30-11-2011
		SI 1968631 T1	31-07-2015
		TW I415622 B	21-11-2013
		TW 201350129 A	16-12-2013
		US 2009010959 A1	08-01-2009
		US 2009017059 A1	15-01-2009
		US 2009017072 A1	15-01-2009
		US 2010074922 A1	25-03-2010
		US 2015190521 A1	09-07-2015
US 2013273098 A1	17-10-2013	AR 084158 A1	24-04-2013
		AU 2011338723 A1	30-05-2013
		CA 2819366 A1	14-06-2012
		CN 103391714 A	13-11-2013
		EP 2648506 A1	16-10-2013
		JP 2014502595 A	03-02-2014
		KR 20140005892 A	15-01-2014
		RU 2013131795 A	20-01-2015
		TW 201304803 A	01-02-2013
		US 2013273098 A1	17-10-2013
		WO 2012078482 A1	14-06-2012
US 5623057 A	22-04-1997	AT 169825 T	15-09-1998

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2015/050316

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 additional sheet

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 an 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.:

7-26(completely); 1-3, 5, 77-80(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 5(all partially)

   An isolated Streptococcus pneumoniae serotype 15B capsular polysaccharide comprising acetate as defined in claims 1 and 5

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2. claims: 2, 3(partially)

   An isolated Streptococcus pneumoniae serotype 15B capsular polysaccharide having a molecular weight as defined in claims 2 and 3

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3. claims: 4, 6(all partially)

   An isolated Streptococcus pneumoniae serotype 15B capsular polysaccharide comprising glycerol as defined in claims 4 and 6

   ---

4. claims: 1-3, 5(all partially)

   An isolated Streptococcus pneumoniae serotype 15B capsular polysaccharide comprising acetate wherein the capsular polysaccharide is having the molecular weight defined in claims 2 and 3

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5. claims: 1, 4-6(all partially)

   An isolated Streptococcus pneumoniae serotype 15B capsular polysaccharide comprising acetate and glycerol

   ---

6. claims: 7-26(completely); 77-80(partially)

   An immunogenic conjugate comprising an isolated Streptococcus pneumoniae serotype 15B capsular polysaccharide covalently linked to a carrier protein. Methods according to claims 77-80 involving the use of said conjugate.

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7. claim: 27

   A process according to claim 27

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8. claims: 28-30

   A process according to claims 28 and 29. A polysaccharide

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2015/050316

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		AU 651656 B2	28-07-1994
		CA 2059692 A1	29-07-1992
		CN 1064217 A	09-09-1992
		CS 9200199 A3	14-10-1992
		DE 69226668 D1	24-09-1998
		DE 69226668 T2	22-04-1999
		DK 0497525 T3	26-10-1998
		EP 0497525 A2	05-08-1992
		ES 2121820 T3	16-12-1998
		FI 920353 A	29-07-1992
		HU 216101 B	28-04-1999
		IE 920244 A1	29-07-1992
		IL 100716 A	22-12-1999
		IL 119961 A	31-01-2000
		IL 119962 A	31-01-2000
		JP H0565300 A	19-03-1993
		JP H0794472 B2	11-10-1995
		LV 12309 A	20-07-1999
		NO 920350 A	29-07-1992
		NZ 241367 A	27-04-1994
		SI 9210081 A	29-02-1996
		SK 280659 B6	16-05-2000
		US 5623057 A	22-04-1997
		YU 8192 A	28-12-1994
WO 2011110531 A2	15-09-2011	AU 2011226160 A1	25-10-2012
		CA 2791926 A1	15-09-2011
		CN 102883745 A	16-01-2013
		DK 2544708 T3	24-08-2015
		EA 201290692 A1	28-06-2013
		EP 2544708 A2	16-01-2013
		EP 2815762 A2	24-12-2014
		JP 5698770 B2	08-04-2015
		JP 2013521394 A	10-06-2013
		KR 20130038233 A	17-04-2013
		SG 183476 A1	27-09-2012
		US 2012321660 A1	20-12-2012
		US 2014186389 A1	03-07-2014
		US 2014186390 A1	03-07-2014
		WO 2011110531 A2	15-09-2011

obtained or obtainable by said processes.

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9. claims: 31-40

Am activated serotype 15B capsular polysaccharide according to claims 31-40. Further lack of unity could exist in this group depending on what "activated" means.

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10. claims: 41-76(completely); 77-80(partially)

Process for the preparation of an immunonenic conjugate comprising S. pneumoniae serotype 15B capsular polypeptide covalently linked to a carrier protein according to claim 41. Methods according to claims 77-80 involving the use of said conjugate.

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(19)中华人民共和国国家知识产权局

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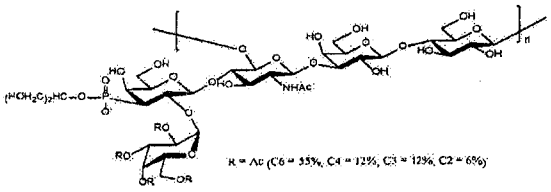
权利要求书4页 说明书30页
序列表5页 附图1页

(54)发明名称

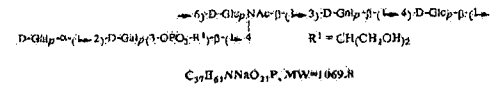
肺炎链球菌荚膜多糖及其缀合物

(57)摘要

本发明涉及分离的肺炎链球菌血清型15B荚膜多糖和它们的制备方法。本发明还涉及包含共价连接到载体蛋白的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物，它们的制备方法和包含它们的免疫原性组合物。



R = Ac (C6 = 55%, C4 = 12%, C3 = 12%, C2 = 6%)



$C_{59}H_{93}NNaO_{31}P$, MW=1369.3

Ac= 乙酰基

CN 106413747 A

1.分离的肺炎链球菌血清型15B荚膜多糖，每mM所述血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM乙酸盐。

2.分离的肺炎链球菌血清型15B荚膜多糖，其具有5kDa-500kDa的分子量。

3.根据权利要求2的多糖，其具有100kDa-350kDa的分子量。

4.分离的肺炎链球菌血清型15B荚膜多糖，每mM所述血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM甘油。

5.根据权利要求1-4任一项的多糖，每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐。

6.根据权利要求1-5任一项的多糖，每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。

7.免疫原性缀合物，其包含共价连接到载体蛋白的根据权利要求1-6任一项的分离的肺炎链球菌血清型15B荚膜多糖。

8.根据权利要求7的免疫原性缀合物，其中所述载体蛋白是CRM_{197}。

9.根据权利要求7或8的免疫原性缀合物，其中所述免疫原性缀合物包含相比血清型15B荚膜多糖的总量小于约50%、45%、40%、35%、30%、25%、20%或15%的游离血清型15B荚膜多糖。

10.根据权利要求7-9任一项的免疫原性缀合物，其中所述免疫原性缀合物包含相比血清型15B荚膜多糖的总量小于约40%的游离血清型15B荚膜多糖。

11.根据权利要求7-10任一项的免疫原性缀合物，其中所述免疫原性缀合物包含相比血清型15B荚膜多糖的总量小于约20%的游离血清型15B荚膜多糖。

12.根据权利要求7-11任一项的免疫原性缀合物，其中所述免疫原性缀合物具有3000-20000kDa、8000-20000kDa、8000-16000KDa或10000-16000KDa的分子量。

13.根据权利要求7-12任一项的免疫原性缀合物，其中所述免疫原性缀合物具有10000-16000KDa的分子量。

14.根据权利要求7-13任一项的免疫原性缀合物，其中缀合物中血清型15B荚膜多糖与载体蛋白的比例是0.4-2。

15.根据权利要求7-13任一项的免疫原性缀合物，其中缀合物中血清型15B荚膜多糖与载体蛋白的比例是0.7-0.9。

16.根据权利要求7-15任一项的免疫原性缀合物，其中至少40%的免疫原性缀合物在CL-4B柱中具有小于或等于0.3的Kd。

17.根据权利要求7-15任一项的免疫原性缀合物，其中至少50%的免疫原性缀合物在CL-4B柱中具有小于或等于0.3的Kd。

18.根据权利要求7-15任一项的免疫原性缀合物，其中至少60%的免疫原性缀合物在CL-4B柱中具有小于或等于0.3的Kd。

19.根据权利要求7-17任一项的免疫原性缀合物，其中缀合度是2-15、2-13、2-10、2-8、2-6、2-5、2-4、3-15、3-13、3-10、3-8、3-6、3-5、3-4、5-15、5-10、8-15、8-12、10-15或10-12。

20.根据权利要求7-17任一项的免疫原性缀合物，其中所述缀合度是2-6。

21.根据权利要求7-17任一项的免疫原性缀合物，其中所述缀合度是3-5。

22.免疫原性组合物，其包含根据权利要求7-21任一项的免疫原性缀合物和生理上可接受的媒介物。

23.根据权利要求22的免疫原性组合物，其进一步包含至少一种额外的抗原。

24.根据权利要求22或23的免疫原性组合物，其进一步包含佐剂。

25.根据权利要求24的免疫原性组合物，其中所述佐剂是磷酸铝。

26.包含根据权利要求22-25任一项的免疫原性组合物的疫苗。

27.生产根据权利要求1-6任一项的多糖的方法，所述方法包括下列步骤：

(a)制备血清型15B肺炎链球菌细菌细胞的发酵培养物；

(b)裂解所述发酵培养物中的细菌细胞；

(c)从发酵培养物纯化肺炎链球菌血清型15B荚膜多糖；

(d)通过高压均质裁剪纯化的肺炎链球菌血清型15B荚膜多糖。

28.用于生产活化的肺炎链球菌血清型15B荚膜多糖的方法，所述方法包括使权利要求1-6任一项的分离的肺炎链球菌血清型15B荚膜多糖与氧化剂反应的步骤。

29.根据权利要求28的方法，其中所述方法包括使所述分离的肺炎链球菌血清型15B荚膜多糖与0.1-0.3摩尔当量的高碘酸钠反应，在20-25℃的温度持续15-20小时。

30.活化的血清型15B荚膜多糖，其通过权利要求28或29的方法获得或可通过权利要求28或29的方法获得。

31.活化的血清型15B荚膜多糖，其具有约5-500kDa、约50-450kDa、约100-400kDa、约100-350kDa、约100-300kDa的分子量。

32.根据权利要求31的活化的多糖，每mM所述血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM乙酸盐。

33.根据权利要求31的活化的多糖，每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐。

34.根据权利要求31-33任一项的活化的多糖，每mM所述血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM甘油。

35.根据权利要求31-34任一项的活化的多糖，每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。

36.根据权利要求31-35任一项的活化的多糖，其特征在于氧化度是2-20、2-15、2-10、2-5、5-20、5-15、5-10、10-20、10-15、或15-20。

37.根据权利要求31-36任一项的活化的多糖，其特征在于氧化度是5-15。

38.根据权利要求31-36任一项的活化的多糖，其特征在于氧化度是10-20。

39.根据权利要求31-36任一项的活化的多糖，其特征在于氧化度是15-20。

40.根据权利要求31-36任一项的活化的多糖，其特征在于氧化度是10-15。

41.包含共价连接到载体蛋白的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物的制备方法，所述方法包括以下步骤：

(a)混合权利要求30-40任一项的活化的多糖与载体蛋白；

和

(b)使所述混合的活化的多糖和载体蛋白与还原剂反应以形成血清型15B荚膜多糖-载体蛋白缀合物。

42.根据权利要求41的方法，其中所述载体蛋白是CRM_{197}。

43.根据权利要求41或42的方法，其中步骤(a)和步骤(b)在DMSO中进行。

44.根据权利要求41或42的方法，其中步骤(a)和步骤(b)在水溶液中进行。

45.根据权利要求41-44任一项的方法，其中步骤(b)中活化的血清型15B荚膜多糖的浓度为0.1-10mg/mL、0.5-5mg/mL、0.5-2mg/mL。

46.根据权利要求41-44任一项的方法，其中步骤(b)中活化的血清型15B荚膜多糖的浓度为0.1、0.2、0.3、0.4、0.5、0.6、0.7、0.8、0.9、1、1.1、1.2、1.3、1.4、1.5、1.6、1.7、1.8、1.9、2、2.1、2.2、2.3、2.4、2.5、2.6、2.7、2.8、2.9或3mg/mL。

47.根据权利要求41-44任一项的方法，其中步骤(b)中活化的血清型15B荚膜多糖的浓度为约0.5mg/mL。

48.根据权利要求41-44任一项的方法，其中步骤(b)中活化的血清型15B荚膜多糖的浓度为约1mg/mL。

49.根据权利要求41-44任一项的方法，其中步骤(b)中活化的血清型15B荚膜多糖的浓度为约1.5mg/mL。

50.根据权利要求41-44任一项的方法，其中步骤(b)中活化的血清型15B荚膜多糖的浓度为约2mg/mL。

51.根据权利要求41-50任一项的方法，其中活化的血清型15B荚膜多糖与载体蛋白的初始输入比例是5:1-0.1:1、2:1-0.1:1、2:1-1:1、1.5:1-1:1、0.1:1-1:1、0.3:1-1:1、0.6:1-1:1或0.6:1-1.5:1。

52.根据权利要求41-50任一项的方法，其中活化的血清型15B荚膜多糖与载体蛋白的初始输入比例是0.4:1、0.5:1、0.6:1、0.7:1、0.8:1、0.9:1、1:1、1.1:1、1.2:1、1.3:1、1.4:1、1.5:1、1.6:1、1.7:1、1.8:1、1.9:1或2:1。

53.根据权利要求41-50任一项的方法，其中活化的血清型15B荚膜多糖与载体蛋白的初始输入比例是约0.6:1。

54.根据权利要求41-50任一项的方法，其中活化的血清型15B荚膜多糖与载体蛋白的初始输入比例是约0.8:1。

55.根据权利要求41-50任一项的方法，其中活化的血清型15B荚膜多糖与载体蛋白的初始输入比例是约1:1。

56.根据权利要求41-50任一项的方法，其中活化的血清型15B荚膜多糖与载体蛋白的初始输入比例是约1.5:1。

57.根据权利要求41-50任一项的方法，其中活化的血清型15B荚膜多糖与载体蛋白的初始输入比例是约2:1。

58.根据权利要求41-57任一项的方法，其中在步骤(b)中，活化的多糖与约1-2摩尔当量的氰基硼氢化钠反应，在约20-26℃的温度持续约40-50小时。

59.根据权利要求41-58任一项的方法，其中所述方法包括额外的下列步骤：

(c)通过加入$NaBH_4$使未反应的醛加帽。

60.根据权利要求41-59任一项的方法，其中所述方法还包括配制多价疫苗中的缀合物的步骤。

61.根据权利要求41-60任一项的方法，其中缀合步骤(b)的产率大于50%。

62.根据权利要求41-60任一项的方法，其中缀合步骤(b)的产率大于60%。

63.免疫原性缀合物，其通过或可通过权利要求41-62任一项的方法获得。

64.根据权利要求63的免疫原性缀合物，其中所述免疫原性缀合物包含相比血清型15B荚膜多糖的总量小于约40％的游离血清型15B荚膜多糖。

65.根据权利要求63-64任一项的免疫原性缀合物，其中所述免疫原性缀合物包含相比血清型15B荚膜多糖的总量小于约20％的游离血清型15B荚膜多糖。

66.根据权利要求63-65任一项的免疫原性缀合物，其中所述免疫原性缀合物具有3000-20000kDa、5000-10000kDa、5000-20000kDa、8000-20000kDa、8000-16000KDa或10000-16000KDa的分子量。

