

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(10) International Publication Number
WO 2015/110940 A2

(43) International Publication Date
30 July 2015 (30.07.2015)

(51) International Patent Classification:
A61K 39/02 (2006.01)

(21) International Application Number:
PCT/IB2015/050314

(22) International Filing Date:
15 January 2015 (15.01.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/929,598 21 January 2014 (21.01.2014) US

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,

DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

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))

Published:

- without international search report and to be republished
upon receipt of that report (Rule 48.2(g))
- with sequence listing part of description (Rule 5.2(a))

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

(57) Abstract: The invention relates to activated Streptococcus pneumoniae serotype 10A, 22F or 33F polysaccharides and pro-
cesses for their preparation. The invention also relates to immunogenic conjugates comprising Streptococcus pneumoniae serotype
10A, 22F or 33F polysaccharides covalently linked to a carrier protein, processes for their preparation and immunogenic composi-
tions and vaccines comprising them.



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Streptococcus pneumoniae capsular polysaccharides and conjugates thereof**Field of the invention**

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The invention relates to activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharides and processes for their preparation. The invention also relates to immunogenic conjugates comprising *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharides covalently linked to a carrier protein, processes for their preparation and

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immunogenic compositions and vaccines comprising them.

Background

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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

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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

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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.

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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.

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None of the currently marketed pneumococcal vaccine provides an appropriate protection against serotype 10A, 22F or 33F *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 10A, 22F or 33F *Streptococcus pneumoniae*.

Summary of the invention

In one aspect the present disclosure provides a process for preparing an activated
5 *Streptococcus pneumoniae* serotype 10A, 22F or 33F capsular polysaccharide, the process comprising the steps of:

(a) reacting an isolated serotype 10A, 22F or 33F capsular polysaccharide with an oxidizing agent; and;

10 (b) quenching the oxidation reaction by addition of a quenching agent resulting in an activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharide.

In a further aspect, the invention relates to activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F capsular polysaccharide obtained or obtainable by the activation process disclosed herein.

In a further aspect, the present disclosure provides a process for the preparation of an
15 immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharide covalently linked to a carrier protein, the process comprising the steps of:

(a) compounding an activated serotype 10A, 22F or 33F polysaccharide obtained or obtainable by a process disclosed herein with a carrier protein; and,

20 (b) reacting the compounded, activated serotype 10A, 22F or 33F polysaccharide and carrier protein with a reducing agent to form a serotype 10A, 22F or 33F polysaccharide:carrier protein conjugate.

In a further aspect, the present disclosure provides serotype 10A, 22F or 33F polysaccharide:carrier protein conjugate obtained or obtainable by the process disclosed herein.

25 In a further aspect, the present disclosure provides immunogenic compositions and vaccines comprising immunogenic conjugate disclosed herein.

In a further aspect, the present disclosure a method of treating or preventing a *Streptococcus pneumoniae* infection, disease or condition associated with serotype 10A, 22F or 33F
30 *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

35 Figure 1 shows the structure of *Streptococcus pneumoniae* capsular polysaccharide Serotype 10A Repeat Unit.

Figure 2 shows the structure of *Streptococcus pneumoniae* capsular polysaccharide Serotype 22F Repeat Unit.

Figure 3 shows the structure of *Streptococcus pneumoniae* capsular polysaccharide Serotype 33F Repeat Unit.

5 Figure 4 shows the molecular weight of activated serotype 22F polysaccharide as a function of the amount of oxidizing agent at 23°C with and without quenching step.

Figure 5 shows the molecular weight of activated serotype 22F polysaccharide as a function of the amount of oxidizing agent at 4°C with and without quenching step.

10 Figure 6 shows the molecular weight of activated serotype 33F polysaccharide as a function of the amount of oxidizing agent at 2 to 8°C with or without quenching step or at 23°C without quenching step.

Detailed description of the invention

15 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
20 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.

25 As used herein, the singular forms "a", "an", and "the" include plural references unless indicated otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to one of ordinary skill in the art upon reading this disclosure.

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

As used herein, the term "degree of oxidation" (DO) refers to the number of sugar repeat
35 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.

It is noted that in this disclosure, terms such as "comprises," "comprised," "comprising," "contains," "containing" and the like can have the meaning attributed to them in U.S. patent law; e.g., they can mean "includes," "included," "including" and the like. Such terms refer to the inclusion of a particular ingredients or set of ingredients without excluding any other ingredients. Terms such as "consisting essentially of" and "consists essentially of" have the meaning attributed to them in U.S. patent law, e.g., they allow for the inclusion of additional ingredients or steps that do not detract from the novel or basic characteristics of the invention, i.e., they exclude additional unrecited ingredients or steps that detract from the novel or basic characteristics of the invention. The terms "consists of" and "consisting of" have the meaning ascribed to them in U.S. patent law; namely, that these terms are closed ended. Accordingly, these terms refer to the inclusion of a particular ingredient or set of ingredients and the exclusion of all other ingredients.

As used herein, the term "conjugates" or "glycoconjugates" as used herein refers to a polysaccharide covalently conjugated to a carrier protein. Glycoconjugates of the invention and immunogenic compositions comprising them may contain some amount of free polysaccharide.

As used herein, the term "serotype 10A glycoconjugate" or "serotype 10A conjugate" refers to an isolated *Streptococcus pneumoniae* serotype 10A capsular polysaccharide covalently conjugated to a carrier protein.

As used herein, the term "serotype 22F glycoconjugate" or "serotype 22F conjugate" refers to an isolated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide covalently conjugated to a carrier protein.

As used herein, the term "serotype 33F glycoconjugate" or "serotype 33F conjugate" refers to an isolated *Streptococcus pneumoniae* serotype 33F capsular polysaccharide covalently conjugated to a carrier protein.

As used herein, the term "serotype 10A polysaccharide" refers to a *Streptococcus pneumoniae* serotype 10A capsular polysaccharide.

As used herein, the term "serotype 22F polysaccharide" refers to a *Streptococcus pneumoniae* serotype 22F capsular polysaccharide.

As used herein, the term "serotype 33F polysaccharide" refers to a *Streptococcus pneumoniae* serotype 33F capsular polysaccharide.

As used herein, the term "free polysaccharide" means a capsular polysaccharide that is not covalently conjugated to the carrier protein, but is nevertheless present in the 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 10A, 22F or 33F 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, "to conjugate," "conjugated" and "conjugating" refer to a process whereby a *Streptococcus pneumoniae* capsular polysaccharide, is covalently attached to a carrier protein.

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.

In the preparation of multivalent conjugate pneumococcal vaccines directed to the prevention of invasive diseases caused by the organism *Streptococcus pneumoniae* (also known as pneumococcus), selected *Streptococcus pneumoniae* serotypes are grown to supply polysaccharides needed to produce the vaccine. The cells are grown in fermentors with lysis induced at the end of the fermentation by addition of sodium deoxycholate or an alternate lysing agent. The lysate broth is then harvested for downstream purification and the recovery of the capsular polysaccharide which surrounds the bacterial cells. After activation and conjugation with a carrier protein, the polysaccharide is included in the final vaccine product and confers immunity in the vaccine's target population to the selected *Streptococcus pneumoniae* serotypes.

The immunogenicity of a polysaccharide:carrier protein conjugate depends on several factors including the size of the polysaccharide. Polysaccharide size is a quality attribute assayed for in each preparation batch and must be appropriately controlled. High molecular weight capsular polysaccharides are able to induce certain antibody immune responses due to a higher valence of the epitopes present on the antigenic surface. It is generally preferable to prevent or to limit undesirable reduction of the size of the polysaccharide during the preparation of the conjugate to retain the immunogenicity of the polysaccharide. In addition, it is important to reduce the batch to batch variability of the polysaccharide Molecular Weight during the activation step and subsequent conjugation in order to maintain the consistency of the quality attributes of the conjugate.

Reductive amination chemistry (RAC) has been demonstrated by the inventors as a suitable process for preparing the *Streptococcus pneumoniae* serotype 10A, 22F or 33F capsular polysaccharide-protein carrier conjugate. The RAC approach involves activation of the polysaccharide by oxidation and subsequent conjugation of the activated polysaccharide to a protein carrier by reduction. Serotype 10A, 33F and 22F polysaccharides have proven to be particularly sensitive polysaccharides and are susceptible to degradation and size reduction during the oxidation step of the preparation of the conjugate. In addition, the use of hitherto known activation process produced activated serotype 10A, 33F and 22F polysaccharide of variable molecular weights.

Therefore, there is an important need for a process for preparing activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharides having a controlled and consistent molecular weight.

An object of the present invention is a process for preparing activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharides. In particular, an object of the invention is a process for preparing an activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharide, said process comprising the steps of:

- (a) reacting isolated serotype 10A, 22F or 33F polysaccharide with an oxidizing agent; and,
- (b) quenching the oxidation reaction by addition of a quenching agent resulting in an activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharide.

Isolation and purification of serotype 10A, 22F or 33F polysaccharide

The structures of serotype 10A, 22F and 33F polysaccharides are disclosed in the literature (see for example Kamerling J P C. Pneumococcal polysaccharides: a chemical view. In:

Tomasz A, editor. *Streptococcus pneumoniae*, molecular biology and mechanisms of disease. New York, N.Y: Mary Ann Liebert, Inc.; 2000. pp. 81–114).

As shown in figure 1, the polysaccharide repeating unit of serotype 22F consists of a
5 branched pentasaccharide backbone (one glucuronic acid (GlcA), one glucopyranose (GlcP) and one galactofuranose (GalF) and two rhamnopyranoses (Rhap)) with a α GlcP branch linked to the C3 hydroxyl group of β Rhap 45. Approximately 80% of the C2 hydroxyl groups of the β Rhap residue in the polysaccharide repeating unit are O-acetylated.

10 As shown in figure 2, the polysaccharide repeating unit of serotype 10A consists of a two D-galactosefuranose, three D-galactopyranose, one N-acetylgalactosamine, and a phosphate bond linked with saccharide chain through one D-ribitol.

As shown in figure 3, the polysaccharide repeating unit of serotype 33F consists of three D-
15 galactopyranose, two D-galactofuranose, and one D-glucopyranose. Notably, 5-D-galactofuranosyl residues are O-acetylated in about 90% of 33F polysaccharide repeating units.

Serotype 10A, 22F and 33F capsular polysaccharides can be obtained directly from bacteria
20 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.

25 Serotype 10A, 22F and 33F *Streptococcus pneumoniae* strains used to make the respective polysaccharides that are used in the immunogenic conjugates of the invention may be obtained from established culture collections or clinical specimens.

The purified serotype 10A, 22F and 33F polysaccharides are obtained by methods well
30 known to the man skilled in the art. The bacterial cells are preferably grown in a soy based medium. Following fermentation of bacterial cells that produce *Streptococcus pneumoniae* serotype 10A, 22F or 33F 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
35 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 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 capsular polysaccharides may then be purified 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 10A, 22F or 33F polysaccharides can then be used for the preparation of immunogenic conjugates.

Preferably, in order to generate serotype 22F conjugates or serotype 33F 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 22F polysaccharide or serotype 33F 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 22F polysaccharide or serotype 33F polysaccharide is reduced by mechanical homogenization.

- 5 In a preferred embodiment, the size of the purified 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.

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The high pressure homogenization process is particularly appropriate for reducing the size of the purified serotype 22F polysaccharide or serotype 33F polysaccharide while preserving the structural features of the polysaccharide such as the presence of O-acetyl groups.

- 15 The isolated serotype 22F polysaccharide obtained by purification of serotype 22F capsular 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, and the mM of acetate per mM of serotype 22F polysaccharide.

In a preferred embodiment, the sized serotype 22F polysaccharide has a molecular weight
20 comprised between 400 and 700kDa.

The isolated serotype 33F polysaccharide obtained by purification of serotype 33F capsular polysaccharide from the *Streptococcus pneumoniae* lysate and optionally sizing of the purified polysaccharide can be characterized by different parameters including for example
25 the molecular weight, and the mM of acetate per mM of serotype 33F polysaccharide.

The degree of O-acetylation of the polysaccharide can be determined by any method known in the art, for example, by proton NMR (Lemercinier and Jones (1996) Carbohydrate Research 296; 83-96, Jones and Lemercinier (2002) J. Pharmaceutical and Biomedical
30 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.

In a preferred embodiment, the isolated serotype 22F polysaccharide is obtained by a
35 process comprising the steps of:

- preparing a fermentation culture of serotype 22F *Streptococcus pneumonia* bacterial cells;
- lysing the bacterial cells in said fermentation culture; and,

- purifying serotype 22F polysaccharide from the fermentation culture.

In a preferred embodiment, the isolated serotype 22F polysaccharide is obtained by a process comprising the steps of:

- 5 - preparing a fermentation culture of serotype 22F *Streptococcus pneumonia* bacterial cells;
 - lysing the bacterial cells in said fermentation culture;
 - purifying serotype 22F polysaccharide from the fermentation culture; and,
 - sizing the serotype 22F polysaccharide by mechanical sizing, preferably by high pressure homogenization.
- 10 In a preferred embodiment, the isolated serotype 22F polysaccharide is obtained by a process comprising the steps of:
- preparing a fermentation culture of serotype 22F *Streptococcus pneumonia* bacterial cells;
 - lysing the bacterial cells in said fermentation culture;
 - purifying serotype 22F polysaccharide from the fermentation culture; and,
 - 15 - sizing the serotype 22F polysaccharide by mechanical sizing, preferably by high pressure homogenization,
- wherein the isolated serotype 22F polysaccharide has a molecular weight comprised between 400 and 700kDa.

- 20 In a preferred embodiment, the isolated serotype 33F polysaccharide is obtained by a process comprising the steps of:
- preparing a fermentation culture of serotype 33F *Streptococcus pneumonia* bacterial cells;
 - lysing the bacterial cells in said fermentation culture; and,
 - purifying serotype 33F polysaccharide from the fermentation culture.

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In a preferred embodiment, the isolated serotype 33F polysaccharide is obtained by a process comprising the steps of:

- preparing a fermentation culture of serotype 33F *Streptococcus pneumonia* bacterial cells;
- lysing the bacterial cells in said fermentation culture;
- 30 - purifying serotype 33F polysaccharide from the fermentation culture; and,
- sizing the serotype 33F polysaccharide by mechanical sizing, preferably by high pressure homogenization.

- 35 The isolated serotype 10A polysaccharide obtained by purification of serotype 10A capsular 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. Preferably, the isolated serotype 10A polysaccharide is not sized.

In a preferred embodiment, the isolated serotype 10A polysaccharide is obtained by a process comprising the steps of:

- preparing a fermentation culture of serotype 10A *Streptococcus pneumonia* bacterial cells;
- 5 - lysing the bacterial cells in said fermentation culture; and,
- purifying serotype 10A polysaccharide from the fermentation culture.

Activation of serotype 10A, 22F or 33F polysaccharide

- 10 The isolated serotype 10A, 22F or 33F polysaccharide is activated by a process comprising the step of:

(a) reacting an isolated serotype 10A, 22F or 33F polysaccharide with an oxidizing agent; and,

- (b) quenching the oxidation reaction by addition of a quenching agent resulting in an
15 activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharide.

In a preferred embodiment, the concentration of serotype 10A, 22F or 33F polysaccharide in step (a) is between 0.1 and 10 mg/mL. In a preferred embodiment, the concentration of serotype 10A, 22F or 33F polysaccharide in step (a) is between 0.5 and 5 mg/mL. In a
20 preferred embodiment, the concentration of serotype 10A, 22F or 33F polysaccharide in step (a) is between 1 and 3 mg/mL. In a preferred embodiment, the concentration of serotype 22F or 33F polysaccharide in step (a) is about 2 mg/mL. In a preferred embodiment, the concentration of serotype 10A polysaccharide in step (a) is about 2.5 mg/mL.

- 25 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
30 embodiment, the oxidizing agent is sodium periodate. In a preferred embodiment, the periodate used for the oxidation of serotype 10A, 22F or 33F polysaccharide is metaperiodate. In a preferred embodiment the periodate used for the oxidation of serotype 10A, 22F or 33F polysaccharide is sodium metaperiodate.

- 35 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, 0.01 to 0.2, 0.05 to 0.5, 0.05 to 0.2, 0.1 to 0.3 molar equivalents of oxidizing agent. In a preferred embodiment, the polysaccharide is reacted with about 0.05,

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, In a preferred embodiment, the polysaccharide is reacted with about 0.10 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 0.8 molar equivalent of oxidizing agent.

10 In a preferred embodiment, the duration of the reaction in step (a) is between 1 and 50, 1 and 40, 1 and 30, 1 and 25, 1 and 20, 1 and 10, 10 to 50, 10 to 40, 10 to 30, 10 to 20 hours. In a preferred embodiment, when the polysaccharide is serotype 10A polysaccharide, the duration of the reaction in step (a) is between 1 to 10 hours. In a preferred embodiment, when the polysaccharide is serotype 10A polysaccharide, the duration of the reaction in step (a) is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 hours. In a preferred embodiment, when the polysaccharide is serotype 10A polysaccharide, the duration of the reaction in step (a) is about 4 hours. In a preferred embodiment, when the polysaccharide is serotype 22F polysaccharide, the duration of the reaction in step (a) is between 10 and 20 hours. In a preferred embodiment, when the polysaccharide is serotype 22F polysaccharide, the duration of the reaction in step (a) is about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 hours. In a preferred embodiment, when the polysaccharide is serotype 33F polysaccharide, the duration of the reaction in step (a) is between 15 to 25 hours. In a preferred embodiment, when the polysaccharide is serotype 33F polysaccharide, the duration of the reaction in step (a) is about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 hours. In a preferred embodiment, when the polysaccharide is serotype 33F polysaccharide, the duration of the reaction in step (a) is about 20 hours.

In a preferred embodiment, the temperature of the reaction in step (a) is maintained between 1 to 30°C, 1 to 10°C, 10 to 20°C, 20 to 30°C, 2 to 8°C. In a preferred embodiment, the temperature of the reaction in step (a) is maintained at about 5°C.

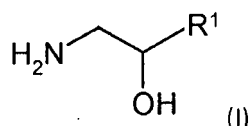
In a preferred embodiment, step (a) is carried out in a buffer selected from sodium phosphate, potassium phosphate, 2-(N-morpholino)ethanesulfonic acid (MES) or Bis-Tris. In a preferred embodiment step (a) is carried out in potassium phosphate buffer. In a preferred embodiment step (a) is carried out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

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

- 5 In a preferred embodiment the pH in step (a) is between 4 and 8, 5 and 7, 5.5 and 6.5. In a preferred embodiment the pH in step (a) is about 6. In a preferred embodiment the pH in step (a) is about 5.8.

- 10 In one embodiment, the quenching agent is selected from vicinal diols, 1,2-aminoalcohols, amino acids, glutathione, sulfite, bisulfate, dithionite, metabisulfite, thiosulfate, phosphites, hypophosphites or phosphorous acid.

In one embodiment, the quenching agent is a 1,2-aminoalcohols of formula



- 15 where R^1 is selected from H, methyl, ethyl, propyl or isopropyl.

In one embodiment, the quenching agent is selected from sodium and potassium salts of sulfite, bisulfate, dithionite, metabisulfite, thiosulfate, phosphites, hypophosphites or phosphorous acid.

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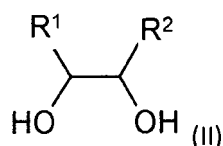
In one embodiment, the quenching agent is an amino acid. In one embodiment, said amino acid is selected from serine, threonine, cysteine, cystine, methionine, proline, hydroxyproline, tryptophan, tyrosine, and histidine.

- 25 In one embodiment, the quenching agent is a sulfite such as bisulfate, dithionite, metabisulfite, thiosulfate.

In one embodiment, the quenching agent is a compound comprising two vicinal hydroxyl groups (vicinal diols), i.e two hydroxyl groups covalently linked to two adjacent carbon atoms.

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Preferably, the quenching agent is a compound of formula (II)



wherein R¹ and R² are each independently selected from H, methyl, ethyl, propyl or isopropyl.

In a preferred embodiment, the quenching agent is glycerol, ethylene glycol, propan-1,2-diol, butan-1,2-diol or butan-2,3-diol, ascorbic acid. In a preferred embodiment, the quenching agent is butan-2,3-diol.

In a preferred embodiment, the oxidizing agent is neutralized by the addition of 0.1 to 10, 0.5 to 5, 0.5 to 3, 0.5 to 2 molar equivalents of quenching agent. In a preferred embodiment, the oxidizing agent is neutralized by the addition of about 0.5, 1, 1.5, 2, 2.5, 3 molar equivalents of quenching agent. In a preferred embodiment, the oxidizing agent is neutralized by the addition of about 2 molar equivalents of quenching agent.

In a preferred embodiment, the duration of step (b) is between 0.1 and 10, 0.5 and 5, or 0.5 and 2 hours. In a preferred embodiment, the duration of step (b) is about 0.5, 1, 1.5, 2.5 or 3 hours.

In a preferred embodiment, the temperature of the reaction in step (b) is maintained between 1 and 30°C, 1 and 25°C, 1 and 20°C, 1 and 10°C, 10 and 20°C, 2 and 8°C. In a preferred embodiment, the temperature of the reaction in step (b) is maintained at about 1, 2, 3, 4, 5, 6, 7 or 8°C.

In a preferred embodiment the pH in step (b) is between 4 and 8, 5 and 7, 5.5 and 6.5. In a preferred embodiment the pH in step (a) is about 6.

In a preferred embodiment, the activated serotype 10A, 22F or 33F polysaccharide is purified. The activated serotype 10A, 22F or 33F polysaccharide can be 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 polysaccharide is purified by concentration and diafiltration using an ultrafiltration device.

In preferred embodiment, the isolated serotype 10A polysaccharide is activated by a process comprising the step of:

- (a) reacting isolated serotype 10A polysaccharide with periodate; and,
- (b) quenching the oxidation reaction by addition of butan-2,3-diol resulting in an activated serotype 10A polysaccharide.

In preferred embodiment, the isolated serotype 10A polysaccharide is activated by a process comprising the step of:

(a) reacting isolated serotype 10A polysaccharide with periodate at a temperature between 2 and 8°C; and,

- 5 (b) quenching the oxidation reaction by addition of butan-2,3-diol at a temperature between 2 and 8°C resulting in an activated serotype 10A polysaccharide.

In preferred embodiment, the isolated serotype 10A polysaccharide is activated by a process comprising the step of:

- 10 (a) reacting isolated serotype 10A polysaccharide with 0.2 to 0.3 molar equivalent periodate at a temperature between 2 and 8°C; and,

(b) quenching the oxidation reaction by addition of 0.5 to 2 molar equivalent of butan-2,3-diol at a temperature between 2 and 8°C resulting in an activated serotype 10A polysaccharide.

- 15 In preferred embodiment, the isolated serotype 22F polysaccharide is activated by a process comprising the step of:

(a) reacting isolated serotype 22F polysaccharide with periodate; and,

(b) quenching the oxidation reaction by addition of butan-2,3-diol resulting in an activated serotype 22F polysaccharide.

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In preferred embodiment, the isolated serotype 22F polysaccharide is activated by a process comprising the step of:

(a) reacting isolated serotype 22F polysaccharide with periodate at a temperature between 2 and 8°C; and,

- 25 (b) quenching the oxidation reaction by addition of butan-2,3-diol at a temperature between 2 and 8°C resulting in an activated serotype 22F polysaccharide.

In preferred embodiment, the isolated serotype 22F polysaccharide is activated by a process comprising the step of:

- 30 (a) reacting isolated serotype 22F polysaccharide with 0.05 to 0.2 molar equivalent periodate at a temperature between 2 and 8°C; and,

(b) quenching the oxidation reaction by addition of 2 to 3, preferably 2, molar equivalents of butan-2,3-diol at a temperature between 2 and 8°C resulting in an activated serotype 22F polysaccharide.

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In preferred embodiment, the isolated serotype 33F polysaccharide is activated by a process comprising the step of:

- (a) reacting isolated serotype 33F polysaccharide with periodate; and,
- (b) quenching the oxidation reaction by addition of butan-2,3-diol resulting in an activated serotype 33F polysaccharide.

- 5 In preferred embodiment, the isolated serotype 33F polysaccharide is activated by a process comprising the step of:
- (a) reacting isolated serotype 33F polysaccharide with periodate at a temperature between 2 and 8°C; and,
 - (b) quenching the oxidation reaction by addition of butan-2,3-diol at a temperature between 2 and 8°C resulting in an activated serotype 33F polysaccharide.
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In preferred embodiment, the isolated serotype 33F polysaccharide is activated by a process comprising the step of:

- (a) reacting isolated serotype 33F polysaccharide with 0.05 to 0.2 molar equivalent periodate at a temperature between 2 and 8°C; and,
 - (b) quenching the oxidation reaction by addition of 0.5 to 1.5 molar equivalent of butan-2,3-diol at a temperature between 2 and 8°C resulting in an activated serotype 33F polysaccharide.
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- 20 In a preferred embodiment, the invention relates to an activated serotype 10A capsular polysaccharide obtained or obtainable by the above disclosed process.

In a preferred embodiment, the invention relates to an activated serotype 22F capsular polysaccharide obtained or obtainable by the above disclosed process.

- 25 In a preferred embodiment, the invention relates to an activated serotype 33F capsular polysaccharide obtained or obtainable by the above disclosed process.

In a preferred embodiment the activated serotype 10A polysaccharide obtained from step (b) has a molecular weight between 20 and 100%, 30 and 95%, 40 and 95%, 60 to 95%, 85 to 95%, or about 90%, of the molecular weight of the isolated polysaccharide used in step (a).

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In a preferred embodiment the activated serotype 10A polysaccharide obtained from step (b) has a molecular weight of at least 20, 25, 30, 35, 40 or 45% of the molecular weight of the isolated polysaccharide used in step (a). In a preferred embodiment the activated serotype 10A polysaccharide obtained from step (b) has a molecular weight of at least 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% of the molecular weight of the isolated polysaccharide used in step (a).

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In a preferred embodiment the activated serotype 22F polysaccharide obtained from step (b) has a molecular weight between 50 and 100%, 60 to 95%, 85 to 95% or about 90% of the molecular weight of the isolated polysaccharide used in step (a).

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In a preferred embodiment the activated serotype 22F polysaccharide obtained from step (b) has a molecular weight of at least 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% of the molecular weight of the isolated polysaccharide used in step (a).

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In a preferred embodiment the activated serotype 33F polysaccharide obtained from step (b) has a molecular weight between 50 and 100%, 60 to 95%, 85 to 95%, or about 90% of the molecular weight of the isolated polysaccharide used in step (a).

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In a preferred embodiment the activated serotype 33F polysaccharide obtained from step (b) has a molecular weight of at least 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% of the molecular weight of the isolated polysaccharide used in step (a).

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In a preferred embodiment the degree of oxidation of the activated serotype 10A, 22F or 33F polysaccharide is between 2 and 30, 2 and 25, 2 and 20, 2 and 15, 2 and 10, 2 and 5, 5 and 30, 5 and 25, 5 and 20, 5 and 15, 5 and 10, 10 and 30, 10 and 25, 10 and 20, 10 and 15, 5 and 25, 10 and 30, 15 and 30, 15 and 25, 15 and 20, 20 to 30, 20 to 25. In a preferred embodiment the degree of oxidation of the activated serotype 10A, 22F or 33F polysaccharide is between 2 and 10, 4 and 8, 4 and 6, 6 and 8, 6 and 12, 8 and 14, 9 and 11, 10 and 16, 12 and 16, 14 and 18, 16 and 20, 16 and 18, 18 and 22, 18 and 20.

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In a preferred embodiment, the activated serotype 10A polysaccharide has a molecular weight between 50 and 400, 50 and 350, 50 and 300, 50 and 250, 50 and 200, 100 and 300, 100 and 250 or 100 and 200kDa. In a preferred embodiment, the activated serotype 10A polysaccharide has a molecular weight between 50 and 300kDa. In a preferred embodiment, the activated serotype 10A polysaccharide has a molecular weight between 100 and 200kDa. In a preferred embodiment, the activated serotype 10A polysaccharide has a molecular weight between 100 and 200kDa and a degree of oxidation between 5 and 20, 5 and 15, 8 and 14, 8 and 12 or 9 and 11. In a preferred embodiment, the activated serotype

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10A polysaccharide has a molecular weight between 100 and 200kDa and a degree of oxidation between 9 and 11.

5 In a preferred embodiment, the activated serotype 22F polysaccharide has a molecular weight between 25 and 1000, 100 and 1000, 300 and 800, 300 and 700, 300 and 600, 400 and 1000, 400 and 800, 400 and 700 or 400 and 600kDa. In a preferred embodiment, the activated serotype 22F polysaccharide has a molecular weight between 300 and 800kDa. In a preferred embodiment, the activated serotype 22F polysaccharide has a molecular weight between 400 and 800kDa. In a preferred embodiment, the activated serotype 22F polysaccharide has a molecular weight between 400 and 600kDa. In a preferred embodiment, the activated serotype 22F polysaccharide has a molecular weight between 400 and 800kDa and a degree of oxidation between 10 and 25, 10 and 20, 12 and 20 or 14 and 18. In a preferred embodiment, the activated serotype 22F polysaccharide has a molecular weight between 400 and 800kDa and a degree of oxidation between 10 and 20.

15 In a preferred embodiment, the activated serotype 22F polysaccharide comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 or 0.7 or about 0.8 mM acetate per mM serotype 22F polysaccharide. In a preferred embodiment, the activated serotype 22F polysaccharide comprises at least 0.5, 0.6 or 0.7 mM acetate per mM serotype 22F polysaccharide. In a preferred embodiment, the activated serotype 22F polysaccharide comprises at least 0.6 mM acetate per mM serotype 22F polysaccharide. In a preferred embodiment, the activated serotype 22F polysaccharide comprises at least 0.7 mM acetate per mM serotype 22F polysaccharide.

25 In a preferred embodiment, the activated serotype 22F polysaccharide has a molecular weight between 400 and 800 kDa and comprises at least 0.6 mM acetate per mM serotype 22F polysaccharide.

30 In a preferred embodiment, the activated serotype 22F polysaccharide has a molecular weight between 400 and 800kDa, a degree of oxidation between 12 and 20 and comprises at least 0.6 mM acetate per mM serotype 22F polysaccharide.

35 In a preferred embodiment, the activated serotype 33F polysaccharide has a molecular weight between 50 and 1250, 200 and 1200, 500 and 1200, 500 and 1000, 700 and 1200, 800 and 1200, 800 and 1100, 900 and 1200, 800 and 1000Da. In a preferred embodiment, the activated serotype 33F polysaccharide has a molecular weight between 500 and

1000kDa. In another preferred embodiment, the activated serotype 33F polysaccharide has a molecular weight between 50 and 600, 50 and 500 or 50 and 400kDa.

In a preferred embodiment, the activated serotype 33F polysaccharide comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 or about 0.9 mM acetate per mM serotype 33F polysaccharide. In a preferred embodiment, the activated serotype 33F polysaccharide comprises at least 0.6, 0.7 or 0.8 mM acetate per mM serotype 33F polysaccharide. In a preferred embodiment, the activated serotype 33F polysaccharide comprises at least 0.6 mM acetate per mM serotype 33F polysaccharide. In a preferred embodiment, the activated serotype 33F polysaccharide comprises at least 0.7 mM acetate per mM serotype 33F polysaccharide.

In a preferred embodiment, the activated serotype 33F polysaccharide has a molecular weight between 800 and 1200 kDa and comprises at least 0.7 mM acetate per mM serotype 33F polysaccharide. In a preferred embodiment, the activated serotype 33F polysaccharide has a molecular weight between 800 and 1200 kDa, comprises at least 0.7 mM acetate per mM serotype 33F polysaccharide and has a degree of oxidation between 10 and 20.

In a preferred embodiment, the activated serotype 33F polysaccharide has a molecular weight between 50 and 200 kDa and comprises at least 0.7 mM acetate per mM serotype 33F polysaccharide.

In a preferred embodiment, the activated serotype 33F polysaccharide has a molecular weight between 50 and 200 kDa, comprises at least 0.7 mM acetate per mM serotype 33F polysaccharide and has a degree of oxidation between 10 and 20.

