

Adjuvanted immunogenic compositions comprising conjugated pneumococcal capsular saccharide antigens and uses thereof

Field of the Invention

5 The present invention relates to new adjuvanted immunogenic compositions comprising conjugated pneumococcal capsular saccharide antigens (glycoconjugates) and a saponin containing liposomal adjuvant. Immunogenic compositions of the present invention will typically comprise glycoconjugates, wherein the saccharides are derived from serotypes of *Streptococcus pneumoniae*. The invention also relates to vaccination of human subjects, in particular infants and
10 elderly, against pneumococcal infections using said novel immunogenic compositions.

Background of the Invention

Infections caused by pneumococci are a major cause of morbidity and mortality all over the world. Pneumonia, febrile bacteraemia and meningitis are the most common manifestations of invasive
15 pneumococcal disease, whereas bacterial spread within the respiratory tract may result in middle-ear infection, sinusitis or recurrent bronchitis. Compared with invasive disease, the non-invasive manifestations are usually less severe, but considerably more common.

In Europe and the United States, pneumococcal pneumonia is the most common community-acquired bacterial pneumonia, estimated to affect approximately 100 per 100,000 adults each
20 year. The corresponding figures for febrile bacteraemia and meningitis are 15–19 per 100 000 and 1–2 per 100,000, respectively. The risk for one or more of these manifestations is much higher in infants and elderly people, as well as immune compromised persons of any age. Even in economically developed regions, invasive pneumococcal disease carries high mortality; for adults with pneumococcal pneumonia the mortality rate averages 10%–20%, whilst it may exceed
25 50% in the high-risk groups. Pneumonia is by far the most common cause of pneumococcal death worldwide.

The etiological agent of pneumococcal diseases, *Streptococcus pneumoniae* (pneumococcus), is a Gram-positive encapsulated coccus, surrounded by a polysaccharide capsule. Differences in the composition of this capsule permit serological differentiation between about 91 capsular types,
30 some of which are frequently associated with pneumococcal disease, others rarely. Invasive pneumococcal infections include pneumonia, meningitis and febrile bacteraemia; among the common non-invasive manifestations are otitis media, sinusitis and bronchitis.

Pneumococcal conjugate vaccines (PCVs) are pneumococcal vaccines used to protect against disease caused by *S. pneumoniae* (pneumococcus). There are currently five PCV vaccines
35 available on the global market: Prevnar® (called Prevenar in some countries) (heptavalent vaccine), SYNFLORIX® (a decavalent vaccine), Prevnar 13® (tridecavalent vaccine), Vaxneuvance™ (a 15-valent vaccine) and Prevnar 20™ (a 20-valent vaccine).

The recent development of widespread microbial resistance to essential antibiotics and the increasing number of immunocompromised persons underline the need for pneumococcal vaccines with even broader protection.

In particular, there is a need to address remaining unmet medical need for coverage of pneumococcal disease due to serotypes not found in Prevnar 13® and potential for serotype replacement over time. The specific serotypes causing disease beyond the 13 in Prevnar 13® vary by region, population, and may change over time due to acquisition of antibiotic resistance, pneumococcal vaccine introduction and secular trends of unknown origin. There is a need for immunogenic compositions that can be used to induce an immune response against additional *Streptococcus pneumoniae* serotypes in humans and in particular in children less than 2 years old.

An object of the new immunogenic compositions of the present invention is to provide for appropriate protection against additional *S. pneumoniae* serotypes not found in Prevnar 20™. In one aspect, an object of the immunogenic compositions of the present invention is to provide for appropriate protection against additional *S. pneumoniae* serotypes not found in Vaxneuvance™ and/or Prevnar 20™ while maintaining an immune response against serotypes currently covered by said vaccines.

Figures

Figure 1 Effect of QS-21 containing liposomal adjuvant on quarter dose PCV20 OPA titer. ST3 specific Opsonophagocytic titers measured from sera collected at 4 weeks post dose 1 from infant rhesus macaques. Each dot represents individual animal and data expressed as geomean titers for OPA with 95% confidence interval. LLOQ - lower limit of quantitation. QS-21 adj - QS-21 containing liposomal adjuvant.

Figure 2 Effect of QS-21 containing liposomal adjuvant on quarter dose PCV20 IgG titer. ST3 specific IgG titers measured from sera collected at 4 weeks post dose 1 from infant rhesus macaques. Each dot represents individual animal and data expressed as µg/ml for IgG with 95% confidence interval. LLOQ - lower limit of quantitation. QS-21 adj - QS-21 containing liposomal adjuvant.

1 Pneumococcal glycoconjugates of the invention

The present invention is directed in part to conjugated pneumococcal capsular saccharide antigens (also named glycoconjugates). For the purpose of the invention the term 'glycoconjugate' indicates a pneumococcal capsular saccharide conjugated to a carrier protein via covalent or non-covalent bonds. In an embodiment, the pneumococcal capsular saccharide is conjugated to a carrier protein via non-covalent bonds (such as the rhizavidin/biotin system, see e.g. WO2012155007, WO2020056202). Preferably, the capsular saccharide is conjugated via covalent bonds. In one embodiment the capsular saccharide is conjugated directly to a carrier

protein. In a second embodiment the capsular saccharide is conjugated to a carrier protein through a spacer/linker.

1.1 Pneumococcal capsular saccharides of the invention

5 The term "saccharide" throughout this specification may indicate polysaccharide or oligosaccharide and includes both. In an embodiment, saccharides of the invention may be oligosaccharides. Oligosaccharides have a low number of repeat units (typically 5-15 repeat units) and are typically derived synthetically or by hydrolysis of polysaccharides. Preferably though, all of the saccharides of the present invention and in the immunogenic compositions of the present invention are polysaccharides. High molecular weight polysaccharides are able to induce certain
10 antibody immune responses due to the epitopes present on the antigenic surface. The isolation and purification of high molecular weight capsular polysaccharides is preferably contemplated for use in the conjugates, compositions and methods of the present invention. Therefore, in a preferred embodiment of the present invention, the saccharide is a polysaccharide.

Preferably, the saccharides used in the present invention are pneumococcal capsular
15 saccharides (also named 'S. *pneumoniae* capsular saccharide' or 'capsular saccharide' herein). Capsules are found in several bacteria of medical importance. Bacterial capsules are largely composed of polysaccharides. Capsular saccharides are prepared by standard techniques known to those of ordinary skill in the art.

In an embodiment, the S. *pneumoniae* capsular saccharides used in the present invention
20 are synthetic carbohydrates.

In a preferred embodiment though, the source of S. *pneumoniae* capsular saccharides according to this invention can be S. *pneumoniae* cells. S. *pneumoniae* strains which can be used as source of capsular saccharide may be obtained from established culture collections (such as for example from the American Type Culture Collection (ATCC, Manassas, VA USA) or the
25 Streptococcal Reference Laboratory (Centers for Disease Control and Prevention, Atlanta, GA USA)) or clinical specimens.

S. *pneumoniae* capsular saccharides can be obtained directly from bacteria using isolation procedures known to one of ordinary skill in the art (see for example methods disclosed in US2006/0228380, US2006/0228381, US2007/0184071, US2007/0184072, US2007/0231340,
30 and US2008/0102498 and WO2008/118752). They can also be produced using synthetic protocols known to the man skilled in the art.

In case the S. *pneumoniae* capsular saccharides are obtained directly from bacteria, the bacterial cells can be grown in a medium (e.g., in a soy-based medium). Following fermentation, the bacterial cells can be lysed to produce a cell lysate. The capsular saccharide may then be
35 isolated from the cell lysate using purification techniques known in the art, including the use of centrifugation, depth filtration, precipitation, ultra-filtration, treatment with activate carbon, diafiltration and/or column chromatography (see, for example, US2006/0228380,

US2006/0228381 and WO2008/118752). The purified capsular saccharide can then be used for the preparation of immunogenic conjugates.

The isolated capsular saccharide obtained by purification can be characterized by different parameters including, for example the weight average molecular weight (Mw). The molecular weight of the polysaccharide can be measured by Size Exclusion Chromatography (SEC) combined with Multiangle Laser Light Scattering detector (MALLS).

The capsular saccharides used in the present invention are capsular saccharides from *Streptococcus pneumoniae*.

Preferably, the capsular saccharide used in the present invention are capsular saccharides from *Streptococcus pneumoniae* serotypes selected from serotypes 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9V, 9N, 10A, 10B, 10C, 10F, 11A, 11B, 11C, 11D, 11E, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20A, 20B, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33C, 33D, 33E, 33F, 34, 35A, 35B, 35C, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F and 48.

Preferably, the capsular saccharides used in the present invention are capsular saccharides from *Streptococcus pneumoniae* serotypes selected from serotypes 1, 2, 3, 4, 5, 6A, 6B, 6C, 7C, 7F, 8, 9V, 9N, 10A, 10B, 11A, 12F, 14, 15A, 15B, 15C, 16F, 17F, 18C, 19A, 19F, 20A, 20B, 21, 22A, 22F, 23A, 23B, 23F, 24B, 24F, 27, 29, 31, 33B, 33F, 34, 35B, 35F, 38, 72 and 73.

S. pneumoniae capsular polysaccharides comprise repeating oligosaccharide units which may contain up to 8 sugar residues.

In an embodiment, capsular saccharides of the invention may be one oligosaccharide unit, or a shorter than native length saccharide chain of repeating oligosaccharide units. In an embodiment, a capsular saccharide of the invention is one repeating oligosaccharide unit of the relevant serotype.

In an embodiment, capsular saccharides of the invention may be oligosaccharides. Oligosaccharides have a low number of repeat units (typically 5-15 repeat units) and are typically derived synthetically or by hydrolysis of polysaccharides.

In a preferred embodiment, the capsular saccharides of the present invention are polysaccharides. High molecular weight capsular polysaccharides are able to induce certain antibody immune responses due to the epitopes present on the antigenic surface. The isolation and purification of high molecular weight capsular polysaccharides is preferably contemplated for use in the conjugates, compositions and methods of the present invention.

In a preferred embodiment, the isolated capsular saccharides used in the present invention (purified before further treatment) has a weight average molecular weight between 50 kDa and 5000 kDa. In a preferred embodiment, the capsular saccharide used in the present invention has a weight average molecular weight between 200 kDa and 4000 kDa.

Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

1.2 Attributes of the *Streptococcus pneumoniae* glycoconjugates of the invention

5 In general, conjugation of saccharides to carriers enhances the immunogenicity of saccharides as it converts them from T-independent antigens to T-dependent antigens, thus allowing priming for immunological memory. Conjugation is particularly useful for pediatric vaccines.

10 The isolated capsular saccharides described above may be activated (e.g., chemically activated) to make them capable of reacting (e.g. with a linker or directly with the carrier protein) and then incorporated into glycoconjugates, as further described herein.

Before activation, the size of the isolated polysaccharides can be reduced while preserving critical features of the structure of the polysaccharide. Mechanical or chemical sizing maybe employed. In an embodiment, the size of the isolated polysaccharide is reduced by chemical hydrolysis. Chemical hydrolysis maybe conducted using a mild acid (e.g. acetic acid, formic acid, 15 propanoic acid). In an embodiment, chemical hydrolysis is conducted using formic acid. In an embodiment, chemical hydrolysis is conducted using propanoic acid. In a preferred embodiment, chemical hydrolysis is conducted using acetic acid. Chemical hydrolysis may also be conducted using a diluted strong acid (such as diluted hydrochloric acid, diluted sulfuric acid, diluted phosphoric acid, diluted nitric acid or diluted perchloric acid). In an embodiment, chemical 20 hydrolysis is conducted using diluted hydrochloric acid. In an embodiment, chemical hydrolysis is conducted using diluted sulfuric acid. In an embodiment, chemical hydrolysis is conducted using diluted phosphoric acid. In an embodiment, chemical hydrolysis is conducted using diluted nitric acid. In an embodiment, chemical hydrolysis is conducted using diluted perchloric acid.

The size of the isolated polysaccharide can also be reduced by mechanical homogenization. 25 In an embodiment, the size of the isolated polysaccharide is reduced by high pressure homogenization. High pressure homogenization achieves high shear rates by pumping the process stream through a flow path with sufficiently small dimensions. The shear rate is increased by using a larger applied homogenization pressure, and exposure time can be increased by recirculating the feed stream through the homogenizer.

30 The weight average molecular weight (Mw) of the saccharide before conjugation refers to the Mw before the activation of the saccharide (i.e. after an eventual sizing step but before reacting the saccharide with an activating agent). In the context of the present invention the Mw of the saccharide is not substantially modified by the activation step and the Mw of the saccharide incorporated in the conjugate is similar to the Mw of the saccharide as measured before activation.

35 In an embodiment, the pneumococcal glycoconjugates of the present invention comprise *S. pneumoniae* capsular saccharides wherein the weight average molecular weights (Mw) of said saccharides before conjugation are between 50 kDa and 1,000 kDa.

In an embodiment, the pneumococcal glycoconjugates of the present invention comprise *S. pneumoniae* capsular saccharides wherein the weight average molecular weights (Mw) of said saccharides before conjugation are between 50 kDa and 600 kDa.

5 In an embodiment, the pneumococcal glycoconjugates of the present invention comprise *S. pneumoniae* capsular saccharides wherein the weight average molecular weights (Mw) of said saccharides before conjugation are between 50 kDa and 500 kDa.

In some embodiments, the pneumococcal glycoconjugates of the invention have a weight average molecular weight (Mw) of between 250 kDa and 20,000 kDa.

10 In some embodiments, the pneumococcal glycoconjugates of the invention have a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa.

Another way to characterize the pneumococcal glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM₁₉₇, SCP, DT or TT) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent
15 linkages to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation may results in a reduction in the number of lysine residues recovered compared to the carrier protein starting material used to generate the conjugate materials. In a preferred embodiment, the degree of conjugation of the pneumococcal glycoconjugates of the invention is between 2 and 15.

20 The pneumococcal glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein. In some embodiments, the ratio of *S. pneumoniae* capsular saccharides to carrier protein in the glycoconjugate (w/w) is between 0.5 and 3.0. In other embodiments, the saccharide to carrier protein ratio (w/w) is between 0.5 and 2.0. In other
25 embodiments, the saccharide to carrier protein ratio (w/w) is between 0.5 and 1.5. In other embodiments, the saccharide to carrier protein ratio (w/w) is between 0.8 and 1.2. In other embodiments, the saccharide to carrier protein ratio (w/w) is between 0.5 and 1.0. In other embodiments, the saccharide to carrier protein ratio (w/w) is between 1.0 and 1.5. In other embodiments, the saccharide to carrier protein ratio (w/w) is between 1.0 and 2.0. In further
30 embodiments, the saccharide to carrier protein ratio (w/w) is between 0.8 and 1.2.

35 The pneumococcal glycoconjugates and immunogenic compositions of the invention may contain free saccharide that is not conjugated to the carrier protein but is nevertheless present in the glycoconjugate composition. The free saccharide may be noncovalently associated with (i.e., noncovalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate. In a preferred embodiment, each pneumococcal glycoconjugate of the invention comprises less than about 50% of free pneumococcal saccharide compared to the total amount of saccharide. In a preferred
embodiment, each pneumococcal glycoconjugate of the invention comprises less than about 25% of free pneumococcal saccharide compared to the total amount of saccharide.

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

In a preferred embodiment, at least 30% of the pneumococcal glycoconjugate of the invention has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 40% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment,
 15 between 40% and 80% of the pneumococcal glycoconjugate of the invention has a K_d below or equal to 0.3 in a CL-4B column.

1.3 Mode of preparation of the *Streptococcus pneumoniae* glycoconjugates of the invention

The pneumococcal glycoconjugates of the present invention can be prepared by any coupling technique known to those of ordinary skill in the art.

20 In an embodiment, the *S. pneumoniae* capsular saccharide is coupled to the carrier protein via non-covalent bonds (see e.g. WO2012155007, WO2020056202).

In an embodiment, the *S. pneumoniae* capsular saccharide is conjugated via covalent bonds. In one embodiment the capsular saccharide is conjugated directly to a carrier protein. In a second embodiment the capsular saccharide is conjugated to a carrier protein through a
 25 spacer/linker.

In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention are 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, two reactive amino groups or two reactive carboxylic
 30 acid groups. The linker has for example between 4 and 20, 4 and 12, 5 and 10 carbon atoms.

A possible linker is adipic acid dihydrazide (ADH). 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).

35 In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention is conjugated directly to the carrier protein (without a linker).

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

- 1) Amino group (for instance via lysine). In one embodiment this group is linked to carboxyl groups on saccharides directly or to a carboxyl group on a linker with carbodiimide chemistry e.g. with EDAC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide). In another embodiment this group is linked to hydroxyl groups activated with CDAP or CNBr on saccharides directly or to such groups on a linker; to saccharides or linkers having an aldehyde group; to saccharides or linkers having a succinimide ester group.
- 2) Carboxyl (for instance via aspartic acid or glutamic acid). In one embodiment this group is linked to amino groups on saccharides directly or to an amino group on a linker with carbodiimide chemistry e.g. with EDAC.
- 3) Sulphydryl (for instance via cysteine). In one embodiment this group is linked to a bromo or chloro acetylated saccharide or linker with maleimide chemistry. In one embodiment this group is activated/modified with bis diazobenzidine.
- 4) Hydroxyl group (for instance via tyrosine). In one embodiment this group is activated/modified with bis diazobenzidine.
- 5) Imidazolyl group (for instance via histidine). In one embodiment this group is activated/modified with bis diazobenzidine.
- 6) Guanidyl group (for instance via arginine).
- 7) Indolyl group (for instance via tryptophan).

On the pneumococcal 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.

In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention are prepared using CDAP chemistry. In said embodiment, the pneumococcal saccharide is activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated saccharide can then 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 can be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using N-[γ-maleimidobutyroxy]succinimide ester (GMBS)) or a haloacetylated carrier protein (for example using iodoacetamide, N-succinimidyl bromoacetate (SBA; SIB), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB), N-succinimidyl iodoacetate (SIA), or succinimidyl 3-[bromoacetamido]propionate (SBAP)).

In a preferred embodiment, the cyanate ester of the activated saccharide is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO 93/15760, WO 95/08348 and WO 96/129094.

In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention are prepared using carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may
 5 be formed by reaction of a free hydroxyl group of the saccharide with CDI (see Bethell et al. (1979) 1. Biol. Chern. 254:2572-2574; Hearn et al. (1981) J. Chromatogr. 218:509-518) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and
 10 coupling the CDI carbamate intermediate with an amino group on a protein.

Direct reductive amination

In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention is prepared by direct reductive amination (see e.g. US 4365170, US 4673574,
 15 WO2006/110381, WO2008/079653, WO2008/143709, WO2008/079732, WO2011/110531, WO2012/119972, WO2015110941, WO2015110940, WO2018/144439, WO2018/156491). According to the present invention, reductive amination involves two steps, (1) oxidation (activation) of the purified pneumococcal saccharide, (2) reduction of the activated saccharide and the carrier protein (e.g., CRM₁₉₇, TT or SCP) to form a glycoconjugate.
 20 As mentioned above, before oxidation, sizing of the pneumococcal saccharide to a target molecular weight (MW) range can be performed. Therefore, in an embodiment, the isolated polysaccharide is sized before oxidation.

In an embodiment, the pneumococcal saccharide of the invention is conjugated to a carrier protein by a process comprising the step of:
 25 (a) reacting said pneumococcal saccharide with an oxidizing agent;
 (b) compounding the activated saccharide of step (a) with a carrier protein; and
 (c) reacting the compounded activated saccharide and carrier protein with a reducing agent to form a glycoconjugate.

In an embodiment, the pneumococcal saccharide of the invention is conjugated to a carrier
 30 protein by a process comprising the step of:
 (a) reacting said pneumococcal saccharide with an oxidizing agent;
 (a') quenching the oxidation reaction by addition of a quenching agent;
 (b) compounding the activated saccharide of step (a') with a carrier protein; and
 (c) reacting the compounded activated saccharide and carrier protein with a reducing agent to
 35 form a glycoconjugate.

Following the oxidation step (a) the saccharide is said to be activated and is referred to as "activated saccharide".

In an embodiment, the oxidizing agent is any oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde. In an embodiment, the oxidizing agent is periodate. For the purpose of the present invention, 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 an embodiment, the oxidizing agent is periodate in the presence of bivalent cations (see WO2008/143709).

In an embodiment, the oxidizing agent is periodic acid. In an embodiment, the oxidizing agent is periodic acid in the presence of bivalent cations. In an embodiment, the oxidizing agent is periodic acid in the presence of Mg^{2+} . In an embodiment, the oxidizing agent is periodic acid in the presence of Ca^{2+} . In an embodiment, the oxidizing agent is orthoperiodate.

In a preferred embodiment, the oxidizing agent is sodium periodate. In an embodiment, the periodate used for the oxidation is metaperiodate. In an embodiment the periodate used for the oxidation is sodium metaperiodate.

When a polysaccharide reacts with periodate, periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. For this reason, the term "reacting a polysaccharide with periodate" includes oxidation of vicinal hydroxyl groups by periodate.

In one embodiment step a) comprises reacting the polysaccharide with 0.01-2 molar equivalents of periodate. In one embodiment step a) comprises reacting the polysaccharide with 0.1-1.0 molar equivalents of periodate. In one embodiment step a) comprises reacting the polysaccharide with 0.1-0.5 molar equivalents of periodate.

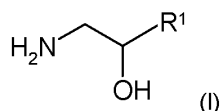
In an embodiment, the oxidizing agent is a mixture of a stable nitroxyl radical compound with an oxidant (see WO2014097099).

In an aspect, said stable nitroxyl radical compound is a molecule bearing a TEMPO or a PROXYL (2,2,5,5-tetramethyl-1-pyrrolidinyloxy) moiety. Preferably said molecule has the ability to selectively oxidize primary alcohol in the presence of an oxidant, to generate aldehyde groups, without affecting secondary hydroxyl groups. More preferably said molecule has the ability to selectively oxidize primary alcohol in the presence of an oxidant, to generate aldehyde groups, without over oxidation to carboxyl groups. In an aspect, said stable nitroxyl radical compound is TEMPO, 2,2,6,6-Tetramethyl-4-(methylsulfonyloxy)-1-piperidinoxy, 4-Phosphonoxy-TEMPO, 4-Oxo-TEMPO, 4-Methoxy-TEMPO, 4-Isothiocyanato-TEMPO, 4-(2-Iodoacetamido)-TEMPO free radical, 4-Hydroxy-TEMPO, 4-Cyano-TEMPO, 4-Carboxy-TEMPO, 4-(2-Bromoacetamido)-TEMPO or 4-Amino-TEMPO, 4-Acetamido-2,2,6,6-tetramethylpiperidine 1-oxyl. Preferably said stable nitroxyl radical compound is TEMPO. In an aspect, said stable nitroxyl radical compound is selected from the groups consisting of TEMPO, 2,2,6,6-Tetramethyl-4-(methylsulfonyloxy)-1-piperidinoxy, 4-Phosphonoxy-TEMPO, 4-Oxo-TEMPO, 4-Methoxy-TEMPO, 4-Isothiocyanato-TEMPO, 4-(2-Iodoacetamido)-TEMPO free radical, 4-Hydroxy-TEMPO, 4-Cyano-TEMPO, 4-

- Carboxy-TEMPO, 4-(2-Bromoacetamido)-TEMPO, 4-Amino-TEMPO, 4-Acetamido-2,2,6,6-tetramethylpiperidine 1-oxyl. Preferably said stable nitroxyl radical compound is TEMPO. In a further aspect, said stable nitroxyl radical compound is 3 β -DOXYL-5 α -cholestane, 5-DOXYL-stearic acid, 16-DOXYL-stearic acid, Methyl 5-DOXYL-stearate, 3-(Aminomethyl)-PROXYL, 3-Carbamoyl-PROXYL, 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl, 3-Carboxy-PROXYL or 3-Cyano-PROXYL. In a further aspect, said stable nitroxyl radical compound is selected from the groups consisting of 3 β -DOXYL-5 α -cholestane, 5-DOXYL-stearic acid, 16-DOXYL-stearic acid, Methyl 5-DOXYL-stearate, 3-(Aminomethyl)-PROXYL, 3-Carbamoyl-PROXYL, 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl, 3-Carboxy-PROXYL, 3-Cyano-PROXYL.
- 10 In an aspect, the oxidant is a molecule bearing a N-halo moiety. Preferably said molecule has the ability to selectively oxidize primary alcohol in the presence of a nitroxyl radical compound. In an aspect, said oxidant is N-Chlorosuccinimide, N-Bromosuccinimide, N-Iodosuccinimide, Dichloroisocyanuric acid, 1,3,5-trichloro-1,3,5-triazinane-2,4,6-trione, Dibromoisocyanuric acid, 1,3,5-tribromo-1,3,5-triazinane-2,4,6-trione, Diiodoisocyanuric acid or 1,3,5-triiodo-1,3,5-triazinane-2,4,6-trione. In an aspect, said oxidant is selected from the group consisting of N-Chlorosuccinimide, N-Bromosuccinimide, N-Iodosuccinimide, Dichloroisocyanuric acid, 1,3,5-trichloro-1,3,5-triazinane-2,4,6-trione, Dibromoisocyanuric acid, 1,3,5-tribromo-1,3,5-triazinane-2,4,6-trione, Diiodoisocyanuric acid and 1,3,5-triiodo-1,3,5-triazinane-2,4,6-trione. Preferably said oxidant is N-Chlorosuccinimide.
- 20 In an aspect, said stable nitroxyl radical compound is 2,2,6,6-Tetramethyl-1-piperidinyloxy free radical (TEMPO) and said oxidant is N-Chlorosuccinimide (NCS).

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

- 25 In one embodiment, the quenching agent is a 1,2-aminoalcohols of formula (I):



wherein R¹ is selected from H, methyl, ethyl, propyl or isopropyl.

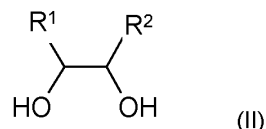
- In one embodiment, the quenching agent is selected from sodium and potassium salts of sulfite, bisulfate, dithionite, metabisulfite, thiosulfate, phosphites, hypophosphites or phosphorous acid.
- 30

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

- In one embodiment, the quenching agent is a sulfite such as bisulfate, dithionite, metabisulfite, thiosulfate.
- 35

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

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



- 5 wherein R¹ and R² are each independently selected from H, methyl, ethyl, propyl or isopropyl. In a preferred embodiment, the quenching agent is glycerol, ethylene glycol, propan-1,2-diol, butan-1,2-diol or butan-2,3-diol, or ascorbic acid. In an even preferred embodiment, the quenching agent is butan-2,3-diol.

- 10 In a preferred embodiment the degree of oxidation (also named "degree of activation" in the present document) of the activated pneumococcal saccharide is between 2 and 30. In an embodiment the degree of oxidation (DO) of the activated pneumococcal polysaccharide is between 10 and 25.

In one embodiment the activated saccharide and the carrier protein are lyophilised before step b).

- 15 In an embodiment the initial input ratio (weight by weight) of activated pneumococcal saccharide to carrier protein at step b) is between 4:1 and 0.1:1. In an embodiment the initial input ratio (weight by weight) of activated pneumococcal saccharide to carrier protein at step b) is between 1.5:1 and 0.5:1.

- 20 In an embodiment, the reduction reaction (c) is carried out in aqueous solvent. In another embodiment, the reduction reaction (c) is carried out in aprotic solvent.

In an embodiment, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO) or dimethylformamide (DMF). In an embodiment, the reduction reaction (c) is carried out in the presence of dimethylformamide (DMF). In an embodiment, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO).

- 25 In one embodiment the reduction reaction (c) is carried out in a solution consisting essentially of dimethylsulphoxide (DMSO) or dimethylformamide (DMF). In one embodiment the reduction reaction (c) is carried out in a solution consisting essentially of dimethylformamide (DMF). In one embodiment the reduction reaction (c) is carried out in a solution consisting essentially of dimethylsulphoxide (DMSO).

- 30 In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) or in DMF (dimethylformamide) solvent. In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent.

- 35 In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxyborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMeⁱPrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane

(PEMB). In an embodiment, the reducing agent is sodium triacetoxyborohydride. In a preferred embodiment, the reducing agent is sodium cyanoborohydride. In an embodiment, the reducing agent is sodium cyanoborohydride in the presence of nickel (see WO2018144439).

5 In one embodiment between 0.2 and 20 molar equivalents of reducing agent is used at step c). In one embodiment between 0.5 and 10 molar equivalents of reducing agent is used at step c). In one embodiment between 1.0 and 5 molar equivalents of reducing agent is used at step c).

At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this
10 capping agent is sodium borohydride (NaBH₄). In an embodiment capping is achieved by mixing the product of step c) with 1 to 20 molar equivalents of sodium borohydride. In an embodiment capping is achieved by mixing the product of step c) with 1 to 10 molar equivalents of sodium borohydride. In an embodiment capping is achieved by mixing the product of step c) with 1 to 5 molar equivalents of sodium borohydride.

15

CDI and/or CDT chemistry

In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention are prepared by CDI and/or CDT chemistry as disclosed in WO2022249107.

CDI and/or CDT chemistry involves two steps, (1) reacting the pneumococcal saccharide
20 with CDI and/or CDT in an aprotic solvent to produce an activated saccharide (activation), (2) reacting the activated saccharide with a carrier protein (e.g. CRM₁₉₇, TT or SCP) to form a glycoconjugate.

In an embodiment, the activating agent of step (1) is 1,1'-carbonyldiimidazole (CDI). In an embodiment, the activating agent of step (1) is 1,1'-Carbonyl-di-(1,2,4-triazole) (CDT).

25 As mentioned above, before activation with CDI and/or CDT, sizing of the pneumococcal saccharide to a target molecular weight (MW) range can be performed.

Therefore, in an embodiment, the pneumococcal saccharide is sized before activation with CDI. In an embodiment, the isolated polysaccharide is sized before activation with CDT. In an embodiment, the pneumococcal saccharide is sized to any of the target molecular weight (MW)
30 range defined above.

