



- (51) **International Patent Classification:**
A61K 39/09 (2006.01) *A61K 39/00* (2006.01)
- (21) **International Application Number:**
PCT/EP2016/075045
- (22) **International Filing Date:**
19 October 2016 (19.10.2016)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
1518684.4 21 October 2015 (21.10.2015) GB
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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, DJ, 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, KW, 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 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))
- of inventorship (Rule 4.17(iv))

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))



WO 2017/067962 A1

(54) **Title:** VACCINE

(57) **Abstract:** The present invention is in the field of pneumococcal capsular saccharide conjugate vaccines. Specifically, the present invention relates to sized Streptococcus pneumoniae serotype 6A capsular polysaccharides, in particular Streptococcus pneumoniae serotype 6A capsular polysaccharides having the average size (e.g. M_w) of the Streptococcus pneumoniae serotype 6A capsular polysaccharide is between 100-1000 kDa, suitably conjugated to a carrier protein.

VACCINE

Field of the Invention

The present invention relates to sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharides, in particular *Streptococcus pneumoniae* serotype 6A capsular polysaccharides having an average size (e.g. M_w) between 100-1000, 110-750, 150-500, 180-600, 210-490, 210-450, 180-400, 210-400, 210-370, 220-360, 230-350, 240-340, 240-320, 240-310 or 250-310 kDa. It additionally relates to sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharides conjugated to a carrier protein, immunogenic compositions, vaccines and processes for making the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharides. It also relates to the use of the immunogenic compositions and vaccines of the invention in therapy and methods of immunising against *Streptococcus pneumoniae* infection.

15 Background of the Invention

Streptococcus pneumoniae (*S. pneumoniae*) is a Gram-positive bacterium responsible for considerable morbidity and mortality (particularly in infants and the elderly), causing invasive diseases such as bacteraemia and meningitis, pneumonia and other non-invasive diseases, such as acute otitis media. About 800,000 children die annually due to pneumococcal disease, especially in emerging countries (O'Brien *et al.* 2009 Lancet 374:893-902). The increasing number of antibiotic-resistant strains (Linares *et al.* 2010 Cin. Microbiol. Infect. 16:402-410) and the severity of pneumococcal diseases make vaccination the most effective intervention.

The major clinical syndromes caused by *S. pneumoniae* are widely recognized and discussed in all standard medical textbooks (Fedson DS, Muscher DM. In: Plotkin SA, Orenstein WA, editors. Vaccines. 4th edition. Philadelphia WB Saunders Co, 2004a: 529-588). For instance, Invasive pneumococcal disease (IPD) is defined as any infection in which *S. pneumoniae* is isolated from the blood or another normally sterile site (Musher DM. *Streptococcus pneumoniae*. In Mandell GL, Bennett JE, Dolin R (eds). Principles and Practice of Infectious diseases (5th ed). New York, Churchill Livingstone, 2001, p2128-2147). Chronic obstructive pulmonary disease (COPD) is recognised as encompassing several conditions (airflow obstruction, chronic bronchitis, bronchiolitis or small airways disease and emphysema) that often coexist (Wilson *et al.*, Eur. Respir. J. 2001; 17: 995-1007). Patients suffer exacerbations of their condition that are usually associated with increased breathlessness, and often have increased cough that may be productive of mucus or purulent sputum (Wilson, Eur Respir J 2001 17:995-1007). COPD is defined

physiologically by the presence of irreversible or partially reversible airway obstruction in patients with chronic bronchitis and/or emphysema (Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society. Am J Respir Crit Care Med. 1995 Nov;152(5 Pt 2):S77-121). Exacerbations of COPD are often
5 caused by bacterial (e.g. pneumococcal) infection (Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clin Microbiol Rev. 2001 Apr;14(2):336-63).

Pneumococcus is encapsulated with a chemically linked polysaccharide which confers serotype specificity. There are more than 90 known serotypes of pneumococci,
10 and the capsule is the principle virulence determinant for pneumococci, as the capsule not only protects the inner surface of the bacteria from complement, but is itself poorly immunogenic. An anti-polysaccharide antibody level has been regarded as predictive of the protection against invasive pneumococcal disease (Jodar et al. Vaccine, (21) 2003, p. 3264-3272). After initial licensure of a 7-valent conjugate vaccine containing serotypes 4,
15 6B, 9V, 14, 18C, 19F, 23F (PCV7), two pneumococcal conjugate vaccines (PCVs) designed to broaden coverage have been licensed. The 10-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PCV10) contains serotypes 1, 4, 5, 6B, 7F, 9V, 14 and 23F conjugated to nontypeable *H. influenzae* protein D, plus serotype 18C conjugated to tetanus toxoid and serotype 19F conjugated to diphtheria toxoid. The
20 13-valent pneumococcal conjugate vaccine (PCV13) contains the PCV7 (4, 6B, 9V, 14, 18C, 19F, 23F) serotypes plus serotypes 1, 3, 5, 6A, 7F and 19A, conjugated to cross-reactive material CRM197.

It is an object of the present invention to develop improved *Streptococcus pneumoniae* polysaccharides and improved *Streptococcus pneumoniae* polysaccharide
25 conjugate vaccines.

S. pneumoniae serogroup 6 isolates, including isolates of serotypes 6A, 6B, 6C, and 6D, are important because they are commonly found in infections (Song et al. JOURNAL OF CLINICAL MICROBIOLOGY, May 2011, p. 1758–1764).
WO2009/000826A2 discloses a *Streptococcus pneumoniae* serotype 6A capsular
30 polysaccharide having a size 1100-1540 (Da x 10³) conjugated to protein D and a *Streptococcus pneumoniae* serotype 6B capsular polysaccharide having a size 1069-1391 (Da x 10³) conjugated to protein D (see Table 2 of WO2009/000826A2). Both the *Streptococcus pneumoniae* serotype 6A capsular polysaccharide and the *Streptococcus pneumoniae* serotype 6B capsular polysaccharide described in WO2009/000826A2 were
35 native polysaccharides. The chemical structure of serotypes 6A and 6B only differs by the link between rhamnose and ribitol units. As the structure of polysaccharide 6A is very

similar to polysaccharide 6B it was originally thought that a native PS6A (polysaccharide 6A) should be used for conjugation as a native PS6B (polysaccharide 6B) is used for conjugation. In the FinIP trial, PHID-CV10 containing 6B conjugated to protein D was demonstrated to be effective against serotype 6B (Palmu *et al.* Lancet 2013; 381: 214–
5 22). However, surprisingly, the present invention provides a sized *Streptococcus pneumoniae* 6A polysaccharide with improved properties. The inventors have found that by using a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide (PS6A) of a particular size, a conjugate may be obtained having both high immunogenicity and which is filterable.

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Brief description of Figures:

Figure 1 Evaluation of PS6A conjugates in 14-valent (14V) AIPO4 formulation in the Balb/c mouse with co- administration of Infanrix™ Hexa model. ELISA anti-PS6A.

Figure 2(A) Immunogenicity of PS6A-CRM197 in the Balb/c mouse model ELISA results

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Figure 2(B) Immunogenicity of PS6A-CRM197 in the Balb/c mouse model OPA results

Figure 3(A) Evaluation of PS6A-CRM197 ELISA results

Figure 3(B) Evaluation of PS6A-CRM197 OPA results

Description of the Invention

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The present invention provides a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide. In one aspect, the present invention provides a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide wherein the average size (M_w) of the *S. pneumoniae* serotype 6A polysaccharide is between 100-1000, 110-750, 150-500, 180-
25 600, 210-490, 210-450, 180-400, 210-400, 210-370, 220-360, 230-350, 240-340, 240-320, 240-310 or 250-310 kDa.

The term “polysaccharide” throughout this specification refers to a complex carbohydrate composed of a chain of saccharides joined together by glycosidic bonds. The
30 polysaccharide may contain at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40 or 50 or more saccharides.

For the purposes of the invention, “native polysaccharide” refers to a polysaccharide that has not been subjected to a process (e.g. post-purification), the purpose of which is to
35 reduce the size of the polysaccharide. A polysaccharide can become slightly reduced in size during normal purification procedures or by degradation during conjugation. Such a

saccharide is still native. Only if the polysaccharide has been subjected to sizing techniques would the polysaccharide not be considered native.

For the purposes of the invention, "sized polysaccharide" refers to a polysaccharide that has been subjected to a process (e.g. post-purification), the purpose of which is to reduce the size of the polysaccharide. Polysaccharides may be sized by mechanical or chemical sizing techniques. Mechanical sizing techniques that may be used include high pressure techniques (such as microfluidization, Emulsiflex™, high pressure homogenization, or Gaulin homogenization) and sonication. Chemical sizing techniques that may be used include acid hydrolysis (e.g. treatment with acetic acid) or treatment with periodate. The term "periodate" includes both periodate and periodic acid; the term also includes both metaperiodate (IO_4^-) and orthoperiodate (IO_6^{5-}) and the various salts of periodate (e.g., sodium periodate and potassium periodate). The molecular weight ranges described herein refer to purified polysaccharides before conjugation (e.g. before activation where activation is carried out).

For the purposes of the invention, "sized by a factor up to x2" means that the saccharide is subject to a process intended to reduce the size of the saccharide but to retain a size more than half the size of the native polysaccharide. Terms such as "sized by a factor up to" x3, x4 etc. are to be interpreted in the same way, i.e. the saccharide is subject to a process intended to reduce the size of the polysaccharide but to retain a size more than a third, a quarter etc., respectively, the size of the native polysaccharide.

The term "Molecular weight" or "average molecular weight" or "average size" of a polysaccharide as used herein refers to the weight-average molecular weight (M_w) of the polysaccharide measured prior to conjugation measured by MALLS (Multi-Angle Laser Light Scattering).

The MALLS technique is known in the art and is typically carried out as described in below. For MALLS analysis of pneumococcal polysaccharides, two columns (TSKG6000 and 5000PWxl) may be used in combination and the polysaccharides are eluted in 0.2M NaCl. Polysaccharides are detected using a light scattering detector (for instance Wyatt Dawn DSP (Digital Signal Processing) equipped with a 10mW argon laser at 488nm) and an interferometric refractometer (for instance Wyatt Otilab DSP (Digital Signal Processing) equipped with a P100 cell and a red filter at 498nm).

The laser light scattering detector measures the light intensities scattered at 16 angles by the macromolecular solution and on the other hand, an interferometric refractometer placed on-line allows the determination of the quantity of sample eluted. From these intensities, the size and shape of the macromolecules in solution can be determined.

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The mean molecular weight in weight (M_w) is defined as the sum of the weights of all the species multiplied by their respective molecular weight and divided by the sum of weights of all the species.

10 a) Weight-average molecular weight: $-M_w-$

$$M_w = \frac{\sum W_i \cdot M_i}{\sum W_i} = \frac{m_2}{m_1}$$

b) Number-average molecular weight: $-M_n-$

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$$M_n = \frac{\sum N_i \cdot M_i}{\sum N_i} = \frac{m_1}{m_0}$$

c) Root mean square radius: $-R_w-$ and r^2_w is the square radius defined by:

$$R^2_w \text{ or } (r^2)_w = \frac{\sum m_i \cdot r_i^2}{\sum m_i}$$

($-m_i-$ is the mass of a scattering centre i and $-r_i-$ is the distance between the scattering centre i and the center of gravity of the macromolecule).

25 d) The polydispersity is defined as the ratio $-M_w / M_n-$.

As used herein, the term "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,

30 (iii) the delay in recurrence of symptoms, and (iii) the substantial or complete elimination

of the pathogen or disorder in question in a subject. Hence, treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

For the purposes of this invention, "treatment or prevention of exacerbations of COPD" or "reduction in severity of COPD exacerbations" refers to a reduction in incidence or rate of COPD exacerbations (for instance a reduction in rate of 0.1, 0.5, 1, 2, 5, 10, 20% or more) or a reduction in severity of COPD exacerbations (e.g. airflow obstruction, chronic bronchitis, bronchiolitis or small airways disease and emphysema), for instance within a patient group immunized with the immunogenic compositions or vaccines of the invention.

Sized *Streptococcus pneumoniae* Serotype 6A Capsular Polysaccharides

Streptococcus pneumoniae serotype 6A capsular polysaccharides may be sized by mechanical or chemical sizing techniques. In an embodiment, the *Streptococcus pneumoniae* serotype 6A capsular polysaccharides of the invention are sized by a chemical sizing technique. Chemical sizing techniques that may be used include treatment with acetic acid or treatment with periodate. In one aspect, the *Streptococcus pneumoniae* serotype 6A capsular polysaccharides of the invention are sized by treatment with periodate. The term "periodate" includes both periodate and periodic acid; the term also includes both metaperiodate (IO_4^-) and orthoperiodate (IO_6^{5-}) and the various salts of periodate (e.g., sodium periodate and potassium periodate). In another aspect, the *Streptococcus pneumoniae* serotype 6A capsular polysaccharides of the invention are sized by treatment with acetic acid. In an embodiment, the *Streptococcus pneumoniae* serotype 6A capsular polysaccharides of the invention are sized by a mechanical sizing technique, for example using a high pressure technique. High pressure techniques include microfluidization, high pressure homogenization, or Gaulin homogenization. In one aspect, the *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is sized by high pressure homogenization. High pressure homogenization achieves high shear rates by pumping the process stream through a flow path with sufficiently small dimensions. The shear rate is increased by using a larger applied homogenization pressure, and exposure time can be increased by recirculating the feed stream through the homogenizer. The technique of high pressure homogenization is described in Cho et al. (*Int. J. Mol. Sci.* **2014**, 15). In another aspect of the invention, the *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is sized by Gaulin homogenization. Gaulin homogenization may be carried out using the technique described in Lander et al. (*Biotechnol. Prog.* **2000**, 16, 80-85). In another aspect, the

Streptococcus pneumoniae serotype 6A capsular polysaccharide of the invention is sized by microfluidization (for example as described in the Examples below).

Sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharides of the present invention have an average size (M_w) between 100-1000, 110-750, 150-500, 180-600, 210-490, 210-450, 180-400, 210-400, 210-370, 220-360, 230-350, 240-340, 240-320, 240-310 or 250-310 kDa. Sizing is by a factor of no more than x20, x10, x8, x6, x5, x4, x3 or x2. For example, sizing may be from a factor of between x2 to x6, x2 to x5, x2 to x4, or x3 to x6, x3 to x5 or x3 to x4. In one aspect, the *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is sized by a factor of no more than x5. The molecular weight ranges described herein refer to molecular weight of the purified *Streptococcus pneumoniae* serotype 6A capsular polysaccharides before conjugation (e.g., before activation where activation is carried out).

In an embodiment, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is antigenic (as determined by ELISA), for example having an antigenicity index of between 70 to 200 %, preferably between 90% and 150% (e.g. between 120 and 144%). As explained in the Examples below, the antigenicity index is measured relative to native *Streptococcus pneumoniae* serotype 6A capsular polysaccharide which is assigned an antigenicity index of 100% (also represented as 1.0 in the tables below).

Conjugated *Streptococcus pneumoniae* Serotype 6A Capsular Polysaccharides

Suitably, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is conjugated to a carrier protein. The carrier protein may be selected from the group consisting of TT (tetanus toxoid), DT (diphtheria toxoid), CRM197, fragment C of TT, PhtD (pneumococcal histidine triad protein D), PhtDE fusions (a fusion of PhtD and PhtE (pneumococcal histidine protein E) particularly those described in WO 01/98334 and WO 03/54007), detoxified pneumolysin and protein D. In one aspect of the invention, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is conjugated to a carrier protein selected from the group consisting of TT (tetanus toxoid), DT (diphtheria toxoid), CRM197, fragment C of TT, and PhtD (pneumococcal histidine triad protein D). In another aspect of the invention, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is conjugated DT or CRM197. In another aspect

of the invention, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is conjugated to CRM197.

5 CRM197 is a non-toxic form of the diphtheria toxin but is immunologically indistinguishable from the diphtheria toxin (DT). Genetically detoxified analogues of diphtheria toxin include CRM197 and other mutants described in US 4,709,017, US 5,843,711, US 5,601,827, and US 5,917,017. CRM197 is produced by *C. diphtheriae* infected by the nontoxigenic phase β 197tox- created by nitrosoguanidine mutagenesis of the toxigenic caryophage b (Uchida et al Nature New Biology (1971) 233; 8-11). The
10 CRM197 protein has the same molecular weight as the diphtheria toxin but differs from it by a single base change in the structural gene. This leads to a glycine to glutamine change of amino acid at position 52 which makes fragment A unable to bind NAD and therefore non-toxic (Pappenheimer 1977, Ann Rev, Biochem. 46; 69-94, Rappuoli Applied and Environmental Microbiology Sept 1983 p560-564).

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Fragment C of TT is the non-toxic carboxy-terminal fragment of the tetanus toxin heavy chain. Tetanus toxin is a single peptide of approximately 150 kDa, which consists of 1315 amino-acid residues. Cleavage of tetanus-toxin by papain yields two fragments; one of them, fragment C, is approximately 50 kDa. Fragment C of TT is further described in
20 Neubauer et al. *Biochim. Biophys.Acta* **1981**, 27, 141–148.

In an embodiment, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is conjugated to the carrier protein via a linker, for instance a bifunctional linker (having two reactive ends). The linker is optionally
25 heterobifunctional (having different reactive groups at either end) or homobifunctional (having identical reactive groups at either end of a spacer arm), for example a reactive amino group and a reactive carboxylic acid group, 2 reactive amino groups or two reactive carboxylic acid groups. The linker has for example between 4 and 20, 4 and 12, 5 and 10 carbon atoms. A possible linker is ADH (adipic acid dihydrazide). Other linkers include B-propionamido (WO 00/10599), nitrophenyl-ethylamine (Gever et al (1979) Med. Microbiol. Immunol. 165; 171-288), haloalkyl halides (US4057685), glycosidic linkages (US4673574, US4808700), hexane diamine and 6-aminocaproic acid (US4459286). In another
30 embodiment, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is directly conjugated to the carrier protein. In one aspect, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is linked
35 to carrier protein via an isourea link. An isourea link is formed by the reaction between a

cyanate ester on polysaccharide and an amino group on the carrier. Reference to an "isourea link" herein refers to a stable link.

Immunogenic Compositions

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In an embodiment, the present invention provides an immunogenic composition comprising a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide (or conjugate) of the invention.

10 Typically the immunogenic compositions of the invention will comprise capsular polysaccharide antigens (suitably conjugated), wherein the polysaccharides are derived from at least ten serotypes of *S. pneumoniae*. The number of *S. pneumoniae* capsular polysaccharides can range from 10 different serotypes (or valences "v") to 23 different serotypes (23v, a 23 valent composition). In one embodiment the immunogenic
15 composition comprises 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more or 15 or more capsular polysaccharides from different *S. pneumoniae* serotypes. In one embodiment, there are 10, 11, 12, 13, 14 or 15 different *S. pneumoniae* serotypes. In another embodiment, the immunogenic composition of the invention may comprise conjugated *S. pneumoniae* polysaccharides and unconjugated *S. pneumoniae*
20 polysaccharides. In an embodiment, the total number of saccharide serotypes is less than or equal to 23.

In one embodiment the multivalent pneumococcal vaccine of the invention will comprise polysaccharides (suitably conjugated) selected from the following serotypes 1, 2, 3, 4, 5,
25 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 15C, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, although it is appreciated that one or two other serotypes could be substituted depending on the age of the recipient receiving the vaccine and the geographical location where the vaccine will be administered. In an embodiment, the vaccine may be an 11-valent vaccine. For example, a 11-valent vaccine may comprise polysaccharides from
30 serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F and 23F. In an embodiment, the vaccine may be an 12-valent or 13-valent vaccine. A 12 or 13-valent paediatric (infant) vaccine may also include the 11 valent formulation supplemented with serotypes 19A, or 22F, or 15, or 19A and 22F, or 19A and 15, or 22F and 15, whereas a 13-valent elderly vaccine may include the 11 valent formulation supplemented with serotypes 19A and 22F, 8 and
35 12F, or 8 and 15, or 8 and 19A, or 8 and 22F, or 12F and 15, or 12F and 19A, or 12F and 22F, or 15 and 19A, or 15 and 22F. In an embodiment, the vaccine may be a 14-valent or

15-valent vaccine. A 14 or 15-valent paediatric vaccine may include the 11 valent formulation described above supplemented with serotypes 3, 19A and 22F; serotypes 8, 19A and 22F; serotypes 12F, 19A and 22F; serotypes 15, 19A and 22F; serotypes 3, 8, 19A and 22F; serotypes 3, 12F, 19A and 22F; serotypes 3, 15, 19A and 22F. In an
5 embodiment, the vaccine may be a 16-valent vaccine. A 16 valent vaccine may include the 11 valent formulation described above supplemented with serotypes 3, 15B, 19A, 22F and 23F. A 16 valent vaccine may include the 11 valent formulation described above supplemented with serotypes 3, 15B, 19A, 22F and 33F. In an embodiment, the vaccine may be a 19-valent vaccine. A 19 valent vaccine may include the 11 valent formulation
10 described above supplemented with serotypes 8, 10A, 11A, 12F, 15B, 19A, 22F and 23F. A 19 valent vaccine may include the 11 valent formulation described above supplemented with serotypes 8, 10A, 11A, 12F, 15B, 19A, 22F and 33F. In an embodiment, the vaccine may be a 20-valent vaccine. A 20 valent vaccine may include the 11 valent formulation described above supplemented with serotypes 3, 8, 10A, 11A, 12F, 15B, 19A, 22F and
15 23F. A 20 valent vaccine may include the 11 valent formulation described above supplemented with serotypes 3, 8, 10A, 11A, 12F, 15B, 19A, 22F and 33F. In an embodiment, the vaccine may be a 21-valent vaccine.

In one embodiment, the immunogenic composition of the invention comprises capsular
20 polysaccharides derived from serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F and 23F (suitably conjugated). In a further embodiment of the invention at least 12 saccharide antigens (suitably conjugated) are included, for example capsular polysaccharides derived from serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F. In a further embodiment of the invention at least 12 saccharide antigens (suitably conjugated) are
25 included, for example capsular polysaccharides derived from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F and 23F. In a further embodiment of the invention, at least 13 polysaccharide antigens (suitably conjugated) are included, for example a vaccine may comprise capsular polysaccharides derived from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F, although further saccharide antigens, for example 23 valents
30 (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), are also contemplated by the invention. In a further embodiment of the invention, at least 15 saccharide antigens (suitably conjugated) are included, for example capsular polysaccharides derived from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F. In a further embodiment of the invention, at
35 least 15 saccharide antigens (suitably conjugated) are included, for example capsular polysaccharides derived from serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 14, 15B, 18C, 19A, 19F

22F, 23F and 33F. In a further embodiment of the invention, at least 16 saccharide antigens (suitably conjugated) are included, for example capsular polysaccharides derived from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 15B, 18C, 19A, 19F 22F, 23F and 33F. In another embodiment, the immunogenic composition comprises (conjugated) capsular (poly)saccharide of serotype 33F of *S. pneumoniae*. In another embodiment, the immunogenic composition comprises (conjugated) capsular (poly)saccharide of serotype 15C of *S. pneumoniae*. In another embodiment, the immunogenic composition comprises (conjugated) capsular (poly)saccharide of serotype 12F of *S. pneumoniae*. In an embodiment, there are 10 to 23 different *S. pneumoniae* capsular polysaccharide serotypes (suitably conjugated).