In an embodiment, the activated serotype 10A, 22F or 33F polysaccharide is lyophilized, optionally in the presence of a cryoprotectant/lyoprotectant. In a preferred embodiment, the cryoprotectant/lyoprotectant is selected from sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit. In a preferred embodiment, the cryoprotectant/lyoprotectant is sucrose. The lyophilized activated polysaccharide can then be compounded with a solution comprising the carrier protein.

In another embodiment, the activated serotype 10A, 22F or 33F polysaccharide is compounded with the carrier protein and lyophilized optionally in the presence of a cryoprotectant/lyoprotectant. In a preferred embodiment, the cryoprotectant/lyoprotectant is selected from sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol

and palatinit. In a preferred embodiment, the cryoprotectant/lyoprotectant is sucrose. The co-lyophilized polysaccharide and carrier protein can then be resuspended in solution and reacted with a reducing agent.

- 5 In an embodiment, the invention relates to a lyophilized activated serotype 10A polysaccharide. In an embodiment, the invention relates to a lyophilized activated serotype 22F polysaccharide. In an embodiment, the invention relates to a lyophilized activated serotype 33F polysaccharide.
- 10 In an embodiment the invention relates to the co-lyophilized activated serotype 10A polysaccharide and protein carrier. In a preferred embodiment, the protein carrier is CRM₁₉₇. In an embodiment the invention relates to the co-lyophilized activated serotype 22F polysaccharide and protein carrier. In a preferred embodiment, the protein carrier is CRM₁₉₇. In an embodiment the invention relates to the co-lyophilized activated serotype 33F polysaccharide and protein carrier. In a preferred embodiment, the protein carrier is CRM₁₉₇.
- 15

Conjugation of activated serotype 10A, 22F or 33F polysaccharide with a carrier protein

- The activated serotype 10A, 22F or 33F polysaccharide disclosed herein can be conjugated to a carrier protein by a process comprising the steps of:
- 20 (a) compounding the activated serotype 10A, 22F or 33F polysaccharide with a carrier protein; and,
- (b) reacting the compounded activated serotype 10A, 22F or 33F polysaccharide and carrier protein with a reducing agent to form a serotype 10A, 22F or 33F polysaccharide:carrier protein conjugate.
- 25

- The conjugation of activated serotype 22F or 33F 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 phase 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.
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- In a preferred embodiment, step (a) comprises dissolving lyophilized serotype 10A, 22F or 33F polysaccharide in an aqueous solution comprising a carrier protein or in a solution comprising a carrier protein and DMSO. In a preferred embodiment, step (a) comprises dissolving co-lyophilized serotype 10A, 22F or 33F polysaccharide and carrier protein in an aqueous solution or in DMSO.
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When step (a) and (b) are carried out in an aqueous solution, said solution comprises a buffer, preferably selected from 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 10A, 22F or 33F polysaccharide in step (a) 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 10A, 22F or 33F polysaccharide in step (a) 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 ratio (weight by weight) of activated serotype 10A, 22F or 33F 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 1:1.2. In a preferred embodiment the initial ratio of activated serotype 10A, 22F or 33F polysaccharide to carrier protein is about 0.6:1 and 1:1.2. In a preferred embodiment the initial ratio of activated serotype 10A, 22F or 33F 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, 2:1.

In a preferred embodiment, in step (b), the activated serotype 10A, 22F or 33F polysaccharide is reacted with between 0.1 and 10 molar equivalents, 0.5 and 5 molar equivalents 0.5 and 2.5 molar equivalents of reducing agent. In a preferred embodiment, in step (b), the activated serotype 10A, 22F or 33F is reacted with about 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 or 2.5 molar equivalents of reducing agent.

In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxymethylborohydride, 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-BuMe²PrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane (PEMB). In a preferred embodiment, the reducing agent is sodium cyanoborohydride.

In a preferred embodiment, the duration of step (b) is between 1 and 50, 5 and 30, 10 and 30, 15 and 30, 20 and 30, 5 and 25, 10 and 25, 15 and 25 hours. In a preferred embodiment, the duration of step (b) is about 20, 21, 22, 23, 24, 25, 26, 27, 28 hours.

In a preferred embodiment, the temperature of the reaction in step (b) is maintained between 10 and 40°C, 15 and 30°C, 20 and 26°C, 21 and 25°C. In a preferred embodiment, the temperature of the reaction in step (b) is maintained at about 21, 22, 23, 24 or 25°C.

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In a preferred embodiment, the process for the preparation of an immunogenic conjugate comprising serotype 10A, 22F or 33F polysaccharide covalently linked to a carrier protein further comprises a step (step c) of capping unreacted aldehyde (quenching) by addition of NaBH₄.

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In a preferred embodiment, in step (c), the unreacted aldehydes are capped by the addition of 0.1 to 10 molar equivalents, 0.5 to 5 molar equivalents or 1 to 3 molar equivalents of NaBH₄. In a preferred embodiment, in step (c), the unreacted aldehydes are capped by the addition of about 2 molar equivalents of NaBH₄.

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

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

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After conjugation of serotype 10A, 22F or 33F 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.

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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.

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In a preferred embodiment, the activated serotype 10A, 22F or 33F polysaccharide is conjugated to a carrier protein which is selected in the group consisting of: DT (Diphtheria toxin), TT (tetanus toxoid) or fragment C of TT, CRM197 (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, WO2009/000826), 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 10A, 22F or 33F polysaccharide is conjugated to DT (Diphtheria toxoid). In another embodiment, the activated serotype 10A, 22F or 33F polysaccharide is conjugated to TT (tetanus toxoid). In another embodiment, the activated serotype 10A, 22F or 33F polysaccharide is conjugated to fragment C of TT. In another embodiment, the activated serotype 10A, 22F or 33F polysaccharide is conjugated to PD (Haemophilus influenza protein D - see, e.g., EP 0 594 610 B).

In a preferred embodiment, the activated serotype 10A, 22F or 33F polysaccharide is conjugated to CRM₁₉₇ protein. The CRM₁₉₇ protein is a nontoxic form of diphtheria toxin but is immunologically indistinguishable from the diphtheria toxin. CRM₁₉₇ is produced by C. diphtheriae infected by the nontoxigenic phage β 197^{tox-} created by nitrosoguanidine mutagenesis of the toxigenic corynebacteriophage beta (Uchida, T. et al. 1971, Nature New Biology 233:8-11). CRM₁₉₇ is purified through ultrafiltration, ammonium sulfate precipitation, and ion-exchange chromatography. The CRM₁₉₇ 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 CRM₁₉₇ protein is a safe and effective T-cell dependent carrier for saccharides. Further details about CRM₁₉₇ and production thereof can be found e.g. in US 5,614,382.

5 In a preferred embodiment, the activated serotype 10A, 22F or 33F polysaccharide disclosed herein is conjugated to CRM₁₉₇ by a process comprising the step of:

(a) compounding the activated serotype 10A, 22F or 33F polysaccharide with CRM₁₉₇;

(b) reacting the compounded activated serotype 10A, 22F or 33F polysaccharide and CRM₁₉₇ with sodium cyanoborohydride to form a serotype 10A, 22F or 33F polysaccharide:CRM₁₉₇

10 conjugate.

In a preferred embodiment, the activated serotype 10A, 22F or 33F polysaccharide disclosed herein is conjugated to CRM₁₉₇ by a process comprising the step of:

(a) compounding the activated serotype 10A, 22F or 33F polysaccharide with a CRM₁₉₇;

15 (b) reacting the compounded activated serotype 10A, 22F or 33F polysaccharide and CRM₁₉₇ with sodium cyanoborohydride to form a serotype 10A, 22F or 33F polysaccharide:CRM₁₉₇ conjugate;

wherein steps (a) and (b) are carried out in DMSO.

20 In a preferred embodiment, the activated serotype 10A, 22F or 33F polysaccharide disclosed herein is conjugated to CRM₁₉₇ by a process comprising the step of:

(a) compounding the activated serotype 10A, 22F or 33F polysaccharide with a CRM₁₉₇;

(b) reacting the compounded activated serotype 10A, 22F or 33F polysaccharide and CRM₁₉₇ with 0.5 to 2 molar equivalent sodium cyanoborohydride to form a serotype 10A, 22F or 33F

25 polysaccharide:CRM₁₉₇ conjugate;

wherein steps (a) and (b) are carried out in DMSO.

In an embodiment, the invention relates to an immunogenic conjugate obtained or a obtainable by a process disclosed herein. In a preferred embodiment, the invention relates to

30 an immunogenic conjugate obtained or obtainable by conjugating a serotype 10A, 22F or 33F activated polysaccharide as disclosed herein to a carrier protein by reductive amination.

In a preferred embodiment, the invention relates to an immunogenic conjugate obtained or obtainable by conjugating a serotype 10A, 22F or 33F activated polysaccharide as disclosed herein to a carrier protein by reductive amination in DMSO. In a preferred embodiment, the

35 carrier protein is CRM₁₉₇.

Serotype 10A immunogenic conjugate

In a preferred embodiment, the serotype 10A immunogenic conjugate has a molecular weight between 500 and 15000; 500 and 10000; 2000 and 10000; or 3000 and 8000kDa. In

5 a preferred embodiment, the serotype 10A immunogenic conjugate has a molecular weight between 3000 and 8000kDa. The molecular weight of the immunogenic conjugate is measured by SEC-MALLS.

10 In a preferred embodiment, the serotype 10A immunogenic conjugate comprises less than about 50, 45, 40, 35, 30, 25, 20 or 15% of free serotype 10A polysaccharide compared to the total amount of serotype 10A polysaccharide. In a preferred embodiment the serotype 10A immunogenic conjugate comprises less than about 25% of free serotype 10A polysaccharide compared to the total amount of serotype 10A polysaccharide. In a preferred embodiment the serotype 10A immunogenic conjugate comprises less than about 20% of free serotype
15 10A polysaccharide compared to the total amount of serotype 10A polysaccharide. In a preferred embodiment the serotype 10A immunogenic conjugate comprises less than about 15% of free serotype 10A polysaccharide compared to the total amount of serotype 10A polysaccharide.

20 In a preferred embodiment, the ratio (weight by weight) of serotype 10A polysaccharide to carrier protein in the conjugate is between 0.5 and 3. In a preferred embodiment, the ratio of serotype 10A polysaccharide to carrier protein in the conjugate is between 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 10A polysaccharide to carrier protein in the conjugate is between 0.8 and 1.4. In a preferred
25 embodiment, the ratio of serotype 10A capsular polysaccharide to carrier protein in the conjugate is between 0.8 and 1.2.

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
30 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), ($K_d=0$), and the fraction representing the
35 maximum retention (V_i), ($K_d=1$). The fraction at which a specified sample attribute is reached (V_e), is related to Kd by the expression, $K_d = (V_e - V_0) / (V_i - V_0)$.

In a preferred embodiment, at least 30% of the serotype 10A 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 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85% of the serotype 10A 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 serotype 10A immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 80% of the serotype 10A immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

The degree of conjugation is the number of lysine residues in the carrier protein that are conjugated to the polysaccharide of interest. 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 CRM₁₉₇ 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 and 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 6 and 8.

Serotype 22F immunogenic conjugate

In a preferred embodiment, the immunogenic conjugate comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 or 0.7 or about 0.8 mM acetate per mM serotype 22F polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.5, 0.6 or 0.7 mM acetate per mM serotype 22F polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.6 mM acetate per mM serotype 22F polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.7 mM acetate per mM serotype 22F polysaccharide.

In a preferred embodiment, the ratio of mM acetate per mM serotype 22F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 22F 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 22F polysaccharide in the

immunogenic conjugate to mM acetate per mM serotype 22F polysaccharide in the isolated polysaccharide is at least 0.7. In a preferred embodiment, the ratio of mM acetate per mM serotype 22F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 22F polysaccharide in the isolated polysaccharide is at least 0.9.

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In a preferred embodiment, the ratio of mM acetate per mM serotype 22F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 22F 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 22F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 22F polysaccharide in the activated polysaccharide is at least 0.7. In a preferred embodiment, the ratio of mM acetate per mM serotype 22F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 22F polysaccharide in the activated polysaccharide is at least 0.9.

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In a preferred embodiment, the serotype 22F immunogenic conjugate has a molecular weight between 400 and 15000; 500 and 10000; 2000 and 10000kDa; 3000 and 8000kDa; or 3000 and 5000kDa. In a preferred embodiment, the serotype 22F immunogenic conjugate has a molecular weight between 3000 and 5000kDa. The molecular weight of the immunogenic conjugate is measured by SEC-MALLS.

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In a preferred embodiment, the serotype 22F immunogenic conjugate comprises less than about 50, 45, 40, 35, 30, 25, 20 or 15% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide. . In a preferred embodiment the serotype 22F immunogenic conjugate comprises less than about 40% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide. In a preferred embodiment the serotype 22F immunogenic conjugate comprises less than about 25% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide. In a preferred embodiment the serotype 22F immunogenic conjugate comprises less than about 20% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide. In a preferred embodiment the serotype 22F immunogenic conjugate comprises less than about 15% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide.

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In a preferred embodiment, the ratio (weight by weight) of serotype 22F polysaccharide to carrier protein in the conjugate is between 0.5 and 3. In a preferred embodiment, the ratio of serotype 22F polysaccharide to carrier protein in the conjugate is between 0.5 and 2, 0.5 and 1.5, 0.8 and 1.2, 0.5 and 1, 1 and 1.5, or 1 and 2. In a preferred embodiment, the ratio of

serotype 22F polysaccharide to carrier protein in the conjugate is between 0.8 and 1.2. In a preferred embodiment, the ratio of serotype 22F capsular polysaccharide to carrier protein in the conjugate is between 0.9 and 1.1.

- 5 In a preferred embodiment, at least 30% of the serotype 22F 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 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85% of the serotype 22F 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 serotype 22F immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 80% of the serotype 22F 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 22F immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

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- 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, 4 and 7, 5 and 15, 5 and 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 4 and 7.

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Serotype 33F immunogenic conjugate

- In a preferred embodiment, the immunogenic conjugate comprises at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 or 0.7 or about 0.8 mM acetate per mM serotype 33F polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.5, 0.6 or 0.7 mM acetate per mM serotype 33F polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.6 mM acetate per mM serotype 33F polysaccharide. In a preferred embodiment, the immunogenic conjugate comprises at least 0.7 mM acetate per mM serotype 33F polysaccharide.

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- In a preferred embodiment, the ratio of mM acetate per mM serotype 33F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 33F 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 33F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 33F polysaccharide in the isolated polysaccharide is at least 0.7. In a preferred embodiment, the ratio of mM acetate per mM

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serotype 33F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 33F polysaccharide in the isolated polysaccharide is at least 0.9.

In a preferred embodiment, the ratio of mM acetate per mM serotype 33F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 33F 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 33F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 33F polysaccharide in the activated polysaccharide is at least 0.7. In a preferred embodiment, the ratio of mM acetate per mM serotype 33F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 33F polysaccharide in the activated polysaccharide is at least 0.9.

In a preferred embodiment, the serotype 33F immunogenic conjugate has a molecular weight between 500 and 30000; 500 and 25000; 500 and 20000; 500 and 15000; 500 and 10000; 1000 and 10000; 1000 and 8000; 1000 and 5000; 2000 and 10000kDa; 2000 and 8000; or 2000 and 5000kDa. In a preferred embodiment, the serotype 33F immunogenic conjugate has a molecular weight between 1000 and 5000kDa. The molecular weight of the immunogenic conjugate is measured by SEC-MALLS.

In a preferred embodiment, the serotype 33F immunogenic conjugate comprises less than about 50, 45, 40, 35, 30, 25, 20 or 15% of free serotype 33F polysaccharide compared to the total amount of serotype 33F polysaccharide. In a preferred embodiment the serotype 33F immunogenic conjugate comprises less than about 25% of free serotype 33F polysaccharide compared to the total amount of serotype 33F polysaccharide. In a preferred embodiment the serotype 33F immunogenic conjugate comprises less than about 20% of free serotype 33F polysaccharide compared to the total amount of serotype 33F polysaccharide. In a preferred embodiment the serotype 33F immunogenic conjugate comprises less than about 15% of free serotype 33F polysaccharide compared to the total amount of serotype 33F polysaccharide.

In a preferred embodiment, the ratio (weight by weight) of serotype 33F polysaccharide to carrier protein in the conjugate is between 0.4 and 3. In a preferred embodiment, the ratio of serotype 33F polysaccharide to carrier protein in the conjugate is between 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 33F polysaccharide to carrier protein in the conjugate is between 0.5 and 1.5. In a preferred embodiment, the ratio of serotype 33F capsular polysaccharide to carrier protein in the conjugate is between 0.5 and 1.2.

In a preferred embodiment, at least 30% of the serotype 33F 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
5 embodiment, at least 45%, 50%, 55%, 60%, 65% of the serotype 33F 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 serotype 33F immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 40% and 80% of the serotype 33F immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred
10 embodiment, between 45% and 65% of the serotype 33F immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

In a preferred embodiment, the degree of conjugation of the immunogenic conjugate is between 1 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
15 13, 3 and 10, 3 and 8, 3 and 6, 3 and 5, 3 and 4, 5 and 15, 5 and 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 3 and 6.

Immunogenic composition

20 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),
25 such as a bacterial capsular polysaccharide, or a glycoconjugate 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 a preferred embodiment, the immunogenic composition comprises a serotype 10A, 22F or
30 33F conjugate obtained by a process disclosed herein. In a preferred embodiment, the immunogenic composition comprising a serotype 10A, 22F or 33F conjugate obtainable by a process disclosed herein.

In an embodiment, the immunogenic composition disclosed herein, when administered to a
35 subject, induces the formation of antibodies capable of binding to serotype 10A *Streptococcus pneumoniae*. In an embodiment, the immunogenic composition disclosed

herein, when administered to a subject, induces the formation of antibodies capable of binding to serotype 10A *Streptococcus pneumonia* as measured by a standard ELISA assay.

5 In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of killing serotype 10A *Streptococcus pneumonia* in an opsonophagocytosis assay as disclosed herein.

10 In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of binding to serotype 22F *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 22F *Streptococcus pneumonia* as measured by a standard ELISA assay.

15 In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of killing serotype 22F *Streptococcus pneumonia* in an opsonophagocytosis assay as disclosed herein.

20 In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of binding to serotype 33F *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 33F *Streptococcus pneumonia* as measured by a standard ELISA assay.

25 In an embodiment, the immunogenic composition disclosed herein, when administered to a subject, induces the formation of antibodies capable of killing serotype 33F *Streptococcus pneumonia* in an opsonophagocytosis assay as disclosed herein.

30 Formulation of the immunogenic composition of the present invention can be accomplished using art-recognized methods. For instance, the serotype 10A, 22F or 33F conjugates 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.

35 In a preferred embodiment, the immunogenic composition may comprise at least one additional antigen. In a preferred embodiment, 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 CRM₁₉₇.

- 5 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:
- 10 (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,
- 15 (a) MF59 (PCT Pub. No. WO 90/14837), containing 5% Squalene, 0.5% 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 110Y microfluidizer (Microfluidics, Newton, MA),
- (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and
- 20 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-deacylated monophosphorylipid A (MPL™) described in U.S. Patent No. 4,912,094 (Corixa),
- 25 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) bacterial lipopolysaccharides, synthetic lipid A analogs such as aminoalkyl glucosamine
- 30 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-O-phosphono-3-O-[(R)-3-tetradecanoyloxytetradecanoyl]-2-[(R)-3-tetradecanoyloxytetradecanoylamino]-β-D-glucopyranoside, which is also known as 529 (formerly known as RC529), which is formulated
- 35 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.;

5 (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
10 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
15 (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
20 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
25 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,
30 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;
35 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' X₁X₂CGX₃X₄ 3', wherein X₁, X₂, X₃, and X₄ are nucleotides. In one embodiment, X₂ is adenine, guanine, or thymine. In another embodiment, X₃ 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*C*G*G*T*G*C*T*T*T 3' (SEQ ID NO: 8), or

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

5' T*C*G*T*C*G*T*T*T*T*G*T*C*G*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*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*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' TCGCGTCGTTTCGGCGCGGCCG 3' (SEQ ID NO: 13), or

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

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

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

5' TCGCGTCGTTTCGGCGCGGCCG 3' (SEQ ID NO: 17), or

5' TCGACGTTTCGGCGCGGCCG 3' (SEQ ID NO: 18), or

5' TCGACGTTTCGGCGCGGCCG 3' (SEQ ID NO: 19), or

5' TCGCGTCGTTTCGGCGGCCG 3' (SEQ ID NO: 20), or

5' TCGCGACGTTTCGGCGCGGCCG 3' (SEQ ID NO: 21), or

5' TCGTCGTTTTTCGGCGCGGCCG 3' (SEQ ID NO: 22), or

5' TCGTCGTTTTTCGGCGGCCGCCG 3' (SEQ ID NO: 23), or

5' TCGTCGTTTTACGGCGCCGTGCCG 3' (SEQ ID NO: 24), or

5' TCGTCGTTTTTCGGCGCGGCCGT 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*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*G*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*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.

- 10 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
 15 oligonucleotide includes at least one YpR dinucleotide. In an embodiment, said oligonucleotide 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,
 20 TTTCG, TTTTCG, 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).

- 25 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.

- 30 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
 35 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 a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include carriers approved by a regulatory agency of a Federal, a state government, or other regulatory agency, or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, including humans as well as non-human mammals. The term carrier may be used to refer to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Water, saline solutions and aqueous dextrose and glycerol solutions can be employed as liquid carriers, particularly for injectable solutions. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. The formulation should suit the mode of administration.

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), with preferred components 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 10A *Streptococcus pneumoniae*, or a method of preventing infection with serotype 10A *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 10A *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 10A *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 10A *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 10A *Streptococcus pneumoniae* and/or preventing infection with serotype 10A *Streptococcus pneumoniae*, and/or reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by serotype 10A *Streptococcus pneumoniae*.

One embodiment of the disclosure provides a method of protecting a subject against an infection with serotype 22F *Streptococcus pneumoniae*, or a method of preventing infection with serotype 22F *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 22F *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 22F *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 22F *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 22F *Streptococcus pneumoniae* and/or preventing infection with serotype 22F *Streptococcus pneumoniae*, and/or reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by serotype 22F *Streptococcus pneumoniae*.

One embodiment of the disclosure provides a method of protecting a subject against an infection with serotype 33F *Streptococcus pneumoniae*, or a method of preventing infection with serotype 33F *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 33F *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 33F *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 33F *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 33F *Streptococcus pneumoniae* and/or preventing infection with serotype 33F *Streptococcus pneumoniae*, and/or reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by serotype 33F *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 10A *Streptococcus pneumoniae* infection. 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 22F *Streptococcus pneumoniae* infection. 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 33F

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.

5 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.

10 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.

15 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
20 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
25 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
30 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 5×10^9 cells per liter, or below 4×10^9 cells per liter, or below 3×10^9 cells per liter, or below 2×10^9 cells per liter, or below 1×10^9 cells per liter, or below 0.5×10^9 cells per liter, or below 0.3×10^9 cells per liter, or below 0.1×10^9 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 $4.3 - 10.8 \times 10^9$ cells per liter.

5 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 2×10^9 cells per liter, or below 1×10^9 cells per liter, or below 0.5×10^9 cells per liter, or below 0.1×10^9 cells per liter, or below 0.05×10^9 cells per liter.

10 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.

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

CD4 cell tests are normally reported as the number of cells in mm^3 . Normal CD4 counts are
20 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.

25 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 mcg of conjugated polysaccharide and about 20 mcg of non-conjugated polysaccharide in a 100 mcg polysaccharide dose. The protein contribution to the conjugate is usually not considered
30 when calculating the dose of a conjugate. Generally, each dose will comprise 0.1 to 100 mcg of polysaccharide, particularly 0.1 to 10 mcg, and more particularly 1 to 10 mcg 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.

5

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 glycoconjugates of the present disclosure. In some embodiments, antibodies are generated upon administration to a subject of the capsular polysaccharides or glycoconjugates of the present disclosure. In some embodiments, the disclosure provides purified or isolated antibodies directed against one or more of the capsular polysaccharides or glycoconjugates 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

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Example 1: preparation of serotype 22F polysaccharide – CRM₁₉₇ conjugate

1.1. Preparation of isolated *Streptococcus pneumoniae* serotype 22F polysaccharide

Serotype 22F 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 22F *Streptococcus pneumoniae* 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 broth was inactivated by the addition of N-lauroyl sarcosine and purified by ultrafiltration and diafiltration.

The purified *Streptococcus pneumoniae* serotype 22F polysaccharide was sized by high pressure homogenization using a PANDA 2K homogenizer ® (GEA Niro Soavi) to produce the isolated *Streptococcus pneumoniae* serotype 22F polysaccharide.

1.2. Oxidation of Isolated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide

Oxidation of Polysaccharide was carried out in 100 mM potassium phosphate buffer (pH 5.8 ± 0.2) obtained by sequential addition of calculated amount of 500 mM potassium phosphate buffer (pH 5.8) and WFI to give final polysaccharide concentration of 2.0 g/L. If required, the reaction pH was adjusted to 5.8, approximately. After pH adjustment, the reaction temperature was lowered to 5 ± 3 °C. Oxidation was initiated by the addition of 0.10 ± 0.02 molar equivalents (MEq) of sodium periodate. The target oxidation reaction time is 16 ± 1 hrs at 5 ± 3 °C.

The oxidation reaction was quenched with 2 MEq of 2,3-butanediol under continuous stirring at 5 ± 3 °C for 1-2 hrs.

Concentration and diafiltration of the activated polysaccharide was carried out using 100K MWCO ultrafiltration cassettes. Diafiltration was performed against 35-fold diavolume of WFI. The purified activated polysaccharide was stored at 5 ± 3°C. The purified activated

saccharide is characterized inter alia by (i) Molecular Weight by SEC-MALLS (ii) presence of O-acetyl and (iii) Degree of Oxidation.

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, such as for example by using Anthrone method. 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.

1.3. Conjugation an activated *Streptococcus pneumoniae* serotype 22F polysaccharide with CRM₁₉₇

The conjugation process consists of the following steps:

- a. Compounding with sucrose excipient, and lyophilization.
- b. Reconstitution of the lyophilized polysaccharide and CRM₁₉₇.
- c. Conjugation of activated polysaccharide to CRM₁₉₇ and capping
- d. Purification of the conjugate

- a. Compounding with Sucrose and Lyophilization

The activated polysaccharide was compounded with sucrose (50% w/v in WFI) 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 $-20 \pm 5^{\circ}\text{C}$. Calculated amount of CRM₁₉₇ protein (target S/P input ratio = 1) was shelffrozen and lyophilized separately. Lyophilized CRM₁₉₇ was stored at $-20 \pm 5^{\circ}\text{C}$.

b. Reconstitution of lyophilized activated polysaccharide and CRM₁₉₇ 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 CRM₁₉₇ for reconstitution.

c. Conjugation of activated polysaccharide to CRM₁₉₇ and capping

Reconstituted CRM₁₉₇ (in DMSO) was combined in the conjugation reaction vessel with the reconstituted activated polysaccharide. The final polysaccharide concentration in reaction solution is 1 g/L. Conjugation was initiated by adding 1.5 ± 0.1 MEq of sodium cyanoborohydride to the reaction mixture and the reaction was incubated at $23 \pm 2^{\circ}\text{C}$ for 20 ± 2 hrs. Termination of conjugation reaction is done by adding 2 MEq of sodium borohydride. The capping reaction was incubated at $23 \pm 2^{\circ}\text{C}$ for 3 ± 1 hrs.

d. Purification of Conjugate

The conjugate solution was diluted 1:5 with chilled 5 mM succinate-0.9% saline (pH 6.0) in preparation for purification by tangential flow filtration using 100K MWCO membranes and a 20X diafiltration was performed using 5 mM succinate-0.9% saline (pH6.0) as the medium. After the diafiltration was completed, the conjugate retentate was further diluted, filtered through a 0.22 μm filter and stored at $2-8^{\circ}\text{C}$.

Table 1 comprises characterizing data for serotype 22F polysaccharide-CRM₁₉₇ conjugates obtained by the method of the invention. In particular, conjugates 5 and 6 of the table were obtained as disclosed in example 1.

Serotype 22F polysaccharide-CRM₁₉₇ conjugates generated by the RAC-DMSO process provided better yields and exhibited better batch to batch consistency in the MW along with significantly lower %Free saccharide levels and higher carrier protein (Lysine) modification compared to the conjugates generated by the RAC-aqueous process, as shown in Table 1.

Table 1 - Serotype 22F Conjugates						
Conjugate No	1	2	3	4	5	6
	Aqueous	Aqueous	Aqueous	DMSO	DMSO	DMSO
Act Poly MW, kDa	488	803	410	639	709	416
DO	17.3	15	10.3	14.6	14.4	13.7
Input Ratio	0.8	0.8	1	0.8	1	1
% Conjugate Yield	66	63	46	70	62	75
%Free Saccharide	18	43	45	< 5	< 5	8
Conjugate MW	1024	3544	462	3911	3734	4453
Saccharide-Protein Ratio	1.6	1.1	1.45	0.8	0.65	1.0
Modified Lys (AAA)	2.3	N/A	N/A	6.1	N/A	4.7

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

Example 2: preparation of serotype 10A polysaccharide – CRM₁₉₇ conjugate

5

2.1. Preparation of isolated *Streptococcus pneumoniae* serotype 10A polysaccharide

Isolated Serotype 10A polysaccharides was obtained as disclosed in example 1.1 except that the purified polysaccharide was not sized.

10 2.2. Oxidation of Isolated *Streptococcus pneumoniae* serotype 10A capsular polysaccharide

A calculated volume of 0.1M potassium phosphate buffer (pH 6.0) and water-for-injection (WFI) was added to the polysaccharide solution to achieve a final polysaccharide concentration of 2.5g/L and a final concentration of 25mM potassium phosphate buffer, If required pH was adjusted to 6.0, approximately. The diluted polysaccharide was then cooled to 5 ± 3°C. Oxidation was initiated by the addition of 0.25± 0.02 molar equivalents (MEq) of sodium periodate solution. The oxidation reaction time was approximately 4 hrs at 5 ± 3°C. The oxidation reaction was quenched with 1 MEq of 2,3-butanediol under continuous stirring at 5 ± 3 °C for 1-2 hrs.

20 After reaching the target reaction time, the activated polysaccharide was concentrated using 30K MWCO Millipore ultrafiltration cassettes. The diafiltration was then performed against 20-fold diavolume of WFI. The purified activated polysaccharide was stored at 5 ± 3°C. The

purified activated saccharide is characterized inter alia by (i) Molecular Weight by SEC-MALLS and (ii) Degree of Oxidation.

2.3 Conjugation an activated *Streptococcus pneumoniae* serotype 10A polysaccharide with CRM₁₉₇

The conjugation process consists of the following steps:

- a. Compounding with sucrose excipient, and lyophilization.
- b. Reconstitution of the lyophilized polysaccharide and CRM197.
- c. Conjugation of activated polysaccharide to CRM197 and capping
- d. Purification of the conjugate

a. Compounding with Sucrose

The activated polysaccharide is compounded with sucrose to a ratio of 25 ± 2.5 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 $-20 \pm 5^\circ\text{C}$.

b. Reconstitution of Lyophilized Activated Polysaccharide and CRM₁₉₇ Protein

Lyophilized activated polysaccharide was reconstituted in anhydrous dimethyl sulfoxide (DMSO). Upon complete dissolution of polysaccharide, the same amount of anhydrous DMSO was added to the calculated CRM₁₉₇ for reconstitution.

c. Conjugation of Activated Polysaccharide to CRM₁₉₇ and Capping

Reconstituted CRM₁₉₇ (in DMSO) was added to the reconstituted activated polysaccharide in the conjugation reactor. The final polysaccharide concentration is 1 g/L. Conjugation was performed by adding 1.2 ± 0.1 MEq of sodium cyanoborohydride to the reaction mixture. The reaction was incubated and at $23 \pm 2^\circ\text{C}$ for 24 ± 2 hrs. Termination of conjugation reaction is done by adding 2 MEq of sodium borohydride. The capping reaction was incubated at $23 \pm 2^\circ\text{C}$ for 3 ± 1 hrs.