Therefore, in an embodiment, the pneumococcal saccharide is conjugated to a carrier protein by a process comprising the step of:

(a) reacting said isolated polysaccharide with CDI and/or CDT in an aprotic solvent;
(b) reacting the activated polysaccharide of step (a) with a carrier protein in an aprotic solvent to
35 form a glycoconjugate.

Following step (a) the polysaccharide is said to be activated and is referred to as "activated polysaccharide".

In one embodiment step a) comprises reacting the pneumococcal saccharide with CDI.

In one embodiment step a) comprises reacting the pneumococcal saccharide with an amount of CDI that is between 0.5-10 molar equivalent to the amount of pneumococcal saccharide present in the reaction mixture.

In one embodiment step a) comprises reacting the pneumococcal saccharide with CDT.

5 In one embodiment step a) comprises reacting the pneumococcal saccharide with an amount of CDT that is between 0.5-10 molar equivalent to the amount of pneumococcal saccharide present in the reaction mixture.

10 In an embodiment, the activating reaction a) is carried out in the presence of dimethylsulphoxide (DMSO), dimethylformamide (DMF), dimethylacetamide, N-methyl-2-pyrrolidone or hexamethylphosphoramide (HMPA). In an embodiment, the activating reaction a) is carried out in the presence of dimethylsulphoxide (DMSO).

15 In one embodiment the activating reaction a) is carried out in a solution consisting essentially of dimethylsulphoxide (DMSO) or dimethylformamide (DMF). In one embodiment the activating reaction a) is carried out in a solution consisting essentially of dimethylsulphoxide (DMSO).

In an embodiment, the conjugation reaction b) is carried out in the presence of dimethylsulphoxide (DMSO), dimethylformamide (DMF), dimethylacetamide, N-methyl-2-pyrrolidone or hexamethylphosphoramide (HMPA).

20 In an embodiment, the conjugation reaction b) is carried out in the presence of dimethylsulphoxide (DMSO).

In one embodiment the conjugation reaction b) is carried out in a solution consisting essentially of dimethylsulphoxide (DMSO) or dimethylformamide (DMF). In one embodiment the conjugation reaction b) is carried out in a solution consisting essentially of dimethylsulphoxide (DMSO).

25 In one embodiment, weak organic base can be added to the reaction mixture after the activating reaction a) but before the conjugation reaction b). The weak organic base can be added before or after the carrier protein is introduced to the reaction mixture. Therefore, in one embodiment, the weak organic base is added to the reaction mixture before the carrier protein is introduced. In another embodiment, the weak organic base is added to the reaction mixture after
30 the carrier protein is introduced. Weak organic base can be selected from alkanamines, imidazole, triazole, pyridine, histidine and guanidine. Alkanamines include alkyl primary amines such as methyl amine, ethylamine, propylamine, isopropylamine; alkyl secondary amines such as dimethyl amine, diethylamine, dipropylamine, diisopropylamine; alkyl tertiary amines such as trimethyl amine, triethylamine, tri-isopropylamine, di-N,N'-isopropylethylamine, et al. In an
35 embodiment, the weak organic base is an alkanamine. In an embodiment, the weak organic base is an imidazole. In an embodiment, the weak organic base is a triazole. In an embodiment, the weak organic base is pyridine. In an embodiment, the weak organic base is histidine. In an embodiment, the weak organic base is guanidine.

In one embodiment following the conjugation reaction b) unconjugated reactive sites of the activated polysaccharide are hydrolyzed. In one embodiment unconjugated reactive sites are hydrolyzed by addition to the conjugation solution of an aqueous solution. In one embodiment unconjugated reactive sites are hydrolyzed by addition to the conjugation solution of an aqueous buffered solution. In one embodiment unconjugated reactive sites are hydrolyzed by addition to the conjugation solution of an aqueous buffered solution and adjustment of the pH to between about 3.0 to about 10.0. In one embodiment unconjugated reactive sites are hydrolyzed by addition to the conjugation solution of an aqueous buffered solution and adjustment of the pH to between about 7.0 to about 10.0. In one embodiment unconjugated reactive sites are hydrolyzed by addition to the conjugation solution of an aqueous buffered solution and adjustment of the pH to between about 3.0 to about 7.0. In one embodiment unconjugated reactive sites are hydrolyzed by addition to the conjugation solution of an aqueous buffered solution and adjustment of the pH to about 4.0. In one embodiment unconjugated reactive sites are hydrolyzed by addition to the conjugation solution of an aqueous buffered solution and adjustment of the pH to about 9.0.

15

eTEC chemistry

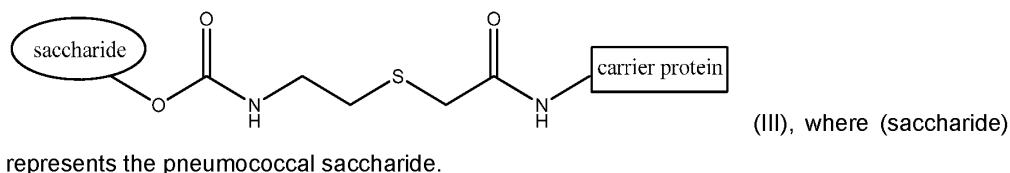
In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention is prepared by eTEC chemistry as disclosed WO2014027302

The eTEC spacer includes seven linear atoms (i.e., $-\text{C}(\text{O})\text{NH}(\text{CH}_2)_2\text{SCH}_2\text{C}(\text{O})-$) and provides stable thioether and amide bonds between the saccharide and carrier protein. Synthesis of the eTEC linked glycoconjugate involves reaction of an activated hydroxyl group of the saccharide with the amino group of a thioalkylamine reagent, e.g., cystamine or cysteamine or a salt thereof, forming a carbamate linkage to the saccharide to provide a thiolated saccharide. Generation of one or more free sulfhydryl groups is accomplished by reaction with a reducing agent to provide an activated thiolated saccharide. Reaction of the free sulfhydryl groups of the activated thiolated saccharide with an activated carrier protein having one or more α -haloacetamide groups on amine containing residues generates a thioether bond to form the conjugate, wherein the carrier protein is attached to the eTEC spacer through an amide bond.

Therefore, in an embodiment, the pneumococcal glycoconjugate of the present invention comprises a pneumococcal saccharide covalently conjugated to a carrier protein through a (2-((2-oxoethyl)thio)ethyl)carbamate (eTEC) spacer.

In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention comprises a pneumococcal saccharide conjugated to a carrier protein through a (2-((2-oxoethyl)thio)ethyl)carbamate (eTEC) spacer, wherein the saccharide is covalently linked to the eTEC spacer through a carbamate linkage, and wherein the carrier protein is covalently linked to the eTEC spacer through an amide linkage.

The eTEC linked glycoconjugates of the invention may be represented by the general formula (III):



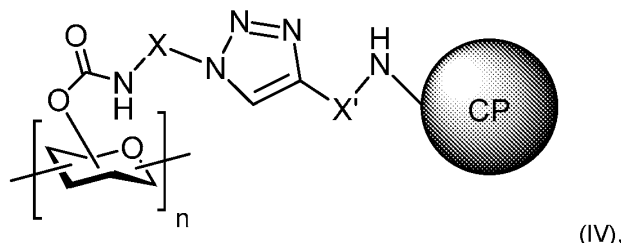
represents the pneumococcal saccharide.

- 5 Formula (III) is a schematic representation of glycoconjugates of the invention. It should not be understood that only one linkage is present between the saccharide and the carrier protein. Rather, an individual carrier protein molecule may be linked to more than one pneumococcal saccharide molecule and an individual saccharide molecule can be linked to more than one individual carrier protein molecule. Additionally, a majority of the saccharide repeating unit
- 10 remains unmodified and covalent linkages between the carrier protein and the saccharide is for a minority of the saccharide repeat units.

Click chemistry

- 15 In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention is prepared by click chemistry (see e.g. PCT/IB2023/050202).

Therefore, in an embodiment, part or all of the pneumococcal glycoconjugates of the present invention comprises a pneumococcal saccharide covalently conjugated to a carrier protein (CP) through a spacer and having the general formula (IV):

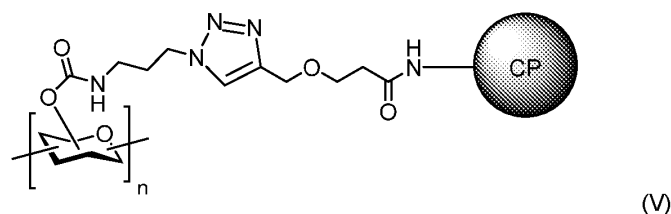


- 20 wherein X is selected from the group consisting of $\text{CH}_2(\text{CH}_2)_{n'}$, $(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{NHCO}(\text{CH}_2)_{n'}$, $\text{NHCO}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{OCH}_2(\text{CH}_2)_{n'}$ and $\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$; where n' is selected from 1 to 10 and m is selected from 1 to 4,
- wherein X' is selected from the group consisting of $\text{CH}_2\text{O}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$, $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_{m'}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10 and m' is selected from 0
- 25 to 4,
- wherein the structure in square bracket represents a repeat unit of the pneumococcal saccharide and wherein n represents the number of repeating units.

In a particular aspect, the invention is directed to a pneumococcal glycoconjugate comprising a pneumococcal saccharide covalently conjugated to a carrier protein (CP) through a

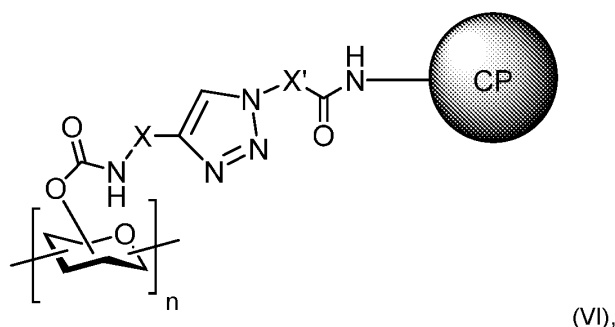
spacer and having the general formula (IV), wherein X is $\text{CH}_2(\text{CH}_2)_{n'}$, where n' is 2 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$ where n'' is 1.

- In a particular aspect, the invention pertains to a pneumococcal glycoconjugate comprising a pneumococcal saccharide covalently conjugated to a carrier protein (CP) through a spacer and having the general formula (V),



wherein the structure in square bracket represents a repeat unit of the pneumococcal saccharide and wherein n represents the number of repeating units.

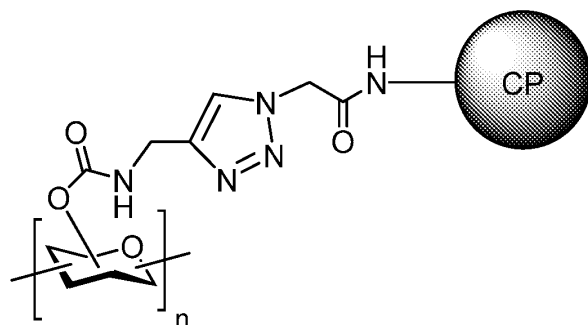
- In an aspect, part or all of the pneumococcal glycoconjugates of the present invention comprises a pneumococcal saccharide covalently conjugated to a carrier protein (CP) through a spacer and having the general formula (VI):



- wherein X is selected from the group consisting of $\text{CH}_2(\text{CH}_2)_{n'}$, $(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{NHCO}(\text{CH}_2)_{n'}$, $\text{NHCO}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{OCH}_2(\text{CH}_2)_{n'}$ and $\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$; where n' is selected from 0 to 10 and m is selected from 1 to 4,
- wherein X' is selected from the group consisting of $\text{CH}_2(\text{CH}_2)_{n''}$, $\text{CH}_2\text{O}(\text{CH}_2)_{n''}\text{CH}_2$, $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_{m'}(\text{CH}_2)_{n''}\text{CH}_2$, where n'' is selected from 0 to 10 and m' is selected from 0 to 4 and wherein the structure in square bracket represents a repeat unit of the pneumococcal saccharide and wherein n represents the number of repeating units.

In a particular aspect, the invention is directed to a pneumococcal glycoconjugate comprising a pneumococcal saccharide covalently conjugated to a carrier protein (CP) through a spacer and having the general formula (VI), wherein X is $\text{CH}_2(\text{CH}_2)_{n'}$, where n' is 0 and wherein X' is $\text{CH}_2(\text{CH}_2)_{n''}$ where n'' is 0.

In a particular aspect, the invention pertains to a pneumococcal glycoconjugate comprising a pneumococcal saccharide covalently conjugated to a carrier protein (CP) through a spacer and having the general formula (VII),



(VII)

- 5 wherein the structure in square bracket represents a repeat unit of the pneumococcal saccharide and wherein n represents the number of repeating units.

Formulas (IV), (V), (VI) and (VII) are schematic representations of glycoconjugates of the invention. It should not be understood that a linkage is present at every repeating unit of the
 10 saccharide (the structure in square brackets). Rather, a majority of the saccharide repeating unit remains unmodified and covalent linkages between the carrier protein and the saccharide is for a minority of the saccharide repeat units. Additionally, an individual carrier protein (CP) molecule may be linked to more than one saccharide molecule and an individual saccharide molecule can be linked to more than one individual carrier protein (CP) molecule. The structure in square
 15 brackets represents a repeat unit of the pneumococcal saccharide.

Following conjugation to the carrier protein, the pneumococcal glycoconjugates of the invention can be purified (enriched with respect to the amount of saccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis,
 20 concentration/diafiltration operations, tangential flow filtration precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. Therefore, in one embodiment the process for producing the glycoconjugate of the present invention comprises the step of purifying the glycoconjugate after it is produced.

25 1.4 *S. pneumoniae* serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 15B, 14, 18C, 19A, 19F, 22F, 23F and 33F glycoconjugates of the invention

In an aspect the invention relates to compositions comprising *S. pneumoniae* serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F glycoconjugate(s).

The structures of *Streptococcus pneumoniae* serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F polysaccharides are known in the art (see e.g. Geno K *et al.* (2015) Clin Microbiol Rev Vol 28:3, p 871-899).

5 In an embodiment, the *S. pneumoniae* serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular saccharide used in the present invention is a synthetic carbohydrate. Preparation of a synthetic *S. pneumoniae* type 1 capsular saccharide can for example be conducted as disclosed in WO2015004041. Preparation of a synthetic *S. pneumoniae* type 4 capsular saccharide can for example be conducted as disclosed in WO2016091399. Preparation of a synthetic *S. pneumoniae* type 5 capsular saccharide can for
10 example be conducted as disclosed in WO2016198170. Preparation of a synthetic *S. pneumoniae* type 8 capsular saccharide can for example be conducted as disclosed in WO2017220753.

In a preferred embodiment though, the source of bacterial polysaccharides according to this invention can be *S. pneumoniae* bacterial cells. Bacterial strains which can be used as source
15 of *S. pneumoniae* capsular polysaccharides may be obtained from established culture collections (such as for example from the Streptococcal Reference Laboratory (Centers for Disease Control and Prevention, Atlanta, GA USA)) or clinical specimens.

S. pneumoniae serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharides can be obtained directly from bacteria using
20 isolation procedures known to one of ordinary skill in the art. They can also be purchased (such as for example from the American Type Culture Collection (ATCC, Manassas, VA USA) (e.g., reference No. ATCC 13-X, ATCC 36-X, ATCC 41-X, ATCC 280-X, ATCC 107-X, ATCC 284-X, ATCC 505-X, ATCC 331-X, ATCC 511-X, ATCC 514-X, ATCC 517-X, ATCC 520-X, ATCC 81-X, ATCC 289-X, ATCC 304-X, ATCC 101-X, ATCC 527-X, ATCC 104-X, ATCC 535-X)).

25 In case the capsular polysaccharide is obtained directly from bacteria, the bacterial cells can be grown in a medium, preferably in a soy based medium. Following fermentation of bacterial cells that produce *S. pneumoniae* capsular polysaccharides, the bacterial cells can be lysed to produce a cell lysate. The capsular polysaccharides may then be isolated from the cell lysate using purification techniques known in the art, including the use of centrifugation, depth filtration,
30 precipitation, ultra-filtration, treatment with activate carbon, diafiltration and/or column chromatography (see, for example, US2006/0228380, US2006/0228381, WO2008/118752 and WO2020170190). The purified capsular polysaccharide can then be used for the preparation of glycoconjugates.

The isolated capsular saccharides can be characterized by different parameters including,
35 for example the weight average molecular weight (Mw).

The molecular weight of the capsular saccharides can be measured by Size Exclusion Chromatography (SEC) combined with Multiangle Laser Light Scattering detector (MALLS).

In a preferred embodiment, the isolated capsular polysaccharides (i.e. purified before further treatment) have a weight average molecular weight between 5 kDa and 5,000 kDa. In an embodiment, the isolated capsular polysaccharide has a weight average molecular weight between 10 kDa and 3,000 kDa. In an embodiment, the isolated capsular polysaccharide has a weight average molecular weight between 50 kDa and 1,000 kDa.

Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In order to generate conjugates with advantageous filterability characteristics, immunogenicity and/or yields, sizing of the capsular polysaccharide to a target molecular weight range can be performed prior to the conjugation to a carrier protein. Advantageously, the size of the purified serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide is reduced while preserving critical features of the structure of the polysaccharide. Mechanical or chemical sizing maybe employed (see e.g. WO2006/110381, WO2015110941).

In an embodiment, the size of the purified capsular polysaccharide is reduced by chemical hydrolysis. Chemical hydrolysis maybe conducted using a mild acid (e.g. acetic acid, formic acid, propanoic acid). Chemical hydrolysis may also be conducted using a diluted strong acid (such as diluted hydrochloric acid, diluted sulfuric acid, diluted phosphoric acid, diluted nitric acid or diluted perchloric acid).

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

In an embodiment, the isolated serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide is not sized.

The isolated serotype 1 capsular polysaccharide may be de-O-acetylated (see e.g. WO 2008/079653). Therefore, in an embodiment, the isolated serotype 1 capsular polysaccharide is partially de-O-acetylated. In an embodiment, de-O-acetylation is conducted using a mild base. The partial de-O-acetylation can be performed using sodium bicarbonate/carbonate buffer.

In an embodiment, the isolated serotype 1 capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 1 capsular polysaccharide before conjugation has a weight average molecular weight between 150 kDa and 900 kDa. In a preferred embodiment, the isolated serotype 1 capsular polysaccharide before conjugation has a weight average molecular weight between 150 kDa and 700 kDa. The weight average molecular weight (Mw) of the isolated saccharide before

conjugation refers to the Mw before activation of the polysaccharide (i.e. after an eventual sizing step but before reacting the polysaccharide with an activating agent).

In an embodiment, the isolated serotype 4 capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 4 capsular polysaccharide before conjugation has a weight average molecular weight between 300 kDa and 900 kDa.

In an embodiment, the isolated serotype 5 capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1,000 kDa. In an embodiment, the isolated serotype 5 capsular polysaccharide before conjugation has a weight average molecular weight between 200 kDa and 600 kDa.

In an embodiment, the isolated serotype 6A capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 6A capsular polysaccharide before conjugation has a weight average molecular weight between 300 kDa and 900 kDa.

In an embodiment, the isolated serotype 6B capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 6B capsular polysaccharide before conjugation has a weight average molecular weight between 200 kDa and 900 kDa.

In an embodiment, the isolated serotype 7F capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 7F capsular polysaccharide before conjugation has a weight average molecular weight between 200 kDa and 900 kDa.

In an embodiment, the isolated serotype 8 capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 8 capsular polysaccharide before conjugation has a weight average molecular weight between 200 kDa and 400 kDa.

In an embodiment, the isolated serotype 9V capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 9V capsular polysaccharide before conjugation has a weight average molecular weight between 200 kDa and 900 kDa.

In an embodiment, the isolated serotype 10A capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 10A capsular polysaccharide before conjugation has a weight average molecular weight between 200 kDa and 900 kDa.

In an embodiment, the isolated serotype 11A capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 11A capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 400 kDa.

In an embodiment, the isolated serotype 12F capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 12F capsular polysaccharide before conjugation has a weight average molecular weight between 150 kDa and 400 kDa.

5 In an embodiment, the isolated serotype 14 capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 14 capsular polysaccharide before conjugation has a weight average molecular weight between 200 kDa and 900 kDa.

10 In an embodiment, the isolated serotype 15B capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 15B capsular polysaccharide before conjugation has a weight average molecular weight between 150 kDa and 300 kDa.

15 In an embodiment, the isolated serotype 18C capsular polysaccharide before conjugation has a weight average molecular weight between 20 kDa and 1000 kDa. In an embodiment, the isolated serotype 18C capsular polysaccharide before conjugation has a weight average molecular weight between 20 kDa and 500 kDa.

20 In an embodiment, the isolated serotype 19A capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 19A capsular polysaccharide before conjugation has a weight average molecular weight between 250 kDa and 700 kDa.

In an embodiment, the isolated serotype 19F capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 19F capsular polysaccharide before conjugation has a weight average molecular weight between 250 kDa and 800 kDa.

25 In an embodiment, the isolated serotype 22F capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 22F capsular polysaccharide before conjugation has a weight average molecular weight between 400 kDa and 700 kDa.

30 In an embodiment, the isolated serotype 23F capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 1000 kDa. In an embodiment, the isolated serotype 23F capsular polysaccharide before conjugation has a weight average molecular weight between 200 kDa and 800 kDa.

35 In an embodiment, the isolated serotype 33F capsular polysaccharide before conjugation has a weight average molecular weight between 300 kDa and 2000 kDa. In an embodiment, the isolated serotype 33F capsular polysaccharide before conjugation has a weight average molecular weight between 500 kDa and 2000 kDa.

In an embodiment, the serotype 1 glycoconjugate of the present invention comprises a serotype 1 capsular polysaccharide wherein the weight average molecular weight (Mw) of said

polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 200 kDa and 750 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 250 kDa and 600 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 4 glycoconjugate of the present invention comprises a serotype 4 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 200 kDa and 1,000 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 400 kDa and 900 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 5 glycoconjugate of the present invention comprises a serotype 5 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 100 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 150 kDa and 800 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 200 kDa and 500 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 6A glycoconjugate of the present invention comprises a serotype 6A capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 200 kDa and 1,000 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 300 kDa and 800 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 6B glycoconjugate of the present invention comprises a serotype 6B capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 200 kDa and 1,000 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 300 kDa and 800 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 7F glycoconjugate of the present invention comprises a serotype 7F capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 200 kDa and 1,000 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 300 kDa and 800 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 8 glycoconjugate of the present invention comprises a serotype 8 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 200 kDa and 800 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 300 kDa and 600 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 200 kDa and 400 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 9V glycoconjugate of the present invention comprises a serotype 9V capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 200 kDa and 900 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 300 kDa and 600 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 100 kDa and 400 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 10A glycoconjugate of the present invention comprises a serotype 10A capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 200 kDa and 800 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 300 kDa and 600 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 100 kDa and 400 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 11A glycoconjugate of the present invention comprises a serotype 11A capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the

weight average molecular weight (Mw) is between 75 kDa and 600 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 100 kDa and 400 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 12F glycoconjugate of the present invention comprises a serotype 12F capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 100 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 150 kDa and 600 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 150 kDa and 400 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 250 kDa and 350 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 14 glycoconjugate of the present invention comprises a serotype 14 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 100 kDa and 800 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 200 kDa and 600 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 15B glycoconjugate of the present invention comprises a serotype 15B capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 100 kDa and 600 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 150 kDa and 300 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 18C glycoconjugate of the present invention comprises a serotype 18C capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 20 kDa and 800 kDa. In an embodiment, the weight average molecular weight (Mw) is between 20 kDa and 400 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 20 kDa and 200 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 19A glycoconjugate of the present invention comprises a serotype 19A capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 100 kDa and 700 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 250 kDa and 500 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 19F glycoconjugate of the present invention comprises a serotype 19F capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 100 kDa and 900 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 250 kDa and 600 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 22F glycoconjugate of the present invention comprises a serotype 22F capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 100 kDa and 900 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 400 kDa and 700 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 23F glycoconjugate of the present invention comprises a serotype 23F capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. In an embodiment, the weight average molecular weight (Mw) is between 100 kDa and 900 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 200 kDa and 600 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In an embodiment, the serotype 33F glycoconjugate of the present invention comprises a serotype 33F capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 2,500 kDa. In an embodiment, the weight average molecular weight (Mw) is between 100 kDa and 2,000 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 600 kDa and 2,000 kDa. Where the weight average molecular weight (Mw) before conjugation refers to the Mw after

activation of the polysaccharide (i.e. after an eventual sizing step and after reacting the polysaccharide with an activating agent).

In some embodiments, the serotype 1 glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 1 glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 1 glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,000 kDa.

In some embodiments, the serotype 4 glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 4 glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 4 glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,000 kDa.

In some embodiments, the serotype 5 glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 5 glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 5 glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,000 kDa.

In some embodiments, the serotype 6A glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 6A glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 6A glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,000 kDa.

In some embodiments, the serotype 6B glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 6B glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 6B glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,500 kDa.

In some embodiments, the serotype 7F glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 7F glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 7F glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,500 kDa.

In some embodiments, the serotype 8 glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 8 glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 8 glycoconjugate has a weight average molecular weight (Mw) of between 2,500 kDa and 8,000 kDa.

In some embodiments, the serotype 9V glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 9V glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 9V glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,500 kDa.

In some embodiments, the serotype 10A glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 10A glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 10A glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,500 kDa.

In some embodiments, the serotype 11A glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 11A glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 11A glycoconjugate has a weight average molecular weight (Mw) of between 700 kDa and 4,500 kDa.

In some embodiments, the serotype 12F glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 12F glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 12F glycoconjugate has a weight average molecular weight (Mw) of between 1,500 kDa and 4,000 kDa.

In some embodiments, the serotype 14 glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 14 glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. In preferred embodiments, the serotype 14 glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,500 kDa.

In some embodiments, the serotype 15B glycoconjugate of the invention has a weight average molecular weight (Mw) of between 1,000 kDa and 25,000 kDa. In other embodiments, the serotype 15B glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 20,000 kDa. In preferred embodiments, the serotype 15B glycoconjugate has a weight average molecular weight (Mw) of between 5,000 kDa and 15,000 kDa.

In some embodiments, the serotype 18C glycoconjugate of the invention has a weight average molecular weight (Mw) of between 150 kDa and 15,000 kDa. In other embodiments, the serotype 18C glycoconjugate has a weight average molecular weight (Mw) of between 250 kDa and 7,000 kDa. In preferred embodiments, the serotype 18C glycoconjugate has a weight average molecular weight (Mw) of between 300 kDa and 4,000 kDa.

In some embodiments, the serotype 19A glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 19A glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa

and 10,000 kDa. In preferred embodiments, the serotype 19A glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 7,500 kDa.

In some embodiments, the serotype 19F glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 19F glycoconjugate has a weight average molecular weight (Mw) of between 750 kDa and 10,000 kDa. In preferred embodiments, the serotype 19F glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 7,500 kDa.

In some embodiments, the serotype 22F glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 10,000 kDa. In other embodiments, the serotype 22F glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 7,500 kDa. In preferred embodiments, the serotype 22F glycoconjugate has a weight average molecular weight (Mw) of between 2,500 kDa and 5,500 kDa.

In some embodiments, the serotype 23F glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 23F glycoconjugate has a weight average molecular weight (Mw) of between 750 kDa and 10,000 kDa. In preferred embodiments, the serotype 23F glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 7,500 kDa.

In some embodiments, the serotype 33F glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In other embodiments, the serotype 33F glycoconjugate has a weight average molecular weight (Mw) of between 750 kDa and 10,000 kDa. In preferred embodiments, the serotype 33F glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 6,000 kDa.

The glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein.

In some embodiments, the ratio of serotype 1 polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.6 and 2.0. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 4 polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.9 and 2.1. Even more preferably, the saccharide to carrier protein ratio (w/w) is between 1.0 and 1.9. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 5 polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 1.3 and 2.5. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 6A polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.7 and 1.6. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

5 In some embodiments, the ratio of serotype 6B polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.4 and 0.8. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

10 In some embodiments, the ratio of serotype 7F polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.7 and 1.5. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

15 In some embodiments, the ratio of serotype 8 polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.6 and 1.6. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

20 In some embodiments, the ratio of serotype 9V polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 1.2 and 2.3. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 10A polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.7 and 1.6. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

25 In some embodiments, the ratio of serotype 11A polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.9 and 1.5. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

30 In some embodiments, the ratio of serotype 12F polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.7 and 1.5. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

35 In some embodiments, the ratio of serotype 14 polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 1.4 and 2.6. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 15B polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio

(w/w) is between 0.6 and 1.6. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 18C polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.7 and 1.5. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 19A polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.4 and 0.9. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 19F polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.5 and 1.0. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 22F polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.8 and 1.2. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 23F polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.4 and 1.0. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

In some embodiments, the ratio of serotype 33F polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 1.0 and 2.2. In some such embodiments, the carrier protein is TT. Preferably, the carrier protein is CRM₁₉₇.

Another way to characterize the glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM₁₉₇, DT or TT) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent linkages to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered compared to the carrier protein starting material used to generate the conjugate materials.

In a preferred embodiment, the degree of conjugation of the serotype 1 glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 4 glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 5 glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 6A glycoconjugate of the invention is between 2 and 15.

5 In a preferred embodiment, the degree of conjugation of the serotype 6B glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 7F glycoconjugate of the invention is between 2 and 15.

10 In a preferred embodiment, the degree of conjugation of the serotype 8 glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 9V glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 10A glycoconjugate of the invention is between 2 and 15.

15 In a preferred embodiment, the degree of conjugation of the serotype 11A glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 12F glycoconjugate of the invention is between 2 and 15.

20 In a preferred embodiment, the degree of conjugation of the serotype 14 glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 15B glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 18C glycoconjugate of the invention is between 2 and 15.