Carrier Proteins

Examples of carrier proteins which may be used in the present invention are DT (Diphtheria toxoid), TT (tetanus toxoid) or fragment C of TT, DT, CRM197 other DT point mutants, such as CRM176, CRM228, CRM 45 (Uchida et al J. Biol. Chem. 218; 3838-3844, 1973); CRM 9, CRM 45, 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 of DT disclosed in US 5843711, pneumococcal pneumolysin (Kuo et al (1995) Infect Immun 63; 2706-13) including pneumolysin (Ply) detoxified in some fashion for example GMBS detoxified pneumolysin (dPly-GMBS) (WO 04081515, PCT/EP2005/010258) or formaldehyde detoxified pneumolysin (dPly-formol), Pht (pneumococcal histidine triad) family proteins (PhtX), including PhtA, PhtB, PhtD, PhtE and fusions of Pht proteins for example PhtDE fusions, PhtBE fusions (WO 01/98334 and WO 03/54007), (Pht A-E are described in more detail below) OMPC (meningococcal outer membrane protein – usually extracted from *N. meningitidis* serogroup B – EP0372501), *Neisseria meningitidis* porin PorB, PD (*Haemophilus influenzae* 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, EP0471177), cytokines, lymphokines, growth factors or hormones (WO 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 *Clostridium difficile* (WO 00/61761).

In an embodiment, in the immunogenic composition of the invention each *Streptococcus pneumoniae* capsular saccharide is conjugated to a carrier protein independently selected from the group consisting of DT, CRM 197, TT, Fragment C of TT, dPly (detoxified pneumolysin), PhtA, PhtB, PhtD, PhtE, PhtDE, OmpC, PorB and *Haemophilus influenzae* Protein D. In a further embodiment, each *Streptococcus pneumoniae* capsular saccharide is conjugated to a carrier protein independently selected from the group consisting of TT, DT, CRM197, fragment C of TT, PhtD, PhtDE fusions (particularly those described in WO 01/98334 and WO 03/54007), detoxified pneumolysin and protein D. In a further embodiment, each *Streptococcus pneumoniae* capsular saccharide is conjugated to a carrier protein independently selected from the group consisting of TT, DT, CRM197, PhtD, detoxified pneumolysin and protein D. In a further embodiment, each *Streptococcus pneumoniae* capsular saccharide is conjugated to a carrier protein independently selected from the group consisting of TT, DT, CRM197, PhtD and protein D. In a further embodiment, each *Streptococcus pneumoniae* capsular saccharide is conjugated to a carrier protein independently selected from the group consisting of TT, DT, CRM197 and protein D.

In an embodiment, the immunogenic composition of the invention comprises two or more different carrier proteins. In an embodiment, the immunogenic composition of the invention comprises 2, 3, 4, 5 or 6 different carrier proteins. Each type of carrier protein may act as carrier for more than one polysaccharide, which polysaccharides may be from the same or different serotypes. In one embodiment, two or more different polysaccharide serotypes may be conjugated to the same carrier protein, either to the same molecule of carrier protein (carrier molecules having 2 or more different polysaccharide serotypes conjugated to it) [see for instance WO 04/083251] or to different molecules of the same carrier protein (each molecule of protein carrier only having one serotype of saccharide conjugated to it).

In an embodiment, the immunogenic composition of the invention comprises protein D from *Haemophilus influenzae* (PD), for example, protein D sequence from Figure 9 (Figure 9a and 9b together, 364 amino acids) of EP 0594610 (SEQ ID NO: 1). Inclusion of this protein in the immunogenic composition may provide a level of protection against *Haemophilus influenzae* related otitis media (Pyrmula et al Lancet 367; 740-748 (2006)). Protein D may be used as a full length protein or as a fragment (for example, Protein D

may be as described in WO0056360). For example, a protein D sequence may comprise (or consist) of the protein D fragment described in EP0594610 which begins at the sequence SSHSSNMANT (SerSerHisSerSerAsnMetAlaAsnThr) (SEQ ID NO. 3), and lacks the 19 N-terminal amino acids from Fig 9 of EP0594610, optionally with the tripeptide MDP from NS1 fused to the N-terminal of said protein D fragment (348 amino acids) (SEQ ID NO:2). In one aspect, the protein D or fragment of protein D is unlipidated. The protein D could be present in the immunogenic composition as a free protein or as a carrier protein. In one aspect, protein D is present in the immunogenic composition as free protein. In another aspect, protein D is present both as a carrier protein and as free protein. In a further aspect, protein D is present as a carrier protein for one or more of the polysaccharides. In a further aspect, 2-9 of the capsular polysaccharides selected from different serotypes are conjugated to protein D. In a further aspect, protein D is present as a carrier protein for the majority of the polysaccharides, for example 6, 7, 8, 9 or more of the polysaccharides may be conjugated to protein D.

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In an embodiment, the immunogenic composition of the invention contains 2-8, 2-7, 2-6, 2-5, 3-5, 4-5, 2-4, 2-3, 3-4 or 2, 3, 4, 5, 6, 7 or 8 capsular saccharide serotype conjugates in which protein D is the carrier protein. For example, 2-8, 2-7, 2-6, 2-5, 3-5, 4-5, 2-4, 2-3, 3-4 or 2, 3, 4, 5, 6, 7 or 8 polysaccharides selected from serotype 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F are conjugated to protein D. For example, polysaccharides from serotypes 1, 4, 5, 6B, 7F, 9V, 14 and 23F are conjugated to protein D.

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In an embodiment, polysaccharides from at least serotypes 1 and 3, 1 and 4, 1 and 5, 1 and 6A, 1 and 6B, 1 and 7, 1 and 9V, 1 and 14, 1 and 22F, 1 and 23F, 3 and 4, 3 and 5, 3 and 6A, 3 and 6B, 3 and 7F, 3 and 9V, 3 and 14, 3 and 22F, 3 and 23F, 4 and 5, 4 and 6A, 4 and 6B, 4 and 7F, 4 and 9V, 4 and 14, 4 and 22F, 4 and 23F, 5 and 6A, 5 and 6B, 5 and 7F, 5 and 9V, 5 and 14, 5 and 22F, 5 and 23F, 6A and 6B, 6A and 7F, 6A and 9V, 6A and 14, 6A and 22F, 6A and 23F, 6B and 7F, 6B and 9V, 6B and 14, 6B and 22F, 6B and 23F, 7F and 9V, 7F and 14, 7F and 22F, 7F and 23F, 9V and 14, 9V and 22F, 9V and 23F, 14 and 22F, 14 and 23F or 22F and 23F are conjugated to protein D.

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In an embodiment, polysaccharides from at least serotypes 1, 3 and 4; 1, 3 and 5; 1, 3 and 6A; 1, 3 and 6B; 1, 3 and 7F; 1, 3 and 9V; 1, 3 and 14; 3, 4 and 7F; 3, 4 and 5; 3, 4 and 7F; 3, 4 and 9V; 3, 4 and 14; 4, 5 and 7F; 4, 5 and 9V; 4, 5, and 14; 5, 7F and 9V; 5, 7F and 14; 7F, 9V and 14; 1, 3, 4 and 5; 3, 4, 5 and 7F; 4, 5, 7F and 9V; 4, 5, 7F and 14;

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4, 5, 9V and 14; 4, 7F, 9V and 14; 5, 7F, 9V and 14; or 4, 5, 7F, 9V and 14 are conjugated to protein D.

For example, in a 10 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7 or 8
5 of the capsular polysaccharides from different serotypes are conjugated to protein D. For example, in a 11 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7 or 8 of the capsular polysaccharides from different serotypes are conjugated to protein D. For example, in a 12 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7 or 8 of the capsular polysaccharides from different serotypes are conjugated to protein D. For
10 example, in a 13 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7 or 8 of the capsular polysaccharides from different serotypes are conjugated to protein D. For example, in a 14 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7 or 8 of the capsular polysaccharides from different serotypes are conjugated to protein D. For example, in a 15 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7 or 8 of
15 the capsular polysaccharides from different serotypes are conjugated to protein D. For example, in a 16 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7 or 8 of the capsular polysaccharides from different serotypes are conjugated to protein D. For example, in a 17 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7 or 8 of the capsular polysaccharides from different serotypes are conjugated to protein D. For
20 example, in a 18 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7, 8 or 9 of the capsular polysaccharides from different serotypes are conjugated to protein D. For example, in a 19 valent *S. pneumoniae* immunogenic composition, 2, 3, 4, 5, 6, 7, 8 or 9 of the capsular polysaccharides from different serotypes are conjugated to protein D. Optionally, the serotypes conjugated to protein D are selected from the groups described
25 above.

In an embodiment, the immunogenic composition of the invention comprises at least one capsular saccharide conjugated to tetanus toxoid (TT). In another embodiment, capsular saccharide 18C is conjugated to TT, optionally wherein 18C is the only saccharide in the
30 composition conjugated to tetanus toxoid (TT).

In an aspect of the present invention, serotype 19F is conjugated to DT or CRM197. In another aspect, serotype 19F is conjugated to DT. In one aspect, the remaining saccharide serotypes of the immunogenic composition may all be conjugated to one or
35 more carrier proteins that are not DT (i.e. only 19F is conjugated to DT). In one embodiment, 19F is conjugated to DT or CRM197, and the remaining serotypes are

conjugated to carrier proteins independently selected from PhtD, PD (Protein D), TT (Tetanus Toxoid), DT (Diphtheria Toxoid) and CRM197. In another embodiment, 19F is conjugated to DT or CRM 197, and the remaining serotypes are conjugated to carrier proteins independently selected from PD, TT, DT and CRM197. In a further embodiment, 5 19F is conjugated to DT or CRM 197, and the remaining serotypes are conjugated to carrier proteins independently selected from PD, TT and CRM197 (for example as described in WO2007/071710A2 and WO2007/071707A2).

In an embodiment, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention and *Streptococcus pneumoniae* serotype 6B capsular polysaccharide are conjugated to different carrier proteins. In a further embodiment, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention is conjugated to CRM 197. In a further embodiment a *Streptococcus pneumoniae* serotype 6B capsular polysaccharide conjugate is present, but is not conjugated to DT or CRM 15 197. In a further embodiment, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is conjugated to CRM197 and *Streptococcus pneumoniae* serotype 6B capsular polysaccharide *Streptococcus pneumoniae* serotype is conjugated to a carrier protein other than CRM197. In a further embodiment, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is conjugated to CRM197 and 20 *Streptococcus pneumoniae* serotype 6B capsular polysaccharide *Streptococcus pneumoniae* serotype is conjugated to a carrier protein selected from PhtD, PD (Protein D), TT (Tetanus Toxoid) or DT (Diphtheria Toxoid). In a further embodiment, the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is conjugated to CRM197 and *Streptococcus pneumoniae* serotype 6B capsular polysaccharide is 25 conjugated to protein D.

In an embodiment of the invention, the carrier protein conjugated to one or more of the *S. pneumoniae* capsular polysaccharides is a member of the polyhistidine triad family (Pht) proteins, fragments or fusion proteins thereof. The PhtA, PhtB, PhtD or PhtE proteins may 30 have an amino acid sequence sharing 80%, 85%, 90%, 95%, 98%, 99% or 100% identity with a sequence disclosed in WO 00/37105 or WO 00/39299 (e.g. with amino acid sequence 1-838 or 21-838 of SEQ ID NO: 4 of WO 00/37105 for PhtD). For example, fusion proteins are composed of full length or fragments of 2, 3 or 4 of PhtA, PhtB, PhtD, PhtE. Examples of fusion proteins are PhtA/B, PhtA/D, PhtA/E, PhtB/A, PhtB/D, PhtB/E, 35 PhtD/A, PhtD/B, PhtD/E, PhtE/A, PhtE/B and PhtE/D, wherein the proteins are linked with the first mentioned at the N-terminus (see for example WO01/98334).

Where fragments of Pht proteins are used (separately or as part of a fusion protein), each fragment optionally contains one or more histidine triad motif(s) and/or coiled coil regions of such polypeptides. A histidine triad motif is the portion of polypeptide that has the sequence HxxHxH where H is histidine and x is an amino acid other than histidine. A
5 sequence HxxHxH where H is histidine and x is an amino acid other than histidine. A coiled coil region is a region predicted by “Coils” algorithm Lupus, A et al (1991) Science 252; 1162-1164. In an embodiment, the fragment includes one or more histidine triad motif as well as at least one coiled coil region. In an embodiment, the fragment contains exactly or at least 2, 3, 4 or 5 histidine triad motifs (optionally, with native Pht sequence
10 between the 2 or more triads, or intra-triad sequence that is more than 50, 60, 70, 80, 90 or 100 % identical to a native pneumococcal intra-triad Pht sequence – e.g. the intra-triad sequence shown in SEQ ID NO: 4 of WO 00/37105 for PhtD). In an embodiment, the fragment contains exactly or at least 2, 3 or 4 coiled coil regions. In an embodiment, a Pht protein disclosed herein includes the full length protein with the signal sequence attached,
15 the mature full length protein with the signal peptide (for example 20 amino acids at N-terminus) removed, naturally occurring variants of Pht protein and immunogenic fragments of Pht protein (e.g. fragments as described above or polypeptides comprising at least 15 or 20 contiguous amino acids from an amino acid sequence in WO00/37105 (SEQ ID NOs 4, 6, 8 or 10) or WO00/39299 (SEQ ID NOs 2, 4, 6, 8, 10 or 14) wherein
20 said polypeptide is capable of eliciting an immune response specific for said amino acid sequence in WO00/37105 or WO00/39299).

In particular, the term “PhtD” as used herein includes the full length protein with the signal sequence attached, the mature full length protein with the signal peptide (for example 20
25 amino acids at N-terminus) removed, naturally occurring variants of PhtD and immunogenic fragments of PhtD (e.g. fragments as described above or polypeptides comprising at least 15 or 20 contiguous amino acids from a PhtD amino acid sequence in WO00/37105 or WO00/39299) wherein said polypeptide is capable of eliciting an immune response specific for said PhtD amino acid sequence in WO00/37105 or WO00/39299
30 (e.g. SEQ ID NO: 4 of WO 00/37105 or SEQ ID NO: 14 of WO 00/39299 for PhtD). All forms of PhtD mentioned above can be used in the present invention.

Conjugation Processes

35 The saccharide conjugates present in the immunogenic compositions of the invention may be prepared by the conjugation methods of the present invention or any known coupling

technique. The conjugation method may rely on activation of the saccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated saccharide may thus be coupled directly or via a spacer (linker) group to an amino group on the carrier protein. For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using GMBS (4-Maleimidobutyric acid N-hydroxysuccinimide ester)) or a haloacetylated carrier protein (for example using SIAB (succinimidyl (4-iodoacetyl)aminobenzoate), or SIA (succinimidyl iodoacetate), or SBAP (succinimidyl-3-(bromoacetamide)propionate)). In an embodiment, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or ADH (adipic acid dihydrazide) and the amino-derivatised saccharide is conjugated to the carrier protein using carbodiimide (e.g. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC or EDC)) chemistry via a carboxyl group on the protein carrier. Such conjugates are described in PCT published application WO 93/15760 Uniformed Services University and WO 95/08348 and WO 96/29094.

In an embodiment, at least one of the *S. pneumoniae* capsular polysaccharides is directly conjugated to a carrier protein (e.g. using one of the chemistries described above). In an embodiment, at least one of the *S. pneumoniae* capsular polysaccharides is directly conjugated by 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP). In an embodiment, the majority of the capsular polysaccharides for example 5, 6, 7, 8, 9 or more are directly linked to the carrier protein by CDAP (see WO 95/08348 and WO 96/29094)

In an embodiment, the *Streptococcus pneumoniae polysaccharide* is conjugated to the carrier protein via a linker, for instance a bifunctional linker. The linker is optionally heterobifunctional or homobifunctional, having for example a reactive amino group and a reactive carboxylic acid group, 2 reactive amino groups or two reactive carboxylic acid groups. The linker has for example between 4 and 20, 4 and 12, 5 and 10 carbon atoms. A possible linker is ADH (adipic acid dihydrazide). Other linkers include B-propionamido (WO 00/10599), nitrophenyl-ethylamine (Gever et al (1979) Med. Microbiol. Immunol. 165; 171-288), haloalkyl halides (US4057685), glycosidic linkages (US4673574, US4808700), hexane diamine and 6-aminocaproic acid (US4459286). In an embodiment, the immunogenic composition of the invention may comprise 18C capsular polysaccharide conjugated to the carrier protein via a linker, optionally the linker is ADH. In an

embodiment, the immunogenic composition of the invention may comprise 22F capsular polysaccharide conjugated to the carrier protein via a linker, optionally the linker is ADH (for example as described in WO2007/071711A2).

5 Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, N-hydroxysulfosuccinimide (S-NHS), EDC, O-(N-Succinimidyl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (TSTU). Many are described in WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the polysaccharide with
10 Carbonyldiimidazole (CDI) (Bethell et al J. Biol. Chem. 1979, 254; 2572-4, Hearn et al J. Chromatogr. 1981. 218; 509-18) followed by reaction of with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling
15 the CDI carbamate intermediate with an amino group on a protein.

The conjugates can also be prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in
20 EP-0-161-188, EP-208375 and EP-0-477508.

A further method involves the coupling of a cyanogen bromide (or CDAP) activated polysaccharide derivatised with adipic acid dihydrazide (ADH) to the protein carrier by Carbodiimide condensation (Chu C. et al Infect. Immunity, 1983 245 256), for example using EDAC.

25 In an embodiment, at least one *S. pneumoniae* polysaccharide is conjugated to a carrier protein via a linker using CDAP and EDAC. For example, 18C or 22F may be conjugated to a protein via a linker (for example those with two hydrazino groups at its ends such as ADH) using CDAP and EDAC as described above. When a linker is used, CDAP may be
30 used to conjugate the polysaccharide to a linker and EDAC may then be used to conjugate the linker to a protein or, alternatively EDAC may be used first to conjugate the linker to the protein, after which CDAP may be used to conjugate the linker to the saccharide.

35 In an embodiment, a hydroxyl group (suitably an activated hydroxyl group for example a hydroxyl group activated to make a cyanate ester [e.g. with CDAP]) on a polysaccharide

is linked to an amino or carboxylic group on a protein either directly or indirectly (through a linker). Where a linker is present, a hydroxyl group on a polysaccharide is suitably linked to an amino group on a linker, for example by using CDAP conjugation. A further amino group in the linker for example ADH) may be conjugated to a carboxylic acid group on a protein, for example by using carbodiimide chemistry, for example by using EDAC. In an embodiment, the pneumococcal capsular saccharide(s) is conjugated to the linker first before the linker is conjugated to the carrier protein. Alternatively the linker may be conjugated to the carrier before conjugation to the saccharide.

10 A combination of techniques may also be used, with some saccharide-protein conjugates being prepared by CDAP, and some by reductive amination.

In general the following types of chemical groups on a protein carrier can be used for coupling / conjugation:

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A) Carboxyl (for instance via aspartic acid or glutamic acid). In one embodiment this group is linked to amino groups on polysaccharides directly or to an amino group on a linker with carbodiimide chemistry e.g. with EDAC.

20 B) Amino group (for instance via lysine). In one embodiment this group is linked to carboxyl groups on polysaccharides directly or to a carboxyl group on a linker with carbodiimide chemistry e.g. with EDAC. In another embodiment this group is linked to hydroxyl groups activated with CDAP or CNBr on polysaccharides directly or to such groups on a linker; to polysaccharides or linkers having an aldehyde group; to
25 polysaccharides or linkers having a succinimide ester group.

C) Sulphydryl (for instance via cysteine). In one embodiment this group is linked to a bromo or chloro acetylated polysaccharide or linker with maleimide chemistry. In one embodiment this group is activated/modified with bis diazobenzidine.

30

D) Hydroxyl group (for instance via tyrosine). In one embodiment this group is activated/modified with bis diazobenzidine.

E) Imidazolyl group (for instance via histidine). In one embodiment this group is
35 activated/modified with bis diazobenzidine.

F) Guanidyl group (for instance via arginine).

G) Indolyl group (for instance via tryptophan).

- 5 On a saccharide, in general the following groups can be used for a coupling: OH, COOH or NH₂. Aldehyde groups can be generated after different treatments known in the art such as: periodate, acid hydrolysis, hydrogen peroxide, etc.

Direct coupling approaches:

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Saccharide-OH + CNBr or CDAP ----> cyanate ester + NH₂-Protein ----> conjugate

Saccharide-aldehyde + NH₂-Protein ----> Schiff base + NaCNBH₃ ----> conjugate

Saccharide-COOH + NH₂-Protein + EDAC ----> conjugate

Saccharide-NH₂ + COOH-Protein + EDAC ----> conjugate

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Indirect coupling via spacer (linker) approaches:

Saccharide-OH + CNBr or CDAP ---> cyanate ester + NH₂----NH₂ ----> saccharide----NH₂
+ COOH-Protein + EDAC ----> conjugate

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Saccharide-OH + CNBr or CDAP ----> cyanate ester + NH₂----SH ----> saccharide----SH
+ SH-Protein (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance) ----> saccharide-S-S-Protein

25

Saccharide-OH + CNBr or CDAP ---> cyanate ester + NH₂----SH -----> saccharide----SH
+ maleimide-Protein (modification of amino groups) ----> conjugate

Saccharide-OH + CNBr or CDAP ---> cyanate ester + NH₂----SH ---> Saccharide-SH +
haloacetylated-Protein ----> Conjugate

30

Saccharide-COOH + EDAC + NH₂----NH₂ ---> saccharide-----NH₂ + EDAC + COOH-Protein ----> conjugate

35

Saccharide-COOH + EDAC+ NH₂----SH ----> saccharide----SH + SH-Protein (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance) ----> saccharide-S-S-Protein

Saccharide-COOH + EDAC+ NH₂---SH ----> saccharide---SH + maleimide-Protein
(modification of amino groups) ----> conjugate

5 Saccharide-COOH + EDAC + NH₂---SH ---> Saccharide-SH + haloacetylated-Protein ----
> Conjugate

Saccharide-Aldehyde + NH₂---NH₂ ----> saccharide---NH₂ + EDAC + COOH-Protein ----
> conjugate

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Note: instead of EDAC above, any suitable carbodiimide may be used.