67.根据权利要求61-66任一项的免疫原性缀合物，其中缀合物中血清型15B荚膜多糖与载体蛋白的比例是0.4-2。

68.根据权利要求61-65任一项的免疫原性缀合物，其中缀合物中血清型15B荚膜多糖与载体蛋白的比例是0.7-0.9。

69.根据权利要求61-68任一项的免疫原性缀合物，其中至少40％的免疫原性缀合物在CL-4B柱中具有小于或等于0.3的Kd。

70.根据权利要求61-68任一项的免疫原性缀合物，其中至少60％的免疫原性缀合物在CL-4B柱中具有小于或等于0.3的Kd。

71.根据权利要求61-70任一项的免疫原性缀合物，其中缀合度是2-15、2-13、2-10、2-8、2-6、2-5、2-4、3-15、3-13、3-10、3-8、3-6、3-5、3-4、5-15、5-10、8-15、8-12、10-15或10-12。

72.根据权利要求61-70任一项的免疫原性缀合物，其中所述缀合度是2-6。

73.根据权利要求61-72任一项的免疫原性缀合物，其中所述免疫原性缀合物中每mM血清型15B荚膜多糖的乙酸盐的mM与在活化的多糖中每mM血清型15B荚膜多糖的乙酸盐的mM的比例是至少0.6、0.65、0.7、0.75、0.8、0.85、0.9或0.95，优选至少0.7或至少0.9。

74.根据权利要求61-72任一项的免疫原性缀合物，其中所述免疫原性缀合物中每mM血清型15B荚膜多糖的乙酸盐的mM与在活化的多糖中每mM血清型15B荚膜多糖的乙酸盐的mM的比例是至少0.6、0.65、0.7、0.75、0.8、0.85、0.9或0.95，优选至少0.7或至少0.9。

75.免疫原性组合物，其包含根据权利要求61-74任一项的免疫原性缀合物和生理上可接受的媒介物。

76.疫苗，其包括根据权利要求75的免疫原性组合物。

77.保护受试者免于血清型15B肺炎链球菌感染的方法，所述方法包括给受试者施用免疫原性量的权利要求22-25或75任一项的免疫原性组合物或权利要求26或76的疫苗。

78.治疗或预防在受试者中与血清型15A、15B和/或15C肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法，所述方法包括施用治疗或预防有效量的权利要求22-25或75任一项的免疫原性组合物或权利要求26或76的疫苗的步骤。

79.治疗或预防在受试者中与血清型15B和/或15C肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法，所述方法包括施用治疗或预防有效量的权利要求22-25或75任一项的免疫原性组合物或权利要求26或76的疫苗的步骤。

80.治疗或预防在受试者中与血清型15B肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法，所述方法包括施用治疗或预防有效量的权利要求22-25或75任一项的免疫原性组合物或权利要求26或76的疫苗的步骤。

肺炎链球菌荚膜多糖及其缀合物

技术领域

[0001] 本发明涉及分离的肺炎链球菌(Streptococcus pneumoniae)血清型15B荚膜多糖和它们的制备方法。本发明还涉及包含共价连接到载体蛋白的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物,它们的制备方法和包含它们的免疫原性组合物和疫苗。

背景技术

[0002] 肺炎链球菌是革兰氏阳性、柳叶刀形球菌,其通常成对出现(双球菌),也可以短链或作为单细胞出现。它们很容易生长在血琼脂平板上,具有发光的菌落并显示α溶血,除非厌氧培养,它们表现出β溶血。大多数肺炎球菌血清型的细胞具有荚膜,其是每个细胞周围的多糖包衣。此荚膜是在人类中毒力的决定因素,因为其通过阻止抗体附着到细菌细胞而干扰吞噬作用。目前鉴定了超过90种已知的肺炎球菌荚膜血清型,其中23种最常见的血清型占全球侵袭性疾病的大约90%。作为疫苗,肺炎球菌多糖包衣在具有发达的或未受损害的免疫系统的个体中可赋予合理程度的对肺炎链球菌的免疫性,而缀合到合适的载体蛋白的荚膜多糖允许在婴儿和也对于肺炎球菌感染风险最大的老年人中的免疫应答。

[0003] 自从2000年推出第一款7价肺炎球菌缀合疫苗(PCV7或Prevnar)以来,来自那七个血清型(4、6B、9V、14、18C、19F、和23F)的侵袭性疾病已几乎消失。在Prevnar 13中加入血清型1、3、5、6A、7F和19A进一步降低了侵袭性肺炎球菌疾病的数目。

[0004] 然而,由非疫苗血清型(诸如肺炎链球菌血清型15A、15B和15C)导致的侵袭性肺炎球菌疾病的发病率最近已增加(参见例如Beall B.等,Journal of Clinical Microbiology.44(3):999-1017,2006,或Jacobs等,Clin Infect Dis.(2008)47(11):1388-1395)。目前市售的肺炎球菌疫苗无一提供在人以及特别是在小于2岁的儿童中针对血清型15B肺炎链球菌的适当的保护。因此,存在对可用于诱导针对血清型15B肺炎链球菌的免疫反应的免疫原性组合物的需要。如果这样的免疫原性组合物可用于保护受试者免受血清型15C和/或15A肺炎链球菌的侵害,也将是额外的益处。

[0005] 发明概述

[0006] 在一方面,本公开内容提供了一种具有5kDa-500kDa的分子量的分离的肺炎链球菌血清型15B荚膜多糖。

[0007] 在进一步的方面,本公开内容提供了分离的肺炎链球菌血清型15B荚膜多糖,每mM所述肺炎链球菌血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM,优选至少0.6mM乙酸盐。

[0008] 在进一步的方面,本公开内容提供了分离的肺炎链球菌血清型15B荚膜多糖,每mM所述肺炎链球菌血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM,优选至少0.6mM甘油。

[0009] 在进一步的方面,本公开内容提供一种包含共价连接到载体蛋白的本文所公开的分离的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物。在一方面,所述载体蛋白是CRM_{197}。

[0010] 在进一步的方面，本公开内容提供了包含本文所公开的免疫原性缀合物和生理上可接受的媒介物的免疫原性组合物。在一方面，所述免疫原性组合物还包含至少一种额外的抗原。在一方面，所述免疫原性组合物还包含佐剂。

[0011] 在进一步的方面，本公开内容提供了包含如本文所公开的免疫原性组合物的疫苗。

[0012] 在进一步的方面，本公开内容提供了用于生产如本文所公开的分离的血清型15B多糖的方法，该方法包括以下步骤：

[0013] (a)制备肺炎链球菌血清型15B细菌细胞的发酵培养物；

[0014] (b)裂解所述发酵培养物中的细菌细胞；

[0015] (c)从发酵培养物纯化肺炎链球菌血清型15B荚膜多糖；和，

[0016] (d)通过高压均质裁剪(sizing)纯化的肺炎链球菌血清型15B荚膜多糖。

[0017] 在进一步的方面，本公开内容提供一种用于生产活化的肺炎链球菌血清型15B荚膜多糖的方法，所述方法包括使如本文所公开的分离的肺炎链球菌血清型15B荚膜多糖与氧化剂反应的步骤。在一方面，本公开内容提供了通过上述方法获得或可获得的活化的血清型15B荚膜多糖。

[0018] 在进一步的方面，本公开内容提供了包含共价连接到载体蛋白的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物的制备方法，所述方法包括以下步骤：

[0019] (a)混合如本文公开的活化的多糖与载体蛋白；

[0020] (b)使所述混合的活化的多糖和载体蛋白与还原剂反应以形成血清型15B荚膜多糖-载体蛋白缀合物。在一方面，本公开内容提供了通过上述方法获得或可获得的免疫原性结合物。

[0021] 在进一步的方面，本公开内容提供了保护受试者免于血清型15B肺炎链球菌感染的方法，所述方法包括给受试者施用免疫原性量的本文所公开的免疫原性组合物或疫苗。

[0022] 在进一步的方面，本公开内容提供了治疗或预防在受试者中与血清型15A、15B和/或15C肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法，所述方法包括施用治疗或预防有效量的本文中公开的免疫原性组合物或疫苗的步骤。

[0023] 附图简述

[0024] 图1—肺炎球菌荚膜多糖血清型15B重复单元的结构。

[0025] 发明详述

[0026] 本发明可以通过参考以下本发明优选实施方案和本文包括的实施例的详细描述而更容易地理解。除非另有定义，否则本文所使用的所有技术和科学术语具有与此发明所属领域技术人员通常理解的相同的含义。虽然与本文所述的那些类似或等效的方法和材料可以用于实践或测试本发明，本文仍然描述了某些优选的方法和材料。在描述实施方案和主张本发明中，可以根据下列定义使用某些术语。

[0027] 定义

[0028] 如本文所用，多糖或多肽-载体蛋白缀合物的“分子量”指通过尺寸排阻色谱法(SEC)结合多角度激光散射检测器(MALLS)计算得到的分子量。

[0029] 如本文所用，术语“游离多糖”指没有共价缀合到载体蛋白但是却在血清型15B荚膜多糖-载体蛋白缀合物组合物中存在的血清型15B荚膜多糖。游离多糖可以非共价连接

(即,非共价结合、吸附、或被捕获于其中或用其捕获)多糖载体蛋白缀合物。

[0030] 游离多糖的百分比在血清型15B荚膜多糖-载体蛋白缀合物的最终纯化后测量。优选地,其在最终纯化后4周内测量。其表示为样品中总的多糖的百分比。

[0031] 如本文所用,术语"血清型15B多糖"或"血清型15B荚膜多糖"指肺炎链球菌血清型15B荚膜多糖。

[0032] 如本文所用,术语"血清型15B糖缀合物"或"血清型15B缀合物"指共价缀合到载体蛋白的分离的血清型15B多糖。

[0033] 如本文所用,术语"氧化度"(DO)指当分离的多糖用氧化剂活化时每个生成的醛基的糖重复单元数目。多糖的氧化度可以使用本领域技术人员已知的常规方法确定。

[0034] 如本文所用,术语"受试者"指哺乳动物,包括人,或指鸟、鱼、爬行动物、两栖动物或任何其它动物。术语"受试者"还包括家庭宠物或研究动物。家庭宠物和研究动物的非限制性实例包括:狗、猫、猪、兔、大鼠、小鼠、沙土鼠、仓鼠、豚鼠、雪貂、猴、鸟、蛇、蜥蜴、鱼、龟和蛙。术语"受试者"还包括家畜动物。家畜动物的非限制性实例包括:羊驼、野牛、骆驼、牛、鹿、猪、马、美洲驼、骡、驴、绵羊、山羊、兔、驯鹿、牦牛、鸡、鹅和火鸡。

[0035] 分离的血清型15B荚膜多糖

[0036] 如在图1中所示,血清型15B的多糖重复单元由分支的三糖主链(一个N-乙酰葡糖胺(Glc_pNAc)、一个吡喃半乳糖(Gal_p)和一个吡喃葡萄糖(Glc_p))与连接于Glc_pNAc的C4羟基的αGal_p-βGal_p二糖分支组成。磷酸甘油连接到二糖分支中βGal_p残基的C3羟基。血清型15B荚膜多糖是O-乙酰化的并且O-乙酰化总量是每个多糖重复单元约为0.8至0.9个O-乙酰基(参见例如C.Jones等,Carbohydrate Research,340(2005)403-409)。来自血清型15C血清型的荚膜多糖具有与血清型15B相同的主链结构但是缺少O-乙酰化。

[0037] 本发明的分离的血清型15B多糖可以通过包含下列步骤的方法获得:

[0038] (a)制备血清型15B肺炎链球菌细菌细胞的发酵培养物;

[0039] (b)裂解所述发酵培养物中的细菌细胞;

[0040] (c)从发酵培养物纯化血清型15B多糖;和,

[0041] (d)通过高压均质裁剪纯化的血清型15B多糖。

[0042] 血清型15B多糖可以使用本领域技术人员已知的分离方法直接从细菌获得(参见,例如美国专利申请公开号20060228380、20060228381、20070184071、20070184072、20070231340和20080102498或WO2008118752公开的方法)。此外,它们可以使用合成方案生产。

[0043] 血清型15B肺炎链球菌菌株可以获自建立的培养物集合(诸如例如ATCC保藏菌株No ATCC10354或可获自疾病控制和预防中心的链球菌参考实验室,亚特兰大,佐治亚州(Streptococcal Reference Laboratory of the Center for disease control and prevention,Atlanta,GA)的菌株)或临床样本。

[0044] 细菌细胞优选在基于大豆的培养基中生长。生产肺炎链球菌血清型15B的细菌细胞发酵后,裂解细菌细胞以产生细胞裂解物。细菌细胞可以使用任意裂解剂进行裂解。"裂解剂"是帮助细胞壁破裂和导致细胞裂解的自溶素释放的试剂,包括例如,去垢剂。如本文所用,术语"去垢剂"指能够诱导细菌细胞裂解的任何阴离子或阳离子去垢剂。用于在本发明的方法中使用的此类去垢剂的代表性实例包括脱氧胆酸钠(DOC)、N-月桂酰肌氨酸、鹅脱

氧胆酸钠和皂苷。

[0045] 在本发明的一个实施方案中，用于裂解细菌细胞的裂解剂是DOC。DOC是胆汁酸脱氧胆酸的钠盐，其通常来源于生物来源，诸如母牛或公牛。DOC活化LytA蛋白，其是参与肺炎链球菌细胞壁生长和分裂的自溶素。LytA蛋白在其C-末端部分具有胆碱结合域，并且已知lytA基因的突变产生对DOC裂解有抗性的LytA突变体。

[0046] 在本发明的一个实施方案中，用于溶解细菌细胞的裂解剂是非动物来源的裂解剂。用于在本发明的方法中使用的非动物来源的裂解剂包括来自非动物来源的与DOC具有类似作用模式的试剂(即，所述作用模式是影响LytA功能并导致肺炎链球菌细胞裂解)。此类非动物来源的裂解剂包括但不限于DOC的类似物、表面活性剂、去垢剂和胆碱结构类似物。在一个实施方案中，非动物来源的裂解剂选自癸磺酸、叔辛基苯氧基聚(氧乙烯)乙醇(例如**Igepal®**CA-630，CAS#:9002-93-1，可购自Sigma Aldrich，St.Louis，MO)，辛基酚环氧乙烷缩合物(例如Triton X-100，可购自Sigma Aldrich，St.Louis，MO)、N-月桂酰肌氨酸、N-月桂酰肌氨酸钠、月桂基亚氨基二丙酸、十二烷基硫酸钠、鹅脱氧胆酸盐、猪脱氧胆酸盐、甘氨脱氧胆酸盐、牛磺脱氧胆酸盐、牛磺鹅脱氧胆酸盐和胆酸盐。在另一个实施方案中，非动物来源的裂解剂是N-月桂酰肌氨酸。在另一个实施方案中，所述裂解剂是N-月桂酰肌氨酸钠。

[0047] 血清型15B多糖然后可以使用本领域中已知的纯化技术(包括使用离心、深度过滤、沉淀、超滤、活性炭处理、渗滤和/或柱色谱法)从细胞裂解物中分离(参见，例如美国专利申请公开号20060228380、20060228381、20070184071、20070184072、20070231340和20080102498或WO2008118752)。可以然后使用纯化的血清型15B荚膜多糖用于免疫原性缀合物的制备。

[0048] 优选地，为了生成具有有利的滤过性特征和/或收率的缀合物，在缀合到载体蛋白之前进行多糖的裁剪成低分子量(MW)范围。有利地，纯化的血清型15B多糖的大小减小同时保留多糖结构的关键特征，诸如例如存在O-乙酰基。优选地，纯化的血清型15B多糖的大小通过机械均质来减小。

[0049] 在优选的实施方案中，纯化的血清型15B多糖的大小通过高压均质来减小。高压均质通过将工艺流泵送通过具有足够小尺寸的流路实现高剪切率。通过使用更大的施加的均质压力增加剪切率并且暴露时间可以通过将进料流再循环通过均质机而增加。