25 In a preferred embodiment, the degree of conjugation of the serotype 19A glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 19F glycoconjugate of the invention is between 2 and 15.

30 In a preferred embodiment, the degree of conjugation of the serotype 22F glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 23F glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 33F glycoconjugate of the invention is between 2 and 15.

35 The serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F or 33F glycoconjugate of the invention and immunogenic compositions comprising said glycoconjugate(s) may contain free saccharide that is not covalently conjugated to the carrier protein but is nevertheless present in the glycoconjugate composition. The free saccharide may

be noncovalently associated with (i.e., noncovalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.

5 In an embodiment, the serotype 1 glycoconjugate of the invention comprises less than about 40% of free serotype 1 polysaccharide compared to the total amount of serotype 1 polysaccharide. In a preferred embodiment the serotype 1 glycoconjugate comprises less than about 20% of free serotype 1 polysaccharide compared to the total amount of serotype 1 polysaccharide.

10 In an embodiment, the serotype 1 glycoconjugate of the invention comprises less than about 40% of free serotype 1 polysaccharide compared to the total amount of serotype 1 polysaccharide. In a preferred embodiment the serotype 1 glycoconjugate comprises less than about 20% of free serotype 1 polysaccharide compared to the total amount of serotype 1 polysaccharide.

15 In an embodiment, the serotype 4 glycoconjugate of the invention comprises less than about 40% of free serotype 4 polysaccharide compared to the total amount of serotype 4 polysaccharide. In a preferred embodiment the serotype 4 glycoconjugate comprises less than about 30% of free serotype 4 polysaccharide compared to the total amount of serotype 4 polysaccharide.

20 In an embodiment, the serotype 5 glycoconjugate of the invention comprises less than about 45% of free serotype 5 polysaccharide compared to the total amount of serotype 5 polysaccharide. In a preferred embodiment the serotype 5 glycoconjugate comprises less than about 40% of free serotype 5 polysaccharide compared to the total amount of serotype 5 polysaccharide.

25 In an embodiment, the serotype 6A glycoconjugate of the invention comprises less than about 40% of free serotype 6A polysaccharide compared to the total amount of serotype 6A polysaccharide. In a preferred embodiment the serotype 6A glycoconjugate comprises less than about 30% of free serotype 6A polysaccharide compared to the total amount of serotype 6A polysaccharide.

30 In an embodiment, the serotype 6B glycoconjugate of the invention comprises less than about 30% of free serotype 6B polysaccharide compared to the total amount of serotype 6B polysaccharide. In a preferred embodiment the serotype 6B glycoconjugate comprises less than about 20% of free serotype 6B polysaccharide compared to the total amount of serotype 6B polysaccharide.

35 In an embodiment, the serotype 7F glycoconjugate of the invention comprises less than about 30% of free serotype 7F polysaccharide compared to the total amount of serotype 7F polysaccharide. In a preferred embodiment the serotype 7F glycoconjugate comprises less than about 20% of free serotype 7F polysaccharide compared to the total amount of serotype 7F polysaccharide.

In an embodiment, the serotype 8 glycoconjugate of the invention comprises less than about 30% of free serotype 8 polysaccharide compared to the total amount of serotype 8 polysaccharide. In a preferred embodiment the serotype 8 glycoconjugate comprises less than about 20% of free serotype 8 polysaccharide compared to the total amount of serotype 8 polysaccharide.

In an embodiment, the serotype 9V glycoconjugate of the invention comprises less than about 40% of free serotype 9V polysaccharide compared to the total amount of serotype 9V polysaccharide. In a preferred embodiment the serotype 9V glycoconjugate comprises less than about 35% of free serotype 9V polysaccharide compared to the total amount of serotype 9V polysaccharide.

In an embodiment, the serotype 10A glycoconjugate of the invention comprises less than about 40% of free serotype 10A polysaccharide compared to the total amount of serotype 10A polysaccharide. In a preferred embodiment the serotype 10A glycoconjugate comprises less than about 20% of free serotype 10A polysaccharide compared to the total amount of serotype 10A polysaccharide.

In an embodiment, the serotype 11A glycoconjugate of the invention comprises less than about 40% of free serotype 11A polysaccharide compared to the total amount of serotype 11A polysaccharide. In a preferred embodiment the serotype 11A glycoconjugate comprises less than about 30% of free serotype 11A polysaccharide compared to the total amount of serotype 11A polysaccharide.

In an embodiment, the serotype 12F glycoconjugate of the invention comprises less than about 40% of free serotype 12F polysaccharide compared to the total amount of serotype 12F polysaccharide. In a preferred embodiment the serotype 12F glycoconjugate comprises less than about 30% of free serotype 12F polysaccharide compared to the total amount of serotype 12F polysaccharide.

In an embodiment, the serotype 14 glycoconjugate of the invention comprises less than about 40% of free serotype 14 polysaccharide compared to the total amount of serotype 14 polysaccharide. In a preferred embodiment the serotype 14 glycoconjugate comprises less than about 35% of free serotype 14 polysaccharide compared to the total amount of serotype 14 polysaccharide.

In an embodiment, the serotype 15B glycoconjugate of the invention comprises less than about 40% of free serotype 15B polysaccharide compared to the total amount of serotype 15B polysaccharide. In a preferred embodiment the serotype 15B glycoconjugate comprises less than about 35% of free serotype 15B polysaccharide compared to the total amount of serotype 15B polysaccharide.

In an embodiment, the serotype 18C glycoconjugate of the invention comprises less than about 30% of free serotype 18C polysaccharide compared to the total amount of serotype 18C polysaccharide. In a preferred embodiment the serotype 18C glycoconjugate comprises less than

about 20% of free serotype 18C polysaccharide compared to the total amount of serotype 18C polysaccharide.

5 In an embodiment, the serotype 19A glycoconjugate of the invention comprises less than about 40% of free serotype 19A polysaccharide compared to the total amount of serotype 19A polysaccharide. In a preferred embodiment the serotype 19A glycoconjugate comprises less than about 30% of free serotype 19A polysaccharide compared to the total amount of serotype 19A polysaccharide.

10 In an embodiment, the serotype 19F glycoconjugate of the invention comprises less than about 30% of free serotype 19F polysaccharide compared to the total amount of serotype 19F polysaccharide. In a preferred embodiment the serotype 19F glycoconjugate comprises less than about 20% of free serotype 19F polysaccharide compared to the total amount of serotype 19F polysaccharide.

15 In an embodiment, the serotype 22F glycoconjugate of the invention comprises less than about 40% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide. In a preferred embodiment the serotype 22F glycoconjugate comprises less than about 20% of free serotype 22F polysaccharide compared to the total amount of serotype 22F polysaccharide.

20 In an embodiment, the serotype 23F glycoconjugate of the invention comprises less than about 30% of free serotype 23F polysaccharide compared to the total amount of serotype 23F polysaccharide. In a preferred embodiment the serotype 23F glycoconjugate comprises less than about 20% of free serotype 23F polysaccharide compared to the total amount of serotype 23F polysaccharide.

25 In an embodiment, the serotype 33F glycoconjugate of the invention comprises less than about 30% of free serotype 33F polysaccharide compared to the total amount of serotype 33F polysaccharide. In a preferred embodiment the serotype 33F glycoconjugate comprises less than about 20% of free serotype 33F polysaccharide compared to the total amount of serotype 33F polysaccharide.

30 The serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F or 33F glycoconjugate of the invention may also be characterized by their molecular size distribution (Kd). Size exclusion chromatography media (CL-4B) can be used to determine the relative molecular size distribution of the conjugate. Size Exclusion Chromatography (SEC) is used in gravity fed columns to profile the molecular size distribution of conjugates. Large molecules excluded from the pores in the media elute more quickly than small molecules. Fraction collectors are used to collect the column eluate. The fractions are tested colorimetrically
35 by saccharide assay. For the determination of Kd, columns are calibrated to establish the fraction at which molecules are fully excluded (V0), (Kd=0), and the fraction representing the maximum retention (Vi), (Kd=1). The fraction at which a specified sample attribute is reached (Ve), is related to Kd by the expression, $Kd = (Ve - V0) / (Vi - V0)$.

In an embodiment, at least 40% of the serotype 1 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 50% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 80% of the serotype 1 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

5 In an embodiment, at least 30% of the serotype 4 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 40% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 40% and 80% of the serotype 4 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

10 In an embodiment, at least 40% of the serotype 5 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 55% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 55% and 80% of the serotype 5 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

15 In an embodiment, at least 50% of the serotype 6A glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 60% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 60% and 90% of the serotype 6A glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

20 In an embodiment, at least 30% of the serotype 6B glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 35% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 35% and 80% of the serotype 6B glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 50% of the serotype 7F glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 65% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 65% and 90% of the serotype 7F glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

25 In an embodiment, at least 60% of the serotype 8 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 70% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 70% and 90% of the serotype 8 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

30 In an embodiment, at least 30% of the serotype 9V glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 40% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 40% and 80% of the serotype 9V glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

35 In an embodiment, at least 40% of the serotype 10A glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 55% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 55% and 85% of the serotype 10A glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 50% of the serotype 11A glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 60% of the glycoconjugate

has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 60% and 85% of the serotype 11A glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 40% of the serotype 12F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 50% of the glycoconjugate
5 has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 80% of the serotype 12F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 40% of the serotype 14 glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 50% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and
10 80% of the serotype 14 glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 30% of the serotype 15B glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 40% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 40% and
15 80% of the serotype 15B glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 30% of the serotype 18C glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 40% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 40% and
20 80% of the serotype 18C glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 40% of the serotype 19A glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 50% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and
25 80% of the serotype 19A glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 40% of the serotype 19F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 50% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and
30 80% of the serotype 19F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 30% of the serotype 22F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 40% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 40% and
35 80% of the serotype 22F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 30% of the serotype 23F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 35% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 35% and
40 80% of the serotype 23F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, at least 50% of the serotype 33F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 60% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 60% and
45 95% of the serotype 33F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, the serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F saccharide is activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide is then coupled directly or via a spacer (linker) group to an amino group on the carrier protein. For example, the
5 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 N-[γ-maleimidobutyroxy]succinimide ester (GMBS)) or a haloacetylated carrier protein (for example using iodoacetimide, N-succinimidyl bromoacetate (SBA; SIB), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl(4-
10 iodoacetyl)aminobenzoate (sulfo-SIAB), N-succinimidyl iodoacetate (SIA) or succinimidyl 3-[bromoacetamido]propionate (SBAP)). Preferably, the cyanate ester is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein (e.g., CRM₁₉₇) using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO 93/15760, WO
15 95/08348 and WO 96/129094.

Other suitable techniques for conjugation use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a
20 carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with 1,1'-carbonyldiimidazole (CDI) (see Bethell et al. (1979) J. Biol. Chem. 254:2572-2574; Hearn et al. (1981) J. Chromatogr. 218:509-518) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with
25 CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

In an embodiment, the serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F glycoconjugates of the invention are prepared using reductive amination chemistry. According to the present invention, reductive amination involves two steps,
30 (1) oxidation (activation) of a purified saccharide, (2) reduction of the activated saccharide and a carrier protein to form a glycoconjugate (see e.g. WO2006/110381, WO2008/079653, WO2008/143709, WO2008/079732, WO2011/110531, WO2012/119972, WO2015110941, WO2015110940, WO2018/144439, WO2018/156491).

In an embodiment, the serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F glycoconjugates of the invention are prepared using reductive
35 amination chemistry.

In another embodiment, the serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F and 23F glycoconjugates of the invention are prepared using reductive amination chemistry.

As mentioned above, before oxidation, sizing of the isolated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide to a target molecular weight (MW) range can be performed. Therefore, in an embodiment, the isolated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide is sized before oxidation.

Therefore, in an embodiment, the isolated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F capsular polysaccharide are sized before oxidation.

In an embodiment, the isolated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide is conjugated to a carrier protein by a process comprising the step of:

(a) reacting said isolated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide with an oxidizing agent to produce an activated polysaccharide; (b) compounding the activated polysaccharide of step (a) with a carrier protein; and (c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

Following the oxidation step (a) the saccharide is said to be activated and is referred to as "activated polysaccharide".

In an embodiment, the oxidizing agent is any oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde. In an embodiment, the oxidizing agent is periodate. For the purpose of the present invention, 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 an embodiment, the isolated serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F capsular polysaccharides are reacted with periodate.

In an embodiment, the isolated serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 19A, 19F, 22F and 23F capsular polysaccharides are reacted with periodate.

In a preferred embodiment, the oxidizing agent is sodium periodate. In an embodiment, the periodate used for the oxidation is metaperiodate. In a most preferred embodiment the periodate used for the oxidation is sodium metaperiodate.

When a polysaccharide reacts with periodate, periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. For this reason, the term "reacting a polysaccharide with periodate" includes oxidation of vicinal hydroxyl groups by periodate.

In one embodiment step a) comprises reacting the serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F polysaccharide with periodate.

In one embodiment step a) comprises reacting the serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F polysaccharide with 0.05-2 molar equivalents of periodate.

5 In one embodiment step a) comprises reacting the serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F polysaccharide with 0.05-0.2 molar equivalents of periodate.

In one embodiment step a) comprises reacting the serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F polysaccharide with 0.2-0.5 molar equivalents of periodate

10 In one embodiment step a) comprises reacting the serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F polysaccharide with 0.5-1.5 molar equivalents of periodate

In one embodiment step a) comprises reacting the serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F polysaccharide with 1.5-2.0 molar equivalents of periodate.

15 In some embodiment, the reaction of step (a) is quenched. Therefore, following step (a) a quenching step (a') can be performed (see WO2015110940). Therefore, in an embodiment, the isolated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide is conjugated to a carrier protein by a process comprising the step of:

(a) reacting said isolated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide with an oxidizing agent to produce an activated polysaccharide; (a') quenching the oxidation reaction by addition of a quenching agent resulting in an activated polysaccharide; (b) compounding the activated polysaccharide of step (a') with a carrier protein; and (c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

20 In an embodiment, the oxidizing agent is 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) free radical and N-Chlorosuccinimide (NCS) as the cooxidant. This oxidizing agent is particularly suitable for oxidizing serotype 12F capsular polysaccharides. In such embodiment, the glycoconjugates from *S. pneumoniae* serotype 12F are prepared using 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) free radical to oxidize primary alcohols of the saccharide to aldehydes using N-Chlorosuccinimide (NCS) as the cooxidant (hereinafter "TEMPO/NCS oxidation"), such as described at Example 7 and in WO 2014/097099. Therefore, in one aspect, the glycoconjugates from *S. pneumoniae* serotype 12F of the invention are obtainable by a method comprising the steps of: a) reacting an isolated serotype 12F capsular polysaccharide with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and N-chlorosuccinimide (NCS) to produce an activated polysaccharide; (b) compounding the activated polysaccharide of step (a) with a carrier protein and (c) reacting the compounded activated polysaccharide and carrier protein with a

reducing agent to form a glycoconjugate (hereinafter "TEMPO/NCS-reductive amination"). In one aspect, the glycoconjugates from *S. pneumoniae* serotype 12F are obtained by said method.

In an embodiment, the isolated serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F capsular polysaccharides are reacted with periodate and the isolated serotype 12F capsular polysaccharide is reacted with TEMPO/NCS.

In an embodiment, the isolated serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 14, 15B, 18C, 19A, 19F, 22F and 23F capsular polysaccharides are reacted with periodate and the isolated serotype 12F capsular polysaccharide is reacted with TEMPO/NCS.

In a preferred embodiment the degree of oxidation (also named "degree of activation" in the present document) of the activated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F polysaccharide is between 2 and 30.

In an embodiment the degree of oxidation of the activated serotype 1 polysaccharide is between 1 and 15 when the carrier protein is TT (see e.g. Table 2 of WO2019/152921).

In a preferred embodiment the degree of oxidation of the activated serotype 1 polysaccharide is between 4 and 10 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

In an embodiment the degree of oxidation of the activated serotype 4 polysaccharide is between 1 and 15. In a preferred embodiment the degree of oxidation of the activated serotype 4 polysaccharide is between 1 and 5 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

In an embodiment the degree of oxidation of the activated serotype 5 polysaccharide is between 1 and 15. In a preferred embodiment the degree of oxidation of the activated serotype 5 polysaccharide is between 2 and 6 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

In an embodiment the degree of oxidation of the activated serotype 5 polysaccharide is between 1 and 15 when the carrier protein is TT. See Table 2 of WO2019/152921.

In an embodiment the degree of oxidation of the activated serotype 6A polysaccharide is between 1 and 15. In a preferred embodiment the degree of oxidation of the activated serotype 6A polysaccharide is between 5 and 15 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

In an embodiment the degree of oxidation of the activated serotype 6B polysaccharide is between 1 and 15. In a preferred embodiment the degree of oxidation of the activated serotype 6B polysaccharide is between 7 and 13 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

In an embodiment the degree of oxidation of the activated serotype 7F polysaccharide is between 1 and 15. In a preferred embodiment the degree of oxidation of the activated serotype 7F polysaccharide is between 2 and 8 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

In an embodiment the degree of oxidation of the activated serotype 8 polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 8 polysaccharide is between 1 and 17 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

5 In an embodiment the degree of oxidation of the activated serotype 9V polysaccharide is between 1 and 15. In a preferred embodiment the degree of oxidation of the activated serotype 9V polysaccharide is between 4 and 9 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

10 In an embodiment the degree of oxidation of the activated serotype 10A polysaccharide is between 1 and 15. In a preferred embodiment the degree of oxidation of the activated serotype 10A polysaccharide is between 1 and 12 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

15 In an embodiment the degree of oxidation of the activated serotype 11A polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 11A polysaccharide is between 1 and 15 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

20 In an embodiment the degree of oxidation of the activated serotype 12F polysaccharide is between 1 and 15. In a preferred embodiment the degree of oxidation of the activated serotype 12F polysaccharide is between 1 and 9 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

In an embodiment the degree of oxidation of the activated serotype 14 polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 14 polysaccharide is between 6 and 13 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

25 In an embodiment the degree of oxidation of the activated serotype 15B polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 15B polysaccharide is between 1 and 17 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

30 In an embodiment the degree of oxidation of the activated serotype 15B polysaccharide is between 1 and 15 when the carrier protein is TT. See Table 2 of WO2019/152925.

In an embodiment the degree of oxidation of the activated serotype 18C polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 18C polysaccharide is between 6 and 14 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

35 In an embodiment the degree of oxidation of the activated serotype 19A polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 19A polysaccharide is between 7 and 13 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

In an embodiment the degree of oxidation of the activated serotype 19F polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 19F polysaccharide is between 6 and 12 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

5 In an embodiment the degree of oxidation of the activated serotype 22F polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 22F polysaccharide is between 1 and 16 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

10 In an embodiment the degree of oxidation of the activated serotype 22F polysaccharide is between 1 and 20 when the carrier protein is TT. See Table 2 of WO2019/152925.

In an embodiment the degree of oxidation of the activated serotype 23F polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 23F polysaccharide is between 6 and 14 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

15 In an embodiment the degree of oxidation of the activated serotype 33F polysaccharide is between 1 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 33F polysaccharide is between 1 and 15 when the carrier protein is CRM₁₉₇. See Table 1 of WO2019/152921.

20 The activated polysaccharide and the carrier protein may be lyophilized (freeze-dried), either independently (discrete lyophilization) or together (co-lyophilized). In one embodiment the activated polysaccharide and the carrier protein are co-lyophilized. In another embodiment the activated polysaccharide and the carrier protein are lyophilized independently.

25 In one embodiment the lyophilization takes place in the presence of a non-reducing sugar, possible non-reducing sugars include sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinin.

30 In an embodiment the initial input ratio (weight by weight) of activated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide to carrier protein at step b) is between 4:1 and 0.1:1. In an embodiment the initial input ratio (weight by weight) of activated serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F capsular polysaccharide to carrier protein is between 1.5:1 and 0.5:1.

35 In an embodiment, the reduction reaction (c) is carried out in aprotic solvent. In one embodiment the reduction reaction (c) is carried out in a solution consisting essentially of dimethylsulphoxide (DMSO). In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent.

In an embodiment, the reduction reaction (c) is carried out in aqueous solvent.

In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent for serotypes 6A, 6B, 7F, 8, 10A, 15B, 19A, 19F, 22F and 23F and the reduction reaction (c) is carried out in aqueous solvent for serotypes 1, 4, 5, 9V, 11A, 12F, 14 and 18C.

5 In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent for serotypes 6A, 6B, 7F, 8, 10A, 15B, 19A, 19F, 22F and 23F and the reduction reaction (c) is carried out in aqueous solvent for serotypes 1, 4, 5, 9V, 11A, 12F, 14, 18C and 33F.

In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent for serotypes 6A, 6B, 7F, 18C, 19A, 19F and 23F and the reduction reaction (c) is carried out in aqueous solvent for serotypes 1, 4, 5, 9V, 14, 22F and 33F.

10 In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent for serotypes 6A, 6B, 7F, 11A, 12F, 19A, 19F and 23F and the reduction reaction (c) is carried out in aqueous solvent for serotypes 1, 4, 5, 8, 9V, 10A, 14, 15B, 18C, 22F and 33F.

In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxymethylborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMeiPrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane (PEMB). In an embodiment, the reducing agent is sodium triacetoxymethylborohydride. In a preferred embodiment, the reducing agent is sodium cyanoborohydride. In an embodiment, the reducing agent is sodium cyanoborohydride in the presence of nickel (see WO2018144439).

20 In one embodiment between 0.2 and 10 molar equivalents of reducing agent is used in step c). Preferably, between 0.5 and 5 molar equivalents of reducing agent is used in step c).

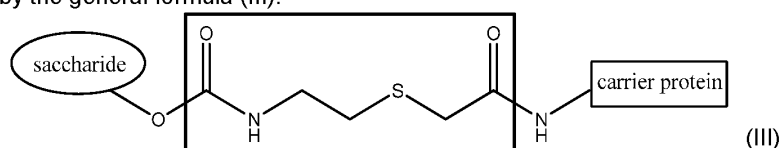
At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride (NaBH₄).

25 In an embodiment capping is achieved by mixing the product of step c) with 1 to 20 molar equivalents of sodium borohydride. In an embodiment capping is achieved by mixing the product of step c) with 1 to 3 molar equivalents of sodium borohydride.

Following conjugation to the carrier protein, the serotype 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and/or 33F glycoconjugate can be purified (enriched with respect to the amount of saccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. Therefore, in one embodiment the process for producing the serotype 1 glycoconjugate of the present invention comprises the step of purifying the glycoconjugate after it is produced.

35 In certain embodiments, the serotype 33F glycoconjugates of the invention are prepared using reductive amination.

In other embodiments, the serotype 33F glycoconjugates of the invention are prepared using eTEC conjugation (hereinafter “serotype 33F eTEC linked glycoconjugates”), such as described in WO 2014/027302 or WO2015110941 (see Examples 1, 2 and 3). Said 33F glycoconjugates comprise a saccharide covalently conjugated to a carrier protein through one or more eTEC spacers, wherein the saccharide is covalently conjugated to the eTEC spacer through a carbamate linkage, and wherein the carrier protein is covalently conjugated to the eTEC spacer through an amide linkage. The eTEC linked glycoconjugates of the invention may be represented by the general formula (III):



wherein the atoms that comprise the eTEC spacer are contained in the central box. The eTEC spacer includes seven linear atoms (i.e., $-\text{C}(\text{O})\text{NH}(\text{CH}_2)_2\text{SCH}_2\text{C}(\text{O})-$) and provides stable thioether and amide bonds between the saccharide and carrier protein. Synthesis of the eTEC linked glycoconjugate involves reaction of an activated hydroxyl group of the saccharide with the amino group of a thioalkylamine reagent, e.g., cystamine or cysteinamine or a salt thereof, forming a carbamate linkage to the saccharide to provide a thiolated saccharide. Generation of one or more free sulfhydryl groups is accomplished by reaction with a reducing agent to provide an activated thiolated saccharide. Reaction of the free sulfhydryl groups of the activated thiolated saccharide with an activated carrier protein having one or more α -haloacetamide groups on amine containing residues generates a thioether bond to form the conjugate, wherein the carrier protein is attached to the eTEC spacer through an amide bond.

In the serotype 33F eTEC linked glycoconjugates of the invention, the saccharide may be a polysaccharide or an oligosaccharide. The carrier protein may be selected from any suitable carrier as described herein or known to those of skill in the art. Preferably, the saccharide is a polysaccharide. In some such embodiments, the carrier protein is CRM₁₉₇. In some such embodiments, the eTEC linked glycoconjugate comprises a *S. pneumoniae* serotype 33F capsular polysaccharide.

In particularly preferred embodiments, the eTEC linked glycoconjugate comprises a Pn-33F capsular polysaccharide, which is covalently conjugated to CRM₁₉₇ through an eTEC spacer (serotype 33F eTEC linked glycoconjugates).

In an embodiment, the serotypes 1, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F and 23F glycoconjugates of the invention are prepared using reductive amination chemistry and the serotype 33F glycoconjugate of the invention is prepared using eTEC conjugation.

1.5 *S. pneumoniae* serotype 15A glycoconjugate of the invention

In an aspect the invention relates to *S. pneumoniae* serotype 15A glycoconjugates.

The structure of *Streptococcus pneumoniae* serotype 15A polysaccharide is known in the art (see e.g. Geno K *et al.* (2015) Clin Microbiol Rev Vol 28:3, p 871-899).

In an embodiment, the capsular *S. pneumoniae* serotype 15A saccharide used in the present invention is a synthetic carbohydrate.

5 In a preferred embodiment though, the source of bacterial polysaccharide according to this invention can be *Streptococcus pneumoniae* serotype 15A bacterial cells. Bacterial strains which can be used as source of *Streptococcus pneumoniae* serotype 15A polysaccharides may be obtained from established culture collections (such as for example from the Streptococcal Reference Laboratory (Centers for Disease Control and Prevention, Atlanta, GA USA)) or clinical
10 specimens.

Serotype 15A saccharides can be obtained directly from bacteria using isolation procedures known to one of ordinary skill in the art. They can also be purchased (such as for example from the American Type Culture Collection (ATCC, Manassas, VA USA) (e.g., reference No. ATCC (537-X)).

15 In case the serotype 15A saccharide is obtained directly from bacteria, the bacterial cells can be grown in a medium, preferably in a soy based medium. Following fermentation of bacterial cells that produce *S. pneumoniae* serotype 15A capsular polysaccharides, the bacterial cells can be lysed to produce a cell lysate. The serotype 15A polysaccharide may then be isolated from the cell lysate using purification techniques known in the art, including the use of centrifugation, depth
20 filtration, precipitation, ultra-filtration, treatment with activate carbon, diafiltration and/or column chromatography (see, for example, US2006/0228380, US2006/0228381, WO2008/118752 and WO2020170190). The purified serotype 15A capsular polysaccharide can then be used for the preparation of glycoconjugates.

The isolated serotype 15A capsular saccharide obtained by purification of serotype 15A
25 polysaccharide from the *S. pneumoniae* lysate and optionally sizing of the purified polysaccharide can be characterized by different parameters including, for example the weight average molecular weight (Mw).

The molecular weight of the polysaccharide can be measured by Size Exclusion Chromatography (SEC) combined with Multiangle Laser Light Scattering detector (MALLS).

30 In a preferred embodiment, the isolated serotype 15A capsular polysaccharide (i.e. purified before further treatment) has a weight average molecular weight between 100 kDa and 2500 kDa. In an embodiment, the isolated serotype 15A capsular polysaccharide has a weight average molecular weight between 250 kDa and 1500 kDa. In an embodiment, the isolated serotype 15A capsular polysaccharide has a weight average molecular weight between 500 kDa
35 and 1000 kDa.

Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In order to generate serotype 15A conjugates with advantageous filterability characteristics, immunogenicity and/or yields, sizing of the polysaccharide to a target molecular weight range can be performed prior to the conjugation to a carrier protein. Advantageously, the size of the purified serotype 15A polysaccharide is reduced while preserving critical features of the structure of the polysaccharide. Mechanical or chemical sizing may be employed.

In an embodiment, the size of the purified serotype 15A polysaccharide is reduced by chemical hydrolysis. Chemical hydrolysis may be conducted using a mild acid (e.g. acetic acid, formic acid, propanoic acid). Chemical hydrolysis may also be conducted using a diluted strong acid (such as diluted hydrochloric acid, diluted sulfuric acid, diluted phosphoric acid, diluted nitric acid or diluted perchloric acid).

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

In an embodiment, the isolated serotype 15A capsular polysaccharide is sized to a weight average molecular weight of between 50 kDa and 500 kDa. In a preferred embodiment, the isolated serotype 15A capsular polysaccharide is sized to a weight average molecular weight of between 75 kDa and 250 kDa. Preferably, the isolated serotype 15A capsular polysaccharide is sized to a weight average molecular weight below 175 kDa. In an even preferred embodiment, the isolated serotype 15A capsular polysaccharide is sized to a weight average molecular weight of between 75 kDa and 175 kDa. In a most preferred embodiment, the isolated serotype 15A capsular polysaccharide is sized to a weight average molecular weight of between 100 kDa and 175 kDa. Preferably, the isolated serotype 15A polysaccharide is sized by mechanical homogenization, preferably by high pressure homogenization.

In an embodiment, the isolated serotype 15A capsular polysaccharide is not sized.

In an embodiment, the isolated serotype 15A capsular polysaccharide before conjugation has a weight average molecular weight between 50 kDa and 500 kDa. In an embodiment, the isolated serotype 15A capsular polysaccharide before conjugation has a weight average molecular weight between 75 kDa and 250 kDa. In a preferred embodiment, the isolated serotype 15A capsular polysaccharide before conjugation has a weight average molecular weight between 75 kDa and 175 kDa. In an even preferred embodiment, the isolated serotype 15A capsular polysaccharide before conjugation has a weight average molecular weight between 90 kDa and 150 kDa. In a most preferred embodiment, the isolated serotype 15A capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 175 kDa.