In summary, the types of protein carrier chemical group that may be generally used for
coupling with a polysaccharide are amino groups (for instance on lysine residues), COOH
15 groups (for instance on aspartic and glutamic acid residues) and SH groups (if accessible)
(for instance on cysteine residues).

In an embodiment, the *Streptococcus pneumoniae* serotype 6A capsular polysaccharide
of the invention is conjugated to the carrier protein (e.g. CRM-197) using CDAP
20 chemistry. In one aspect, the CDAP chemistry uses a CDAP:PS6A ratio between 1:2 to
3:1, 1:1.5 to 2:1, e.g. 1:1. In another aspect, the CDAP conjugation is carried out using a
coupling time of between 50-130 minutes, 60-130 minutes, or 110-130 minutes. Thus the
present invention also provides, a process for preparing a *Streptococcus pneumoniae*
serotype 6A capsular polysaccharide conjugate (e.g. a sized *Streptococcus pneumoniae*
25 serotype 6A capsular polysaccharide of the invention) comprising conjugating a sized
Streptococcus pneumoniae serotype 6A capsular polysaccharide to a carrier protein (e.g.
CRM-197) or to a linker (e.g. ADH) using CDAP chemistry using a CDAP:PS
(polysaccharide) ratio between 1:2 to 3:1, 1:1.5 to 2:1, e.g. 1:1.

In another aspect, the present invention provides, a process for preparing a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide conjugate (e.g. a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention) comprising conjugating a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide to a carrier protein (e.g. CRM-197) using CDAP chemistry using a CDAP:PS (polysaccharide) ratio between 1:2 to 3:1, 1:1.5 to 2:1, e.g. 1:1. In one aspect, the present invention also provides, a process for preparing a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide conjugate (e.g. a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention, such as a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide having an average size (e.g. M_w) between 100-1000, 110-750, 150-500, 180-600, 210-490, 210-450, 180-400, 210-400, 210-370, 220-360, 230-350, 240-340, 240-320, 240-310 or 250-310 kDa) comprising conjugating a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide to a carrier protein (e.g. CRM-197) or to a linker (e.g. ADH) using CDAP chemistry using a coupling time of between 50-130 minutes, 60-130 minutes, or 110-130 minutes.

In another aspect, the present invention provides, a process for preparing a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide conjugate (e.g. a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention, such as a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide having an average size (e.g. M_w) between 100-1000, 110-750, 150-500, 180-600, 210-490, 210-450, 180-400, 210-400, 210-370, 220-360, 230-350, 240-340, 240-320, 240-310 or 250-310 kDa) comprising conjugating a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide to a carrier protein (e.g. CRM-197) using CDAP chemistry using a coupling time of between 50-130 minutes, 60-130 minutes, or 110-130 minutes. In one aspect, the pH for activation and coupling is between pH 8 to pH9, suitably pH9.5. In another aspect, the conjugation is carried out in the presence of NaCl. For example, in 0.1-3M NaCl, 0.1-2.5M NaCl, 1.5-2.5M NaCl or 2M NaCl.

The present invention also provides, a process for preparing a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide conjugate comprising (a) conjugation of a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide (e.g. a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide of the invention) to a carrier protein (e.g. CRM-197) and (b) diafiltration against a solution having a concentration of NaCl below 150mM (e.g. below 100mM NaCl, below 50mM NaCl, below 10mM NaCl) or using water (e.g. WFI, water for injection).

In another aspect, the present invention provides, a solution comprising 6A-CRM197 in less than 150mM NaCl. For example, less than 100mM NaCl, less than 50mM NaCl, less than 10nM NaCl, or in the absence of sodium chloride. In another aspect, the present invention provides, an immunogenic composition of the invention (e.g. 6A-CRM197) comprising less than 150mM NaCl. For example, less than 100mM NaCl, less than 50mM NaCl, less than 10mM NaCl, or in the absence of sodium chloride.

Ratio of Carrier Protein to Polysaccharide

In an embodiment, the ratio of carrier protein to *S. pneumoniae polysaccharide* is between 1:5 and 5:1; e.g. between 1:0.5-4:1, 1:1-3.5:1, 1.2:1-3:1, 1.5:1-2.5:1; e.g. between 1:2 and 2.5:1; 1:1 and 2:1 (w/w; weight/weight). In an embodiment, the majority of the conjugates, for example 6, 7, 8, 9 or more of the conjugates have a ratio of carrier protein to polysaccharide that is greater than 1:1, for example 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1 or 1.6:1.

In an embodiment, the ratio of carrier protein to *Streptococcus pneumoniae* serotype 6A capsular polysaccharide in immunogenic compositions of the invention is between 5:1 and 1:5, 4:1 and 1:1 or 2:1 and 1:1, 1.5:1 and 1:1, 1.4:1 and 1.3:1 (for example 1.2:1, 1.5:1) (w/w).

The ratio of polysaccharide to carrier protein (w/w) in a conjugate may be determined using the conjugate. The amount of protein is determined using a Lowry assay (for example Lowry *et al.* (1951) J. Biol. Chem. 193, 265-275 or Peterson *et al.* Analytical Biochemistry 100, 201-220 (1979)) and the amount of polysaccharide is determined using resorcinol assay (Monsigny *et al.* (1988) Anal. Biochem. 175, 525-530). The final Protein/Polysaccharide ratio (w/w) on the sterilized conjugate is determined by the ratio of the Lowry/resorcinol concentrations.

Size of Capsular Polysaccharides in the Immunogenic Composition

Capsular polysaccharides of *Streptococcus pneumoniae* comprise repeating oligosaccharide units which may contain up to 8 sugar residues. For a review of the oligosaccharide units for the key *Streptococcus pneumoniae* serotypes see JONES, Christopher. **Vaccines based on the cell surface carbohydrates of pathogenic bacteria.** *An. Acad. Bras. Ciênc.*, June 2005, vol.77, no.2, p.293-324. Table II ISSN 0001-

3765. In one embodiment, a capsular polysaccharide may be a full length polysaccharide, however in others it may be a shorter than native length polysaccharide chain of repeating units. In one embodiment, the *Streptococcus pneumoniae* serotype capsular polysaccharide conjugates post conjugation should be readily filterable through a 0.2
5 micron filter such that a yield of more than 95% is obtained post filtration compared with the pre filtration sample.

In addition to the sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide conjugate of the invention, the immunogenic composition of the invention may comprise
10 one or more (poly)saccharide conjugates from *Streptococcus pneumoniae* serotypes other than 6A (e.g. 6B and/or 23F) wherein the average size (e.g. weight-average molecular weight; M_w) of the (poly)saccharide before conjugation is above 80kDa, 100kDa, 200kDa, 300kDa, 400kDa, 500kDa, 700kDa or 1000kDa. For example, the immunogenic composition of the invention may comprise one or more (poly)saccharide
15 conjugates from *Streptococcus pneumoniae* serotypes other than 6A wherein the average size (e.g. weight-average molecular weight; M_w) of the (poly)saccharide before conjugation is between 80-100kDa, 100-200kDa, 200-300kDa, 300-400kDa, 400-500kDa, 500-1000kDa or 1000-1400kDa. In one embodiment, the immunogenic composition comprises (i) sized a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide
20 conjugate and (ii) one or more (poly)saccharide conjugates with an average size of saccharide before conjugation of 50-1600, 80-1400, 100-1000, 150-500, or 200-400 kDa (note that where average size is M_w , 'kDa' units should be replaced herein with ' $\times 10^3$ ').

In an embodiment, the immunogenic composition of the invention comprises a serotype 1
25 *S. pneumoniae* polysaccharide having an average size (M_w) of between 100-1000, 200-800, 250-600, or 300-400 kDa. In an embodiment, the immunogenic composition of the invention comprises a serotype 4 *S. pneumoniae* polysaccharide having an average size (M_w) of between 50-500, 60-300, 70-150, or 75-125 kDa. In an embodiment, the immunogenic composition of the invention comprises a serotype 5 *S. pneumoniae*
30 polysaccharide having an average size (M_w) of between 100-1000, 100-700, 100-350, or 150-300 kDa. In an embodiment, the immunogenic composition of the invention comprises a serotype 6B *S. pneumoniae* polysaccharide having an average size (M_w) of between 200-1800, 500-1800, 600-1800, 900-1660, or 1000-1400 kDa. In an embodiment, the immunogenic composition of the invention comprises a serotype 7F *S.*
35 *pneumoniae* polysaccharide having an average size (M_w) of between 50-1000, 100-750, 150-500, or 200-300 kDa. In an embodiment, the immunogenic composition of the

invention comprises a serotype 9V *S. pneumoniae* polysaccharide having an average size (M_w) of between 50-1000, 100-750, 150-500, 200-400, or 250-300 kDa. In an embodiment, the immunogenic composition of the invention comprises a serotype 14 *S. pneumoniae* polysaccharide having an average size (M_w) of between 50-1000, 100-750, 150-500, or 200-250 kDa. In an embodiment, the immunogenic composition of the invention comprises a 18C *S. pneumoniae* polysaccharide having an average size (M_w) of between 50-1000, 50-750, 50-500, 50-190, 50-150 or 80-110 kDa. In an embodiment, the immunogenic composition of the invention comprises a 19A *S. pneumoniae* polysaccharide having an average size (M_w) of between 50-800, 110-700, 110-300, 120-200, 130-180, 140-160 or 80-130 kDa. In an embodiment, the immunogenic composition of the invention comprises a serotype 19F *S. pneumoniae* polysaccharide having an average size (M_w) of between 50-1000, 100-750, 100-500, 100-190 or 120-180 kDa. In an embodiment, the immunogenic composition of the invention comprises a serotype 23F *S. pneumoniae* polysaccharide having an average size (M_w) of between 500-1500, 600-1500, 700-1300, 900-1250, 800-1100, or 900-1000 kDa. In an embodiment, the immunogenic composition of the invention comprises a serotype 22F *S. pneumoniae* polysaccharide having an average size (M_w) of between 50- 800, 110- 700, 110-300, 120-200, 130-180, 150-170, 100-190, 100-150, 95-125 or 100-115 kDa.

In an embodiment, the immunogenic composition of the invention comprises 1 or more, native capsular polysaccharides from different *S. pneumoniae* serotypes. In another embodiment, the immunogenic composition comprises *Streptococcus pneumoniae* polysaccharides from at least 10 serotypes conjugated to a carrier protein, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, or 9 of the *S. pneumoniae* polysaccharide serotypes is native polysaccharide. In another embodiment, the immunogenic composition of the invention comprises native *Streptococcus pneumoniae* capsular serotype 6B polysaccharide. In another embodiment, the immunogenic composition of the invention comprises native *Streptococcus pneumoniae* capsular serotype 23F polysaccharide.

In an aspect of the invention, the immunogenic composition comprises *Streptococcus pneumoniae* polysaccharides from at least 10 serotypes conjugated to a carrier protein, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, or 9 of the *S. pneumoniae* polysaccharide serotypes is sized by a factor up to x2, x3, x4, x5, x6, x7, x8, x9 or x10. In one embodiment of this aspect, the majority of the polysaccharides, for example 6, 7, 8 or more of the polysaccharide serotypes are sized by a factor up to x2, x3, x4, x5, x6, x7, x8, x9 or x 10.

For example, sizing may be from a factor of between x2 to x6, x2 to x5, x2 to x4, or x3 to x6, x3 to x5 or x3 to x4.

5 In an embodiment, the majority of *S. pneumoniae* polysaccharides in the immunogenic composition are sized. In an embodiment, the majority of *S. pneumoniae* polysaccharide serotypes in the immunogenic composition are sized. In one aspect, *S. pneumoniae* polysaccharides in the immunogenic composition are sized mechanical cleavage, for instance by microfluidisation or sonication. In another aspect, *S. pneumoniae* polysaccharides in the immunogenic composition are sized by chemical cleavage, e.g.
10 treatment with acetic acid or periodate. Sizing is by a factor of no more than x20, x10, x8, x6, x5, x4, x3 or x2.

In an embodiment, the immunogenic composition comprises *S. pneumoniae* conjugates that are a mixture of native polysaccharides and polysaccharides that are sized by a
15 factor of no more than x20, x10, x8, x6, x5, x4, x3 or x2. In one aspect of this embodiment, the majority of the polysaccharides, for example 6, 7, 8, 9, 10 or more of the polysaccharides are sized by a factor of up to x2, x3, x4, x5 or x6.

20 Dosage

In general, the immunogenic composition of the invention may comprise a dose of each saccharide conjugate between 0.1 and 20 μ g, 1 and 10 μ g or 1 and 3 μ g of saccharide.

In an embodiment, in the immunogenic composition of the present invention the dose of
25 the *Streptococcus pneumoniae* 6A polysaccharide conjugate is between 1 and 10 μ g, 1 and 5 μ g, or 1 and 3 μ g of saccharide (e.g. 2 μ g).

In an embodiment, the immunogenic composition of the invention contains each *S. pneumoniae* capsular saccharide at a dose of between 0.1-20 μ g; 0.5-10 μ g; 0.5- 5 μ g or 1-
30 3 μ g of saccharide. In an embodiment, capsular polysaccharides may be present at different dosages, for example some capsular polysaccharides may be present at a dose of around or exactly 1 μ g or some capsular polysaccharides may be present at a dose of around or exactly 3 μ g. In an embodiment, polysaccharides from serotypes 3, 18C and 19F are present at a higher dose than other polysaccharides. In an embodiment,
35 polysaccharides from serotypes 4, 18C and 19F are present at a higher dose than other

polysaccharides. In one aspect of this embodiment, serotypes 3, 18C and 19F are present at a dose of around or exactly 3 μ g whilst other polysaccharides in the immunogenic composition are present at a dose of around or exactly 1 μ g. In one aspect of this embodiment, serotypes 4, 18C and 19F are present at a dose of around or exactly 3 μ g whilst other polysaccharides in the immunogenic composition are present at a dose of around or exactly 1 μ g.

“Around” or “approximately” are defined as within 10% more or less of the given figure for the purposes of the invention.

***Streptococcus pneumoniae* Proteins**

The immunogenic composition of the invention may also comprise *Streptococcus pneumoniae* proteins, herein termed *Streptococcus pneumoniae* proteins of the invention. Such proteins may be used as carrier proteins, or may be present as free proteins, or may be present both as carrier proteins and as free proteins. In an embodiment, the immunogenic composition of the invention further comprises one or more unconjugated or conjugated *S. pneumoniae* proteins. In an embodiment, the immunogenic composition of the invention further comprises one or more unconjugated *S. pneumoniae* proteins. For example, immunogenic compositions of the invention may comprise unconjugated pneumolysin, e.g. dPly, and unconjugated pneumococcal PhtD.

The *Streptococcus pneumoniae* proteins of the invention are either surface exposed, at least during part of the life cycle of the pneumococcus, or are proteins which are secreted or released by the pneumococcus. In an embodiment, the proteins of the invention are selected from the following categories, such as proteins having a Type II Signal sequence motif of LXXC (where X is any amino acid, e.g., the polyhistidine triad family (PhtX)), choline binding proteins (e.g. CbpX, PcpA), proteins having a Type I Signal sequence motif (e.g., Sp101), proteins having a LPXTG motif (where X is any amino acid, e.g., Sp128, Sp130), and toxins (e.g., Ply). Preferred examples within these categories (or motifs) are the following proteins, or immunologically functional equivalents thereof. Thus, the immunogenic composition of the invention may comprise one or more *S. pneumoniae* proteins selected from Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, pneumococcal autolysin LytX family (LytA (*N*-acetylmuramoyl-L-alanine amidase), LytB, LytC), LytX truncates, CbpX truncate-LytX truncate chimeric

proteins, detoxified pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133. In a further embodiment, the immunogenic composition of the invention comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX
5 truncates, CbpXtruncate-LytXtruncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, and Sp128. In a further embodiment, the immunogenic composition comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin
10 (Ply), and Sp128.

The Pht (Poly Histidine Triad) family comprises proteins PhtA, PhtB, PhtD, and PhtE. The family is characterized by a lipidation sequence, two domains separated by a proline-rich region and several histidine triads, possibly involved in metal or nucleoside binding or
15 enzymatic activity, (3-5) coiled-coil regions, a conserved N-terminus and a heterogeneous C terminus. It is present in all strains of pneumococci tested. Homologous proteins have also been found in other Streptococci and Neisseria. In one embodiment of the invention, the immunogenic composition comprises PhtD. It is understood, however, that the terms Pht A, B, D, and E refer to proteins having sequences disclosed in the citations below as
20 well as variants thereof that have a sequence homology that is at least 90% identical to the proteins described below, e.g. amino acids 21-838 of SEQ ID NO: 4 of WO00/37105. In an embodiment it is at least 95% identical and in another embodiment it is 97% identical.

25 With regards to the PhtX proteins, PhtA is disclosed in WO 98/18930, and is also referred to Sp36. As noted above, it is a protein from the polyhistidine triad family and has the type II signal motif of LXXC. PhtD is disclosed in WO 00/37105, and is also referred to Sp036D. As noted above, it also is a protein from the polyhistidine triad family and has the type II LXXC signal motif. PhtB is disclosed in WO 00/37105, and is also referred to
30 Sp036B. Another member of the PhtB family is the C3-Degrading Polypeptide, as disclosed in WO 00/17370. This protein also is from the polyhistidine triad family and has the type II LXXC signal motif. A preferred immunologically functional equivalent is the protein Sp42 disclosed in WO 98/18930. A PhtB truncate (approximately 79kD) is disclosed in WO99/15675 which is also considered a member of the PhtX family. PhtE is
35 disclosed in WO00/30299 and is referred to as BVH-3. Where any Pht protein is referred to herein, it is meant that immunogenic fragments or fusions thereof of the Pht protein can

be used. For example, a reference to PhtX includes immunogenic fragments or fusions thereof from any Pht protein.

In one embodiment, the *S. pneumoniae* protein selected from member(s) of the Polyhistidine Triad family is PhtD. The term "PhtD" as used herein includes the full length protein with the signal sequence attached or the mature full length protein with the signal peptide (for example 20 amino acids at N-terminus) removed, and immunogenic fragments, variants and/or fusion proteins thereof, e.g. SEQ ID NO: 4 of WO00/37105. In one aspect, PhtD is the full length protein with the signal sequence attached e.g. SEQ ID NO: 4 of WO00/37105. In another aspect, PhtD is a sequence comprising the mature full length protein with the signal peptide (for example 20 amino acids at N-terminus) removed, e.g. amino acids 21-838 of SEQ ID NO: 4 of WO00/37105. Suitably, the PhtD sequence comprises an N-terminal methionine. The present invention also includes PhtD polypeptides which are immunogenic fragments of PhtD, variants of PhtD and/or fusion proteins of PhtD. For example, as described in WO00/37105, WO00/39299, US6699703 and WO09/12588.

Where immunogenic fragments of PhtD proteins are used (separately or as part of a fusion protein), these immunogenic fragments will be at least about 15, at least about 20, at least about 40, or at least about 60 contiguous amino acid residues in length, e.g. from a PhtD amino acid sequence in WO00/37105 or WO00/39299, such as SEQ ID NO: 4 of WO00/37105. In an embodiment of the invention, immunogenic fragments of PhtD protein comprise at least about 15, at least about 20, at least about 40, or at least about 60 contiguous amino acid residues of the sequence shown in SEQ ID NO: 4 of WO00/37105, wherein said polypeptide is capable of eliciting an immune response specific for said amino acid sequence. In an embodiment, the immunogenic composition of the invention comprises an immunogenic fragment of PhtD, for example described in WO09/12601, WO01/98334 and WO09/12588. Where immunogenic fragments of PhtD proteins are used (separately or as part of a fusion protein), each immunogenic fragment optionally contains one or more histidine triad motif(s) of such polypeptides. A histidine triad motif is the portion of polypeptide that has the sequence HxxHxH where H is histidine and x is an amino acid other than histidine. In an embodiment of the present invention, the or each immunogenic fragment contains exactly or at least 2, 3, 4 or 5 histidine triad motifs (optionally, with native PhtD sequence between the 2 or more triads, or intra-triad sequence) where the immunogenic fragment is more than 50, 60, 70, 80, 90 or 100% identical to a native pneumococcal intra-triad PhtD sequence (e.g. the intra-triad

sequence shown in SEQ ID NO: 4 of WO00/37105). Immunogenic fragments of PhtD proteins optionally contain one or more coiled coil regions of such polypeptides. A coiled coil region is a region predicted by "Coils" algorithm Lupus, A et al (1991) Science 252; 1162-1164. In an embodiment of the present invention, each immunogenic fragment
5 contains exactly or at least 2, 3 or 4 coiled coil regions. In an embodiment of the present invention, the or each immunogenic fragment contains exactly or at least 2, 3 or 4 coiled coil regions where the immunogenic fragment is more than 50, 60, 70, 80, 90, 95, 96 or 100% identical to a native pneumococcal PhtD sequence (e.g. the sequence shown in SEQ ID NO: 4 of WO00/37105). In another embodiment of the present invention, the
10 immunogenic fragment includes one or more histidine triad motif as well as at least 1, 2, 3 or 4 coiled coil regions.

In the case where the PhtD polypeptide is a variant, the variation is generally in a portion thereof other than the histidine triad residues and the coiled-coil region, although
15 variations in one or more of these regions may be made. In accordance with the present invention, a polypeptide variant includes sequences in which one or more amino acids are substituted and/or deleted and/or inserted compared to the wild type sequence. Amino acid substitution may be conservative or non-conservative. In one aspect, amino acid substitution is conservative. Substitutions, deletions, insertions or any combination thereof
20 may be combined in a single variant so long as the variant is an immunogenic polypeptide. Variants of PhtD typically include any immunogenic fragment or variation of PhtD which shares at least 80, 90, 95, 96, 98, or 99% amino acid sequence identity with a wild-type PhtD sequence, e.g. SEQ ID NO: 4 of WO00/37105. In an embodiment, the present invention includes immunogenic fragments and/or variants in which several, 5 to
25 10, 1 to 5, 1 to 3, 1 to 2 or 1 amino acid(s) are substituted, deleted, or added in any combination. In another embodiment, the present invention includes immunogenic fragments and/or variants which comprise a B-cell or T-cell epitope. Such epitopes may be predicted using a combination of 2D-structure prediction, e.g. using the PSIPRED program (from David Jones, Brunel Bioinformatics Group, Dept. Biological Sciences,
30 Brunel University, Uxbridge UB8 3PH, UK) and antigenic index calculated on the basis of the method described by Jameson and Wolf (CABIOS 4:181-186 [1988]).