Termination of conjugation reaction is done by adding 2 MEq of sodium borohydride. This capping reaction proceeded for 3 ± 1 hrs at $23 \pm 2^\circ\text{C}$.

d. Purification of Conjugate

The conjugate solution was then diluted into 5x (by volume) chilled 5 mM succinate-0.9% saline (pH 6.0 and a 20X diafiltration was performed using 5 mM succinate-0.9% saline (pH6.0). After the initial 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), and after the final 0.22µm filtration step it was stored at 2-8 °C.

Table 2 comprises characterizing data for serotype 10A polysaccharide-CRM₁₉₇ conjugates obtained by the method of the invention. In particular, conjugates 4 to 6 of table 2 were obtained as disclosed in example 2.

Serotype 10A polysaccharide-CRM₁₉₇ conjugates generated by the RAC-DMSO process provided better yields and exhibited better batch to batch consistency in the MW along with significantly lower %Free saccharide levels and higher carrier protein (Lysine) modification compared to the conjugates generated by the RAC-aqueous process, as shown in Table 2.

Table 2. Serotype 10A Conjugates						
Conjugate No	1	2	3	4	5	6
	Aqueous	Aqueous	Aqueous	DMSO	DMSO	DMSO
DO	12.2	12.2	12.8	10.3	10.8	10.5
Activated Saccharide MW, kda	157	157	163	170	170	170
Input Ratio	1.0	1.0	1.0	1.1	1.1	1.1
%Yield	71	34	50	82	73	66
%Free Saccharide	20	31	26	6.8	6.4	9.7
Conjugate MW, kDa	1550	968	3050	4034	3463	5540
Saccharide-protein ratio	1.6	2.6	1.6	1.1	1.2	1.0
Lys modification AAA	2.6	N/A	N/A	6.9	6.7	6.1

Example 3: preparation of serotype 33F polysaccharide – CRM₁₉₇ conjugate

3.1. Preparation of isolated *Streptococcus pneumoniae* serotype 33F polysaccharide

Isolated Serotype 33F polysaccharide was obtained as disclosed in example 1.1.

3.2. Oxidation of Isolated *Streptococcus pneumoniae* serotype 33F capsular polysaccharide

A calculated volume of 0.1M sodium phosphate buffer (pH 6.0) and water-for-injection (WFI) was added to the polysaccharide solution to achieve a final polysaccharide concentration of 2g/L. If required pH was adjusted to 6.0, approximately. The diluted polysaccharide was then cooled to $5 \pm 3^\circ\text{C}$. Oxidation was initiated by the addition of 0.1 molar equivalent (MEq) of sodium periodate solution. The oxidation reaction time was approximately 20 hrs at $5 \pm 3^\circ\text{C}$. The oxidation reaction was quenched with 1 MEq of 2,3-butanediol under continuous stirring at $5 \pm 3^\circ\text{C}$ for about 1 hour. After reaching the target reaction time, the activated polysaccharide was concentrated using 100K MWCO Millipore ultrafiltration cassettes. The diafiltration was then performed against 40-fold diavolume of WFI. The purified activated polysaccharide was stored at $5 \pm 3^\circ\text{C}$. The purified activated saccharide is characterized inter alia by (i) Molecular Weight by SEC-MALLS and (ii) Degree of Oxidation.

3.3 Conjugation an activated *Streptococcus pneumoniae* serotype 33F polysaccharide with CRM₁₉₇

The serotype 33F polysaccharide-CRM197 conjugate was obtained by a process similar to the process of example 1.3, using the activated 33F polysaccharide obtained in example 3.2.

Serotype 33F polysaccharide-CRM₁₉₇ conjugates generated by the RAC-DMSO process provided better yields and exhibited better batch to batch consistency in terms of preservation of O-Acetyl levels along with overall lower %Free saccharide levels and higher carrier protein (Lysine) modification compared to the conjugates generated by the RAC-aqueous process, as shown in Table 3.

Table 3 - Serotype 33F Conjugates						
Conjugate No	1	2	3	4	5	6
	Aq	Aq	Aq	DMSO	DMSO	DMSO
DO	15.2	15.5	15.7	17	9	14
Activated Poly MW	1126	573	574	120	328	128
%Yield	65	51	48	78	86	66
%Free Saccharide	8.5	19	13	18	5	< 5
Conjugate MW, kDa	1341	2137	3674	3158	2165	2001
Saccharide-protein ratio	1.7	1.4	1.3	1.1	1.1	0.79
% O-Ac preserved	< LOQ	< LOQ	< LOQ	82	100%	100%
Conj Lys (AAA)	N/A	2.3	2.3	4.5	3.4	5.2

LOQ = Limit of Quantitation

Example 4: effect of quenching on molecular weight of activated serotype 22F and 33F polysaccharides

5 Activation of 22F and 33F polysaccharides was carried out at either 4 °C or 22 °C. Both temperatures gave similar DO values. However, due to the fact that elevated temperature resulted in faster breakdown of activated polysaccharide thereby lowering activated poly MW (as shown when comparing for example figure 4 (22 °C) and figure 5 (4 °C)), 4 °C was generally preferred for activation reaction. In addition, activation data showed that quenching
10 of un-reacted NaIO₄ after oxidation is an essential step for activation, especially for activation using higher amount of NaIO₄. The graph shown in Figure 6 indicate that the MW of the activated 33F polysaccharide is relatively stable when increasing concentrations of periodate are used for activation (with quenching) while such MW is highly variable when no quenching step is used. At specific conditions set up to obtain a targeted degree of oxidation, the
15 molecular weight of the activated polysaccharide is less variable when the quenching step is used. Addition of quenching reagent after oxidation helps to keep the structural integrity of activated polysaccharide until the completion of purification for example by ultrafiltration and diafiltration.

20 **Example 5: Opsonophagocytic activity assay (OPA)**

The immunogenicity of the conjugates obtained by the processes disclosed herein can be assessed using the opsonophagocytic assay (OPA) described below.

25 Groups of thirty 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.

30 Validated opsonophagocytic activity (OPA) assays are used to measure functional antibodies in murine sera specific for *S. pneumonia* serotype 10A, 22F or 33F. 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
35 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.

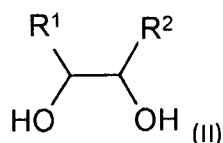
OPA procedures were based on methods described in Hu et al., Clin Diagn Lab Immunol 2005;12(February (2)):287–95 with the following modifications. Test serum was serially diluted 2.5-fold and added to microtiter assay plates. Live serotype 10A, 22F and 33F target bacterial strains were added to the wells and the plates were shaken at either 25°C (serotype 22F) or 37°C (serotype 10A and 33F) for 30 minutes. Differentiated HL-60 cells (phagocytes) and baby rabbit serum (3- to 4-week old, Pel-Freez®, 12.5% final concentration) were added to the wells, and the plates were shaken at 37°C for 45 minutes (serotype 22F and 33F) or 60 minutes (serotype 10A). 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% CO₂ 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.

Serotype 10A polysaccharide-CRM₁₉₇ conjugates, Serotype 22F polysaccharide-CRM₁₉₇ conjugates and Serotype 33F polysaccharide-CRM₁₉₇ conjugates obtained as disclosed in examples 1 to 3 were tested in the OPA assay disclosed above and were found to be immunogenic.

Claims

1. A process for preparing an activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F capsular polysaccharide, the process comprising the steps of:
- 5 (a) reacting an isolated serotype 10A, 22F or 33F capsular polysaccharide with an oxidizing agent; and
- (b) quenching the oxidation reaction by addition of a quenching agent resulting in an activated *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharide.
- 10 2. A process according to claim 1 wherein the concentration of serotype 10A, 22F or 33F polysaccharide in step (a) is between 0.5 and 5 mg/mL.
3. A process according to claim 1 or 2 wherein said 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, 0.01 to 0.2, 0.05 to 0.5, 0.05 to 0.2, 0.1 to 0.3 molar equivalents of oxidizing agent in step (a).
- 15 4. A process according to any one of claims 1 to 3 wherein said polysaccharide is reacted with 0.05 to 0.3 molar equivalents of oxidizing agent in step (a).
- 20 5. A process according to any one of claims 1 to 4 where the oxidizing agent is periodate.
6. A process according to any one of claims 1 to 4 where the oxidizing agent is sodium metaperiodate.
- 25 7. A process according to any one of claims 1 to 6 wherein the temperature of the reaction in step (a) is maintained between 1 and 10°C.
8. A process according to any one of claims 1 to 7 wherein the temperature of the reaction in step (a) is about °C or about 5°C.
- 30 9. A process according to any one of claims 1 to 8 wherein the duration of step (a) is between 1 and 25 hours.
- 35 10. A process according to any one of claims 1 to 9 where the quenching agent is selected from vicinal diols, 1,2-aminoalcohols, amino acids, glutathione, sulfite, bisulfate, dithionite, metabisulfite, thiosulfate, phosphites, hypophosphites or phosphorous acid.

11. A process according to any one of claims 1 to 9 where the quenching agent is a compound of formula (II)



5 wherein R¹ and R² are each independently selected from H, methyl, ethyl, propyl or isopropyl.

12. A process according to any one of claims 1 to 9 where the quenching agent is selected from glycerol, ethylene glycol, propan-1,2-diol, butan-1,2-diol or butan-2,3-diol.

10 13. A process according to any one of claims 1 to 9 where the quenching agent is butan-2,3-diol.

14. A process according to any one of claims 1 to 13 wherein the oxidation reaction is quenched by the addition of 0.1 to 10, 0.5 to 5, 0.5 to 3 or 0.5 to 2 molar equivalents of
15 quenching agent.

15. A process according to any one of claims 1 to 13 wherein the oxidation reaction is quenched by the addition of 0.5 to 2 molar equivalents of quenching agent.

20 16. A process according to any one of claims 1 to 15 wherein the temperature of the reaction in step (b) is maintained between 1 and 10°C.

17. A process according to any one of claims 1 to 16 wherein the temperature of the reaction in step (b) is about 4°C.

25 18. A process according to any one of claims 1 to 17 wherein the duration of step (b) is between 0.5 and 2 hours.

19. A process according to any one of claims 1 to 18 wherein the activated polysaccharide is
30 purified after step (b).

20. A process according to any one of claims 1 to 19 wherein said isolated polysaccharide is an isolated *Streptococcus pneumoniae* serotype 10A polysaccharide.

21. A process according to claim 20 wherein said isolated *Streptococcus pneumoniae* serotype 10A polysaccharide is obtained by a process comprising the steps of:

(a) preparing a fermentation culture of serotype 10A *Streptococcus pneumoniae* bacterial cells;

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

(c) purifying serotype 10A polysaccharide from the fermentation culture.

22. An activated *Streptococcus pneumoniae* serotype 10A capsular polysaccharide obtained by a process of any one of claims 1 to 21.

23. An activated *Streptococcus pneumoniae* serotype 10A capsular polysaccharide obtainable by a process of any one of claims 1 to 21.

24. An activated *Streptococcus pneumoniae* serotype 10A capsular polysaccharide according to any one of claims 22 or 23, where said polysaccharide has a molecular weight between 50 and 400, 50 and 350, 50 and 300, 50 and 250, 50 and 200, 100 and 300, 100 and 250, or 100 and 200kDa.

25. An activated *Streptococcus pneumoniae* serotype 10A capsular polysaccharide according to any one of claims 22 or 23, where said polysaccharide has a molecular weight between 50 and 300kDa.

26. An activated *Streptococcus pneumoniae* serotype 10A capsular polysaccharide according to any one of claims 22 or 23, where said polysaccharide has a molecular weight between 100 and 200kDa.

27. An activated *Streptococcus pneumoniae* serotype 10A capsular polysaccharide according to any one of claims 22 to 26, where said polysaccharide has a degree of oxidation between 5 and 20, 5 and 15, 8 and 14, 8 and 12, or 9 and 11.

28. An activated *Streptococcus pneumoniae* serotype 10A capsular polysaccharide according to any one of claims 22 to 26, where said polysaccharide has a degree of oxidation between 9 and 11.

29. A process according to any one of claims 1 to 19 wherein said isolated polysaccharide is an isolated *Streptococcus pneumoniae* serotype 22F polysaccharide.

30. A process according to claim 29 wherein said isolated *Streptococcus pneumoniae* serotype 22F polysaccharide is obtained by a process comprising the steps of:

(a) preparing a fermentation culture of serotype 22F *Streptococcus pneumoniae* bacterial cells;

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

(c) purifying serotype 22F polysaccharide from the fermentation culture; and

(d) sizing the serotype 22F polysaccharide by mechanical sizing, preferably by high pressure homogenization.

10 31. An activated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide obtained by a process of any one of claims 1 to 19 or 29 or 30.

32. An activated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide obtainable by a process of any one of claims 1 to 19 or 29 or 30.

15

33. An activated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide according to any one of claims 31 or 32, where said polysaccharide has a molecular weight between 25 and 1000, 100 and 1000, 300 and 800, 300 and 700, 300 and 600, 400 and 1000, 400 and 800, 400 and 700, or 400 and 600kDa.

20

34. An activated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide according to any one of claims 31 or 32, where said polysaccharide has a molecular weight between 300 and 800kDa.

25 35. An activated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide according to any one of claims 31 or 32, where said polysaccharide has a molecular weight between 400 and 600kDa.

36. An activated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide
30 according to any one of claims 31 to 35, where said polysaccharide has a degree of oxidation between 10 and 25, 10 and 20, 12 and 20, or 14 and 18.

37. An activated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide according to any one of claims 31 to 35, where said polysaccharide has a degree of
35 oxidation between 10 and 20.

38. An activated *Streptococcus pneumoniae* serotype 22F capsular polysaccharide according to any one of claims 31 to 37, where said polysaccharide comprises at least 0.6 mM acetate per mM serotype 22F polysaccharide.

5 39. A process according to any one of claims 1 to 19 wherein said isolated polysaccharide is an isolated *Streptococcus pneumoniae* serotype 33F polysaccharide.

40. A process according to claim 39 wherein said isolated *Streptococcus pneumoniae* serotype 33F polysaccharide is obtained by a process comprising the steps of:

- 10 (a) preparing a fermentation culture of serotype 33F *Streptococcus pneumoniae* bacterial cells;
(b) lysing the bacterial cells in said fermentation culture;
(c) purifying serotype 33F polysaccharide from the fermentation culture; and
(d) sizing the serotype 33F polysaccharide by mechanical sizing, preferably by high pressure
15 homogenization.

41. An activated *Streptococcus pneumoniae* serotype 33F capsular polysaccharide obtained by a process of any one of claims 1 to 19 or 39 or 40.

20 42. An activated *Streptococcus pneumoniae* serotype 33F capsular polysaccharide obtainable by a process of any one of claims 1 to 19 or 39 or 40.

43. An activated *Streptococcus pneumoniae* serotype 33F capsular polysaccharide according to claim 39 or 40, where said polysaccharide has a molecular weight between 50
25 and 1250, 200 and 1200, 500 and 1200, 500 and 1000, 700 and 1200, 800 and 1200, 800 and 1100, 900 and 1200, or 800 and 1000Da.

44. An activated *Streptococcus pneumoniae* serotype 33F capsular polysaccharide according to claim 39 or 40, where said polysaccharide has a molecular weight between 500
30 and 1000kDa.

45. An activated *Streptococcus pneumoniae* serotype 33F capsular polysaccharide according to claim 39 or 40, where said polysaccharide has a molecular weight between 50
and 400kDa.

35

46. An activated *Streptococcus pneumoniae* serotype 33F capsular polysaccharide according to any one of claims 41 to 45, where said polysaccharide has a degree of oxidation between 10 and 20.

5 47. An activated *Streptococcus pneumoniae* serotype 33F capsular polysaccharide according to any one of claims 41 to 34, where said polysaccharide comprises at least 0.7 mM acetate per mM serotype 33F polysaccharide.

10 48. A process according to any one of claims 1 to 47 wherein the activated polysaccharide is lyophilized after step (b).

49. A process for the preparation of an immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 10A, 22F or 33F polysaccharide covalently linked to a carrier protein, the process comprising the steps of:

15 (a) compounding an activated serotype 10A, 22F or 33F polysaccharide obtained or obtainable by a process of any one of claims 1 to 19 with a carrier protein; and,
(b) reacting the compounded, activated serotype 10A, 22F or 33F polysaccharide and carrier protein with a reducing agent to form a serotype 10A, 22F or 33F polysaccharide:carrier protein conjugate.

20

50. A process according to claim 49, said process further comprising the step of:

(c) capping unreacted aldehydes in the serotype 10A, 22F or 33F polysaccharide:carrier protein conjugate.

25 51. A process according to claim 49 or 50 where step (a) and (b) are carried out in DMSO.

52. A process according to claim 49 or 50 where step (a) and (b) are carried out in an aqueous solution.

30 53. A process according to anyone of claims 49 to 52 where the activated polysaccharide and the protein carrier are co-lyophilized after step (a).

54. A process according to claim 53 where step (b) is carried out in DMSO.

35 55. A process according to claim 53 where step (b) is carried out in an aqueous solution.

56. A process according to anyone of claims 49 to 55 where the concentration of activated 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.

5 57. A process according to anyone of claims 49 to 55 where the concentration of activated polysaccharide in step (b) is between 0.5 and 2 mg/mL.

58. A process according to anyone of claims 49 to 57 where the initial ratio of activated serotype 10A, 22F or 33F polysaccharide to carrier protein is between 5:1 and 0.1:1, 2:1 and 10 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, or 0.6:1 and 1.2:1.

59. A process according to anyone of claims 49 to 57 where the initial ratio of activated serotype 10A, 22F or 33F polysaccharide to carrier protein is between 0.6:1 and 1.2:1.

15 60. A process according to anyone of claims 49 to 59 where, in step (b), the activated polysaccharide is reacted with between 0.5 to 2.5 molar equivalents of reducing agent.

61. A process according to anyone of claims 49 to 60 where the reducing agent is sodium cyanoborohydride.

20

62. A process according to anyone of claims 49 to 61 wherein the duration of step (d) is between 20 and 30 hours.

63. A process according to any one of claims 49 to 62 wherein the temperature of the 25 reaction in step d is maintained between 20 and 26°C.

64. A process according to claim 53 where the unreacted aldehydes are capped by the addition of about 2 molar equivalents of NaBH₄.

30 65. A process according to any one of claims 49 to 64 further comprising a step of purifying the polysaccharide:carrier protein conjugate.

66. A process according to any one of claims 49 to 65 further comprising a step of purifying the polysaccharide:carrier protein conjugate by tangential flow filtration and diafiltration.

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67. An immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 10A polysaccharide covalently linked to a carrier protein obtained by the process of any one of claims 49 to 66.

5 68. An immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 10A polysaccharide covalently linked to a carrier protein obtainable by the process of any one of claims 49 to 66.

69. An immunogenic conjugate according to claim 67 or 68, where said conjugate has a
10 molecular weight between 500 and 15000; 500 and 10000; 2000 and 10000; or 3000 and 8000kDa.

70. An immunogenic conjugate according to claim 67 or 68, where said conjugate has a molecular weight between 3000 and 8000kDa.

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71. An immunogenic conjugate according to any one of claims 67 to 70, where said conjugate comprises less than about 50, 45, 40, 35, 30, 25, 20 or 15% of free serotype 10A polysaccharide compared to the total amount of serotype 10A polysaccharide.

20 72. An immunogenic conjugate according to any one of claims 67 to 70, where said conjugate comprises less than about 20% of free serotype 10A polysaccharide compared to the total amount of serotype 10A polysaccharide.

73. An immunogenic conjugate according to any one of claims 67 to 72, where the ratio of
25 serotype 10A polysaccharide to carrier protein in the conjugate is between 0.5 and 3.

74. An immunogenic conjugate according to any one of claims 67 to 72, where the ratio of serotype 10A polysaccharide to carrier protein in the conjugate is between 0.8 and 1.4.

30 75. An immunogenic conjugate according to any one of claims 67 to 74, where at least 40% of said immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

76. An immunogenic conjugate according to any one of claims 67 to 74, where between 50% and 80% of the serotype 10A immunogenic conjugate has a Kd below or equal to 0.3 in a
35 CL-4B column.

77. An immunogenic conjugate according to any one of claims 67 to 76, where the degree of conjugation of said 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 and 10, 8 and 15, 8 and 12, 10 and 15, or 10 and 12.

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78. An immunogenic conjugate according to any one of claims 67 to 76, where the degree of conjugation of said immunogenic conjugate is between 6 and 8.

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79. An immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 22F polysaccharide covalently linked to a carrier protein obtained by the process of any one of claims 49 to 66.

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80. An immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 22F polysaccharide covalently linked to a carrier protein obtainable by the process of any one of claims 49 to 66.

81. An immunogenic conjugate according to claim 79 or 80, where said conjugate comprises at least 0.5, 0.6 or 0.7 mM acetate per mM serotype 22F polysaccharide.

20

82. An immunogenic conjugate according to claim 79 or 80, where the ratio of mM acetate per mM serotype 22F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 22F polysaccharide in the isolated polysaccharide is at least 0.7.

25

83. An immunogenic conjugate according to any one of claims 79 to 82, where the ratio of mM acetate per mM serotype 22F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 22F polysaccharide in the activated polysaccharide is at least 0.7.

30

84. An immunogenic conjugate according to any one of claims 79 to 83, where said conjugate has a molecular weight between 400 and 15000; 500 and 10000; 2000 and 10000kDa; 3000 and 8000kDa; or 3000 and 5000kDa.

85. An immunogenic conjugate according to any one of claims 79 to 83, where said conjugate has a molecular weight between 3000 and 5000kDa.

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86. An immunogenic conjugate according to any one of claims 79 to 85, where said conjugate comprises less than about 50, 45, 40, 35, 30, 25, 20 or 15% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide.

87. An immunogenic conjugate according to any one of claims 79 to 83, where said conjugate comprises less than about 20% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide.

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88. An immunogenic conjugate according to any one of claims 79 to 87, where the ratio of serotype 22F polysaccharide to carrier protein in the conjugate is between 0.5 and 2,

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89. An immunogenic conjugate according to any one of claims 79 to 87, where the ratio of serotype 22F polysaccharide to carrier protein in the conjugate is between 0.8 and 1.2.

90. An immunogenic conjugate according to any one of claims 79 to 89, where at least 40% of said immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

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91. An immunogenic conjugate according to any one of claims 79 to 89, where between 50% and 80% of the serotype 22F immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

20

92. An immunogenic conjugate according to any one of claims 79 to 91, where the degree of conjugation of said 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, 4 and 7, 5 and 15, 5 and 10, 8 and 15, 8 and 12, 10 and 15, or 10 and 12.

25

93. An immunogenic conjugate according to any one of claims 79 to 91, where the degree of conjugation of said immunogenic conjugate is between 4 and 7.

30

94. An immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 33F polysaccharide covalently linked to a carrier protein obtained by the process of any one of claims 49 to 66.

95. An immunogenic conjugate comprising *Streptococcus pneumoniae* serotype 33F polysaccharide covalently linked to a carrier protein obtainable by the process of any one of claims 49 to 66.

35

96. An immunogenic conjugate according to claim 94 or 95, where said conjugate comprises at least 0.5, 0.6 or 0.7 mM acetate per mM serotype 33F polysaccharide.

97. An immunogenic conjugate according to claim 94 or 95, where the ratio of mM acetate per mM serotype 33F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 33F polysaccharide in the isolated polysaccharide is at least 0.7.

5 98. An immunogenic conjugate according to any one of claims 94 to 97, where the ratio of mM acetate per mM serotype 33F polysaccharide in the immunogenic conjugate to mM acetate per mM serotype 33F polysaccharide in the activated polysaccharide is at least 0.7.

10 99. An immunogenic conjugate according to any one of claims 94 to 98, where said conjugate has a molecular weight between 500 and 30000; 500 and 25000; 500 and 20000; 500 and 15000; 500 and 10000; 1000 and 10000; 1000 and 8000; 1000 and 5000; 2000 and 10000kDa; 2000 and 8000; or 2000 and 5000kDa.

15 100. An immunogenic conjugate according to any one of claims 94 to 98, where said conjugate has a molecular weight between 1000 and 5000kDa.

20 101. An immunogenic conjugate according to any one of claims 94 to 100, where said conjugate comprises less than about 50, 45, 40, 35, 30, 25, 20 or 15% of free serotype 33F polysaccharide compared to the total amount of serotype 33F polysaccharide.

102. An immunogenic conjugate according to any one of claims 94 to 100, where said conjugate comprises less than about 20% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide.

25 103. An immunogenic conjugate according to any one of claims 94 to 102, where the ratio of serotype 33F polysaccharide to carrier protein in the conjugate is between 0.5 and 2.

104. An immunogenic conjugate according to any one of claims 94 to 102, where the ratio of serotype 22F polysaccharide to carrier protein in the conjugate is between 0.5 and 1.5.

30 105. An immunogenic conjugate according to any one of claims 94 to 104, where at least 40% of said immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

35 106. An immunogenic conjugate according to any one of claims 94 to 104, where between 40% and 80% of the serotype 33F immunogenic conjugate has a Kd below or equal to 0.3 in a CL-4B column.

107. An immunogenic conjugate according to any one of claims 94 to 106, where the degree of conjugation of said 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 and 10, 8 and 15, 8 and 12, 10 and 15, or 10 and 12.

5

108. An immunogenic conjugate according to any one of claims 94 to 106, where the degree of conjugation of said immunogenic conjugate is between 4 and 6.

109. An immunogenic conjugate according to any one of claims 67 to 108 where the carrier protein is CRM₁₉₇.

10

110. A process according to any one of claims 49 to 66 where the carrier protein is CRM₁₉₇.

111. A process according to any one of claims 49 to 66 where the activated polysaccharide is of serotype 10A.

15

112. A process according to any one of claims 49 to 66 where the activated polysaccharide is of serotype 22F.

113. A process according to any one of claims 49 to 66 where the activated polysaccharide is of serotype 33F.

20

114. A process according to anyone of claims 49 to 66 and 110 to 113 wherein said process further comprises the step of formulating the conjugate in a multivalent vaccine.

25

115. An immunogenic composition comprising an immunogenic conjugate according to any one of claims 67 to 109 and a physiologically acceptable vehicle.

116. An immunogenic composition according to claim 115 further comprising at least one additional antigen.

30

117. An immunogenic composition according to claim 115 or 116 further comprising an adjuvant.

118. An immunogenic composition according to claim 117 where the adjuvant is aluminium phosphate.

35

119. A vaccine comprising an immunogenic composition according to any one of claims 115 to 118.

- 5 120. A method of treating or preventing a *Streptococcus pneumoniae* infection, disease or condition associated with serotype 10A, 22F or 33F *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 115 to 118, or the vaccine of claim 119.