[0050] 高压均质方法特别适用于减小纯化的血清型15B多糖的大小，同时保留多糖的结构特征，诸如O-乙酰基的存在。

[0051] 通过从肺炎链球菌裂解物纯化血清型15B多糖和任选地纯化的多糖的裁剪所获得的分离的血清型15B荚膜多糖可以通过不同的参数来表征，包括例如分子量、每mM所述血清型15B荚膜多糖的甘油的mM、每mM所述血清型15B荚膜多糖的乙酸盐的mM。

[0052] 多糖的O-乙酰化程度可通过本领域中已知的任何方法确定，例如通过质子NMR(参见例如Lemercinier和Jones(1996)Carbohydrate Research 296;83-96、Jones和Lemercinier(2002)J.Pharmaceutical and Biomedical Analysis 30;1233-1247、WO 05/033148或WO00/56357)。另一种常用方法描述于Hestrin(1949)J.Biol.Chem.180;249-261中。优选地，O-乙酰基的存在通过离子HPLC分析确定。

[0053] 在纯化的、分离的或活化的血清型15B荚膜多糖中或在血清型15B多糖-载体蛋白

缀合物中O-乙酰基的存在表示为每mM所述多糖的乙酸盐的mM数或作为每个多糖重复单元O-乙酰基的数目。

[0054] 甘油磷酸侧链的存在可以在通过用氢氟酸(HF)处理多使其释放后，通过使用具有脉冲安培检测法的高效阴离子交换色谱法(HPAEC-PAD)测量甘油来确定。在纯化的分离的或活化的血清型15B荚膜多糖中或在血清型15B多糖-载体蛋白缀合物中甘油的存在表示为每mM血清型15B多糖的甘油的mM数。

[0055] 分离的血清型15B荚膜多糖也可以使用本领域技术人员已知的方法合成产生。

[0056] 在优选的实施方案中，分离的血清型15B荚膜多糖具有5-500kDa、50-500kDa、50-450kDa、100-400kDa、100-350kDa的分子量。在优选的实施方案中，分离的血清型15B荚膜多糖具有100-350kDa的分子量。在优选的实施方案中，分离的血清型15B荚膜多糖具有100-300kDa的分子量。在优选的实施方案中，分离的血清型15B荚膜多糖具有150-300kDa的分子量。

[0057] 在优选的实施方案中，所述分离的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM乙酸盐。在优选的实施方案中，所述分离的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.5、0.6、或0.7mM乙酸盐。在优选的实施方案中，所述分离的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐。在优选的实施方案中，所述分离的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.7mM乙酸盐。在优选的实施方案中，O-乙酰基的存在通过离子-HPLC分析确定。

[0058] 在优选的实施方案中，所述分离的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM甘油。在优选的实施方案中，所述分离的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.5、0.6、或0.7mM甘油。在优选的实施方案中，所述分离的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。在优选的实施方案中，所述分离的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.7mM甘油。

[0059] 在优选的实施方案中，所述分离的血清型15B荚膜多糖具有100-350kDa、优选150-350kDa的分子量，并且每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐。

[0060] 在优选的实施方案中，所述分离的血清型15B荚膜多糖具有100-350kDa、优选150-350kDa的分子量，并且每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。

[0061] 在优选的实施方案中，所述分离的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐和每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。

[0062] 在优选的实施方案中，所述分离的血清型15B荚膜多糖具有100-350kDa、优选150-350kDa的分子量，并且每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐和每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。

[0063] 血清型15B荚膜多糖-载体蛋白缀合物

[0064] 分离的血清型15B荚膜多糖可以缀合到载体蛋白以获得免疫原性缀合物。分离的多糖可以通过本领域技术人员已知的方法缀合到载体蛋白(参见例如美国专利申请公开号20060228380、20070184071、20070184072、20070231340或WO2011/100151)。

[0065] 在一个实施方案中，可以用1-氰基-4-二甲基氨基-吡啶鎓四氟硼酸盐(CDAP)活化

多糖以形成氰酸酯。活化的多糖可以与载体蛋白上的氨基直接偶联或通过间隔子(接头)基团偶联。例如,间隔可为胱胺或半胱胺以提供硫醇化多糖,其可通过与马来酰亚胺活化的载体蛋白(例如,使用GMBS)或卤乙酰化的载体蛋白(例如使用碘乙酰亚胺或溴乙酸N-琥珀酰亚胺酯或SIAB,或SIA,或SBAP)反应后获得的硫醚键与载体偶联。优选地,氰酸酯(任选地通过CDAP化学反应制得)与己二胺或己二酸二酰肼(ADH)偶联,并且氨基衍生化的糖采用碳二亚胺(例如EDAC或EDC)化学反应通过蛋白载体上的羧基与载体蛋白偶联。此类缀合物描述于例如WO93/15760、WO 95/08348和WO 96129094。

[0066] 其他合适的技术使用碳二亚胺、酰肼、活性酯、降冰片烷、对硝基苯甲酸、N-羟基琥珀酰亚胺、S-NHS、EDC、TSTU。很多描述于国际专利申请公开号WO98/42721中。偶联可以涉及羰基接头,其可以通过使糖的游离羟基与CDI反应形成(参见Bethell等,1979,1.Biol.Chern.254:2572-4;Hearn等,1981,J.Chromatogr.218:509-18),之后与蛋白反应以形成氨基甲酸酯键。这可涉及到异头末端还原到伯羟基,任选地,伯羟基基团的保护/脱保护,伯羟基基团与CDI反应形成CDI氨基甲酸酯中间体,以及CDI氨基甲酸酯中间体与蛋白质氨基的偶联。

[0067] 在优选的实施方案中,分离的血清型15B荚膜多糖通过还原性胺化缀合到载体蛋白。还原性胺化涉及通过氧化活化的多糖和活化的多糖通过还原与蛋白载体缀合。

[0068] 血清型15B荚膜多糖的活化

[0069] 活化的血清型15B荚膜多糖通过使分离的血清型15B荚膜多糖与氧化剂反应获得。例如,所述活化的血清型15B荚膜多糖可以通过包括以下步骤的方法获得:

[0070] (a)制备血清型15B肺炎链球菌细菌细胞的发酵培养物;

[0071] (b)裂解所述发酵培养物中的细菌细胞;

[0072] (c)从发酵培养物纯化血清型15B多糖;

[0073] (d)通过高压均质裁剪纯化的血清型15B多糖。

[0074] (e)使经裁剪的血清型15B多糖与氧化剂反应。

[0075] 在优选的实施方案中,与氧化剂反应的分离的血清型15B荚膜多糖的浓度为0.1-10mg/mL、0.5-5mg/mL、1-3mg/mL,或约2mg/mL。

[0076] 在优选的实施方案中,所述氧化剂是高碘酸盐。高碘酸盐氧化邻位羟基以形成羰基或醛基并导致切割C-C键。术语'高碘酸盐'既包括高碘酸盐也包括高碘酸。该术语还既包括偏高碘酸盐(IO_4^-)也包括正高碘酸盐(IO_6^{5-})。术语'高碘酸盐'还包括高碘酸盐的各种盐,包括高碘酸钠和高碘酸钾。在优选的实施方案中,所述氧化剂是高碘酸钠。在优选的实施方案中,用于氧化血清型15B荚膜多糖的高碘酸盐是偏高碘酸盐。在优选的实施方案中,用于氧化血清型15B荚膜多糖的高碘酸盐是偏高碘酸钠。

[0077] 在优选的实施方案中,多糖与0.01-10、0.05-5、0.1-1、0.5-1、0.7-0.8、0.05-0.5、0.1-0.3摩尔当量的氧化剂反应。在优选的实施方案中,多糖与约0.1、0.15、0.2、0.25、0.3、0.35、0.4、0.45、0.5、0.55、0.6、0.65、0.7、0.75、0.8、0.85、0.9、0.95摩尔当量的氧化剂反应。在优选的实施方案中,多糖与约0.15摩尔当量的氧化剂反应。在优选的实施方案中,多糖与约0.25摩尔当量的氧化剂反应。在优选的实施方案中,多糖与约0.5摩尔当量的氧化剂反应。在优选的实施方案中,多糖与约0.6摩尔当量的氧化剂反应。在优选的实施方案中,多糖与约0.7摩尔当量的氧化剂反应。

[0078] 在优选的实施方案中，反应的持续时间是1-50、10-30、15-20、15-17小时或约16小时。

[0079] 在优选的实施方案中，反应的温度维持在15-45℃、15-30℃、20-25℃。在优选的实施方案中，反应的温度维持在约23℃。

[0080] 在优选的实施方案中，氧化反应在选自磷酸钠、磷酸钾、2-(N-吗啉代)乙磺酸(MES)或Bis-Tris的缓冲液中进行。在优选的实施方案中，缓冲液是磷酸钾。

[0081] 在优选的实施方案中，缓冲液具有1-500mM、1-300mM、50-200mM的浓度。在优选的实施方案中，缓冲液具有约100mM的浓度。在优选的实施方案中，氧化反应在4-8、5-7、5.5和6.5的pH下进行。在优选的实施方案中，pH是约6。

[0082] 在优选的实施方案中，活化的血清型15B荚膜多糖通过使0.5-5mg/mL分离的血清型15B荚膜多糖与0.2-0.3摩尔当量的高碘酸盐在20-25℃的温度下反应获得。

[0083] 在优选的实施方案中，活化的血清型15B荚膜多糖是纯化的。活化的血清型15B荚膜多糖根据本领域技术人员已知的方法纯化，诸如凝胶渗透色谱法(GPC)、透析或超滤/渗滤。例如，活化的荚膜多糖通过使用超滤装置浓缩和渗滤。

[0084] 在优选的实施方案中，本发明涉及通过上述公开的方法获得或可获得的活化的血清型15B荚膜多糖。

[0085] 在优选的实施方案中，活化的血清型15B荚膜多糖的氧化度是2-20、2-15、2-10、2-5、5-20、5-15、5-10、10-20、10-15、15-20。在优选的实施方案中，活化的血清型15B荚膜多糖的氧化度是2-10、4-8、4-6、6-8、6-12、8-12、9-11、10-16、12-16、14-18、16-20、16-18或18-20。

[0086] 在优选的实施方案中，活化的血清型15B荚膜多糖具有5-500kDa、50-500kDa、50-450kDa、100-400kDa、100-350kDa的分子量。在优选的实施方案中，活化的血清型15B荚膜多糖具有100-350kDa的分子量。在优选的实施方案中，活化的血清型15B荚膜多糖具有100-300kDa的分子量。在优选的实施方案中，活化的血清型15B荚膜多糖具有150-300kDa的分子量。在优选的实施方案中，活化的血清型15B荚膜多糖具有100-250kDa的分子量。

[0087] 在优选的实施方案中，所述活化的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM乙酸盐。在优选的实施方案中，所述活化的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.5、0.6或0.7mM乙酸盐。在优选的实施方案中，所述活化的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐。在优选的实施方案中，所述活化的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.7mM乙酸盐。在优选的实施方案中，O-乙酰基的存在通过离子-HPLC分析确定。

[0088] 在优选的实施方案中，所述活化的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM甘油。在优选的实施方案中，所述活化的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.5、0.6或0.7mM甘油。在优选的实施方案中，所述活化的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。在优选的实施方案中，所述活化的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.7mM甘油。

[0089] 在优选的实施方案中，所述活化的血清型15B荚膜多糖具有100-250kDa的分子量，

并且每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐。

[0090] 在优选的实施方案中，所述活化的血清型15B荚膜多糖具有100-250kDa的分子量，并且每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。

[0091] 在优选的实施方案中，所述活化的血清型15B荚膜多糖每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐和每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。

[0092] 在优选的实施方案中，所述活化的血清型15B荚膜多糖具有100-250kDa的分子量，并且每mM所述血清型15B荚膜多糖包含至少0.6mM乙酸盐和每mM所述血清型15B荚膜多糖包含至少0.6mM甘油。

[0093] 在实施方案中，活化的血清型15B荚膜多糖是冻干的，任选地在冷冻保护剂/冻干保护剂的存在下冻干。在实施方案中，所述冷冻保护剂/冻干保护剂是糖。在优选的实施方案中，所述糖选自蔗糖、海藻糖、棉子糖、水苏糖、松三糖、葡聚糖、甘露醇、乳糖醇及帕拉金糖醇。在优选的实施方案中，所述糖是蔗糖。冻干的活化的荚膜多糖可以然后与包含载体蛋白的溶液混合。

[0094] 在另一个实施方案中，活化的血清型15B荚膜多糖与载体蛋白混合并冻干，任选地在冷冻保护剂/冻干保护剂的存在下冻干。在实施方案中，所述冷冻保护剂/冻干保护剂是糖。在优选的实施方案中，所述糖选自蔗糖、海藻糖、棉子糖、水苏糖、松三糖、葡聚糖、甘露醇、乳糖醇及帕拉金糖醇。在优选的实施方案中，所述糖是蔗糖。共冻干的多糖和载体蛋白可以然后重悬在溶液中并与还原剂反应。

[0095] 在实施方案中，本发明涉及冻干的活化的血清型15B荚膜多糖。

[0096] 在实施方案中，本发明涉及共冻干的活化的血清型15B荚膜多糖和蛋白载体。在优选的实施方案中，所述蛋白载体是CRM_{197}。

[0097] 活化的血清型15B荚膜多糖和载体蛋白的缀合

[0098] 活化的血清型15B荚膜多糖可以通过包含下列步骤的方法缀合到载体蛋白：

[0099] (a)混合活化的血清型15B荚膜多糖和载体蛋白，和，

[0100] (b)使所述混合的活化的血清型15B荚膜多糖和载体蛋白与还原剂反应以形成血清型15B荚膜多糖-载体蛋白缀合物。

[0101] 与例如在水溶液中的还原胺化(其中多糖的O-乙酰化水平显著降低)相比，通过在二甲亚砜(DMSO)中还原胺化活化的血清型15B荚膜多糖和蛋白载体的缀合适合于保持多糖的O-乙酰基含量。在优选的实施方案中，步骤(a)和步骤(b)在DMSO中进行。

[0102] 在优选的实施方案中，步骤(a)包括在包含载体蛋白和DMSO的溶液中溶解冻干的血清型15B荚膜多糖。在优选的实施方案中，步骤(a)包括在DMSO中溶解共冻干的血清型15B荚膜多糖和载体蛋白。

[0103] 当步骤(a)和(b)在水溶液中进行时，步骤(a)和(b)在缓冲液中在6.0-8.5、7-8或7-7.5的pH下进行，所述缓冲液优选选自PBS、MES、HEPES、Bis-Tris、ADA、PIPES、MOPSO、BES、MOPS、DIPSO、MOBS、HEPPSO、POPSO、TEA、EPPS、Bicine或HEPB。在优选的实施方案中，所述缓冲液是PBS。在优选的实施方案中，pH是约7.3。

[0104] 在优选的实施方案中，步骤(b)中活化的血清型15B荚膜多糖的浓度为0.1-10mg/mL、0.5-5mg/mL、0.5-2mg/mL。在优选的实施方案中，步骤(b)中活化的血清型15B荚膜多糖的浓度为0.1、0.2、0.3、0.4、0.5、0.6、0.7、0.8、0.9、1、1.1、1.2、1.3、1.4、1.5、1.6、1.7、

1.8、1.9、2、2.1、2.2、2.3、2.4、2.5、2.6、2.7、2.8、2.9或3mg/mL。

[0105] 在优选的实施方案中，活化的血清型15B荚膜多糖与载体蛋白的初始输入比例(重量比)是5:1-0.1:1、2:1-0.1:1、2:1-1:1、1.5:1-1:1、0.1:1-1:1、0.3:1-1:1、0.6:1-1:1。