In an embodiment of the invention, PhtD and its immunogenic fragments, variants and/or fusion proteins thereof comprise an amino acid sequence sharing at least 80, 85, 90, 95,
35 96, 97, 98, 99 or 100% identity with amino acid sequence 21 to 838 of SEQ ID NO:4 of WO00/37105. In another embodiment of the invention, PhtD and its immunogenic

fragments, variants and/or fusion proteins thereof have an amino acid sequence sharing at least 80, 85, 90, 95, 96, 97, 98, 99 or 100% identity with amino acid sequence 21 to 838 of SEQ ID NO:4 of WO00/37105. Suitably, PhtD and its immunogenic fragments, variants and/or fusion proteins thereof comprise an amino acid sequence having an N-terminal methionine. In another embodiment of the invention, PhtD and its immunogenic fragments, variants and/or fusion proteins thereof comprise at least about 15, at least about 20, at least about 40, or at least about 60 or at least about 100, or at least about 200, or at least about 400 or at least about 800 contiguous amino acid residues of the sequence shown in SEQ ID NO: 4 of WO00/37105.

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Pneumolysin (Ply) is a multifunctional toxin with a distinct cytolytic (hemolytic) and complement activation activities (Rubins et al., Am. Respir. Crit Care Med, 153:1339-1346 (1996)). The toxin is not secreted by pneumococci, but it is released upon lysis of pneumococci under the influence of autolysin. Its effects include e.g., the stimulation of the production of inflammatory cytokines by human monocytes, the inhibition of the beating of cilia on human respiratory epithelial, the decrease of bactericidal activity and migration of neutrophils, and in the lysis of red blood cells, which involves binding to cholesterol. Because it is a toxin, it needs to be detoxified (i.e., non-toxic to a human when provided at a dosage suitable for protection) before it can be administered *in vivo*.

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Expression and cloning of wild-type or native pneumolysin is known in the art. See, for example, Walker et al. (Infect Immun, 55:1184-1189 (1987)), Mitchell et al. (Biochim Biophys Acta, 1007:67-72 (1989) and Mitchell et al (NAR, 18:4010 (1990)). Detoxification of Ply can be conducted by chemical means, e.g., subject to formalin or glutaraldehyde treatment or a combination of both (WO 04081515, PCT/EP2005/010258). Such methods are known in the art for various toxins. Alternatively, Ply can be genetically detoxified. Thus, the invention encompasses derivatives of pneumococcal proteins which may be, for example, mutated proteins. The term "mutated" is used herein to mean a molecule which has undergone deletion, addition or substitution of one or more amino acids using known techniques for site directed mutagenesis or any other conventional method. For example, as described above, a mutant Ply protein may be altered so that it is biologically inactive whilst still maintaining its immunogenic epitopes, see, for example, WO90/06951, Berry et al. (Infect Immun, 67:981-985 (1999)) and WO99/03884.

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As used herein, it is understood that the term "Ply" encompasses mutated pneumolysin and detoxified pneumolysin (dPly) suitable for medical use (i.e., non toxic).

Concerning the Choline Binding Protein family (CbpX), members of that family were originally identified as pneumococcal proteins that could be purified by choline-affinity chromatography. All of the choline-binding proteins are non-covalently bound to phosphorylcholine moieties of cell wall teichoic acid and membrane-associated lipoteichoic acid. Structurally, they have several regions in common over the entire family, although the exact nature of the proteins (amino acid sequence, length, etc.) can vary. In general, choline binding proteins comprise an N terminal region (N), conserved repeat regions, a proline rich region (P) and a conserved choline binding region (C), made up of multiple repeats, that comprises approximately one half of the protein. As used in this application, the term "Choline Binding Protein family (CbpX)" is selected from the group consisting of Choline Binding Proteins as identified in WO97/41151, , Choline binding protein A, CbpA (also referred to as PbcA (C3-binding protein A), SpsA (*Streptococcus pneumoniae* secretory IgA binding protein), PspC (pneumococcal surface protein C)), Choline binding protein D (CbpD), and Choline binding protein G (CbpG). CbpA is disclosed in WO97/41151. CbpD and CbpG are disclosed in WO00/29434. PspC is disclosed in WO97/09994. PbcA is disclosed in WO98/21337. SpsA is a Choline binding protein disclosed in WO 98/39450. In an embodiment, the Choline Binding Proteins is CbpA. Another Choline Binding Protein is pneumococcal choline-binding protein A (PcpA) (Sanchez-Beato et al FEMS Microbiology Letters 164 (1998) 207-214).

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Another preferred embodiment is CbpX truncates wherein "CbpX" is CbpA, CbpD or CbpG and "truncates" refers to CbpX proteins lacking 50% or more of the Choline binding region (C). Another preferred embodiment is PcpA truncates wherein "truncates" refers to PcpA proteins lacking 50% or more of the Choline binding region (C). In an embodiment, CbpX truncates or PcpA truncates lack the entire choline binding region. In another embodiment, the CbpX truncates or PcpA truncates lack (i) the choline binding region and (ii) a portion of the N-terminal half of the protein as well, yet retain at least one repeat region. In another embodiment, the truncate has at least 2 repeat regions. Examples of such preferred embodiments are illustrated in WO99/51266 or WO99/51188, however, other choline binding proteins lacking a similar choline binding region are also contemplated within the scope of this invention.

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The LytX family is membrane associated proteins associated with cell lysis. The N-terminal domain comprises choline binding domain(s), however the LytX family does not have all the features found in the CbpA family noted above and thus for the present invention, the LytX family is considered distinct from the CbpX family. In contrast with the

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CbpX family, the C-terminal domain contains the catalytic domain of the LytX protein family. The family comprises LytA, LytB and LytC. With regards to the LytX family, LytA is disclosed in Ronda et al., Eur J Biochem, 164:621-624 (1987). LytB is disclosed in WO 98/18930, and is also referred to as Sp46. LytC is also disclosed in WO 98/18930, and is also referred to as Sp91. A preferred member of that family is LytC.

Another preferred embodiment are LytX truncates wherein "LytX" is LytA, LytB or LytC and "truncates" refers to LytX proteins lacking 50% or more of the Choline binding region. Suitably such proteins lack the entire choline binding region. Yet another preferred embodiment of this invention are CbpX truncate-LytX truncate chimeric proteins (or fusions). In an embodiment, the CbpX truncate-LytX truncate chimeric protein comprises the repeat regions of CbpX and the C-terminal portion (Cterm, i.e., lacking the choline binding domains) of LytX (e.g., LytCCterm or Sp91Cterm). In another embodiment, CbpX is selected from the group consisting of CbpA, PbcA, SpsA and PspC. In another embodiment, it is CbpA. In an embodiment, LytX is LytC (also referred to as Sp91). Another embodiment of the present invention is a PspA or PsaA truncates lacking the choline binding domain (C) and expressed as a fusion protein with LytX. In an embodiment, LytX is LytC.

PsaA and transmembrane deletion variants thereof have been described by Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62. PspA and transmembrane deletion variants thereof have been disclosed in, for example, US 5804193, WO 92/14488, and WO 99/53940.

Sp128 and Sp130 are disclosed in WO00/76540. Sp125 is an example of a pneumococcal surface protein with the Cell Wall Anchored motif of LPXTG (i.e. leucine-proline-X-threonine-glycine where X is any amino acid). Any protein within this class of pneumococcal surface protein with this motif has been found to be useful within the context of this invention, and is therefore considered a further protein of the invention. Sp125 itself is disclosed in WO 98/18930, and is also known as ZmpB – a zinc metalloproteinase. Sp101 is disclosed in WO 98/06734 (where it has the reference # y85993). It is characterized by a Type I signal sequence. Sp133 is disclosed in WO 98/06734 (where it has the reference # y85992). It is also characterized by a Type I signal sequence.

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The proteins of the invention may also be beneficially combined. By combined is meant that the immunogenic composition comprises all of the proteins from within the following combinations, either as carrier proteins or as free proteins or a mixture of the two. For example, in a combination of two proteins as set out hereinafter, both proteins may be
5 used as carrier proteins, or both proteins may be present as free proteins, or both may be present as carrier and as free protein, or one may be present as a carrier protein and a free protein whilst the other is present only as a carrier protein or only as a free protein, or one may be present as a carrier protein and the other as a free protein. Where a combination of three proteins is given, similar possibilities exist. Preferred combinations
10 include, but are not limited to PhtD + CbpX repeat regions, , PhtD + Ply, PhtD + Sp128, PhtD + PsaA, PhtD + PspA, PhtA + CbpX repeat regions , PhtA + CbpX repeat regions - Sp91Cterm chimeric or fusion proteins, PhtA + Ply, PhtA + Sp128, PhtA + PsaA, PhtA + PspA, CbpX repeat regions + LytC, CbpX repeat regions + PspA, CbpX repeat regions + PsaA, CbpX repeat regions + Sp128, CbpX repeat regions + LytC, CbpX repeat regions +
15 PspA, CbpX repeat regions + PsaA, CbpX repeat regions + Sp128, CbpX repeat regions + PhtD, CbpX repeat regions + PhtA. In an embodiment, CbpX repeat regions is from CbpA. In another embodiment, it is from CbpA. Other combinations include 3 protein combinations such as PhtD + CbpX repeat regions + Ply, and PhtA + CbpX repeat regions + PhtD. In one embodiment, the immunogenic composition comprises detoxified pneumolysin and PhtD or PhtDE as carrier proteins. In a further embodiment, the
20 immunogenic composition comprises detoxified pneumolysin and PhtD or PhtDE as free proteins.

The total content of protein antigens in the vaccine will typically be in the range 1-100µg,
25 or 5-80µg, e.g. in the range 50-70µg. For example, in one aspect, the immunogenic composition of the invention comprises 26µg-45µg (for example 26µg-40µg, 28µg-35µg or around 30µg) of each *S. pneumoniae* protein, per human dose. In another aspect, the immunogenic composition comprises 26µg-45µg (for example 26µg-40µg, 28µg-35µg or around 30µg) of PhtD, per human dose. In another aspect, the immunogenic composition
30 of the invention comprises 26µg-45µg (for example 26µg-40µg, 28µg-35µg or around 30µg) of pneumolysin (e.g. dPly), per human dose.

By the term "human dose" is meant a dose which is in a volume suitable for human use. Generally this is between 0.25 and 1.5 ml. In one embodiment, a human dose is 0.5 ml. In
35 a further embodiment, a human dose is higher than 0.5 ml, for example 0.6, 0.7, 0.8, 0.9 or 1 ml. In a further embodiment, a human dose is between 1 ml and 1.5 ml. In another

embodiment, in particular when the immunogenic composition is for the paediatric population, a human dose may be less than 0.5 ml such as between 0.25 and 0.5 ml.

Adjuvants

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The immunogenic compositions of the present invention may be adjuvanted, particularly when intended for use in an elderly population but also for use in infant populations. Thus, a further aspect is an immunogenic composition of the invention which further comprises an adjuvant. Suitable adjuvants include an aluminum salt such as aluminum hydroxide gel
10 or aluminum phosphate or alum, but a suitable adjuvant may also be a salt of calcium, magnesium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatized polysaccharides, or polyphosphazenes. In one aspect of the invention, the adjuvant is an aluminium salt, e.g. aluminium phosphate. In a further aspect, the adjuvant comprises (per 0.5 mL dose) 100-
15 750, 200-500, or 300-400 µg Al (aluminium) as aluminium phosphate.

In one embodiment, the adjuvant is a preferential inducer of a Th1 type of response. Such high levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to a given antigen, whilst high levels of Th2-type cytokines tend to favour the
20 induction of humoral immune responses to the antigen. Suitable adjuvant systems which promote a predominantly Th1 response include: Monophosphoryl lipid A or a derivative thereof, particularly 3-de-O-acylated monophosphoryl lipid A (3D-MPL) (for its preparation see GB 2220211 A); and a combination of monophosphoryl lipid A, suitably 3-de-O-acylated monophosphoryl lipid A, together with either an aluminum salt (for instance
25 aluminum phosphate or aluminum hydroxide) or an oil-in-water emulsion. In such combinations, antigen and 3D-MPL are contained in the same particulate structures, allowing for more efficient delivery of antigenic and immunostimulatory signals. Studies have shown that 3D-MPL is able to further enhance the immunogenicity of an alum-adsorbed antigen [Thoelen *et al.* Vaccine (1998) 16:708-14; EP 689454-B1].

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The immunogenic composition may comprise a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide (and optionally an immunostimulant) adsorbed onto a metal salt (such as an aluminium salt, for example Aluminium phosphate or Aluminium hydroxide). The term "immunostimulant" as used herein means a substance that
35 stimulates an immune response, in particular an adjuvant which stimulates the immune system of a host animal (e.g. human) to which it is administered and thereby increases

the protective effect produced by a antigen administered to that animal, as compared to the effect which would be produced by administration of the antigen alone. For aluminium based vaccine formulations wherein the antigen is typically adsorbed onto aluminium salt for one hour at room temperature under agitation.

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Adjuvants comprising Additional Immunostimulants

The adjuvant of the invention may comprise immunostimulants, such as saponins (e.g. QS21) and/or 3D-MPL. Examples of immunostimulants are described herein and in
10 "Vaccine Design – The Subunit and Adjuvant Approach" 1995, Pharmaceutical Biotechnology, Volume 6, Eds. Powell, M.F., and Newman, M.J., Plenum Press, New York and London, ISBN 0-306-44867-X. In one aspect of the present invention the adjuvant comprises QS21, monophosphoryl lipid A (MPL), phospholipid and sterol, presented in the form of a liposome.

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QS-21 is a purified saponin fraction from the bark extracts of the South American tree *Quillaja saponaria*. QS21 typically comprises two principal isomers that share a triterpene, a branched trisaccharide, and a glycosylated pseudodimeric acyl chain. The two isomeric forms differ in the constitution of the terminal sugar within the linear tetrasaccharide
20 segment, wherein the major isomer, QS-21-Api incorporates a β -D-apiose residue, and the minor isomer, QS-21-Xyl terminates in a β -D-xylose substituent. (Cleland, J. L. et al. J. Pharm. Sci. 1996, 85, 22–28). QS21 may be prepared by HPLC purification from Quil ATM. Quil ATM was described as having adjuvant activity by Dalsgaard *et al.* in 1974 ("Saponin adjuvants", Archiv. für die gesamte Virusforschung, Vol. 44, Springer Verlag, Berlin, p243-
25 254). Methods for production of QS21 are described in US5057540 (where QS21 is described as QA21) and EP0362278. In an embodiment, immunogenic compositions of the invention contain QS21 in substantially pure form, that is to say, the QS21 comprises at least 90%, for example at least 95%, or at least 98% of the immunogenic composition (i.e. the QS21 composition contains at least 90%, for example at least 95%, or at least
30 98% QS21). The dose of QS21 is suitably able to enhance an immune response to an antigen in a human. In particular a suitable QS21 amount is that which improves the immunological potential of the composition compared to the unadjuvanted composition, or compared to the composition adjuvanted with another QS21 amount, whilst being acceptable from a reactogenicity profile. QS21 can be used, for example, at an amount of
35 1 to 100 μ g per composition dose, for example in an amount of 10 to 50 μ g per composition dose.

Monophosphoryl lipid A (MPL) is a nontoxic derivative of the lipopolysaccharide (LPS) of gram-negative bacteria, e.g. *Salmonella minnesota* R595. It retains adjuvant properties of the LPS while demonstrating a reduced toxicity (Johnson et al. 1987 Rev. Infect. Dis. 9 Suppl:S512-S516). MPL is composed of a series of 4'-monophosphoryl lipid A species that vary in the extent and position of fatty acid substitution. It may be prepared by treating LPS with mild acid and base hydrolysis followed by purification of the modified LPS. For example, LPS may be refluxed in mineral acid solutions of moderate strength (e.g. 0.1 M HCl) for a period of approximately 30 minutes. This process results in dephosphorylation at the 1 position, and decarbohydration at the 6' position. The term "monophosphoryl lipid A (MPL)" as used herein includes derivatives of monophosphoryl lipid A. Derivatives of monophosphoryl lipid A include 3D-MPL and synthetic derivatives.

3D-MPL is 3-O-deacylated monophosphoryl lipid A (or 3 De-O-acylated monophosphoryl lipid A). Chemically it is a mixture of 3- deacylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. 3D-MPL is available under the trademark MPL® by GlaxoSmithKline Biologicals North America. 3-O-deacylated monophosphoryl lipid A (3D-MPL). It has a further reduced toxicity while again maintaining adjuvanticity, and may typically be prepared by mild alkaline hydrolysis, see for example US4912094. Alkaline hydrolysis is typically performed in organic solvent, such as a mixture of chloroform/methanol, by saturation with an aqueous solution of weak base, such as 0.5 M sodium carbonate at pH 10.5. For further information on the preparation of 3D-MPL see GB2220211A and WO02078637 (Corixa Corporation). In one aspect of the present invention small particle 3 D-MPL may be used. Small particle 3D-MPL has a particle size such that it may be sterile-filtered through a 0.22µm filter. Such preparations are described in International Patent Application No. WO94/21292. In an embodiment, immunogenic compositions of the invention comprise 3-O-Deacylated monophosphoryl lipid A (3D-MPL).

The dose of monophosphoryl lipid A (MPL), e.g. 3D-MPL, is suitably able to enhance an immune response to an antigen in a human. In particular a suitable monophosphoryl lipid A (MPL), e.g. 3D-MPL, amount is that which improves the immunological potential of the composition compared to the unadjuvanted composition, or compared to the composition adjuvanted with another MPL amount, whilst being acceptable from a reactogenicity profile. Monophosphoryl lipid A (MPL), e.g. 3D-MPL, can be used, for example, at an amount of 1 to 100 µg per composition dose, for example in an amount of 10 to 50 µg per composition dose.

Liposomes may be made from phospholipids (such as dioleoyl phosphatidyl choline, DOPC) and sterol, e.g. cholesterol, using techniques known in the art. Such liposome carriers may carry the QS21 and/or monophosphoryl lipid A (MPL), e.g. 3D-MPL. Suitable compositions of the invention are those wherein liposomes are initially prepared without MPL (as described in WO96/33739), and MPL is then added, suitably as small particles of below 100 nm particles or particles that are susceptible to sterile filtration through a 0.22 µm membrane. The MPL is therefore not contained within the vesicle membrane (known as MPL out). Compositions where the MPL is contained within the vesicle membrane (known as MPL in) also form an aspect of the invention. The unconjugated *S. pneumoniae* proteins can be contained within the vesicle membrane or contained outside the vesicle membrane. Suitably soluble antigens are outside and hydrophobic or lipidated antigens are either contained inside or outside the membrane. Encapsulation within liposomes is described in US4235877.

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The liposomes of the present invention may comprise a phospholipid, for example a phosphatidylcholine, which may be non-crystalline at room temperature, for example egg yolk phosphatidylcholine, dioleoyl phosphatidylcholine or dilauryl phosphatidylcholine. Suitably, the phospholipid is dioleoylphosphatidylcholine (DOPC). A further aspect is an immunogenic composition of the invention comprising 0.1 to 10mg, 0.2 to 7, 0.3 to 5, 0.4 to 2, or 0.5 to 1 mg (e.g. 0.4 to 0.6, 0.9 to 1.1, 0.5 or 1 mg) phospholipid.

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The liposomes of the present invention may comprise a sterol. The sterol increases the stability of the liposome structure. Suitable sterols include β-sitosterol, stigmasterol, ergosterol, ergocalciferol and cholesterol. These sterols are described in the art, for example cholesterol is disclosed in the Merck Index, 11th Edn., page 341, as a naturally occurring sterol found in animal fat. In one particular embodiment of the invention, the sterol is cholesterol. Typically, the sterol may be added during formulation of the antigen preparation using QS21 quenched with the sterol as described in WO96/33739. In an embodiment, the immunogenic compositions of the invention comprise 0.025 to 2.5, 0.05 to 1.5, 0.075 to 0.75, 0.1 to 0.3, or 0.125 to 0.25 mg (e.g. 0.2 to 0.3, 0.1 to 0.15, 0.25 or 0.125 mg) sterol.

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In one embodiment the the adjuvant comprises (per 0.5 mL dose) 0.1-10mg, 0.2-7, 0.3-5, 0.4-2, or 0.5-1 mg (e.g. 0.4-0.6, 0.9-1.1, 0.5 or 1 mg) phospholipid (for instance DOPC), 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or

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0.125 mg) sterol (for instance cholesterol), 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) lipid A derivative (for instance 3D-MPL), and 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) saponin (for instance QS21).

- 5 Liposomes of the invention will suitably be comprised in a liquid medium. The liquid medium comprises physiologically acceptable liquids such as water, aqueous salt solutions and buffer solutions, e.g phosphate buffered saline (PBS) etc. For example, immunogenic compositions of the invention may comprise water and PBS.
- 10 In one aspect of the invention, the adjuvant is AS01B (see e.g. WO96/33739). In another aspect of the invention, the adjuvant is AS01E (see e.g. WO2007/068907).

In some cases it may be advantageous that the immunogenic compositions and vaccines of the present invention will further contain a stabiliser, for example other
15 emulsifiers/surfactants, including caprylic acid (Merck index 10th Edition, entry no. 1739), of which Tricaprylin is particularly preferred.

Oil in Water Emulsion Adjuvants

- 20 Oil in water emulsion adjuvants *per se* have been suggested to be useful as adjuvant compositions (EP 0 399 843B), also combinations of oil in water emulsions and other active agents have been described as adjuvants for vaccines (WO 95/17210; WO 98/56414; WO 99/12565; WO 99/11241). Other oil emulsion adjuvants have been described, such as water in oil emulsions (US 5,422,109; EP 0 480 982 B2) and water in
25 oil in water emulsions (US 5,424,067; EP 0 480 981 B). All of which form preferred oil emulsion systems (in particular when incorporating tocolds) that are suitable as adjuvants for use in compositions of the present invention.

A suitable oil emulsion (for example an oil-in-water emulsion) comprises a metabolisable,
30 non-toxic oil, such as squalane, a tocopherol such as alpha tocopherol and optionally an emulsifier (or surfactant) such as polysorbate 80 (TweenTM 80). A sterol (for example cholesterol) may also be included. In one aspect of the invention, there is provided a vaccine or immunogenic composition comprising a sized *S. pneumoniae* serotype 6A capsular polysaccharide and an adjuvant composition comprising an oil in water emulsion,
35 wherein the oil in water emulsion comprises 0.5 - 11mg metabolisable oil, (such as

squalene), 0.5 - 12 mg tocol (such as alpha-tocopherol) and 0.4 - 5 mg emulsifying agent (such as polyoxyethylene sorbitan monooleate), per human dose.