FIG. 1

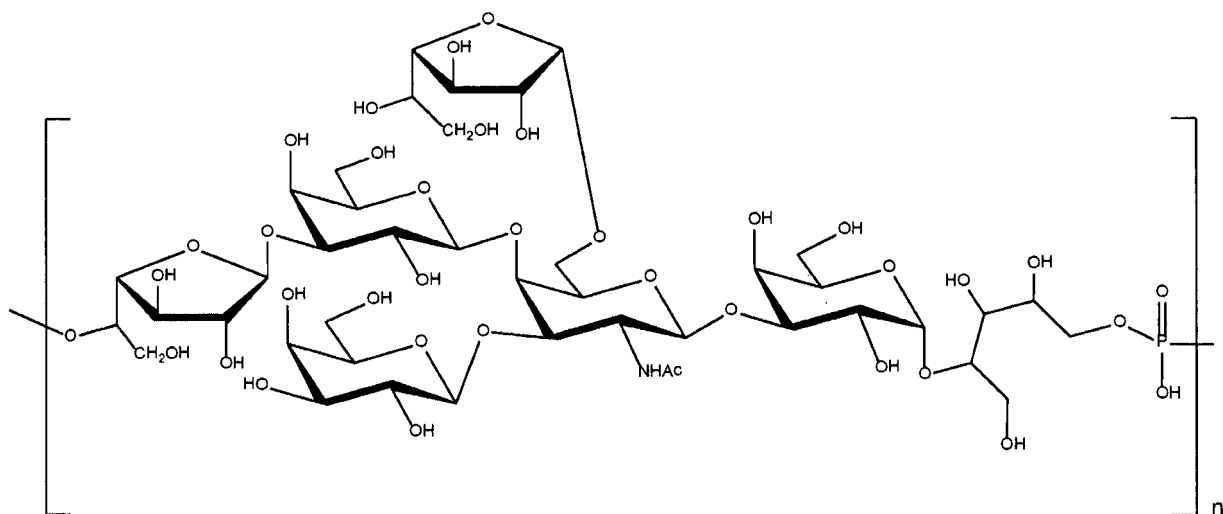


FIG. 2

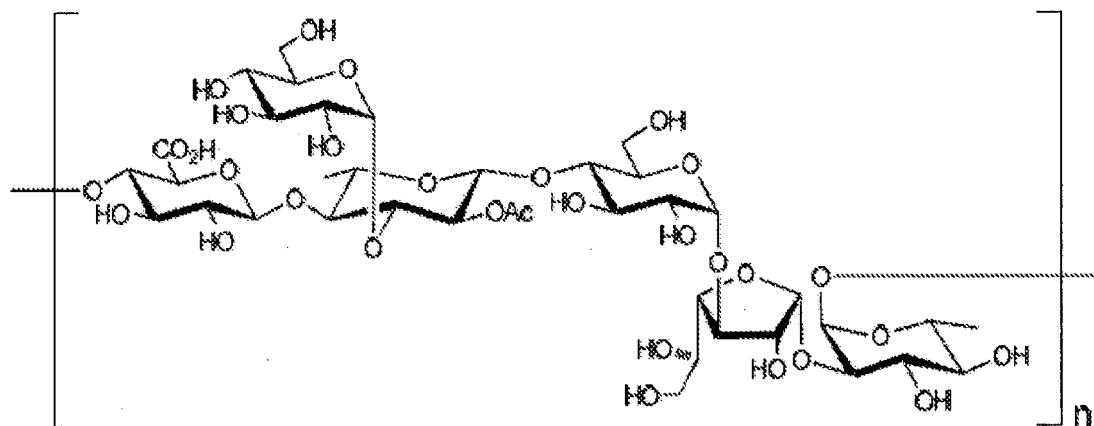


FIG. 3

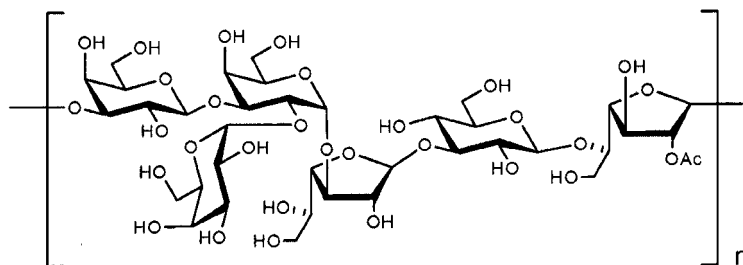


FIG. 4

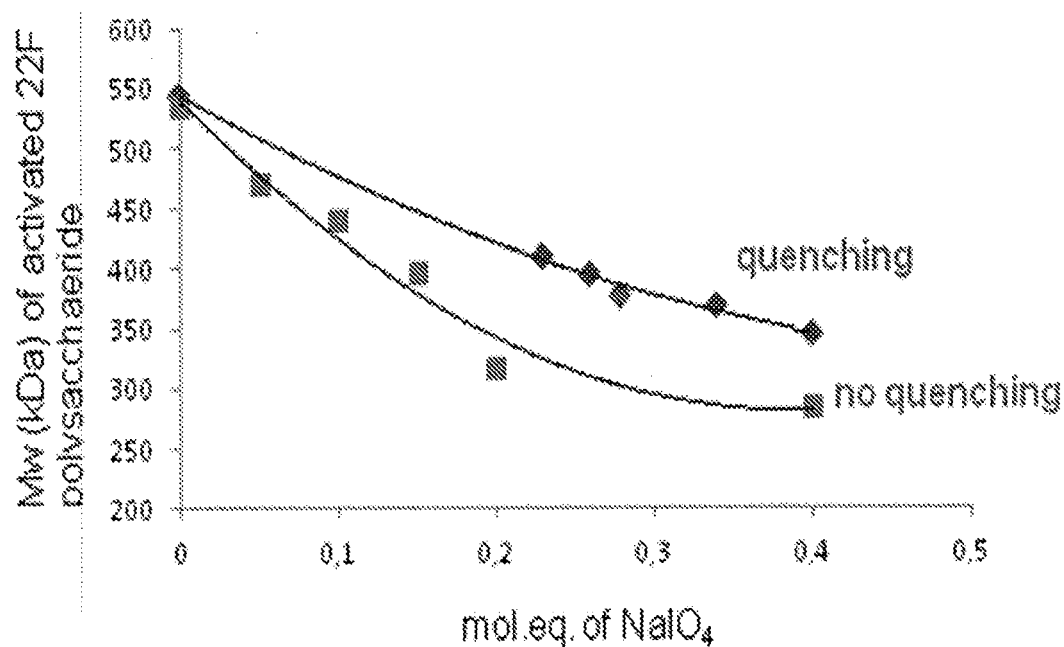


FIG. 5

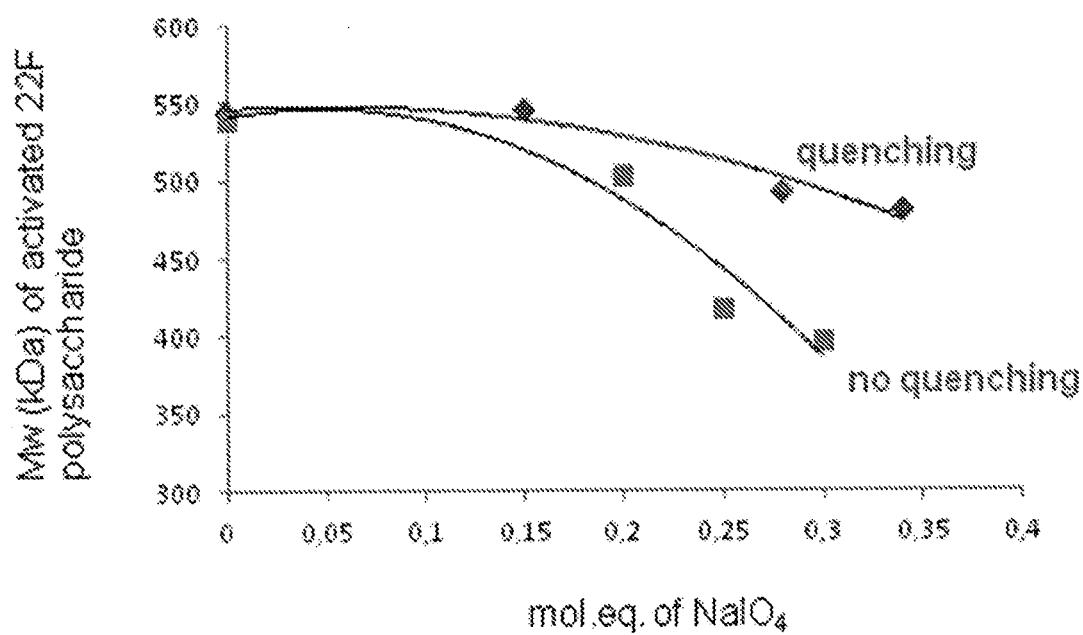
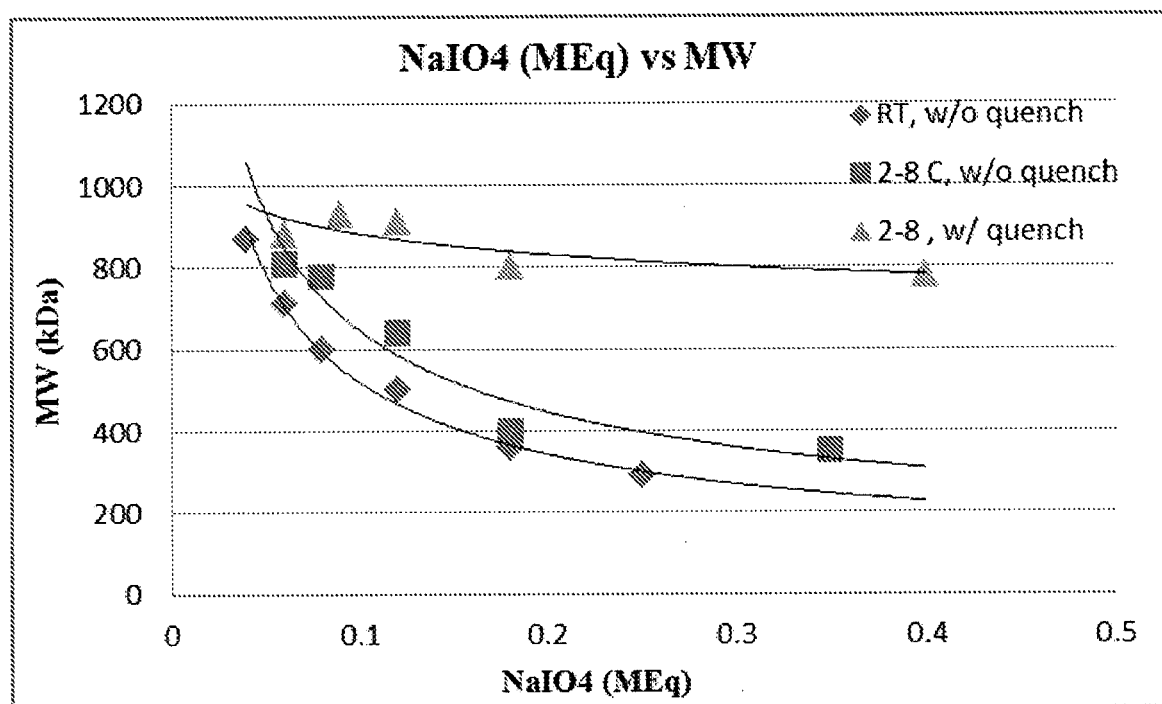


FIG. 6





(51) International Patent Classification:

A61K 39/02 (2006.01) A61K 39/09 (2006.01)
A61K 39/385 (2006.01) A61K 39/00 (2006.01)

(21) International Application Number:

PCT/IB2015/050314

(22) International Filing Date:

15 January 2015 (15.01.2015)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/929,598 21 January 2014 (21.01.2014) US

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,

DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

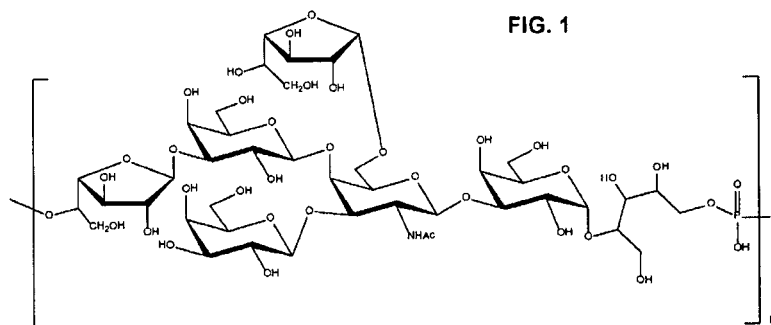
- as to the identity of the inventor (Rule 4.17(i))
- as to applicant's entitlement to apply for and be granted a
patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the
earlier application (Rule 4.17(iii))

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(88) Date of publication of the international search report:
26 November 2015

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



(57) Abstract: The invention relates to activated Streptococcus pneumoniae serotype 10A, 22F or 33F polysaccharides and processes for their preparation. The invention also relates to immunogenic conjugates comprising Streptococcus pneumoniae serotype 10A, 22F or 33F polysaccharides covalently linked to a carrier protein, processes for their preparation and immunogenic compositions and vaccines comprising them.

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2015/050314

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/02 A61K39/385 A61K39/09 A61K39/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

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

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

EP0-Internal, WPI Data, EMBASE, FSTA, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R. E. BIAGINI ET AL: "Method for Simultaneous Measurement of Antibodies to 23 Pneumococcal Capsular Polysaccharides", CLINICAL AND VACCINE IMMUNOLOGY, vol. 10, no. 5, 1 September 2003 (2003-09-01), pages 744-750, XP055085087, ISSN: 1556-6811, DOI: 10.1128/CDLI.10.5.744-750.2003 See e.g. the abstract; page 745, left column, first paragraph; page 745 section bridging left and right columns ----- -/-	1-6,14, 15, 19-23, 29-32, 39-42



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

6 May 2015

Date of mailing of the international search report

04/08/2015

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Authorized officer

Valcárcel, Rafael

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2015/050314

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.:
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.:
1-6, 14, 15, 19-23, 29-32, 39-42(all partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2015/050314

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRASCH ET AL: "Preparation of bacterial polysaccharide-protein conjugates: Analytical and manufacturing challenges", VACCINE, ELSEVIER LTD, GB, vol. 27, no. 46, 30 October 2009 (2009-10-30), pages 6468-6470, XP026704485, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2009.06.013 [retrieved on 2009-06-24] See e.g. the abstract; Table 1; Figure 1; page 6469, left column, second paragraph -----	1-6,14, 15, 19-23, 29-32, 39-42
X	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 or paragraph [0059] -----	1-6,14, 15, 19-23, 29-32, 39-42

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. claims: 1-6, 14, 15, 19-23, 29-32, 39-42(all partially)

A process as defined in claim 1: wherein the oxidizing agent is e.g. sodium metaperiodate (NaIO_4); wherein the quenching agent is not specified; wherein the Mw of the polysaccharides is not specified; wherein the degree of oxidation is not specified; wherein the temperature and duration of steps a) and b) are not specified

2. claims: 7-9(completely); 1-6, 14, 15, 19-23, 29-32, 39-42(partially)

As subject 1 but wherein the temperature or duration of step a) is as described in claims 7-9

- 3-14. claims: 1-6, 10, 14, 15, 19-23, 29-32, 39-42(all partially)

As subject 1 but when the quenching agent is one of the ones specified in claim 10.

15. claims: 11(completely); 1-6, 14, 15, 19-23, 29-32, 39-42(partially)

As subject 1 but when the quenching agent is one of the one specified in claim 11.

- 16-20. claims: 1-6, 12, 14, 15, 19-23, 29-32, 39-42(all partially)

As subject 1 but when the quenching agent is one of the ones specified in claim 12.

21. claims: 13(completely); 1-6, 14, 15, 19-23, 29-32, 39-42(partially)

As subject 1 but when the quenching agent is the one specified in claim 13

22. claims: 16-18(completely); 1-6, 14, 15, 19-23, 29-32, 39-42(partially)

As subject 1 but wherein the temperature or duration of step b) is as described in claims 16-18

23. claims: 24-26, 33-35, 43-45(completely); 1-6, 14, 15, 19-23, 29-32, 39-42(partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

As subject 1 but wherein the molecular weight of the polysaccharides is as described in claims 24-26,33-35 and 43-45

24. claims: 27, 28, 36, 37, 46, 47(completely); 1-6, 14, 15, 19-23, 29-32, 39-42(partially)

As subject 1 but wherein the degree of oxidation is as specified in claims 27, 28, 36, 37, 46 and 47.

25. claims: 38(completely); 1-6, 14, 15, 19-23, 29-32, 39-42(partially)

As subject 1 but as defined in claim 38

26. claims: 48(completely); 1-6, 14, 15, 19-23, 29-32, 39-42(partially)

As subject 1 but as defined in claim 48

27. claims: 49-120

A process as defined in claim 49 and dependent claims. Compositions defined by said processes. A method of treating or preventing a S. pneumonia infection comprising the use of these compositions. There is further lack of unity in this group since the concept of conjugating pneumococcal capsular polysaccharides to carrier proteins by activating the polysaccharides by periodate oxidation, quenching and conjugation to the carrier protein is well known in the art of polysaccharide-protein conjugates. However, since almost each dependent claim would constitute a potential separate invention, for the sake of efficiency, at present all inventions are grouped under the present subject.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2015/050314

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 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
		W0 2008157590 A1	24-12-2008

(19)中华人民共和国国家知识产权局



(12)发明专利申请



(10)申请公布号 CN 105934251 A

(43)申请公布日 2016.09.07

(21)申请号 201580005247.5

(22)申请日 2015.01.15

(30)优先权数据

61/929,598 2014.01.21 US

(85)PCT国际申请进入国家阶段日

2016.07.21

(86)PCT国际申请的申请数据

PCT/IB2015/050314 2015.01.15

(87)PCT国际申请的公布数据

W02015/110940 EN 2015.07.30

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(51)Int.Cl.

A61K 39/09(2006.01)

A61K 39/385(2006.01)

A61K 39/39(2006.01)

A61P 31/04(2006.01)

C08B 37/00(2006.01)

C12P 19/04(2006.01)

C12R 1/46(2006.01)

权利要求书7页 说明书35页

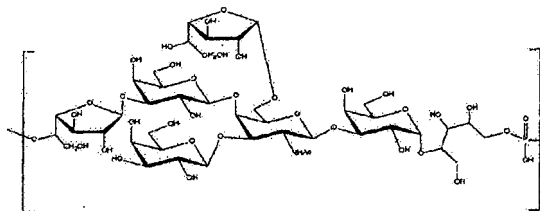
序列表5页 附图3页

(54)发明名称

肺炎链球菌荚膜多糖及其缀合物

(57)摘要

本发明涉及活化的肺炎链球菌血清型10A、22F或33F多糖及其制备方法。本发明还涉及包含共价连接于载体蛋白的肺炎链球菌血清型10A、22F或33F多糖的免疫原性缀合物、其制备方法以及包含其的免疫原性组合物和疫苗。



1. 一种用于制备活化的肺炎链球菌血清型10A、22F或33F荚膜多糖的方法,所述方法包括以下步骤:

- (a)将分离的血清型10A、22F或33F荚膜多糖与氧化剂反应;和
- (b)通过添加猝灭剂来猝灭氧化反应,从而导致活化的肺炎链球菌血清型10A、22F或33F多糖。

2. 权利要求1的方法,其中步骤(a)中的血清型10A、22F或33F多糖的浓度在0.5mg/mL与5mg/mL之间。

3. 权利要求1或2的方法,其中在步骤(a)中将所述多糖与0.01至10摩尔当量、0.05至5摩尔当量、0.1至1摩尔当量、0.5至1摩尔当量、0.7至0.8摩尔当量、0.01至0.2摩尔当量、0.05至0.5摩尔当量、0.05至0.2摩尔当量、0.1至0.3摩尔当量的氧化剂反应。

4. 权利要求1至3的任一项的方法,其中在步骤(a)中将所述多糖与0.05至0.3摩尔当量的氧化剂反应。

5. 权利要求1至4的任一项的方法,其中所述氧化剂为高碘酸盐。

6. 权利要求1至4的任一项的方法,其中所述氧化剂为偏高碘酸钠。

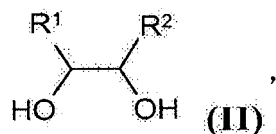
7. 权利要求1至6的任一项的方法,其中将步骤(a)中的反应温度维持在1°C与10°C之间。

8. 权利要求1至7的任一项的方法,其中步骤(a)中的反应温度为约°C或约5°C。

9. 权利要求1至8的任一项的方法,其中步骤(a)的持续时间在约1小时与25小时之间。

10. 权利要求1至9的任一项的方法,其中所述猝灭剂选自邻二醇、1,2-氨基醇、氨基酸、谷胱甘肽、亚硫酸盐、硫酸氢盐、连二亚硫酸盐、焦亚硫酸盐、硫代硫酸盐、亚磷酸盐、次磷酸盐或亚磷酸。

11. 权利要求1至9的任一项的方法,其中所述猝灭剂为式(II)的化合物



其中R¹和R²各自独立地选自H、甲基、乙基、丙基或异丙基。

12. 权利要求1至9的任一项的方法,其中所述猝灭剂选自甘油、乙二醇、丙-1,2-二醇、丁-1,2-二醇或丁-2,3-二醇。

13. 权利要求1至9的任一项的方法,其中所述猝灭剂为丁-2,3-二醇。

14. 权利要求1至13的任一项的方法,其中通过添加0.1至10、0.5至5、0.5至3或0.5至2摩尔当量的猝灭剂来猝灭氧化反应。

15. 权利要求1至13的任一项的方法,其中通过添加0.5至2摩尔当量的猝灭剂来猝灭氧化反应。

16. 权利要求1至15的任一项的方法,其中将步骤(b)中的反应温度维持在1°C与10°C之间。

17. 权利要求1至16的任一项的方法,其中步骤(b)中的反应温度为约4°C。

18. 权利要求1至17的任一项的方法,其中步骤(b)的持续时间在0.5与2小时之间。

19. 权利要求1至18的任一项的方法,其中在步骤(b)后纯化所述活化的多糖。

20. 权利要求1至19的任一项的方法,其中所述分离的多糖为分离的肺炎链球菌血清型10A多糖。

21. 权利要求20的方法,其中通过包含以下步骤的方法获得所述分离的肺炎链球菌血清型10A多糖:

- (a) 制备血清型10A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;和
- (c) 从所述发酵培养物纯化血清型10A多糖。

22. 一种活化的肺炎链球菌血清型10A荚膜多糖,其通过权利要求1至21的任一项的方法获得。

23. 一种活化的肺炎链球菌血清型10A荚膜多糖,其可通过权利要求1至21的任一项的方法获得。

24. 权利要求22或23的任一项的活化的肺炎链球菌血清型10A荚膜多糖,其中所述多糖具有50kDa与400kDa之间、50kDa与350kDa之间、50kDa与300kDa之间、50kDa与250kDa之间、50kDa与200kDa之间、100kDa与300kDa之间、100kDa与250kDa之间、或100kDa与200kDa之间的分子量。

25. 权利要求22或23的任一项的活化的肺炎链球菌血清型10A荚膜多糖,其中所述多糖具有50kDa与300kDa之间的分子量。

26. 权利要求22或23的任一项的活化的肺炎链球菌血清型10A荚膜多糖,其中所述多糖具有100kDa与200kDa之间的分子量。

27. 权利要求22至26的任一项的活化的肺炎链球菌血清型10A荚膜多糖,其中所述多糖具有5与20之间、5与15之间、8与14之间、8与12之间或9与11之间的氧化程度。

28. 权利要求22至26的任一项的活化的肺炎链球菌血清型10A荚膜多糖,其中所述多糖具有9与11之间的氧化程度。

29. 权利要求1至19的任一项的方法,其中所述分离的多糖为分离的肺炎链球菌血清型22F多糖。

30. 权利要求29的方法,其中通过包含以下步骤的方法获得所述分离的肺炎链球菌血清型22F多糖:

- (a) 制备血清型22F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) 从所述发酵培养物纯化血清型22F多糖;和
- (d) 通过机械裁剪,优选通过高压均化来裁剪所述血清型22F多糖。

31. 一种活化的肺炎链球菌血清型22F荚膜多糖,其通过权利要求1至19或29或30的任一项的方法获得。

32. 一种活化的肺炎链球菌血清型22F荚膜多糖,其可通过权利要求1至19或29或30的任一项的方法获得。

33. 权利要求31或32的任一项的活化的肺炎链球菌血清型22F荚膜多糖,其中所述多糖具有25kDa与1000kDa之间、100kDa与1000kDa之间、300kDa与800kDa之间、300kDa与700kDa之间、300kDa与600kDa之间、400kDa与1000kDa之间、400kDa与800kDa之间、400kDa与700kDa之间或400kDa与600kDa之间的分子量。

34. 权利要求31或32的任一项目的活化的肺炎链球菌血清型22F荚膜多糖,其中所述多糖具有300kDa与800kDa之间的分子量。

35. 权利要求31或32的任一项目的活化的肺炎链球菌血清型22F荚膜多糖,其中所述多糖具有400kDa与600kDa之间的分子量。

36. 权利要求31至35的任一项目的活化的肺炎链球菌血清型22F荚膜多糖,其中所述多糖具有10与25之间、10与20之间、12与20之间或14与18之间的氧化程度。

37. 权利要求31至35的任一项目的活化的肺炎链球菌血清型22F荚膜多糖,其中所述多糖具有10与20之间的氧化程度。

38. 权利要求31至37的任一项目的活化的肺炎链球菌血清型22F荚膜多糖,其中所述多糖包含每mM血清型22F多糖至少0.6mM的醋酸盐。

39. 权利要求1至19的任一项目的方法,其中所述分离的多糖是分离的肺炎链球菌血清型33F多糖。

40. 权利要求39的方法,其中所述分离的肺炎链球菌血清型33F多糖通过包含以下步骤的方法获得:

- (a) 制备血清型33F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) 从发酵培养物纯化血清型33F多糖;和
- (d) 通过机械裁剪,优选通过高压均化来裁剪所述血清型33F多糖。

41. 一种活化的肺炎链球菌血清型33F荚膜多糖,其通过权利要求1至19或39或40的任一项目的方法获得。

42. 一种活化的肺炎链球菌血清型33F荚膜多糖,其可通过权利要求1至19或39或40的任一项目的方法获得。

43. 权利要求39或40的活化的肺炎链球菌血清型33F荚膜多糖,其中所述多糖具有50kDa与1250kDa之间、200kDa与1200kDa之间、500kDa与1200kDa之间、500kDa与1000kDa之间、700kDa与1200kDa之间、800kDa与1200kDa之间、800kDa与1100kDa之间、900kDa与1200kDa之间或800kDa与1000kDa之间的分子量。

44. 权利要求39或40的活化的肺炎链球菌血清型33F荚膜多糖,其中所述多糖具有500kDa与1000kDa之间的分子量。

45. 权利要求39或40的活化的肺炎链球菌血清型33F荚膜多糖,其中所述多糖具有50kDa与400kDa之间的分子量。

46. 权利要求41至45的任一项目的活化的肺炎链球菌血清型33F荚膜多糖,其中所述多糖具有10与20之间的氧化程度。

47. 权利要求41至34的任一项目的活化的肺炎链球菌血清型33F荚膜多糖,其中所述多糖包含每mM血清型33F多糖至少0.7mM的醋酸盐。

48. 权利要求1至47的任一项目的方法,其中在步骤(b)后冷冻干燥所述活化的多糖。

49. 一种用于制备包含共价连接于载体蛋白的肺炎链球菌血清型10A、22F或33F多糖的免疫原性缀合物的方法,所述方法包括步骤:

(a) 将通过或可通过权利要求1至19的任一项目的方法获得的活化的血清型10A、22F或33F多糖与载体蛋白混合;和,

(b)将混合的活化的血清型10A、22F或33F多糖和载体蛋白与还原剂反应,以形成血清型10A、22F或33F多糖:载体蛋白缀合物。

50.权利要求49的方法,所述方法还包括步骤:

(c)将所述血清型10A、22F或33F多糖:载体蛋白缀合物中的未反应的醛加帽。

51.权利要求49或50的方法,其中在DMSO中进行步骤(a)和(b)。

52.权利要求49或50的方法,其中在水溶液中进行步骤(a)和(b)。

53.权利要求49至52的任一项的方法,其中在步骤(a)后共同冷冻干燥所述活化的多糖和蛋白质载体。

54.权利要求53的方法,其中在DMSO中进行步骤(b)。

55.权利要求53的方法,其中在水溶液中进行步骤(b)。

56.权利要求49至55的任一项的方法,其中步骤(b)中的活化的多糖的浓度在0.1mg/mL与10mg/mL之间、0.5mg/mL与5mg/mL之间或0.5mg/mL与2mg/mL之间。

57.权利要求49至55的任一项的方法,其中步骤(b)中的活化的多糖的浓度在0.5mg/mL与2mg/mL之间。

58.权利要求49至57的任一项的方法,其中活化的血清型10A、22F或33F多糖对载体蛋白的初始比率在5:1与0.1:1之间、2:1与0.1:1之间、2:1与1:1之间、1.5:1与1:1之间、0.1:1与1:1之间、0.3:1与1:1之间或0.6:1与1.2:1之间。

59.权利要求49至57的任一项的方法,其中活化的血清型10A、22F或33F多糖对载体蛋白的初始比率在0.6:1与1.2:1之间。

60.权利要求49至59的任一项的方法,其中在步骤(b)中,将所述活化的多糖与0.5与2.5摩尔当量之间的还原剂反应。

61.权利要求49至60的任一项的方法,其中所述还原剂为氰基硼氢化钠。

62.权利要求49至61的任一项的方法,其中步骤(d)的持续时间在20小时与30小时之间。

63.权利要求49至62的任一项的方法,其中将步骤d中的反应温度维持在20℃与26℃之间。

64.权利要求53的方法,其中通过添加约2摩尔当量的NaBH₄来使未反应的醛加帽。

65.权利要求49至64的任一项的方法,其还包括纯化所述多糖:载体蛋白缀合物的步骤。

66.权利要求49至65的任一项的方法,其还包括通过切向流过滤和渗滤纯化所述多糖:载体蛋白缀合物的步骤。

67.一种包含共价连接于载体蛋白的肺炎链球菌血清型10A多糖的免疫原性缀合物,其通过权利要求49至66的任一项的方法获得。

68.一种包含共价连接于载体蛋白的肺炎链球菌血清型10A多糖的免疫原性缀合物,其可通过权利要求49至66的任一项的方法获得。

69.权利要求67或68的免疫原性缀合物,其中所述缀合物具有500kDa与15000kDa之间、500kDa与10000kDa之间、2000kDa与10000kDa之间或3000kDa与8000kDa之间的分子量。

70.权利要求67或68的免疫原性缀合物,其中所述缀合物具有3000kDa与8000kDa之间的分子量。

71. 权利要求67至70的任一项的免疫原性缀合物,其中所述缀合物包含相较于血清型10A多糖的总量少于约50%、45%、40%、35%、30%、25%、20%或15%的游离血清型10A多糖。

72. 权利要求67至70的任一项的免疫原性缀合物,其中所述缀合物包含相较于血清型10A多糖的总量少于约20%的游离血清型10A多糖。

73. 权利要求67至72的任一项的免疫原性缀合物,其中所述缀合物中的血清型10A多糖对载体蛋白的比率在0.5与3之间。

74. 权利要求67至72的任一项的免疫原性缀合物,其中所述缀合物中的血清型10A多糖对载体蛋白的比率在0.8与1.4之间。

75. 权利要求67至74的任一项的免疫原性缀合物,其中至少40%的所述免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。

76. 权利要求67至74的任一项的免疫原性缀合物,其中50%与80%之间的所述血清型10A免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。

77. 权利要求67至76的任一项的免疫原性缀合物,其中所述免疫原性缀合物的缀合程度在2与15之间、2与13之间、2与10之间、2与8之间、2与6之间、2与5之间、2与4之间、3与15之间、3与13之间、3与10之间、3与8之间、3与6之间、3与5之间、3与4之间、5与15之间、5与10之间、8与15之间、8与12之间、10与15之间或10与12之间。

78. 权利要求67至76的任一项的免疫原性缀合物,其中所述免疫原性缀合物的缀合程度在6与8之间。

79. 一种包含共价连接于载体蛋白的肺炎链球菌血清型22F多糖的免疫原性缀合物,其通过权利要求49至66的任一项的方法获得。

80. 一种包含共价连接于载体蛋白的肺炎链球菌血清型22F多糖的免疫原性缀合物,其可通过权利要求49至66的任一项的方法获得。

81. 权利要求79或80的免疫原性缀合物,其中所述缀合物包含每mM血清型22F多糖至少0.5、0.6或0.7mM的醋酸盐。

82. 权利要求79或80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22F多糖的醋酸盐的mM数对分离的多糖中每mM血清型22F多糖的醋酸盐的mM数的比率为至少0.7。

83. 权利要求79至82的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22F多糖的醋酸盐的mM数对活化的多糖中每mM血清型22F多糖的醋酸盐的mM数的比率为至少0.7。

84. 权利要求79至83的任一项的免疫原性缀合物,其中所述缀合物具有400kDa与15000kDa之间、500kDa与10000kDa之间、2000kDa与10000kDa之间、3000kDa与8000kDa之间或3000kDa与5000kDa之间的分子量。

85. 权利要求79至83的任一项的免疫原性缀合物,其中所述缀合物具有3000kDa与5000kDa之间的分子量。

86. 权利要求79至85的任一项的免疫原性缀合物,其中所述缀合物包含相较于血清型22F多糖的总量少于约50%、45%、40%、35%、30%、25%、20%或15%的游离血清型22F多糖。

87. 权利要求79至83的任一项的免疫原性缀合物,其中所述缀合物包含相较于血清型

22F多糖的总量少于约20%的游离血清型22F多糖。

88. 权利要求79至87的任一项的免疫原性缀合物, 其中所述缀合物中血清型22F多糖对载体蛋白的比率在0.5与2之间。

89. 权利要求79至87的任一项的免疫原性缀合物, 其中所述缀合物中血清型22F多糖对载体蛋白的比率在0.8与1.2之间。

90. 权利要求79至89的任一项的免疫原性缀合物, 其中至少40%的所述免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。

91. 权利要求79至89的任一项的免疫原性缀合物, 其中50%与80%之间的所述血清型22F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。

92. 权利要求79至91的任一项的免疫原性缀合物, 其中所述免疫原性缀合物的缀合程度在2与15之间、2与13之间、2与10之间、2与8之间、2与6之间、2与5之间、2与4之间、3与15之间、3与13之间、3与10之间、3与8之间、3与6之间、3与5之间、3与4之间、4与7之间、5与15之间、5与10之间、8与15之间、8与12之间、10与15之间或10与12之间。

93. 权利要求79至91的任一项的免疫原性缀合物, 其中所述免疫原性缀合物的缀合程度在4与7之间。

94. 一种包含共价连接于载体蛋白的肺炎链球菌血清型33F多糖的免疫原性缀合物, 其通过权利要求49至66的任一项的方法获得。

95. 一种包含共价连接于载体蛋白的肺炎链球菌血清型33F多糖的免疫原性缀合物, 其可通过权利要求49至66的任一项的方法获得。

96. 权利要求94或95的免疫原性缀合物, 其中所述缀合物包含每mM血清型33F多糖至少0.5mM、0.6mM或0.7mM的醋酸盐。

97. 权利要求94或95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33F多糖的醋酸盐的mM数对分离的多糖中每mM血清型33F多糖的醋酸盐的mM数的比率为至少0.7。

98. 权利要求94至97的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33F多糖的醋酸盐的mM数对活化的多糖中每mM血清型33F多糖的醋酸盐的mM数的比率为至少0.7。

99. 权利要求94至98的任一项的免疫原性缀合物, 其中所述缀合物具有500kDa与30000kDa之间、500kDa与25000kDa之间、500kDa与20000kDa之间、500kDa与15000kDa之间、500kDa与10000kDa之间、1000kDa与10000kDa之间、1000kDa与8000kDa之间、1000kDa与5000kDa之间、2000kDa与10000kDa之间、2000kDa与8000kDa之间或2000kDa与5000kDa之间的分子量。

100. 权利要求94至98的任一项的免疫原性缀合物, 其中所述缀合物具有1000kDa与5000kDa之间的分子量。

101. 权利要求94至100的任一项的免疫原性缀合物, 其中所述缀合物含有相较于血清型33F多糖的总量少于约50%、45%、40%、35%、30%、25%、20%或15%的游离血清型33F多糖。

102. 权利要求94至100的任一项的免疫原性缀合物, 其中所述缀合物包含相较于血清型22F多糖的总量少于约20%的游离血清型22F多糖。

103. 权利要求94至102的任一项的免疫原性缀合物, 其中所述缀合物中血清型33F多糖

对载体蛋白的比率在0.5与2之间。

104. 权利要求94至102的任一项的免疫原性缀合物, 其中所述缀合物中血清型22F多糖对载体蛋白的比率在0.5与1.5之间。

105. 权利要求94至104的任一项的免疫原性缀合物, 其中至少40%的所述免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。

106. 权利要求94至104的任一项的免疫原性缀合物, 其中40%与80%之间的所述血清型33F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。