[0106] 在优选的实施方案中，活化的血清型15B荚膜多糖与载体蛋白的初始输入比例约0.6:1-1.5:1，优选0.6:1-1:1。这样的初始输入比例特别适合在免疫原性缀合物中获得低水平的游离多糖。

[0107] 在优选的实施方案中，活化的血清型15B荚膜多糖与载体蛋白的初始输入比例是0.4:1、0.5:1、0.6:1、0.7:1、0.8:1、0.9:1、1:1、1.1:1、1.2:1、1.3:1、1.4:1、1.5:1、1.6:1、1.7:1、1.8:1、1.9:1或2:1。

[0108] 在实施方案中，还原剂是氰基硼氢化钠，三乙酰氧基硼氢化钠，布朗斯台德或路易斯酸存在下的硼氢化钠或硼氢化锌，胺合硼烷(诸如吡啶硼烷、2-甲基吡啶硼烷、2,6-二硼烷-甲醇、二甲胺-硼烷、t-BuMe^{i}PrN-BH_3、苄胺-BH_3或5-乙基-2-甲基吡啶硼烷(PEMB)。在优选的实施方案中，所述还原剂是氰基硼氢化钠。在优选的实施方案中，所述还原剂是2-甲基吡啶硼烷钠。

[0109] 在优选的实施方案中，在步骤(b)使用的还原剂的量是大约0.1-10摩尔当量、0.5-5摩尔当量，1-2摩尔当量。在优选的实施方案中，在步骤(b)使用的还原剂的量是大约1、1.1、1.2、1.3、1.4、1.5、1.6、1.7、1.8、1.9或2摩尔当量。

[0110] 在优选的实施方案中，步骤(b)的持续时间是1-60小时、10-50小时、40-50小时、42-46小时。在优选的实施方案中，步骤(b)的持续时间是约44小时。

[0111] 在优选的实施方案中，步骤(b)中反应的温度维持在10-40℃、15-30℃或20-26℃。在优选的实施方案中，步骤(b)中反应的温度维持在约23℃。

[0112] 在优选的实施方案中，包含共价连接到载体蛋白的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物的制备方法进一步包括通过加入$NaBH_4$对未反应的醛加帽(淬灭)的步骤(步骤(c))。

[0113] 在优选的实施方案中，在步骤(c)使用的$NaBH_4$的量是大约0.1-10摩尔当量、0.5-5摩尔当量，1-3摩尔当量。在优选的实施方案中，在步骤(c)使用的$NaBH_4$的量是大约2摩尔当量。

[0114] 在优选的实施方案中，步骤(c)的持续时间是0.1-10小时、0.5-5小时、2-4小时。在优选的实施方案中，步骤(c)的持续时间是约3小时。

[0115] 在优选的实施方案中，步骤(c)中反应的温度维持在15-45℃、15-30℃或20-26℃。在优选的实施方案中，步骤(c)中反应的温度维持在约23℃。

[0116] 在优选的实施方案中，缀合步骤(步骤b)的产率是大于50%、55%、60%、65%、70%、75%、80%、85%或90%。在优选的实施方案中，缀合步骤(步骤b)的产率是大于60%。在优选的实施方案中，缀合步骤(步骤b)的产率是大于70%。该产率是在缀合物中血清型15B多糖的量×100/缀合步骤中使用的活化的多糖的量。

[0117] 在优选的实施方案中，包含共价连接到载体蛋白的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物的制备方法包括以下步骤：

[0118] (a)制备血清型15B肺炎链球菌细菌细胞的发酵培养物；

[0119] (b)裂解所述发酵培养物中的细菌细胞；

[0120] (c)从发酵培养物纯化血清型15B多糖；

[0121] (d)通过高压均质裁剪纯化的血清型15B多糖；

[0122] (e)使经裁剪的血清型15B多糖与氧化剂反应；

[0123] (f)混合活化的血清型15B多糖和载体蛋白，和，

[0124] (g)使所述混合的活化的血清型15B多糖和载体蛋白与还原剂反应以形成血清型15B多糖-载体蛋白缀合物；和，

[0125] (h)通过加入$NaBH_4$使未反应的醛加帽(淬灭)。

[0126] 在优选的实施方案中，上述方法的缀合步骤(步骤g)的产率是大于50％、55％、60％、65％、70％、75％、80％、85％或90％。在优选的实施方案中，所述缀合步骤(步骤g)的产率是大于60％。在优选的实施方案中，所述缀合步骤(步骤g)的产率是大于70％。该产率是在缀合物中血清型15B多糖的量×100)/缀合步骤中使用的活化的多糖的量。

[0127] 血清型15B荚膜多糖缀合到载体蛋白之后，可以通过本领域技术人员已知的各种技术纯化多糖-蛋白缀合物(使多糖-蛋白缀合物的量富集)。这些技术包括透析、浓缩/渗滤操作、切向流过滤、沉淀/洗脱、柱色谱法(DEAE或疏水相互作用色谱法)和深度过滤。

[0128] 在优选的实施方案中，载体蛋白是无毒和非反应性的并可以足够的量和纯度获得的。载体蛋白应当适合标准的缀合程序。

[0129] 在优选的实施方案中，活化的血清型15B荚膜多糖缀合选自下列的载体蛋白：DT(白喉毒素)；TT(破伤风类毒素)或TT的片段C；CRM_{197}(白喉毒素的无毒但抗原性相同的变体)；其它DT点突变体诸如CRM176、CRM228、CRM45(Uchida等J.BioI.Chem.218；3838-3844，1973)，CRM 9、CRM102、CRM 103和CRM107和其它由Nicholls和Youle描述于Genetically Engineered Toxins，Ed:Frankel，Maecel Dekker Inc，1992中的突变，Glu-148缺失或至Asp、Gln或Ser和/或Ala 158至Gly的突变和描述于US 4709017或US 4950740中的其它突变，至少一个或多个残基Lys 516、Lys 526、Phe 530和/或Lys 534的突变和US 5917017或US 6455673中公开的其它突变，或US 5843711中公开的片段；肺炎球菌的肺炎球菌溶血素(Kuo等(1995)Infect Immun 63；2706-13)包括以某种方式脱毒的ply例如dPLY-GMBS(WO 04081515，PCT/EP2005/010258)或dPLY-formol、PhtX，包括PhtA、PhtB、PhtD或PhtE(PhtA、PhtB、PhtD或PhtE的序列公开于WO 00/37105或WO 00/39299)和Pht蛋白的融合蛋白例如PhtDE融合蛋白、PhtBE融合蛋白、Pht A-E(WO 01/98334、WO 03/54007、W02009/000826)；OMPC(脑膜炎球菌外膜蛋白-通常从脑膜炎奈瑟球菌血清组B提取-EP0372501)；PorB(来自脑膜炎奈瑟氏球菌)；PD(流感嗜血杆菌蛋白D-参见例如，EP 0 594 610B)，或其免疫功能等价物；合成肽(EP0378881，EP0427347)；热休克蛋白(WO93/17712，WO 94/03208)；百日咳蛋白(WO98/58668，EP0471 177)；细胞因子；淋巴因子；生长因子或激素(WO10 91/01146)；包含来自各种病原体来源的抗原的多种人CD4+T细胞表位的人工蛋白(Falugi等(2001)Eur J Immunol 31；3816-3824)，诸如N19蛋白(Baraldoi等(2004)Infect Immun 72；4884-7)；肺炎球菌表面蛋白PspA(WO 02/091998)；铁摄取蛋白(WO 01/72337)；艰难梭菌的毒素A或B(WO 00/61761)。在实施方案中，活化的血清型15B荚膜多糖缀合至DT(白喉类毒素)。在另一个实施方案中，活化的血清型15B荚膜多糖缀合至TT(破伤风类毒素)。在另一个实施方案中，活化的血清型15B荚膜多糖缀合至TT的片段C。在另一个实施方案中，活化的血清型15B荚膜多糖缀合至PD(流感嗜血杆菌蛋白D——参见例如EP 0 594 610B)。

[0130] 在优选的实施方案中，本发明的活化的血清型15B荚膜多糖缀合至CRM_{197}蛋白。CRM_{197}蛋白是白喉毒素的无毒形式，但是与白喉毒素免疫原性没有区别。CRM_{197}由被通过产毒的β-棒状杆菌噬菌体(Corynephage beta)的亚硝基胍诱变产生的不产毒的噬菌体$\beta 197^{tox}$感染的白喉杆菌产生(Uchida,T.等1971,Nature New Biology 233:8-11)。通过超滤，硫酸铵沉淀和离子交换色谱法纯化CRM_{197}。CRM_{197}蛋白与白喉毒素具有相同的分子量但是与其区别在于结构基因中的单个碱基改变(鸟嘌呤至腺嘌呤)。此单碱基改变导致成熟蛋白中的氨基酸取代(甘氨酸被谷氨酸取代)，并消除了白喉毒素的毒性。CRM_{197}蛋白是用于糖的安全和有效的T细胞依赖性载体。关于CRM_{197}和其生产的进一步细节可以见于例如US 5,614,382中。

[0131] 在实施方案中，本发明涉及包含共价连接到载体蛋白的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物。在实施方案中，本发明涉及包含通过还原胺化共价连接到载体蛋白的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物。在实施方案中，本发明涉及包含通过在DMSO中还原胺化共价连接到载体蛋白的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物。在优选的实施方案中，所述载体蛋白是CRM_{197}。在优选的实施方案中，所述多糖是如本文所定义的分离的血清型15B荚膜多糖。在优选的实施方案中，所述多糖是如本文所定义的分离的血清型15B荚膜多糖，其已通过高压均质进行裁剪。

[0132] 在优选的实施方案中，所述免疫原性缀合物包含相比血清型15B荚膜多糖的总量小于约50、45、40、35、30、25、20或15%的游离血清型15B荚膜多糖。在优选的实施方案中，所述免疫原性缀合物包含相比血清型15B荚膜多糖的总量小于约25%的游离血清型15B荚膜多糖。在优选的实施方案中，所述免疫原性缀合物包含相比血清型15B荚膜多糖的总量小于约20%的游离血清型15B荚膜多糖。在优选的实施方案中，所述免疫原性缀合物包含相比血清型15B荚膜多糖的总量小于约15%的游离血清型15B荚膜多糖。

[0133] 在优选的实施方案中，免疫原性缀合物具有3000-20000kDa、5000-10000kDa、5000-20000kDa、8000-20000kDa、8000-16000KDa或10000-16000KDa的分子量。所述免疫原性缀合物的分子量通过SEC-MALLS测量。

[0134] 在优选的实施方案中，缀合物中血清型15B荚膜多糖与载体蛋白的比例(重量比)是0.5-3。在优选的实施方案中，缀合物中血清型15B荚膜多糖与载体蛋白的比例是0.4-2、0.5-2、0.5-1.5、0.5-1、1-1.5、1-2。在优选的实施方案中，缀合物中血清型15B荚膜多糖与载体蛋白的比例是0.7-0.9。

[0135] 尺寸排阻色谱介质(CL-4B)可用来确定缀合物的相对分子大小分布。尺寸排阻色谱法(SEC)在重力给料柱中使用以分析缀合物的分子大小分布。在介质中被孔排除的大分子比小分子洗脱更加迅速。级分收集器用于收集柱洗脱液。级分通过糖类测定进行比色测试。对于Kd值的确定，校准柱子，以确立分子完全排除在外的级分(V_0)，(Kd＝0)，并且该级分代表最大保留(V_i)，(Kd＝1)。特定样品属性所达到的级分(V_e)通过表达式$Kd=(V_e-V_0)/(V_i-V_0)$与Kd相关。

[0136] 在优选的实施方案中，至少20%免疫原性缀合物在CL-4B柱中具有低于或等于0.3的Kd。在优选的实施方案中，至少30%免疫原性缀合物在CL-4B柱中具有低于或等于0.3的Kd。在优选的实施方案中，至少40%免疫原性缀合物在CL-4B柱中具有低于或等于0.3的Kd。在优选的实施方案中，至少40%、45%、50%、55%、60%、65%、70%、75%、80%或85%免疫

原性缀合物在CL-4B柱中具有低于或等于0.3的Kd。在优选的实施方案中，至少60％免疫原性缀合物在CL-4B柱中具有低于或等于0.3的Kd。在优选的实施方案中，至少70％免疫原性缀合物在CL-4B柱中具有低于或等于0.3的Kd。

[0137] 在优选的实施方案中，40％-90％的血清型15B免疫原性缀合物在CL-4B柱中具有低于或等于0.3的Kd。在优选的实施方案中，50％-90％的血清型15B免疫原性缀合物在CL-4B柱中具有低于或等于0.3的Kd。在优选的实施方案中，65％-80％的血清型15B免疫原性缀合物在CL-4B柱中具有低于或等于0.3的Kd。

[0138] 在优选的实施方案中，所述免疫原性缀合物每mM血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM乙酸盐。在优选的实施方案中，所述免疫原性缀合物每mM血清型15B荚膜多糖包含至少0.5、0.6或0.7mM乙酸盐。在优选的实施方案中，所述免疫原性缀合物每mM血清型15B荚膜多糖包含至少0.6mM乙酸盐。在优选的实施方案中，所述免疫原性缀合物每mM血清型15B荚膜多糖包含至少0.7mM乙酸盐。在优选的实施方案中，O-乙酰基的存在通过离子-HPLC分析确定。

[0139] 在优选的实施方案中，免疫原性缀合物中每mM血清型15B荚膜多糖的乙酸盐的mM与在分离的多糖中每mM血清型15B荚膜多糖的乙酸盐的mM的比例是至少0.6、0.65、0.7、0.75、0.8、0.85、0.9或0.95。在优选的实施方案中，免疫原性缀合物中每mM血清型15B荚膜多糖的乙酸盐的mM与在分离的多糖中每mM血清型15B荚膜多糖的乙酸盐的mM的比例是至少0.7。在优选的实施方案中，免疫原性缀合物中每mM血清型15B荚膜多糖的乙酸盐的mM与在分离的多糖中每mM血清型15B荚膜多糖的乙酸盐的mM的比例是至少0.9。在优选的实施方案中，O-乙酰基的存在通过离子-HPLC分析确定。

[0140] 在优选的实施方案中，免疫原性缀合物中每mM血清型15B荚膜多糖的乙酸盐的mM与在活化的多糖中每mM血清型15B荚膜多糖的乙酸盐的mM的比例是至少0.6、0.65、0.7、0.75、0.8、0.85、0.9或0.95。在优选的实施方案中，免疫原性缀合物中每mM血清型15B荚膜多糖的乙酸盐的mM与在活化的多糖中每mM血清型15B荚膜多糖的乙酸盐的mM的比例是至少0.7。在优选的实施方案中，免疫原性缀合物中每mM血清型15B荚膜多糖的乙酸盐的mM与在活化的多糖中每mM血清型15B荚膜多糖的乙酸盐的mM的比例是至少0.9。在优选的实施方案中，O-乙酰基的存在通过离子-HPLC分析确定。

[0141] 在优选的实施方案中，所述免疫原性缀合物每mM血清型15B荚膜多糖包含至少0.1、0.2、0.3、0.4、0.5、0.6、0.7或0.8mM甘油。在优选的实施方案中，所述免疫原性缀合物每mM血清型15B荚膜多糖包含至少0.5、0.6或0.7mM甘油。在优选的实施方案中，所述免疫原性缀合物每mM血清型15B荚膜多糖包含至少0.6mM甘油。在优选的实施方案中，所述免疫原性缀合物每mM血清型15B荚膜多糖包含至少0.7mM甘油。