5 In order for any oil in water composition to be suitable for human administration, the oil phase of the emulsion system has to comprise a metabolisable oil. The meaning of the term metabolisable oil is known in the art. Metabolisable can be defined as 'being capable of being transformed by metabolism' (Dorland's Illustrated Medical Dictionary, W.B. Sanders Company, 25th edition (1974)). The oil may be any vegetable oil, fish oil, animal
10 oil or synthetic oil, which is not toxic to the recipient and is capable of being transformed by metabolism. Nuts, seeds, and grains are common sources of vegetable oils. Synthetic oils are also part of this invention and can include commercially available oils such as NEOBEE® and others. A particularly suitable metabolisable oil is squalene. Squalene (2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene) is an unsaturated oil
15 which is found in large quantities in shark-liver oil, and in lower quantities in olive oil, wheat germ oil, rice bran oil, and yeast, and is a particularly preferred oil for use in this invention. Squalene is a metabolisable oil, that it is an intermediate in the biosynthesis of cholesterol (Merck index, 10th Edition, entry no.8619). Suitably the metabolisable oil is present in the adjuvant composition in an amount of 0.5-10 mg, for example 1-10, 2-10, 3-
20 9, 4-8, 5-7, or 5-6 mg (e.g. 2-3, 5-6, or 9-10mg).

The oil in water emulsion suitably comprises a tocol. Tocols are known in the art and are described in EP0382271. Suitably the tocol is alpha-tocopherol or a derivative thereof such as alpha-tocopherol succinate (also known as vitamin E succinate). Said tocol is
25 suitably present in the adjuvant composition in an amount of 0.5-11 mg, for example 1-11, 2-10, 3-9, 4-8, 5-7, 5-6 (e.g. 10-11, 5-6, 2.5-3.5 or 1-3 mg). In a specific embodiment the tocol is present in an amount of 5.94 mg or 2.38 mg. In a further embodiment, said tocol is suitably present in the vaccine (or immunogenic) composition in an amount of 0.5-11 mg, for example 1-11, 2-10, 3-9, 4-8, 5-7, 5-6 (e.g. 10-11, 5-6, 2.5-3.5 or 1-3 mg).

30 The oil in water emulsion may further comprise an emulsifying agent. The emulsifying agent may suitably be polyoxyethylene sorbitan monooleate. In a particular embodiment the emulsifying agent may be selected from the group comprising: Polysorbate 80 (Tween™ 80). Said emulsifying agent is suitably present in the adjuvant composition in an
35 amount of 0.1-5, 0.2-5, 0.3-4, 0.4-3 or 2-3 mg (e.g. 0.4-1.2, 2-3 or 4-5 mg) emulsifying agent.

The method of producing oil-in-water emulsions is known to the person skilled in the art. Commonly, the method comprises mixing the tocol-containing oil phase with a surfactant such as a PBS/Tween™80 solution, followed by homogenisation using a homogenizer. A method comprising passing the mixture twice through a syringe needle would be suitable for homogenising small volumes of liquid. Equally, the emulsification process in microfluidiser (M110S Microfluidics machine, maximum of 50 passes, for a period of 2 minutes at maximum pressure input of 6 bar (output pressure of about 850 bar)) could be adapted by the man skilled in the art to produce smaller or larger volumes of emulsion.

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In an oil in water emulsion, the oil and emulsifier should be in an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline.

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In an embodiment, the oil-in-water emulsion systems of the present invention have a small oil droplet size in the sub-micron range. Suitably the droplet sizes will be in the range 120 to 750 nm, or from 120 to 600 nm in diameter. In an embodiment, the oil-in water emulsion contains oil droplets of which at least 70% by intensity are less than 500 nm in diameter, or at least 80% by intensity are less than 300 nm in diameter, or at least 90% by intensity are in the range of 120 to 200 nm in diameter.

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The oil droplet size, i.e. diameter, according to the present invention is given by intensity. There are several ways of determining the diameter of the oil droplet size by intensity. Intensity is measured by use of a sizing instrument, suitably by dynamic light scattering such as the Malvern Zetasizer 4000 or suitably the Malvern Zetasizer 3000HS. A first possibility is to determine the z average diameter (ZAD) by dynamic light scattering (PCS, Photon correlation spectroscopy); this method additionally gives the polydispersity index (PDI), and both the ZAD and PDI are calculated with the cumulants algorithm. These values do not require the knowledge of the particle refractive index. A second mean is to calculate the diameter of the oil droplet by determining the whole particle size distribution by another algorithm, either the Contin, or non-negative least squares (NNLS), or the automatic "Malvern" one (the default algorithm provided for by the sizing instrument). Most of the time, as the particle refractive index of a complex composition is unknown, only the intensity distribution is taken into consideration, and if necessary the intensity means originating from this distribution.

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Lipopolysaccharide (LPS) or lipooligosaccharide (LOS) derivatives or mutations or lipid A derivatives described herein are designed to be less toxic (e.g. 3D-MPL) than native lipopolysaccharides

5 In one embodiment the adjuvant used for the compositions of the invention comprises an oil in water emulsion made from a metabolisable oil (such as squalene), an emulsifier (such as Tween™ 80) and optionally a tocol (such as alpha tocopherol). In one embodiment the adjuvant comprises (per 0.5 mL dose) 0.5-15, 1-13, 2-11, 4-8, or 5-6mg (e.g. 2-3, 5-6, or 10-11 mg) metabolisable oil (such as squalene), 0.1-10, 0.3-8, 0.6-6, 0.9-
10 5, 1-4, or 2-3 mg (e.g. 0.9-1.1, 2-3 or 4-5 mg) emulsifier (such as Tween™ 80) and optionally 0.5-20, 1-15, 2-12, 4-10, 5-7 mg (e.g. 11-13, 5-6, or 2-3 mg) tocol (such as alpha tocopherol).

The adjuvant may optionally further comprise 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50,
15 10, 20, 30, 40 or 50 µg) lipid A derivative (for instance 3D-MPL).

The adjuvant may optionally contain 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or 0.125 mg) sterol (for instance cholesterol), 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) lipid A derivative (for
20 instance 3D-MPL), and 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) saponin (for instance QS21).

In one embodiment, the adjuvant used for the compositions of the invention comprises aluminium phosphate and a lipid A derivative (such as 3D-MPL). This adjuvant may
25 comprise (per 0.5 mL dose) 100-750, 200-500, or 300-400 µg Al as aluminium phosphate, and 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) lipid A derivative (for instance 3D-MPL).

Method of Administration

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The vaccine preparations containing immunogenic compositions of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via a systemic or mucosal route. These administrations may include injection *via* the intramuscular (IM), intraperitoneal (IP), intradermal (ID) or
35 subcutaneous (SC) routes; or *via* mucosal administration to the oral/alimentary,

respiratory, genitourinary tracts. Although the vaccine of the invention may be administered as a single dose, components thereof may also be co-administered together at the same time or at different times (for instance pneumococcal saccharide conjugates could be administered separately, at the same time or 1-2 weeks after the administration of the any bacterial protein component of the vaccine for optimal coordination of the immune responses with respect to each other). For co-administration, the optional Th1 adjuvant may be present in any or all of the different administrations. In addition to a single route of administration, 2 different routes of administration may be used. For example, polysaccharide conjugates may be administered IM (or ID) and bacterial proteins may be administered IN (or ID). In addition, the vaccines of the invention may be administered IM for priming doses and IN for booster doses.

Following an initial vaccination, subjects may receive one or several booster immunizations adequately spaced.

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Vaccine

The present invention further provides a vaccine containing the immunogenic compositions of the invention and a pharmaceutically acceptable excipient or carrier.

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Pharmaceutically acceptable excipients and carriers are well known and can be selected by those of skill in the art. For example, the pharmaceutically acceptable excipient or carrier can include a buffer, such as Tris (trimethamine), phosphate (e.g. sodium phosphate), acetate, borate (e.g. sodium borate), citrate, glycine, histidine and succinate (e.g. sodium succinate), suitably sodium chloride, histidine, sodium phosphate or sodium succinate. The pharmaceutically acceptable excipient may include a salt, for example sodium chloride, potassium chloride or magnesium chloride. Optionally, the pharmaceutically acceptable excipient contains at least one component that stabilizes solubility and/or stability. Examples of solubilizing/stabilizing agents include detergents, for example, laurel sarcosine and/or tween (e.g. Tween 80). Examples of stabilizing agents also include poloxamer (e.g. poloxamer 124, poloxamer 188, poloxamer 237, poloxamer 338 and poloxamer 407). The pharmaceutically acceptable excipient may include a non-ionic surfactant, for example polyoxyethylene sorbitan fatty acid esters, Polysorbate-80 (Tween 80), Polysorbate-60 (Tween 60), Polysorbate-40 (Tween 40) and Polysorbate-20 (Tween 20), or polyoxyethylene alkyl ethers (suitably polysorbate-80). Alternative solubilizing/stabilizing agents include arginine, and glass forming polyols (such

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as sucrose, trehalose and the like). The pharmaceutically excipient may be a preservative, for example phenol, 2-phenoxyethanol, or thiomersal. Other pharmaceutically acceptable excipients include sugars (e.g. lactose, sucrose), and proteins (e.g. gelatine and albumin). Pharmaceutically acceptable carriers include water, saline solutions, aqueous dextrose and glycerol solutions. Numerous pharmaceutically acceptable excipients and carriers are known in the art and are described, e.g., in Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 5th Edition (975).

10 According to a further aspect of the invention there is provided a process for making the immunogenic composition or vaccine of the invention comprising the step of mixing *S. pneumoniae* capsular polysaccharide (conjugates) of the invention, optionally with a pharmaceutically acceptable excipient or carrier.

15 Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The vaccines of the present invention may be stored in solution or lyophilized. In an embodiment, the solution is lyophilized in the presence of a sugar such as sucrose or lactose. It is still further preferable that they are lyophilized and extemporaneously reconstituted prior to use. Lyophilizing may result in a more stable composition (vaccine) and may possibly lead to higher antibody titers in the presence of 3D-MPL and in the absence of an aluminium based adjuvant.

25 In one aspect of the invention is provided a vaccine kit, comprising a vial containing an immunogenic composition of the invention, optionally in lyophilised form, and further comprising a vial containing an adjuvant as described herein. It is envisioned that in this aspect of the invention, the adjuvant will be used to reconstitute the lyophilised immunogenic composition.

30 Although the vaccines of the present invention may be administered by any route, administration of the described vaccines into the skin (ID) forms one embodiment of the present invention. Human skin comprises an outer "horny" cuticle, called the stratum corneum, which overlays the epidermis. Underneath this epidermis is a layer called the dermis, which in turn overlays the subcutaneous tissue. Researchers have shown that

injection of a vaccine into the skin, and in particular the dermis, stimulates an immune response, which may also be associated with a number of additional advantages. Intradermal vaccination with the vaccines described herein forms a preferred feature of the present invention.

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The conventional technique of intradermal injection, the “mantoux procedure”, comprises steps of cleaning the skin, and then stretching with one hand, and with the bevel of a narrow gauge needle (26-31 gauge) facing upwards the needle is inserted at an angle of between 10-15°. Once the bevel of the needle is inserted, the barrel of the needle is
10 lowered and further advanced whilst providing a slight pressure to elevate it under the skin. The liquid is then injected very slowly thereby forming a bleb or bump on the skin surface, followed by slow withdrawal of the needle.

More recently, devices that are specifically designed to administer liquid agents into or
15 across the skin have been described, for example the devices described in WO 99/34850 and EP 1092444, also the jet injection devices described for example in WO 01/13977; US 5,480,381, US 5,599,302, US 5,334,144, US 5,993,412, US 5,649,912, US 5,569,189, US 5,704,911, US 5,383,851, US 5,893,397, US 5,466,220, US 5,339,163, US 5,312,335, US 5,503,627, US 5,064,413, US 5,520, 639, US 4,596,556, US 4,790,824, US
20 4,941,880, US 4,940,460, WO 97/37705 and WO 97/13537. Alternative methods of intradermal administration of the vaccine preparations may include conventional syringes and needles, or devices designed for ballistic delivery of solid vaccines (WO 99/27961), or transdermal patches (WO 97/48440; WO 98/28037); or applied to the surface of the skin (transdermal or transcutaneous delivery WO 98/20734 ; WO 98/28037).

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When the vaccines of the present invention are to be administered to the skin, or more specifically into the dermis, the vaccine is in a low liquid volume, particularly a volume of between about 0.05 ml and 0.2 ml.

30 The content of the immunogenic composition in the skin or intradermal vaccines of the present invention may be similar to conventional doses as found in intramuscular vaccines (see above). However, it is a feature of skin or intradermal vaccines that the formulations may be “low dose”. Accordingly the protein antigens in “low dose” vaccines are suitably present in as little as 0.1 to 10µg, or 0.1 to 5 µg per dose; and the

polysaccharide (suitably conjugated) antigens may be present in the range of 0.01-1 μ g, and suitably between 0.01 to 0.5 μ g of saccharide per dose.

As used herein, the term "intradermal delivery" means delivery of the vaccine or immunogenic composition to the region of the dermis in the skin. However, the vaccine or immunogenic composition will not necessarily be located exclusively in the dermis. The dermis is the layer in the skin located between about 1.0 and about 2.0 mm from the surface in human skin, but there is a certain amount of variation between individuals and in different parts of the body. In general, it can be expected to reach the dermis by going 1.5 mm below the surface of the skin. The dermis is located between the stratum corneum and the epidermis at the surface and the subcutaneous layer below. Depending on the mode of delivery, the vaccine or immunogenic composition may ultimately be located solely or primarily within the dermis, or it may ultimately be distributed within the epidermis and the dermis.

The present invention further provides an improved vaccine for the prevention or amelioration of otitis media caused by *Haemophilus influenzae* by the addition of *Haemophilus influenzae* proteins, for example protein D in conjugated form. One or more *Moraxella catarrhalis* protein antigens can also be included in the vaccine or immunogenic composition of the invention in a free or conjugated form. Thus, the present invention is an improved method to elicit an immune response against otitis media in infants.

Examples of preferred *Moraxella catarrhalis* protein antigens which can be included in a combination vaccine or immunogenic composition of the invention (especially for the prevention of otitis media) are: outer membrane protein 106 (OMP106) [WO 97/41731 (Antex) & WO 96/34960 (PMC)]; outer membrane protein 21 (OMP21) or fragments thereof (WO 0018910); lactoferrin binding protein A (LbpA) &/or lactoferrin binding protein B (LbpB) [WO 98/55606 (PMC)]; transferrin binding protein A (TbpA) &/or transferring binding protein B (TbpB) [WO 97/13785 & WO 97/32980 (PMC)]; *Moraxella catarrhalis* CopB protein [Helminen ME, *et al.* (1993) Infect. Immun. 61:2003-2010]; ubiquitous surface protein A1 (UspA1) and/or ubiquitous surface protein A2 (UspA2) [WO 93/03761 (University of Texas)]; outer membrane protein CD (OmpCD); HasR (PCT/EP99/03824); PilQ (PCT/EP99/03823); outer membrane protein 85 (OMP85) (PCT/EP00/01468); lipo06 (GB 9917977.2); lipo10 (GB 9918208.1); lipo11 (GB 9918302.2); lipo18 (GB 9918038.2); outer membrane protein P6 (P6) (PCT/EP99/03038); D15 surface antigen (D15) (PCT/EP99/03822); outer membrane protein A1 (OmpA1) (PCT/EP99/06781); Hly3

(PCT/EP99/03257); and outer membrane protein E (OmpE). Examples of non-typeable *Haemophilus influenzae* proteins or fragments thereof which can be included in a combination vaccine (especially for the prevention of otitis media) include: Fimbrin protein [(US 5766608 - Ohio State Research Foundation)] and fusions comprising peptides therefrom [eg LB1(f) peptide fusions; US 5843464 (OSU) or WO 99/64067]; outer membrane protein 26 (OMP26) [WO 97/01638 (Cortecs)]; P6 [EP 281673 (State University of New York)]; TbpA and/or TbpB; *H. influenzae* adhesin (Hia); *Haemophilus* surface fibrils (Hsf); *Haemophilus influenzae* Hin47 protein; *Haemophilus influenzae* Hif protein; *Haemophilus influenzae* Hmw1 protein; *Haemophilus influenzae* Hmw2 protein; *Haemophilus influenzae* Hmw3 protein; *Haemophilus influenzae* Hmw4 protein; *Haemophilus influenzae* autotransporter adhesin (Hap); D15 (WO 94/12641); P2; and P5 (WO 94/26304).

Methods of Treatment and Use

The present invention provides a method for the treatment or prevention of *Streptococcus pneumoniae* infection in a subject in need thereof comprising administering to said subject a therapeutically effective amount of an immunogenic composition or the vaccine of the invention. The present invention also provides a method of immunising a human host against *Streptococcus pneumoniae* infection comprising administering to the host an immunoprotective dose of the immunogenic composition or vaccine of the invention. The present invention also provides a method of inducing an immune response to *Streptococcus pneumoniae* (e.g. *Streptococcus pneumoniae* serotype 6A) in a subject, the method comprising administering a therapeutically effective amount of the immunogenic composition or vaccine of the invention.

In an embodiment, the present invention is an improved method to elicit an immune response in infants (defined as 0-2 years old in the context of the present invention) by administering a therapeutically effective amount of an immunogenic composition or vaccine of the invention. In one embodiment, the immune response is protective (i.e. it can prevent or reduce infection caused by *S. pneumoniae*). In one embodiment, the vaccine is a paediatric vaccine.

In an embodiment, the present invention is an improved method to elicit a (protective) immune response in the elderly population (in the context of the present invention a patient is considered elderly if they are 50 years or over in age, typically over 55 years

and more generally over 60 years) by administering a therapeutically effective amount of the immunogenic composition or vaccine of the invention.

5 In one embodiment, the present invention provides a method of protecting a subject against a disease caused by 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 an immunogenic composition or vaccine of the invention.

10

In an embodiment, the present invention provides immunogenic compositions and vaccines of the invention for use in the prevention or treatment of a disease caused by *S. pneumoniae* infection. In an embodiment, the present invention provides the use of an immunogenic composition or vaccine of the invention in the manufacture of a medicament for the prevention (or treatment) of a disease caused by *S. pneumoniae* infection.

15

The disease caused by *Streptococcus pneumoniae* infection may be selected from pneumonia, invasive pneumococcal disease (IPD), exacerbations of chronic obstructive pulmonary disease (COPD), otitis media, meningitis, bacteraemia, pneumonia and/or conjunctivitis. Where the human host is an infant, the disease may be selected from otitis media, meningitis, bacteraemia, pneumonia and/or conjunctivitis. Where the human host is elderly, the disease may be selected from pneumonia, invasive pneumococcal disease (IPD), and/or exacerbations of chronic obstructive pulmonary disease (COPD).

20

25 Embodiments herein relating to “vaccine compositions” of the invention are also applicable to embodiments relating to “immunogenic compositions” of the invention, and vice versa.

Embodiments of the invention are further described in the subsequent numbered paragraphs:

30

Paragraph 1: A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide wherein the average size (Mw) of the *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is between 180-400, 210-400, 210-370, 220-360, 230-350, 240-340, 240-320, 240-310 or 250-310 kDa.

35

Paragraph 2: A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to paragraph 1 which has been sized by a mechanical sizing technique.

5 Paragraph 3: A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to paragraph 1 or paragraph 2 conjugated to a carrier protein (e.g. CRM-197).

10 Paragraph 4: A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to paragraph 3 wherein the *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is directly conjugated to the carrier protein.

Paragraph 5: A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to any one of paragraphs 3-4 wherein the 6A polysaccharide is conjugated to the carrier protein or to a linker using CDAP chemistry.

15 Paragraph 6: A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to any one of paragraphs 3-5 wherein the serotype 6A capsular polysaccharide (PS6A) is conjugated to the carrier protein or to a linker using CDAP chemistry using a CDAP:PS6A ratio between 1:2 to 3:1, 1:1.5 to 2:1, or 1:1.

20 Paragraph 7: A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to any one of paragraphs 3-6 wherein the serotype 6A capsular polysaccharide is conjugated to the carrier protein or to a linker using CDAP chemistry wherein the reaction was carried out using a coupling time of between 50-130 minutes, 60-130 minutes, or 110-130 minutes.

25 Paragraph 8: A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to any one of paragraphs 3-7 wherein the ratio of carrier protein to serotype 6A capsular polysaccharide is between 5:1 and 1:5, 4:1 and 1:1 or 2:1 and 1:1, 1.5:1 and 1:1, 1.4:1 and 1.3:1 (for example 1.2:1, 1.5:1) (w/w).

30 Paragraph 9: A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to paragraphs 1-8 wherein the 6A capsular polysaccharide is sized by a factor of no more than x5.

35 Paragraph 10: A process for preparing a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide conjugate comprising conjugating a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide to a carrier protein or to a linker using CDAP chemistry using a CDAP:PS ratio between 1:2 to 3:1, 1:1.5 to 2:1, or 1:1.

40 Paragraph 11: A process according to paragraph 10 wherein the reaction was carried out using a coupling time of between 50-130 minutes, 60-130 minutes, or 110-130 minutes.

Paragraph 12: A process for preparing a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide conjugate comprising (a) conjugation of a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide to a carrier protein and (b) diafiltration against a solution having a concentration of NaCl below 150mM.

5

Paragraph 13: An immunogenic composition comprising a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to paragraphs 1-9 or *Streptococcus pneumoniae* serotype 6A capsular polysaccharide obtained by a process of any one of paragraphs 10-12 conjugated to a carrier protein.

10

Paragraph 14: An immunogenic composition according to paragraph 13 comprising 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, or 16 or more capsular polysaccharides conjugates from different *S. pneumoniae* serotypes.

15

Paragraph 15: An immunogenic composition according to paragraph 13 or paragraph 14 comprising 1 or more, native capsular polysaccharides conjugates from *S. pneumoniae*.

Paragraph 16: An immunogenic composition according to paragraph 15 comprising native *Streptococcus pneumoniae* capsular serotype 6B polysaccharide.

20

Paragraph 17: An immunogenic composition according to paragraph 15 or 16 comprising native *Streptococcus pneumoniae* capsular serotype 23F polysaccharide.

25

Paragraph 18: An immunogenic composition according to any one of paragraphs 13 to 17 which comprises *Streptococcus pneumoniae* capsular serotype 6B polysaccharide having an average size (M_w) of between 500-1800, 900-1660, or 1000- 1400 kDa.