107. 权利要求94至106的任一项的免疫原性缀合物, 其中所述免疫原性缀合物的缀合程度在2与15之间、2与13之间、2与10之间、2与8之间、2与6之间、2与5之间、2与4之间、3与15之间、3与13之间、3与10之间、3与8之间、3与6之间、3与5之间、3与4之间、5与15之间、5与10之间、8与15之间、8与12之间、10与15之间或10与12之间。

108. 权利要求94至106的任一项的免疫原性缀合物, 其中所述免疫原性缀合物的缀合程度在4与6之间。

109. 权利要求67至108的任一项的免疫原性缀合物, 其中所述载体蛋白为CRM₁₉₇。

110. 权利要求49至66的任一项的方法, 其中所述载体蛋白为CRM₁₉₇。

111. 权利要求49至66的任一项的方法, 其中所述活化的多糖为血清型10A。

112. 权利要求49至66的任一项的方法, 其中所述活化的多糖为血清型22F。

113. 权利要求49至66的任一项的方法, 其中所述活化的多糖为血清型33F。

114. 权利要求49至66和110至113的任一项的方法, 其中所述方法还包括在多价疫苗中配制所述缀合物的步骤。

115. 一种免疫原性组合物, 其包含权利要求67至109的任一项的免疫原性缀合物和生理上可接受的媒介物。

116. 权利要求115的免疫原性组合物, 其还包含至少一种另外的抗原。

117. 权利要求115或116的免疫原性组合物, 其还包含佐剂。

118. 权利要求117的免疫原性组合物, 其中所述佐剂为磷酸铝。

119. 一种疫苗, 其包含权利要求115至118的任一项的免疫原性组合物。

120. 一种治疗或预防受试者的与血清型10A、22F或33F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115至118的任一项的免疫原性组合物或权利要求119的疫苗的步骤。

肺炎链球菌荚膜多糖及其缀合物

发明领域

[0001] 本发明涉及活化的肺炎链球菌(*Streptococcus pneumoniae*)血清型10A、22F或33F多糖及其制备方法。本发明还涉及包含共价连接于载体蛋白的肺炎链球菌血清型10A、22F或33F多糖的免疫原性缀合物、其制备方法以及包含其的免疫原性组合物和疫苗。

[0002] 背景

[0003] 肺炎链球菌为革兰阳性、柳叶刀形球菌,其通常被成对地(双球菌)看见,但也呈短链或作为单个细胞存在。它们容易在血液琼脂板上以闪光菌落生长,并且展示 α 溶血作用,除非厌氧生长(其中它们显示 β 溶血作用)。大多数肺炎球菌血清型的细胞具有作为围绕每一个细胞的多糖包衣的荚膜。该荚膜是在人中的毒力的决定子,因为它通过阻止抗体附着于细菌细胞来干扰吞噬作用。目前存在超过90种已鉴定的已知的肺炎球菌荚膜血清型,其中23种最常见的血清型导致世界范围内约90%的侵袭性疾病。作为疫苗,肺炎球菌多糖包衣可在具有发达的或健全的免疫系统的个体中赋予合理程度的针对肺炎链球菌的免疫,但缀合于合适的载体蛋白的荚膜多糖允许在还处于肺炎球菌感染的最高风险的婴儿和老人中产生免疫应答。

[0004] 由于在2000年引入第一种7-价肺炎球菌缀合物疫苗(PCV7或Pneumovax),来自七种血清型(4、6B、9V、14、18C、19F和23F)的侵袭性疾病已几乎消失。血清型1、3、5、6A、7F和19A在Pneumovax 13中的添加进一步减少了侵袭性肺炎球菌疾病的数量。

[0005] 目前市售的肺炎球菌疫苗均未在人中,尤其是不足2岁的儿童中提供针对血清型10A、22F或33F肺炎链球菌的适当保护。因此,存在对可用于诱导针对血清型10A、22F或33F肺炎链球菌的免疫应答的免疫原性组合物的需要。

[0006] 发明概述

[0007] 在一个方面,本公开内容提供了用于制备活化的肺炎链球菌血清型10A、22F或33F荚膜多糖的方法,所述方法包括以下步骤:

[0008] (a)将分离的血清型10A、22F或33F荚膜多糖与氧化剂反应;和

[0009] (b)通过添加猝灭剂来猝灭氧化反应,从而导致活化的肺炎链球菌血清型10A、22F或33F多糖。

[0010] 在其它方面,本发明涉及通过或可通过本文中公开的活化方法获得的活化的肺炎链球菌血清型10A、22F或33F荚膜多糖。

[0011] 在其它方面,本公开内容提供了用于制备包含共价连接于载体蛋白的肺炎链球菌血清型10A、22F或33F多糖的免疫原性缀合物的方法,所述方法包括以下步骤:

[0012] (a)将通过或可通过本文中公开的方法获得的活化的血清型10A、22F或33F多糖与载体蛋白混合;和,

[0013] (b)将混合的活化的血清型10A、22F或33F多糖和载体蛋白与还原剂反应,以形成血清型10A、22F或33F多糖:载体蛋白缀合物。

[0014] 在其它方面,本公开内容提供了通过或可通过本文中公开的方法获得的血清型10A、22F或33F多糖:载体蛋白缀合物。

[0015] 在其它方面,本公开内容提供了包含本文中公开的免疫原性缀合物的免疫原性组合物和疫苗。

[0016] 在其它方面,本公开内容提供了治疗或预防受试者的与血清型10A、22F或33F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。

[0017] 附图简述

[0018] 图1显示肺炎链球菌荚膜多糖血清型10A重复单元的结构。

[0019] 图2显示肺炎链球菌荚膜多糖血清型22F重复单元的结构。

[0020] 图3显示肺炎链球菌荚膜多糖血清型33F重复单元的结构。

[0021] 图4显示在23℃下在利用和不利用猝灭步骤的情况下作为氧化剂的量的函数的活化的血清型22F多糖的分子量。

[0022] 图5显示在4℃下在利用和不利用猝灭步骤的情况下作为氧化剂的量的函数的活化的血清型22F多糖的分子量。

[0023] 图6显示在2至8℃下在利用或不利用猝灭步骤的情况下或在23℃下在不利用猝灭步骤的情况下作为氧化剂的量的函数的活化的血清型33F多糖的分子量。

[0024] 发明详述

[0025] 通过参考本发明的优选实施方案和本文包括的实施例的以下详细描述可更容易地理解本发明。除非另有定义,否则本文中使用的所有技术和科学术语具有与本发明所属的领域中的普通技术人员通常理解的含义相同的含义。虽然可以在本发明的实践或测试中使用与本文所述的方法和材料类似或等同的任何方法和材料,但本文中描述了某些优选方法和材料。在描述实施方案和主张本发明中,将按照下文所示的定义使用某些术语。

[0026] 如本文中所用,除非另有说明,否则单数形式“一个/种(a)”,“一个/种(an)”和“该(the)”包括复数所指物。因此,例如,对“方法”的引用包括本文所述的和/或在阅读本公开内容后对于本领域普通技术人员来说将变得明显的类型的一个或多个方法和/或步骤。

[0027] 如本文中所用,术语多糖或载体蛋白-多糖缀合物的“分子量”是指通过与多角度激光散射检测器(MALLS)组合的尺寸排阻层析(SEC)计算的分子量。

[0028] 如本文中所用,术语“氧化程度”(DO)是指当用氧化剂活化分离的多糖时产生的每醛基的糖重复单元的数目。可使用本领域中技术人员已知的常规方法测定多糖的氧化程度。

[0029] 应当指出的是,在本公开内容中,术语如“包含”、“包括”、“含有”、“具有”、“拥有”等可以具有在美国专利法中赋予它们的含义;例如,它们可意指“包括”、“包含”、“含有”等。这样的术语是指特定成分或成分的组的包含,而不排除任何其它成分。术语诸如“基本上由……组成”和“基本上由……构成”具有在美国专利法中赋予它们的含义,例如,它们允许包含不减损本发明的新型或基本特征的另外的成分或步骤,即,它们排除减损本发明的新型或基本特征的另外的未引述的成分或步骤。术语“由……组成”和“由……构成”具有在美国专利法中赋予它们的含义;即,这些术语是封闭式的。因此,这些术语是指特定成分或成分的组的包含以及排除所有其它组分。

[0030] 如本文中所用,术语“缀合物”或“糖缀合物”是指共价缀合于载体蛋白的多糖。本发明的糖缀合物和包含其的免疫原性组合物可含有一定量的游离多糖。

[0031] 如本文所用,术语“血清型10A糖缀合物”或“血清型10A缀合物”是指共价缀合于载体蛋白的分离的肺炎链球菌血清型10A荚膜多糖。

[0032] 如本文中所用,术语“血清型22F糖缀合物”或“血清型22F缀合物”是指共价缀合于载体蛋白的分离的肺炎链球菌血清型22F荚膜多糖。

[0033] 如本文中所用,术语“血清型33F糖缀合物”或“血清型33F缀合物”是指共价缀合于载体蛋白的分离的肺炎链球菌血清型33F荚膜多糖。

[0034] 如本文中所用,术语“血清型10A多糖”是指肺炎链球菌血清型10A荚膜多糖。

[0035] 如本文中所用,术语“血清型22F多糖”是指肺炎链球菌血清型22F荚膜多糖。

[0036] 如本文中所用,术语“血清型33F多糖”是指肺炎链球菌血清型33F荚膜多糖。

[0037] 如本文中所用,术语“游离多糖”意指未共价缀合于载体蛋白,但仍然存在于荚膜多糖-载体蛋白缀合物组合物中的荚膜多糖。游离多糖可与多糖-载体蛋白缀合物非共价地缔合(即,非共价结合于、吸附于或捕捉于或与其非共价结合、吸附或捕捉)。

[0038] 在血清型10A、22F或33F荚膜多糖-载体蛋白缀合物的最终纯化后,测量游离多糖的百分比。优选地在最终纯化后4周内测定其。将其表达为样品中的总多糖的百分比。

[0039] 如本文中所用,“缀合”、“被缀合”和“缀合的”是指籍以将肺炎链球菌荚膜多糖籍共价连接于载体蛋白的方法。

[0040] 术语“受试者”是指哺乳动物,包括人,或指鸟、鱼、爬行类动物、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例子包括:羊驼、美洲野牛、骆驼、牛、鹿、猪、马、驼马、骡、驴、绵羊、山羊、兔、驯鹿、牦牛、鸡、鹅和火鸡。

[0041] 在用于预防由生物肺炎链球菌(也称为肺炎球菌)引起的侵袭性疾病的多价缀合肺炎球菌疫苗的制备中,将选定的肺炎链球菌血清型生长以提供产生疫苗所需的多糖。将细胞在发酵罐中生长,在发酵结束时通过添加脱氧胆酸钠或替代裂解剂来诱导裂解。随后收获裂解肉汤以用于下游纯化和包围细菌细胞的荚膜多糖的回收。在活化和与载体蛋白缀合后,多糖被包含在最终的疫苗产品中,并且在疫苗的目标人群中赋予针对所选定的肺炎链球菌血清型的免疫。

[0042] 多糖:载体蛋白缀合物的免疫原性取决于几个因素,包括多糖的大小。多糖大小是在每一个制备批次中测定的质量属性,并且必须受到适当地控制。高分子量荚膜多糖因存在于抗原表面上的表位的较高效价而能够诱导一定的抗体免疫应答。通常优选在缀合物的制备过程中防止或限制多糖大小的不期望的减小,以保留多糖的免疫原性。此外,重要的是在活化步骤和随后的缀合过程中减小多糖分子量的批次间的变化性,以维持缀合物的质量属性的一致性。

[0043] 还原胺化化学(RAC)已被本发明人证明为用于制备肺炎链球菌血清型10A、22F或33F荚膜多糖-蛋白载体缀合物的合适方法。RAC法牵涉通过氧化活化多糖和随后通过还原缀合活化的多糖与蛋白质载体。血清型10A、33F和22F多糖已被证明是特别敏感的多糖并且在缀合物的制备的氧化步骤过程中易受降解和大小减小。另外,迄今已知的活化方法的使用产生了具有可变分子量的活化血清型10A、33F和22F多糖。

[0044] 因此,存在对用于制备具有受控和一致的分子量的活化的肺炎链球菌血清型10A、

22F或33F多糖的方法的重要需要。

[0045] 本发明的目的是用于制备活化的肺炎链球菌血清型10A、22F或33F多糖的方法。具体地,本发明的目的是用于制备活化的肺炎链球菌血清型10A、22F或33F多糖的方法,所述方法包括以下步骤:

[0046] (a)将分离的血清型10A、22F或33F多糖与氧化剂反应;和,

[0047] (b)通过添加猝灭剂来猝灭氧化反应,从而导致活化的肺炎链球菌血清型10A、22F或33F多糖。

[0048] 血清型10A、22F或33F多糖的分离和纯化

[0049] 血清型10A、22F和33F多糖的结构公开于文献中(参见例如Kamerling J P C. *Pneumococcal polysaccharides: a chemical view*. In: Tomasz A, editor. *Streptococcus pneumoniae, molecular biology and mechanisms of disease*. New York, N.Y: Mary Ann Liebert, Inc.; 2000. 第81-114页)中。

[0050] 如在图1中所示,血清型22F的多糖重复单元由分枝多糖骨架(一个葡萄糖醛酸(GlcpA)、一个吡喃葡萄糖(Glcp)和一个呋喃半乳糖(Galf)以及两个吡喃鼠李糖(Rhap))组成,其中 α Glcp分支连于 β Rhap 45的C3羟基。多糖重复单元中的 β Rhap残基的约80%的C2羟基是O-乙酰化的。

[0051] 如在图2中所示,血清型10A的多糖重复单元由两个D-呋喃半乳糖、三个D-吡喃半乳糖、一个N-乙酰半乳糖胺以及通过一个D-核糖醇与糖链连接的磷酸键组成。

[0052] 如在图3中所示,血清型33F的多糖重复单元由三个D-吡喃半乳糖、二个D-呋喃半乳糖和一个D-吡喃葡萄糖组成。值得注意的是,在约90%的33F多糖重复单元中5-D-呋喃半乳糖残基是O-乙酰化的。

[0053] 可使用本领域普通技术人员已知的分离方法(参见例如美国专利申请公开号20060228380、20060228381、20070184071、20070184072、20070231340和20080102498或W02008118752中公开的方法)直接从细菌获得血清型10A、22F和33F荚膜多糖。另外,可使用合成方案产生它们。

[0054] 用于产生用于本发明的免疫原性缀合物的各自多糖的血清型10A、22F和33F肺炎链球菌菌株可从已建立的培养物保藏或临床样品获得。

[0055] 纯化的血清型10A、22F和33F多糖通过本领域技术人员公知的方法获得。优选将细菌细胞在基于大豆的培养基中生长。在产生肺炎链球菌血清型10A、22F或33F荚膜多糖的细菌细胞的发酵后,裂解所述细菌细胞以产生细胞裂解物。使用任何裂解剂裂解细菌细胞。“裂解剂”是有助于细胞壁破裂和释放引起细胞裂解的自溶素的任何试剂,包括例如,去垢剂。如本文中所用,“去垢剂”是指能够诱导细菌细胞的裂解的任何阴离子或阳离子去垢剂。用于在本发明的方法内使用的这样的去垢剂的代表性的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。

[0056] 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牛。DOC活化LytA蛋白,所述LytA蛋白为参与肺炎链球菌的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结构域,并且已知lytA基因的突变产生抵抗使用DOC的裂解的LytA突变体。

[0057] 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非动物来源的裂解

剂。用于在本发明的方法内使用的非动物来源的裂解剂包括来自非动物来源的具有与DOC的作用模式(即,影响LytA功能并导致肺炎链球菌细胞的裂解)类似的作用模式的试剂。这样的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、去垢剂和胆碱的结构类似物。在一个实施方案中,非动物来源的裂解剂选自癸烷磺酸、叔-辛基苯氧基聚(氧乙烯)乙醇(例如**Igepal®**CA-630,CAS#:9002-93-1,可获自Sigma Aldrich,St.Louis,MO)、辛基酚环氧乙烷缩合物(例如**Triton®**X-100,,可获自Sigma Aldrich,St.Louis,MO)、N-月桂酰肌氨酸钠、月桂亚氨基二丙酸盐、十二烷基硫酸钠、鹅脱氧胆酸盐、猪脱氧胆酸盐、甘氨酸脱氧胆酸盐、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,所述非动物来源的裂解剂为N-月桂酰肌氨酸。在另一个实施方案中,所述裂解剂为N-月桂酰肌氨酸钠。

[0058] 随后可使用本领域中已知的纯化技术从细胞裂解物纯化荚膜多糖,所述技术包括离心、深度过滤、沉淀、超滤、利用活性碳的处理、渗滤和/或柱层析的使用(参见例如美国专利申请公开号20060228380、20060228381、20070184071、20070184072、20070231340和20080102498或W02008118752)。随后可将纯化的血清型10A、22F或33F多糖用于免疫原性缀合物的制备。

[0059] 优选地,为了产生具有有利的过滤性特征和/或产率的血清型22F缀合物或血清型33F缀合物,在缀合于载体蛋白之前进行多糖至较低分子量(MW)范围的裁剪。有利地,减小纯化的血清型22F多糖或血清型33F多糖的大小,同时保留多糖结构的关键特征,诸如例如O-乙酰基的存在。优选地,纯化的血清型22F多糖或血清型33F多糖的大小通过机械均化来减小。

[0060] 在优选实施方案中,通过高压均化来减小纯化的多糖的大小。高压均化通过将加工流泵送通过具有足够小的尺寸的流路来实现高剪切率。剪切率通过使用较大的施加的均化压力来增加,并且可通过使进料流再循环通过均化器来增加暴露时间。

[0061] 高压均化方法特别适合用于减小纯化的血清型22F多糖或血清型33F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。

[0062] 可通过不同的参数(包括例如分子量、每mM血清型22F多糖的醋酸盐的mM数)来表征通过从肺炎链球菌裂解物纯化血清型22F荚膜多糖和任选地裁剪纯化的多糖获得的分离的血清型22F多糖。

[0063] 在优选实施方案中,经裁剪的血清型22F多糖具有400kDa与700kDa之间的分子量。

[0064] 可通过不同的参数(包括例如分子量和每mM血清型33F多糖的醋酸盐的mM数)来表征通过从肺炎链球菌裂解物纯化血清型33F荚膜多糖和任选地裁剪纯化的多糖获得的分离的血清型33F多糖。

[0065] 可通过本领域已知的任何方法,例如,通过质子NMR(Lemercinier和Jones(1996) Carbohydrate Research 296:83-96, Jones和Lemercinier(2002) J. Pharmaceutical and Biomedical Analysis 30:1233-1247, W0 05/033148或W000/56357)测定多糖的O-乙酰化的程度。另一种常用的方法描述于Hestrin(1949) J. Biol. Chem. 180:249-261中。优选地,O-乙酰基的存在通过离子HPLC分析来测定。

[0066] 在优选实施方案中,通过包括以下步骤的方法获得分离的血清型22F多糖:

[0067] -制备血清型22F肺炎链球菌细菌细胞的发酵培养物;

- [0068] -裂解所述发酵培养物中的细菌细胞;和,
- [0069] -从发酵培养物纯化血清型22F多糖。
- [0070] 在优选实施方案中,通过包括以下步骤的方法获得分离的血清型22F多糖:
- [0071] -制备血清型22F肺炎链球菌细菌细胞的发酵培养物;
- [0072] -裂解所述发酵培养物中的细菌细胞;
- [0073] -从发酵培养物纯化血清型22F多糖;和,
- [0074] -通过机械裁剪,优选通过高压均化来裁剪血清型22F多糖。
- [0075] 在优选实施方案中,分离的血清型22F多糖通过包含以下步骤的方法获得:
- [0076] -制备血清型22F肺炎链球菌细菌细胞的发酵培养物;
- [0077] -裂解所述发酵培养物中的细菌细胞;
- [0078] -从发酵培养物纯化血清型22F多糖;和
- [0079] -通过机械裁剪,优选通过高压均化来裁剪血清型22F多糖,
- [0080] 其中所述分离的血清型22F多糖具有400kDa与700kDa之间的分子量。
- [0081] 在优选实施方案中,通过包括以下步骤的方法获得分离的血清型33F多糖:
- [0082] -制备血清型33F肺炎链球菌细菌细胞的发酵培养物;
- [0083] -裂解所述发酵培养物中的细菌细胞;和,
- [0084] -从发酵培养物纯化血清型33F多糖。
- [0085] 在优选实施方案中,通过包括以下步骤的方法获得分离的血清型33F多糖:
- [0086] -制备血清型33F肺炎链球菌细菌细胞的发酵培养物;
- [0087] -裂解所述发酵培养物中的细菌细胞;
- [0088] -从发酵培养物纯化血清型33F多糖;和,
- [0089] -通过机械裁剪,优选通过高压均化来裁剪血清型33F多糖。
- [0090] 可通过不同的参数(包括例如分子量)来表征通过从肺炎链球菌裂解物纯化血清型10A荚膜多糖和任选地裁剪纯化的多糖获得的分离的血清型10A多糖。优选地,不裁剪分离的血清型10A多糖。
- [0091] 在优选实施方案中,通过包括以下步骤的方法获得分离的血清型10A多糖:
- [0092] -制备血清型10A肺炎链球菌细菌细胞的发酵培养物;
- [0093] -裂解所述发酵培养物中的细菌细胞;和,
- [0094] -从发酵培养物纯化血清型10A多糖。
- [0095] 血清型10A、22F或33F多糖的活化
- [0096] 通过包括以下步骤的方法来活化分离的血清型10A、22F或33F多糖:
- [0097] (a)将分离的血清型10A、22F或33F多糖与氧化剂反应;和,
- [0098] (b)通过添加猝灭剂来猝灭氧化反应,从而导致活化的肺炎链球菌血清型10A、22F或33F多糖。
- [0099] 在优选实施方案中,步骤(a)中的血清型10A、22F或33F多糖的浓度在0.1mg/mL与10mg/mL之间。在优选实施方案中,步骤(a)中的血清型10A、22F或33F多糖的浓度在0.5mg/mL与5mg/mL之间。在优选实施方案中,步骤(a)中的血清型10A、22F或33F多糖的浓度在1mg/mL与3mg/mL之间。在优选实施方案中,步骤(a)中的血清型22F或33F多糖的浓度为约2mg/mL。在优选实施方案中,步骤(a)中的血清型10A多糖的浓度为约2.5mg/mL。

[0100] 在优选实施方案中,所述氧化剂为高碘酸盐。所述高碘酸盐氧化邻位羟基以形成羰基或醛基,并引起C-C键的断裂。

[0101] 术语“高碘酸盐”包括高碘酸盐和高碘酸。该术语还包括偏高碘酸盐(IO_4^-)和正高碘酸盐(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。在优选实施方案中,氧化剂为高碘酸钠。在优选实施方案中,用于血清型10A、22F或33F多糖的氧化的高碘酸盐为偏高碘酸盐。在优选实施方案中,用于血清型10A、22F或33F多糖的氧化的高碘酸盐为偏高碘酸钠。

[0102] 在优选实施方案中,将多糖与0.01至10摩尔当量、0.05至5摩尔当量、0.1至1摩尔当量、0.5至1摩尔当量、0.7至0.8摩尔当量、0.01至0.2摩尔当量、0.05至0.5摩尔当量、0.05至0.2摩尔当量、0.1至0.3摩尔当量的氧化剂反应。在优选实施方案中,将多糖与约0.05摩尔当量、0.1摩尔当量、0.15摩尔当量、0.2摩尔当量、0.25摩尔当量、0.3摩尔当量、0.35摩尔当量、0.4摩尔当量、0.45摩尔当量、0.5摩尔当量、0.55摩尔当量、0.6摩尔当量、0.65摩尔当量、0.7摩尔当量、0.75摩尔当量、0.8摩尔当量的氧化剂反应。在优选实施方案中,将多糖与约0.10摩尔当量的氧化剂反应。在优选实施方案中,将多糖与约0.15摩尔当量的氧化剂反应。在优选实施方案中,将多糖与约0.25摩尔当量的氧化剂反应。在优选实施方案中,将多糖与约0.5摩尔当量的氧化剂反应。在优选实施方案中,将多糖与约0.8摩尔当量的氧化剂反应。

[0103] 在优选实施方案中,步骤(a)中的反应持续时间在1与50小时之间、1与40小时之间、1与30小时之间、1与25小时之间、1与20小时之间、1与10小时之间、10至50小时、10至40小时、10至30小时、10至20小时。在优选实施方案中,当多糖为血清型10A多糖时,步骤(a)中的反应持续时间在1与10小时之间。在优选实施方案中,当多糖为血清型10A多糖时,步骤(a)中的反应持续时间为约1小时、2小时、3小时、4小时、5小时、6小时、7小时、8小时、9小时、10小时。在优选实施方案中,当多糖为血清型10A多糖时,步骤(a)中的反应持续时间为约4小时。在优选实施方案中,当多糖为血清型22F多糖时,步骤(a)中的反应持续时间在10小时与20小时之间。在优选实施方案中,当多糖为血清型22F多糖时,步骤(a)中的反应持续时间为约10小时、11小时、12小时、13小时、14小时、15小时、16小时、17小时、18小时、19小时、20小时。在优选实施方案中,当多糖为血清型33F多糖时,步骤(a)中的反应持续时间在15小时与25小时之间。在优选实施方案中,当多糖为血清型33F多糖时,步骤(a)中的反应持续时间为约15小时、16小时、17小时、18小时、19小时、20小时、21小时、22小时、23小时、24小时或25小时。在优选实施方案中,当多糖为血清型33F多糖时,步骤(a)中的反应持续时间为约20小时。

[0104] 在优选实施方案中,将步骤(a)的反应温度维持在1°C与30°C之间、1°C与10°C之间、10°C与20°C之间、20°C与30°C之间、2°C与8°C之间。在优选实施方案中,将步骤(a)的反应温度维持在约5°C。

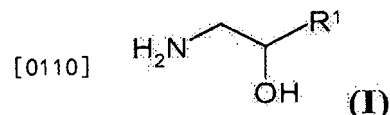
[0105] 在优选实施方案中,在选自磷酸钠、磷酸钾、2-(N-吗啉代)乙磺酸(MES)或Bis-Tris的缓冲液中进行步骤(a)。在优选实施方案中,在磷酸钾缓冲液中进行步骤(a)。在优选实施方案中,在磷酸钠缓冲液中进行步骤(a)。

[0106] 在优选实施方案中,所述缓冲液具有在1mM与500mM之间、1mM与300mM之间、50mM与200mM之间的浓度。在优选实施方案中,所述缓冲液具有约100mM的浓度。

[0107] 在优选实施方案中,步骤(a)中的pH在4与8之间、5与7之间、5.5与6.5之间。在优选实施方案中,步骤(a)中的pH为约6。在优选实施方案中,步骤(a)中的pH为约5.8。

[0108] 在一个实施方案中,猝灭剂选自邻二醇、1,2-氨基醇、氨基酸、谷胱甘肽、亚硫酸盐、硫酸氢盐、连二亚硫酸盐、焦亚硫酸盐、硫代硫酸盐、亚磷酸盐、次磷酸盐或亚磷酸。

[0109] 在一个实施方案中,猝灭剂为下式的1,2-氨基醇



[0111] 其中R¹选自H、甲基、乙基、丙基或异丙基。

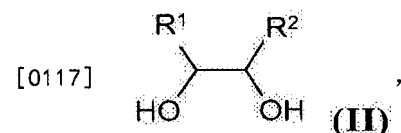
[0112] 在一个实施方案中,猝灭剂选自亚硫酸、硫酸氢、连二亚硫酸、焦亚硫酸、硫代硫酸、亚磷酸、次磷酸或亚磷酸的钠盐和钾盐。

[0113] 在一个实施方案中,猝灭剂为氨基酸。在一个实施方案中,所述氨基酸选自丝氨酸、苏氨酸、半胱氨酸、胱氨酸、甲硫氨酸、脯氨酸、羟脯氨酸、色氨酸、酪氨酸和组氨酸。

[0114] 在一个实施方案中,猝灭剂为亚硫酸盐诸如硫酸氢盐、连二亚硫酸盐、焦亚硫酸盐、硫代硫酸盐。

[0115] 在一个实施方案中,猝灭剂为包含两个邻位羟基(即共价连接于两个相邻碳原子的两个羟基)的化合物(邻二醇)。

[0116] 优选地,猝灭剂为式(II)的化合物



[0118] 其中R¹和R²各自独立地选自H、甲基、乙基、丙基或异丙基。

[0119] 在优选实施方案中,猝灭剂是甘油、乙二醇、丙-1,2-二醇、丁-1,2-二醇或丁-2,3-二醇、抗坏血酸。在优选实施方案中,猝灭剂为丁-2,3-二醇。

[0120] 在优选实施方案中,通过添加0.1至10摩尔当量、0.5至5摩尔当量、0.5至3摩尔当量、0.5至2摩尔当量的猝灭剂来中和氧化剂。在优选实施方案中,通过添加约0.5摩尔当量、1摩尔当量、1.5摩尔当量、2摩尔当量、2.5摩尔当量、3摩尔当量的猝灭剂来中和氧化剂。在优选实施方案中,通过添加约2摩尔当量的猝灭剂来中和氧化剂。

[0121] 在优选实施方案中,步骤(b)的持续时间在0.1小时与10小时之间、0.5小时与5小时之间或0.5小时与2小时之间。在优选实施方案中,步骤(b)的持续时间为约0.5小时、1小时、1.5小时、2.5小时或3小时。

[0122] 在优选实施方案中,将步骤(b)中的反应温度维持在1℃与30℃之间、1℃与25℃之间、1℃与20℃之间、1℃与10℃之间、10℃与20℃之间、2℃与8℃之间。在优选实施方案中,将步骤(b)中的反应温度维持在约1℃、2℃、3℃、4℃、5℃、6℃、7℃或8℃。

[0123] 在优选实施方案中,步骤(b)中的pH在4与8之间、5与7之间、5.5与6.5之间。在优选实施方案中,步骤(a)中的pH为约6。

[0124] 在优选实施方案中,纯化活化的血清型10A、22F或33F多糖。可按照本领域技术人员已知的方法诸如凝胶渗透层析法(GPC)、透析或超滤/渗滤纯化活化的血清型10A、22F或

33F多糖。例如,通过使用超滤装置进行浓缩和渗滤来纯化活化的多糖。

[0125] 在优选实施方案中,通过包括以下步骤的方法来活化分离的血清型10A多糖:

[0126] (a)将分离的血清型10A多糖与高碘酸盐反应;和,

[0127] (b)通过添加丁-2,3-二醇来猝灭氧化反应,从而导致活化的血清型10A多糖。

[0128] 在优选实施方案中,通过包括以下步骤的方法来活化分离的血清型10A多糖:

[0129] (a)在2°C与8°C之间的温度下将分离的血清型10A多糖与高碘酸盐反应;和,

[0130] (b)通过在2°C与8°C之间的温度下添加丁-2,3-二醇来猝灭氧化反应,从而导致活化的血清型10A多糖。

[0131] 在优选实施方案中,通过包括以下步骤的方法来活化分离的血清型10A多糖:

[0132] (a)在2°C与8°C之间的温度下将分离的血清型10A多糖与0.2至0.3摩尔当量的高碘酸盐反应;和,

[0133] (b)通过在2°C与8°C之间的温度下添加0.5至2摩尔当量的丁-2,3-二醇来猝灭氧化反应,从而导致活化的血清型10A多糖。

[0134] 在优选实施方案中,通过包括以下步骤的方法来活化分离的血清型22F多糖:

[0135] (a)将分离的血清型22F多糖与高碘酸盐反应;和,

[0136] (b)通过添加丁-2,3-二醇来猝灭氧化反应,从而导致活化的血清型22F多糖。

[0137] 在优选实施方案中,通过包括以下步骤的方法来活化分离的血清型22F多糖:

[0138] (a)在2°C与8°C之间的温度下将分离的血清型22F多糖与高碘酸盐反应;和,

[0139] (b)通过在2°C与8°C之间的温度下添加丁-2,3-二醇来猝灭氧化反应,从而导致活化的血清型22F多糖。

[0140] 在优选实施方案中,通过包括以下步骤的方法来活化分离的血清型22F多糖:

[0141] (a)在2°C与8°C之间的温度下将分离的血清型22F多糖与0.05至0.2摩尔当量的高碘酸盐反应;和,

[0142] (b)通过在2°C与8°C之间的温度下添加2至3摩尔当量,优选2摩尔当量的丁-2,3-二醇来猝灭氧化反应,从而导致活化的血清型22F多糖。

[0143] 在优选实施方案中,通过包括以下步骤的方法来活化分离的血清型33F多糖:

[0144] (a)将分离的血清型33F多糖与高碘酸盐反应;和,

[0145] (b)通过添加丁-2,3-二醇来猝灭氧化反应,从而导致活化的血清型33F多糖。

[0146] 在优选实施方案中,通过包括以下步骤的方法来活化分离的血清型33F多糖:

[0147] (a)在2°C与8°C之间的温度下将分离的血清型33F多糖与高碘酸盐反应;和,

[0148] (b)通过在2°C与8°C之间的温度下添加丁-2,3-二醇来猝灭氧化反应,从而导致活化的血清型33F多糖。

[0149] 在优选实施方案中,通过包括以下步骤的方法来活化分离的血清型33F多糖:

[0150] (a)在2°C与8°C之间的温度下将分离的血清型33F多糖与0.05至0.2摩尔当量的高碘酸盐反应;和,

[0151] (b)通过在2°C与8°C之间的温度下添加0.5至1.5摩尔当量的丁-2,3-二醇来猝灭氧化反应,从而导致活化的血清型33F多糖。

[0152] 在优选实施方案中,本发明涉及通过或可通过上文中公开的方法获得的活化的血清型10A荚膜多糖。

[0153] 在优选实施方案中,本发明涉及通过或可通过上文中公开的方法获得的活化的血清型22F荚膜多糖。

[0154] 在优选实施方案中,本发明涉及通过或可通过上文中公开的方法获得的活化的血清型33F荚膜多糖。

[0155] 在优选实施方案中,获自步骤(b)的活化的血清型10A多糖具有步骤(a)中使用的分离的多糖的分子量的20%与100%之间、30%与95%之间、40%与95%之间、60%至95%、85%至95%或约90%的分子量。

[0156] 在优选实施方案中,获自步骤(b)的活化的血清型10A多糖具有步骤(a)中使用的分离的多糖的分子量的至少20%、25%、30%、35%、40%或45%的分子量。在优选实施方案中,获自步骤(b)的活化的血清型10A多糖具有步骤(a)中使用的分离的多糖的分子量的至少50%、51%、52%、53%、54%、55%、56%、57%、58%、59%、60%、61%、62%、63%、64%、65%、66%、67%、68%、69%、70%、71%、72%、73%、74%、75%、76%、77%、78%、79%、80%、81%、82%、83%、84%、85%、86%、87%、88%、89%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99%的分子量。

[0157] 在优选实施方案中,获自步骤(b)的活化的血清型22F多糖具有步骤(a)中使用的分离的多糖的分子量的50%与100%之间、60%至95%、85%至95%或约90%的分子量。

[0158] 在优选实施方案中,获自步骤(b)的活化的血清型22F多糖具有步骤(a)中使用的分离的多糖的分子量的至少50%、51%、52%、53%、54%、55%、56%、57%、58%、59%、60%、61%、62%、63%、64%、65%、66%、67%、68%、69%、70%、71%、72%、73%、74%、75%、76%、77%、78%、79%、80%、81%、82%、83%、84%、85%、86%、87%、88%、89%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99%的分子量。

[0159] 在优选实施方案中,获自步骤(b)的活化的血清型33F多糖具有步骤(a)中使用的分离的多糖的分子量的50%与100%之间、60%至95%、85%至95%或约90%的分子量。

[0160] 在优选实施方案中,获自步骤(b)的活化的血清型33F多糖具有步骤(a)中使用的分离的多糖的分子量的至少50%、51%、52%、53%、54%、55%、56%、57%、58%、59%、60%、61%、62%、63%、64%、65%、66%、67%、68%、69%、70%、71%、72%、73%、74%、75%、76%、77%、78%、79%、80%、81%、82%、83%、84%、85%、86%、87%、88%、89%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99%的分子量。

[0161] 在优选实施方案中,活化的血清型10A、22F或33F多糖的氧化程度在2与30之间、2与25之间、2与20之间、2与15之间、2与10之间、2与5之间、5与30之间、5与25之间、5与20之间、5与15之间、5与10之间、10与30之间、10与25之间、10与20之间、10与15之间、5与25之间、10与30之间、15与30之间、15与25之间、15与20之间、20至30、20至25。在优选实施方案中,活化的血清型10A、22F或33F多糖的氧化程度在2与10之间、4与8之间、4与6之间、6与8之间、6与12之间、8与14之间、9与11之间、10与16之间、12与16之间、14与18之间、16与20之间、16与18之间、18与22之间、18与20之间。

[0162] 在优选实施方案中,活化的血清型10A多糖具有在50kDa与400kDa之间、50kDa与350kDa之间、50kDa与300kDa之间、50kDa与250kDa之间、50kDa与200kDa之间、100kDa与300kDa之间、100kDa与250kDa之间或100kDa与200kDa之间的分子量。在优选实施方案中,活化的血清型10A多糖具有50kDa与300kDa之间的分子量。在优选实施方案中,活化的血清型

10A多糖具有100kDa与200kDa之间的分子量。在优选实施方案中,活化的血清型10A多糖具有100kDa与200kDa之间的分子量以及5与20之间、5与15之间、8与14之间、8与12之间或9与11之间的氧化程度。在优选实施方案中,活化的血清型10A多糖具有100kDa与200kDa之间的分子量以及9与11之间的氧化程度。

[0163] 在优选实施方案中,活化的血清型22F多糖具有25kDa与1000kDa之间、100kDa与1000kDa之间、300kDa与800kDa之间、300kDa与700kDa之间、300kDa与600kDa之间、400kDa与1000kDa之间、400kDa与800kDa之间、400kDa与700kDa之间或400kDa与600kDa之间的分子量。在优选实施方案中,活化的血清型22F多糖具有300kDa与800kDa之间的分子量。在优选实施方案中,活化的血清型22F多糖具有400kDa与800kDa之间的分子量。在优选实施方案中,活化的血清型22F多糖具有400kDa与600kDa之间的分子量。在优选实施方案中,活化的血清型22F多糖具有400kDa与800kDa之间的分子量以及10与25之间、10与20之间、12与20之间或14与18之间的氧化程度。在优选实施方案中,活化的血清型22F多糖具有400kDa与800kDa之间的分子量以及10与20之间的氧化程度。

[0164] 在优选实施方案中,活化的血清型22F多糖包含每mM血清型22F多糖至少0.1mM、0.2mM、0.3mM、0.4mM、0.5mM、0.6mM或0.7mM或约0.8mM的醋酸盐。在优选实施方案中,活化的血清型22F多糖包含每mM血清型22F多糖至少0.5、0.6或0.7mM的醋酸盐。在优选实施方案中,活化的血清型22F多糖包含每mM血清型22F多糖至少0.6mM的醋酸盐。在优选实施方案中,活化的血清型22F多糖包含每mM血清型22F多糖至少0.7mM的醋酸盐。

[0165] 在优选实施方案中,活化的血清型22F多糖具有400kDa与800kDa之间的分子量并且包含每mM血清型22F多糖至少0.6mM的醋酸盐。

[0166] 在优选实施方案中,活化的血清型22F多糖具有在400kDa与800kDa之间的分子量、12与20之间的氧化程度,并且包含每mM血清型22F多糖至少0.6mM的醋酸盐。

[0167] 在优选实施方案中,活化的血清型33F多糖具有50kDa与1250kDa之间、200kDa与1200kDa之间、500kDa与1200kDa之间、500kDa与1000kDa之间、700kDa与1200kDa之间、800kDa与1200kDa之间、800kDa与1100kDa之间、900kDa与1200kDa之间、800kDa与1000kDa之间的分子量。在优选实施方案中,活化的血清型33F多糖具有500kDa与1000kDa之间的分子量。在另一个优选实施方案中,活化的血清型33F多糖具有50kDa与600kDa之间、50kDa与500kDa之间或50kDa与400kDa之间的分子量。

[0168] 在优选实施方案中,活化的血清型33F多糖包含每mM血清型33F多糖至少0.1mM、0.2mM、0.3mM、0.4mM、0.5mM、0.6mM、0.7mM或0.8或约0.9mM的醋酸盐。在优选实施方案中,活化的血清型33F包含每mM血清型33F多糖至少0.6mM、0.7mM或0.8mM的醋酸盐。在优选实施方案中,活化的血清型33F多糖包含每mM血清型33F多糖至少0.6mM的醋酸盐。在优选实施方案中,活化的血清型33F多糖包含每mM血清型33F多糖至少0.7mM的醋酸盐。

[0169] 在优选实施方案中,活化的血清型33F多糖具有800kDa与1200kDa之间的分子量并且包含每mM血清型33F多糖至少0.7mM的醋酸盐。在优选实施方案中,活化的血清型33F多糖具有800kDa与1200kDa之间的分子量,包含每mM血清型33F多糖至少0.7mM的醋酸盐并且具有10与20之间的氧化程度。

[0170] 在优选实施方案中,活化的血清型33F多糖具有50kDa与200kDa之间的分子量并且包含每mM血清型33F多糖至少0.7mM的醋酸盐。

[0171] 在优选实施方案中,活化的血清型33F多糖具有50kDa与200kDa之间的分子量,包含每mM血清型33F多糖至少0.7mM的醋酸盐,并且具有10与20之间的氧化程度。

[0172] 在实施方案中,任选地在冷冻保护剂/冻干保护剂存在的情况下冷冻干燥活化的血清型10A、22F或33F多糖。在优选实施方案中,冷冻保护剂/冻干保护剂选自蔗糖、海藻糖、棉子糖、水苏糖、松三糖、葡聚糖、甘露醇、乳糖醇和帕拉金糖醇。在优选实施方案中,所述冷冻保护剂/冻干保护剂为蔗糖。随后可将冻干的活化的多糖与包含载体蛋白的溶液混合。

[0173] 在另一个实施方案中,将活化的血清型10A、22F或33F多糖与载体蛋白混合,并且任选地在冷冻保护剂/冻干保护剂存在的情况下将其冷冻干燥。在优选实施方案中,所述冷冻保护剂/冻干保护剂选自蔗糖、海藻糖、棉子糖、水苏糖、松三糖、葡聚糖、甘露醇、乳糖醇和帕拉金糖醇。在优选实施方案中,所述冷冻保护剂/冻干保护剂为蔗糖。可随后将共冻干的多糖和载体蛋白重悬浮于溶液中并与还原剂反应。

[0174] 在实施方案中,本发明涉及冻干的活化的血清型10A多糖。在实施方案中,本发明涉及冻干的活化的血清型22F多糖。在实施方案中,本发明涉及冻干的活化的血清型33F多糖。

[0175] 在实施方案中,本发明涉及共冻干的活化的血清型10A多糖和蛋白载体。在优选实施方案中,所述蛋白载体为CRM₁₉₇。在实施方案中,本发明涉及共冻干的活化的血清型22F多糖和蛋白载体。在优选实施方案中,所述蛋白载体为CRM₁₉₇。在实施方案中,本发明涉及共冻干的活化的血清型33F多糖和蛋白载体。在优选实施方案中,所述蛋白载体为CRM₁₉₇。

[0176] 利用载体蛋白缀合活化的血清型10A、22F或33F多糖

[0177] 可通过包括以下步骤的方法将本文中公开的活化的血清型10A、22F或33F多糖缀合于载体蛋白:

[0178] (a)将活化的血清型10A、22F或33F多糖与载体蛋白混合;和,

[0179] (b)将混合的活化的血清型10A、22F或33F多糖和载体蛋白与还原剂反应以形成血清型10A、22F或33F多糖:载体蛋白缀合物。

[0180] 相较于在水相中的还原胺化(其中多糖的O-乙酰化水平被显著降低),通过在二甲基亚砜(DMSO)中进行还原胺化的活化的血清型22F或33F多糖与蛋白质载体的缀合适于保留多糖的O-乙酰基含量。在优选实施方案中,在DMSO中进行步骤(a)和步骤(b)。

[0181] 在优选实施方案中,步骤(a)包括将冻干的血清型10A、22F或33F多糖溶解在包含载体蛋白的水溶液中或包含载体蛋白和DMSO的溶液中。在优选实施方案中,步骤(a)包括将共冻干的血清型10A、22F或33F多糖和载体蛋白溶解在水溶液中或DMSO中。

[0182] 当在水溶液中进行步骤(a)和(b)时,所述溶液包含缓冲液,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,pH在6.0与8.5之间、7与8之间或7与7.5之间。在优选实施方案中,所述缓冲液为PBS。在优选实施方案中,pH为约7.3。

[0183] 在优选实施方案中,步骤(a)中的活化的血清型10A、22F或33F多糖的浓度在0.1mg/mL与10mg/mL之间、0.5mg/mL与5mg/mL之间、0.5mg/mL与2mg/mL之间。在优选实施方案中,步骤(a)中的活化的血清型10A、22F或33F多糖的浓度为约0.1mg/mL、0.2mg/mL、0.3mg/mL、0.4mg/mL、0.5mg/mL、0.6mg/mL、0.7mg/mL、0.8mg/mL、0.9mg/mL、1mg/mL、1.1mg/mL、1.2mg/mL、1.3mg/mL、1.4mg/mL、1.5mg/mL、1.6mg/mL、1.7mg/mL、1.8mg/mL、1.9mg/mL、

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

[0184] 在优选实施方案中,活化的血清型10A、22F或33F多糖对载体蛋白的初始比率(重量/重量)在5:1与0.1:1之间、2:1与0.1:1之间、2:1与1:1之间、1.5:1与1:1之间、0.1:1与1:1之间、0.3:1与1:1之间、0.6:1与1:1.2之间。在优选实施方案中,活化的血清型10A、22F或33F多糖对载体蛋白的初始比率为约0.6:1和1:1.2。在优选实施方案中,活化的血清型10A、22F或33F多糖对载体蛋白的初始比率为约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、2:1。

[0185] 在优选实施方案中,在步骤(b)中,将活化的血清型10A、22F或33F多糖与0.1摩尔当量与10摩尔当量之间、0.5摩尔当量与5摩尔当量之间、0.5摩尔当量与2.5摩尔当量之间的还原剂反应。在优选实施方案中,在步骤(b)中,将活化的血清型10A、22F或33F与约0.5摩尔当量、0.6摩尔当量、0.7摩尔当量、0.8摩尔当量、0.9摩尔当量、1摩尔当量、1.1摩尔当量、1.2摩尔当量、1.3摩尔当量、1.4摩尔当量、1.5摩尔当量、1.6摩尔当量、1.7摩尔当量、1.8摩尔当量、1.9摩尔当量、2摩尔当量、2.1摩尔当量、2.2摩尔当量、2.3摩尔当量、2.4或2.5摩尔当量的还原剂反应。

[0186] 在实施方案中,所述还原剂为氰基硼氢化钠、三乙酰氧基硼氢化钠、硼氢化钠或锌(在布朗斯台德酸或路易斯酸存在的情况下)、胺硼烷诸如吡啶硼烷、2-甲基吡啶硼烷、2,6-二硼烷-甲醇、二甲胺-硼烷、 $t\text{-BuMe}^i\text{PrN-BH}_3$ 、苄胺- BH_3 或5-乙基-2-甲基吡啶硼烷(PEMB)。在优选实施方案中,所述还原剂是氰基硼氢化钠。

[0187] 在优选实施方案中,步骤(b)的持续时间在1小时与50小时、5小时与30小时、10小时与30小时、15小时与30小时、20小时与30小时、5小时与25小时、10小时与25小时、15小时与25小时之间。在优选实施方案中,步骤(b)的持续时间为约20小时、21小时、22小时、23小时、24小时、25小时、26小时、27小时、28小时。

[0188] 在优选实施方案中,将步骤(b)中的反应温度维持在10°C与40°C之间、15°C与30°C之间、20°C与26°C之间、21°C与25°C之间。在优选实施方案中,将步骤(b)中的反应温度维持在约21°C、22°C、23°C、24°C或25°C。

[0189] 在优选实施方案中,用于制备包含共价连接于载体蛋白的血清型10A、22F或33F多糖的免疫原性缀合物的方法还包括通过添加 NaBH_4 来对未反应的醛加帽(猝灭)的步骤(步骤c)。

[0190] 在优选实施方案中,在步骤(c)中,通过添加0.1摩尔当量至10摩尔当量、0.5摩尔当量至5摩尔当量或1摩尔当量至3摩尔当量的 NaBH_4 来对未反应的醛进行加帽。在优选实施方案中,在步骤(c)中,通过添加约2摩尔当量的 NaBH_4 来对未反应的醛进行加帽。

[0191] 在优选实施方案中,步骤(c)的持续时间在0.1小时与10小时之间、0.5小时与5小时之间或2小时与4小时之间。在优选实施方案中,步骤(c)的持续时间为约3小时。

[0192] 在优选实施方案中,将步骤(c)中的反应温度维持在10至40°C之间、15至30°C之间或20至26°C之间。在优选实施方案中,将步骤(c)中的反应温度维持在约23°C。

[0193] 在将血清型10A、22F或33F多糖缀合于载体蛋白之后,可通过本领域技术人员已知的各种技术来纯化(针对多糖-蛋白质缀合物进行富集)多糖-蛋白质缀合物。这些技术包括透析、浓缩/渗滤操作、切向流过滤沉淀/洗脱、柱层析(DEAE或疏水相互作用层析)和深度过

滤。

[0194] 在优选实施方案中,所述载体蛋白是无毒的和非反应原性的,并且可以以足够的量和纯度获得。载体蛋白应当顺从于标准缀合方法。

[0195] 在优选实施方案中,将活化的血清型10A、22F或33F多糖缀合于载体蛋白,载体蛋白选自DT(白喉毒素)、TT(破伤风类毒素)或TT的片段C、CRM197(白喉毒素的无毒性的但抗原性上相同的变体)、其它DT点突变体,诸如CRM176、CRM228、CRM 45(Uchida等J.Biol.Chem.218:3838-3844,1973);CRM 9、CRM102、CRM 103和CRM107以及由Nicholls和Youle在Genetically Engineered Toxins,Ed:Frankel,Maecel Dekker Inc,1992中描述的其它突变;Glu-148的缺失或Glu-148至Asp、Gln或Ser的突变和/或Ala 158的缺失或Ala 158至Gly的突变以及US 4709017或US 4950740中公开的其它突变;至少一个或多个残基Lys 516、Lys 526、Phe 530和/或Lys 534的突变以及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 Immun63:2706-13),包括以一定方式脱毒的ply例如dPLY-GMBS(W004081515,PCT/EP2005/010258)或dPLY-甲醛、PhtX,包括PhtA、PhtB、PhtD、PhtE(PhtA,PhtB,PhtD或PhtE的序列公开于W000/37105或W0 00/39299中)以及Pht蛋白的融合物例如PhtDE融合物、PhtBE融合物、Pht A-E(W0 01/98334,W0 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群B提取的-EP0372501)、PorB(来自脑膜炎奈瑟球菌)、PD(流感嗜血杆菌蛋白D-参见,例如,EP 0594610B)、或其免疫功能等同物、合成肽(EP0378881,EP0427347)、热休克蛋白(W0 93/17712,W094/03208)、百日咳蛋白(W0 98/58668,EP0471177)、细胞因子、淋巴因子、生长因子或激素(W01091/01146)、包含来自各种病原体来源的抗原的多个人CD4+T细胞表位的人T.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(W0 02/091998)、铁摄取蛋白(W0 01/72337)、艰难梭菌(C.difficile)的毒素A或B(W000/61761)。在实施方案中,将活化的血清型10A、22F或33F多糖缀合于DT(白喉类毒素)。在另一个实施方案中,将活化的血清型10A、22F或33F多糖缀合于TT(破伤风类毒素)。在另一个实施方案中,将活化的血清型10A、22F或33F多糖缀合于TT的片段C。在另一个实施方案中,将活化的血清型10A、22F或33F多糖缀合于PD(流感嗜血杆菌蛋白D-参见,例如,EP 0594610B)。

[0196] 在优选实施方案中,将活化的血清型10A、22F或33F多糖缀合于CRM197蛋白。所述CRM197蛋白为白喉毒素的无毒形式,但其在免疫上与白喉毒素无区别。CRM197由被通过产毒 β 棒状噬菌体的亚硝基胍诱变产生的非产毒噬菌体 β 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层析纯化CRM197。CRM197蛋白具有与白喉毒素相同的分子量但与其相异在于结构基因中的单碱基变化(鸟嘌呤至腺嘌呤)。该单碱基变化在成熟蛋白质中引起氨基酸取代(谷氨酸对甘氨酸的取代)并且消除白喉毒素的毒性性质。CRM197蛋白是糖类的安全有效的T细胞依赖性载体。关于CRM197及其生产的其它详细内容可见于例如US5614382中。

[0197] 在优选实施方案中,通过包括以下步骤的方法将本文中公开的活化的血清型10A、22F或33F多糖缀合于CRM197:

[0198] (a)将活化的血清型10A、22F或33F多糖与CRM197混合;

[0199] (b)将混合的活化的血清型10A、22F或33F多糖和CRM₁₉₇与氰基硼氢化钠反应,以形成血清型10A、22F或33F多糖:CRM₁₉₇缀合物。

[0200] 在优选实施方案中,通过包括以下步骤的方法将本文中公开的活化的血清型10A、22F或33F多糖缀合于CRM₁₉₇:

[0201] (a)将活化的血清型10A、22F或33F多糖与CRM₁₉₇混合;

[0202] (b)将混合的活化的血清型10A、22F或33F多糖和CRM₁₉₇与氰基硼氢化钠反应,以形成血清型10A、22F或33F多糖:CRM₁₉₇缀合物;

[0203] 其中在DMSO中进行步骤(a)和(b)。

[0204] 在优选实施方案中,通过包括以下步骤的方法将本文中公开的活化的血清型10A、22F或33F多糖缀合于CRM₁₉₇:

[0205] (a)将活化的血清型10A、22F或33F多糖与CRM₁₉₇混合;

[0206] (b)将混合的活化的血清型10A、22F或33F多糖和CRM₁₉₇与0.5摩尔当量至2摩尔当量的氰基硼氢化钠反应,以形成血清型10A、22F或33F多糖:CRM₁₉₇缀合物;

[0207] 其中在DMSO中进行步骤(a)和(b)。

[0208] 在实施方案中,本发明涉及通过或可通过本文中公开的方法获得的免疫原性缀合物。在优选实施方案中,本发明涉及通过或可通过将如本文中公开的血清型10A、22F或33F活化的多糖缀合(通过还原胺化)于载体蛋白获得的免疫原性缀合物。在优选实施方案中,本发明涉及通过或可通过将本文中公开的血清型10A、22F或33F活化的多糖缀合(通过在DMSO中进行还原胺化)于载体蛋白获得的免疫原性缀合物。在优选实施方案中,所述载体蛋白为CRM₁₉₇。

[0209] 血清型10A免疫原性缀合物

[0210] 在优选实施方案中,血清型10A免疫原性缀合物具有500kDa与15000kDa之间、500kDa与10000kDa之间、2000kDa与10000kDa之间或3000kDa与8000kDa之间的分子量。在优选实施方案中,血清型10A免疫原性缀合物具有3000kDa与8000kDa之间的分子量。免疫原性缀合物的分子量通过SEC-MALLS来测量。

[0211] 在优选实施方案中,血清型10A免疫原性缀合物包含相较于血清型10A多糖的总量少于约50%、45%、40%、35%、30%、25%、20%或15%的游离血清型10A多糖。在优选实施方案中,血清型10A免疫原性缀合物包含相较于血清型10A多糖的总量少于约25%的游离血清型10A多糖。在优选实施方案中,血清型10A免疫原性缀合物包含相较于血清型10A多糖的总量少于约20%的游离血清型10A多糖。在优选实施方案中,血清型10A免疫原性缀合物包含相较于血清型10A多糖的总量少于约15%的游离血清型10A多糖。

[0212] 在优选实施方案中,缀合物中的血清型10A多糖对载体蛋白的比率(重量/重量)在0.5与3之间。在优选实施方案中,缀合物中的血清型10A多糖对载体蛋白的比率在0.5与2之间、0.5与1.5之间、0.5与1之间、1与1.5之间、1与2之间。在优选实施方案中,缀合物中的血清型10A多糖对载体蛋白的比率在0.8与1.4之间。在优选实施方案中,缀合物中的血清型10A荚膜多糖对载体蛋白的比率在0.8与1.2之间。

[0213] 大小排阻层析介质(CL-4B)可被用来测定缀合物的相对分子大小分布。将大小排阻层析(SEC)用于重力进料柱中以分析缀合物的分子大小分布。从介质中的孔排斥的大分子比小分子更快地洗脱。将级分收集器用于收集柱洗脱液。通过糖类测定来比色测试级分。

为了测定 K_d ,将柱校准以确定分子被完全排斥时级分(V_0),($K_d=0$),和代表最大持留量的级分(V_i),($K_d=1$)。达到指定的样品属性时的级分(V_e)按照表达式 $K_d=(V_e-V_0)/(V_i-V_0)$ 与 K_d 相关。

[0214] 在优选实施方案中,至少30%的血清型10A免疫原性缀合物具有在CL-4B柱中低于或等于0.3的 K_d 。在优选实施方案中,至少40%的血清型10A免疫原性缀合物具有在CL-4B柱中低于或等于0.3的 K_d 。在优选实施方案中,至少45%、50%、55%、60%、65%、70%、75%、80%或85%的血清型10A免疫原性缀合物具有在CL-4B柱中低于或等于0.3的 K_d 。在优选实施方案中,至少60%的血清型10A免疫原性缀合物具有在CL-4B柱中低于或等于0.3的 K_d 。在优选实施方案中,50%与80%之间的血清型10A免疫原性缀合物具有在CL-4B柱中低于或等于0.3的 K_d 。

[0215] 缀合程度是载体蛋白中缀合于目标多糖的赖氨酸残基的数目。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因与多糖的共价键联而引起的载体蛋白的赖氨酸修饰的证据。缀合导致相较于用于产生缀合物材料的CRM197蛋白起始材料,回收的赖氨酸残基的数目的减少。

[0216] 在优选实施方案中,免疫原性缀合物的缀合程度在2与15之间、2与13之间、2与10之间、2与8之间、2与6之间、2与5之间、2与4之间、3与15之间、3与13之间、3与10之间、3与8之间、3与6之间、3与5之间、3与4之间、5与15之间、5与10之间、8与15之间、8与12之间、10与15之间或10与12之间。在优选实施方案中,免疫原性缀合物的缀合程度在6与8之间。

[0217] 血清型22F免疫原性缀合物

[0218] 在优选实施方案中,免疫原性缀合物包含每mM血清型22F多糖至少0.1mM、0.2mM、0.3mM、0.4mM、0.5mM、0.6mM或0.7mM或约0.8mM的醋酸盐。在优选实施方案中,免疫原性缀合物包含每mM血清型22F多糖至少0.5mM、0.6mM或0.7mM的醋酸盐。在优选实施方案中,免疫原性缀合物包含每mM血清型22F多糖至少0.6mM的醋酸盐。在优选实施方案中,免疫原性缀合物包含每mM血清型22F多糖至少0.7mM的醋酸盐。

[0219] 在优选实施方案中,免疫原性缀合物中每mM血清型22F多糖的醋酸盐的mM数对分离的多糖中每mM血清型22F多糖的醋酸盐的mM数的比率为至少0.6、0.65、0.7、0.75、0.8、0.85、0.9或0.95。在优选实施方案中,免疫原性缀合物中每mM血清型22F多糖的醋酸盐的mM数对分离的多糖中每mM血清型22F多糖的醋酸盐的mM数的比率为至少0.7。在优选实施方案中,免疫原性缀合物中每mM血清型22F多糖的醋酸盐的mM数对分离的多糖中每mM血清型22F多糖的醋酸盐的mM数的比率为至少0.9。

[0220] 在优选实施方案中,免疫原性缀合物中每mM血清型22F多糖的醋酸盐的mM数对活化的多糖中每mM血清型22F多糖的醋酸盐的mM数的比率为至少0.6、0.65、0.7、0.75、0.8、0.85、0.9或0.95。在优选实施方案中,免疫原性缀合物中每mM血清型22F多糖的醋酸盐的mM数对活化的多糖中每mM血清型22F多糖的醋酸盐的mM数的比率为至少0.7。在优选实施方案中,免疫原性缀合物中每mM血清型22F多糖的醋酸盐的mM数对活化的多糖中每mM血清型22F多糖的醋酸盐的mM数的比率为至少0.9。

[0221] 在优选实施方案中,血清型22F免疫原性缀合物具有在400kDa与15000kDa之间、500kDa与10000kDa之间、2000kDa与10000kDa之间、3000kDa与8000kDa之间或3000kDa与5000kDa之间的分子量。在优选实施方案中,血清型22F免疫原性缀合物具有在3000kDa与

5000kDa之间的分子量。通过SEC-MALLS测量所述免疫原性缀合物的分子量。

[0222] 在优选实施方案中,血清型22F免疫原性缀合物包含相较于血清型22F多糖的总量少于约50%、45%、40%、35%、30%、25%、20%或15%的游离血清型22F多糖。在优选实施方案中,血清型22F免疫原性缀合物包含相较于血清型22F多糖的总量少于约40%的游离血清型22F多糖。在优选实施方案中,血清型22F免疫原性缀合物包含相较于血清型22F多糖的总量少于约25%的游离血清型22F多糖。在优选实施方案中,血清型22F免疫原性缀合物包含相较于血清型22F多糖的总量少于约20%的游离血清型22F多糖。在优选实施方案中,血清型22F免疫原性缀合物包含相较于血清型22F多糖的总量少于约15%的游离血清型22F多糖。

[0223] 在优选实施方案中,缀合物中的血清型22F多糖对载体蛋白的比率(重量/重量)在0.5与3之间。在优选实施方案中,缀合物中的血清型22F多糖对载体蛋白的比率在0.5与2之间、0.5与1.5之间、0.8与1.2之间、0.5与1之间、1与1.5之间或1与2之间。在优选实施方案中,缀合物中的血清型22F多糖对载体蛋白的比率在0.8与1.2之间。在优选实施方案中,缀合物中的血清型22F荚膜多糖对载体蛋白的比率在0.9与1.1之间。

[0224] 在优选实施方案中,至少30%的血清型22F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,至少40%的免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,至少45%、50%、55%、60%、65%、70%、75%、80%、或85%的血清型22F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,至少60%的血清型22F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,50%与80%之间的血清型22F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,65%与80%之间的血清型22F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。

[0225] 在优选实施方案中,免疫原性缀合物的缀合程度在2与15之间、2与13之间、2与10之间、2与8之间、2与6之间、2与5之间、2与4之间、3与15之间、3与13之间、3与10之间、3与8之间、3与6之间、3与5之间、3与4之间、4与7之间、5与15之间、5与10之间、8与15之间、8与12之间、10与15之间或10与12之间。在优选实施方案中,免疫原性缀合物的缀合程度在4与7之间。

[0226] 血清型33F免疫原性缀合物

[0227] 在优选实施方案中,免疫原性缀合物包含每mM血清型33F多糖至少0.1mM、0.2mM、0.3mM、0.4mM、0.5mM、0.6mM或0.7mM或约0.8mM的醋酸盐。在优选实施方案中,免疫原性缀合物包含每mM血清型33F多糖至少0.5mM、0.6mM或0.7mM的醋酸盐。在优选实施方案中,免疫原性缀合物包含每mM血清型33F多糖至少0.6mM的醋酸盐。在优选实施方案中,免疫原性缀合物包含每mM血清型33F多糖至少0.7mM的醋酸盐。