The weight average molecular weight (Mw) of the saccharide before conjugation refers to the Mw before activation of the polysaccharide (i.e. after an eventual sizing step but before

reacting the polysaccharide with an activating agent). In the context of the present invention the Mw of the 15A polysaccharide is not substantially modified by the activation step and the Mw of the 15A polysaccharide incorporated in the conjugate is similar to the Mw of the polysaccharide as measured before activation.

5 In an embodiment, the serotype 15A glycoconjugate of the present invention comprises a serotype 15A capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 500 kDa. In an embodiment, the weight average molecular weight (Mw) is between 75 kDa and 250 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 75 kDa and 175 kDa. In a most preferred
10 embodiment, the weight average molecular weight (Mw) is between 90 kDa and 150 kDa.

In some embodiments, the serotype 15A glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 10,000 kDa. In other embodiments, the serotype 15A glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 10,000 kDa. Preferably, the serotype 15A glycoconjugate has a weight average molecular
15 weight (Mw) of between 1,000 kDa and 6,000 kDa.

The serotype 15A glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein. In some embodiments, the ratio of serotype 15A polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.5 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.5 and 1.5. Even more preferably, the
20 saccharide to carrier protein ratio (w/w) is between 0.7 and 1.1.

Another way to characterize the serotype 15A glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM197, DT or TT) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent linkages
25 to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered compared to the carrier protein starting material used to generate the conjugate materials. In a preferred embodiment, the degree of conjugation of the serotype 15A glycoconjugate of the invention is between 2 and 20. Preferably, the degree of conjugation of the
30 serotype 15A glycoconjugate of the invention is between 5 and 10.

The serotype 15A glycoconjugates and immunogenic compositions of the invention may contain free saccharide that is not covalently conjugated to the carrier protein but is nevertheless present in the glycoconjugate composition. The free saccharide may be noncovalently associated with (i.e., noncovalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.

35 In an embodiment, the serotype 15A glycoconjugate comprises less than about 40% of free serotype 15A polysaccharide compared to the total amount of serotype 15A polysaccharide. In a preferred embodiment the serotype 15A glycoconjugate comprises less than about 25% of free serotype 15A polysaccharide compared to the total amount of serotype 15A polysaccharide.

The serotype 15A glycoconjugates may also be characterized by their molecular size distribution (Kd). Size exclusion chromatography media (CL-4B) can be used to determine the relative molecular size distribution of the conjugate. Size Exclusion Chromatography (SEC) is used in gravity fed columns to profile the molecular size distribution of conjugates. Large molecules excluded from the pores in the media elute more quickly than small molecules. Fraction collectors are used to collect the column eluate. The fractions are tested colorimetrically by saccharide assay. For the determination of Kd, columns are calibrated to establish the fraction at which molecules are fully excluded (V0), (Kd=0), and the fraction representing the maximum retention (Vi), (Kd=1). The fraction at which a specified sample attribute is reached (Ve), is related to Kd by the expression, $Kd = (Ve - V0) / (Vi - V0)$.

In an embodiment, at least 40% of the serotype 15A glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 50% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 90% of the serotype 15A glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, the serotype 15A saccharide is activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide is then coupled directly or via a spacer (linker) group to an amino group on the carrier protein (preferably CRM₁₉₇). 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 N-[γ-maleimidobutyroxy]succinimide ester (GMBS)) or a haloacetylated carrier protein (for example using iodoacetamide, N-succinimidyl bromoacetate (SBA; SIB), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB), N-succinimidyl iodoacetate (SIA) or succinimidyl 3-[bromoacetamido]propionate (SBAP)). Preferably, the cyanate ester is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein (e.g., CRM₁₉₇) using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO 93/15760, WO 95/08348 and WO 96/129094.

Other suitable techniques for conjugation use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with 1,1'-carbonyldiimidazole (CDI) (see Bethell et al. (1979) J. Biol. Chem. 254:2572-2574; Hearn et al. (1981) J. Chromatogr. 218:509-518) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

In a preferred embodiment, the serotype 15A glycoconjugates of the invention are prepared using reductive amination chemistry. According to the present invention, reductive amination involves two steps, (1) oxidation (activation) of a purified saccharide, (2) reduction of the activated saccharide and a carrier protein to form a glycoconjugate (see e.g. 5 WO2006/110381, WO2008/079653, WO2008/143709, WO2008/079732, WO2011/110531, WO2012/119972, WO2015110941, WO2015110940, WO2018/144439, WO2018/156491).

In an embodiment, the serotype 15A glycoconjugates of the invention are prepared by conjugating an isolated serotype 15A capsular polysaccharide to a carrier protein by a process comprising the step of:

- 10 (a) reacting said isolated serotype 15A capsular polysaccharide with an oxidizing agent; (b) compounding the activated polysaccharide of step (a) with a carrier protein; and (c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

As mentioned above, before oxidation, sizing of the isolated serotype 15A capsular 15 polysaccharide to a target molecular weight (MW) range can be performed. Therefore, in an embodiment, the isolated serotype 15A capsular polysaccharide is sized before oxidation.

In an embodiment, the isolated serotype 15A capsular polysaccharide is not sized before oxidation.

In an embodiment, the oxidation step (a) is carried out at a pH of between 4.0 and 6.0. 20 Preferably, the oxidation step (a) is carried out at a pH of between 4.5 and 5.5.

In an embodiment, the oxidation step (a) is carried out at a pH of about 5.0.

Following the oxidation step (a) the saccharide is said to be activated and is referred to as "activated polysaccharide".

In an embodiment, the activated serotype 15A polysaccharide of the present invention has 25 a weight average molecular weight (Mw) of between 50 kDa and 500 kDa. In an embodiment, the weight average molecular weight (Mw) is between 75 kDa and 250 kDa. In a preferred embodiment, the weight average molecular weight (Mw) is between 75 kDa and 175 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 90 kDa and 150 kDa.

30 In an embodiment, the oxidizing agent is any oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde. In an embodiment, the oxidizing agent is periodate. For the purpose of the present invention, 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).

35 In a preferred embodiment, the oxidizing agent is sodium periodate. In an embodiment, the periodate used for the oxidation is metaperiodate. In a most preferred embodiment, the periodate used for the oxidation is sodium metaperiodate.

When a polysaccharide reacts with periodate, periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. For this reason, the term "reacting a polysaccharide with periodate" includes oxidation of vicinal hydroxyl groups by periodate.

5 In one embodiment step a) comprises reacting the polysaccharide with 0.1-2 molar equivalents of periodate. Preferably step a) comprises reacting the polysaccharide with 0.5-1.5 molar equivalents of periodate. Most preferably, step a) comprises reacting the polysaccharide with 0.8-1.2 molar equivalents of periodate.

10 In an embodiment the degree of oxidation (also named "degree of activation" in the present document) of the activated serotype 15A polysaccharide is between 2 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 15A polysaccharide is between 2 and 8. In a most preferred embodiment the degree of oxidation of the activated serotype 15A polysaccharide is 5 ± 2.5 .

15 In one embodiment the activated serotype 15A polysaccharide and the carrier protein are lyophilised before step b). Preferably lyophilisation occurs after step a). In one embodiment the activated polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised. In one embodiment the activated serotype 15A polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised, and the activated polysaccharide and the carrier protein are reconstituted in the same solution.

20 In an embodiment, the activated serotype 15A polysaccharide and the carrier protein are lyophilized independently (discrete lyophilization). In an embodiment, the activated serotype 15A polysaccharide and the carrier protein are lyophilized together (co-lyophilized).

In one embodiment the lyophilization takes place in the presence of a non-reducing sugar, possible non-reducing sugars include sucrose, trehalose, raffinose, stachyose, melezitose, 25 dextran, mannitol, lactitol and palatinit. In an embodiment the sugar is selected from the group consisting of sucrose, trehalose, and mannitol. In an embodiment the sugar is sucrose, trehalose or mannitol. In an embodiment the sugar is sucrose.

30 In an embodiment the initial input ratio (weight by weight) of activated serotype 15A capsular polysaccharide to carrier protein at step b) is between 3:1 and 0.5:1. In an embodiment the initial input ratio (weight by weight) of activated serotype 15A capsular polysaccharide to carrier protein is between 1.5:1 and 0.5:1. Preferably, the initial input ratio (weight by weight) of activated serotype 15A capsular polysaccharide to carrier protein is between 1.1:1 and 0.9:1.

In an embodiment, the reduction reaction (c) is carried out in aqueous solvent. Preferably, the reduction reaction (c) is carried out in aprotic solvent.

35 In an embodiment, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO) or dimethylformamide (DMF). Preferably, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO).

In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) or in DMF (dimethylformamide)) solvent.

Most preferably, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent.

5 In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxyborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMeiPrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane (PEMB). In an embodiment, the reducing agent is sodium triacetoxyborohydride. In an
10 embodiment, the reducing agent is sodium cyanoborohydride in the present of nickel (see WO2018144439). In a preferred embodiment, the reducing agent is sodium cyanoborohydride.

In one embodiment between 0.2 and 5 molar equivalents of reducing agent is used in step c). Preferably, between 0.5 and 2 molar equivalents of reducing agent is used in step c). Most preferably, between 0.9 and 1.1 molar equivalents of reducing agent is used in step c).

15 At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride (NaBH₄).

In an embodiment capping is achieved by mixing the product of step c) with 1 to 20 molar equivalents of sodium borohydride. In an embodiment capping is achieved by mixing the product
20 of step c) with 1 to 3 molar equivalents of sodium borohydride.

Following conjugation to the carrier protein, the serotype 15A glycoconjugate can be purified (enriched with respect to the amount of saccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration precipitation/elution, column
25 chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. Therefore, in one embodiment the process for producing the serotype 15A glycoconjugate of the present invention comprises the step of purifying the glycoconjugate after it is produced.

1.6 *S. pneumoniae* serotype 23A glycoconjugate of the invention

30 In an aspect the invention relates to *S. pneumoniae* serotype 23A glycoconjugates.

The structure of *Streptococcus pneumoniae* serotype 23A polysaccharide is known in the art (see e.g. Ravenscroft N *et al.* (2017) Carbohydrate Res. Vol. 450, p 19-29 and WO2019050814). The structure of the serotype 23A capsular polysaccharide is: →4)-β-D-Glcp-(1→3)-[[α-L-Rhap-(1→2)]-[Gro-(2→P→3)]-β-D-Galp-(1→4)]-β-L-Rhap-(1→.

35 In an embodiment, the capsular *S. pneumoniae* serotype 23A saccharide used in the present invention is a synthetic carbohydrate.

In a preferred embodiment though, the source of bacterial polysaccharide according to this invention can be *Streptococcus pneumoniae* serotype 23A bacterial cells. Bacterial strains

which can be used as source of *Streptococcus pneumoniae* serotype 23A polysaccharides may be obtained from established culture collections (such as for example from the Streptococcal Reference Laboratory (Centers for Disease Control and Prevention, Atlanta, GA USA)) or clinical specimens.

5 Serotype 23A saccharides can be obtained directly from bacteria using isolation procedures known to one of ordinary skill in the art. They can also be purchased (such as for example from the American Type Culture Collection (ATCC, Manassas, VA USA) (e.g., reference No. ATCC 545-X, No. ATCC 546-X, No. ATCC 547-X)).

10 In case the serotype 23A saccharide is obtained directly from bacteria, the bacterial cells can be grown in a medium, preferably in a soy based medium. Following fermentation of bacterial cells that produce *S. pneumoniae* serotype 23A capsular polysaccharides, the bacterial cells can be lysed to produce a cell lysate. The serotype 23A polysaccharide may then be isolated from the cell lysate using purification techniques known in the art, including the use of centrifugation, depth filtration, precipitation, ultra-filtration, treatment with activate carbon, diafiltration and/or column chromatography (see, for example, US2006/0228380, US2006/0228381, WO2008/118752 and
15 WO2020170190). The purified serotype 23A capsular polysaccharide can then be used for the preparation of glycoconjugates.

20 The isolated serotype 23A capsular saccharide obtained by purification of serotype 23A polysaccharide from the *S. pneumoniae* lysate and optionally sizing of the purified polysaccharide can be characterized by different parameters including, for example the weight average molecular weight (Mw).

 The molecular weight of the polysaccharide can be measured by Size Exclusion Chromatography (SEC) combined with Multiangle Laser Light Scattering detector (MALLS).

25 In a preferred embodiment, the isolated serotype 23A capsular polysaccharide (i.e. purified before further treatment) has a weight average molecular weight between 100 kDa and 2500 kDa. In an embodiment, the isolated serotype 23A capsular polysaccharide has a weight average molecular weight between 250 kDa and 1500 kDa. In an embodiment, the isolated serotype 23A capsular polysaccharide has a weight average molecular weight between 500 kDa and 1000 kDa.

30 Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

 In order to generate serotype 23A conjugates with advantageous filterability characteristics, immunogenicity and/or yields, sizing of the polysaccharide to a target molecular weight range can be performed prior to the conjugation to a carrier protein. Advantageously, the
35 size of the purified serotype 23A polysaccharide is reduced while preserving critical features of the structure of the polysaccharide. Mechanical or chemical sizing maybe employed.

 Most preferably, the size of the purified serotype 23A polysaccharide is reduced by mechanical homogenization.

It has been discovered that the use of acid hydrolysis, as for example recommended in WO2019050814 or in WO2019050818 is not appropriate to reduce the size of serotype 23A polysaccharide.

5 It has been found that acid hydrolysis can affect serotype 23A polysaccharide structure integrity. Even relatively mild hydrolysis (e.g. treatment with acetic acid at 100mM at 80°C for 2 hours) has been found to lead to a 25% loss of the branched rhamnose (α -L-Rhap-(1→2)) (see figure 1).

Mechanical sizing allows not to lose this residue, which can be important from an immunological point of view. Furthermore, this residue is the main activation site for periodate oxidation. It is therefore important to retain it in case periodate oxidation of the polysaccharide is used (e.g. activation step in the reductive amination chemistry).

Therefore, in a preferred embodiment, the size of the purified serotype 23A polysaccharide is reduced by mechanical homogenization.

15 In an embodiment, the size of the purified serotype 23A polysaccharide is reduced by high pressure homogenization. High pressure homogenization achieves high shear rates by pumping the process stream through a flow path with sufficiently small dimensions. The shear rate is increased by using a larger applied homogenization pressure, and exposure time can be increased by recirculating the feed stream through the homogenizer.

20 In a most preferred embodiment, the process to prepare serotype 23A glycoconjugate of the invention does not comprise a step of sizing of the serotype 23A polysaccharide by acid hydrolysis.

In an embodiment, the isolated serotype 23A capsular polysaccharide is sized to a weight average molecular weight of between 50 kDa and 500 kDa. In a preferred embodiment, the isolated serotype 23A capsular polysaccharide is sized to a weight average molecular weight of between 75 kDa and 400 kDa. In an even preferred embodiment, the isolated serotype 23A capsular polysaccharide is sized to a weight average molecular weight of between 100 kDa and 350 kDa. In a most preferred embodiment, the isolated serotype 23A capsular polysaccharide is sized to a weight average molecular weight of between 125 kDa and 225 kDa. Preferably, the isolated serotype 23A polysaccharide is sized by mechanical homogenization, most preferably by high pressure homogenization.

30 In an embodiment, the isolated serotype 23A capsular polysaccharide is not sized.

In an embodiment, the isolated serotype 23A capsular polysaccharide before conjugation has a weight average molecular weight between 50 kDa and 500 kDa. In an embodiment, the isolated serotype 23A capsular polysaccharide before conjugation has a weight average molecular weight between 75 kDa and 400 kDa. In an even preferred embodiment, the isolated serotype 23A capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 350 kDa. In a most preferred embodiment, the isolated serotype 23A

capsular polysaccharide before conjugation has a weight average molecular weight of between 125 kDa and 225 kDa.

5 The weight average molecular weight (Mw) of the saccharide before conjugation refers to the Mw before activation of the polysaccharide (i.e. after an eventual sizing step but before reacting the polysaccharide with an activating agent). In the context of the present invention the Mw of the 23A polysaccharide is not substantially modified by the activation step and the Mw of the 23A polysaccharide incorporated in the conjugate is similar to the Mw of the polysaccharide as measured before activation.

10 In an embodiment, the serotype 23A glycoconjugate of the present invention comprises a serotype 23A capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 400 kDa. In an embodiment, the weight average molecular weight (Mw) is between 100 kDa and 300 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 120 kDa and 240 kDa.

15 In some embodiments, the serotype 23A glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 10,000 kDa. In other embodiments, the serotype 23A glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 7,500 kDa. Preferably, the serotype 23A glycoconjugate has a weight average molecular weight (Mw) of between 2,000 kDa and 5,000 kDa.

20 The serotype 23A glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein. In some embodiments, the ratio of serotype 23A polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.5 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.7 and 1.5. Even more preferably, the saccharide to carrier protein ratio (w/w) is between 0.8 and 1.4.

25 Another way to characterize the serotype 23A glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM197, DT or TT) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent linkages to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered compared to the carrier protein starting material used to generate the conjugate materials. In a preferred embodiment, the degree of conjugation of the serotype 23A glycoconjugate of the invention is between 2 and 20. Preferably, the degree of conjugation of the serotype 23A glycoconjugate of the invention is between 5 and 15.

35 The serotype 23A glycoconjugates and immunogenic compositions of the invention may contain free saccharide that is not covalently conjugated to the carrier protein but is nevertheless present in the glycoconjugate composition. The free saccharide may be noncovalently associated with (i.e., noncovalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.

In an embodiment, the serotype 23A glycoconjugate comprises less than about 40% of free serotype 23A polysaccharide compared to the total amount of serotype 23A polysaccharide. In a preferred embodiment the serotype 23A glycoconjugate comprises less than about 25% of free serotype 23A polysaccharide compared to the total amount of serotype 23A polysaccharide.

5 The serotype 23A glycoconjugates may also be characterized by their molecular size distribution (Kd). Size exclusion chromatography media (CL-4B) can be used to determine the relative molecular size distribution of the conjugate. Size Exclusion Chromatography (SEC) is used in gravity fed columns to profile the molecular size distribution of conjugates. Large molecules excluded from the pores in the media elute more quickly than small molecules.
10 Fraction collectors are used to collect the column eluate. The fractions are tested colorimetrically by saccharide assay. For the determination of Kd, columns are calibrated to establish the fraction at which molecules are fully excluded (V_0), ($K_d=0$), and the fraction representing the maximum retention (V_i), ($K_d=1$). The fraction at which a specified sample attribute is reached (V_e), is related to Kd by the expression, $K_d = (V_e - V_0) / (V_i - V_0)$.

15 In an embodiment, at least 40% of the serotype 23A glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 50% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 90% of the serotype 23A glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

20 The serotype 23A glycoconjugates may also be characterized by the amount of branched Rhamnose residue which remains in the 23A polysaccharide. As mentioned above, it has been found that serotype 23A polysaccharide can lose the branched Rhamnose residue (see figure 1).

25 Therefore, in an embodiment, the *S. pneumoniae* serotype 23A glycoconjugate of the present invention comprises a *S. pneumoniae* serotype 23A capsular polysaccharide wherein said *S. pneumoniae* serotype 23A capsular polysaccharide has a branched Rhamnose content greater than 75% when compared to native *S. pneumoniae* serotype 23A capsular polysaccharide wherein the branched Rhamnose content is considered to be about 100%. In an embodiment, the *S. pneumoniae* serotype 23A glycoconjugate of the present invention comprises a *S. pneumoniae* serotype 23A capsular polysaccharide wherein said *S. pneumoniae* serotype 23A capsular polysaccharide has a branched Rhamnose content greater than 90% when compared to native *S. pneumoniae* serotype 23A capsular polysaccharide wherein the branched Rhamnose content is considered to be about 100%. Preferably, the *S. pneumoniae* serotype 23A glycoconjugate of the present invention comprises a *S. pneumoniae* serotype 23A capsular polysaccharide wherein said *S. pneumoniae* serotype 23A capsular polysaccharide has a branched Rhamnose content greater than 95% when compared to native *S. pneumoniae* serotype 23A capsular polysaccharide wherein the branched Rhamnose content is considered to be about 100%.

35 In a most preferred embodiment, the *S. pneumoniae* serotype 23A glycoconjugate of the present invention comprises a *S. pneumoniae* serotype 23A capsular polysaccharide wherein

said *S. pneumoniae* serotype 23A capsular polysaccharide has a branched Rhamnose content of about 100% when compared to native *S. pneumoniae* serotype 23A capsular polysaccharide wherein the branched Rhamnose content is considered to be about 100%.

In an embodiment, the serotype 23A saccharide is activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide is then coupled directly or via a spacer (linker) group to an amino group on the carrier protein (preferably CRM₁₉₇). 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 N-[γ-maleimidobutyroxy]succinimide ester (GMBS)) or a haloacetylated carrier protein (for example using iodoacetamide, N-succinimidyl bromoacetate (SBA; SIB), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB), N-succinimidyl iodoacetate (SIA) or succinimidyl 3-[bromoacetamido]propionate (SBAP)). Preferably, the cyanate ester is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein (e.g., CRM₁₉₇) using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO 93/15760, WO 95/08348 and WO 96/129094.

Other suitable techniques for conjugation use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with 1,1'-carbonyldiimidazole (CDI) (see Bethell et al. (1979) J. Biol. Chem. 254:2572-2574; Hearn et al. (1981) J. Chromatogr. 218:509-518) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

In a preferred embodiment, the serotype 23A glycoconjugates of the invention are prepared using reductive amination chemistry. According to the present invention, reductive amination involves two steps, (1) oxidation (activation) of a purified saccharide, (2) reduction of the activated saccharide and a carrier protein to form a glycoconjugate (see e.g. WO2006/110381, WO2008/079653, WO2008/143709, WO2008/079732, WO2011/110531, WO2012/119972, WO2015110941, WO2015110940, WO2018/144439, WO2018/156491).

In an embodiment, the serotype 23A glycoconjugates of the invention are prepared by conjugating an isolated serotype 23A capsular polysaccharide to a carrier protein by a process comprising the step of:

- (a) reacting said isolated serotype 23A capsular polysaccharide with an oxidizing agent;
- (b) compounding the activated polysaccharide of step (a) with a carrier protein; and (c) reacting

the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

As mentioned above, before oxidation, sizing of the isolated serotype 23A capsular polysaccharide to a target molecular weight (MW) range can be performed. Therefore, in an embodiment, the isolated serotype 23A capsular polysaccharide is sized before oxidation.

In a preferred embodiment, the size of the isolated serotype 23A capsular polysaccharide is reduced by mechanical homogenization. In an embodiment, the size of the isolated serotype 23A polysaccharide is reduced by high pressure homogenization. In a most preferred embodiment, the isolated serotype 23A capsular polysaccharide is not sized by acid hydrolysis.

In an embodiment, the isolated serotype 23A capsular polysaccharide is not sized before oxidation.

In an embodiment, the oxidation step (a) is carried out at a pH of between 4.5 and 6.5. Preferably, the oxidation step (a) is carried out at a pH of between 5.0 and 6.0.

In an embodiment, the oxidation step (a) is carried out at a pH of about 5.0.

Following the oxidation step (a) the saccharide is said to be activated and is referred to as "activated polysaccharide".

In an embodiment, the activated serotype 23A polysaccharide of the present invention has a weight average molecular weight (Mw) of between 50 kDa and 400 kDa. In an embodiment, the weight average molecular weight (Mw) is between 100 kDa and 250 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 120 kDa and 200 kDa.

In an embodiment, the activated serotype 23A polysaccharide of the present invention retains at least 80% of the branched Rhamnose. In an embodiment, the activated serotype 23A polysaccharide of the present invention retains at least 85% of the branched Rhamnose. In an embodiment, the activated serotype 23A polysaccharide of the present invention retains at least 90% of the branched Rhamnose. In a preferred embodiment, the activated serotype 23A polysaccharide of the present invention retains at least 95% of the branched Rhamnose.

In a preferred embodiment, the activated serotype 23A polysaccharide of the present invention retains at least 96% of the branched Rhamnose.

In an embodiment, the oxidizing agent is any oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde. In an embodiment, the oxidizing agent is periodate. For the purpose of the present invention, the term "periodate" includes both periodate and periodic acid; the term also includes both metaperiodate (IO_4^-) and orthoperiodate (IO_5^{5-}) and the various salts of periodate (e.g., sodium periodate and potassium periodate).

In a preferred embodiment, the oxidizing agent is sodium periodate. In an embodiment, the periodate used for the oxidation is metaperiodate. In a most preferred embodiment, the periodate used for the oxidation is sodium metaperiodate.

When a polysaccharide reacts with periodate, periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. For this reason, the

term "reacting a polysaccharide with periodate" includes oxidation of vicinal hydroxyl groups by periodate.

In one embodiment step a) comprises reacting the polysaccharide with 0.1-2 molar equivalents of periodate. Preferably step a) comprises reacting the polysaccharide with 0.2-1.5 molar equivalents of periodate. Most preferably, step a) comprises reacting the polysaccharide with 0.3-0.5 molar equivalents of periodate.

In an embodiment the degree of oxidation (also named "degree of activation" in the present document) of the activated serotype 23A polysaccharide is between 2 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 23A polysaccharide is between 2 and 8. In a most preferred embodiment the degree of oxidation of the activated serotype 23A polysaccharide is 5 ± 2.5 .

In one embodiment the activated serotype 23A polysaccharide and the carrier protein are lyophilised before step b). Preferably lyophilisation occurs after step a). In one embodiment the activated polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised. In one embodiment the activated serotype 23A polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised, and the activated polysaccharide and the carrier protein are reconstituted in the same solution.

In an embodiment, the activated serotype 23A polysaccharide and the carrier protein are lyophilized independently (discrete lyophilization). In an embodiment, the activated serotype 23A polysaccharide and the carrier protein are lyophilized together (co-lyophilized).

In one embodiment the lyophilization takes place in the presence of a non-reducing sugar, possible non-reducing sugars include sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinin. In an embodiment the sugar is selected from the group consisting of sucrose, trehalose, and mannitol. In an embodiment the sugar is sucrose, trehalose or mannitol. In an embodiment the sugar is sucrose.

In an embodiment the initial input ratio (weight by weight) of activated serotype 23A capsular polysaccharide to carrier protein at step b) is between 2:1 and 0.5:1. In an embodiment the initial input ratio (weight by weight) of activated serotype 23A capsular polysaccharide to carrier protein is between 1.2:1 and 0.6:1. Preferably, the initial input ratio (weight by weight) of activated serotype 23A capsular polysaccharide to carrier protein is between 0.9:1 and 0.7:1.

In an embodiment, the reduction reaction (c) is carried out in aqueous solvent. Preferably, the reduction reaction (c) is carried out in aprotic solvent.

In an embodiment, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO) or dimethylformamide (DMF). Preferably, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO).

In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) or in DMF (dimethylformamide) solvent.

Most preferably, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent.

In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxyborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMeiPrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane (PEMB). In an embodiment, the reducing agent is sodium triacetoxyborohydride. In an embodiment, the reducing agent is sodium cyanoborohydride in the present of nickel (see WO2018144439). In a preferred embodiment, the reducing agent is sodium cyanoborohydride.

In one embodiment between 0.2 and 5 molar equivalents of reducing agent is used in step c). Preferably, between 0.2 and 1.5 molar equivalents of reducing agent is used in step c). Most preferably, between 0.5 and 1.0 molar equivalent of reducing agent is used in step c).

At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride (NaBH₄).

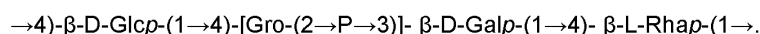
In an embodiment capping is achieved by mixing the product of step c) with 1 to 20 molar equivalents of sodium borohydride. In an embodiment capping is achieved by mixing the product of step c) with 1 to 3 molar equivalents of sodium borohydride.

Following conjugation to the carrier protein, the serotype 23A glycoconjugate can be purified (enriched with respect to the amount of saccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. Therefore, in one embodiment the process for producing the serotype 23A glycoconjugate of the present invention comprises the step of purifying the glycoconjugate after it is produced.

1.7 *S. pneumoniae* serotype 23B glycoconjugate of the invention

In an aspect the invention relates to *S. pneumoniae* serotype 23B glycoconjugates.

The structure of *Streptococcus pneumoniae* serotype 23B polysaccharide is known in the art (see e.g. Ravenscroft N *et al.* (2017) Carbohydrate Res. Vol. 450, p 19-29 and WO2019050814). The structure of the serotype 23A capsular polysaccharide is:



In an embodiment, the capsular *S. pneumoniae* serotype 23B saccharide used in the present invention is a synthetic carbohydrate.

In a preferred embodiment though, the source of bacterial polysaccharide according to this invention can be *Streptococcus pneumoniae* serotype 23B bacterial cells. Bacterial strains which can be used as source of *Streptococcus pneumoniae* serotype 23B polysaccharides may be obtained from established culture collections (such as for example from the Streptococcal

Reference Laboratory (Centers for Disease Control and Prevention, Atlanta, GA USA)) or clinical specimens.

Serotype 23B saccharides can be obtained directly from bacteria using isolation procedures known to one of ordinary skill in the art. They can also be purchased (such as for
5 example from the American Type Culture Collection (ATCC, Manassas, VA USA) (e.g., reference No. ATCC 548-X, No. ATCC 549-X, No. ATCC 550-X)).

In case the serotype 23B saccharide is obtained directly from bacteria, the bacterial cells can be grown in a medium, preferably in a soy based medium. Following fermentation of bacterial cells that produce *S. pneumoniae* serotype 23B capsular polysaccharides, the bacterial cells can
10 be lysed to produce a cell lysate. The serotype 23B polysaccharide may then be isolated from the cell lysate using purification techniques known in the art, including the use of centrifugation, depth filtration, precipitation, ultra-filtration, treatment with activate carbon, diafiltration and/or column chromatography (see, for example, US2006/0228380, US2006/0228381, WO2008/118752 and WO2020170190). The purified serotype 23B capsular polysaccharide can then be used for the
15 preparation of glycoconjugates.