[0142] 缀合度是载体蛋白中缀合到血清型15B荚膜多糖的赖氨酸残基的数目。通过使用本领域技术人员已知的常规方法通过氨基酸分析获得由于共价连接至多糖的载体蛋白的赖氨酸修饰的证据。相比用于产生缀合材料的CRM_{197}蛋白原料，缀合导致回收的赖氨酸残基的数目减少。

[0143] 在优选的实施方案中，免疫原性缀合物的缀合度是2-15、2-13、2-10、2-8、2-6、2-5、2-4、3-15、3-13、3-10、3-8、3-6、3-5、3-4、5-15、5-10、8-15、8-12、10-15或10-12。在优选的实施方案中，免疫原性缀合物的缀合度是2-5。

[0144] 免疫原性组合物

[0145] 术语“免疫原性组合物”涉及含有抗原(例如微生物或其组分)的任何药物组合物，所述组合物可以用于引发受试者中的免疫应答。

[0146] 如本文中所用，“免疫原性”是指抗原(或该抗原的表位)(诸如细菌荚膜多糖)或免疫原性缀合物或免疫原性组合物在宿主(诸如哺乳动物)中引发体液免疫应答或细胞介导的免疫应答或两者的能力。

[0147] 在实施方案中，本公开内容涉及包含本文中公开的免疫原性的血清型15B荚膜多糖-载体蛋白缀合物的免疫原性组合物。

[0148] 在实施方案中，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够结合血清型15B肺炎链球菌的抗体的形成。在实施方案中，如通过标准ELISA测定所测量的，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够结合血清型15B肺炎链球菌的抗体的形成。

[0149] 在实施方案中，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够结合血清型15B和15A和/或15C肺炎链球菌的抗体的形成。在实施方案中，如通过标准ELISA测定所测量的，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够结合血清型15B和15A和/或15C肺炎链球菌的抗体的形成。

[0150] 在实施方案中，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够结合血清型15B和15C肺炎链球菌的抗体的形成。在实施方案中，如通过标准ELISA测定所测量的，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够结合血清型15B和15C肺炎链球菌的抗体的形成。

[0151] 在ELISA(酶联免疫吸附测定)方法中，来自接种受试者的血清的抗体与已吸附到固体支持物的多糖一起孵育。结合的抗体用酶缀合的二级检测抗体进行检测。

[0152] 在实施方案中，所述ELISA测定法是标准化的(WHO)ELISA测定法，如由WHO在‘Training manual for Enzyme linked immunosorbent assay for the quantitation of Streptococcus pneumoniae serotype specific IgG(Pn PS ELISA).’中定义(可于http://www.vaccine.uab.edu/ELISA%20protocol.pdf访问；2014年3月31日访问)。

[0153] ELISA测量人血清中存在的类型特异性IgG抗-肺炎链球菌荚膜多糖(PS)抗体。当人血清的稀释液添加到类型特异性荚膜PS包被的微量滴定板上时，特异性针对该荚膜PS的抗体结合到微量滴定板上。结合到板上的抗体使用山羊抗人IgG碱性磷酸酶标记的抗体，然后用磷酸对硝基苯酯底物进行检测。有色终产物的光密度与血清中存在抗荚膜PS抗体的量成正比。

[0154] 在实施方案中，如通过ELISA测定法所确定，本发明的免疫原性组合物能够引发人中IgG抗体，其能够以下列浓度结合肺炎链球菌血清型15B多糖：至少为0.05、0.1、0.2、0.3、0.35、0.4或0.5μg/ml。

[0155] 在实施方案中，如通过ELISA测定法所确定，本发明的免疫原性组合物能够引发人中IgG抗体，其能够以下列浓度结合肺炎链球菌血清型15C多糖：至少为0.05、0.1、0.2、0.3、0.35、0.4或0.5μg/ml。

[0156] 在实施方案中，如通过ELISA测定法所确定，本发明的免疫原性组合物能够引发人中IgG抗体，其能够以下列浓度结合肺炎链球菌血清型15B和15C多糖：至少为0.05、0.1、

0.2、0.3、0.35、0.4或0.5μg/ml。

[0157] 在实施方案中，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够在如本文所公开的调理吞噬作用测定(OPA)中杀死血清型15B肺炎链球菌的抗体的形成。在实施方案中，本文所公开的免疫原性组合物，当在如本文所公开的OPA测定中测试时，具有高于未缀合的天然肺炎链球菌血清型15B荚膜多糖所获得的OPA滴度的OPA滴度。

[0158] 在实施方案中，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够在如本文所公开的调理吞噬作用测定中杀死血清型15C肺炎链球菌的抗体的形成。在实施方案中，本文所公开的免疫原性组合物，当在如本文所公开的OPA测定中测试时，具有OPA滴度高于未缀合的天然肺炎链球菌血清型15C荚膜多糖所获得的OPA滴度的OPA滴度。

[0159] 在实施方案中，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够在如本文所公开的调理吞噬作用测定中杀死血清型15B和15C和/或15A肺炎链球菌的抗体的形成。

[0160] 在实施方案中，本文所公开的免疫原性组合物，当施用于受试者时，诱导能够杀死血清型15B和15C的抗体的形成。

[0161] 肺炎球菌调理吞噬测定(OPA)(其在功能性抗体和补体的存在下测量吞噬效应细胞杀死肺炎链球菌细胞)被认为是评估肺炎球菌疫苗的有效性的重要替代物(surrogate)。

[0162] 调理吞噬测定(OPA)可通过一起孵育肺炎链球菌细胞，待测试的热灭活人血清、分化的HL-60细胞(吞噬细胞)和外源性补体来源(例如幼兔补体)的混合物来进行。调理吞噬作用在孵育期间进行，表面覆盖抗体和补体的细菌细胞在调理吞噬作用下被杀死。从调理吞噬作用逃逸的存活的细菌的集落形成单位(cfu)通过将测定混合物铺板来确定。OPA滴度定义为稀释度倒数，其导致相对无测试血清的对照孔菌数减少50％。OPA滴度是来自涵盖此50％杀死截止值的两个稀释度的插值。

[0163] 认为1:8或更高的终点滴度是这些杀死类型OPA中的阳性结果。

[0164] 在实施方案中，如通过调理吞噬作用杀伤测定(OPA)所确定的，本发明的免疫原性组合物能够在至少50％受试者中引发至少1:8的针对肺炎链球菌血清型15B的滴度。在实施方案中，如通过调理吞噬作用杀伤测定(OPA)所确定的，本发明的免疫原性组合物能够在至少60％、70％、80％、90％或至少93％受试者中引发至少1:8的针对肺炎链球菌血清型15B的滴度。

[0165] 在实施方案中，如通过调理吞噬作用杀伤测定(OPA)所确定的，本发明的免疫原性组合物能够在至少50％受试者中引发至少1:8的针对肺炎链球菌血清型15C的滴度。在实施方案中，如通过调理吞噬作用杀伤测定(OPA)所确定的，本发明的免疫原性组合物能够在至少60％、70％、80％、90％或至少95％受试者中引发至少1:8的针对肺炎链球菌血清型15C的滴度。

[0166] 本发明的免疫原性组合物的配制可以使用现有技术承认的方法实现。例如，可以使用生理上可接受的媒介物来配制本发明的免疫原性缀合物以制备组合物。这些媒介物的实例包括但不限于水、缓冲盐水、多元醇(例如，甘油、丙二醇、液体聚乙二醇)和右旋糖溶液。

[0167] 在优选的实施方案中，所述免疫原性组合物可包含至少一种额外的抗原。在优选的实施方案中，所述免疫原性组合物可包含至少一种额外的肺炎链球菌荚膜多糖。

[0168] 在优选的实施方案中，所述免疫原性组合物可包含至少一种额外的缀合到载体蛋白的肺炎链球菌荚膜多糖。在优选的实施方案中，所述载体蛋白是CRM_{197}。

[0169] 在某些实施方案中，免疫原性组合物包含一种或多种佐剂。如本文所定义的，"佐剂"是指用于增强本发明的免疫原性组合物的免疫原性的物质。因此，经常给予佐剂以加强免疫应答，并且佐剂是本领域技术人员所熟知的。增强组合物有效性的合适的佐剂包括但不限于：

[0170] (1)铝盐(明矾)，诸如氢氧化铝、磷酸铝、硫酸铝等；

[0171] (2)水包油乳液制剂(含或不含其它具体的免疫刺激剂诸如胞壁酰肽(定义见下文)或细菌细胞壁成分)，诸如，例如

[0172] (a)MF59(PCT公开号WO 90/14837)，含有5%角鲨烯、0.5%5吐温80和0.5%司盘85(任选地含有不同量的MTP-PE(见下文，虽然不是必须的))，使用微流化器，诸如110Y型微流化器(Microfluidics,Newton,MA)配制成亚微米颗粒，

[0173] (b)SAF，含有10%角鲨烯、0.4%吐温80、5%普朗尼克嵌段聚合物L121和thr-MDP(见下文)微流化成亚微米乳剂或涡旋产生较大粒度的乳液，和

[0174] (c)RiBi™佐剂系统(RAS)(Corixa,Hamilton,MT)含有2%角鲨烯、0.2%吐温80和选自3-O-脱酰基单磷脂A(MPL™)(描述于美国专利号4,912,094)(Corixa)、海藻糖二霉菌酸酯(TDM)和细胞壁骨架(CWS)，优选MPL+CWS(Detox™)的一种或多种细菌细胞壁组分；

[0175] (3)可使用皂苷佐剂，诸如奎尔(Quil)A或STIMULON™ QS-21(Antigenics,Framingham,MA)(美国专利号5,057,540)或其生成的粒子诸如ISCOM(免疫刺激复合物)；

[0176] (4)细菌脂多糖，合成的脂质A类似物，诸如氨基烷基葡糖胺磷酸酯化合物(AGP)，或者其衍生物或类似物，它们可购自Corixa，并且描述于美国专利号6,113,918中；一个这样的AGP是2-[(R)-3-十四酰氧基十四酰氨基]乙基2-脱氧-4-O-膦酰基-3-O-[(R)-3-十四酰氧基十四酰]-2-[(R)-3-十四酰氧基十四酰氨]-b-D-吡喃葡糖苷，其也称为529(以前称为RC529)，其被配制成含水形式或作为稳定的乳液，合成的多核苷酸，诸如含有CpG基序的寡核苷酸(美国专利号6,207,646)；

[0177] (5)细胞因子，诸如白介素(例如，IL-1、IL-2、IL-4、IL-5、IL-6、IL-7、IL-12、IL-15、IL-18等)，干扰素(例如，γ-干扰素)，粒细胞巨噬细胞集落刺激因子(GM-CSF)，巨噬细胞集落刺激因子(M-CSF)，肿瘤坏死因子(TNF)，共刺激分子87-1和87-2，等；

[0178] (6)细菌ADP-核糖基化毒素的脱毒突变体，诸如野生型或突变体形式霍乱毒素(CT)，所述突变形式例如其中氨基酸位置29上的谷氨酸被另一氨基酸(优选组氨酸)取代(根据公开的国际专利申请号WO00/18434(也参见WO02/098368和WO02/098369))；百日咳毒素(PT)；或大肠杆菌不耐热毒素(LT)，特别是LT-K63、LT-R72、CT-S109、PT-K9/G129(参见，例如WO 93/13302和WO 92/19265)；和

[0179] (7)充当免疫刺激剂以增强该组合物的效力的其它物质。

[0180] 胞壁酰肽包括、但不限于，N-乙酰-胞壁酰-L-苏-D-异谷氨酰胺(thr-MDP)、N-乙酰基-去甲胞壁酰基-L-丙氨酸-2-(1'-2'-二棕榈酰-sn-甘油基-3-羟基磷酰)-乙胺(MTP-PE)等。

[0181] 在本发明的实施方案中，如本文所公开免疫原性组合物包含CpG寡核苷酸作为佐剂。如本文中所使用的CpG寡核苷酸指免疫刺激性CpG寡聚脱氧核苷酸(CpG ODN)，因此，除

非另有说明，否则这些术语可互换使用。免疫刺激性剂CpG寡脱氧核苷酸含有一个或多个免疫刺激性CpG基序，其是非甲基化胞嘧啶-鸟嘌呤二核苷酸，任选在某些优选的碱基背景内。CpG免疫刺激性基序的甲基化状态一般是指在二核苷酸中的胞嘧啶残基。含有至少一个非甲基化的CpG二核苷酸的免疫刺激性寡核苷酸是含有通过磷酸酯键连接至3'鸟嘌呤的5'非甲基化胞嘧啶的寡核苷酸，并且其通过结合于Toll-样受体9(TLR-9)激活免疫系统。在另一个实施方案中，免疫刺激性寡核苷酸可含有一个或多个甲基化的CpG二核苷酸，其会通过TLR9激活免疫系统而不像如果CpG基序是非甲基化的那么强烈。CpG免疫刺激性寡核苷酸可包含一个或多个回文序列，其进而可包括CpG二核苷酸。CpG寡核苷酸已描述于许多授权的专利、公开的专利申请和其它出版物，包括美国专利号6,194,388、6,207,646、6,214,806、6,218,371、6,239,116和6,339,068。

[0182] 在本发明的实施方案中，如本文所公开的免疫原性组合物包括在WO2010/125480的第3页第22行至第12页第36行所述任何CpG寡核苷酸。

[0183] 已鉴定了不同类的CpG免疫刺激性寡核苷酸。这些称为A、B、C和P类，并且更详细地描述于WO2010/125480的第3页第22行至第12页第36行。本发明的方法涵盖这些不同类的CpG免疫刺激性寡核苷酸的用途。

[0184] 在本发明的实施方案中，如本文所公开免疫原性组合物包含A类CpG寡核苷酸。优选地，本发明的"A类"CpG寡核苷酸具有以下核酸序列：5'GGGGACGACGTCGTGGGGGGG 3'(SEQ ID NO:1)。A类寡核苷酸的一些非限制性实例包括：5'G*G*G_G_A_C_G_A_C_G_T_C_G_T_G_G*G*G*G*G*G 3'(SEQ ID NO:2)；其中，*是指硫代磷酸酯键并且_指磷酸二酯键。

[0185] 在本发明的实施方案中，如本文所公开的免疫原性组合物包含B类CpG寡核苷酸。在一个实施方案中，用于本发明的CpG寡核苷酸是B类CpG寡核苷酸，其由至少下式表示：

[0186] 5'$X_1X_2CGX_3X_4$ 3'，其中X1、X2、X3和X4是核苷酸。在一个实施方案中，X_2是腺嘌呤、鸟嘌呤或胸腺嘧啶。在另一个实施方案中，X_3是胞嘧啶、腺嘌呤或胸腺嘧啶。

[0187] 本发明的B类CpG寡核苷酸序列是广泛描述于下列专利中的那些序列：美国专利号6,194,388、6,207,646、6,214,806、6,218,371、6,239,116和6,339,068。示例性序列包括但不限于在这些后来的申请和专利中公开的那些序列。

[0188] 在实施方案中，本发明的"B类"CpG寡核苷酸具有下列核酸序列：

[0189] 5'TCGTCGTTTTTCGGTGCTTTT 3'(SEQ ID NO:3)，或

[0190] 5'TCGTCGTTTTTCGGTCGTTTT 3'(SEQ ID NO:4)，或

[0191] 5'TCGTCGTTTTGTCGTTTTGTCGTT 3'(SEQ ID NO:5)，或

[0192] 5'TCGTCGTTTCGTCGTTTTGTCGTT 3'(SEQ ID NO:6)，或

[0193] 5'TCGTCGTTTTGTCGTTTTTTTCGA 3'(SEQ ID NO:7)。

[0194] 在任何这些序列中，所有的键可以都是硫代磷酸酯键。在另一个实施方案中，在任何这些序列中，一个或多个键可以是磷酸二酯，优选在CpG基序的"C"和"G"之间，形成半柔性的CpG寡核苷酸。在任何这些序列中，乙基-尿苷或卤素可以取代5'T；卤素取代的实例包括但不限于溴尿苷或碘尿苷取代。