[0228] 在优选实施方案中,免疫原性缀合物中每mM血清型33F多糖的醋酸盐的mM数对分离的多糖中每mM血清型33F多糖的醋酸盐的mM数的比率为至少0.6、0.65、0.7、0.75、0.8、0.85、0.9或0.95。在优选实施方案中,免疫原性缀合物中每mM血清型33F多糖的醋酸盐的mM数对分离的多糖中每mM血清型33F多糖的醋酸盐的mM数的比率为至少0.7。在优选实施方案中,免疫原性缀合物中每mM血清型33F多糖的醋酸盐的mM数对分离的多糖中每mM血清型33F多糖的醋酸盐的mM数的比率为至少0.9。

[0229] 在优选实施方案中,免疫原性缀合物中每mM血清型33F多糖的醋酸盐的mM数对活化的多糖中每mM血清型33F多糖的醋酸盐的mM数的比率为至少0.6、0.65、0.7、0.75、0.8、0.85、0.9或0.95。在优选实施方案中,免疫原性缀合物中每mM血清型33F多糖的醋酸盐的mM数对活化的多糖中每mM血清型33F多糖的醋酸盐的mM数的比率为至少0.7。在优选实施方案中,免疫原性缀合物中每mM血清型33F多糖的醋酸盐的mM数对活化的多糖中每mM血清型33F多糖的醋酸盐的mM数的比率为至少0.9。

[0230] 在优选实施方案中,血清型33F免疫原性缀合物具有在500kDa与30000kDa之间、500kDa与25000kDa之间、500kDa与20000kDa之间、500kDa与15000kDa之间、500kDa与10000kDa之间、1000kDa与10000kDa之间、1000kDa与8000kDa之间、1000kDa与5000kDa之间、2000kDa与10000kDa之间、2000kDa与8000kDa之间或2000kDa与5000kDa之间的分子量。在优选实施方案中,血清型33F免疫原性缀合物具有在1000kDa与5000kDa之间的分子量。通过SEC-MALLS测量所述免疫原性缀合物的分子量。

[0231] 在优选实施方案中,血清型33F免疫原性缀合物包含相较于血清型33F多糖的总量少于约50%、45%、40%、35%、30%、25%、20%或15%的游离血清型33F多糖。在优选实施方案中,血清型33F免疫原性缀合物包含相较于血清型33F多糖的总量少于约25%的游离血清型33F多糖。在优选实施方案中,血清型33F免疫原性缀合物包含相较于血清型33F多糖的总量少于约20%的游离血清型33F多糖。在优选实施方案中,血清型33F免疫原性缀合物包含相较于血清型33F多糖的总量少于约15%的游离血清型33F多糖。

[0232] 在优选实施方案中,缀合物中血清型33F多糖对载体蛋白的比率(重量/重量)在0.4与3之间。在优选实施方案中,缀合物中的血清型33F多糖对载体蛋白的比率在0.5与2之间、0.5与1.5之间、0.5与1之间、1与1.5之间、1与2之间。在优选实施方案中,缀合物中的血清型33F多糖对载体蛋白的比率在0.5与1.5之间。在优选实施方案中,缀合物中的血清型33F荚膜多糖对载体蛋白的比率在0.5与1.2之间。

[0233] 在优选实施方案中,至少30%的血清型33F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,至少40%的免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,至少45%、50%、55%、60%、65%的血清型33F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,至少60%的血清型33F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,40%与80%之间的血清型33F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。在优选实施方案中,45%与65%之间的血清型33F免疫原性缀合物具有在CL-4B柱中低于或等于0.3的Kd。

[0234] 在优选实施方案中,免疫原性缀合物的缀合程度在1与15之间、2与13之间、2与10之间、2与8之间、2与6之间、2与5之间、2与4之间、3与15之间、3与13之间、3与10之间、3与8之间、3与6之间、3与5之间、3与4之间、5与15之间、5与10之间、8与15之间、8与12之间、10与15之间或10与12之间。在优选实施方案中,免疫原性缀合物的缀合程度在3与6之间。

[0235] 免疫原性组合物

[0236] 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,所述组合物可用于引发受试者的免疫应答。

[0237] 如本文中所用,“免疫原性”意指抗原(或抗原的表位)(诸如细菌荚膜多糖)或包含抗原的糖缀合物或免疫原性组合物引发宿主诸如哺乳动物的免疫应答(体液或细胞介导的

或两者)的能力。

[0238] 在优选实施方案中,免疫原性组合物包含通过本文中公开的方法获得的血清型10A、22F或33F缀合物。在优选实施方案中,免疫原性组合物包含可通过本文中公开的方法获得的血清型10A、22F或33F缀合物。

[0239] 在实施方案中,当向受试者施用,本文中公开的免疫原性组合物诱导能够结合血清型10A肺炎链球菌的抗体的形成。在实施方案中,当向受试者施用,本文中公开的免疫原性组合物诱导如通过标准ELISA测定测量的能够结合血清型10A肺炎链球菌的抗体的形成。

[0240] 在实施方案中,当向受试者施用,本文中公开的免疫原性组合物诱导在如本文公开的调理吞噬测定中能够杀死血清型10A肺炎链球菌的抗体的形成。

[0241] 在实施方案中,当向受试者施用,本文中公开的免疫原性组合物诱导能够结合血清型22F肺炎链球菌的抗体的形成。在实施方案中,当向受试者施用,本文中公开的免疫原性组合物诱导如通过标准ELISA测定测量的能够结合血清型22F肺炎链球菌的抗体的形成。

[0242] 在实施方案中,当向受试者施用,本文中公开的免疫原性组合物诱导在如本文公开的调理吞噬测定中能够杀死血清型22F肺炎链球菌的抗体的形成。

[0243] 在实施方案中,当向受试者施用,本文中公开的免疫原性组合物诱导能够结合血清型33F肺炎链球菌的抗体的形成。在实施方案中,当向受试者施用,本文中公开的免疫原性组合物诱导如通过标准ELISA测定测量的能够结合血清型33F肺炎链球菌的抗体的形成。

[0244] 在实施方案中,当向受试者施用,本文中公开的免疫原性组合物诱导在如本文中公开的调理吞噬测定中能够杀死血清型33F肺炎链球菌的抗体的形成。

[0245] 可使用本领域公认的方法实现本发明的免疫原性组合物的配制。例如,可用生理上可接受的媒介物配制血清型10A、22F或33F缀合物,以制备组合物。这样的媒介物的实例包括,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)和葡萄糖溶液。

[0246] 在优选实施方案中,免疫原性组合物可包含至少一种另外的抗原。在优选实施方案中,免疫原性组合物可包含至少一种另外的肺炎链球菌荚膜多糖。

[0247] 在优选实施方案中,免疫原性组合物可包含至少一种另外的缀合于载体蛋白的肺炎链球菌荚膜多糖。在优选实施方案中,所述载体蛋白为CRM₁₉₇。

[0248] 在某些实施方案中,免疫原性组合物包含一种或多种佐剂。如本文中所定义的,“佐剂”为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,佐剂通常被给予来加强免疫应答,并且对于本领域技术人员来说是公知的。增强组合物的功效的合适的佐剂包括,但不限于:

[0249] (1)铝盐(明矾),诸如氢氧化铝、磷酸铝、硫酸铝等;

[0250] (2)水包油乳液制剂(具有或不具有其它特定的免疫刺激剂诸如胞壁酰肽(下文中定义的)或细菌细胞壁组分),诸如,例如,

[0251] (a)MF59(PCT公开号WO 90/14837),其含有5%角鲨烯,0.5%5Tween 80和0.5%Span 85(任选地含有不同量的MTP-PE(参见下文,尽管不是必需的)),通过使用微流化器诸如Model 110Y微流化器(Microfluidics, Newton, MA)配制成亚微米颗粒,

[0252] (b)SAF,其含有10%角鲨烯,0.4%Tween 80,5%pluronic-嵌段聚合物L121和thr-MDP(参见下文),其被微流化成亚微米乳剂或经涡旋以产生较大粒径的乳剂,和

[0253] (c)Ribi™佐剂系统(RAS),(Corixa,Hamilton,MT),其含有2%角鲨烯,0.2%Tween 80和一种或多种细菌细胞壁组分,所述细菌细胞壁组分来自美国专利No.4,912,094(Corixa)中描述的3-O-脱酰基单磷酸脂质A(MPL™)、海藻糖二霉菌酸酯(TDM)和细胞壁骨架(CWS),优选为MPL+CWS(Detox™);

[0254] (3)皂苷佐剂,诸如可使用Quil A或STIMULON™ QS-21(Antigenics,Framingham,MA)(美国专利No.5,057,540)或从其产生的颗粒诸如ISCOM(免疫刺激复合物);

[0255] (4)细菌脂多糖、合成的脂质A类似物诸如氨基烷基氨基葡萄糖胺磷酸盐化合物(AGP),或其衍生物或类似物,其可获自Corixa,并且其描述于美国专利No.6,113,918中;一种这样的AGP为2-[(R)-3-十四酰氧基十四酰氨基]乙基2-脱氧-4-O-磷酸基-3-O-[(R)-3-十四酰氧基十四酰]-2-[(R)-3-十四酰氧基十四酰氨基]-β-D-吡喃葡萄糖苷,其也被称为529(先前称为RC529),其被配制为含水形式或稳定的乳剂,合成的多核苷酸诸如含CpG基序的寡核苷酸(美国专利No.6,207,646);

[0256] (5)细胞因子,诸如白细胞介素(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;

[0257] (6)野生型或突变体形式的细菌ADP-核糖基化毒素诸如霍乱毒素(CT)的脱毒突变体,例如,其中氨基酸位置29上的谷氨酸被另一种氨基酸优选组氨酸替代(根据公开的国际专利申请号WO 00/18434(也参见WO 02/098368和WO 02/098369)),百日咳毒素(PT)或大肠杆菌(E.coli)不耐热毒素(LT),尤其是LT-K63、LT-R72、CT-S109、PT-K9/G129(参见,例如,WO 93/13302和WO 92/19265);和

[0258] (7)用作增强组合物的功效的免疫刺激剂的其它物质。

[0259] 胞壁酰肽包括,但不限于,N-乙酰基-胞壁酰-L-苏氨酸-D-异谷酰胺(thr-MDP)、N-乙酰基-正胞壁酰-L-丙氨酸-2-(1'-2'-二棕榈酰-sn-甘油-3-羟基磷酸氧基)-β-胺(MTP-PE)等。

[0260] 在本发明的实施方案中,如本文公开的免疫原性组合物包含CpG寡核苷酸作为佐剂。如本文所用的CpG寡核苷酸是指免疫刺激性CpG寡脱氧核苷酸(CpG ODN),因此,除非另有所指,否则这些术语可互换使用。免疫刺激性CpG寡脱氧核苷酸含有(任选地在某些优选的碱基背景中)一个或多个为未甲基化的胞嘧啶-鸟嘌呤二核苷酸的免疫刺激性CpG基序。所述CpG免疫刺激性基序的甲基化状态通常是指二核苷酸中的胞嘧啶残基。含有至少一个未甲基化的CpG二核苷酸的免疫刺激性寡核苷酸为含有通过磷酸键连接于3'鸟嘌呤的5'未甲基化的胞嘧啶,并且通过结合于Toll-样受体9(TLR-9)激活免疫系统的寡核苷酸。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,其将通过TLR9激活免疫系统,但不如CpG基序未被甲基化那样强。CpG免疫刺激性寡核苷酸可包含一个或多个回文序列,所述回文序列继而可包含CpG二核苷酸。已在许多颁布的专利、公开的专利申请以及其它出版物,包括美国专利No.6,194,388;6,207,646;6,214,806;6,218,371;6,239,116和6,339,068中描述了CpG寡核苷酸。

[0261] 在本发明的实施方案中,如本文公开的免疫原性组合物包含在WO2010/125480的

第3页第22行至第12页第36行描述的CpG寡核苷酸的任一个。

[0262] 已鉴定了不同种类的CpG免疫刺激性寡核苷酸。这些种类被称为A、B、C和P类,并且更详细地描述于W02010/125480的第3页第22行至第12页第36行。本发明的方法包括这些不同种类的CpG免疫刺激性寡核苷酸的使用。

[0263] 在本发明的实施方案中,如本文公开的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,_是指磷酸二酯键。

[0264] 在本发明的实施方案中,如本文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寡核苷酸为由至少下式代表的B类CpG寡核苷酸:

[0265] 5'X₁X₂CGX₃X₄ 3',其中X₁、X₂、X₃和X₄为核苷酸。在一个实施方案中,X₂为腺嘌呤、鸟嘌呤或胸腺嘧啶。在另一个实施方案中,X₃为胞嘧啶、腺嘌呤或胸腺嘧啶。

[0266] 本发明的B类CpG寡核苷酸序列是上文中广泛描述的美国专利No.6,194,388、6,207,646、6,214,806、6,218,371、6,239,116和6,339,068中的那些。示例性序列包括但不限于后面这些的申请和专利中公开的那些序列。

[0267] 在实施方案中,本发明的“B类”CpG寡核苷酸具有以下核酸序列:

[0268] 5'TCGTCGTTTTTCGGTCGTTTT 3'(SEQ ID NO:3),或

[0269] 5'TCGTCGTTTTTCGGTCGTTTT 3'(SEQ ID NO:4),或

[0270] 5'TCGTCGTTTTGTCGTTTTGTCGTT 3'(SEQ ID NO:5),或

[0271] 5'TCGTCGTTTCGTCGTTTTGTCGTT 3'(SEQ ID NO:6),或

[0272] 5'TCGTCGTTTTGTCGTTTTTTTCGA 3'(SEQ ID NO:7)。

[0273] 在任何这些序列中,所有键联可以均为硫代磷酸酯键。在另一个实施方案中,在任何这些序列中,一个或多个键联可以是磷酸二酯键,优选在产生半软CpG寡核苷酸的CpG基序的“C”与“G”之间。在任何这些序列中,乙基-尿苷或卤素可取代5'T;卤素取代的实例包括但不限于:溴-尿苷或碘-尿苷取代。

[0274] B-类寡核苷酸的一些非限制性实例包括:

[0275] 5'T*C*G*T*C*G*T*T*T*T*T*T*C*G*G*T*G*C*T*T*T*T 3'(SEQ ID NO:8),或

[0276] 5'T*C*G*T*C*G*T*T*T*T*T*T*T*C*G*G*T*C*G*T*T*T*T 3'(SEQ ID NO:9),或

[0277] 5'

[0278] 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),或

[0279] 5'

[0280] T*C*G*T*C*G*T*T*T*T*T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T 3'(SEQ ID NO:11),或

[0281] 5'

[0282] T*C*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T*T*T*T*T*T*T*T*C*G*A 3'(SEQ ID NO:12),

[0283] 其中*是指硫代磷酸酯键。

[0284] 在本发明的实施方案中,如本文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寡核苷酸具有以下核酸序列:

[0285] 5'TCGCGTCGTTCCGCGCGCGCCG 3'(SEQ ID NO:13),或

[0286] 5'TCGTCGACGTTCCGCGCGCGCCG 3'(SEQ ID NO:14),或

- [0287] 5'TCGGACGTTTCGGCGCGCGCCG 3'(SEQ ID NO:15),或
 [0288] 5'TCGGACGTTTCGGCGCGCGCCG 3'(SEQ ID NO:16),或
 [0289] 5'TCGCGTCGTTTCGGCGCGCGCCG 3'(SEQ ID NO:17),或
 [0290] 5'TCGACGTTTCGGCGCGCGCCG 3'(SEQ ID NO:18),或
 [0291] 5'TCGACGTTTCGGCGCGCGCCG 3'(SEQ ID NO:19),或
 [0292] 5'TCGCGTCGTTTCGGCGCGCCG 3'(SEQ ID NO:20),或
 [0293] 5'TCGCGACGTTTCGGCGCGCGCCG 3'(SEQ ID NO:21),或
 [0294] 5'TCGTCGTTTTTCGGCGCGCGCCG 3'(SEQ ID NO:22),或
 [0295] 5'TCGTCGTTTTTCGGCGCGCGCCG 3'(SEQ ID NO:23),或
 [0296] 5'TCGTCGTTTTACGGCGCGCGTGCCG 3'(SEQ ID NO:24),或
 [0297] 5'TCGTCGTTTTTCGGCGCGCGCCGT 3'(SEQ ID NO:25)。

[0298] 在任何这些序列中,所有键联可以均为硫代磷酸酯键。在另一个实施方案中,在任何这些序列中,一个或多个键联可以是磷酸二酯键,优选在产生半软CpG寡核苷酸的CpG基序的“C”与“G”之间。

[0299] C-类寡核苷酸的一些非限定性实例包括:

- [0300] 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
 [0301] 5'
 [0302] 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
 [0303] 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
 [0304] 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
 [0305] 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
 [0306] 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
 [0307] 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
 [0308] 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
 [0309] 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
 [0310] 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
 [0311] 5'T*C*G*T*C*G*T*T*T*T*T*C*G*G*C*G*G*C*C*G*C*C*G3'(SEQ ID NO:36),或
 [0312] 5'
 [0313] 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
 [0314] 5'
 [0315] T*C_G*T*C*G*T*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G*T 3'(SEQ ID NO:38),
 [0316] 其中*是指硫代磷酸酯键,_是指磷酸二酯键。

[0317] 在任何这些序列中,乙基-尿苷或卤素可取代5'T;卤素取代的实例包括但不限于溴-尿苷或碘-尿苷取代。

[0318] 在本发明的实施方案中,如本文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寡核苷酸为P类CpG寡核苷酸,其含有5'TLR活化结构域和至少2个回文区,一个回文区为长度为至少6个核苷酸的5'回文区,其直接或通过间隔子连接至长度为至少8个核苷酸的3'回文区,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

[0319] (SEQ ID NO:27)。在一个实施方案中,P类CpG寡核苷酸包括至少一个未甲基化的CpG二核苷酸。在另一个实施方案中,所述TLR活化结构域为TCG、TTCG、TTTCG、TYpR、TTYpR、TTTYpR、UCG、UUCG、UUUCG、TTT或TTTT。在另一个实施方案中,所述TLR活化结构域在5'回文区内。在另一个实施方案中,所述TLR活化结构域紧接所述5'回文区的5'。

[0320] 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)。

[0321] 在所述序列中,所有键联可以均为硫代磷酸酯键。在另一个实施方案中,一个或多个键联可以是磷酸二酯键,优选在产生半软CpG寡核苷酸的CpG基序的“C”与“G”之间。在任何这些序列中,乙基-尿苷或卤素可取代5'T;卤素取代的实例包括但不限于溴-尿苷或碘-尿苷取代。

[0322] P-类寡核苷酸的非限制性实例包括:

[0323] 5'

[0324] 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)

[0325] ,其中*是指硫代磷酸酯键,_是指磷酸二酯键。

[0326] 在一个实施方案中,寡核苷酸包括至少一个硫代磷酸酯键联。在另一个实施方案中,寡核苷酸的所有核苷酸间键联为硫代磷酸酯键联。在另一个实施方案中,寡核苷酸包括至少一个磷酸二酯样键联。在另一个实施方案中,所述磷酸二酯样键联为磷酸二酯键联。在另一个实施方案中,将亲脂性基团缀合于所述寡核苷酸。在一个实施方案中,所述亲脂性基团为胆固醇。

[0327] 在实施方案中,本文公开的CpG寡核苷酸的所有核苷酸间键联为磷酸二酯键(“软”寡核苷酸,如PCT申请W02007/026190中描述的)。在另一个实施方案中,使本发明的CpG寡核苷酸被赋予对降解的抗性(例如,被稳定化)。“稳定化的寡核苷酸”是指对体内降解(例如经由外切或内切核酸酶)相对耐受的寡核苷酸。可通过主链修饰实现核酸稳定化。具有硫代磷酸酯键联的寡核苷酸提供了最大活性并且保护寡核苷酸免受细胞内外切和内切核酸酶的降解。

[0328] 免疫刺激性寡核苷酸可具有嵌合主链,其具有磷酸二酯键联与硫代磷酸酯键联的组合。为本发明的目的,嵌合主链是指部分稳定化的主链,其中至少一个核苷酸间键联是磷酸二酯或磷酸二酯样键联,其中至少一个其它的核苷酸间键联是稳定化的核苷酸间键联,其中所述至少一个磷酸二酯键联或磷酸二酯样键联和所述至少一个稳定化的键联是不同的。当所述磷酸二酯键联优选位于CpG基序内时,这样的分子被称为“半软的”,如PCT申请W02007/026190中描述的。

[0329] 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)也可贡献寡核苷酸的刺激活性。为了有利于至细胞内的摄取,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,则具有大于6个核苷酸的任何大小(甚至许多kb长)的寡核苷酸能够诱导免疫应答,因为较大的寡核苷酸在细胞内被降解。在某些实施方案中,所述CpG寡核苷酸为6至100个核苷酸长,优选8至30个核苷酸长。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。

[0330] 在实施方案中,本文公开的CpG寡核苷酸包含诸如在碱基和/或糖中的取代或修饰,如在W02007/026190的段落134至147中描述的。

[0331] 在实施方案中,本发明的CpG寡核苷酸被化学修饰。化学修饰的实例对于本领域技术人员来说是已知的,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; 和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,其中相较于由天然DNA或RNA组成的相同序列的寡核苷酸,每一个修饰位于特定的磷酸二酯核苷间桥上和/或特定的β-D-核糖单元上和/或在特定的天然核苷碱基位置上。

[0332] 在本发明的一些实施方案中,可按照本领域技术人员已知的方法将含CpG的核酸与免疫原性载体简单地混合(参见,例如,W003/024480)。

[0333] 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至100mg的CpG寡核苷酸,优选0.1mg至50mg CpG寡核苷酸,优选0.2mg至10mg CpG寡核苷酸,优选0.3mg至5mg CpG寡核苷酸,优选0.5至2mg CpG寡核苷酸,优选0.75至1.5mg CpG寡核苷酸。在优选实施方案中,本文公开的免疫原性组合物包含约1mg CpG寡核苷酸。

[0334] 在优选实施方案中,佐剂为选自磷酸铝、硫酸铝和氢氧化铝的基于铝的佐剂。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。

[0335] 在优选实施方案中,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、非离子去垢剂、自由基氧化的抑制剂、稀释剂或载体的至少一种。

[0336] 免疫原性组合物任选地可包含药学上可接受的载体。药学上可接受的载体包括用于动物(包括人以及非人动物)的由联邦监管机构、州政府或其它管理机构批准的,或美国药典或其它通常公认的药典中所列的载体。术语载体可用于指与药物组合物一起施用的稀释剂、佐剂、赋形剂或媒介物。可将水、盐水溶液以及含水葡萄糖和甘油溶液用作液体载体,特别是用于可注射溶液。在E. W. Martin的“Remington's Pharmaceutical Sciences”中描述了合适的药物载体的实例。所述制剂应当适应于施用模式。

[0337] 免疫原性组合物任选地可包含选自但不限于Tris(三甲胺)、磷酸盐、醋酸盐、硼酸盐、柠檬酸盐、甘氨酸、组氨酸和琥珀酸盐的一种或多种生理上可接受的缓冲剂。在某些实施方案中,将制剂缓冲至约5.0至约7.0,优选约5.5至约6.5的pH范围内。

[0338] 免疫原性组合物任选地可包含一种或多种非离子性表面活性剂,包括但不限于聚氧乙烯山梨聚糖脂肪酸酯、聚山梨醇酯-80(Tween 80)、聚山梨醇酯-60(Tween 60)、聚山梨醇酯-40(Tween 40)和聚山梨醇酯-20(Tween 20)、聚氧乙烯烷基醚,包括但不限于Brij 58、Brij 35以及其它物质诸如Triton X-100; Triton X-114、NP40、Span 85和非离子性表面活性剂的Pluronic系列(例如,Pluronic 121),其中优选的组分聚山梨醇酯-80的浓度为约0.001%至约2%(高至约0.25%是优选的)或聚山梨醇酯-40的浓度为约0.001%至1%(高至约0.5%是优选的)。

[0339] 本发明还涉及包含本发明的免疫原性组合物的疫苗。

[0340] 用于诱导免疫应答和保护免受感染的方法

[0341] 本公开内容还包括用于本文所述的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,其包括向受试者施用免疫原性量的本文所述的任何免疫原性组合物。

[0342] 本公开内容的一个实施方案提供了保护受试者免受肺炎链球菌感染的方法,或预

防肺炎链球菌感染的方法,或减轻与由肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作的方法,所述方法包括向受试者施用免疫原性量的本文所述的任何免疫原性组合物。

[0343] 本公开内容的一个实施方案提供了保护受试者免受血清型10A肺炎链球菌感染的方法,或预防血清型10A肺炎链球菌感染的方法,或减轻与由血清型10A肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作的方法,所述方法包括向受试者施用产生免疫原性的量的本文所述的任何免疫原性组合物。

[0344] 本公开内容的一个实施方案提供了治疗或预防受试者的与血清型10A肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法,所述方法包括向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的与血清型10A肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法,所述方法包括从本文所述的免疫原性组合物产生多克隆或单克隆抗体试剂,和使用所述抗体试剂以给受试者赋予被动免疫。

[0345] 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造药剂的用途,所述药剂用于保护受试者免受肺炎链球菌感染,和/或预防肺炎链球菌感染,和/或减轻与由肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作,和/或保护受试者免受血清型10A肺炎链球菌感染和/或预防血清型10A肺炎链球菌感染,和/或减轻与由血清型10A肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作。

[0346] 本公开内容的一个实施方案提供了保护受试者免受血清型22F肺炎链球菌感染的方法,或预防血清型22F肺炎链球菌感染的方法,或减轻与由血清型22F肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作的方法,所述方法包括向受试者施用免疫原性量的本文所述的任何免疫原性组合物。

[0347] 本公开内容的一个实施方案提供了治疗或预防受试者的与血清型22F肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法,所述方法包括向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的与血清型22F肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法,所述方法包括从本文所述的免疫原性组合物产生多克隆或单克隆抗体试剂,以及使用所述抗体试剂以给受试者赋予被动免疫。

[0348] 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造药剂的用途,所述药剂用于保护受试者免受肺炎链球菌感染,和/或预防肺炎链球菌感染,和/或减轻与由肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作,和/或保护受试者免受血清型22F肺炎链球菌感染和/或预防血清型22F肺炎链球菌感染,和/或减轻与由血清型22F肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作。

[0349] 本公开内容的一个实施方案提供了保护受试者免受血清型33F肺炎链球菌感染的方法,或预防血清型33F肺炎链球菌感染的方法,或减轻与由血清型33F肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作的方法,所述方法包括向受试者施用免疫原性量的本文所述的任何免疫原性组合物。

[0350] 本公开内容的一个实施方案提供了治疗或预防受试者的与血清型33F肺炎链球菌相关的肺炎链球菌感染、疾病或病况的方法,所述方法包括向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的与血清型33F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,以及使用所述抗体制剂以给受试者赋予被动免疫。

[0351] 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造药剂的用途,所述药剂用于保护受试者免受肺炎链球菌感染,和/或预防肺炎链球菌感染,和/或减轻与由肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作,和/或保护受试者免受血清型33F肺炎链球菌感染和/或预防血清型33F肺炎链球菌感染,和/或减轻与由血清型33F肺炎链球菌引起的感染相关的至少一个症状的严重度或延迟其发作。

[0352] 针对免疫原性组合物的“免疫应答”是受试者中针对存在于目标免疫原性组合物或疫苗组合物中的分子的体液和/或细胞介导的免疫应答的发生。为了本公开内容的目的,“体液免疫应答”是抗体介导的免疫应答,并且牵涉诱导和产生识别本公开内容的免疫原性组合物或疫苗中的抗原并以一定的亲和力与其结合的抗体,而“细胞介导的免疫应答”是由T细胞和/或其它白血细胞介导的免疫应答。“细胞介导的免疫应答”通过与主要组织相容性复合物(MHC)的I类或II类分子、CD1或其它非经典的MHC-样分子缔合的抗原性表位的呈递引发。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具有针对与由经典或非经典MHC编码并在细胞表面上表达的蛋白质缔合呈递的肽抗原的特异性。CTL帮助诱导和促进细胞内微生物的细胞内破坏,或被这样的微生物感染的细胞的裂解。细胞免疫的另一个方面牵涉通过辅助T细胞的抗原特异性应答。辅助T-细胞用于帮助刺激针对在其表面上展示与经典或非经典MHC分子缔合的肽或其它抗原的细胞的非特异性效应细胞的功能和聚焦其活性。“细胞介导的免疫应答”还指细胞因子、趋化因子以及由活化的T细胞和/或其它白血细胞(包括来源于CD4+和CD8+T细胞的那些白血细胞)产生的其它这样的分子的产生。特定抗原或组合物刺激细胞介导的免疫应答的能力可通过许多测定,诸如通过淋巴细胞增殖(淋巴细胞活化)测定、CTL细胞毒性细胞测定,通过测定致敏受试者中特异于抗原的T淋巴细胞,或通过测量由T细胞响应于利用抗原的再刺激产生的细胞因子来测定。这样的测定在本领域中公知的。参见,例如,Erickson等(1993)J. Immunol. 151:4189-4199和Doe等(1994)Eur. J. Immunol. 24:2369-2376。

[0353] 如本文中所用,“治疗”(包括其变型,例如,“医治”或“治疗的”)意指以下的任一个或多个:(i)感染或再感染的预防,如在常规疫苗中,(ii)症状的严重度的减轻或症状消除,和(iii)所涉及的病原体或病症的基本或完全消除。因此,可预防性(在感染前)或治疗性(在感染后)实现治疗。在本公开内容中,预防性治疗是优选模式。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)宿主动物对抗血清型10A肺炎链球菌感染的组合物和方法。根据本发明的具体实施方案,提供了治疗(包括预防性和/或治疗性免疫)宿主动物对抗血清型22F肺炎链球菌感染的组合物和方法。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)宿主动物对抗血清型33F肺炎链球菌感染的组合物和方法。本公开内容的方法用于赋予受试者预防性和/或治疗性免疫。还可对受试

者实施本公开内容的方法以进行生物医学研究应用。

[0354] “免疫原性量”和“免疫上有效的量”这两者在本文中可互换使用,是指足以引发免疫应答(细胞(T-细胞)或体液(B-细胞或抗体)应答,或两者)的抗原或免疫原性组合物的量,如通过本领域技术人员已知的标准测定测量的。

[0355] 在优选实施方案中,所述受试者为人。在最优选实施方案中,所述受试者是新生儿(即小于3个月)、婴儿(从3个月至1岁)或幼儿(即从1岁至4岁)。

[0356] 在实施方案中,本文公开的免疫原性组合物用作疫苗。

[0357] 在这样的实施方案中,待接种的受试者可小于1岁。例如,待接种的受试者可为约1个月、2个月、3个月、4个月、5个月、6个月、7个月、8个月、9个月、10个月、11个月或12个月大。在实施方案中,待接种的受试者为约2、4或6个月大。在另一个实施方案中,待接种的受试者小于2岁。例如,待接种的受试者可为约12-15个月大。在一些情况下,需要少至一个剂量的根据本发明的免疫原性组合物,但在一些情况下,可给予第二、第三或第四剂量(参见方案部分)。

[0358] 在本发明的实施方案中,待接种的受试者为50岁或更老的成年人,更优选55岁或更老的成年人。在实施方案中,待接种的受试者为65岁或更老、70岁或更老、75岁或更老或80岁或更老的成年人。

[0359] 在实施方案中,待接种的受试者为免疫力低下个体,特别是人。免疫力低下个体通常被定义为表现出减弱的或降低的引发针对感染原攻击的正常体液或细胞防御的能力的人。

[0360] 在本发明的实施方案中,待接种的免疫力低下受试者患有削弱免疫系统并导致不足以保护免受肺炎球菌疾病或治疗肺炎球菌疾病的抗体应答的疾病或病况。

[0361] 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、抗体缺乏、明确定义的综合征、免疫失调疾病、吞噬细胞紊乱、固有免疫缺陷、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W02010/125480的第24页第11行至第25页第19行中公开的原发性免疫缺陷症。

[0362] 在本发明的具体实施方案中,待接种的免疫力低下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、慢性阻塞性肺疾病(COPD)、脾功能障碍(诸如镰状细胞病)、脾功能缺乏(无脾)、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。

[0363] 在本发明的实施方案中,待接种的免疫力低下受试者患有营养不良。

[0364] 在本发明的具体实施方案中,待接种的免疫力低下受试者正在服用降低身体对感染的抗性的药物或进行降低身体对感染的抗性的治疗。在实施方案中,所述药物选自PCT申请W02010/125480的第26页第33行至第26页第40行中公开的药物。