The isolated serotype 23B capsular saccharide obtained by purification of serotype 23B polysaccharide from the *S. pneumoniae* lysate and optionally sizing of the purified polysaccharide can be characterized by different parameters including, for example the weight average molecular weight (Mw).

20 The molecular weight of the polysaccharide can be measured by Size Exclusion Chromatography (SEC) combined with Multiangle Laser Light Scattering detector (MALLS).

In a preferred embodiment, the isolated serotype 23B capsular polysaccharide (i.e. purified before further treatment) has a weight average molecular weight between 100 kDa and 2500 kDa. In an embodiment, the isolated serotype 23B capsular polysaccharide has a weight
25 average molecular weight between 250 kDa and 2000 kDa. In an embodiment, the isolated serotype 23B capsular polysaccharide has a weight average molecular weight between 300 kDa and 1000 kDa.

Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

30 In order to generate serotype 23B conjugates with advantageous filterability characteristics, immunogenicity and/or yields, sizing of the polysaccharide to a target molecular weight range can be performed prior to the conjugation to a carrier protein. Advantageously, the size of the purified serotype 23B polysaccharide is reduced while preserving critical features of the structure of the polysaccharide. Mechanical or chemical sizing maybe employed.

35 In an embodiment, the size of the purified serotype 23B polysaccharide is reduced by chemical hydrolysis. Chemical hydrolysis maybe conducted using a mild acid (e.g. acetic acid, formic acid, propanoic acid). Chemical hydrolysis may also be conducted using a diluted strong

acid (such as diluted hydrochloric acid, diluted sulfuric acid, diluted phosphoric acid, diluted nitric acid or diluted perchloric acid).

Preferably though, the size of the purified serotype 23B polysaccharide is reduced by mechanical homogenization. In an embodiment, the size of the purified serotype 23B polysaccharide is reduced by high pressure homogenization. High pressure homogenization achieves high shear rates by pumping the process stream through a flow path with sufficiently small dimensions. The shear rate is increased by using a larger applied homogenization pressure, and exposure time can be increased by recirculating the feed stream through the homogenizer.

In an embodiment, the isolated serotype 23B capsular polysaccharide is sized to a weight average molecular weight of between 50 kDa and 750 kDa. In a preferred embodiment, the isolated serotype 23B capsular polysaccharide is sized to a weight average molecular weight of between 75 kDa and 400 kDa. In an even preferred embodiment, the isolated serotype 23B capsular polysaccharide is sized to a weight average molecular weight of between 100 kDa and 250 kDa. Preferably, the isolated serotype 23B polysaccharide is sized by mechanical homogenization, most preferably by high pressure homogenization.

In an embodiment, the isolated serotype 23B capsular polysaccharide is not sized.

In an embodiment, the isolated serotype 23B capsular polysaccharide before conjugation has a weight average molecular weight between 50 kDa and 750 kDa. In an embodiment, the isolated serotype 23B capsular polysaccharide before conjugation has a weight average molecular weight between 75 kDa and 400 kDa. In an even preferred embodiment, the isolated serotype 23B capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 250 kDa.

The weight average molecular weight (Mw) of the saccharide before conjugation refers to the Mw before activation of the polysaccharide (i.e. after an eventual sizing step but before reacting the polysaccharide with an activating agent). In the context of the present invention the Mw of the 23B polysaccharide is not substantially modified by the activation step and the Mw of the 23B polysaccharide incorporated in the conjugate is similar to the Mw of the polysaccharide as measured before activation.

In an embodiment, the serotype 23B glycoconjugate of the present invention comprises a serotype 23B capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 40 kDa and 600 kDa. In an embodiment, the weight average molecular weight (Mw) is between 50 kDa and 300 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 100 kDa and 200 kDa.

In some embodiments, the serotype 23B glycoconjugate of the invention has a weight average molecular weight (Mw) of between 250 kDa and 7,500 kDa. In other embodiments, the serotype 23B glycoconjugate has a weight average molecular weight (Mw) of between 500 kDa and 4,000 kDa. Preferably, the serotype 23B glycoconjugate has a weight average molecular weight (Mw) of between 700 kDa and 2,000 kDa.

The serotype 23B glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein. In some embodiments, the ratio of serotype 23B polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.5 and 1.5. Even more preferably, the
5 saccharide to carrier protein ratio (w/w) is between 0.6 and 1.3.

Another way to characterize the serotype 23B glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM197, DT or TT) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent linkages
10 to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered compared to the carrier protein starting material used to generate the conjugate materials. In a preferred embodiment, the degree of conjugation of the serotype 23B glycoconjugate of the invention is between 2 and 15. Preferably, the degree of conjugation of the
15 serotype 23B glycoconjugate of the invention is between 5 and 12.

The serotype 23B glycoconjugates and immunogenic compositions of the invention may contain free saccharide that is not covalently conjugated to the carrier protein but is nevertheless present in the glycoconjugate composition. The free saccharide may be noncovalently associated with (i.e., noncovalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.
20

In an embodiment, the serotype 23B glycoconjugate comprises less than about 40% of free serotype 23B polysaccharide compared to the total amount of serotype 23B polysaccharide. In a preferred embodiment the serotype 23B glycoconjugate comprises less than about 25% of free serotype 23B polysaccharide compared to the total amount of serotype 23B polysaccharide.

The serotype 23B glycoconjugates may also be characterized by their molecular size distribution (Kd). Size exclusion chromatography media (CL-4B) can be used to determine the relative molecular size distribution of the conjugate. Size Exclusion Chromatography (SEC) is used in gravity fed columns to profile the molecular size distribution of conjugates. Large molecules excluded from the pores in the media elute more quickly than small molecules. Fraction collectors are used to collect the column eluate. The fractions are tested colorimetrically
25 by saccharide assay. For the determination of Kd, columns are calibrated to establish the fraction at which molecules are fully excluded (V₀), (K_d=0), and the fraction representing the maximum retention (V_i), (K_d=1). The fraction at which a specified sample attribute is reached (V_e), is related to Kd by the expression, $K_d = (V_e - V_0) / (V_i - V_0)$.
30

In an embodiment, at least 30% of the serotype 23B glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 35% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 40% and 60% of the serotype 23B glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.
35

In an embodiment, the serotype 23B saccharide is activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide is then coupled directly or via a spacer (linker) group to an amino group on the carrier protein (preferably CRM₁₉₇). 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 N-[γ-maleimidobutyroxy]succinimide ester (GMBS)) or a haloacetylated carrier protein (for example using iodoacetamide, N-succinimidyl bromoacetate (SBA; SIB), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB), N-succinimidyl iodoacetate (SIA) or succinimidyl 3-[bromoacetamido]propionate (SBAP)). Preferably, the cyanate ester is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein (e.g., CRM₁₉₇) using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO 93/15760, WO 95/08348 and WO 96/129094.

Other suitable techniques for conjugation use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with 1,1'-carbonyldiimidazole (CDI) (see Bethell et al. (1979) J. Biol. Chem. 254:2572-2574; Hearn et al. (1981) J. Chromatogr. 218:509-518) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

In a preferred embodiment, the serotype 23B glycoconjugates of the invention are prepared using reductive amination chemistry. According to the present invention, reductive amination involves two steps, (1) oxidation (activation) of a purified saccharide, (2) reduction of the activated saccharide and a carrier protein to form a glycoconjugate (see e.g. WO2006/110381, WO2008/079653, WO2008/143709, WO2008/079732, WO2011/110531, WO2012/119972, WO2015110941, WO2015110940, WO2018/144439, WO2018/156491).

In an embodiment, the serotype 23B glycoconjugates of the invention are prepared by conjugating an isolated serotype 23B capsular polysaccharide to a carrier protein by a process comprising the step of:

- (a) reacting said isolated serotype 23B capsular polysaccharide with an oxidizing agent;
- (b) compounding the activated polysaccharide of step (a) with a carrier protein; and
- (c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

As mentioned above, before oxidation, sizing of the isolated serotype 23B capsular polysaccharide to a target molecular weight (MW) range can be performed. Therefore, in an embodiment, the isolated serotype 23B capsular polysaccharide is sized before oxidation.

5 In a preferred embodiment, the size of the isolated serotype 23B capsular polysaccharide is reduced by mechanical homogenization. In an embodiment, the size of the isolated serotype 23B polysaccharide is reduced by high pressure homogenization.

In an embodiment, the isolated serotype 23B capsular polysaccharide is not sized before oxidation.

10 In an embodiment, the oxidation step (a) is carried out at a pH of between 4.0 and 6.5. Preferably, the oxidation step (a) is carried out at a pH of between 4.5 and 5.5.

In an embodiment, the oxidation step (a) is carried out at a pH of about 5.0.

Following the oxidation step (a) the saccharide is said to be activated and is referred to as "activated polysaccharide".

15 In an embodiment, the activated serotype 23B polysaccharide of the present invention has a weight average molecular weight (Mw) of between 40 kDa and 600 kDa. In an embodiment, the weight average molecular weight (Mw) is between 50 kDa and 300 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 100 kDa and 200 kDa.

20 In an embodiment, the oxidizing agent is any oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde. In an embodiment, the oxidizing agent is periodate. For the purpose of the present invention, 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).

25 In a preferred embodiment, the oxidizing agent is sodium periodate. In an embodiment, the periodate used for the oxidation is metaperiodate. In a most preferred embodiment, the periodate used for the oxidation is sodium metaperiodate.

When a polysaccharide reacts with periodate, periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. For this reason, the term "reacting a polysaccharide with periodate" includes oxidation of vicinal hydroxyl groups by periodate.

30 In one embodiment step a) comprises reacting the polysaccharide with 0.05-1 molar equivalents of periodate. Preferably step a) comprises reacting the polysaccharide with 0.1-0.3 molar equivalents of periodate. Most preferably, step a) comprises reacting the polysaccharide with about 0.2 molar equivalents of periodate.

35 In an embodiment the degree of oxidation (also named "degree of activation" in the present document) of the activated serotype 23B polysaccharide is between 2 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 23B polysaccharide is between 4 and 15. In a most preferred embodiment the degree of oxidation of the activated serotype 23B polysaccharide is 9 ± 3 .

In one embodiment the activated serotype 23B polysaccharide and the carrier protein are lyophilised before step b). Preferably lyophilisation occurs after step a). In one embodiment the activated polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised.

5 In one embodiment the activated serotype 23B polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised, and the activated polysaccharide and the carrier protein are reconstituted in the same solution.

In an embodiment, the activated serotype 23B polysaccharide and the carrier protein are lyophilized independently (discrete lyophilization). In an embodiment, the activated serotype 23B polysaccharide and the carrier protein are lyophilized together (co-lyophilized).

10 In one embodiment the lyophilization takes place in the presence of a non-reducing sugar, possible non-reducing sugars include sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit. In an embodiment the sugar is selected from the group consisting of sucrose, trehalose, and mannitol. In an embodiment the sugar is sucrose, trehalose or mannitol. In an embodiment the sugar is sucrose.

15 In an embodiment the initial input ratio (weight by weight) of activated serotype 23B capsular polysaccharide to carrier protein at step b) is between 2:1 and 0.5:1. In an embodiment the initial input ratio (weight by weight) of activated serotype 23B capsular polysaccharide to carrier protein is between 1.2:1 and 0.6:1. Preferably, the initial input ratio (weight by weight) of activated serotype 23B capsular polysaccharide to carrier protein is between 0.9:1 and 0.7:1.

20 In an embodiment, the reduction reaction (c) is carried out in aqueous solvent. Preferably, the reduction reaction (c) is carried out in aprotic solvent.

In an embodiment, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO) or dimethylformamide (DMF). Preferably, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO).

25 In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) or in DMF (dimethylformamide) solvent.

Most preferably, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent.

30 In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxymethylborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMeiPrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane (PEMB). In an embodiment, the reducing agent is sodium triacetoxymethylborohydride. In an embodiment, the reducing agent is sodium cyanoborohydride in the present of nickel (see WO2018144439). In a preferred embodiment, the reducing agent is sodium cyanoborohydride.

35 In one embodiment between 0.2 and 5 molar equivalents of reducing agent is used in step c). Preferably, between 0.5 and 1.5 molar equivalents of reducing agent is used in step c). Most preferably, between 0.9 and 1.1 molar equivalent of reducing agent is used in step c).

At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride (NaBH₄).

5 In an embodiment capping is achieved by mixing the product of step c) with 1 to 20 molar equivalents of sodium borohydride. In an embodiment capping is achieved by mixing the product of step c) with 1 to 3 molar equivalents of sodium borohydride.

Following conjugation to the carrier protein, the serotype 23B glycoconjugate can be purified (enriched with respect to the amount of saccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, 10 concentration/diafiltration operations, tangential flow filtration precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. Therefore, in one embodiment the process for producing the serotype 23B glycoconjugate of the present invention comprises the step of purifying the glycoconjugate after it is produced.

15 1.8 *S. pneumoniae* serotype 24F glycoconjugate of the invention

In an aspect the invention relates to *S. pneumoniae* serotype 24F glycoconjugates.

The structure of *Streptococcus pneumoniae* serotype 24F polysaccharide is known in the art (see e.g. WO2019050815).

20 In an embodiment, the capsular *S. pneumoniae* serotype 24F saccharide used in the present invention is a synthetic carbohydrate.

In a preferred embodiment though, the source of bacterial polysaccharide according to this invention can be *Streptococcus pneumoniae* serotype 24F bacterial cells. Bacterial strains which can be used as source of *Streptococcus pneumoniae* serotype 24F polysaccharides may be obtained from established culture collections (such as for example from the Streptococcal 25 Reference Laboratory (Centers for Disease Control and Prevention, Atlanta, GA USA)) or clinical specimens.

Serotype 24F saccharides can be obtained directly from bacteria using isolation procedures known to one of ordinary skill in the art. They can also be purchased (such as for example from the American Type Culture Collection (ATCC, Manassas, VA USA) (e.g., reference 30 No. ATCC 551-X, No. ATCC 552-X, No. ATCC 553-X)).

In case the serotype 24F saccharide is obtained directly from bacteria, the bacterial cells can be grown in a medium, preferably in a soy based medium. Following fermentation of bacterial cells that produce *S. pneumoniae* serotype 24F capsular polysaccharides, the bacterial cells can be lysed to produce a cell lysate. The serotype 24F polysaccharide may then be isolated from the 35 cell lysate using purification techniques known in the art, including the use of centrifugation, depth filtration, precipitation, ultra-filtration, treatment with activate carbon, diafiltration and/or column chromatography (see, for example, US2006/0228380, US2006/0228381, WO2008/118752 and

WO2020170190). The purified serotype 24F capsular polysaccharide can then be used for the preparation of glycoconjugates.

The isolated serotype 24F capsular saccharide obtained by purification of serotype 24F polysaccharide from the *S. pneumoniae* lysate and optionally sizing of the purified polysaccharide can be characterized by different parameters including, for example the weight average molecular weight (Mw).

The molecular weight of the polysaccharide can be measured by Size Exclusion Chromatography (SEC) combined with Multiangle Laser Light Scattering detector (MALLS).

In a preferred embodiment, the isolated serotype 24F capsular polysaccharide (i.e. purified before further treatment) has a weight average molecular weight between 100 kDa and 2500 kDa. In an embodiment, the isolated serotype 24F capsular polysaccharide has a weight average molecular weight between 250 kDa and 2000 kDa. In an embodiment, the isolated serotype 24F capsular polysaccharide has a weight average molecular weight between 500 kDa and 1000 kDa.

Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In order to generate serotype 24F conjugates with advantageous filterability characteristics, immunogenicity and/or yields, sizing of the polysaccharide to a target molecular weight range can be performed prior to the conjugation to a carrier protein. Advantageously, the size of the purified serotype 24F polysaccharide is reduced while preserving critical features of the structure of the polysaccharide. Mechanical or chemical sizing maybe employed.

Most preferably, the size of the purified serotype 24F polysaccharide is reduced by mechanical homogenization.

It has been discovered that the use of acid hydrolysis, as for example recommended in WO2019050815 or in WO2019050818 is not appropriate to reduce the size of serotype 24F polysaccharide.

It has been found that acid hydrolysis can affect serotype 24F polysaccharide structure integrity. Even relatively mild hydrolysis (e.g. treatment with acetic acid at 100mM at 80°C for about 2 hour) has been found to lead to a 25% loss of the branched ribose residue (see figure 2).

Mechanical sizing allows not to loose this residue, which can be important from an immunological point of view.

Therefore, in a preferred embodiment, the size of the purified serotype 24F polysaccharide is reduced by mechanical homogenization.

In an embodiment, the size of the purified serotype 24F polysaccharide is reduced by high pressure homogenization. High pressure homogenization achieves high shear rates by pumping the process stream through a flow path with sufficiently small dimensions. The shear rate is increased by using a larger applied homogenization pressure, and exposure time can be increased by recirculating the feed stream through the homogenizer.

In a most preferred embodiment, the process to prepare serotype 24F glycoconjugate of the invention does not comprise a step of sizing of the serotype 24F polysaccharide by acid hydrolysis.

5 In an embodiment, the isolated serotype 24F capsular polysaccharide is sized to a weight average molecular weight of between 50 kDa and 500 kDa. In a preferred embodiment, the isolated serotype 24F capsular polysaccharide is sized to a weight average molecular weight of between 75 kDa and 400 kDa. In an even preferred embodiment, the isolated serotype 24F capsular polysaccharide is sized to a weight average molecular weight of between 125 kDa and 275 kDa. In a most preferred embodiment, the isolated serotype 24F capsular polysaccharide is
10 sized to a weight average molecular weight of between 125 kDa and 225 kDa. Preferably, the isolated serotype 24F polysaccharide is sized by mechanical homogenization, most preferably by high pressure homogenization.

In an embodiment, the isolated serotype 24F capsular polysaccharide is not sized.

In an embodiment, the isolated serotype 24F capsular polysaccharide before conjugation
15 has a weight average molecular weight between 50 kDa and 500 kDa. In an embodiment, the isolated serotype 24F capsular polysaccharide before conjugation has a weight average molecular weight between 75 kDa and 400 kDa. In an even preferred embodiment, the isolated serotype 24F capsular polysaccharide before conjugation has a weight average molecular weight between 125 kDa and 275 kDa. In a most preferred embodiment, the isolated serotype 24F
20 capsular polysaccharide before conjugation has a weight average molecular weight between 125 kDa and 225 kDa.

The weight average molecular weight (Mw) of the saccharide before conjugation refers to the Mw before activation of the polysaccharide (i.e. after an eventual sizing step but before reacting the polysaccharide with an activating agent). In the context of the present invention the
25 Mw of the 24F polysaccharide is not substantially modified by the activation step and the Mw of the 24F polysaccharide incorporated in the conjugate is similar to the Mw of the polysaccharide as measured before activation.

In an embodiment, the serotype 24F glycoconjugate of the present invention comprises a serotype 24F capsular polysaccharide wherein the weight average molecular weight (Mw) of said
30 polysaccharide before conjugation is between 50 kDa and 400 kDa. In an embodiment, the weight average molecular weight (Mw) is between 120 kDa and 250 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 120 kDa and 200 kDa.

In some embodiments, the serotype 24F glycoconjugate of the invention has a weight average molecular weight (Mw) of between 500 kDa and 10,000 kDa. In other embodiments, the
35 serotype 24F glycoconjugate has a weight average molecular weight (Mw) of between 1,500 kDa and 7,500 kDa. Preferably, the serotype 24F glycoconjugate has a weight average molecular weight (Mw) of between 3,000 kDa and 6,000 kDa.

The serotype 24F glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein. In some embodiments, the ratio of serotype 24F polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.5 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.7 and 1.5. Even more preferably, the
5 saccharide to carrier protein ratio (w/w) is between 0.8 and 1.4.

Another way to characterize the serotype 24F glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM197, DT or TT) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent linkages
10 to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered compared to the carrier protein starting material used to generate the conjugate materials. In a preferred embodiment, the degree of conjugation of the serotype 24F glycoconjugate of the invention is between 2 and 15. Preferably, the degree of conjugation of the
15 serotype 24F glycoconjugate of the invention is between 5 and 12.

The serotype 24F glycoconjugates and immunogenic compositions of the invention may contain free saccharide that is not covalently conjugated to the carrier protein but is nevertheless present in the glycoconjugate composition. The free saccharide may be noncovalently associated with (i.e., noncovalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.
20

In an embodiment, the serotype 24F glycoconjugate comprises less than about 40% of free serotype 24F polysaccharide compared to the total amount of serotype 24F polysaccharide. In a preferred embodiment the serotype 24F glycoconjugate comprises less than about 25% of free serotype 24F polysaccharide compared to the total amount of serotype 24F polysaccharide.

The serotype 24F glycoconjugates may also be characterized by their molecular size distribution (Kd). Size exclusion chromatography media (CL-4B) can be used to determine the relative molecular size distribution of the conjugate. Size Exclusion Chromatography (SEC) is used in gravity fed columns to profile the molecular size distribution of conjugates. Large molecules excluded from the pores in the media elute more quickly than small molecules. Fraction collectors are used to collect the column eluate. The fractions are tested colorimetrically
25 by saccharide assay. For the determination of Kd, columns are calibrated to establish the fraction at which molecules are fully excluded (V_0), ($K_d=0$), and the fraction representing the maximum retention (V_i), ($K_d=1$). The fraction at which a specified sample attribute is reached (V_e), is related to Kd by the expression, $K_d = (V_e - V_0) / (V_i - V_0)$.
30

In an embodiment, at least 40% of the serotype 24F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 50% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 90% of the serotype 24F glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.
35

The serotype 24F glycoconjugates may also be characterized by the amount of branched Ribose residue which remains in the 24F polysaccharide. As mentioned above, it has been found that serotype 24F polysaccharide can lose the branched ribose residue (see figure 7).

Therefore, in an embodiment, the *S. pneumoniae* serotype 24F glycoconjugate of the present invention comprises a *S. pneumoniae* serotype 24F capsular polysaccharide wherein said *S. pneumoniae* serotype 24F capsular polysaccharide has a Ribose content greater than 75% when compared to native *S. pneumoniae* serotype 24F capsular polysaccharide wherein the Ribose content is considered to be about 100%. In an embodiment, the *S. pneumoniae* serotype 24F glycoconjugate of the present invention comprises a *S. pneumoniae* serotype 24F capsular polysaccharide wherein said *S. pneumoniae* serotype 24F capsular polysaccharide has a Ribose content greater than 90% when compared to native *S. pneumoniae* serotype 24F capsular polysaccharide wherein the Ribose content is considered to be about 100%. Preferably, the *S. pneumoniae* serotype 24F glycoconjugate of the present invention comprises a *S. pneumoniae* serotype 24F capsular polysaccharide wherein said *S. pneumoniae* serotype 24F capsular polysaccharide has a Ribose content greater than 95% when compared to native *S. pneumoniae* serotype 24F capsular polysaccharide wherein the Ribose content is considered to be about 100%.

In a most preferred embodiment, the *S. pneumoniae* serotype 24F glycoconjugate of the present invention comprises a *S. pneumoniae* serotype 24F capsular polysaccharide wherein said *S. pneumoniae* serotype 24F capsular polysaccharide has a Ribose content of about 100% when compared to native *S. pneumoniae* serotype 24F capsular polysaccharide wherein the Ribose content is considered to be about 100%.

In an embodiment, the serotype 24F saccharide is activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide is then coupled directly or via a spacer (linker) group to an amino group on the carrier protein (preferably CRM₁₉₇). 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 N-[γ-maleimidobutyroxy]succinimide ester (GMBS)) or a haloacetylated carrier protein (for example using iodoacetamide, N-succinimidyl bromoacetate (SBA; SIB), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB), N-succinimidyl iodoacetate (SIA) or succinimidyl 3-[bromoacetamido]propionate (SBAP)). Preferably, the cyanate ester is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein (e.g., CRM₁₉₇) using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO 93/15760, WO 95/08348 and WO 96/129094.

Other suitable techniques for conjugation use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, TSTU. Many are described

in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with 1,1'-carbonyldiimidazole (CDI) (see Bethell et al. (1979) J. Biol. Chem. 254:2572-2574; Hearn et al. (1981) J. Chromatogr. 218:509-518) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

In a preferred embodiment, the serotype 24F glycoconjugates of the invention are prepared using reductive amination chemistry. According to the present invention, reductive amination involves two steps, (1) oxidation (activation) of a purified saccharide, (2) reduction of the activated saccharide and a carrier protein to form a glycoconjugate.

In an embodiment, the serotype 24F glycoconjugates of the invention are prepared by conjugating an isolated serotype 24F capsular polysaccharide to a carrier protein by a process comprising the step of: (a) reacting said isolated serotype 24F capsular polysaccharide with an oxidizing agent; (b) compounding the activated polysaccharide of step (a) with a carrier protein; and (c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

As mentioned above, before oxidation, sizing of the isolated serotype 24F capsular polysaccharide to a target molecular weight (MW) range can be performed. Therefore, in an embodiment, the isolated serotype 24F capsular polysaccharide is sized before oxidation.

In a preferred embodiment, the size of the isolated serotype 24F capsular polysaccharide is reduced by mechanical homogenization. In an embodiment, the size of the isolated serotype 24F polysaccharide is reduced by high pressure homogenization. In a most preferred embodiment, the isolated serotype 24F capsular polysaccharide is not sized by acid hydrolysis.

In an embodiment, the isolated serotype 24F capsular polysaccharide is not sized before oxidation.

In an embodiment, the oxidation step (a) is carried out at a pH of between 4.5 and 6.5. Preferably, the oxidation step (a) is carried out at a pH of between 5.0 and 6.0.

In an embodiment, the oxidation step (a) is carried out at a pH of about 5.0.

Following the oxidation step (a) the saccharide is said to be activated and is referred to as "activated polysaccharide".

In an embodiment, the activated serotype 24F polysaccharide of the present invention has a weight average molecular weight (Mw) of between 50 kDa and 400 kDa. In an embodiment, the weight average molecular weight (Mw) is between 120 kDa and 250 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 120 kDa and 200 kDa.

In an embodiment, the activated serotype 24F polysaccharide of the present invention retains at least 75% of the branched Ribose. In an embodiment, the activated serotype 24F

polysaccharide of the present invention retains at least 90% of the branched Ribose. In a preferred embodiment, the activated serotype 24F polysaccharide of the present invention retains at least 95% of the branched Ribose.

5 In a most preferred embodiment, the activated serotype 24F polysaccharide of the present invention retains about 100% of the branched Ribose.

In an embodiment, the oxidizing agent is any oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde. In an embodiment, the oxidizing agent is periodate. For the purpose of the present invention, 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
10 of periodate (e.g., sodium periodate and potassium periodate).

In a preferred embodiment, the oxidizing agent is sodium periodate. In an embodiment, the periodate used for the oxidation is metaperiodate. In a most preferred embodiment, the periodate used for the oxidation is sodium metaperiodate.

15 When a polysaccharide reacts with periodate, periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. For this reason, the term "reacting a polysaccharide with periodate" includes oxidation of vicinal hydroxyl groups by periodate.

In one embodiment step a) comprises reacting the polysaccharide with 0.1-2 molar equivalents of periodate. Preferably step a) comprises reacting the polysaccharide with 0.5-1.5
20 molar equivalents of periodate. Most preferably, step a) comprises reacting the polysaccharide with 0.9-1.1 molar equivalents of periodate.

In an embodiment the degree of oxidation (also named "degree of activation" in the present document) of the activated serotype 24F polysaccharide is between 2 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 24F polysaccharide is between 4
25 and 15. In a most preferred embodiment the degree of oxidation of the activated serotype 24F polysaccharide is 9 ± 3 .

In one embodiment the activated serotype 24F polysaccharide and the carrier protein are lyophilised before step b). Preferably lyophilisation occurs after step a). In one embodiment the activated polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised.

30 In one embodiment the activated serotype 24F polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised, and the activated polysaccharide and the carrier protein are reconstituted in the same solution.

In an embodiment, the activated serotype 24F polysaccharide and the carrier protein are lyophilized independently (discrete lyophilization). In an embodiment, the activated serotype 24F
35 polysaccharide and the carrier protein are lyophilized together (co-lyophilized).

In one embodiment the lyophilization takes place in the presence of a non-reducing sugar, possible non-reducing sugars include sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinin. In an embodiment the sugar is selected from the group

consisting of sucrose, trehalose, and mannitol. In an embodiment the sugar is sucrose, trehalose or mannitol. In an embodiment the sugar is sucrose.

5 In an embodiment the initial input ratio (weight by weight) of activated serotype 24F capsular polysaccharide to carrier protein at step b) is between 2:1 and 0.5:1. In an embodiment the initial input ratio (weight by weight) of activated serotype 24F capsular polysaccharide to carrier protein is between 1.2:1 and 0.6:1. Preferably, the initial input ratio (weight by weight) of activated serotype 24F capsular polysaccharide to carrier protein is between 0.9:1 and 0.7:1.

In an embodiment, the reduction reaction (c) is carried out in aqueous solvent. Preferably, the reduction reaction (c) is carried out in aprotic solvent.

10 In an embodiment, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO) or dimethylformamide (DMF). Preferably, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO).

In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) or in DMF (dimethylformamide) solvent.

15 Most preferably, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent.

In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxyborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMeiPrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane (PEMB). In an embodiment, the reducing agent is sodium triacetoxyborohydride. In an embodiment, the reducing agent is sodium cyanoborohydride in the present of nickel (see WO2018144439). In a preferred embodiment, the reducing agent is sodium cyanoborohydride.

25 In one embodiment between 0.2 and 5 molar equivalents of reducing agent is used in step c). Preferably, between 0.5 and 2.5 molar equivalents of reducing agent is used in step c). Most preferably, between 1.5 and 2.5 molar equivalent of reducing agent is used in step c).

In one embodiment about 2 molar equivalents of reducing agent is used in step c).