[0195] B类寡核苷酸的一些非限制性实例包括：

[0196] 5'T*C*G*T*C*G*T*T*T*T*T*C*G*G*T*G*C*T*T*T*T 3'(SEQ ID NO:8)，或

[0197] 5'T*C*G*T*C*G*T*T*T*T*T*C*G*G*T*C*G*T*T*T*T 3'(SEQ ID NO:9)，或

[0198] 5'T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T 3'(SEQ ID NO:10),或

[0199] 5'T*C*G*T*C*G*T*T*T*C*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T 3'(SEQ ID NO:11),或

[0200] 5'T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*T*T*T*T*C*G*A 3'(SEQ ID NO:12)。

[0201] 其中*指硫代磷酸酯键。

[0202] 在本发明的实施方案中,如本文所公开的免疫原性组合物包含C类CpG寡核苷酸。在实施方案中,本发明的"C类"CpG寡核苷酸具有下列核酸序列:

[0203] 5'TCGCGTCGTTCGGCGCGCGCCG 3'(SEQ ID NO:13),或

[0204] 5'TCGTCGACGTTCGGCGCGCGCCG 3'(SEQ ID NO:14),或

[0205] 5'TCGGACGTTCGGCGCGCGCCG 3'(SEQ ID NO:15),或

[0206] 5'TCGGACGTTCGGCGCGCCG 3'(SEQ ID NO:16),或

[0207] 5'TCGCGTCGTTCGGCGCGCCG 3'(SEQ ID NO:17),或

[0208] 5'TCGACGTTCGGCGCGCGCCG 3'(SEQ ID NO:18),或

[0209] 5'TCGACGTTCGGCGCGCCG 3'(SEQ ID NO:19),或

[0210] 5'TCGCGTCGTTCGGCGCCG 3'(SEQ ID NO:20),或

[0211] 5'TCGCGACGTTCGGCGCGCGCCG 3'(SEQ ID NO:21),或

[0212] 5'TCGTCGTTTTCGGCGCGCGCCG 3'(SEQ ID NO:22),或

[0213] 5'TCGTCGTTTTCGGCGGCCGCCG 3'(SEQ ID NO:23),或

[0214] 5'TCGTCGTTTTACGGCGCCGTGCCG 3'(SEQ ID NO:24),或

[0215] 5'TCGTCGTTTTCGGCGCGCGCCGT 3'(SEQ ID NO:25)。

[0216] 在任何这些序列中,所有的键可以都是硫代磷酸酯键。另一个实施方案中,在任何这些序列中,一个或多个键可以是磷酸二酯,优选在CpG基序的"C"和"G"之间,形成半柔性的CpG寡核苷酸。

[0217] C类寡核苷酸的一些非限制性实例包括:

[0218] 5'T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G3'(SEQ ID NO:26),或

[0219] 5'T*C_G*T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3'(SEQ ID NO:27),或

[0220] 5'T*C_G*G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3'(SEQ ID NO:28),或

[0221] 5'T*C_G*G*A*C_G*T*T*C_G*G*C*G*C*G*C*C*G 3'(SEQ ID NO:29),或

[0222] 5'T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C*G*C*C*G 3'(SEQ ID NO:30),或

[0223] 5'T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G 3'(SEQ ID NO:31),或

[0224] 5'T*C_G*A*C_G*T*T*C_G*G*C*G*C*G*C*C*G 3'(SEQ ID NO:32),或

[0225] 5'T*C_G*C_G*T*C_G*T*T*C_G*G*C*G*C*C*G 3'(SEQ ID NO:33),或

[0226] 5'T*C_G*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G3'(SEQ ID NO:34),或

[0227] 5'T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G3'(SEQ ID NO:35),或

[0228] 5'T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*G*C*C*G*C*C*G3'(SEQ ID NO:36),或

[0229] 5'T*C*G*T*C_G*T*T*T*T*A*C_G*G*C*G*C*C_G*T*G*C*C*G 3'(SEQ ID NO:37),或

[0230] 5'T*C_G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G*T3'(SEQ ID NO:38)

[0231] 其中*指硫代磷酸酯键并且_指磷酸二酯键。

[0232] 在任何这些序列中,乙基-尿苷或卤素可以取代5'T;卤素取代的实例包括但不限于溴尿苷或碘尿苷取代。

[0233] 在本发明的实施方案中，如本文所公开的免疫原性组合物包含P类CpG寡核苷酸。在实施方案中，用于本发明的CpG寡核苷酸是P类CpG寡核苷酸，其包含5'TLR活化域和至少两个回文区域，一个回文区域是长度为至少6个核苷酸的5'回文区域并且直接或通过间隔子连接到长度至少8个核苷酸的3'回文区域，其中，所述寡核苷酸包括至少一个YpR二核苷酸。在实施方案中，所述寡核苷酸不是T*C_G*T*C_G*A*C_G*T*T*C_G*G*C*G*C_G*C*G*C*C*G (SEQ ID NO:27)。在一个实施方案中，所述P类CpG寡核苷酸包括至少一个非甲基化的CpG二核苷酸。在另一个实施方案中，所述TLR活化域是TCG、TTCG、TTTCG、TYpR、TTYpR、TTTYpR、UCG、UUCG、UUUCG、TTT或TTTT。在又一个实施方案中，所述TLR活化域在5'回文区域内。在另一个实施方案中，所述TLR活化域在5'紧接着5'回文区域。

[0234] 在实施方案中，本发明的"P类"CpG寡核苷酸具有以下核酸序列：5' TCGTCGACGATCGGCGCGCGCCG 3'(SEQ ID NO:39)。

[0235] 在所述序列中，所有的键可以都是硫代磷酸酯键。在另一个实施方案中，一个或多个键可以是磷酸二酯，优选在CpG基序的"C"和"G"之间，形成半柔性的CpG寡核苷酸。在任何这些序列中，乙基-尿苷或卤素可以取代5'T；卤素取代的实例包括但不限于溴尿苷或碘尿苷取代。

[0236] P类寡核苷酸的非限制性实例包括：

[0237] 5'T*C_G*T*C_G*A*C_G*A*T*C_G*G*C*G*C_G*C*G*C*C*G 3'(SEQ ID NO:40)

[0238] 其中*指硫代磷酸酯键并且_指磷酸二酯键。

[0239] 在一个实施方案中，所述寡核苷酸包括至少一个硫代磷酸酯键。在另一个实施方案中，所述寡核苷酸的所有核苷酸间连接是硫代磷酸酯键。在另一个实施方案中，所述寡核苷酸包括至少一个磷酸二酯样键。在另一个实施方案中，所述磷酸二酯样键是磷酸二酯键。在另一个实施方案中，亲脂基团缀合到寡核苷酸。在一个实施方案中，所述亲脂基团是胆固醇。

[0240] 在实施方案中，本文公开的所有CpG寡核苷酸的核苷酸间键是磷酸二酯键("柔性"的寡核苷酸，如PCT申请WO2007/026190所述)。在另一个实施方案中，本发明的CpG寡核苷酸呈现抗降解(例如，是稳定化的)。"稳定化的寡核苷酸"是指寡核苷酸对体内降解(例如，经由外切核酸酶或内切核酸酶)具有相对抗性。核酸稳定化可以通过主链修饰来实现。具有硫代磷酸酯键的寡核苷酸提供最大活性并保护寡核苷酸免于细胞内外切和内切核酸酶降解。免疫刺激性寡核苷酸可以具有嵌合主链，其具有磷酸二酯键和硫代磷酸酯键的组合。对于本发明的目的，嵌合主链指的是部分稳定化的主链，其中至少一个核苷酸间键是磷酸二酯键或磷酸二酯样键，并且其中至少一个其它核苷酸间键是稳定化的核苷酸间键，其中所述至少一个磷酸二酯键或磷酸二酯样键和所述至少一个稳定化的键是不同的。如在PCT申请WO2007/026190中所描述的，当磷酸二酯键优选地位于CpG基序内时，这样的分子被称为是"半柔性"的。

[0241] 所述CpG寡核苷酸的大小(即，沿着寡核苷酸的长度的核苷酸残基的数目)也可有助于寡核苷酸的刺激活性。为了促进摄取到细胞中，本发明的CpG寡核苷酸优选具有6个核苷酸残基的最小长度。如果存在足够的免疫刺激性基序，任何尺寸大于6个核苷酸(甚至长为许多kb)的寡核苷酸都能够诱导免疫应答，因为更大的寡核苷酸在细胞内降解。在某些实施方案中，所述CpG寡核苷酸长6-100个核苷酸，优选长8-30个核苷酸。在重要的实施方案

中，本发明的核酸和寡核苷酸不是质粒或表达载体。

[0242] 在实施方案中，本文公开的CpG寡核苷酸包含取代或修饰，诸如在碱基和/或糖上，如描述于WO2007/026190的第134-147段。

[0243] 在实施方案中，本发明的CpG寡核苷酸是化学修饰的。化学修饰的实例是本领域技术人员已知的，并且描述于例如Uhlmann E.等(1990)，Chem.Rev.90:543，S.Agrawal，Ed.，Humana Press，Totowa，USA 1993；Crooke，S.T.等(1996) Annu.Rev.Pharmacol.Toxicol.36:107-129；and Hunziker J.等，(1995)，Mod.Synth.Methods 7:331-417中。根据本发明的寡核苷酸可具有一个或多个修饰，其中每个修饰位于特定的核苷间磷酸二酯桥和/或位于特定的β-D-核糖单元和/或位于特定的天然核苷碱基位置，相比由天然DNA或RNA构成的相同序列的寡核苷酸。

[0244] 在本发明的一些实施方案中，含有CpG的核酸可根据本领域技术人员已知的方法简单地与免疫原性载体混合(参见例如WO03/024480)。

[0245] 在本发明的具体实施方案中，本文公开的任何免疫原性组合物包含2μg-100mg的CpG寡核苷酸，优选为0.1mg-50mg的CpG寡核苷酸，优选0.2mg-10mg的CpG寡核苷酸，优选0.3mg-5mg的CpG寡核苷酸，甚至优选0.5-2mg的CpG寡核苷酸，甚至优选0.75-1.5mg的CpG寡核苷酸。在优选的实施方案中，本文公开的免疫原性组合物包含约1mg的CpG寡核苷酸。

[0246] 在优选的实施方案中，佐剂是基于铝的佐剂，其选自磷酸铝、硫酸铝和氢氧化铝。在一个实施方案中，本文所述的免疫原性组合物包含佐剂磷酸铝。

[0247] 在优选的实施方案中，本发明的免疫原性组合物还包含缓冲剂、冷冻保护剂、盐、二价阳离子、非离子型去垢剂、自由基氧化抑制剂、稀释剂或载体中的至少一种。

[0248] 免疫原性组合物任选地可包含一种或多种生理学上可接受的缓冲液，所述缓冲液选自、但不限于Tris(三羟甲基氨基甲烷(trimethamine))、磷酸盐、乙酸盐、硼酸盐、柠檬酸盐、甘氨酸、组氨酸和琥珀酸盐。在某些实施方案中，所述制剂缓冲至约5.0到约7.0，优选约5.5至约6.5的pH范围内。

[0249] 免疫原性组合物任选地可以包含一种或多种非离子表面活性剂，包括但不限于聚氧乙烯脱水山梨醇脂肪酸酯、聚山梨酯80(吐温80)、聚山梨酯60(吐温60)、聚山梨酯40(吐温40)和聚山梨酯-20(吐温20)、聚氧乙烯烷基醚，包括但不限于Brij58、Brij35，以及其它诸如曲拉通X-100、曲拉通X-114、NP40、司盘85和普朗尼克系列非离子表面活性剂(例如，普朗尼克121)。在优选的实施方案中，免疫原性组合物包含聚山梨酯80或聚山梨酯40，优选聚山梨酯80。在优选的实施方案中，免疫原性组合物包含浓度为约0.001％至约2％(优选至多约0.25％)的聚山梨酯80或浓度为约0.001％至1％(优选至多约0.5％)的聚山梨酯40。

[0250] 本发明还涉及包含本发明的免疫原性组合物的疫苗。

[0251] 用于诱导免疫应答和保护免受感染的方法

[0252] 本公开内容还包括本文所述免疫原性组合物的使用方法。例如，本公开内容的一个实施方案提供了诱导针对肺炎链球菌的免疫应答的方法，其包括给受试者施用免疫原性量的任何本文所述的免疫原性组合物。

[0253] 本公开内容的一个实施方案提供了保护受试者免于感染肺炎链球菌的方法，或预防感染肺炎链球菌的方法，或降低肺炎链球菌引起的感染的严重程度或延迟与肺炎链球菌引起的感染相关的至少一种症状发作的方法，所述方法包括给受试者施用免疫原性量的任

何本文所述的免疫原性组合物。

[0254] 本公开内容的一个实施方案提供了保护受试者免于感染血清型15B肺炎链球菌的方法，或预防感染血清型15B肺炎链球菌的方法，或降低血清型15B肺炎链球菌引起的感染的严重程度或延迟与血清型15B肺炎链球菌引起的感染相关的至少一种症状发作的方法，所述方法包括给受试者施用免疫原性量的任何本文所述的免疫原性组合物。

[0255] 本公开内容的一个实施方案提供了保护受试者免于感染血清型15C肺炎链球菌的方法，或预防感染血清型15C肺炎链球菌的方法，或降低血清型15C肺炎链球菌引起的感染的严重程度或延迟与血清型15C肺炎链球菌引起的感染相关的至少一种症状发作的方法，所述方法包括给受试者施用免疫原性量的任何本文所述的免疫原性组合物。

[0256] 本公开内容的一个实施方案提供了保护受试者免于感染血清型15A肺炎链球菌的方法，或预防感染血清型15A肺炎链球菌的方法，或降低血清型15A肺炎链球菌引起的感染的严重程度或延迟与血清型15A肺炎链球菌引起的感染相关的至少一种症状发作的方法，所述方法包括给受试者施用免疫原性量的任何本文所述的免疫原性组合物。

[0257] 本公开内容的一个实施方案提供了治疗或预防受试者中与血清型15A、15B和/或15C(优选15B和/或15C，更优选15B)肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法，所述方法包括对受试者施用治疗或预防有效量的本文所述的免疫原性组合物的步骤。另一个实施方案提供了治疗或预防受试者中与血清型15A、15B和/或15C(优选15B和/或15C，更优选15B)相关的肺炎链球菌感染、疾病或病况的方法，所述方法包括从本文所述的免疫原性组合物产生多克隆或单克隆抗体制剂，并使用所述抗体制剂对受试者赋予被动免疫。

[0258] 在一个实施方案中，本公开内容涉及本文公开的免疫原性缀合物或免疫原性组合物用于制备药物的用途，所述药物用于保护受试者免于肺炎链球菌感染，和/或预防肺炎链球菌感染，和/或降低肺炎链球菌引起的感染的严重程度或延迟肺炎链球菌引起的感染相关的至少一种症状的发作，和/或保护受试者免于血清型15A、15B和/或15C(优选15B和/或15C，更优选15B)肺炎链球菌感染和/或预防血清型15A，15B和/或15C(优选15B和/或15C，更优选15B)，和/或降低血清型15A、15B和/或15C(优选15B和/或15C，更优选15B)肺炎链球菌引起的感染的严重程度或延迟血清型15A、15B和/或15C(优选15B和/或15C，更优选15B)肺炎链球菌引起的感染相关的至少一种症状的发作。