[0365] 在本发明的具体实施方案中,待接种的免疫力低下受试者是吸烟者。

[0366] 在本发明的具体实施方案中,待接种的免疫力低下受试者具有低于 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/升的白血细胞计数(白细胞计数)。

[0367] 白血细胞计数(白细胞计数):血液中的白血细胞(WBC)的数目。WBC通常被测量为CBC(全血计数)的部分。白血细胞是血液中的抗感染细胞,并且与被称为红细胞的红(载氧)血细胞不同。存在不同类型的白血细胞,包括嗜中性粒细胞(多形核白细胞;PMN)、杆状核细胞(轻度未成熟嗜中性粒细胞)、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。所有类型的白血细胞反映于白血细胞计数中。白血细胞计数的正常范围通常在每立方毫米血液4300个细胞与10800个细胞之间。这也可被称为白细胞计数,并且可以以国际单位表示为 $4.3-10.8 \times 10^9$ 个细胞/升。

[0368] 在本发明的具体实施方案中,待接种的免疫力低下受试者患有嗜中性粒细胞减少症。在本发明的具体实施方案中,待接种的免疫力低下受试者具有低于 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/升的嗜中性粒细胞计数。

[0369] 低白血细胞计数或“嗜中性粒细胞减少症”是特征在于循环血液中异常低水平的嗜中性粒细胞的病况。嗜中性粒细胞是帮助预防和抵抗感染的特殊类型的白血细胞。癌症患者经历嗜中性粒细胞减少症的最常见原因是作为化疗的副作用。化疗诱导的嗜中性粒细胞减少症增加了患者的感染风险并使癌症治疗中断。

[0370] 在本发明的具体实施方案中,待接种的免疫力低下受试者具有低于 $500/\text{mm}^3$ 的CD4+细胞计数,或低于 $300/\text{mm}^3$ 的CD4+细胞计数,或低于 $200/\text{mm}^3$ CD4+细胞计数,低于 $100/\text{mm}^3$ 的CD4+细胞计数,低于 $75/\text{mm}^3$ 的CD4+细胞计数,或低于 $50/\text{mm}^3$ 的CD4+细胞计数。

[0371] CD4细胞测试通常被报告为 1mm^3 中的细胞的数目。正常CD4计数在500个与1600个之间,CD8计数在375个与1100个之间。CD4计数在患有HIV的患者中急剧下降。

[0372] 在本发明的实施方案中,本文公开的任何免疫力低下受试者为男人或女人。

[0373] 组合物中缀合物的量通常基于针对该缀合物是缀合的和非缀合的总多糖来计算。例如,具有20%游离多糖的缀合物将在100mcg多糖剂量中具有约80mcg缀合的多糖和约20mcg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。通常地,每一个剂量将包含0.1至100mcg,具体地0.1至10mcg,更具体地1至10mcg以及更具体地1至5 μg 的多糖。优选地,每一个剂量将包含约1.1、2、2.2、3、3.3、4、4.4 μg 多糖。

[0374] 可通过牵涉观察受试者中适当的免疫应答的标准研究来确定特定免疫原性组合物或疫苗的组分量的最佳量。在初始接种后,受试者可接受一次或数次适当间隔的加强免疫。

[0375] 可通过增殖测定,通过测量T细胞裂解其特定靶细胞的能力的细胞溶解测定诸如铬释放测定,或通过测量B细胞活性的水平(通过测量血清中特异于抗原的循环抗体的水平)来测量抗原作为免疫原的功效。还可通过测量在施用抗原后诱导的抗原特异性抗体的血清水平,和更具体地,通过测量所诱导的抗体增强特定白血细胞的调理吞噬能力的能力来检测免疫应答,如本文中所描述的。免疫应答的保护水平可通过用已被施用的抗原攻击经免疫的宿主来测量。例如,如果针对其的免疫应答是期望的抗原为细菌,则由免疫原性量的抗原诱导的保护水平通过在用所述细菌细胞攻击动物后检测百分比存活率或百分比死亡率来测量。在一个实施方案中,可通过测量至少一个与细菌感染相关的症状,例如与感染相关的发热来测量保护量。多抗原或多组分疫苗或免疫原性组合物中的每一种抗原的量可相对每一种其它组分而变化,并且可通过本领域技术人员已知的方法来测定。这样的方法

可包括用于测量免疫原性和/或体内功效的方法。

[0376] 本公开内容还提供了特异性和选择性结合本公开内容的荚膜多糖或糖缀合物的抗体和抗体组合物。在一些实施方案中,在对受试者施用本公开内容的荚膜多糖或糖缀合物后产生抗体。在一些实施方案中,本公开内容提供了针对本公开内容的一种或多种荚膜多糖或糖缀合物的纯化的或分离的抗体。在一些实施方案中,本公开内容的抗体具有功能,如通过在动物功效模型中杀死细菌或经由调理吞噬杀死测定测量的。在一些实施方案中,本公开内容的抗体给受试者赋予被动免疫。通过使用本领域技术人员公知的技术,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,以及产生本公开内容的抗体或抗体组合物的细胞、细胞系(诸如用于抗体的重组产生的杂交瘤细胞或其它工程细胞系)或转基因动物。

实施例

[0377] 实施例1:血清型22F多糖-CRM₁₉₇缀合物的制备

[0378] 1.1.分离的肺炎链球菌血清型22F多糖的制备

[0379] 可使用本领域普通技术人员已知的分离方法直接从细菌获得血清型22F荚膜多糖。(参见例如美国专利申请公开号20060228380,20060228381,20070184071,20070184072,20070231340和20080102498或W02008118752中公开的方法)。将血清型22F肺炎链球菌生长在接种瓶中,随后转移至接种发酵罐。一旦达到靶向光学密度,就将细胞转移至生产发酵罐中。发酵肉汤通过添加N-月桂酰基肌氨酸灭活和通过超滤和渗滤纯化。

[0380] 使用PANDA 2K均化器®(GEA Niro Soavi),通过高压均化裁剪纯化的肺炎链球菌血清型22F多糖,以产生分离的肺炎链球菌血清型22F多糖。

[0381] 1.2.分离的肺炎链球菌血清型22F荚膜多糖的氧化

[0382] 在通过连续添加计算的量的500mM磷酸钾缓冲液(pH 5.8)和WFI(以提供2.0g/L的多糖终浓度)获得的100mM磷酸钾缓冲液(pH5.8±0.2)中进行多糖的氧化。必要时,将反应pH调整至大致5.8。在pH调整后,将反应温度降至5±3℃。通过添加0.10±0.02摩尔当量(MEq)的高碘酸钠来起始氧化。靶氧化反应时间为在5±3℃下16±1小时。

[0383] 通过在5±3℃连续搅拌1-2小时,用2MEq的2,3-丁二醇猝灭氧化反应。

[0384] 使用100K MWCO超滤盒进行活化的多糖的浓缩和渗滤。针对35倍渗滤体积的WFI进行渗滤。将纯化的活化的多糖于5±3℃下贮存。纯化的活化的糖通过除其它以外(i)通过SEC-MALLS测量的分子量,(ii)O-乙酰基的存在和(iii)氧化程度来表征。

[0385] SEC-MALLS用于测定多糖和多糖-蛋白质缀合物的分子量。SEC用于通过流体力学体积分离多糖。折射率(RI)和多角度激光光散射(MALLS)检测器用于测定分子量。当光与物质相互作用时,其散射,并且散射光的量与浓度、dn/dc(特定的折射率增量)的平方和物质的摩尔质量相关。基于来自MALLS检测器的散射光信号和来自RI检测器的浓度信号的读数计算分子量测量值。

[0386] 如下测定活化的多糖的氧化程度(DO=糖重复单元的摩尔数/醛的摩尔数):

[0387] 通过各种比色法,诸如例如通过使用蒽酮法测定糖重复单元的摩尔数。首先通过硫酸和热的作用将多糖分解成单糖。蒽酮测糖试剂与己糖反应以形成黄绿色复合物,其吸光度在625nm处通过分光光度计读取。在测定的范围内,吸光度直接与存在的己糖的量成比

例。

[0388] 还同时使用MBTH比色法测定醛的摩尔数。MBTH测定牵涉通过将醛基团(来自给定的样品)与3-甲基-2-苯并噻唑酮腈(MBTH测定试剂)反应来形成吡嗪化合物。过量的3-甲基-2-苯并噻唑酮腈氧化以形成反应性阳离子。所述反应性阳离子与吡嗪反应以形成蓝色生色团。随后在650nm处通过分光光度计读取所形成的发色团。

[0389] 1.3.活化的肺炎链球菌血清型22F多糖与CRM₁₉₇的缀合

[0390] 缀合方法由以下步骤组成:

[0391] a.与蔗糖赋形剂混合,并冷冻干燥。

[0392] b.重建冻干的多糖和CRM₁₉₇。

[0393] c.将活化的多糖缀合于CRM₁₉₇并加帽

[0394] d.纯化缀合物。

[0395] a.与蔗糖混合和冷冻干燥

[0396] 将活化的多糖与蔗糖(50%w/v,于WFI中)混合至每克活化的多糖25克蔗糖的比率。随后冻干瓶装的混合的混合物。在冻干后,将装有冻干的活化的多糖的瓶子在-20±5℃下贮存。分别地壳式冷冻和冻干计算的量的CRM₁₉₇蛋白(靶S/P输入比率=1)。将冻干的CRM₁₉₇于-20±5℃下贮存。

[0397] b.冻干的活化的多糖和CRM₁₉₇蛋白的重建

[0398] 在无水二甲基亚砜(DMSO)中重建冻干的活化的多糖。在多糖完全溶解后,将等量的无水DMSO添加至冻干的CRM₁₉₇以进行重建。

[0399] c.将活化的多糖缀合于CRM₁₉₇并加帽

[0400] 将重建的CRM₁₉₇(于DMSO中)与重建的活化的多糖在缀合反应容器中组合。反应溶液中的多糖终浓度为1g/L。通过向反应混合物中添加1.5±0.1MEq氰基硼氢化钠来起始缀合,将反应在23±2℃下温育20±2小时。通过添加2MEq硼氢化钠来终止缀合反应。将加帽反应于23±2℃温育3±1小时。

[0401] d.纯化缀合物

[0402] 在制剂中用冰冷的5mM琥珀酸盐-0.9%盐水(pH 6.0)以1:5稀释缀合物溶液,以用于使用100K MWCO膜通过切向流过滤来纯化,使用5mM琥珀酸盐-0.9%盐水(pH6.0)作为介质进行20X渗滤。在完成渗滤后,进一步稀释缀合物渗余物,将其通过0.22μm滤器过滤,并于2-8℃下贮存。

[0403] 表1包含通过本发明的方法获得的血清型22F多糖-CRM₁₉₇缀合物的表征数据。具体地,如实施例1中所公开的获得表中的缀合物5和6。

[0404] 相较于通过RAC-含水法产生的缀合物,通过RAC-DMSO法产生的血清型22F多糖-CRM₁₉₇缀合物提供了更好的产率,并且在MW上表现出更好的批间一致性,以及显著更低的游离糖百分比水平和较高的载体蛋白(赖氨酸)修饰,如表1中显示的。

[0405]

表 1 - 血清型 22F 缀合物						
缀合物编号	1	2	3	4	5	6
	水	水	水	DMSO	DMSO	DMSO
活化的多糖的 MW, kDa	488	803	410	639	709	416
DO	17.3	15	10.3	14.6	14.4	13.7
输入比率	0.8	0.8	1	0.8	1	1
缀合物产率%	66	63	46	70	62	75

[0406]

游离糖%	18	43	45	< 5	< 5	8
缀合物的 MW	1024	3544	462	3911	3734	4453
糖-蛋白质比 率	1.6	1.1	1.45	0.8	0.65	1.0
经修饰的 Lys (AAA)	2.3	N/A	N/A	6.1	N/A	4.7

[0407] 如下计算缀合物产率百分比: (缀合物中的多糖的量 $\times$ 100)/活化的多糖的量。

[0408] 实施例2: 血清型10A多糖-CRM₁₉₇缀合物的制备

[0409] 2.1. 分离的肺炎链球菌血清型10A多糖的制备

[0410] 除不裁剪纯化的多糖外, 如实施例1.1中所公开的获得分离的血清型10A多糖。

[0411] 2.2. 分离的肺炎链球菌血清型10A荚膜多糖的氧化

[0412] 将计算的体积的0.1M磷酸钾缓冲液(pH 6.0)和注射用水(WFI)添加至多糖溶液以获得2.5g/L的多糖终浓度和25mM终浓度的磷酸钾缓冲液, 必要时, 将pH调整至大致6.0。随后将稀释的多糖冷却至 $5 \pm 3^\circ\text{C}$ 。通过添加 0.25 ± 0.02 摩尔当量(MEq)的高碘酸钠溶液起始氧化。氧化反应时间为在 $5 \pm 3^\circ\text{C}$ 约4小时。通过在 $5 \pm 3^\circ\text{C}$ 连续搅拌1-2小时, 用1MEq的2,3-丁二醇猝灭氧化反应。

[0413] 在达到靶反应时间后, 使用30K MWC0 Millipore超滤盒浓缩活化的多糖。随后针对20倍渗滤体积的WFI进行渗滤。将纯化的活化的多糖于 $5 \pm 3^\circ\text{C}$ 下贮存。纯化的活化的多糖通过除其它以外(i)通过SEC-MALLS测量的分子量, 和(ii)氧化程度来表征。

[0414] 2.3活化的肺炎链球菌血清型10A多糖与CRM₁₉₇的缀合

[0415] 缀合反应由以下步骤组成:

[0416] a. 与蔗糖赋形剂混合, 并冷冻干燥。

[0417] b. 重建冻干的多糖和CRM₁₉₇。

- [0418] c. 将活化的多糖缀合于CRM₁₉₇并加帽
- [0419] d. 纯化缀合物。
- [0420] a. 与蔗糖混合
- [0421] 将活化的多糖与蔗糖混合至每克活化的多糖 25 ± 2.5 克蔗糖的比率。随后冻干瓶装的混合的混合物。在冻干后, 将装有冻干的活化的多糖的瓶子在 $-20 \pm 5^\circ\text{C}$ 下贮存。
- [0422] b. 冻干的活化的多糖和CRM₁₉₇蛋白的重建
- [0423] 在无水二甲基亚砜(DMSO)中重建冻干的活化的多糖。在多糖完全溶解后, 将等量的无水DMSO添加至计算的CRM₁₉₇以进行重建。
- [0424] c. 将活化的多糖缀合于CRM₁₉₇并加帽
- [0425] 将重建的CRM₁₉₇(于DMSO中)于缀合反应容器中添加至重建的活化的多糖。多糖终浓度为 1g/L 。通过向反应混合物中添加 $1.2 \pm 0.1\text{MEq}$ 的氰基硼氢化钠来进行缀合。将反应在 $23 \pm 2^\circ\text{C}$ 下温育 24 ± 2 小时。通过添加 2MEq 硼氢化钠来终止缀合反应。将加帽反应于 $23 \pm 2^\circ\text{C}$ 温育 3 ± 1 小时。
- [0426] 通过添加 2MEq 的硼氢化钠来终止缀合反应。将此加帽反应在 $23 \pm 2^\circ\text{C}$ 进行 3 ± 1 小时。
- [0427] d. 缀合物的纯化
- [0428] 随后将缀合物溶液稀释至 $5\times$ (按体积)冰冷的 5mM 琥珀酸盐- 0.9% 盐水($\text{pH } 6.0$)中并且使用 5mM 琥珀酸盐- 0.9% 盐水($\text{pH } 6.0$)进行 $20\times$ 渗滤。在完成初始渗滤后, 将缀合物渗余物转移通过 $0.22\mu\text{m}$ 过滤器。用 5mM 琥珀酸盐/ 0.9% 盐水($\text{pH } 6$)进一步稀释缀合物, 并在最终的 $0.22\mu\text{m}$ 过滤步骤后, 将其于 $2-8^\circ\text{C}$ 下贮存。
- [0429] 表2包含通过本发明的方法获得的血清型10A多糖-CRM₁₉₇缀合物的表征数据。具体地, 如实施例2中所公开的获得表2中的缀合物4至6。
- [0430] 相较于通过RAC-含水法产生的缀合物, 通过RAC-DMSO法产生的血清型10A多糖-CRM₁₉₇缀合物提供了更好的产率, 并且在MW上表现出更好的批间一致性, 以及显著更低的游离糖百分比水平和较高的载体蛋白(赖氨酸)修饰, 如表2中显示的。

[0431]

表 2. 血清型 10A 缀合物						
缀合物编号	1	2	3	4	5	6
	水	水	水	DMSO	DMSO	DMSO
DO	12.2	12.2	12.8	10.3	10.8	10.5
活化的糖的 MW, kda	157	157	163	170	170	170
输入比率	1.0	1.0	1.0	1.1	1.1	1.1
产率%	71	34	50	82	73	66
游离糖%	20	31	26	6.8	6.4	9.7
缀合物的 MW, kDa	1550	968	3050	4034	3463	5540
糖-蛋白质比率	1.6	2.6	1.6	1.1	1.2	1.0
Lys 修饰 AAA	2.6	N/A	N/A	6.9	6.7	6.1

- [0432] 实施例3:血清型33F多糖-CRM₁₉₇缀合物的制备
- [0433] 3.1. 分离的肺炎链球菌血清型33F多糖的制备
- [0434] 如实施例1.1中公开的,获得分离的血清型33F多糖。
- [0435] 3.2. 分离的肺炎链球菌血清型33F荚膜多糖的氧化
- [0436] 将计算的体积的0.1M磷酸钾缓冲液(pH 6.0)和注射用水(WFI)添加至多糖溶液以获得2g/L的多糖终浓度。必要时,将pH调整至大致6.0。随后将稀释的多糖冷却至5±3℃。通过添加0.1摩尔当量(MEq)的高碘酸钠溶液起始氧化。氧化反应时间为在5±3℃约20小时。通过在5±3℃连续搅拌约1小时,用1MEq的2,3-丁二醇猝灭氧化反应。在达到靶反应时间后,使用100K MWCO Millipore超滤盒浓缩活化的多糖。随后针对40倍渗滤体积的WFI进行渗滤。将纯化的活化的多糖于5±3℃贮存。纯化的活化的糖通过除其它以外(i)通过SEC-MALLS测量的分子量和(ii)氧化程度来表征。
- [0437] 3.3活化的肺炎链球菌血清型33F多糖与CRM₁₉₇的缀合
- [0438] 使用实施例3.2中获得的活化的33F多糖,通过与实施例1.3的方法类似的方法获得血清型33F多糖-CRM₁₉₇缀合物。
- [0439] 相较于通过RAC-含水法产生的缀合物,通过RAC-DMSO法产生的血清型33F多糖-CRM₁₉₇缀合物提供更好的产率并且在O-乙酰基水平的保持上表现出更好的批间一致性以及总体更低的游离糖百分比水平和较高的载体蛋白(赖氨酸)修饰,如表3中显示的。

[0440]

表 3-血清型 33F 缀合物						
缀合物编号	1	2	3	4	5	6
	水	水	水	DMSO	DMSO	DMSO
DO	15.2	15.5	15.7	17	9	14
活化的聚合物的 MW	1126	573	574	120	328	128
产率%	65	51	48	78	86	66
游离糖%	8.5	19	13	18	5	< 5
缀合物的 MW, kDa	1341	2137	3674	3158	2165	2001
糖-蛋白质比率	1.7	1.4	1.3	1.1	1.1	0.79
保留的 O-Ac %	< LOQ	< LOQ	< LOQ	82	100%	100%
缀合 Lys (AAA)	N/A	2.3	2.3	4.5	3.4	5.2

[0441] LOQ=定量限

[0442] 实施例4: 猝灭对活化的血清型22F和33F多糖的分子量的影响

[0443] 在4℃或22℃进行22F和33F多糖的活化。两个温度均给出相似的DO值。然而, 由于升高的温度导致活化的多糖较快分解, 从而降低活化的多糖的MW(如当例如比较图4(22℃)与图5(4℃)时显示的), 4℃对于活化反应通常是优选的。另外, 活化数据显示在氧化后未反应的NaIO₄的猝灭对于活化, 尤其是对于使用较高量的NaIO₄的活化是必需步骤。图6中显示的图表明当将递增浓度的高碘酸盐用于活化时(具有猝灭)活化的33F多糖的MW是相对稳定的, 而当不使用猝灭步骤时, 这样的MW是高度可变的。在设置来获得靶向的氧化程度的特定条件下, 当使用猝灭步骤时, 活化的多糖的分子量是较少可变的。氧化后猝灭剂的添加帮助保持活化的多糖的结构完整性, 直至例如通过超滤和渗滤完成纯化。

[0444] 实施例5: 调理吞噬活性测定(OPA)

[0445] 可使用下述调整吞噬测定(OPA)评估通过本文公开的方法获得的缀合物的免疫原性。

[0446] 在第0周通过皮下途径利用0.001μg、0.01μg或0.1μg的测试缀合物免疫30只6-7周龄雌性Swiss Webster小鼠的组。在第3周利用相同剂量的缀合物加强免疫小鼠, 随后在第4周取血。对第4周血清样品进行血清型特异性OPA。

[0447] 将经验证的调整吞噬活性(OPA)测定用于测量鼠血清中的特异于肺炎链球菌血清型10A、22F或33F的功能性抗体。在测量荚膜多糖特异性免疫球蛋白调理细菌、触发补体沉

积,从而促进吞噬作用和吞噬细胞对细菌的杀死的能力的测定反应中建立测试血清。OPA滴度被定义为导致相对于无测试血清的对照孔细菌计数的50%减少的稀释度倒数。从包含该50%杀死截断值的两个稀释度内插OPA滴度。

[0448] OPA法基于Hu等, Clin Diagn Lab Immunol 2005;12(February(2)):287-95中描述的方法并进行了以下修饰。将测试血清进行2.5倍系列稀释,并添加至微量滴定测定板。将活的血清型10A、22F和33F靶细菌菌株添加至孔中,并在25℃(血清型22F)或37℃(血清型10A和33F)下摇动板,进行30分钟。将分化的HL-60细胞(吞噬细胞)和幼兔血清(3至4周龄, **Pel-Freez®**, 12.5%的终浓度)添加至孔中,将板在37℃摇动45分钟(血清型22F和33F)或60分钟(血清型10A)。为了终止反应,将80μL的0.9%NaCl添加至所有孔,混合,并将10μL等分部分转移至含有200μL水的MultiScreen HTS HV过滤器板(**Millipore®**)的孔。将液体在真空下过滤通过板,将150μL HySoy介质添加至每一个孔并过滤通过。随后将过滤板在37℃, 5%CO₂温育过夜,随后用脱色溶液(Bio-Rad)进行固定。随后用考马斯蓝对板进行染色,并将其脱色一次。在Cellular Technology Limited(CTL)ImmunoSpot**分析仪®**上对集落进行成像和计数。将原始集落计数用于绘制杀死曲线和计算OPA滴度。

[0449] 在上述公开的OPA测定中测试如实施例1至3中所公开的获得的血清型10A多糖-CRM₁₉₇缀合物、血清型22F多糖-CRM₁₉₇缀合物和血清型33F多糖-CRM₁₉₇缀合物,并发现其是免疫原性的。

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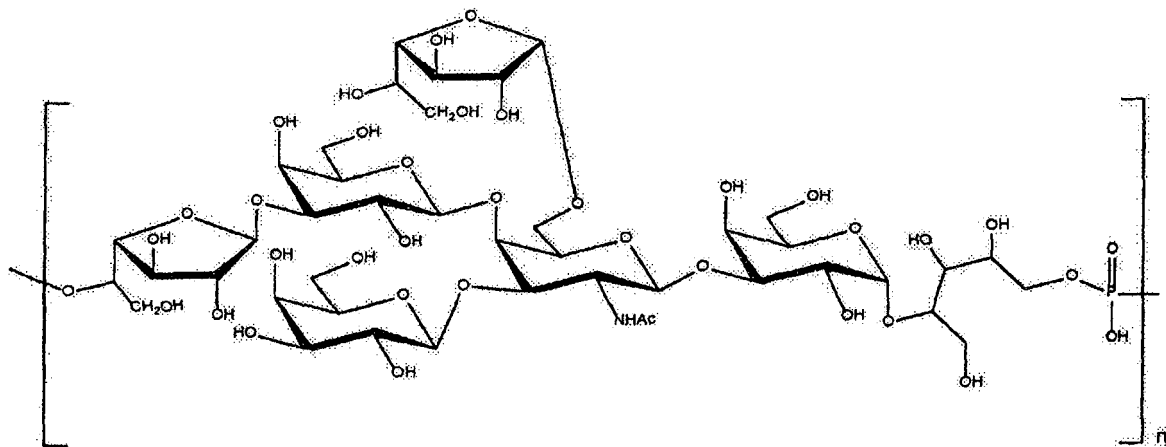


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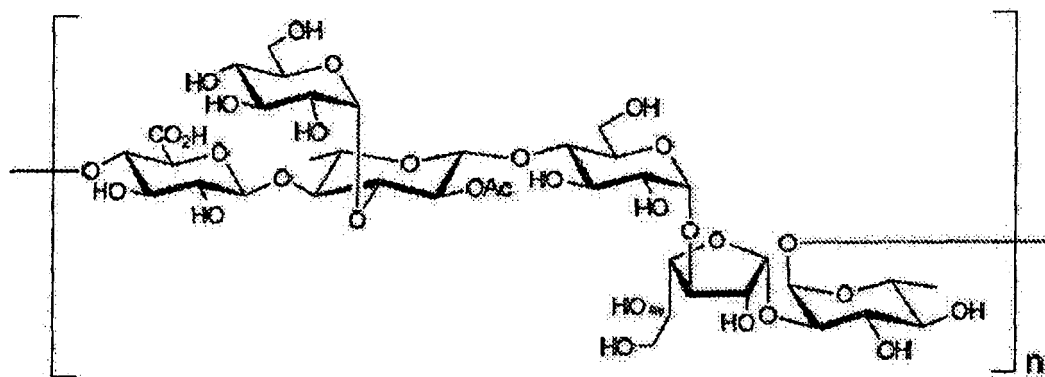


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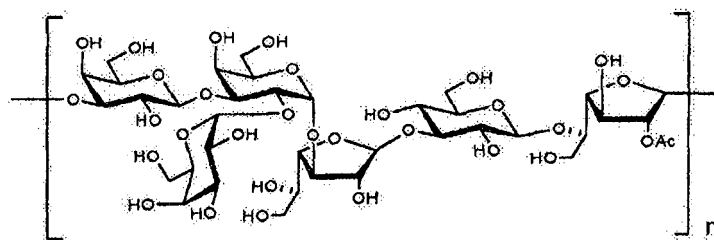


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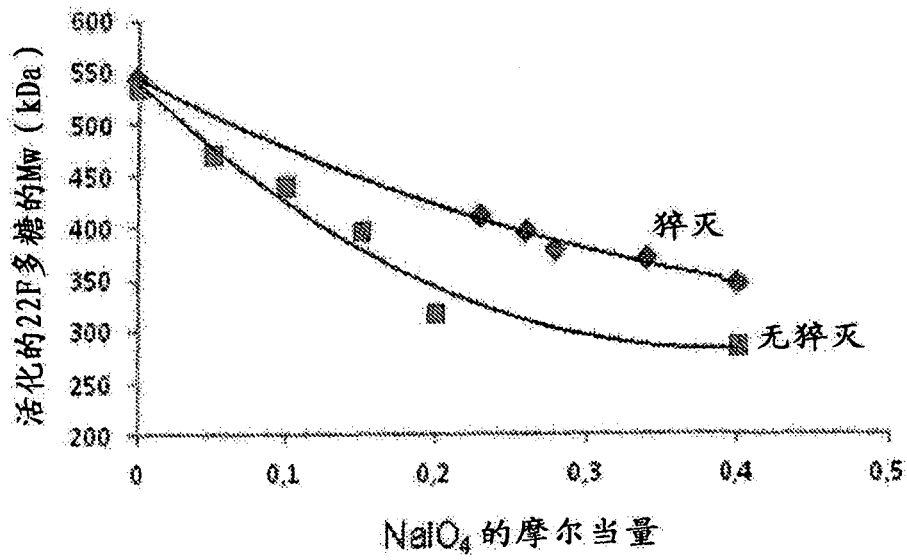


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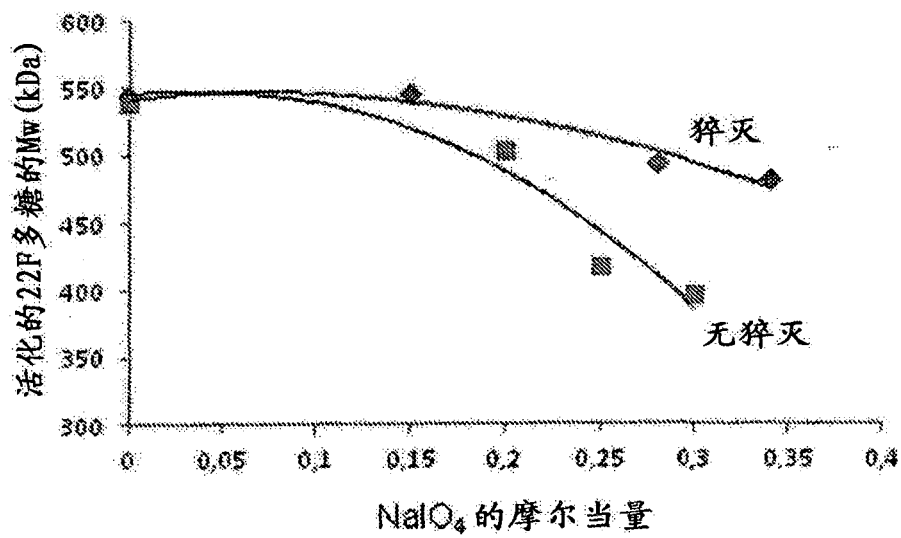


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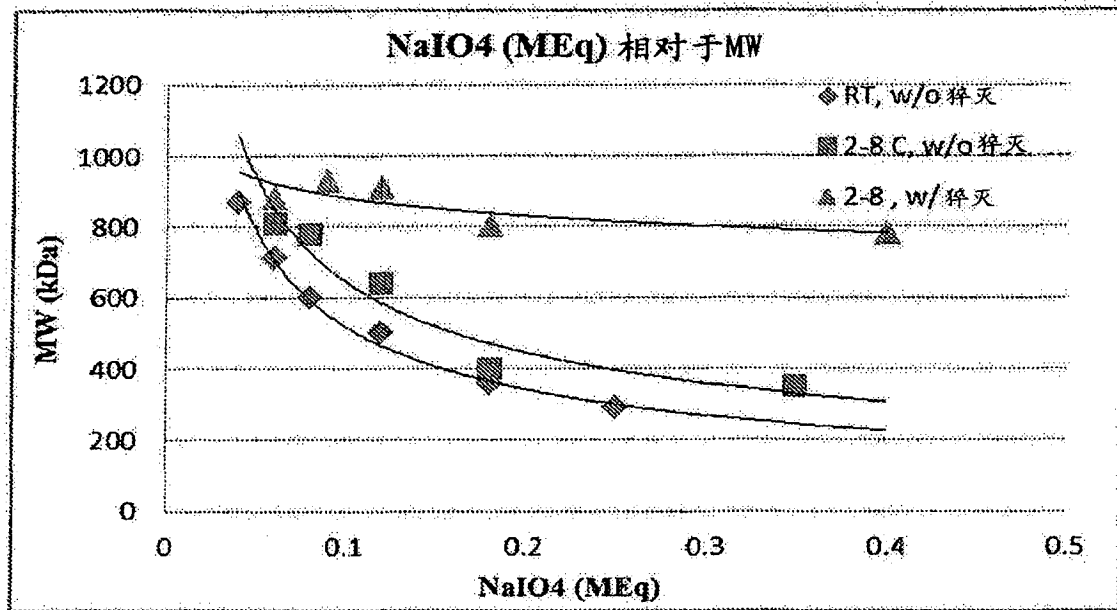


图6