30 At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride (NaBH₄).

In an embodiment capping is achieved by mixing the product of step c) with 1 to 20 molar equivalents of sodium borohydride. In an embodiment capping is achieved by mixing the product of step c) with 1 to 3 molar equivalents of sodium borohydride.

35 In an embodiment capping is achieved by mixing the product of step c) with about 2 molar equivalents of sodium borohydride.

Following conjugation to the carrier protein, the serotype 24F glycoconjugate can be purified (enriched with respect to the amount of saccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis,

concentration/diafiltration operations, tangential flow filtration precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. Therefore, in one embodiment the process for producing the serotype 24F glycoconjugate of the present invention comprises the step of purifying the glycoconjugate after it is produced.

5

1.9 *S. pneumoniae* serotype 35B glycoconjugate of the invention

In an aspect the invention relates to *S. pneumoniae* serotype 23B glycoconjugates.

The structure of *Streptococcus pneumoniae* serotype 35B polysaccharide is known in the art (see e.g. Geno K *et al.* (2015) Clin Microbiol Rev Vol 28:3, p 871-899).

10 In an embodiment, the capsular *S. pneumoniae* serotype 35B saccharide used in the present invention is a synthetic carbohydrate.

In a preferred embodiment though, the source of bacterial polysaccharide according to this invention can be *Streptococcus pneumoniae* serotype 35B bacterial cells. Bacterial strains which can be used as source of *Streptococcus pneumoniae* serotype 35B polysaccharides may
15 be obtained from established culture collections (such as for example from the Streptococcal Reference Laboratory (Centers for Disease Control and Prevention, Atlanta, GA USA)) or clinical specimens.

Serotype 35B saccharides can be obtained directly from bacteria using isolation procedures known to one of ordinary skill in the art. They can also be purchased (such as for
20 example from the American Type Culture Collection (ATCC, Manassas, VA USA) (e.g., reference No. ATCC 539-X, No. ATCC 540-X, No. ATCC 541-X)).

In case the serotype 35B saccharide is obtained directly from bacteria, the bacterial cells can be grown in a medium, preferably in a soy based medium. Following fermentation of bacterial cells that produce *S. pneumoniae* serotype 35B capsular polysaccharides, the bacterial cells can
25 be lysed to produce a cell lysate. The serotype 35B polysaccharide may then be isolated from the cell lysate using purification techniques known in the art, including the use of centrifugation, depth filtration, precipitation, ultra-filtration, treatment with activate carbon, diafiltration and/or column chromatography (see, for example, US2006/0228380, US2006/0228381, WO2008/118752 and WO2020170190). The purified serotype 35B capsular polysaccharide can then be used for the
30 preparation of glycoconjugates.

The isolated serotype 35B capsular saccharide obtained by purification of serotype 35B polysaccharide from the *S. pneumoniae* lysate and optionally sizing of the purified polysaccharide can be characterized by different parameters including, for example the weight average molecular weight (Mw).

35 The molecular weight of the polysaccharide can be measured by Size Exclusion Chromatography (SEC) combined with Multiangle Laser Light Scattering detector (MALLS).

In a preferred embodiment, the isolated serotype 35B capsular polysaccharide (i.e. purified before further treatment) has a weight average molecular weight between 100 kDa and

5000 kDa. In an embodiment, the isolated serotype 35B capsular polysaccharide has a weight average molecular weight between 300 kDa and 2000 kDa. In a preferred embodiment, the isolated serotype 35B capsular polysaccharide has a weight average molecular weight between 500 kDa and 1000 kDa.

5 Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

The size of the purified serotype 35B polysaccharide may be reduced while preserving critical features of the structure of the polysaccharide. Mechanical or chemical sizing maybe employed.

10 However, *S. pneumoniae* serotype 35B polysaccharide has been found to be cleaved during activation with periodate, which is the classical oxidant used in the commonly used reductive amination process. It appears that periodate oxidation occurs on the backbone of serotype 35B polysaccharide and cleaves the mannitol or ribitol, leading to size reduction. The activation with periodate results in a decrease in polysaccharide Mw. Therefore, in the case where
15 activation with periodate, is used, the serotype 35B capsular polysaccharide is not sized.

Therefore, in an embodiment, the isolated serotype 35B capsular polysaccharide is not sized.

In an embodiment, the isolated serotype 35B capsular polysaccharide before conjugation has a weight average molecular weight between 100 kDa and 5,000 kDa. In an embodiment, the
20 isolated serotype 35B capsular polysaccharide before conjugation has a weight average molecular weight between 300 kDa and 2000 kDa. In an even preferred embodiment, the isolated serotype 35B capsular polysaccharide before conjugation has a weight average molecular weight between 500 kDa and 1000 kDa.

The weight average molecular weight (Mw) of the saccharide before conjugation refers to the Mw before activation of the polysaccharide (i.e. before reacting the polysaccharide with an activating agent).
25

In an embodiment, the serotype 35B glycoconjugate of the present invention comprises a serotype 35B capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide is between 15 kDa and 100 kDa. In an embodiment, the weight average molecular
30 weight (Mw) is between 25 kDa and 50 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between about 30 kDa and about 40 kDa.

In some embodiments, the serotype 35B glycoconjugate of the invention has a weight average molecular weight (Mw) of between 250 kDa and 7,500 kDa. In other embodiments, the serotype 35B glycoconjugate has a weight average molecular weight (Mw) of between 500 kDa
35 and 5,000 kDa. Preferably, the serotype 35B glycoconjugate has a weight average molecular weight (Mw) of between 1,000 kDa and 4,000 kDa.

The serotype 35B glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein. In some embodiments, the ratio of serotype 35B

polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.4 and 3.0. Preferably, the saccharide to carrier protein ratio (w/w) is between 0.4 and 2.0. Even more preferably, the saccharide to carrier protein ratio (w/w) is between 0.5 and 1.5.

Another way to characterize the serotype 35B glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM197, DT or TT) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent linkages to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered compared to the carrier protein starting material used to generate the conjugate materials. In a preferred embodiment, the degree of conjugation of the serotype 35B glycoconjugate of the invention is between 2 and 15. Preferably, the degree of conjugation of the serotype 35B glycoconjugate of the invention is between 5 and 10.

The serotype 35B glycoconjugates and immunogenic compositions of the invention may contain free saccharide that is not covalently conjugated to the carrier protein but is nevertheless present in the glycoconjugate composition. The free saccharide may be noncovalently associated with (i.e., noncovalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.

In an embodiment, the serotype 35B glycoconjugate comprises less than about 40% of free serotype 35B polysaccharide compared to the total amount of serotype 35B polysaccharide. In an embodiment, the serotype 35B glycoconjugate comprises less than about 20% of free serotype 35B polysaccharide compared to the total amount of serotype 35B polysaccharide. In a preferred embodiment the serotype 35B glycoconjugate comprises less than about 10% of free serotype 35B polysaccharide compared to the total amount of serotype 35B polysaccharide.

The serotype 35B glycoconjugates may also be characterized by their molecular size distribution (Kd). Size exclusion chromatography media (CL-4B) can be used to determine the relative molecular size distribution of the conjugate. Size Exclusion Chromatography (SEC) is used in gravity fed columns to profile the molecular size distribution of conjugates. Large molecules excluded from the pores in the media elute more quickly than small molecules. Fraction collectors are used to collect the column eluate. The fractions are tested colorimetrically by saccharide assay. For the determination of Kd, columns are calibrated to establish the fraction at which molecules are fully excluded (V_0), ($K_d=0$), and the fraction representing the maximum retention (V_i), ($K_d=1$). The fraction at which a specified sample attribute is reached (V_e), is related to Kd by the expression, $K_d = (V_e - V_0) / (V_i - V_0)$.

In an embodiment, at least 40% of the serotype 35B glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 60% of the glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 80% of the serotype 35B glycoconjugate has a Kd below or equal to 0.3 in a CL-4B column.

In an embodiment, the serotype 35B saccharide is activated with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide is then coupled directly or via a spacer (linker) group to an amino group on the carrier protein (preferably CRM₁₉₇). 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 N-[γ-maleimidobutyroxy]succinimide ester (GMBS)) or a haloacetylated carrier protein (for example using iodoacetamide, N-succinimidyl bromoacetate (SBA; SIB), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-SIAB), N-succinimidyl iodoacetate (SIA) or succinimidyl 3-[bromoacetamido]propionate (SBAP)). Preferably, the cyanate ester is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein (e.g., CRM₁₉₇) using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in WO 93/15760, WO 95/08348 and WO 96/129094.

Other suitable techniques for conjugation use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with 1,1'-carbonyldiimidazole (CDI) (see Bethell et al. (1979) J. Biol. Chem. 254:2572-2574; Hearn et al. (1981) J. Chromatogr. 218:509-518) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

In a preferred embodiment, the serotype 35B glycoconjugates of the invention are prepared using reductive amination chemistry. According to the present invention, reductive amination involves two steps, (1) oxidation (activation) of a purified saccharide, (2) reduction of the activated saccharide and a carrier protein to form a glycoconjugate.

In an embodiment, the serotype 35B glycoconjugates of the invention are prepared by conjugating an isolated serotype 35B capsular polysaccharide to a carrier protein by a process comprising the step of: (a) reacting said isolated serotype 35B capsular polysaccharide with an oxidizing agent; (b) compounding the activated polysaccharide of step (a) with a carrier protein; and (c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

Preferably, the isolated serotype 35B capsular polysaccharide is not sized before oxidation.

In a preferred embodiment, step (a) is quenched by addition of a quenching agent to stop oxidation.

Therefore in a preferred embodiment, the serotype 35B glycoconjugates of the invention are prepared by conjugating an isolated serotype 35B capsular polysaccharide to a carrier protein by a process comprising the step of: (a) reacting said isolated serotype 35B capsular polysaccharide with an oxidizing agent; (a') quenching the oxidation reaction by addition of a quenching agent resulting in an activated serotype 35B capsular polysaccharide; (b) compounding the activated polysaccharide of step (a') with a carrier protein; and (c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

In an embodiment, the oxidation step (a) is carried out at a pH of between 5.0 and 7.0. Preferably, the oxidation step (a) is carried out at a pH of between 5.5 and 6.5.

In an embodiment, the oxidation step (a) is carried out at a pH of about 6.0.

Following the oxidation step (a)-(a') the saccharide is said to be activated and is referred to as "activated polysaccharide".

In an embodiment, the oxidizing agent is any oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde. In an embodiment, the oxidizing agent is periodate. For the purpose of the present invention, 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).

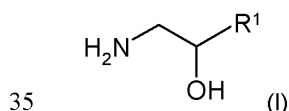
In a preferred embodiment, the oxidizing agent is sodium periodate. In an embodiment, the periodate used for the oxidation is metaperiodate. In a most preferred embodiment, the periodate used for the oxidation is sodium metaperiodate.

When a polysaccharide reacts with periodate, periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. For this reason, the term "reacting a polysaccharide with periodate" includes oxidation of vicinal hydroxyl groups by periodate.

In one embodiment step a) comprises reacting the polysaccharide with 0.05-0.2 molar equivalents of periodate. Preferably step a) comprises reacting the polysaccharide with 0.09-0.11 molar equivalents of periodate. Most preferably, step a) comprises reacting the polysaccharide with about 0.1 molar equivalents of periodate.

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

In one embodiment, the quenching agent is a 1,2-aminoalcohols of formula (I):



wherein R^1 is selected from H, methyl, ethyl, propyl or isopropyl.

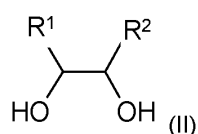
In one embodiment, the quenching agent is selected from sodium and potassium salts of sulfite, bisulfate, dithionite, metabisulfite, thiosulfate, phosphites, hypophosphites or phosphorous acid.

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

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

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

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



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

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

In a preferred embodiment, the isolated serotype 35B polysaccharide is activated by a process comprising the step of:

- (a) reacting the isolated serotype 35B polysaccharide with periodate;
- (b) quenching the oxidation reaction by addition of butan-2,3-diol resulting in an activated serotype 35B polysaccharide.

Following the oxidation step of the polysaccharide, the polysaccharide is said to be activated and is referred to as "activated polysaccharide" here below.

In an embodiment, the activated serotype 35B polysaccharide of the present invention has a weight average molecular weight (Mw) of between 15 kDa and 100 kDa. In an embodiment, the weight average molecular weight (Mw) is between 25 kDa and 50 kDa. In a most preferred embodiment, the weight average molecular weight (Mw) is between 30 kDa and 40 kDa.

In an embodiment the degree of oxidation (also named "degree of activation" in the present document) of the activated serotype 35B polysaccharide is between 2 and 20. In a preferred embodiment the degree of oxidation of the activated serotype 35B polysaccharide is between 4 and 15. In a most preferred embodiment the degree of oxidation of the activated serotype 35B polysaccharide is 9 ± 3 .

In one embodiment the activated serotype 35B polysaccharide and the carrier protein are lyophilised before step b). Preferably lyophilisation occurs after step a). In one embodiment the activated polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised.

In one embodiment the activated serotype 35B polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised, and the activated polysaccharide and the carrier protein are reconstituted in the same solution.

5 In an embodiment, the activated serotype 35B polysaccharide and the carrier protein are lyophilized independently (discrete lyophilization). In an embodiment, the activated serotype 35B polysaccharide and the carrier protein are lyophilized together (co-lyophilized).

In one embodiment the lyophilization takes place in the presence of a non-reducing sugar, possible non-reducing sugars include sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit. In an embodiment the sugar is selected from the group
10 consisting of sucrose, trehalose, and mannitol. In an embodiment the sugar is sucrose, trehalose or mannitol. In an embodiment the sugar is sucrose.

In an embodiment the initial input ratio (weight by weight) of activated serotype 35B capsular polysaccharide to carrier protein at step b) is between 2:1 and 0.5:1. In an embodiment the initial input ratio (weight by weight) of activated serotype 35B capsular polysaccharide to
15 carrier protein is between 1.2:1 and 0.6:1. Preferably, the initial input ratio (weight by weight) of activated serotype 35B capsular polysaccharide to carrier protein is between 0.9:1 and 0.7:1.

In an embodiment, the reduction reaction (c) is carried out in aqueous solvent. Preferably, the reduction reaction (c) is carried out in aprotic solvent.

In an embodiment, the reduction reaction (c) is carried out in the presence of dimethylsulphoxide (DMSO) or dimethylformamide (DMF). Preferably, the reduction reaction (c)
20 is carried out in the presence of dimethylsulphoxide (DMSO).

In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) or in DMF (dimethylformamide)) solvent.

Most preferably, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide)
25 solvent.

In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxyborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMeiPrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane
30 (PEMB). In an embodiment, the reducing agent is sodium triacetoxyborohydride. In an embodiment, the reducing agent is sodium cyanoborohydride in the present of nickel (see WO2018144439). In a preferred embodiment, the reducing agent is sodium cyanoborohydride.

In one embodiment between 0.2 and 5 molar equivalents of reducing agent is used in step c). Preferably, between 0.5 and 1.5 molar equivalents of reducing agent is used in step c). Most
35 preferably, between 0.9 and 1.1 molar equivalent of reducing agent is used in step c).

At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride (NaBH₄).

In an embodiment capping is achieved by mixing the product of step c) with 1 to 20 molar equivalents of sodium borohydride. In an embodiment capping is achieved by mixing the product of step c) with 1 to 3 molar equivalents of sodium borohydride.

5 Following conjugation to the carrier protein, the serotype 35B glycoconjugate can be purified (enriched with respect to the amount of saccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. Therefore, in one embodiment the process for producing the serotype 35B glycoconjugate of the
10 present invention comprises the step of purifying the glycoconjugate after it is produced.

1.10 *S. pneumoniae* serotype 3 glycoconjugates of the invention

In an aspect the invention relates to compositions comprising a *S. pneumoniae* serotype 3 glycoconjugate.

15 The structure of *Streptococcus pneumoniae* serotype 3 polysaccharide is known in the art. The polysaccharide repeating unit of serotype 3 consists of a linear disaccharide unit with one glucopyranose (GlcP) and one glucuronic acid (GlcPA) (see e.g. Geno K et al. (2015) Clin Microbiol Rev Vol 28:3, p 871-899).

In an embodiment, the capsular *S. pneumoniae* serotype 3 saccharide used in the present
20 invention is a synthetic carbohydrate. Preparation of a synthetic *Streptococcus pneumoniae* type 3 capsular saccharide can for example be conducted as disclosed in WO2017178664 or WO2015040140.

In a preferred embodiment though, the source of bacterial polysaccharide according to this invention can be *Streptococcus pneumoniae* serotype 3 bacterial cells. Bacterial strains which
25 can be used as source of *Streptococcus pneumoniae* serotype 3 polysaccharides may be obtained from established culture collections (such as for example from the Streptococcal Reference Laboratory (Centers for Disease Control and Prevention, Atlanta, GA USA)) or clinical specimens.

Serotype 3 polysaccharides can be obtained directly from bacteria using isolation
30 procedures known to one of ordinary skill in the art (see for example methods disclosed in US2006/0228380, US2006/0228381, US2007/0184071, US2007/0184072, US2007/0231340, and US2008/0102498 and WO2008/118752). They can also be produced using synthetic protocols known to the man skilled in the art. They can also be purchased (such as for example from the American Type Culture Collection (ATCC, Manassas, VA USA) (e.g., reference No.
35 ATCC 172-X or ATCC 33-X)).

In case the serotype 3 polysaccharide is obtained directly from bacteria, the bacterial cells can be grown in a medium, preferably in a soy based medium. Following fermentation of bacterial cells that produce *S. pneumoniae* serotype 3 capsular polysaccharides, the bacterial cells can be

lysed to produce a cell lysate. The serotype 3 polysaccharide may then be isolated from the cell lysate using purification techniques known in the art, including the use of centrifugation, depth filtration, precipitation, ultra-filtration, treatment with activate carbon, diafiltration and/or column chromatography (see, for example, US2006/0228380, US2006/0228381 and WO2008/118752).

- 5 The purified serotype 3 capsular polysaccharide can then be used for the preparation of immunogenic conjugates.

The isolated serotype 3 capsular polysaccharide obtained by purification of serotype 3 polysaccharide from the *S. pneumoniae* lysate and optionally sizing of the purified polysaccharide can be characterized by different parameters including, for example the weight average molecular weight (Mw).

10 The molecular weight of the polysaccharide can be measured by Size Exclusion Chromatography (SEC) combined with Multiangle Laser Light Scattering detector (MALLS).

In an embodiment, the isolated serotype 3 capsular polysaccharide (i.e. purified before further treatment) has a weight average molecular weight between 5 kDa and 5,000 kDa. In an embodiment, the isolated capsular polysaccharide has a weight average molecular weight between 100 kDa and 4,000 kDa. In a preferred embodiment, the isolated capsular polysaccharide has a weight average molecular weight between 1,000 kDa and 3,500 kDa.

20 Preferably, in order to generate serotype 3 conjugates with advantageous filterability characteristics, immunogenicity and/or yields, sizing of the polysaccharide to a target molecular weight range is performed prior to the conjugation to a carrier protein. Advantageously, the size of the purified serotype 3 polysaccharide is reduced while preserving critical features of the structure of the polysaccharide. Mechanical or chemical sizing maybe employed.

In an embodiment, the size of the purified serotype 3 polysaccharide is reduced by chemical hydrolysis. Chemical hydrolysis maybe conducted using a mild acid (e.g. acetic acid, formic acid, propanoic acid). In an embodiment, chemical hydrolysis is conducted using formic acid. In an embodiment, chemical hydrolysis is conducted using propanoic acid. In a preferred embodiment, chemical hydrolysis is conducted using acetic acid. Chemical hydrolysis may also be conducted using a diluted strong acid (such as diluted hydrochloric acid, diluted sulfuric acid, diluted phosphoric acid, diluted nitric acid or diluted perchloric acid). In an embodiment, chemical hydrolysis is conducted using diluted hydrochloric acid. In an embodiment, chemical hydrolysis is conducted using diluted sulfuric acid. In an embodiment, chemical hydrolysis is conducted using diluted phosphoric acid. In an embodiment, chemical hydrolysis is conducted using diluted nitric acid. In an embodiment, chemical hydrolysis is conducted using diluted perchloric acid.

35 The size of the purified serotype 3 polysaccharide can also be reduced by mechanical homogenization. In an embodiment, the size of the purified serotype 3 polysaccharide is reduced by high pressure homogenization. High pressure homogenization achieves high shear rates by pumping the process stream through a flow path with sufficiently small dimensions. The shear rate is increased by using a larger applied homogenization pressure, and exposure time can be

increased by recirculating the feed stream through the homogenizer. The high-pressure homogenization process can be appropriate for reducing the size of the purified serotype 3 polysaccharide while preserving the structural features of the polysaccharide.

5 In an embodiment, the isolated serotype 3 capsular polysaccharide is sized to a weight average molecular weight between 5 kDa and 1000 kDa. In an embodiment, the isolated serotype 3 capsular polysaccharide is sized to a weight average molecular weight between 50 kDa and 300 kDa. In a preferred embodiment, the isolated serotype 3 capsular polysaccharide is sized to a weight average molecular weight between 100 kDa and 300 kDa.

10 In an embodiment, the isolated serotype 3 capsular polysaccharide is sized to a weight average molecular weight of between about 200 kDa and about 300 kDa.

In an embodiment, the isolated serotype 3 capsular polysaccharide is sized to a weight average molecular weight of between about 100 kDa and about 200 kDa.

In an embodiment, the isolated serotype 3 capsular polysaccharide is not sized.

15 In an embodiment, the serotype 3 glycoconjugate of the present invention comprises a serotype 3 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 50 kDa and 1,000 kDa. Preferably, the weight average molecular weight (Mw) is between 100 kDa and 300 kDa.

20 The weight average molecular weight (Mw) of the serotype 3 saccharide before conjugation refers to the Mw before the activation of the serotype 3 polysaccharide (i.e. after an eventual sizing step but before reacting the polysaccharide with an activating agent). In the context of the present invention the Mw of the serotype 3 polysaccharide is not substantially modified by the activation step and the Mw of the serotype 3 polysaccharide incorporated in the conjugate is similar to the Mw of the polysaccharide as measured before activation.

25 In an embodiment, the serotype 3 glycoconjugate of the present invention comprises a serotype 3 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 100 kDa and 200 kDa.

30 In an embodiment, the serotype 3 glycoconjugate of the present invention comprises a serotype 3 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide before conjugation is between 200 kDa and 300 kDa.

In some embodiments, the serotype 3 glycoconjugate of the invention has a weight average molecular weight (Mw) of between 250 kDa and 20,000 kDa. In other embodiments, the serotype 3 glycoconjugate has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa. In yet other embodiments, the serotype 3 glycoconjugate has a weight average molecular weight (Mw) of between 500 kDa and 10,000 kDa. Preferably, the serotype 3 glycoconjugate has a weight average molecular weight (Mw) of between 500 kDa and 5,000 kDa.

In an embodiment, the serotype 3 glycoconjugate has a weight average molecular weight (Mw) of between 600 kDa and 3,000 kDa.

The molecular weight of the polysaccharide can be measured by Size Exclusion Chromatography (SEC) combined with Multiangle Laser Light Scattering detector (MALLS).

5 Another way to characterize the serotype 3 glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM₁₉₇ or SCP) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent linkages to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those
10 of skill in the art. Conjugation results in a reduction in the number of lysine residues recovered compared to the carrier protein starting material used to generate the conjugate materials. In a preferred embodiment, the degree of conjugation of the serotype 3 glycoconjugate of the invention is between 2 and 15.

In a preferred embodiment, the degree of conjugation of the serotype 3 glycoconjugate of the
15 invention is between 4 and 7. In some such embodiments, the carrier protein is CRM₁₉₇. In other such embodiments, the carrier protein is SCP.

The serotype 3 glycoconjugates of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein. In some embodiments, the ratio of serotype 3 polysaccharide to carrier protein in the glycoconjugate (w/w) is between 0.5 and 3.0. In other
20 embodiments, the saccharide to carrier protein ratio (w/w) is between 0.5 and 1.5. In a preferred embodiment, the ratio of serotype 3 capsular polysaccharide to carrier protein in the conjugate is between 0.9 and 1.1.

The serotype 3 glycoconjugates of the invention may also be characterized by the number of covalent linkages between the carrier protein and the saccharide as a function of repeat units
25 of the saccharide. In one embodiment, the serotype 3 glycoconjugate of the invention comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 4 saccharide repeat units of the polysaccharide. In another embodiment, the covalent linkage between the carrier protein and the polysaccharide occurs at least once in every 10 saccharide repeat units of the polysaccharide. In another embodiment, the covalent linkage between the
30 carrier protein and the polysaccharide occurs at least once in every 15 saccharide repeat units of the polysaccharide. In a further embodiment, the covalent linkage between the carrier protein and the polysaccharide occurs at least once in every 25 saccharide repeat units of the polysaccharide. In a further embodiment, the covalent linkage between the carrier protein and the polysaccharide occurs at least once in every 50 saccharide repeat units of the polysaccharide. In
35 yet a further embodiment, the covalent linkage between the carrier protein and the polysaccharide occurs at least once in every 100 saccharide repeat units of the polysaccharide.

In other embodiments, the serotype 3 glycoconjugate of the invention comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 5 to 10 saccharide repeat units of the polysaccharide.

5 In other embodiments, the serotype 3 glycoconjugate of the invention comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 10 to 20 saccharide repeat units of the polysaccharide.

In some embodiments, the carrier protein is CRM₁₉₇ and the covalent linkage between the CRM₁₉₇ and the polysaccharide occurs at least once in every 4, 10, 15 or 25 saccharide repeat units of the polysaccharide. In frequent embodiments, the carrier protein is SCP and the covalent linkage
10 between the SCP and the polysaccharide occurs at least once in every 4, 10, 15 or 25 saccharide repeat units of the polysaccharide.

The serotype 3 glycoconjugates and immunogenic compositions of the invention may contain free saccharide that is not covalently conjugated to the carrier protein but is nevertheless present in the glycoconjugate composition. The free saccharide may be noncovalently associated with (i.e.,
15 noncovalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.

In a preferred embodiment, the serotype 3 glycoconjugate comprises less than about 50% of free serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide. In a preferred embodiment the serotype 3 glycoconjugate comprises less than about 40% of free serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide. In a yet
20 preferred embodiment, the serotype 3 glycoconjugate comprises less than about 25% of free serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide. In an even preferred embodiment, the serotype 3 glycoconjugate comprises less than about 20% of free serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide. In a yet preferred embodiment, the serotype 3 glycoconjugate comprises less than about 15% of free
25 serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide.

The serotype 3 glycoconjugates may also be characterized by their molecular size distribution (K_d). Size exclusion chromatography media (CL-4B) can be used to determine the relative molecular size distribution of the conjugate. Size Exclusion Chromatography (SEC) is used in gravity fed columns to profile the molecular size distribution of conjugates. Large molecules
30 excluded from the pores in the media elute more quickly than small molecules. Fraction collectors are used to collect the column eluate. The fractions are tested colorimetrically by saccharide assay. For the determination of K_d , columns are calibrated to establish the fraction at which molecules are fully excluded (V_0), ($K_d=0$), and the fraction representing the maximum retention (V_i), ($K_d=1$). The fraction at which a specified sample attribute is reached (V_e), is related to K_d by the expression, $K_d = (V_e - V_0) / (V_i - V_0)$.
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In a preferred embodiment, at least 30% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 40% of the glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 45%, 50%, 55%,

60%, 65%, 70%, 75%, 80%, or 85% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, at least 60% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 50% and 80% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column. In a preferred embodiment, between 65% and 80% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.

In an embodiment, the serotype 3 glycoconjugates of the present invention are prepared using reductive amination chemistry (see WO2006110381, WO2008143709, PCT/IB2022/054920).

According to the present invention, reductive amination involves two steps, (1) oxidation (activation) of a purified saccharide, (2) reduction of the activated saccharide and a carrier protein (e.g., CRM₁₉₇, TT or SCP) to form a glycoconjugate.

As mentioned above, before oxidation, sizing of the polysaccharide to a target molecular weight (MW) range can be performed.

Therefore, in an embodiment, the isolated polysaccharide is sized before oxidation.

In an embodiment, the isolated polysaccharide is sized to any of the target molecular weight (MW) range defined above.

In an embodiment, the isolated serotype 3 capsular polysaccharide is conjugated to a carrier protein by a process comprising the step of:

- (a) reacting said isolated polysaccharide with an oxidizing agent;
- (b) compounding the activated polysaccharide of step (a) with a carrier protein; and
- (c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

Following the oxidation step (a) the saccharide is said to be activated and is referred to as "activated polysaccharide".

In an embodiment, the oxidizing agent is any oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde. In an embodiment, the oxidizing agent is periodate. For the purpose of the present invention, 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 an embodiment, the oxidizing agent is periodate in the presence of bivalent cations (see WO2008/143709).

In an embodiment, the oxidizing agent is periodic acid. In an embodiment, the oxidizing agent is periodic acid in the presence of bivalent cations. In an embodiment, the oxidizing agent is periodic acid in the presence of Mg^{2+} . In an embodiment, the oxidizing agent is periodic acid in the presence of Ca^{2+} . In an embodiment, the oxidizing agent is orthoperiodate.

In an embodiment, the oxidizing agent is sodium periodate. In an embodiment, the periodate used for the oxidation is metaperiodate. In an embodiment the periodate used for the oxidation is sodium metaperiodate.

When a polysaccharide reacts with periodate, periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. For this reason, the term "reacting a polysaccharide with periodate" includes oxidation of vicinal hydroxyl groups by periodate.

In one embodiment step a) comprises reacting the polysaccharide with 0.01-2 molar equivalents of periodate. Preferably step a) comprises reacting the polysaccharide with 0.1-2 molar equivalents of periodate.

In one embodiment step a) comprises reacting the polysaccharide with 0.01-2 molar equivalents of periodic acid. Preferably step a) comprises reacting the polysaccharide with 0.2-2 molar equivalents of periodic acid.