[0259] 在一个实施方案中，本公开内容涉及本文公开的免疫原性缀合物或免疫原性组合物的用途，所述药物用于保护受试者免于肺炎链球菌感染，和/或预防肺炎链球菌感染，和/或降低肺炎链球菌引起的感染的严重程度或延迟肺炎链球菌引起的感染相关的至少一种症状的发作，和/或保护受试者免于血清型15A、15B和/或15C(优选15B和/或15C，更优选15B)肺炎链球菌感染和/或预防血清型15A，15B和/或15C(优选15B和/或15C，更优选15B)，和/或降低血清型15A、15B和/或15C(优选15B和/或15C，更优选15B)肺炎链球菌引起的感染的严重程度或延迟血清型15A、15B和/或15C(优选15B和/或15C，更优选15B)肺炎链球菌引起的感染相关的至少一种症状的发作。

[0260] 对免疫原性组合物的“免疫应答”是在受试者中发展体液免疫应答和/或细胞介导的免疫应答，所述应答针对存在于所关注的免疫原性组合物或疫苗组合物中的分子。对于本公开的目的，“体液免疫应答”是抗体介导的免疫应答，并涉及对在本公开内容的免疫原

性组合物或疫苗中的抗原进行识别并以一些亲和力进行结合的抗体的诱导和生成，而“细胞介导的免疫应答”是一种由T细胞和/或其它白细胞介导的免疫应答。“细胞介导的免疫应答”是由与I类或II类主要组织相容性复合物分子(MHC)、CD1或其它非经典的MHC样分子结合的抗原表位的呈递引发的。这活化抗原特异性CD4+T辅助细胞或CD8+细胞毒性T淋巴细胞(“CTL”)。CTL对于通过经典或非经典MHC编码并表达在细胞表面上的蛋白结合呈递的肽抗原具有特异性。CTL帮助诱导和促进细胞内微生物的细胞内破坏，或感染此类微生物的细胞的裂解。细胞免疫的另一个方面涉及由辅助T细胞的抗原特异性应答。辅助T细胞作用在于帮助刺激功能，并聚焦非特异性效应细胞的活性针对在其表面上展示与经典或非经典的MHC分子结合的肽或其它抗原的细胞。“细胞介导的免疫应答”还指产生细胞因子、趋化因子和通过活化的T细胞和/或其它白细胞(包括衍生自CD4+和CD8+T细胞的那些)产生的其它此类分子。特定抗原或组合物刺激细胞介导的免疫应答的能力可以通过许多测定法来确定，诸如通过淋巴细胞增殖(淋巴细胞活化)测定法、CTL细胞毒性细胞测定法，通过测定特异性针对敏化受试者中抗原的T淋巴细胞，或通过测量响应抗原再刺激的T细胞产生的细胞因子。此类测定法是本领域熟知的。参见例如Erickson等(1993)J.Immunol.151:4189-4199；和Doe等(1994)Eur.J.Immunol.24:2369-2376。

[0261] 如本文所用，“治疗(treatment)”(包括其变化，例如，“治疗(treat)”或“经治疗的(treated)”)是指下列的任意一种或多种：(i)感染或再感染的预防，如在传统的疫苗中，(ii)症状的严重程度的降低，或症状的消除，和(iii)所讨论的病原体或病症的基本上或完全消除。因此，治疗可以预防性(感染前)或治疗性(感染后)实现。在本公开中，预防性治疗是优选的方式。根据本公开内容的具体实施方案，提供了治疗(包括预防性和/或治疗性免疫)宿主动物免于血清型15A、15B和/或15C(优选15B和/或15C，更优选15B)肺炎链球菌感染的组合物和方法。本公开的方法用于赋予受试者预防性和/或治疗性免疫。本公开的方法也可以在用于生物医学研究应用的受试者上实施。

[0262] “免疫原性量”和“免疫学有效的量”两者在本文中可互换使用，指的是抗原或免疫原性组合物的量，所述量足以引发如通过本领域技术人员已知的标准测定法所测量的免疫应答，即细胞(T细胞)或体液(B细胞或抗体)应答，或两者。

[0263] 在优选的实施方案中，所述受试者是人。在最优选的实施方案中，所述受试者是新生儿(即年龄三个月以下)、婴儿(年龄3个月到一岁)或幼儿(即年龄一岁到四岁)。

[0264] 在实施方案中，本文所公开的免疫原性组合物用作疫苗。

[0265] 在这样的实施方案中，待接种的受试者年龄可以小于1岁。例如，待接种的受试者可以是约1、2、3、4、5、6、7、8、9、10、11或12个月的年龄。在实施方案中，待接种的受试者是约2、4或6个月的年龄。在另一个实施方案中，待接种的受试者年龄小于2岁。例如待接种的受试者可以是约12-15个月的年龄。在一些情况下，需要小至一个剂量的根据本发明的免疫原性组合物，但是在某些情况下，可以给予第二、第三或第四的剂量(参见方案部分)。

[0266] 在本发明的实施方案中，待接种的受试者是50岁或以上的成人，更优选55岁或以上的成人。在实施方案中，待接种的受试者是65岁或以上、70岁或以上、75岁或以上或80岁或以上的成人。

[0267] 在实施方案中，待接种的受试者是免疫功能低下的个体，特别是人。免疫功能低下的个体通常定义为展示受损或降低的针对感染性试剂攻击产生正常体液或细胞防御的能

力的人。

[0268] 在本发明的实施方案中，待接种的免疫功能低下的受试者患有破坏人体免疫系统并导致不足以防止或治疗肺炎球菌疾病的抗体反应的疾病或病况。

[0269] 在实施方案中，所述疾病为原发性免疫缺陷病症。优选地，所述原发性免疫缺陷病症选自：组合的T和B细胞免疫缺陷、抗体缺陷、明确定义的综合症、免疫失调疾病、吞噬细胞紊乱、先天性免疫缺陷、自身炎症性病症以及补体缺陷。在实施方案中，所述原发性免疫缺陷病症选自PCT申请WO2010/125480的第24页第11行-第25页第19行公开的病症。

[0270] 在本发明的具体实施方案中，待接种的免疫功能低下的受试者患有选自下列的疾病：HIV感染、获得性免疫缺陷综合症(AIDS)、癌症、慢性心脏或肺部病症、充血性心脏衰竭、糖尿病、慢性肝病、酒精中毒、肝硬化、脊髓液渗漏、心肌病、慢性支气管炎、肺气肿、慢性阻塞性肺病(COPD)、脾功能障碍(诸如镰状细胞病)、脾功能缺乏(无脾症(asplenia))、血液恶性肿瘤、白血病、多发性骨髓瘤、霍奇金病、淋巴瘤、肾功能衰竭、肾病综合征和哮喘。

[0271] 在本发明的实施方案中，待接种的免疫功能低下受试者患有营养不良。

[0272] 在本发明的具体实施方案中，待接种的免疫功能低下的受试者正在接受降低机体对感染的抵抗力的药物或治疗。在实施方案中，这样的药物选自PCT申请WO2010/125480的第26页第33行-第26页第40行公开的药物。

[0273] 在本发明的具体实施方案中，待接种的免疫功能低下受试者是吸烟者。

[0274] 在本发明的具体实施方案中，待接种的免疫功能低下的受试者具有白细胞计数(白血球计数)低于5×10^9个细胞/升，或低于4×10^9个细胞/升，或低于3×10^9个细胞/升，或低于2×10^9个细胞/升，或低于1×10^9个细胞/升，或低于0.5×10^9个细胞/升，或低于0.3×10^9个细胞/升，或低于0.1×10^9个细胞/升。

[0275] 白细胞计数(白血球计数)：在血液中白血细胞(WBC)的数量。WBC通常作为CBC(全血细胞计数)的一部分进行测量。白血细胞是在血液中的抗感染细胞，并与称为红细胞的红色(携氧)血细胞不同。有不同类型的白细胞，包括中性粒细胞(多形核白细胞；PMN)、杆状核粒细胞(band cell)(略未成熟中性粒细胞)、T型淋巴细胞(T细胞)、B型淋巴细胞(B细胞)、单核细胞、嗜酸性粒细胞和嗜碱性粒细胞。白细胞的所有类型都反映在白细胞计数上。白细胞计数的正常范围是每立方毫米的血液通常4,300-10,800个细胞。这也可以称为白血球计数，并且可以以国际单位表示为$4.3\text{-}10.8\times10^9$个细胞/升。

[0276] 在本发明的具体实施方案中，待接种的免疫功能低下受试者患有中性粒细胞减少症。在本发明的具体实施方案中，待接种的免疫功能低下的受试者具有嗜中性粒细胞计数低于2×10^9个细胞/升，或低于1×10^9个细胞/升，或低于0.5×10^9个细胞/升，或低于0.1×10^9个细胞/升，或低于0.05×10^9个细胞/升。

[0277] 低白细胞计数或“中性粒细胞减少症”是其特征在于在循环血液里中性粒细胞的异常低水平的状况。中性粒细胞是一种帮助防止和抵抗感染的特定的白细胞。癌症患者经历中性粒细胞减少症最常见的原因是作为化疗的副作用。化疗诱导的中性粒细胞减少症增加了患者感染的风险，并破坏癌症的治疗。

[0278] 在本发明的具体实施方案中，待接种的免疫功能低下的受试者具有CD4+细胞计数低于$500/mm^3$，或CD4+细胞计数低于$300/mm^3$，或CD4+细胞计数低于$200/mm^3$，CD4+细胞计数低于$100/mm^3$，CD4+细胞计数低于$75/mm^3$，或CD4+细胞计数低于$50/mm^3$。

[0279] CD4细胞测试通常报道为mm^3中的细胞数量。正常的CD4计数是500-1600，并且CD8计数是375-1100。CD4计数在带有HIV的人中急剧下降。

[0280] 在本发明的实施方案中，本文公开的任何免疫功能低下受试者是男性人类或女性人类。

[0281] 组合物中缀合物的量一般是基于对于该缀合物缀合和非缀合的总多糖进行计算。例如，具有20％游离多糖的缀合物将具有在100μg多糖剂量中约80μg缀合的多糖和约20μg非缀合的多糖。当计算缀合物的剂量时，通常不考虑蛋白质对缀合物的贡献。通常，每个剂量包含0.1-100μg的多糖，特别是0.1-10μg，更特别是1-10μg，更特别是1-5μg。优选每次剂量将包含约1.1、2、2.2、3、3.3、4、4.4μg多糖。

[0282] 对于特定免疫原性组合物或疫苗的组分的最优量可以通过标准研究确定，所述研究涉及受试者中适当免疫应答的观察。初始接种后，受试者可以充分的间隔接受一次或若干次加强免疫。

[0283] 抗原作为免疫原的有效性，可通过如下进行测量，通过增殖测定法，或通过细胞溶解测定法(诸如测量T细胞裂解其特异性目标细胞的能力的铬释放测定)，或通过测量对血清中抗原特异性的循环抗体的水平来测量B细胞活性水平。如本文所述，免疫应答还可以通过测量施用抗原后诱导的抗原特异性抗体的血清水平进行检测，并且更具体地，通过测量如此诱导的抗体的增强特定白血细胞调理吞噬能力的能力进行检测。免疫应答的保护水平可以通过用已施用的抗原攻击免疫的宿主来测量。例如，如果期望免疫应答针对的抗原是细菌，则通过抗原的免疫原性量诱导的保护水平是通过检测用细菌细胞攻击动物后百分比存活或百分比死亡来进行测量。在一个实施方案中，保护的量可以通过测量与细菌感染相关的至少一种症状(例如与感染相关的发热)来测量。多抗原或多组分疫苗或免疫原性组合物中的每个抗原的量将就每种其它组分而变化，并且可以通过本领域技术人员已知的方法来确定。这样的方法将包括用于测量免疫原性和/或体内疗效的方法。

[0284] 本公开内容进一步提供了特异性并选择性结合本公开的荚膜多糖或免疫原性偶联物的抗体和抗体组合物。在一些实施方案中，抗体在向受试者施用本公开内容的荚膜多糖或免疫原性缀合物后产生。在一些实施方案中，本公开内容提供针对一种或多种本公开内容的荚膜多糖或免疫原性缀合物的纯化的或分离的抗体。在一些实施方案中，如通过在动物疗效模型中或经由调理吞噬杀死测定法杀死细菌所测量的，本公开内容的抗体是有功能的。在一些实施方案中，本公开内容的抗体赋予受试者被动免疫。本公开内容还提供编码本公开内容的抗体或抗体片段的多核苷酸分子，和细胞，细胞系(诸如杂交瘤细胞或用于重组生产抗体的其它工程改造的细胞系)，或使用本领域技术人员熟知的技术产生本公开内容的抗体或抗体组合物的转基因动物。

实施例

[0285] 实施例1：分离的肺炎链球菌血清型15B荚膜多糖的制备

[0286] 1.1发酵和纯化

[0287] 血清型15B荚膜多糖可以使用本领域技术人员已知的分离方法直接从细菌获得(参见例如美国专利申请公开号20060228380、20060228381、20070184071、20070184072、20070231340和20080102498或WO2008118752公开的方法)。血清型15B肺炎链球菌在种子瓶

中生长，然后转移到种子发酵罐。一旦目标光密度达到，则将细胞转移到生产发酵罐。发酵肉汤通过加入N-月桂酰肌氨酸进行灭活并通过超滤和渗滤纯化。

[0288] 然后通过使用PANDA 2K均质机®(GEA Niro Soavi)进行高压均质来裁剪纯化的肺炎链球菌血清型15B多糖，以产生分离的肺炎链球菌血清型15B多糖。

[0289] 优选地，通过上述方法得到的分离的肺炎链球菌血清型15B荚膜多糖每mM血清型15B荚膜多糖包含至少0.6mM醋酸盐，并且具有的50kDa-500kDa、优选150-350kDa的分子量。

[0290] 1.2分离的肺炎链球菌血清型15B荚膜多糖的氧化

[0291] 多糖氧化在100mM磷酸钾缓冲液(pH6.0±0.2)中进行，通过依次加入计算量的500mM磷酸钾缓冲液(pH6.0)和WFI，以得到2.0g/L的最终多糖浓度。如果需要的话，将反应pH调节至约pH6.0。pH调节后，将反应温度调节至23±2℃。通过加入约0.25摩尔当量的高碘酸钠开始氧化反应。在23±2℃下进行氧化反应过程，持续约16小时。

[0292] 活化的多糖的浓缩和渗滤使用10K MWCO超滤盒进行。相对20倍渗滤体积的WFI进行渗滤。然后将纯化的活化的多糖贮存于5±3℃。纯化的活化的糖尤其是通过如下进行表征：(i)通过比色测定的糖浓度；(ii)通过比色测定的醛浓度；(iii)氧化度(iv)通过SEC-MALLS的分子量和(v)O-乙酰基和甘油的存在。

[0293] SEC-MALLS用于确定多糖和多糖-蛋白缀合物的分子量。使用SEC通过流体动力学体积分离多糖。使用折射率(RI)和多角度激光光散射(MALLS)检测器确定分子量。当光与物质相互作用时，其散射并且散射光的量与物质的浓度、dn/dc的平方(比折射率增量)和摩尔质量相关。分子量测量基于来自MALLS检测器散射的光信号和来自RI检测器的浓度信号的读数进行计算。

[0294] 如下确定活化的多糖的氧化度(DO=糖重复单元的摩尔数/醛的摩尔数)：

[0295] 糖重复单元的摩尔数通过多种比色法确定，例如通过使用蒽酮法。通过蒽酮法，多糖首先通过硫酸和热的作用分解成单糖。蒽酮试剂与己糖反应形成黄绿有色络合物，其吸光度在625nm以分光光度法读出。在测定的范围内，吸光度与存在的己糖的量成正比。