30

Paragraph 19: An immunogenic composition according to any one of paragraphs 13 to 18 which comprises *Streptococcus pneumoniae* capsular serotype 23F polysaccharide having an average size (M_w) of between 500-1500, 600-1500, 700-1300, 900-1250, 800-1100, or 900-1000 kDa.

35
40

Paragraph 20: An immunogenic composition according to any one of paragraphs 13 to 19 which comprises *Streptococcus pneumoniae* capsular polysaccharide from: (a) serotype 1 having an average size (M_w) of between 100-1000, 200-800, 250-600, or 300-400 kDa; (b) serotype 4 having an average size (M_w) of between 50-500, 60-300, 70-150, or 75-125 kDa; (c) serotype 5 having an average size (M_w) of between 100-1000, 100-700, 100-350, or 150-300 kDa; (d) serotype 7F having an average size (M_w) of between 50-1000, 100-750, 150-500, or 200-300 kDa; (e) serotype 9V having an average size (M_w) of between 50-1000, 100-750, 150-500, 200-400, or 250-300 kDa; (f) serotype 14 having an average size (M_w) of between 50-1000, 100-750, 150-500, or 200-250 kDa; (g) 18C having an

average size (M_w) of between 50-1000, 50-750, 50-500, 50-190, 50-150 or 80-110 kDa (h) serotype 19F having an average size (M_w) of between 50-1000, 100-750, 100-500, 100-190 or 120-180 kDa; and/or (i) serotype 19A having an average size (M_w) of between 50-800kDa, 110-700, 110-300, 120-200, 130-180, 140-160 or 80-130 kDa.

5

Paragraph 21: An immunogenic composition according to any one of paragraphs 13 to 20 which further comprises *Streptococcus pneumoniae* capsular serotype 22F having an average size (M_w) of between 50 and 800 kDa, 110 and 700 kDa, 110-300, 120-200, 130-180, 150-170, 100-190, 100-150, 95-125 or 100-115 kDa.

10

Paragraph 22: An immunogenic composition according to any one of paragraphs 13 to 21 comprising 2, 3, 4, 5 or 6 different carrier proteins.

Paragraph 23: An immunogenic composition according to any one of paragraphs 13 to 22 wherein one or more or all carrier proteins is selected from the group consisting of: diphtheria toxoid (DT), CRM197, tetanus toxoid (TT), Fragment C of TT, dPly, PhtA, PhtB, PhtD, PhtE, PhtDE, OmpC, PorB and *Haemophilus influenzae* Protein D.

15

Paragraph 24: An immunogenic composition of any one of paragraphs 13 to 23 wherein the *Streptococcus pneumoniae* capsular serotype 6B polysaccharide is conjugated to a different carrier protein (e.g. protein D) than the carrier protein to which *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is conjugated.

20

Paragraph 25: An immunogenic composition of any one of paragraphs 13 to 24 comprising serotype 1 saccharide conjugated to protein D, serotype 4 saccharide conjugated to protein D, serotype 5 saccharide conjugated to protein D, serotype 6B saccharide conjugated to protein D, serotype 7F saccharide conjugated to protein D, serotype 9V saccharide conjugated to protein D, serotype 14 saccharide conjugated to protein D and serotype 23F saccharide conjugated to protein D.

25

30

Paragraph 26: An immunogenic composition according to any one of paragraphs 13 to 25 comprising serotype 19F conjugated to Diphtheria toxoid.

Paragraph 27: An immunogenic composition according to any one of paragraphs 13 to 26 wherein the composition comprises capsular saccharide 18C conjugated to tetanus toxoid (TT), optionally wherein 18C is the only saccharide in the composition conjugated to tetanus toxoid (TT), optionally via an ADH linker

35

Paragraph 28: An immunogenic composition according to any of paragraphs 13 to 27 further comprising *S. pneumoniae* capsular saccharide(s) of one or more of: serotype 33F, serotype 15 and serotype 12F, conjugated to carrier protein(s).

40

Paragraph 29: An immunogenic composition according to any of paragraphs 13 to 28 wherein the dose of the 6A saccharide conjugate is between 1 and 10 µg, 1 and 5 µg, or 1 and 3 µg of saccharide (e.g. 2 µg).

- 5 Paragraph 30: An immunogenic composition according to any one of paragraphs 13 to 29 which further comprises one or more unconjugated or conjugated *S pneumoniae* proteins selected from: Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins, detoxified pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and
10 Sp133.

Paragraph 31: An immunogenic composition according to any one of paragraphs 13 to 30 which further comprises an adjuvant.

- 15 Paragraph 32: An immunogenic composition of paragraph 31 wherein the adjuvant comprises (per 0.5 mL dose) 100-750, 200-500, or 300-400 µg Al (aluminium) as aluminium phosphate.

- 20 Paragraph 33: A vaccine comprising the immunogenic composition of any one of paragraphs 13 to 32 and a pharmaceutically acceptable excipient or carrier.

- Paragraph 34: A process for making the vaccine according to paragraph 33 which comprises the step of mixing the immunogenic composition of any of paragraphs 13 to 32 with a pharmaceutically acceptable excipient or carrier.
25

- Paragraph 35: A method for the treatment or prevention of *Streptococcus pneumoniae* infection in a subject in need thereof comprising administering to said subject a therapeutically effective amount of an immunogenic composition of any of paragraphs 13 to 32 or the vaccine of paragraph 33.
30

- Paragraph 36: A method of immunising a human host against *Streptococcus pneumoniae* infection comprising administering to the host an immunoprotective dose of the immunogenic composition of any of paragraphs 13 to 32 or vaccine of paragraph 34.

- 35 Paragraph 37: A method of inducing an immune response to *Streptococcus pneumoniae* serotype 6A in a subject, the method comprising administering a therapeutically or prophylactically effective amount of the immunogenic composition of any of paragraphs 13 to 32 or the vaccine of paragraph 33.

- 40 Paragraph 38: The immunogenic composition of paragraphs 13 to 32 or vaccine of paragraph 33 for use in the treatment or prevention of disease caused by *Streptococcus pneumoniae* infection.

Paragraph 39: A use of the immunogenic composition of paragraphs 12 to 32 or vaccine of paragraph 33 in the manufacture of a medicament for the treatment or prevention of a disease caused by *Streptococcus pneumoniae* infection.

5

All references or patent applications cited within this patent specification are incorporated by reference herein.

In order that this invention may be better understood, the following examples are set forth.

10 These examples are for purposes of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

Examples

15 **Example 1 – preparation of sized polysaccharide**

Sizing

A homogenizer EMULSIFLEX C-50 apparatus was used to reduce the molecular weight and the viscosity of the polysaccharide before the activation step (microfluidization). The efficiency of the sizing depended on the circuit pressure and on the total cycle's number. The homogenizing cell of EMULSIFLEX C-50 was replaced by a cell with a fixed geometry (Microfluidics F20Y-75µm interaction chamber for E01 to E07; Microfluidics F30Y-125µm interaction chamber for E08). The aim of the sizing was to reduce the molecular weight and the viscosity of the polysaccharide without a critical decrease of its antigenicity.

The size reduction was followed *in-process* by viscosimetry (Brookfield Programmable DV III + rheometer). When the target in viscosity was reached, the sized polysaccharide was characterized by HP-SEC-RI (high-performance size-exclusion chromatography, refractive index).

In-process characterizations of the M_w were performed by HP-SEC-RI (TSK5000PW_{XL} column + guard column). The elution was realised using a 50mM Na/K₂PO₄, 500mM NaCl pH 7.6 at a flow rate of 0.6ml/min. The detection was realised by a refractive index detector. This characterization is based on the determination of a relative retention time

(RRT). The RRT is calculated with regard to calibrated dextrans having a molecular weight in the linear range of separation of a TSK5000PW_{XL} column. The RRT is defined by the following formula:

$$5 \quad RRT = \frac{RT_{(sized\ PS)} - RT_{(HMW\ dext)}}{RT_{(LMW\ dext)} - RT_{(HMW\ dext)}}$$

RT_(sized PS) = retention time of sized PS (polysaccharide)

RT_(HMW dext) = retention time of high molecular weight dextran (dextran 1730 Kda)

10 RT_(LMW dext) = retention time of low molecular weight dextran (dextran 150 Kda)

The native serotype 6A polysaccharide was dissolved at 15 mg/ml during 4 hours in WFI (water for injection) at room temperature. After 4 hours of dissolution, the pH of the solution was adjusted at 6.5 +/- 0.5 before its transfer in a cold room to pursue dissolution overnight. Before the sizing, the solution of native serotype 6A polysaccharide was clarified on 5 µm filter.

The serotype 6A polysaccharide was then sized by Emulsiflex with a Microfluidics F30Y-125 µm homogenizing cell at a pressure of between 2900 and 3800. The sizing was stopped when the viscosity of the polysaccharide reached the targeted value for the viscosity (8.35 or 12.4). The number of cycles required depended on the target. For example, 32.5 cycles were needed to reach a viscosity of 12.4 cps using a pressure of 2900 psi. For this sample, in process determination of relative retention time by HP-SEC-RI gave a value of 0.28.

25

The sized 6A polysaccharide was filtered on a Millipak 20 (5 g scale) membrane (cut-off 0.22 µm) at a flow-rate of 10 ml/min.

The polysaccharide content of sized serotype 6A polysaccharide solution was accurately determined by colorimetric method (resorcinol) before its use in conjugation. Sized polysaccharide was characterized either by HP-SEC-MALLS or estimated by dextrans calibration curve and determination of antigenic activity (Table 1).

35 Antigenicity

35

Test and reference samples were incubated in microtiter plates previously coated with monoclonal antibodies raised against *S. pneumoniae* polysaccharide serotype 6A (PS6A). Rabbit polyclonal anti-PS6A antibodies were then added. Antigen-antibody complex were revealed using a goat anti-rabbit Ig peroxidase linked. Colour development was then performed by the system Ortho-phenylenediamine/H₂O₂ reacting with peroxidase. Coloration was measured by spectrophotometry (absorbances at 490 and 620 nm). The polysaccharide curve was compared to the reference curve (one native polysaccharide lot used as reference while another one native polysaccharide lot was used as internal control) in order to determine the polysaccharide concentration.

10 Measurement of Molecular weight and polydispersity by MALLS

The molecular weight (M_w) was determined by Laser Light Scattering (SEC-MALLS). In a first time, the analyses were performed on a TSK5000PW_{XL} (+guard column) using NaCl 0.2M + 0.02% azide solution as elution buffer. Last analyses were performed on a TSKGMPW_{XL} column (+guard column) with a loading of 100 μ l of polysaccharide (1 mg/ml) using 50 mM Na/K₂PO₄, 200 mM NaCl pH 7.0 as elution buffer and a flow-rate of 0.75 ml/min.

The detection was realised with a laser spectrophotometer and an interferometric refractometer (Wyatt Otilab DSP equipped with a P100 cell and a red filter at 498nm).

The average molecular weight in number (M_n) was also obtained by MALLS. The polydispersity of the sized polysaccharide was obtained as the M_w/M_n ratio.

A theoretical dn/dc of 0.14 was first used. When determined, the experimental value of 0.151 was used.

25 Table 1: Sized PS6A characterizations

PS6A Lot	PS content (μ g/ml)	RRT 150- 1730 ¹	Viscosity (cp)	M_w (kDa) ² (estimation versus dextran calibration curve)	MALLS - M_w (kDa) - R_w (nm) - Polydispersity	Antigenicity by ELISA (%)
E01	13650	0.45	8.35	575	ND	124
E02	8570	0.50	6.03	514	ND	ND

E03	14210	0.24	15.6	968	ND	ND
E04	12000	0.21	17.3	1018		120 ³
E05	-	-	-	-	-	-
E06	13910	0.24	12.1	916	T13M / -70°C 256 25.6 1.124	130 ³
E07	14700	0.24	12.2	929	307.4 28.1 1.112	120 ³
E08	13590	0.20	12.3	1004	T12M / -70°C 277 27.7 1.094	144 ³

¹ RRT: relative retention time (HP-SEC-RI) -² HP-SEC-RI - ³ relative value versus corresponding native polysaccharide. Remark: No MALLS values available at T0 for lots E06 and E08. Value at T13M (time = 13 minutes) or T12M (time = 12 minutes) could be considered as representative insofar as relative retention time and viscosity did not change between T0 (time = 0 minutes) and T13M or T12M for both samples. ND means "not determined".

Example 2: Production of 6A conjugates with different carriers

- 10 a) Native *S. pneumoniae* serotype 6A polysaccharide vs. sized *S. pneumoniae* serotype 6A polysaccharide

The activation and coupling conditions used to produce different 6A conjugates are given in Table 2.

15

Table 2: Specific activation/coupling/quenching conditions of *S. pneumoniae* serotype 6A polysaccharide -Protein D/PhtD conjugates

Carrier	PD (lot PD003) (PS6A-PD conjugate)	PhtD (lot PhtD004) (PS6A-PhtD conjugate)	PhtD (lot PhtD008) (PS6A-PhtD conjugate)	dPly (lot dPly010) (PS6A-dPly conjugate)
PS6A conc. (mg/ml)	5.5 Native PS	5.5 Native PS	10.0 Sized PS	10 Sized PS
PS6A dissolution	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M
Carrier protein concentration (mg/ml)	5.0	10.0	20.0	10.0
Initial Carrier protein/PS6A Ratio (w/w)	1/1	3/1	3/1	3/1
CDAP/PS6A ratio (mg/mg PS)	0.75	1.5	1.5	1.5
Coupling time	60 min	45 min	150 min	180 min
pH_a/pH_c/pH_q	9.5/9.5/9.0	9.5/9.5/9.0	9.5/9.5/9.0	9.5/9.5/9.0

The final protein/PS6A ratio (w/w) for the resulting conjugates were:

PS6A-PD003: 0.6/1 (Native PS6A, M_w by MALLS 1106kDa)

PS6A-PhtD004: 1.4/1 (Native PS6A, M_w by MALLS 1106kDa)

5 PS6A-PhtD008: 2.7/1 (Sized PS6A, lot E01)

PS6A-dPly010: 1.65/1 (Sized PS6A, lot E01)

b) Sized polysaccharide conjugated to different carriers

10 The activation and coupling conditions used to produce different PS6A conjugates are given in Table 3.

5

**Table 3: Specific activation/coupling/quenching conditions of PS6A-Protein
D/CRM197/PhtD conjugates**

10

Carrier	PD (lot PDLS001 and lot PDLS002) (PS6A-PD conjugate)	CRM197 (lot CRM025) (PS6A-CRM197 conjugate)
PS6A conc. (mg/ml)	10.0	10.0
PS6A dissolution	NaCl 2M	NaCl 2M
Carrier protein concentration (mg/ml)	10.0	10.0
Initial Carrier protein /PS6A Ratio (w/w)	1.5/1	1.5/1
CDAP conc. (mg/mg PS)	1.5	1
Coupling time	120 min	120 min
pH_a/pH_c/pH_q	9.5/9.5/9.0	9.5/9.5/9.0

The final protein/PS6A ratio (w/w) for the resulting conjugates were:
 PS6A-PD/LS001 and PS6A-PD/LS002: 1.6/1 (Sized PS6A, lot E06)
 PS6A-CRM197/025: 1.3/1 (Sized PS6A, lot E03)

PS6A_{AH}-PhtD conjugate (lot 6A-PhtD106)

In a second conjugation method PS6A was linked to the carrier protein PhtD via a linker – Adipic acid dihydrazide (ADH); this conjugate is designated PS6A_{AH}-PhtD.

5 PS6A derivatization

Activation and coupling were performed at 25°C under continuous stirring in a temperature-controlled waterbath. Microfluidized PS6A was diluted to obtain a final polysaccharide concentration of 10 mg/ml in water for injection (WFI) and the solution was
10 adjusted at pH 6.0 ± 0.2 with 0.1N HCl. CDAP solution (100 mg/ml freshly prepared in acetonitrile/WFI, 50/50) was added to reach the appropriate CDAP/PS ratio (1/1, w/w).

The pH was raised up to the activation pH 9.00 ± 0.05 by the addition of 0.2M NaOH. After 3 minutes, ADH was added to reach the appropriate ADH/PS ratio (8.9/1 w/w); the
15 pH was regulated to coupling pH 9.0. The solution was left for 1 hour under pH regulation. The PS_{AH} derivative was then dialysed against 0.2M NaCl.

Coupling

20 PhtD at 7.5 mg/ml in 0.2M NaCl was added to the PS6A_{AH} derivative (PS6A with the ADH linker) in order to reach a PhtD/PS6A_{AH} ratio of 3/1 (w/w). The pH was adjusted to 5.0 ± 0.05 with HCl. The EDAC solution (50 mg/ml in 0.1M Tris-HCl pH 7.5) was added manually in 10 min (20 µl / min) to reach 0.5 mg EDAC/mg PS6A_{AH}. The resulting solution was incubated for 45 min at room temperature under stirring and pH regulation. The
25 solution was neutralized by addition of 1ml of 1M Tris-HCl pH 7.5 and let 30 min at room temperature.

Prior to the elution on Sephacryl S400HR, the conjugate was clarified using a 5µm Minisart filter. The resulting conjugate PS6A_{AH}-PhtD106 had a final PhtD/PS6A ratio of
30 2.84 (w/w).

A target in relative retention time was chosen (RRT₁₅₀₋₁₇₃₀ around 0.50). Several conjugates (6A-PhtD004 (native polysaccharide) and 6A-PhtD008 (sized polysaccharide)

(as described above) were produced with sized polysaccharide and were compared to conjugates produced with native PS6A.

Example 3: Preclinical evaluation of Anti-PS6A Responses

5

Groups of 40 mice were immunized IM at days 0, 14 and 28 with 14 valent (14V) formulations containing PS6A conjugates (at 1/10 of human dose) using AlPO_4 as adjuvant. Anti-PS6A ELISA IgG titers and Opsonophagocytosis (OP) titers were measured in individual sera collected at day 42.

10

The 14V-formulation contained the following conjugates :

1-PD, 3-PD, 4-PD, 5-PD, 6B-PD, 7F-PD, 9V-PD, 14-PD, 18C-TT_{AH}, 19A-dPly, 19F-DT, 22F-PhtD, 23F-PD. The serotype 6A was conjugated with PD, PhtD or dPly as carrier.

15 Results are summarized in Figure 1 ELISA anti-PS6A response.

Conclusions:

The highest anti-PS6A ELISA IgG titers were obtained with the PS6A-PD conjugate produced with a native polysaccharide (1106kDa). The evaluation showed a trend to a lower immunogenicity for conjugates with sized polysaccharide. Using PhtD as carrier, a lower anti-PS ELISA IgG titer was observed with the conjugate produced with a sized polysaccharide (14V (6A-PhtD/008 using lot E01). Subsequently, as described in the following examples, a change in sizing target was investigated to produce a PS6A with robustness of process and immunogenicity of resulting conjugates. The following experiments were carried out to evaluate sized polysaccharide having a higher molecular weight than PS6A from lot E01.

25

Example 4: Evaluation of Sized PS6A-CRM197 Conjugates

30 For each conjugate, conjugation parameters are given in Table 4 (PS6A and carrier concentrations, initial carrier/PS6A ratio(w/w), coupling time and CDAP/PS6A ratio (w/w)

Preparation of the solutions

- CDAP solution

35 Just before activation, a cyanodiaminopyridinium tetrafluoroborate (CDAP) solution was prepared at 100 mg/ml in acetonitrile/water for injection (50/50 (v/v))

Activation

Native or sized PS6A was diluted at a defined concentration and the pH of the solution was set to 6.0 ± 0.2 . At time 0, the CDAP solution was added manually in order to obtain
5 a defined CDAP/PS6A ratio (w/w).

After 1.5 minutes the pH was raised up to activation pH value by addition of NaOH.

At time 4.5 minutes, the protein solution (at a defined concentration and buffer) was added in order to obtain a fixed protein/PS6A ratio (w/w). The pH of the solution was
10 regulated at coupling pH value during a defined timing (coupling time – See Table 4).

At time $T = \text{coupling time} + 4\text{min } 30$, a solution of Glycine 2M pH 9.0 was added to quench the reaction. After 30 minutes of quenching, the conjugate was directly injected on purification column or let overnight under continuous stirring at $+2$ to $+8^\circ\text{C}$ before
15 purification.

Purification

Before purification, the conjugate solution was filtered through a $5 \mu\text{m}$ or $10 \mu\text{m}$ membrane in order to remove aggregates and particles. Conjugate was then purified on Sephacryl S400HR column (bed height: $100 \text{ cm} \pm 10 \text{ cm}$) using NaCl 150 mM as eluent.
20 The conjugate was then sterile filtered on $0.22 \mu\text{m}$ PVDF (polyvinylidene difluoride) membrane. The sterilised bulk (Conjugated Bulk) was stored at $+2$ - 8°C until formulation.

Characterization

The final Protein/Polysaccharide ratio (w/w) on the sterilized conjugate was determined by
25 the ratio of the Lowry/resorcinol concentrations. Antigenicity and free PS (polysaccharide) content were determined using methods described here below. Data are shown in Table 5.

Antigenicity test

30 Native polysaccharide was assigned an antigenicity index value of 100%. Insofar as correlation between antigenicity index and immunogenicity had not been established, the determination of sized polysaccharide antigenicity was performed as indicative value. The objective was to keep this value as high as possible. This value was determined by
35 ELISA.

The antigenicity of polysaccharide was determined in a sandwich-type ELISA (see Example 1 for measurement of antigenicity).

Free PS content by ELISA

5

After reaction of the conjugate with anti-carrier serum, the complex was precipitated using saturated ammonium sulphate (SAS). After centrifugation, the free PS06A content was performed by ELISA (anti-PS/anti-PS, see part 2.8.1.2) on the supernatant. The percentage of free PS was calculated proportionally to the total PS content measured by resorcinol method.

10

The absence of conjugate in the supernatant was also controlled by a α -carrier/ α -PS06A ELISA.