In a preferred embodiment the degree of oxidation (also named "degree of activation" in the present document) of the activated serotype 3 polysaccharide is between 2 and 30. In a preferred embodiment the degree of oxidation of the activated serotype 3 polysaccharide is between 2 to 20.

In an embodiment the degree of oxidation of the activated serotype 3 polysaccharide is between 2 to 8.

In an embodiment the degree of oxidation of the activated serotype 3 polysaccharide is between 11 to 19.

In one embodiment the activated polysaccharide and the carrier protein are lyophilised before step b). Preferably lyophilisation occurs after step a). In one embodiment the activated polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised.

In one embodiment the activated polysaccharide is lyophilised after step a) and the carrier protein is also lyophilised, and the activated polysaccharide and the carrier protein are reconstituted in the same solution, this acts as compounding the activated polysaccharide and the carrier protein together.

In an embodiment, the activated polysaccharide and the carrier protein are lyophilized independently (discrete lyophilization). In an embodiment, the activated polysaccharide and the carrier protein are lyophilized together (co-lyophilized).

In one embodiment the lyophilization takes place in the presence of a non-reducing sugar, possible non-reducing sugars include sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinin. In an embodiment the sugar is selected from the group consisting of sucrose, trehalose, and mannitol. In an embodiment the sugar is sucrose, trehalose or mannitol. In an embodiment the sugar is trehalose. In an embodiment the sugar is sucrose.

In an embodiment the initial input ratio (weight by weight) of activated serotype 3 capsular polysaccharide to carrier protein at step b) is between 4:1 and 0.1:1. In an embodiment the initial

input ratio (weight by weight) of activated serotype 3 capsular polysaccharide to carrier protein is between 2:1 and 0.4:1.

In an embodiment, the reduction reaction (c) is carried out in aqueous solvent.

In an embodiment, the reduction reaction (c) is carried out in aprotic solvent. In an embodiment, the reduction reaction (c) is carried out in DMSO (dimethylsulfoxide) solvent.

In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxyborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, *t*-BuMePrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane (PEMB). In an embodiment, the reducing agent is sodium triacetoxyborohydride. In a preferred embodiment, the reducing agent is sodium cyanoborohydride. In an embodiment, the reducing agent is sodium cyanoborohydride in the presence of nickel (see WO2018144439).

In one embodiment between 0.2 and 20 molar equivalents of reducing agent is used in step c). In one embodiment between 0.5 and 3 molar equivalents of reducing agent is used in step c).

At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride (NaBH₄).

In an embodiment capping is achieved by mixing the product of step c) with 1 to 20 molar equivalents of sodium borohydride. In an embodiment capping is achieved by mixing the product of step c) with 1 to 3 molar equivalents of sodium borohydride.

In an embodiment, the glycoconjugate of the present invention is prepared using CDI and/or CDT chemistry (see PCT/IB2022/054920).

CDI and/or CDT chemistry involves two steps, (1) reacting the isolated saccharide with CDI and/or CDT in an aprotic solvent to produce an activated saccharide (activation), (2) reacting the activated saccharide with a carrier protein (e.g. CRM₁₉₇ or SCP) to form a glycoconjugate.

In an embodiment, the activating agent of step (1) is 1,1'-carbonyldiimidazole (CDI). In an embodiment, the activating agent of step (1) is 1,1'-Carbonyl-di-(1,2,4-triazole) (CDT).

In an embodiment, the isolated serotype 3 capsular polysaccharide is conjugated to a carrier protein by a process comprising the step of:

(a) reacting said isolated polysaccharide with CDI and/or CDT in an aprotic solvent;

(b) reacting the activated polysaccharide of step (a) with a carrier protein in an aprotic solvent to form a glycoconjugate.

In an embodiment, serotype 3 glycoconjugates of the present invention are prepared using click chemistry (see e.g. PCT/IB2022/054914).

According to the present invention, click chemistry comprises three steps, (a) reacting an isolated serotype 3 capsular polysaccharide with a carbonic acid derivative and an azido linker in an aprotic solvent to produce an activated azido polysaccharide (activation of the polysaccharide), (b) reacting a carrier protein with an agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group where the NHS moiety reacts with the amino groups to form an amide linkage thereby obtaining an alkyne functionalized carrier protein (activation of the carrier protein), (c) reacting the activated azido polysaccharide of step (a) with the activated alkyne-carrier protein of step (b) by Cu⁺¹ mediated azide-alkyne cycloaddition reaction to form a glycoconjugate.

Following step (a) the polysaccharide is said to be activated and is referred to herein as "activated polysaccharide" or "activated azido polysaccharide".

Following step (b) the carrier is said to be activated and is referred to as "activated carrier".

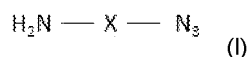
As mentioned above, before the activation (a), sizing of the polysaccharide to a target molecular weight (MW) range can be performed.

Therefore, in an embodiment, the isolated polysaccharide is sized before activation with a carbonic acid derivative and an azido linker.

In an embodiment, the isolated polysaccharide is sized to any of the target molecular weight (MW) range defined above.

In an embodiment, said carbonic acid derivative is 1,1'-carbonyldiimidazole (CDI) or 1,1'-Carbonyl-di-(1,2,4-triazole) (CDT). Preferably, said carbonic acid derivative is 1,1'-carbonyldiimidazole (CDI).

In an embodiment, said azido linker is a compound of formula (I),



wherein X is selected from the group consisting of CH₂(CH₂)_n, (CH₂CH₂O)_mCH₂CH₂, NHCO(CH₂)_n, NHCO(CH₂CH₂O)_mCH₂CH₂, OCH₂(CH₂)_n and O(CH₂CH₂O)_mCH₂CH₂; where n is selected from 1 to 10 and m is selected from 1 to 4.

In an embodiment, said azido linker is a compound of formula (II),

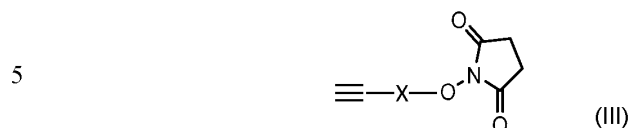


In an embodiment, said azido linker is 3-azido-propylamine.

In an embodiment, said agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group is an agent bearing an N-Hydroxysuccinimide (NHS) moiety and a terminal alkyne.

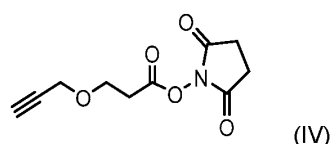
In an embodiment, said agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group is an agent bearing an N-Hydroxysuccinimide (NHS) moiety and a cycloalkyne.

In an embodiment, said agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group is a compound of formula (III),



where X is selected from the group consisting of $\text{CH}_2\text{O}(\text{CH}_2)_n\text{CH}_2\text{C}=\text{O}$ and $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_m(\text{CH}_2)_n\text{CH}_2\text{C}=\text{O}$, where n is selected from 0 to 10 and m is selected from 0 to 4.

10 In an embodiment, said agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group is a compound of formula (IV):



15 In an embodiment, step a) comprises reacting the polysaccharide with a carbonic acid derivative followed by reacting the carbonic acid derivative-activated polysaccharide with an azido linker in an aprotic solvent to produce an activated azido polysaccharide.

In an embodiment, at step a) the isolated polysaccharide is reacted with a carbonic acid derivative in an aprotic solvent.

20 In a preferred embodiment the isolated polysaccharide is reacted with a carbonic acid derivative in a solution consisting essentially of dimethylsulphoxide (DMSO).

In a preferred embodiment the isolated polysaccharide is reacted with CDI in dimethylsulphoxide (DMSO). In an embodiment the isolated polysaccharide is reacted with CDI in anhydrous DMSO.

25 Once the polysaccharide has been reacted with carbonic acid derivative, and following an eventual quenching of carbonic acid derivative with water, the carbonic acid derivative-activated polysaccharide is reacted with an azido linker.

In one embodiment step a) further comprises reacting the carbonic acid derivative-activated polysaccharide with an amount of azido linker that is between 0.01-10 molar equivalent to the amount of polysaccharide Repeat Unit of the activated polysaccharide (molar equivalent of RU).

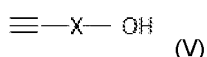
30 In an embodiment, the conjugation reaction c) is carried out in aqueous buffer. In an embodiment, the conjugation reaction c) is carried out in aqueous buffer in the presence of copper (I) as catalyst. In an embodiment, the conjugation reaction c) is carried out in aqueous buffer in the presence of an oxidant and of copper (I) as catalyst. In a preferred embodiment, the conjugation reaction c) is carried out in aqueous buffer in the presence of copper (I) as catalyst and ascorbate as oxidant. In an embodiment, THPTA (tris(3-hydroxypropyltriazolylmethyl)amine) and

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aminoguanidine may be further added to protect the protein from side reactions. Therefore, in a preferred embodiment, the conjugation reaction c) is carried out in aqueous buffer in the presence of copper (I) as catalyst and ascorbate as oxidant, wherein the reaction mixture further comprises THPTA (tris(3-hydroxypropyltriazolylmethyl)amine) and aminoguanidine.

- 5 Following the click conjugation reaction, there may remain unreacted azido groups in the conjugates, these may be capped using a suitable azido group capping agent. Therefore, in an embodiment, following step c), unreacted azido groups in the conjugates, are capped using a suitable azido group capping agent. In one embodiment this azido group capping agent is an agent bearing an alkyne group. In one embodiment this azido group capping agent is an agent bearing a terminal alkyne. In one embodiment this azido group capping agent is an agent bearing a cycloalkyne.

In an embodiment, said azido group capping agent is a compound of formula (V),

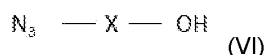


wherein X is $(\text{CH}_2)_n$ wherein n is selected from 1 to 15.

- 15 In one embodiment this azido group capping agent is propargyl alcohol. Therefore, in an embodiment, following step (c) the process further comprises a step of capping the unreacted azido groups remained in the conjugates with an azido group capping agent.

- Following the click conjugation reaction, unreacted alkyne groups may remain present in the conjugates, these may be capped using a suitable alkyne group capping agent. In one embodiment this alkyne group capping agent is an agent bearing an azido group.

In an embodiment, said alkyne group capping agent is a compound of formula (VI),



wherein X is $(\text{CH}_2)_n$ wherein n is selected from 1 to 15.

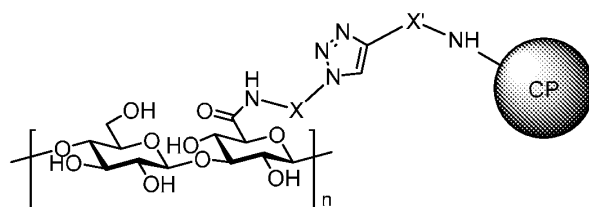
In one embodiment this alkyne group capping agent is 3-azido-1-propanol.

- 25 Therefore, in an embodiment, following step (c) the process further comprises a step of capping the unreacted alkyne groups remained in the conjugates with an alkyne group capping agent.

- 30 Following conjugation to the carrier protein, the glycoconjugate can be purified (enriched with respect to the amount of saccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. Therefore, in one embodiment the process for producing the glycoconjugate of the present invention comprises the step of purifying the glycoconjugate after it is produced.

- 35 In an aspect, the serotype 3 glycoconjugate of the invention is produced according to click chemistry as disclosed above and in application PCT/IB2022/054914, which is incorporated by reference in its entirety. Therefore, in an embodiment, the serotype 3 glycoconjugate of the

invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII):



(VII),

wherein X is selected from the group consisting of $\text{CH}_2(\text{CH}_2)_{n'}$, $(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{NHCO}(\text{CH}_2)_{n'}$, $\text{NHCO}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{OCH}_2(\text{CH}_2)_{n'}$ and $\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$; where n' is selected from 1 to 10 and m is selected from 1 to 4, and wherein X' is selected from the group consisting of $\text{CH}_2\text{O}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$, $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_{m'}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10 and m' is selected from 0 to 4.

Formula (VII) is a schematic representation of serotype 3 glycoconjugates of the invention. It should not be understood that a linkage is present at every repeating unit of the saccharide. Rather, a majority of the *S. pneumoniae* serotype 3 saccharide repeating unit remains unmodified and covalent linkages between the carrier protein and the saccharide is for a minority of the saccharide repeat units. Additionally, an individual carrier protein (CP) molecule may be linked to more than one *S. pneumoniae* serotype 3 saccharide molecule and an individual *S. pneumoniae* serotype 3 saccharide molecule can be linked to more than one individual carrier protein (CP) molecule.

In a preferred embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{CH}_2(\text{CH}_2)_{n'}$, where n' is 2 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$ where n'' is 1.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{CH}_2(\text{CH}_2)_{n'}$, where n' is selected from 1 to 10 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$ where n'' is selected from 0 to 10. In an embodiment, n' is selected from 1 to 5 and n'' is selected from 0 to 10. In an embodiment, n' is selected from 1 to 5 and n'' is selected from 0 to 5. In an embodiment, n' is selected from 1 to 3 and n'' is selected from 0 to 3. In an embodiment, n' is selected from 1 to 2 and n'' is selected from 0 to 2. In a particular embodiment, n' is 1 and n'' is 0. In another embodiment, n' is 2 and n'' is 0.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{CH}_2(\text{CH}_2)_{n'}$, where n' is selected from 1 to 10 and we rein

$\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_m(\text{CH}_2)_{n'}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10 and m' is selected from 0 to 4.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, where m is selected from 1 to 4 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2)_{n'}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10. In an embodiment, m is selected from 1 to 3 and n'' is selected from 0 to 10.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, where m is selected from 1 to 4 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_m(\text{CH}_2)_{n'}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10 and m' is selected from 0 to 4.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{NHCO}(\text{CH}_2)_{n'}$, where n' is selected from 1 to 10 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2)_{n'}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{NHCO}(\text{CH}_2)_{n'}$, where n' is selected from 1 to 10 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_m(\text{CH}_2)_{n'}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10 and m' is selected from 0 to 4.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{NHCO}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, where m is selected from 1 to 4 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2)_{n'}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10. In an embodiment, m is selected from 1 to 3 and n'' is selected from 0 to 10.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{NHCO}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, where m is selected from 1 to 4 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_m(\text{CH}_2)_{n'}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10 and m' is selected from 0 to 4.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{OCH}_2(\text{CH}_2)_{n'}$, where n' is selected from 1 to 10 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2)_{n'}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{OCH}_2(\text{CH}_2)_{n'}$, where n' is selected from 1 to 10 and wherein X' is

$\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_m(\text{CH}_2)_n\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10 and m' is selected from 0 to 4.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, where m is selected from 1 to 4 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_m(\text{CH}_2)_n\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10.

In an embodiment, the serotype 3 glycoconjugate of the invention comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, where m is selected from 1 to 4 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_m(\text{CH}_2)_n\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10 and m' is selected from 0 to 4.

Following conjugation to the carrier protein, the serotype 3 glycoconjugate can be purified (enriched with respect to the amount of saccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration. Therefore, in one embodiment the process for producing the glycoconjugate of the present invention comprises the step of purifying the glycoconjugate after it is produced.

1.11 Carrier Proteins

A component of the glycoconjugate is a carrier protein to which the pneumococcal saccharide is conjugated. The terms "protein carrier" or "carrier protein" or "carrier" may be used interchangeably herein. Carrier proteins should be amenable to standard conjugation procedures.

In a preferred embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate is selected in the group consisting of: DT (Diphtheria Toxoid), TT (Tetanus Toxoid) or fragment C of TT, CRM₁₉₇ (a nontoxic but antigenically identical variant of diphtheria toxin), other DT mutants (such as CRM₁₇₆, CRM₂₂₈, CRM₄₅ (Uchida et al. (1973) J. Biol. Chem. 218:3838-3844), CRM₉, CRM₁₀₂, CRM₁₀₃ or 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 U.S. Patent Nos. 4,709,017 and 4,950,740; mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in U.S. Patent Nos. 5,917,017 and 6,455,673; or fragment disclosed in U.S. Patent No. 5,843,711, pneumococcal pneumolysin (ply) (Kuo et al. (1995) Infect Immun 63:2706-2713) including ply detoxified in some fashion, for example dPLY-GMBS (WO 2004/081515, WO 2006/032499) or dPLY-formol, PhtX, including PhtA, PhtB, PhtD, PhtE (sequences of PhtA, PhtB, PhtD or PhtE are disclosed in WO 00/37105 and WO 00/39299) and fusions of Pht proteins, for example PhtDE fusions, PhtBE fusions, Pht A-E (WO 01/98334,

WO 03/054007, WO 2009/000826), OMPC (meningococcal outer membrane protein), which is usually extracted from *Neisseria meningitidis* serogroup B (EP0372501), PorB (from *N. meningitidis*), PD (*Haemophilus influenzae* protein D; see, e.g., EP0594610 B), or immunologically functional equivalents thereof, synthetic peptides (EP0378881, EP0427347),
 5 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 72:4884-4887) pneumococcal surface protein PspA (WO 02/091998), iron uptake
 10 proteins (WO 01/72337), toxin A or B of *Clostridium difficile* (WO 00/61761), transferrin binding proteins, pneumococcal adhesion protein (PsaA), recombinant *Pseudomonas aeruginosa* exotoxin A (in particular non-toxic mutants thereof (such as exotoxin A bearing a substitution at glutamic acid 553 (Douglas et al. (1987) J. Bacteriol. 169(11):4967-4971)). Other proteins, such as ovalbumin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or purified protein
 15 derivative of tuberculin (PPD) also can be used as carrier proteins. Other suitable carrier proteins include inactivated bacterial toxins such as cholera toxoid (e.g., as described in WO 2004/083251), *Escherichia coli* LT, *E. coli* ST, and exotoxin A from *P. aeruginosa*. Another suitable carrier protein is a C5a peptidase from *Streptococcus* (SCP). Another suitable carrier protein is rhizavidin [aa 45-179J-GGGGSSS-SP1500- AAA-SP0785] (CP1) (WO2020056202).

20 In a preferred embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is selected from the group consisting of TT, DT, DT mutants (such as CRM₁₉₇), and a C5a peptidase from *Streptococcus* (SCP).

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate is DT (Diphtheria Toxoid). In another embodiment, the carrier protein of the pneumococcal
 25 saccharide glycoconjugate is TT (Tetanus Toxoid).

In another embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate is PD (*H. influenzae* protein D; see, e.g., EP0594610 B).

In a preferred embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate is CRM₁₉₇ or a C5a peptidase from *Streptococcus* (SCP).

30 In another embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate is rhizavidin [aa 45-179J-GGGGSSS-SP1500- AAA-SP0785] (CP1).

In a preferred embodiment, part or all of the pneumococcal glycoconjugates of the present invention is conjugated to CRM₁₉₇ protein. The CRM₁₉₇ protein is a nontoxic form of diphtheria toxin but is immunologically indistinguishable from the diphtheria toxin. CRM₁₉₇ is produced by
 35 *Corynebacterium diphtheriae* infected by the nontoxigenic phage β 197^{tox-} created by nitrosoguanidine mutagenesis of the toxigenic corynebacteriophage beta (Uchida et al. (1971) Nature New Biology 233:8-11). The CRM₁₉₇ protein has the same molecular weight as the diphtheria toxin but differs therefrom by a single base change (guanine to adenine) in the structural gene.

This single base change causes an amino acid substitution (glutamic acid for glycine) in the mature protein and eliminates the toxic properties of diphtheria toxin. The CRM₁₉₇ protein is a safe and effective T-cell dependent carrier for saccharides. Further details about CRM₁₉₇ and production thereof can be found, e.g., in U.S. Patent No. 5,614,382.

5 In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention is conjugated to CRM₁₉₇ protein. In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention is conjugated to CRM₁₉₇ protein or the A chain of CRM₁₉₇ (see CN103495161). In an embodiment, part or all of the pneumococcal glycoconjugates of the present invention is conjugated the A chain of CRM₁₉₇ obtained via expression by genetically
10 recombinant *E. coli* (see CN103495161).

In other preferred embodiments, the carrier protein of part or all of the pneumococcal glycoconjugates of the present invention is SCP (Streptococcal C5a Peptidase). See WO2022249106, in particular pages 95-111 which are incorporated by reference.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of
15 the invention is an SCP from GBS (SCP_B). An example of SCP_B is provided at SEQ. ID.NO: 3 of WO97/26008. See also SEQ ID NO: 3 of WO00/34487.

In another preferred embodiments, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an SCP from GAS (SCP_A).
Examples of SCP_A can be found at SEQ.ID.No.1 and SEQ.ID.No.2 of WO97/26008. See also
20 SEQ ID NO: 1, 2 and 23 of WO00/34487.

In a preferred embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCP.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is a fragment of an SCP. In an embodiment, the carrier protein of the pneumococcal
25 saccharide glycoconjugate of the invention is a fragment of an SCP_A. Preferably, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is a fragment of an SCP_B.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is a fragment of an SCP which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not
30 comprise the export signal presequence, the pro-sequence and the cell wall anchor domain.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is a fragment of an SCP which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not
35 comprise the export signal presequence, the pro-sequence and the cell wall anchor domain.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of an SCP which comprises the protease domain, the protease-associated domain (PA domain) and two of the three fibronectin type III

(Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of an SCP. In an embodiment, said enzymatically inactive fragment of SCP comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of an SCPA. In an embodiment, said enzymatically inactive fragment of an SCPA comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain.

In a preferred embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCPB. Preferably, said enzymatically inactive fragment of SCPB comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain.

In an embodiment, the enzymatic activity of SCP is inactivated by replacing at least one amino acid of the wild type sequence. In an embodiment, said replacement is selected from the group consisting of D130A, H193A, N295A and S512A. The numbers indicate the amino acid residue position in the peptidase according to the numbering of SEQ ID NO: 1 of WO00/34487.

Therefore, in an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCP where said inactivation is accomplished by replacing at least one amino acid of the wild type sequence. Preferably, said replacement of at least one amino acid is in the protease domain. In an embodiment, said replacement of at least one amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least one amino acid is in part 2 of the protease domain. In an embodiment, said replacement is selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said replacement is D130A. In another embodiment, said replacement is H193A. In another embodiment, said replacement is N295A. In yet another embodiment, said replacement is S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCPA where said inactivation is accomplished by replacing at least one amino acid of the wild type sequence. Preferably, said replacement of at least one amino acid is in the protease domain. In an embodiment, said replacement of at least one amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least one amino acid is in part 2 of the protease domain. In an embodiment, said replacement is selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said

replacement is D130A. In another embodiment, said replacement is H193A. In another embodiment, said replacement is N295A. In yet another embodiment, said replacement is S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCPB where said inactivation is accomplished by replacing at least one amino acid of the wild type sequence. Preferably, said replacement of at least one amino acid is in the protease domain. In an embodiment, said replacement of at least one amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least one amino acid is in part 2 of the protease domain. In an embodiment, said replacement is selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said replacement is D130A. In another embodiment, said replacement is H193A. In another embodiment, said replacement is N295A. In yet another embodiment, said replacement is S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of an SCP where said inactivation is accomplished by replacing at least one amino acid of the wild type sequence. Preferably, said replacement of at least one amino acid is in the protease domain. In an embodiment, said replacement of at least one amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least one amino acid is in part 2 of the protease domain. In an embodiment, said replacement is selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said replacement is D130A. In another embodiment, said replacement is H193A. In another embodiment, said replacement is N295A. In yet another embodiment, said replacement is S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least one amino acid of the wild type sequence. Preferably, said replacement of at least one amino acid is in the protease domain. In an embodiment, said replacement of at least one amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least one amino acid is in part 2 of the protease domain. In an embodiment, said replacement is selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said replacement is D130A. In another embodiment, said replacement is H193A. In another embodiment, said replacement is N295A. In yet another embodiment, said replacement is S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCPA which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least one amino acid

of the wild type sequence. Preferably, said replacement of at least one amino acid is in the protease domain. In an embodiment, said replacement of at least one amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least one amino acid is in part 2 of the protease domain. In an embodiment, said replacement is selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said replacement is D130A. In another embodiment, said replacement is H193A. In another embodiment, said replacement is N295A. In yet another embodiment, said replacement is S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCPB which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least one amino acid of the wild type sequence. Preferably, said replacement of at least one amino acid is in the protease domain. In an embodiment, said replacement of at least one amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least one amino acid is in part 2 of the protease domain. In an embodiment, said replacement is selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said replacement is D130A. In another embodiment, said replacement is H193A. In another embodiment, said replacement is N295A. In yet another embodiment, said replacement is S512A.

In an embodiment, the enzymatic activity of SCP is inactivated by replacing at least two amino acids of the wild type sequence. In an embodiment, said at least two amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least two amino acids replacements are D130A and H193A. In an embodiment, said at least two amino acids replacements are D130A and N295A. In an embodiment, said at least two amino acids replacements are D130A and S512A. In an embodiment, said at least two amino acids replacements are H193A and N295A. In an embodiment, said at least two amino acids replacements are H193A and S512A. In an embodiment, said at least two amino acids replacements are N295A and S512A.

Therefore, in an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCP where said inactivation is accomplished by replacing at least two amino acids of the wild type sequence. Preferably, said replacement of at least two amino acids is in the protease domain. In an embodiment, said replacement of at least two amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least two amino acid is in part 2 of the protease domain. In an embodiment, said at least two amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least two amino acids replacements are D130A and H193A. In an embodiment, said at least two amino acids replacements are D130A and N295A. Preferably, said at least two amino acids replacements are D130A and S512A. In an

embodiment, said at least two amino acids replacements are H193A and N295A. In an embodiment, said at least two amino acids replacements are H193A and S512A. In an embodiment, said at least two amino acids replacements are N295A and S512A.

5 In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCPA where said inactivation is accomplished by replacing at least two amino acids of the wild type sequence. Preferably, said replacement of at least two amino acids is in the protease domain. In an embodiment, said replacement of at least two amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least two amino acid is in part 2 of the protease domain. In an embodiment, said at least two
10 amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least two amino acids replacements are D130A and H193A. In an embodiment, said at least two amino acids replacements are D130A and N295A. Preferably, said at least two amino acids replacements are D130A and S512A. In an embodiment, said at least two amino acids replacements are H193A and N295A. In an embodiment, said at least two
15 amino acids replacements are H193A and S512A. In an embodiment, said at least two amino acids replacements are N295A and S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCPB where said inactivation is accomplished by replacing at least two amino acids of the wild type sequence. Preferably, said replacement of at least
20 two amino acids is in the protease domain. In an embodiment, said replacement of at least two amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least two amino acid is in part 2 of the protease domain. In an embodiment, said at least two amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least two amino acids replacements are D130A and H193A.
25 In an embodiment, said at least two amino acids replacements are D130A and N295A. Preferably, said at least two amino acids replacements are D130A and S512A. In an embodiment, said at least two amino acids replacements are H193A and N295A. In an embodiment, said at least two amino acids replacements are H193A and S512A. In an embodiment, said at least two amino acids replacements are N295A and S512A.

30 In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of an SCP where said inactivation is accomplished by replacing at least two amino acids of the wild type sequence. Preferably, said replacement of at least two amino acids is in the protease domain. In an embodiment, said replacement of at least two amino acids is in part 1 of the protease domain. In an embodiment,
35 said replacement of at least two amino acid is in part 2 of the protease domain. In an embodiment, said at least two amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least two amino acids replacements are D130A and H193A. In an embodiment, said at least two amino acids replacements are D130A

and N295A. Preferably, said at least two amino acids replacements are D130A and S512A. In an embodiment, said at least two amino acids replacements are H193A and N295A. In an embodiment, said at least two amino acids replacements are H193A and S512A. In an embodiment, said at least two amino acids replacements are N295A and S512A.

5 In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least two amino acids of the wild
10 type sequence. Preferably, said replacement of at least two amino acids is in the protease domain. In an embodiment, said replacement of at least two amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least two amino acid is in part 2 of the protease domain. In an embodiment, said at least two amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least
15 two amino acids replacements are D130A and H193A. In an embodiment, said at least two amino acids replacements are D130A and N295A. Preferably, said at least two amino acids replacements are D130A and S512A. In an embodiment, said at least two amino acids replacements are H193A and N295A. In an embodiment, said at least two amino acids replacements are H193A and S512A. In an embodiment, said at least two amino acids
20 replacements are N295A and S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCPA which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor
25 domain, where said inactivation is accomplished by replacing at least two amino acids of the wild type sequence. Preferably, said replacement of at least two amino acids is in the protease domain. In an embodiment, said replacement of at least two amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least one amino acids is in part 2 of the protease domain. In an embodiment, said at least two amino acids replacements are selected
30 from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least two amino acids replacements are D130A and H193A. In an embodiment, said at least two amino acids replacements are D130A and N295A. Preferably, said at least two amino acids replacements are D130A and S512A. In an embodiment, said at least two amino acids replacements are H193A and N295A. In an embodiment, said at least two amino acids
35 replacements are H193A and S512A. In an embodiment, said at least two amino acids replacements are N295A and S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCPB which comprises the protease

domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least two amino acids of the wild type sequence. Preferably, said replacement of at least two amino acids is in the protease domain. In an embodiment, said replacement of at least two amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least two amino acids is in part 2 of the protease domain. In an embodiment, said at least two amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least two amino acids replacements are D130A and H193A. In an embodiment, said at least two amino acids replacements are D130A and N295A. Preferably, said at least two amino acids replacements are D130A and S512A. In an embodiment, said at least two amino acids replacements are H193A and N295A. In an embodiment, said at least two amino acids replacements are H193A and S512A. In an embodiment, said at least two amino acids replacements are N295A and S512A.

In an embodiment, the enzymatic activity of SCP is inactivated by replacing at least three amino acids of the wild type sequence. In an embodiment, said at least three amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least three amino acids replacements are D130A, H193A and N295A. In an embodiment, said at least three amino acids replacements are D130A, H193A and S512A. In an embodiment, said at least three amino acids replacements are D130A, N295A and S512A. In an embodiment, said at least three amino acids replacements are H193A, N295A and S512A.