[0296] 还同时采用MBTH比色法确定醛的摩尔数。MBTH测定涉及吖嗪化合物的形成，通过使醛基(来自给定样品)与3-甲基-2-苯并噻唑酮腙(MBTH测定试剂)反应形成。过量的3-甲基-2-苯并噻唑酮腙氧化以形成活性阳离子。活性阳离子和吖嗪反应以形成蓝色发色团。然后所形成的发色团在650nm进行光谱读取。

[0297] 优选地，通过上述方法得到的活化的肺炎链球菌血清型15B荚膜多糖每mM血清型15B荚膜多糖包含至少0.6mM乙酸盐，并且具有的50kDa-500kDa、优选100-250kDa的分子量。

[0298] 1.3活化的肺炎链球菌血清型15B荚膜多糖与CRM_{197}的缀合

[0299] 缀合方法由下列步骤组成：

[0300] a)与蔗糖赋形剂混合并冻干

[0301] b)重构冻干的活化的多糖和CRM_{197}

[0302] c)活化的多糖缀合至CRM_{197}并加帽

[0303] d)纯化缀合物

[0304] a)与蔗糖赋形剂混合并冻干

[0305] 活化的多糖与蔗糖混合达到每克活化的多糖25克蔗糖的比例。然后混合的混合物的瓶进行冻干。冻干后，含有冻干的活化的多糖的瓶保存在-20±5℃。计算量的CRM_{197}蛋白

分开进行壳式冷冻(shell-frozen)并冻干。冻干的CRM_{197}保存在-20±5℃。

[0306] b)重构冻干的活化的多糖和CRM_{197}蛋白

[0307] 冻干的活化的多糖在无水二甲亚砜(DMSO)中重构。一旦多糖完全溶解,将等量的无水DMSO添加到冻干的CRM_{197}用于重构。

[0308] c)缀合和加帽

[0309] 重构的活化的多糖与重构的CRM_{197}在反应容器(输入比0.8:1)中合并,随后彻底混合以获得清澈的溶液,然后用氰基硼氢化钠起始缀合。反应溶液中的最终多糖浓度是约1g/L。通过向反应混合物中添加1.0-1.5MEq氰基硼氢化钠起始缀合,并在23±2℃孵育40-48小时。缀合反应通过添加2MEq的硼氢化钠($NaBH_4$)以加帽未反应的醛基而终止。此加帽反应继续在23±2℃持续3±1小时。

[0310] d)纯化缀合物

[0311] 该缀合物溶液用冷却的5mM琥珀酸盐-0.9%盐水(pH6.0)1:10稀释以制备用于通过使用100-300K MWCO滤膜的切向流过滤进行纯化。将稀释的缀合物溶液通过5μm过滤器过滤,并使用5mM琥珀酸盐-0.9%盐水(pH6.0)作为介质进行渗滤。渗滤完成后,缀合物截留物通过0.22μm的过滤器转移。

[0312] 缀合物用5mM琥珀酸盐/0.9%盐水(pH6)进一步稀释,以达到约0.5mg/mL的目标糖浓度。完成最终的0.22μm的过滤步骤,以获得免疫原性缀合物。

[0313] 优选地,通过上述方法得到的缀合物每mM血清型15B荚膜多糖包含至少0.6mM乙酸盐,具有3000-20000kDa的分子量,并具有2-6的缀合度。

[0314] 实施例2:包含共价连接到CRM_{197}的肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物的表征

[0315] 通过实施例1中公开的方法制备缀合物1。使用不同量的氧化剂通过类似方法制备缀合物2和3。缀合物4通过类似的方法制备,除了纯化的血清型15B荚膜多糖没有被裁剪并且被活化到较低的DO(更高氧化水平),并在含水介质中进行缀合。缀合物5通过类似的方法制备,除了纯化的血清型15B荚膜多糖通过化学水解进行裁剪,并且在含水介质中进行缀合。缀合物6和7通过类似的方法制备,除了纯化的血清型15B荚膜多糖没有被裁剪。所得到的缀合物进行了表征,并且结果总结在表1中。

[0316] 表1:肺炎链球菌血清型15B荚膜多糖-CRM_{197}缀合物

[0317]

缀合物	1	2	3	4	5	6	7
多糖	经裁剪的	经裁剪的	经裁剪的	天然的	水解的	天然的	天然的
O-乙酰化；多糖(μmol 乙酸盐/μmol 多糖)	0.69	0.69	0.69	1.01	0.66	0.76	NA
溶剂介质	DMSO	DMSO	DMSO	含水的	含水的	DMSO	DMSO
活化的多糖DO	11.4	5.8	9.7	4.8	8.8	5	12
活化的多糖MW	196KDa	218KDa	235KDa	435 KDa	270KDa	431KDa	460KDa
产率 (%)	87.2	64	63.7	96.2	78.8	24.2	26.2
糖蛋白比例	0.68	0.65	0.71	1.22	1.29	0.9	1.5
游离糖 (%)	< 5	< 5	6.1	18.1	14.2	8.8	18
缀合物 MW,	6190KDa	7090KDa	7937KDa	1766KDa	1029KDa	6293KDa	4466KDa

[0318]

SEC-MALLS							
O-乙酰化，缀合物 μmol 乙酸盐/μmol 多糖)	0.68	0.7	0.68	0.61	0.44	0.85	NA
< 0.3 Kd (%), SEC	NA	73	NA	NA	62	NA	NA
缀合度 (AAA)；修饰的 Lys	3.7	3.9	4.1	NA	3.4	NA	NA
%缀合物中保留的O-乙酰化	99%	100%	99.5%	60%	67%	100%	NA

[0319] 游离多糖的百分比通过利用氢氧化铝凝胶结合蛋白和共价结合的糖以通过离心去除的方法进行测量。样品与磷酸盐缓冲的氢氧化铝凝胶混合并离心。结合的糖与凝胶一起沉淀，而游离糖保留在上清液中。得到的上清液和对照样品通过适当比色测定法来定量，以确定游离糖的比例，并证实充分除去蛋白质和糖的回收。

[0320] 对于氨基酸分析，首先使用6N盐酸(HCl)水解，在真空下加热(160℃，15分钟)，将多糖-蛋白样品水解成它的个体组分，水解为游离氨基酸。水解后，将样品用氨基酸分析仪进行分析。个体氨基酸通过离子交换色谱法分离，使用柠檬酸钠缓冲液的不连续梯度，具有温度和流速的变化。分离后，各氨基酸残基的量使用柱后茚三酮耦合检测系统进行定量确定。在这个系统中，茚三酮与柱后反应器系统中柱洗脱液混合并且混合物进入光度计。茚三酮与洗脱的氨基酸的反应得到在570nm最大吸收的紫色化合物。此吸光度是存在的α-氨基的量的线性响应(函数)并且该反应提供了用于具有α-氨基的所有的有机化合物的定量比色测定。在与不具有游离氨基的亚氨基酸(诸如脯氨酸和羟基脯氨酸)的反应中，产生亮黄色化合物并且在440nm进行监测。使用570和440nm波长输出两者计算每种氨基酸的峰面积。

[0321] 如下计算产率：(在缀合物中多糖的量×100)/活化的多糖的量。

[0322] 使用含水介质产生的缀合物(4和5)表现出O-乙酰基水平显著损失。在DMSO溶剂中

使用天然多糖而未进行分子量裁剪产生的缀合物(6和7)并没有表现出在O-乙酰基水平的损失。然而,除了可过滤性差的特性之外,缀合物产率也非常差。使用通过高压均质裁剪的多糖在DMSO中产生的缀合物(1、2和3)具有较高的产率和更好的可过滤性特性,并显著保存O-乙酰基水平。这些缀合物还具有非常低水平的游离多糖。

[0323] 实施例3:调理吞噬活性(OPA)测定

[0324] 本发明缀合物的免疫原性可以使用下面描述的调理吞噬测定(OPA)进行评估。

[0325] 30只6-7周龄的雌性Swiss Webster小鼠的组用0.001μg、0.01μg或0.1μg的测试缀合物经由皮下途径在第0周进行免疫。将小鼠用相同剂量的缀合物在第3周加强然后在第4周放血。对第4周的血清样品进行血清型特异性OPA。

[0326] OPA用于测量在特异性针对肺炎链球菌血清型15B的鼠类血清中的功能性抗体。在测定反应中设置测试血清,所述反应测量荚膜多糖特异性免疫球蛋白的下列能力:调理细菌,触发补体沉积,从而促进吞噬作用和通过吞噬细胞杀死细菌。OPA滴度定义为稀释度倒数,其导致相对无测试血清的对照孔菌数减少50%。OPA滴度是来自涵盖此50%杀死截止值的两个稀释度的插值。

[0327] OPA方法基于描述于Hu等,Clin Diagn Lab Immunol 2005;12(February(2)):287-95中的方法并进行了以下修改。将测试血清进行2.5倍系列稀释,并加入到微量滴定测定板。将活的血清型15B目标细菌加入到孔中并将板在37℃摇动30分钟。将分化的HL-60细胞(吞噬细胞)和仔兔血清(3-4周龄,**Pel-Freez®**,6.25%的最终浓度)加入到孔中,并将板在37℃下摇动45分钟。为了终止反应,将80μL的0.9%NaCl加入到所有孔中,混合,并将10μL等分试样转移至含200μL水的MultiScreen HTS HV过滤板(**Millipore®**)的孔中。液体在真空下过滤通过板,并且150μL的HySoy介质加入到每个孔中,并过滤通过。过滤板然后在37℃,5%CO_2孵育过夜,并随后用Destain溶液(Bio-Rad)固定。板随后用考马斯蓝染色并脱色一次。集落成像并在Cellular Technology Limited(CTL)ImmunoSpot**Analyzer®**上计数。使用原始集落计数绘制杀死曲线,并计算OPA滴度。

[0328] 缀合物1和2的免疫原性已经根据上述测定法进行测试。一个附加的缀合物和未缀合的天然肺炎链球菌血清型15B荚膜多糖(未缀合的PS)也在相同的测定法中进行测试:

[0329] 缀合物9通过在水溶液中还原胺化通过天然(即没有被裁剪的)血清型15B荚膜多糖缀合至CRM_{197}来制备。

[0330] 结果显示在表2中。

[0331] 表2:动物测试的OPA滴度

[0332]

OPA GMT（几何平均抗体滴度）（95％CI）			
	0.001 μg	0.01 μg	0.1 μg
缀合物1	485 (413, 569)	804 (565, 1145)	1563 (1048, 2330)
缀合物2	556 (438, 707)	871 (609, 1247)	1672 (1054, 2651)
缀合物9	395 (329, 475)	856 (627, 1168)	1802 (1108, 2930)
未缀合的PS	-	-	698 (466, 1045)

[0333] 如在上表中所示，缀合物1和2，当施用于小鼠时，产生能够调理血清15B肺炎链球菌，触发补体沉积，由此促进吞噬作用并通过吞噬细胞杀死细菌的抗体。此外，尽管它们的分子量更低，它们也表现出相对于没有被裁剪的缀合物9类似的免疫原性水平。

[0334] 实施例4：血清型15B及15C血清型之间的交叉功能性OPA应答

[0335] 肺炎球菌血清群15包括四个结构相关的血清型：15A、15B、15C和15F。血清型15B和15C通过基因分型技术无法进行区分并且具有相似的荚膜多糖(PS)组成，除了15B-PS是15C-PS的O-乙酰化变体。为了理解对于血清15B的抗荚膜PS抗体是否在功能上与血清型15C交叉反应，10只兔子用PCV16v和PCV20v疫苗(两者均含有如本文所公开的包含共价连接到CRM_{197}肺炎链球菌血清型15B荚膜多糖的免疫原性缀合物作为它们制剂的一部分)进行免疫。来自接种前后的血清在OPA测定中针对血清型15B和15C目标肺炎球菌菌株进行测试。

[0336] 用血清型15B缀合物免疫后，来自各组的10只兔子中，100％具有对血清型15B的OPA应答。这些相同的样品中，100％对血清型15C也具有OPA应答(表1和表2)。在15C OPA中在接种前的血清中观察到低OPA效价。然而，相比于接种前，接种后的血清具有超过10倍GMT OPA滴度的增加，这表明本发明的免疫原性缀合物在OPA中诱导能够杀死血清型15B和15C肺炎链球菌的抗体的形成。

[0337] PCV16v是16价缀合物组合物，其包含来自肺炎链球菌血清型1、3、4、5、6A、6B、7F、9V、14、15B、18C、19A、19F、22F、23F和33F(16vPnC)的糖缀合物(所有单独缀合至CRM_{197})。

[0338] PCV20v是20价缀合物组合物，其包含来自肺炎链球菌血清型1、3、4、5、6A、6B、7F、8、9V、10A、11A、12F、14、15B、18C、19A、19F、22F、23F和33F(20vPnC)的糖缀合物(所有单独缀合至CRM_{197})。

[0339] 表1.在兔血清中用PCV16v接种前后针对血清型15B和15C菌株的OPA滴度

[0340]

动物	15B OPA		15C OPA	
	wk0	wk4	wk0	wk4
1	4	4129	50	2524
2	4	1645	182	472
3	4	1131	126	818
4	4	3199	50	1189
5	4	2664	36	727
6	4	4589	68	2492
7	11	3601	169	1137
8	4	1838	165	672
9	4	1334	98	528
10	4	1108	204	2425
GMT	**4**	**2222**	**98**	**1075**

[0341]

[0342] 表2.在兔血清中用PCV20v接种前后针对血清型15B和15C菌株的OPA滴度

[0343]

动物	15B OPA		15C OPA	
	wk0	wk4	wk0	wk4
1	4	3784	无法确定*	2353
2	4	862	480	938
3	4	3056	69	1497
4	4	1948	无法确定*	1316
5	4	2360	4	4665
6	4	1594	无法确定*	1835
7	4	4943	172	4085

[0344]

8	4	2419	117	1458
9	4	1245	无法确定*	527
10	4	616	无法确定*	545
GMT	**4**	**1917**	**77**	**1515**

[0345] *由于杀死曲线差而无法确定滴度。

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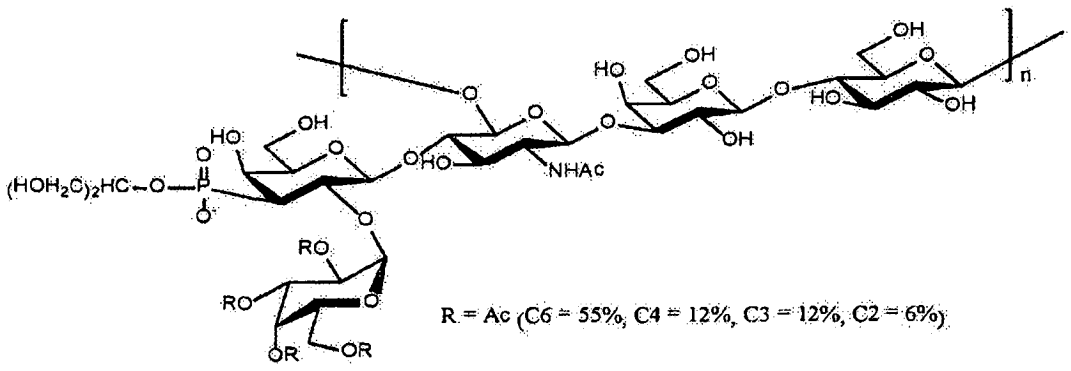

→6)-D-Glc*p*NAc-β-(1→3)-D-Gal*p*-β-(1→4)-D-Glc*p*-β-(1→

D-Gal*p*-α-(1→2)-D-Gal*p*(3-OPO$_3$-R^1)-β-(1→4

R^1 = CH(CH$_2$OH)$_2$

$C_{37}H_{61}NNaO_{31}P$, MW=1069.8

Ac= 乙酰基

图1