Table 4: Process conditions for PS6A-CRM197 conjugates at 50mg scale

Conjugate	PS6A	PS Dissolution (M NaCl)	[mg/ ml]	Carrier Dissolution buffer	[mg/ ml]
PS6A- CRM197/015	native	2	5	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A- CRM197/016	native	2	5	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A- CRM197/017	E01	2	10	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A- CRM197/018	E01	2	10	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A- CRM197/019	E01	2	10	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A- CRM197/020	E01	2	10	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A- CRM197/021	E01	2	10	K/K ₂ PO ₄ 10mM pH7	10

				2NaCl 0.2M	
PS6A-CRM197/022	E03	2	10	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A-CRM197/023	E03	2	10	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A-CRM197/024	E03	2	10	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10

Conjugate	Initial Ratio (w/w)	CDAP (mg/mg PS)	Coupling Time (min)	pHa/ pHc/ PHq	Remark
PS6A-CRM197/015	1.5/1	1.5/1	60	9.5/9.5/9.0	50 mg
PS6A-CRM197/016	1.5/1	0.75/1	60	9.5/9.5/9	50 mg
PS6A-CRM197/017	1.5/1	0.75/1	120	9.5/9.5/9	50 mg
PS6A-CRM197/018	1.5/1	1.5/1	120	9.5/9.5/9	50 mg
PS6A-CRM197/019	1.5/1	1/1	120	9.5/9.5/9	50 mg
PS6A-CRM197/020	2/1	0.75/1	120	9.5/9.5/9	50 mg
PS6A-CRM197/021	1.5/1	1.5/1	120	9.5/9.5/9	200 mg
PS6A-CRM197/022	1.5/1	1.5/1	120	9.5/9.5/9	50 mg
PS6A-CRM197/023	1.5/1	1.5/1	60	9.5/9.5/9	50 mg
PS6A-CRM197/024	1.5/1	1/1	120	9.5/9.5/9	50 mg

Note: Designation such as "PS6ACRM197/024" indicates PS6ACRM197 from lot024.

5 **Table 5: Characterization of PS6A-CRM197 conjugates at 50mg-PS scale**

Conjugate	Final Ratio Carrier/PS	Free polysaccharide	αPS/ αPS (%)	αprot/ αPS (%)	Yield (%)	Remark

	(w/w)	ELISA (%) 4°C	4°C	4°C		
PS6A- CRM197/015	0.91/1				58.8	Filtration issue
PS6A- CRM197/016	0.65/1				45.1	
PS6A- CRM197/017	1.23/1				28.2	Tightened pool/bad separation
PS6A- CRM197/018	1.33/1 (NF)				48.3	
PS6A- CRM197/019	1.10/1				50.2	
PS6A- CRM197/020	1.41/1				50.6	
PS6A- CRM197/021	1.42/1	0.8	35	125	45.4	
PS6A- CRM197/022	1.11/1	1.1	23	67	69.8	Filtration issue
PS6A- CRM197/023	1.24/1	0.8	27	98	61.4	
PS6A- CRM197/024	1.19/1	1.3	31	95	65.3	

The first set of conjugates (PS6A-CRM197/015, PS6A-CRM197/016) were produced with native polysaccharide (M_w by MALLS 990kDa) and showed limitations in term of reachable final CRM/PS ratio and filterability of resulting conjugates (Table 5).

5

A second set of conjugates (PS6A-CRM197/017, PS6A-CRM197/018, PS6A-CRM197/019, PS6A-CRM197/020, PS6A-CRM197/021) were produced using sized polysaccharide (lot E01). By increasing CDAP/PS ratio and/or initial CRM/PS ratio, the desired final CRM/PS ratio and polysaccharide yield could be reached. The best conditions (PS6A-CRM197/021) were reproduced with a new sized polysaccharide lots but having a higher molecular weight (lot E03) than the previous one. However a filtration

10

issue was observed. It was found that by reducing coupling time or CDAP/PS6A ratio, the filtration issue was solved.

A third set of conjugates (PS6A-CRM197/022, PS6A-CRM197/023, PS6A-CRM197/024) were produced using sized serotype 6A polysaccharide (lot E03). The best conditions (PS6A-CRM197/024) were the following: a polysaccharide concentration of 10 mg/ml in 2M NaCl, a CDAP/PS ratio of 1/1 (w/w), a CRM concentration of 10 mg/ml in 10 mM K/K₂ PO₄ pH 7.2, 0.2M NaCl, an initial CRM/PS ratio of 1.5/1 (w/w), a pH for activation and coupling of 9.5 and a coupling time of 120 min. Resulting conjugates had a final CRM/PS ratio around 1.2/1 (w/w) for a global yield around 65 %.

Example 5: Production of Sized 6A-CRM197 Conjugates at 200mg scale

A scale up at 200mg-PS scale was performed (Table 6 and Table 7).

15

Table 6: Process conditions for PS6A-CRM197 conjugates at 200mg-PS scale

Conjugate	Lot	Estimated molecular weight (kDa)*	PS Dissolution (M NaCl)	[mg/ml]	Lot	Carrier Dissolution buffer	[mg/ml]
PS6A-CRM197/025	E03	968	2	10	CRM197-012	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A-CRM197/026	E03	968	2	10	CRM197-012	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A-CRM197/027	E03	968	2	10	CRM197-009	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A-CRM197/028	E07	929	2	10	CRM197-014	K/K ₂ PO ₄ 10mM pH7 2NaCl 2M	10
PS6A-CRM197/029	E07	929	2	10	CRM197-014	K/K ₂ PO ₄ 10mM pH7 2NaCl 0.2M	10
PS6A-CRM197/030	E08	1004	2	10	CRM197-014	K/K ₂ PO ₄ 10mM pH7 2NaCl 2M	10

* Estimation realized using a dextran calibration curve (see Table 1 for MALLS data)

Conjugate	Initial Ratio (w/w)	CDAP (mg/mg PS)	Coupling Time (min)	pHa/pHc/pHq
PS6A-CRM197/025	1.5/1	1/1	120	9.5/ 9.5/ 9
PS6A-CRM197/026	1.5/1	1/1	120	9.5/ 9.5/ 9
PS6A-CRM197/027	1.5/1	1/1	120	9.5/ 9.5/ 9
PS6A-CRM197/028	1.5/1	1/1	120	9.5/ 9.5/ 9
PS6A-CRM197/029	1.5/1	1/1	120	9.5/ 9.5/ 9
PS6A-CRM197/030	1.5/1	1/1	120	9.5/ 9.5/ 9

Table 7: Characterization of PS6A-CRM conjugates at 200mg-PS scale

Conjugate	F.Ratio Carrier/PS (w/w)	Free PS ELISA (%) 4°C	α PS / α PS (%) 4°C	α prot / α PS (%) 4°C	Yield (%)
PS6A-CRM197/025	1.32/1	0.2	56	132	56.6
PS6A-CRM197/026	1.20/1	0.1	50	125	56.2
PS6A-CRM197/027	1.25/1	0.2	49	113	57.6
PS6A-CRM197/028	1.18/1		44	89	60.2
PS6A-CRM197/029	1.22/1		36	101	67.8

PS6A-CRM197/030	1.36/1		28	91	56.4
Remark					
E6A-417			PS6A-CRM197/025		

Data from 200 mg-PS scale lots was consistent with data obtained at small scale. Resulting conjugates had final CRM/PS ratio between 1.25 to 1.32/1, for a global polysaccharide yield of 56 %. In term of stability, no issue appeared in HP-SEC analyses or free polysaccharide content by ELISA (data not available with chemical method).

Based on these results, further lots (see Table 5 below) were produced in the following conditions: a polysaccharide concentration of 10 mg/ml in 2M NaCl, a CDAP/PS ratio of 1/1 (w/w), a CRM concentration of 10 mg/ml in 10 mM K/K₂PO₄ pH 7.2, 2M NaCl, an initial CRM/PS ratio of 1.5/1 (w/w), a pH for activation and coupling of 9.5 and a coupling time of 120 min.

Example 6: Immunogenicity of PS6A conjugates in mice

Different sized PS6A conjugates were evaluated in Balb/c mice (as monovalent formulation): 6A-CRM197 (PS6A-CRM025), 6A-PD (PS6A-PDLS001(E06)), 6A-PD (PS6A-PDLS002(E06)), 6A-PhtD (PS6A-PhtD008) and 6A_{AH}-PhtD (PS6A-PhtD106 (E04)), PS6A-PhtD (PS6A-PhtD100(E06)) conjugates produced with a sized PS.

34 Balb/c Mice were immunized three times (days 0-14-28) with 0.3 µg of different PS6A conjugates adsorbed onto AlPO₄. Anti-PS6A ELISA IgG titers levels and Opsonophagocytosis (OP) titers were measured in individual sera collected at day 42. Results are shown in Figures 2A and 2B. A significantly higher antibody response was induced by PS6A-AH-PhtD (CDAP/EDAC) and PS6A-CRM 025 (CDAP) in ELISA and OPA.

Example 7: Characterisation of sized PS6A

CRM197 concentration

Thawing of CRM197

Purified bulk is stored at -20°C at a concentration of 1.917 mg/ml in 10 mM K/K₂PO₄ pH 7.2. 7.5 g of purified bulk were thawed overnight at +2/+8°C.

Concentration by UF

- 5 The ultrafiltration was realized at room temperature on a centramate device.
The ultrafiltration membrane was an OMEGA medium screen membrane of 0.09 m² with a 10 kDa cut off (2 membranes). The circulation flow-rate was 1200 ml/min and the transmembrane pressure applied during the run was between 7-10 psi.
The CRM197 bulk was 7.5-fold concentrated in order to reach a concentration of around
10 15 mg/ml (target for conjugation >10 mg/ml).

Filtration on 0.22 µm

- After the ultrafiltration, the concentrated bulk was sterile filtered on 0.22 µm Millipack 20 filter (PVDF) at 20 ml/min. It was then stored at -20°C until its use in coupling.
15 The sized PS6A was characterized by the following test: MALLS and antigen content by ELISA. Stability was evaluated following 2 months at -70°C (T=0 compared to T=2 months). No stability issue observed when the sized PS6A is stored at -70°C. Results of sized PS6A characterization are summarized in (Table 8).

Table 8: Characterization data and stability of sized PS6A

	T =0	T=2M at -70°C
Antigen content by resorcinol (IP value)	15487 µg/ml	/
Mean molecular weight in weight by MALLS	302.1 kDa	288.8 kDa
Root mean square radius in weight by MALLS	27.7 nm	27.3 nm
Polydispersity ratio (M_w/M_n) by MALLS	1.102	1.136
Antigen content by ELISA	22250 µg/ml	22911 µg/ml
Ratio ELISA/resorcinol (%)	144 %	148 %

20

Two conjugates (D06ADJA001, D06ADJA002) were produced at 2g-PS scale using lot of sized PS6A and lot as carrier. The conditions of conjugation are described hereafter.

The reaction was performed at 25 +/- 1°C in a 1L-bioreactor.

2g of sized PS6A was diluted at 10 mg/ml in 2M NaCl and the pH of the solution was adjusted at 6.0 +/- 0.2 with 0.05N HCl solution.

5 At T = 0, 2 g of CDAP in solution (solution at 100 mg/ml in CH₃CN/H₂O 50/50) was manually added to the solution (CDAP/PS ratio = 1.0/1 w/w).

At T= 1min 30, pH was increased up to 9.5 +/- 0.05 by addition of 0.5N NaOH. It took approximately 90 sec to reach the target pH.

10 At T = 4min30, 3g of CRM197 (solution at 10 mg/ml in 10 mM KH₂PO₄/K₂HPO₄ pH 7.2, 2M NaCl) was added at the solution of activated PS6A in 1 minute in order to reach a CRM197/PS6A ratio of 1.5/1 w/w. The pH was regulated at 9.5 +/- 0.05 during 120 minutes.

15 At T =124 min 30, 100 ml of a solution of 2M glycine pH 9.0 was added to quench the conjugate (Ratio Gly/PS = 7.5/1 w/w). After 30 minutes of quenching, the pH of the mixture was adjusted to 6.5 +/- 0.2 using 5N HCl. When pH was stabilized, the conjugate was let overnight under continuous stirring at +2/+8°C before clarification and purification.

Purification

20 Prior to the elution on Sephacryl S400HR, the conjugate was clarified using a 10 µm Kleenpack HDCII filter at 50 ml/min

The conjugate was then injected on a Sephacryl S400HR. Elution was done with 0.15M NaCl solution and the collection pool was based on a Kd value. Kd is the distribution coefficient ($K_d = (V_e - V_0) / (V_t - V_0)$) V_e = elution volume, V_0 = void volume, V_t = total volume of the column.

25 The following criteria were used for the pool collection: from OD_{280nm}=0.05 AU to a Kd value of 0.28.

Sterilizing filtration

Before filtration, the bulk was brought back to room temperature.

30 Lots were filtered on Opticap 4" sterilizing membrane (1900 cm² PVDF) at a flow-rate of 40 ml/min. The filter was rinsed by 0.15M NaCl buffer before filtration. After filtration, 200 ml of 0.15M NaCl was passed through filter to limit the loss of material. No issue appeared during filtration.

35 Preclinical evaluation

Groups of 48 female Balb/c mice (4-weeks-old) were immunized IM at days 0,14 and 28 with adsorbed conjugates formulations (AIPO₄) containing 0.1µg of either D06ADJA001, D06ADJA002, PS6A-CRMLS001 (produced with sized PS6A, lot E07), PS6A-CRMLS002 (produced with sized PS6A, lot E07) or PS6A-PDLS001 (as a benchmark).

- 5 Anti-PS6A IgG levels (Figure 3A) and opsono-phagocytosis titers (Figure 3B) were measured in individual sera collected at day 42. No significant difference was observed in antibody responses induced by the PS6A-CRM produced according to Example 7 compared to the PS6A-CRM produced according to Example 5 in ELISA and in OPA.

10 **Example 8: Preparation of 6A Conjugate**

Sized 6A conjugate (lot E06AADJA059) was produced at 15 g PS scale in a 15L reactor using the conjugation parameters of the sized 6A produced at 200 mg PS scale (see Example 5). Conjugate was then purified on Sephacryl S400HR column (BPG450 column
15 (GE Healthcare)) using NaCl 150 mM as eluent. The conjugate was then sterile filtered on 0.22 µm PDVF membrane and stored at 2-8°C. The conjugate was concentrated (5x) and diafiltrated (10 diafiltration volume) against WFI (water for injection) using a 10 kDa MWCO OMEGA PES membrane (T-series, PALL). The retentate was then filtered on 0.22
20 µm membrane.

SEQ ID NO 1: MetLysLeuLysThrLeuAlaLeuSerLeuLeuAlaAlaGlyValLeuAlaGly
 CysSerSerHisSerSerAsnMetAlaAsnThrGlnMetLysSerAspLyslle
 5 llelleAlaHisArgGlyAlaSerGlyTyrLeuProGluHisThrLeuGluSerLysAla
 LeuAlaPheAlaGlnGlnAlaAspTyrLeuGluGlnAspLeuAlaMetThrLysAspGly
 ArgLeuValVallleHisAspHisPheLeuAspGlyLeuThrAspValAlaLysLysPhe
 ProHisArgHisArgLysAspGlyArgTyrTyrVallleAspPheThrLeuLysGlulle
 GlnSerLeuGluMetThrGluAsnPheGluThrLysAspGlyLysGlnAlaGlnValTyr
 10 ProAsnArgPheProLeuTrpLysSerHisPheArglleHisThrPheGluAspGlulle
 GluPhelleGlnGlyLeuGluLysSerThrGlyLysLysValGlylleTyrProGlulle
 LysAlaProTrpPheHisHisGlnAsnGlyLysAsplleAlaAlaGluThrLeuLysVal
 LeuLysLysTyrGlyTyrAspLysLysThrAspMetValTyrLeuGlnThrPheAspPhe
 AsnGluLeuLysArglleLysThrGluLeuLeuProGlnMetGlyMetAspLeuLysLeu
 15 ValGlnLeulleAlaTyrThrAspTrpLysGluThrGlnGluLysAspProLysGlyTyr
 TrpValAsnTyrAsnTyrAspTrpMetPheLysProGlyAlaMetAlaGluValValLys
 TyrAlaAspGlyValGlyProGlyTrpTyrMetLeuValAsnLysGluGluSerLysPro
 AspAsnlleValTyrThrProLeuValLysGluLeuAlaGlnTyrAsnValGluValHis
 ProTyrThrValArgLysAspAlaLeuProGluPhePheThrAspValAsnGlnMetTyr
 20 AspAlaLeuLeuAsnLysSerGlyAlaThrGlyValPheThrAspPheProAspThrGly
 ValGluPheLeuLysGlylleLys

SEQ ID NO. 2: MetAspProSerSerHisSerSerAsnMetAlaAsnThrGlnMetLysSerAspLyslle
 llelleAlaHisArgGlyAlaSerGlyTyrLeuProGluHisThrLeuGluSerLysAla
 25 LeuAlaPheAlaGlnGlnAlaAspTyrLeuGluGlnAspLeuAlaMetThrLysAspGly
 ArgLeuValVallleHisAspHisPheLeuAspGlyLeuThrAspValAlaLysLysPhe
 ProHisArgHisArgLysAspGlyArgTyrTyrVallleAspPheThrLeuLysGlulle
 GlnSerLeuGluMetThrGluAsnPheGluThrLysAspGlyLysGlnAlaGlnValTyr
 ProAsnArgPheProLeuTrpLysSerHisPheArglleHisThrPheGluAspGlulle
 30 GluPhelleGlnGlyLeuGluLysSerThrGlyLysLysValGlylleTyrProGlulle
 LysAlaProTrpPheHisHisGlnAsnGlyLysAsplleAlaAlaGluThrLeuLysVal
 LeuLysLysTyrGlyTyrAspLysLysThrAspMetValTyrLeuGlnThrPheAspPhe
 AsnGluLeuLysArglleLysThrGluLeuLeuProGlnMetGlyMetAspLeuLysLeu
 ValGlnLeulleAlaTyrThrAspTrpLysGluThrGlnGluLysAspProLysGlyTyr
 35 TrpValAsnTyrAsnTyrAspTrpMetPheLysProGlyAlaMetAlaGluValValLys
 TyrAlaAspGlyValGlyProGlyTrpTyrMetLeuValAsnLysGluGluSerLysPro
 AspAsnlleValTyrThrProLeuValLysGluLeuAlaGlnTyrAsnValGluValHis
 ProTyrThrValArgLysAspAlaLeuProGluPhePheThrAspValAsnGlnMetTyr
 AspAlaLeuLeuAsnLysSerGlyAlaThrGlyValPheThrAspPheProAspThrGly
 40 ValGluPheLeuLysGlylleLys

SEQ ID NO. 3: SerSerHisSerSerAsnMetAlaAsnThr

CLAIMS

1. A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide wherein the average size (M_w) of the *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is between 180-400, 210-400, 210-370, 220-360, 230-350, 240-340, 240-320, 240-310 or 250-310 kDa.
2. A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to claim 1 which has been sized by a mechanical sizing technique.
3. A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to claim 1 or claim 2 conjugated to a carrier protein (e.g. CRM-197).
4. A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to claim 3 wherein the *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is directly conjugated to the carrier protein.
5. A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to any one of claims 3-4 wherein the 6A polysaccharide is conjugated to the carrier protein or to a linker using CDAP chemistry.
6. A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to any one of claims 3-5 wherein the serotype 6A capsular polysaccharide (PS6A) is conjugated to the carrier protein or to a linker using CDAP chemistry using a CDAP:PS6A ratio between 1:2 to 3:1, 1:1.5 to 2:1, or 1:1.
7. A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to any one of claims 3-6 wherein the serotype 6A capsular polysaccharide is conjugated to the carrier protein or to a linker using CDAP chemistry wherein the reaction was carried out using a coupling time of between 50-130 minutes, 60-130 minutes, or 110-130 minutes.
8. A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to any one of claims 3-7 wherein the ratio of carrier protein to serotype 6A capsular polysaccharide is between 5:1 and 1:5, 4:1 and 1:1 or 2:1 and 1:1, 1.5:1 and 1:1, 1.4:1 and 1.3:1 (for example 1.2:1, 1.5:1) (w/w).

9. A sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to claims 1-8 wherein the 6A capsular polysaccharide is sized by a factor of no more than x5.
10. A process for preparing a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide conjugate comprising conjugating a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide to a carrier protein or to a linker using CDAP chemistry using a CDAP:PS ratio between 1:2 to 3:1, 1:1.5 to 2:1, or 1:1.
11. A process according to claim 10 wherein the reaction was carried out using a coupling time of between 50-130 minutes, 60-130 minutes, or 110-130 minutes.
12. A process for preparing a *Streptococcus pneumoniae* serotype 6A capsular polysaccharide conjugate comprising (a) conjugation of a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide to a carrier protein and (b) diafiltration against a solution having a concentration of NaCl below 150mM.
13. An immunogenic composition comprising a sized *Streptococcus pneumoniae* serotype 6A capsular polysaccharide according to claims 1-9 or *Streptococcus pneumoniae* serotype 6A capsular polysaccharide obtained by a process of any one of claims 10-12 conjugated to a carrier protein.
14. An immunogenic composition according to claim 13 comprising 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, or 16 or more capsular polysaccharides conjugates from different *S. pneumoniae* serotypes.
15. An immunogenic composition according to claim 13 or claim 14 comprising 1 or more, native capsular polysaccharides conjugates from *S. pneumoniae*.
16. An immunogenic composition according to claim 15 comprising native *Streptococcus pneumoniae* capsular serotype 6B polysaccharide.
17. An immunogenic composition according to claim 15 or 16 comprising native *Streptococcus pneumoniae* capsular serotype 23F polysaccharide.

18. An immunogenic composition according to any one of claims 13 to 17 which comprises *Streptococcus pneumoniae* capsular serotype 6B polysaccharide having an average size (M_w) of between 500-1800, 900-1660, or 1000-1400 kDa.
19. An immunogenic composition according to any one of claims 13 to 18 which comprises *Streptococcus pneumoniae* capsular serotype 23F polysaccharide having an average size (M_w) of between 500-1500, 600-1500, 700-1300, 900-1250, 800-1100, or 900-1000 kDa.
20. An immunogenic composition according to any one of claims 13 to 19 which comprises *Streptococcus pneumoniae* capsular polysaccharide from: (a) serotype 1 having an average size (M_w) of between 100-1000, 200-800, 250-600, or 300-400 kDa; (b) serotype 4 having an average size (M_w) of between 50-500, 60-300, 70-150, or 75-125 kDa; (c) serotype 5 having an average size (M_w) of between 100-1000, 100-700, 100-350, or 150-300 kDa; (d) serotype 7F having an average size (M_w) of between 50-1000, 100-750, 150-500, or 200-300 kDa; (e) serotype 9V having an average size (M_w) of between 50-1000, 100-750, 150-500, 200-400, or 250-300 kDa; (f) serotype 14 having an average size (M_w) of between 50-1000, 100-750, 150-500, or 200-250 kDa; (g) 18C having an average size (M_w) of between 50-1000, 50-750, 50-500, 50-190, 50-150 or 80-110 kDa (h) serotype 19F having an average size (M_w) of between 50-1000, 100-750, 100-500, 100-190 or 120-180 kDa; and/or (i) serotype 19A having an average size (M_w) of between 50-800kDa, 110-700, 110-300, 120-200, 130-180, 140-160 or 80-130 kDa.
21. An immunogenic composition according to any one of claims 13 to 20 which further comprises *Streptococcus pneumoniae* capsular serotype 22F having an average size (M_w) of between 50 and 800 kDa, 110 and 700 kDa, 110-300, 120-200, 130-180, 150-170, 100-190, 100-150, 95-125 or 100-115 kDa.
22. An immunogenic composition according to any one of claims 13 to 21 comprising 2, 3, 4, 5 or 6 different carrier proteins.
23. An immunogenic composition according to any one of claims 13 to 22 wherein one or more or all carrier proteins is selected from the group consisting of: diphtheria toxoid (DT), CRM197, tetanus toxoid (TT), Fragment C of TT, dPly, PhtA, PhtB, PhtD, PhtE, PhtDE, OmpC, PorB and *Haemophilus influenzae* Protein D.

24. An immunogenic composition of any one of claims 13 to 23 wherein the *Streptococcus pneumoniae* capsular serotype 6B polysaccharide is conjugated to a different carrier protein (e.g. protein D) than the carrier protein to which *Streptococcus pneumoniae* serotype 6A capsular polysaccharide is conjugated.
25. An immunogenic composition of any one of claims 13 to 24 comprising serotype 1 saccharide conjugated to protein D, serotype 4 saccharide conjugated to protein D, serotype 5 saccharide conjugated to protein D, serotype 6B saccharide conjugated to protein D, serotype 7F saccharide conjugated to protein D, serotype 9V saccharide conjugated to protein D, serotype 14 saccharide conjugated to protein D and serotype 23F saccharide conjugated to protein D.
26. An immunogenic composition according to any one of claims 13 to 25 comprising serotype 19F conjugated to Diphtheria toxoid.
27. An immunogenic composition according to any one of claims 13 to 26 wherein the composition comprises capsular saccharide 18C conjugated to tetanus toxoid (TT), optionally wherein 18C is the only saccharide in the composition conjugated to tetanus toxoid (TT), optionally via an ADH linker
28. An immunogenic composition according to any of claims 13 to 27 further comprising *S. pneumoniae* capsular saccharide(s) of one or more of: serotype 33F, serotype 15 and serotype 12F, conjugated to carrier protein(s).
29. An immunogenic composition according to any of claims 13 to 28 wherein the dose of the 6A saccharide conjugate is between 1 and 10 µg, 1 and 5 µg, or 1 and 3 µg of saccharide (e.g. 2 µg).
30. An immunogenic composition according to any one of claims 13 to 29 which further comprises one or more unconjugated or conjugated *S pneumoniae* proteins selected from: Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins, detoxified pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133.
31. An immunogenic composition according to any one of claims 13 to 30 which further comprises an adjuvant.

32. An immunogenic composition of claim 31 wherein the adjuvant comprises (per 0.5 mL dose) 100-750, 200-500, or 300-400 µg Al (aluminium) as aluminium phosphate.
33. A vaccine comprising the immunogenic composition of any one of claims 13 to 32 and a pharmaceutically acceptable excipient or carrier.
34. A process for making the vaccine according to claim 33 which comprises the step of mixing the immunogenic composition of any of claims 13 to 32 with a pharmaceutically acceptable excipient or carrier.
35. A method for the treatment or prevention of *Streptococcus pneumoniae* infection in a subject in need thereof comprising administering to said subject a therapeutically effective amount of an immunogenic composition of any of claims 13 to 32 or the vaccine of claim 33.
36. A method of immunising a human host against *Streptococcus pneumoniae* infection comprising administering to the host an immunoprotective dose of the immunogenic composition of any of claims 13 to 32 or vaccine of claim 34.
37. A method of inducing an immune response to *Streptococcus pneumoniae* serotype 6A in a subject, the method comprising administering a therapeutically or prophylactically effective amount of the immunogenic composition of any of claims 13 to 32 or the vaccine of claim 33.
38. The immunogenic composition of claims 13 to 32 or vaccine of claim 33 for use in the treatment or prevention of disease caused by *Streptococcus pneumoniae* infection.
39. A use of the immunogenic composition of claims 12 to 32 or vaccine of claim 33 in the manufacture of a medicament for the treatment or prevention of a disease caused by *Streptococcus pneumoniae* infection.

Figure 1: Evaluation of PS6A conjugates in 14V AIPO4 formulation in the Balb/c mouse with co-administration of Infanrix Hexa model. ELISA anti-PS6A.

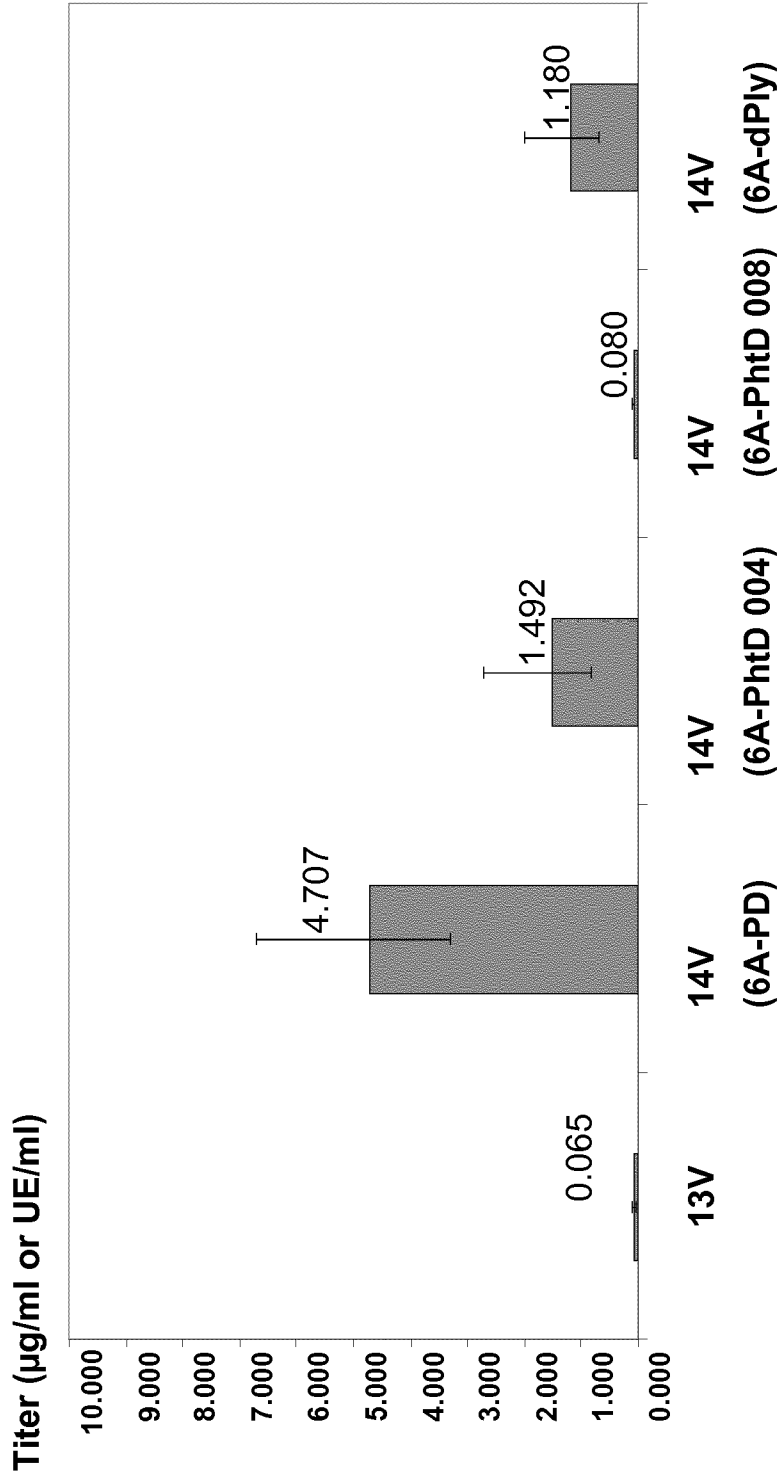
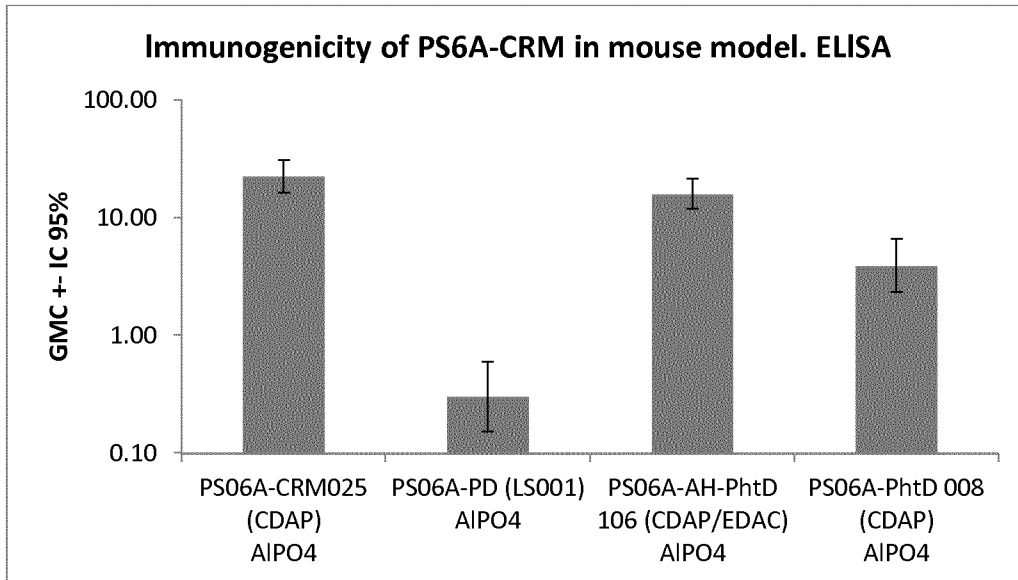


Figure 1

(A)



(B)

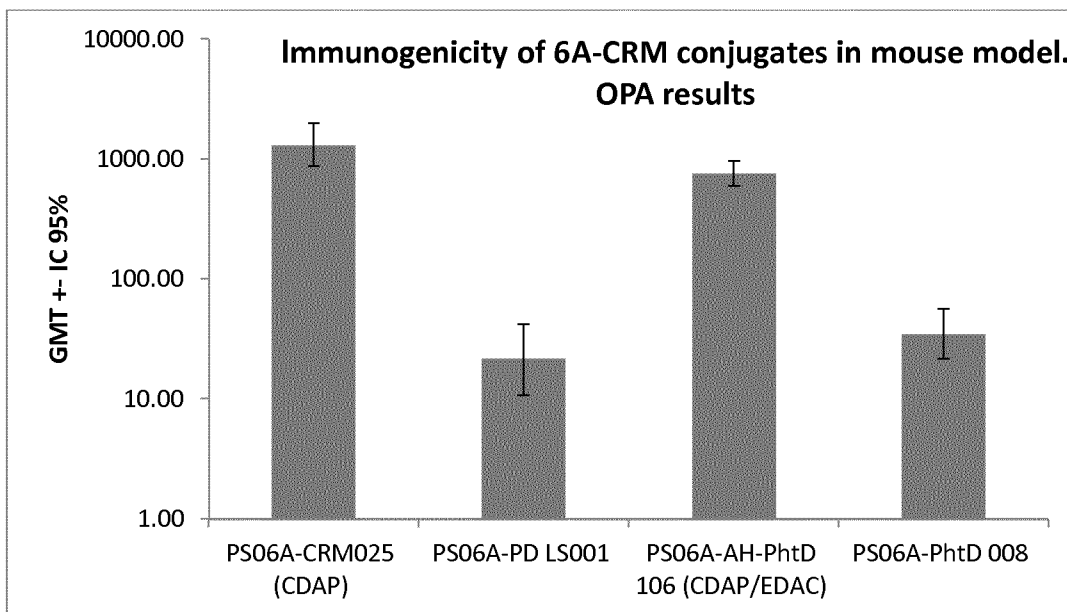


Figure 2

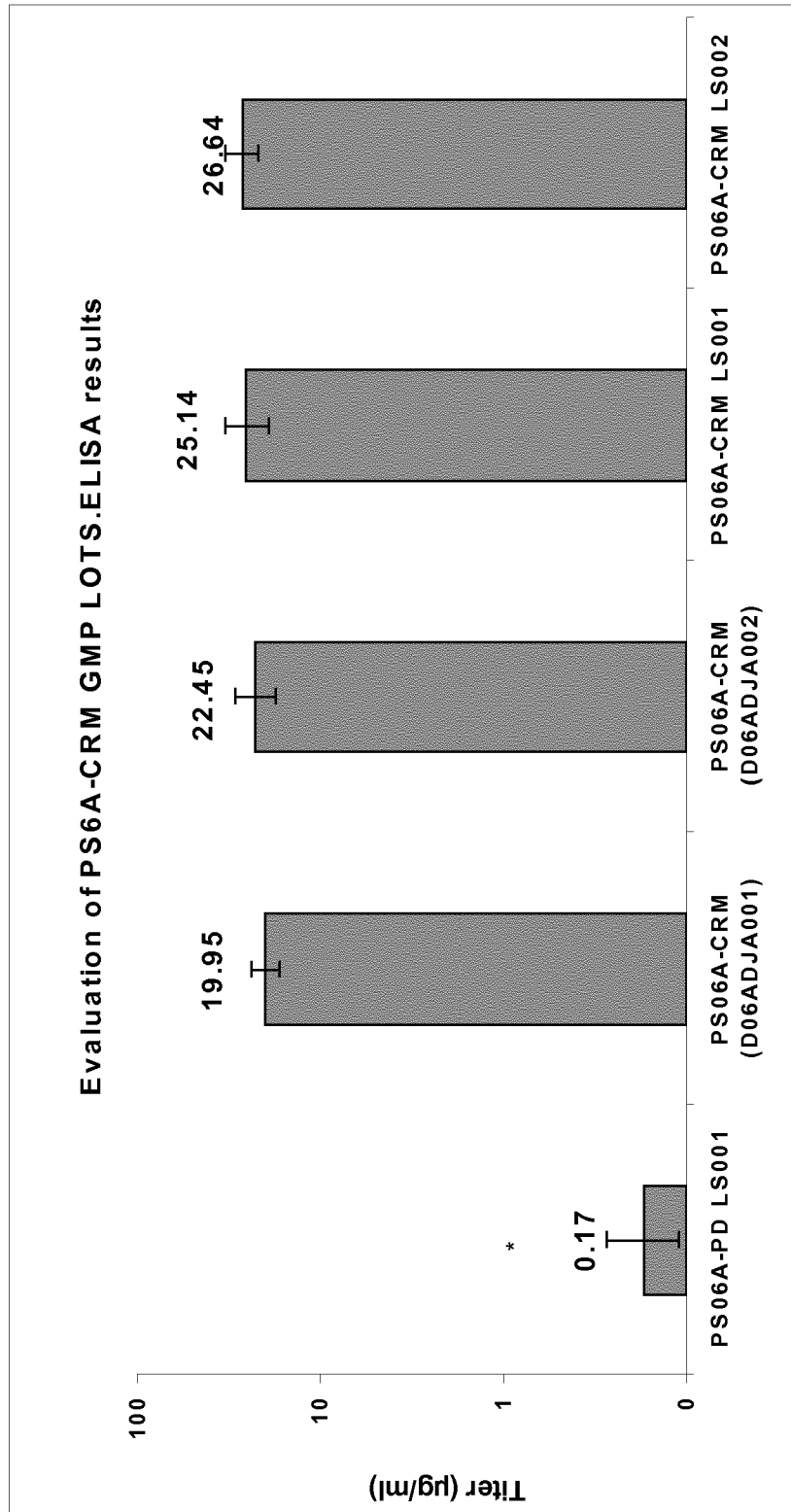


Figure 3A

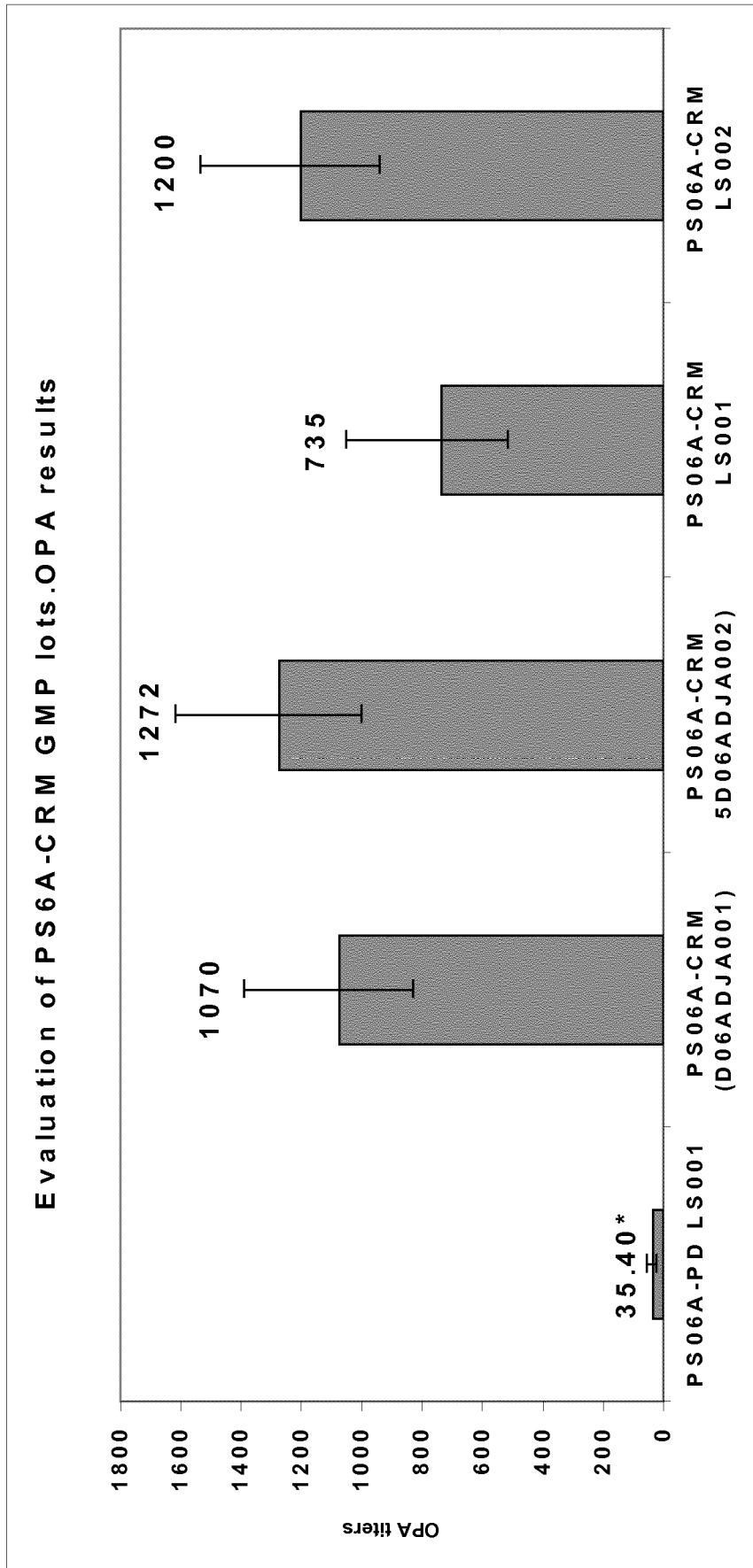


Figure 3B

INTERNATIONAL SEARCH REPORT

International application No PCT/EP2016/075045

A. CLASSIFICATION OF SUBJECT MATTER INV. 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) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/110241 A1 (GLAXOSMITHKLINE BIOLOG SA [BE]; BIEMANS RALPH LEON [BE]; DUVIVIER PIER) 15 September 2011 (2011-09-15) example A1	1-39
X	----- WO 2009/000826 A1 (GLAXOSMITHKLINE BIOLOG SA [BE]; BIEMANS RALPH LEON [BE]; HERMAND PHILI) 31 December 2008 (2008-12-31) example A1	1-39
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X	----- WO 2014/060383 A1 (GLAXOSMITHKLINE BIOLOG SA [BE]) 24 April 2014 (2014-04-24) example A1	1-39
----- -/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search	Date of mailing of the international search report	
3 March 2017	13/03/2017	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Fellows, Edward	

3

INTERNATIONAL SEARCH REPORT

International application No PCT/EP2016/075045

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/202309 A1 (EMINI EMILIO ANTHONY [US] ET AL) 23 July 2015 (2015-07-23) example A11	1-39
X	----- US 2011/019086 A1 (ALMEIDA RUI SERGIO RAINHO [PT] ET AL) 27 January 2011 (2011-01-27) example A11 -----	1-39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/075045

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.:

1-39 (partially)
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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

1. claims: 1-39(partially)

A sized Streptococcus pneumoniae serotype 6A capsular polysaccharide wherein the average size (Mw) of said polysaccharide is 180-400 kDa and related subject-matter.

2. claims: 1-39(partially)

A sized Streptococcus pneumoniae serotype 6A capsular polysaccharide wherein the average size (Mw) of said polysaccharide is 210-400 kDa and related subject-matter.

3. claims: 1-39(partially)

A sized Streptococcus pneumoniae serotype 6A capsular polysaccharide wherein the average size (Mw) of said polysaccharide is 210-370 kDa and related subject-matter.

4. claims: 1-39(partially)

A sized Streptococcus pneumoniae serotype 6A capsular polysaccharide wherein the average size (Mw) of said polysaccharide is 220-360 kDa and related subject-matter.

5. claims: 1-39(partially)

A sized Streptococcus pneumoniae serotype 6A capsular polysaccharide wherein the average size (Mw) of said polysaccharide is 230-350 kDa and related subject-matter.

6. claims: 1-39(partially)

A sized Streptococcus pneumoniae serotype 6A capsular polysaccharide wherein the average size (Mw) of said polysaccharide is 240-340 kDa and related subject-matter.

7. claims: 1-39(partially)

A sized Streptococcus pneumoniae serotype 6A capsular polysaccharide wherein the average size (Mw) of said polysaccharide is 240-320 kDa and related subject-matter.

8. claims: 1-39(partially)

A sized Streptococcus pneumoniae serotype 6A capsular

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

polysaccharide wherein the average size (Mw) of said polysaccharide is 240-310 kDa and related subject-matter.

9. claims: 1-39(partially)

A sized Streptococcus pneumoniae serotype 6A capsular polysaccharide wherein the average size (Mw) of said polysaccharide is 250-310 kDa and related subject-matter.

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