Therefore, in an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCP where said inactivation is accomplished by replacing at least three amino acids of the wild type sequence. Preferably, said replacement of at least three amino acids is in the protease domain. In an embodiment, said replacement of at least three amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least three amino acid is in part 2 of the protease domain. In an embodiment, said at least three amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least three amino acids replacements are D130A, H193A and N295A. In an embodiment, said at least three amino acids replacements are D130A, H193A and S512A. In an embodiment, said at least three amino acids replacements are D130A, N295A and S512A. In an embodiment, said at least three amino acids replacements are H193A, N295A and S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCPA where said inactivation is accomplished by replacing at least three amino acids of the wild type sequence. Preferably, said replacement of at least three amino acids is in the protease domain. In an embodiment, said replacement of at least three amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at

least three amino acid is in part 2 of the protease domain. In an embodiment, said at least three amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least three amino acids replacements are D130A, H193A and N295A. In an embodiment, said at least three amino acids replacements are D130A, H193A and S512A. In an embodiment, said at least three amino acids replacements are D130A, N295A and S512A. In an embodiment, said at least three amino acids replacements are H193A, N295A and S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCPB where said inactivation is accomplished by replacing at least three amino acids of the wild type sequence. Preferably, said replacement of at least three amino acids is in the protease domain. In an embodiment, said replacement of at least three amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least three amino acid is in part 2 of the protease domain. In an embodiment, said at least three amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least three amino acids replacements are D130A, H193A and N295A. In an embodiment, said at least three amino acids replacements are D130A, H193A and S512A. In an embodiment, said at least three amino acids replacements are D130A, N295A and S512A. In an embodiment, said at least three amino acids replacements are H193A, N295A and S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of an SCP where said inactivation is accomplished by replacing at least three amino acids of the wild type sequence. Preferably, said replacement of at least three amino acids is in the protease domain. In an embodiment, said replacement of at least three amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least three amino acid is in part 2 of the protease domain. In an embodiment, said at least three amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least three amino acids replacements are D130A, H193A and N295A. In an embodiment, said at least three amino acids replacements are D130A, H193A and S512A. In an embodiment, said at least three amino acids replacements are D130A, N295A and S512A. In an embodiment, said at least three amino acids replacements are H193A, N295A and S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least three amino acids of the wild type sequence. Preferably, said replacement of at least three amino acids is in the protease domain. In an embodiment, said replacement of at least three amino acids is in part 1 of the

protease domain. In an embodiment, said replacement of at least three amino acid is in part 2 of the protease domain. In an embodiment, said at least three amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least three amino acids replacements are D130A, H193A and N295A. In an embodiment, said
5 at least three amino acids replacements are D130A, H193A and S512A. In an embodiment, said at least three amino acids replacements are D130A, N295A and S512A. In an embodiment, said at least three amino acids replacements are H193A, N295A and S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCPA which comprises the protease
10 domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least three amino acids of the wild type sequence. Preferably, said replacement of at least three amino acids is in the protease domain. In an embodiment, said replacement of at least three amino acids is in part
15 1 of the protease domain. In an embodiment, said replacement of at least three amino acids is in part 2 of the protease domain. In an embodiment, said at least three amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least three amino acids replacements are D130A, H193A and N295A. In an embodiment, said at least three amino acids replacements are D130A, H193A and S512A. In an embodiment,
20 said at least three amino acids replacements are D130A, N295A and S512A. In an embodiment, said at least three amino acids replacements are H193A, N295A and S512A.

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCPB which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn)
25 domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least three amino acids of the wild type sequence. Preferably, said replacement of at least three amino acids is in the protease domain. In an embodiment, said replacement of at least three amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least three amino acids is in part
30 part 2 of the protease domain. In an embodiment, said at least three amino acids replacements are selected from the group consisting of D130A, H193A, N295A and S512A. In an embodiment, said at least three amino acids replacements are D130A, H193A and N295A. In an embodiment, said at least three amino acids replacements are D130A, H193A and S512A. In an embodiment, said at least three amino acids replacements are D130A, N295A and S512A. In an embodiment,
35 said at least three amino acids replacements are H193A, N295A and S512A.

In an embodiment, the enzymatic activity of SCP is inactivated by replacing at least four amino acids of the wild type sequence. In an embodiment, said at least four amino acids replacements are D130A, H193A, N295A and S512A.

Therefore, in an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCP where said inactivation is accomplished by replacing at least four amino acids of the wild type sequence. Preferably, said replacement of at least four amino acids is in the protease domain. In an embodiment, said replacement of at least four amino acid is in part 1 of the protease domain. In an embodiment, said replacement of at least four amino acid is in part 2 of the protease domain. In an embodiment, said at least four amino acids replacements are D130A, H193A, N295A and S512A

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCPA where said inactivation is accomplished by replacing at least four amino acids of the wild type sequence. Preferably, said replacement of at least four amino acids is in the protease domain. In an embodiment, said replacement of at least four amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least four amino acid is in part 2 of the protease domain. In an embodiment, said at least four amino acids replacements are D130A, H193A, N295A and S512A

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive SCPB where said inactivation is accomplished by replacing at least four amino acids of the wild type sequence. Preferably, said replacement of at least four amino acids is in the protease domain. In an embodiment, said replacement of at least four amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least four amino acid is in part 2 of the protease domain. In an embodiment, said at least four amino acids replacements are D130A, H193A, N295A and S512A

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of an SCP where said inactivation is accomplished by replacing at least four amino acids of the wild type sequence. Preferably, said replacement of at least four amino acids is in the protease domain. In an embodiment, said replacement of at least four amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least four amino acid is in part 2 of the protease domain. In an embodiment, said at least four amino acids replacements are D130A, H193A, N295A and S512A

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least four amino acids of the wild type sequence. Preferably, said replacement of at least four amino acids is in the protease domain. In an embodiment, said replacement of at least four amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least four amino acid is in part 2 of the protease domain. In an embodiment, said at least four amino acids replacements are D130A, H193A, N295A and S512A

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCPA which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least four amino acids of the wild type sequence. Preferably, said replacement of at least four amino acids is in the protease domain. In an embodiment, said replacement of at least four amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least one amino acids is in part 2 of the protease domain. In an embodiment, said at least four amino acids replacements are D130A, H193A, N295A and S512A

In an embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCPB which comprises the protease domain, the protease-associated domain (PA domain) and the three fibronectin type III (Fn) domains but does not comprise the export signal presequence, the pro-sequence and the cell wall anchor domain, where said inactivation is accomplished by replacing at least four amino acids of the wild type sequence. Preferably, said replacement of at least four amino acids is in the protease domain. In an embodiment, said replacement of at least four amino acids is in part 1 of the protease domain. In an embodiment, said replacement of at least four amino acids is in part 2 of the protease domain. In an embodiment, said at least four amino acids replacements are D130A, H193A, N295A and S512A

In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP which consists of SEQ ID NO: 1.

In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP which consists of SEQ ID NO: 2.

SEQ ID NO: 1 :

MAKTADTPATSKATIRDNDPSQVKTLQEKGAGTVVAVIAAGFDKNH
EAWRLTDKAKARYQSKEDLEKAKKEHGITYGEWWNDKVAYYHDYSKDGMT
AVDQEHGTHVSGILSGNAPSETKEPYRLEGAMPEAQLLLMRVEIVNGLAD
YARNYAQAIRDAINLGAKVINMSFGNAALAYANLPDETKKAFDYAKSKGV
SIVTSAGNDSSFGGKTRLPLADHPDYGWVGTPAAADSTLTVASYSPPDKQL
TETVTVKTDQDQKEMPVLSTNRFEPNKAYDYAYANRGTKEDDFKDVKGK
IALIERGDIDFKDKIAKAKKAGAVGVLIYDNQDKGFPIELPNVDQMPPAF
ISRKDGLLLKDNPPQKTITFNATPKVLPTASGKLSRFSSWGLTADGNIKP
DIAAPGQDILSSVANNKYAKLSGTAMSAPLVAGIMGLLQEQYETQYPDMT
PSERLDLAKKVLMSATLYDEDEKAYFSRQQGAGAVDAKKASAATMYV

TDKDNTSSKVHLNNVSDKFEVTVTVHNKSDKPQELYYQATVQTDKVDGKH
 FALAPKALYETSWQKITIPANSSKQVTPIDASRFSKDLLAQMKNGYFLE
 GFVRFKQDPKKEELMSIPYIGFRGDFGNLSALEKPIYDSKDGSSYYHEAN
 SDAKDQLDGDGLQFYALKNNFTALTTESNPWTIIKAVKEGVENIEDIESS
 5 EITETIFAGTFAKQDDDSHYIHRHANGKPYAAISPNGDGNRDYVQFQGT
 FLRNAKNLVAEVLDEKGNVVTSEVTEQVVKNNNDLASTLGSTRFEKTR
 WDGKDKDGKVVANGTYTYRVRYTPISSGAKEQHTDFDVVDNTTPEVATS
 ATFSTEDRRLTLASKPKTSQPVYRERIAITYMDEDLPTTEYISPNEGTF
 10 TLPEEAETMEGATVPLKMSDFTYVVEDMAGNITYTPVTKLLEGHSNKPEQ

SEQ ID NO: 1 is 950 amino acids long.

SEQ ID NO: 2 :

AKTADTPATSKATIRDLNDPSQVKTLQEKAGKGAGTVVAVIAAGFDKNH
 15 EAWRLTDKAKARYQSKEDLEKAKKEHGITYGEWVNDKVAYYHDYSDKGKT
 AVDQEHGTHVSGILSGNAPSETKEPYRLEGAMPEAQLLLMRVEIVNGLAD
 YARNYAQAIRDAINLGAKVINMSFGNAALAYANLPDETKKAFDYAKSKGV
 SIVTSAGNDSSFGGKTRLPLADHPDYGVVGTPAAADSTLTVASYSPDKQL
 TETVTVKTADQQDKEMPVLSTNRFEPNKAYDYAYANRGTKEDDFKDVKGK
 20 IALIERGDIDFKDKIAKAKKAGAVGVLIYDNQDKGFPIELPNVDQMPAAF
 ISRKDGLLLKDNPKTITFNATPKVLPTASGTKLSRFSSWGLTADGNIKP
 DIAAPGQDILSSVANNKYAKLSGTAMSAPLVAGIMGLLQEYETQYPDMT
 PSERLDLAKKVLMSATALYDEDEKAYFSRQQGAGAVDAKKASAATMYV
 TDKDNTSSKVHLNNVSDKFEVTVTVHNKSDKPQELYYQATVQTDKVDGKH
 25 FALAPKALYETSWQKITIPANSSKQVTPIDASRFSKDLLAQMKNGYFLE
 GFVRFKQDPKKEELMSIPYIGFRGDFGNLSALEKPIYDSKDGSSYYHEAN
 SDAKDQLDGDGLQFYALKNNFTALTTESNPWTIIKAVKEGVENIEDIESS
 EITETIFAGTFAKQDDDSHYIHRHANGKPYAAISPNGDGNRDYVQFQGT
 FLRNAKNLVAEVLDEKGNVVTSEVTEQVVKNNNDLASTLGSTRFEKTR
 30 WDGKDKDGKVVANGTYTYRVRYTPISSGAKEQHTDFDVVDNTTPEVATS
 ATFSTEDRRLTLASKPKTSQPVYRERIAITYMDEDLPTTEYISPNEGTF
 TLPEEAETMEGATVPLKMSDFTYVVEDMAGNITYTPVTKLLEGHSNKPEQ

SEQ ID NO: 2 is 949 amino acids long.

35

In a particular embodiment, the carrier protein of the pneumococcal saccharide
 glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a
 polypeptide having at least 90% identity with SEQ ID NO: 1.

In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 95% identity with SEQ ID NO: 1.

5 In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 99% identity with SEQ ID NO: 1.

In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 99.5% identity with SEQ ID NO: 1.

10 In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 99.8% identity with SEQ ID NO: 1.

In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 99.85% identity with SEQ ID NO: 1.

15 In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 90% identity with SEQ ID NO: 2.

20 In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 95% identity with SEQ ID NO: 2.

In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 99% identity with SEQ ID NO: 2.

25 In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 99.5% identity with SEQ ID NO: 2.

In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 99.8% identity with SEQ ID NO: 2.

30 In a particular embodiment, the carrier protein of the pneumococcal saccharide glycoconjugate of the invention is an enzymatically inactive fragment of SCP consisting of a polypeptide having at least 99.85% identity with SEQ ID NO: 2.

35

2. Immunogenic compositions of the invention

2.1 Combinations of glycoconjugates of the invention

5 In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from different serotypes of *S. pneumoniae* (such as the ones disclosed at section 1 above) and a saponin containing liposomal adjuvant.

In a preferred aspect, the invention relates to multi-valent pneumococcal immunogenic compositions, in particular highly valent composition.

10 Therefore, in an embodiment the invention relates to an immunogenic composition comprising at least 20 glycoconjugates from different serotypes of *S. pneumoniae* (such as the ones disclosed at section 1 above) and a saponin containing liposomal adjuvant.

Preferably, the number of different *S. pneumoniae* capsular saccharides can range from 20 different serotypes (or "v", valences) to 45 different serotypes (45v).

15 Therefore, in an embodiment the invention relates to an immunogenic composition comprising between 20 and 45 glycoconjugates from different serotypes of *S. pneumoniae* (such as the ones disclosed at section 1 above) and a saponin containing liposomal adjuvant.

If the protein carrier is the same for 2 or more saccharides in the composition, the saccharides can be conjugated to the same molecule of the protein carrier (carrier molecules having 2 or more different saccharides conjugated to it) [see for instance WO2020121159].

20 In a preferred embodiment though, the saccharides are each individually conjugated to different molecules of the protein carrier (each molecule of protein carrier only having one type of saccharide conjugated to it). In said embodiment, the capsular saccharides are said to be individually conjugated to the carrier protein.

25 In an embodiment the invention relates to an immunogenic composition comprising twenty pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising twenty-one pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising twenty-two pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

30 In an embodiment the invention relates to an immunogenic composition comprising twenty-three pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising twenty-four pneumococcal glycoconjugates of the invention and a saponin containing liposomal adjuvant.

35 In an embodiment the invention relates to an immunogenic composition comprising twenty-five pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising twenty-six pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising twenty-seven pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising twenty-eight pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

5 In an embodiment the invention relates to an immunogenic composition comprising twenty-nine pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising thirty pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

10 In an embodiment the invention relates to an immunogenic composition comprising thirty-one pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising thirty-two pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising thirty-three pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

15 In an embodiment the invention relates to an immunogenic composition comprising thirty-four pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising thirty-five pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

20 In an embodiment the invention relates to an immunogenic composition comprising thirty-six pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising thirty-seven pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising thirty-eight pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

25 In an embodiment the invention relates to an immunogenic composition comprising thirty-nine pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising forty pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

30 In an embodiment the invention relates to an immunogenic composition comprising forty-one pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising forty-two pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising forty-three pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

35 In an embodiment the invention relates to an immunogenic composition comprising forty-four pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

In an embodiment the invention relates to an immunogenic composition comprising forty-five pneumococcal glycoconjugates and a saponin containing liposomal adjuvant.

Preferably, the immunogenic compositions of the invention comprise glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F (such as the ones of section 1 above) and further comprise a saponin containing liposomal adjuvant.

5 More preferably, the immunogenic compositions of the invention comprise glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F (such as the ones of section 1 above) and further comprise a saponin containing liposomal adjuvant.

10 Even more, the immunogenic compositions of the invention comprise glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B (such as the ones of section 1 above) and further comprise a saponin containing liposomal adjuvant.

15 In an embodiment the invention relates to an immunogenic composition comprising from 21 to 25 pneumococcal glycoconjugates from different serotypes of *S. pneumoniae*, wherein said serotypes are selected from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and further comprising a saponin containing liposomal adjuvant.

20 In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F and additionally comprising from 1 to 5 glycoconjugates from *S. pneumoniae* serotypes 15A, 23A, 23B, 24F and/or 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 21, 22, 23, 24 or 25-valent pneumococcal conjugate composition.

25 In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F and additionally comprising one glycoconjugate from *S. pneumoniae* serotypes 15A, 23A, 23B, 24F or 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 21-valent pneumococcal conjugate composition.

30 In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F and additionally comprising 2 glycoconjugates selected from *S. pneumoniae* serotypes 15A, 23A, 23B, 24F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

35 In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F and additionally comprising 3 glycoconjugates selected from *S. pneumoniae* serotypes 15A, 23A, 23B, 24F and 35B and further comprising a saponin

containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F and additionally comprising 4 glycoconjugates selected from *S. pneumoniae* serotypes 15A, 23A, 23B, 24F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 24-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 21-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23A, 23F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 21-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23B, 23F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 21-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, 24F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 21-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 21-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23B, 23F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23F, 24F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23A, 23F, 24F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23A, 23F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23B, 23F, 24F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23B, 23F, 33F and 35B and further comprising a saponin containing

liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, 24F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 22-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23F, 24F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23B, 23F, 24F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23B, 23F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23F, 24F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 33F and 35B. and further comprising a saponin containing liposomal adjuvant In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate compositions.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23A, 23F, 24F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23B, 23F, 24F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 23-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F and 33F and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 24-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 24-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23F, 33F, 24F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 24-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23B, 23F, 24F, 33F and 35B and further comprising a saponin

containing liposomal adjuvant. In an embodiment the immunogenic composition is a 24-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 24-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and further comprising a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 25-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least one glycoconjugate from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 26-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least two glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 27-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least three glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 28-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least four glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin

containing liposomal adjuvant. In an embodiment the immunogenic composition is a 29-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least five glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 30-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least six glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 31-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least seven glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 32-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least eight glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 33-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least nine glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 34-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least ten glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 35-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least eleven glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 36-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least twelve glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 37-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least thirteen glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 38-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least fourteen glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 39-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at

least fifteen glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 40-valent pneumococcal conjugate composition.

5 In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least sixteen glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a
10 saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 41-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at
15 least seventeen glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 42-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising
20 glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least eighteen glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 43-
25 valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at
30 least nineteen glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 44-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14,
35 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least twenty glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73 and further comprises a

saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 45-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least one glycoconjugate from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 26-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least two glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 27-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least three glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 28-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least four glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 29-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least five glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 30-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least six glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 31-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least seven glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 32-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least eight glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 33-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least nine glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 34-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least ten glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 35-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 35-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 35-valent pneumococcal conjugate composition.

In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14,

15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment the immunogenic composition is a 35-valent pneumococcal conjugate composition.

5 In an embodiment the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least eleven glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 and further comprises a saponin containing liposomal adjuvant. In an embodiment
10 the immunogenic composition is a 36-valent pneumococcal conjugate composition.

In a preferred embodiment, the saccharides are each individually conjugated to different molecules of the protein carrier (each molecule of protein carrier only having one type of saccharide conjugated to it). In said embodiment, the capsular saccharides are said to be individually conjugated to the carrier protein. Preferably, all the glycoconjugates of the above
15 immunogenic compositions are individually conjugated to the carrier protein.

In an embodiment of any of the above immunogenic compositions, the glycoconjugates of the invention are conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to CRM₁₉₇.

20 In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 22F is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 33F is conjugated
25 to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 15B is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 12F is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 10A is conjugated to CRM₁₉₇. In an embodiment
30 of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 11A is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 8 is conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugates from *S. pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F and 23F are conjugated to CRM₁₉₇. In an embodiment of any
35 of the above immunogenic compositions, the glycoconjugates from *S. pneumoniae* serotypes 1, 5 and 7F are conjugated to CRM₁₉₇. In an embodiment of any of the above immunogenic compositions, the glycoconjugates from *S. pneumoniae* serotypes 6A and 19A are conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugates of any of the above immunogenic compositions are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least one other glycoconjugate is conjugated to TT and the other glycoconjugate(s) is/are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, one other glycoconjugate is conjugated to TT and the other glycoconjugate(s) is/are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least two other glycoconjugates are conjugated to TT and the other glycoconjugate(s) is/are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, two other glycoconjugates are conjugated to TT and the other glycoconjugate(s) is/are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least three other glycoconjugates are conjugated to TT and the other glycoconjugate(s) is/are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, three other glycoconjugates are conjugated to TT and the other glycoconjugate(s) is/are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least four other glycoconjugates are conjugated to TT and the other glycoconjugate(s) is/are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, four other glycoconjugates are conjugated to TT and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least five other glycoconjugates are conjugated to TT and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, five other glycoconjugates are conjugated to TT and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least one other glycoconjugate is conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, one other glycoconjugate is conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

5 In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least two other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, two other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

10 In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least three other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, three other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

15 In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least four other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

20 In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, four other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least five other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

25 In an embodiment of any of the above immunogenic compositions, the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, five other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

In an embodiment, the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B, wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇ and further comprising a saponin containing liposomal adjuvant. In a preferred embodiment the immunogenic composition is a 25-valent pneumococcal conjugate compositions.

35 In an embodiment, the invention relates to an immunogenic composition comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B, wherein the glycoconjugates are all individually conjugated to CRM₁₉₇ and further comprising a saponin containing liposomal

adjuvant. In a preferred embodiment the immunogenic composition is a 25-valent pneumococcal conjugate compositions.

5 Compositions of the invention may include a small amount of free carrier. When a given carrier protein is present in both free and conjugated form in a composition of the invention, the unconjugated form is preferably no more than 5% of the total amount of the carrier protein in the composition as a whole, and more preferably present at less than 2% by weight.

2.2 Saccharide dosage of the immunogenic compositions of the invention

10 The amount of glycoconjugate(s) in each dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees.

The amount of a particular glycoconjugate in an immunogenic composition can be calculated based on total saccharide for that conjugate (conjugated and non-conjugated). For example, a glycoconjugate with 20% free saccharide will have about 80 µg of conjugated
15 saccharide and about 20 µg of nonconjugated saccharide in a 100 µg saccharide dose. The amount of glycoconjugate can vary depending upon the bacteria and bacteria serotype. The saccharide concentration can be determined by the uronic acid assay.

The immunogenic compositions of the invention contain a saponin containing liposomal adjuvant which has been shown to greatly enhance the immune response. Therefore, the dosage
20 used for each glycoconjugate of the immunogenic compositions of the invention is typically lower than currently used for pneumococcal conjugate vaccines in human.

Generally, each dose will comprise about 0.1 µg to about 5 µg of saccharide for a given serotype. In a preferred embodiment each dose will comprise about 0.2 µg to about 4 µg of saccharide for a given serotype. Any whole number integer within any of the above ranges is
25 contemplated as an embodiment of the disclosure.

In an embodiment, each dose will comprise about 0.5 µg to about 1.1 µg of saccharide for each particular glycoconjugate.

In an embodiment, each dose will comprise about 0.5 µg, 0.55 µg, about 1.0 µg, 1.1 µg, about 1.5 µg, about 2.0 µg, about 2.2 µg, about 2.5 µg, about 3.0 µg, about 3.5 µg, about 4.0 µg,
30 about 4.5 µg, or about 5.0 µg of saccharide for each particular glycoconjugate.

In an embodiment, the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates.

In an embodiment, the amount of saccharide for glycoconjugates from *S. pneumoniae* serotype 3 and 6B is about two times the dose of the other glycoconjugates.

35 In an embodiment, the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates and the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 3 is about three times the dose of the other glycoconjugates.

In an embodiment, the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates and the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 3 is about four times the dose of the other glycoconjugates.

5 Therefore, in an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg to 1.1 µg except for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B which is about 1.0 µg to 2.2 µg. Preferably, the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates.

10 In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 1.0 µg.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.55 µg and the dose of saccharide for the glycoconjugate
15 from *S. pneumoniae* serotype 6B is about 1.1 µg.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.0 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.0 µg.

20 In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.1 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.2 µg.

In another embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg to 1.1 µg except for the dose of saccharide for the glycoconjugates from *S. pneumoniae* serotypes 3 and 6B which is about 1.0 µg to 2.2 µg.
25 Preferably, the dose of saccharide for glycoconjugates from *S. pneumoniae* serotypes 3 and 6B is about two times the dose of the other glycoconjugates.

.In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg and the dose of saccharide for the glycoconjugates from *S. pneumoniae* serotypes 3 and 6B is about 1.0 µg.

30 In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.55 µg and the dose of saccharide for the glycoconjugates from *S. pneumoniae* serotypes 3 and 6B is about 1.1 µg.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.0 µg and the dose of saccharide for the glycoconjugates
35 from *S. pneumoniae* serotypes 3 and 6B is about 2.0 µg.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.1 µg and the amount of saccharide for the glycoconjugates from *S. pneumoniae* serotypes 3 and 6B is about 2.2 µg.

In another embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg to 1.1 µg except for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B which is about 1.0 µg to 2.2 µg and for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 which is about 1.5 µg to 3.3 µg. Preferably, the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates and the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 3 is about three times the dose of the other glycoconjugates.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 1.0 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 1.5 µg.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.55 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 1.1 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 1.65 µg.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.0 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.0 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 3.0 µg.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.1 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.2 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 3.3 µg.

In another embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg to 1.1 µg except for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B which is about 1.0 µg to 2.2 µg and for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 which is about 2.0 µg to 4.4 µg. Preferably, the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates and the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 3 is about four times the dose of the other glycoconjugates.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 1.0 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 2.0 µg.

In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.55 µg, the dose of saccharide for the glycoconjugate from

S. pneumoniae serotype 6B is about 1.1 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 2.2 µg.

.In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.0 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.0 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 4.0 µg.

.In an embodiment, each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.1 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.2 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 4.4 µg.

2.3 Carrier amount

Generally, each dose will comprise 10 µg to 150 µg of carrier protein, particularly 15 µg to 100 µg of carrier protein, more particularly 25 µg to 100 µg of carrier protein, and even more particularly 25 µg to 75 µg of carrier protein.

In an embodiment, each dose will comprise about 25 µg, about 30 µg, about 35 µg, about 40 µg, about 45 µg, about 50 µg, about 60 µg or about 70 µg of carrier protein.

Preferably, each dose will comprise from about 30 µg to about 70 µg of carrier protein.

Compositions of the invention may include a small amount of free carrier. When a given carrier protein is present in both free and conjugated form in a composition of the invention, the unconjugated form is preferably no more than 5% of the total amount of the carrier protein in the composition as a whole, and more preferably present at less than 2% by weight.

3. Saponin containing liposomal adjuvant of the invention

The immunogenic compositions of the invention comprise a saponin containing liposomal adjuvant.

In a preferred aspect, the saponin containing liposomal adjuvant of the invention is a monophosphoryl lipid A (MPLA)-containing liposome composition comprising at least one saponin. In one aspect, the saponin containing liposomal adjuvant comprises a monophosphoryl lipid A (MPLA)-containing liposome composition and at least one saponin, wherein the liposome composition comprises i) a lipid bilayer comprising phospholipids and ii) cholesterol.

In one aspect, the saponin may be selected from QS-7, QS-18, QS-21, or a mixture thereof. Preferably, the saponin is QS-21.

In one aspect, the phospholipid is selected from dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG), dipalmitoyl phosphatidylglycerol (DPPG), and distearyl phosphatidylglycerol (DSPG). Preferably the phospholipids are DMPC and DMPG.

In a preferred aspect, the saponin containing liposomal adjuvant comprises Monophosphoryl Lipid A (MPLA), QS-21, DMPC, DMPG and cholesterol.

In a very preferred aspect, the saponin containing liposomal adjuvant comprises Monophosphoryl Lipid A (MPLA), QS-21, DMPC, DMPG and cholesterol in a phosphate buffer.

5 MPLA is preferably Monophosphoryl 3-Deacyl Lipid A (also known as 3D-PHAD). 3D PHAD is a synthetic analogue of MPLA derived from *Salmonella Minnesota*.

In one aspect, the immunogenic composition of the invention comprises about 0.1 to about 1.0 mg/mL or higher of MPLA, including about 0.1 mg/mL, about 0.2 mg/mL, about 0.3 mg/mL, about 0.4 mg/mL, about 0.5 mg/mL, about 0.6 mg/mL, about 0.7 mg/mL, about 0.8 mg/mL, about 0.9 mg/mL or about 1.0 mg/mL or higher of MPLA (preferably Monophosphoryl 3-Deacyl Lipid A).

In one aspect, the composition comprises about 0.2 to about 0.6 mg/mL of MPLA (preferably Monophosphoryl 3-Deacyl Lipid A).

In one preferred aspect, the composition comprises about 0.3 to about 0.5 mg/mL of MPLA (preferably Monophosphoryl 3-Deacyl Lipid A).

15 In one particular aspect, the composition comprises about 0.4 mg/mL of MPLA (preferably Monophosphoryl 3-Deacyl Lipid A).

In one aspect, the immunogenic composition of the invention comprises about 0.05 to about 1.0 mg/mL or higher of QS-21, including about 0.05 mg/mL, about 0.1 mg/mL, about 0.2 mg/mL, about 0.3 mg/mL, about 0.4 mg/mL, about 0.5 mg/mL, about 0.6 mg/mL, about 0.7 mg/mL, about 0.8 mg/mL, about 0.9 mg/mL or about 1.0 mg/mL or higher of QS-21.

In one aspect, the composition comprises about 0.1 to about 0.4 mg/mL of QS-21.

In one particular aspect, the composition comprises about 0.2 mg/mL of QS-21.

In one aspect, the immunogenic composition of the invention comprises about 0.5 to about 20 mg/mL or higher of cholesterol, including about 1.0 mg/mL, about 2.0 mg/mL, about 3.0 mg/mL, about 4.0 mg/mL, about 5.0 mg/mL, about 6.0 mg/mL, about 7.0 mg/mL, about 8.0 mg/mL, about 9.0 mg/mL, about 10.0 mg/mL, about 11.0 mg/mL, about 12.0 mg/mL, about 13.0 mg/mL, about 14.0 mg/mL, about 15.0 mg/mL, about 16.0 mg/mL, about 17.0 mg/mL, about 18.0 mg/mL, about 19.0 mg/mL, about 20.0 mg/mL or higher of cholesterol.

In one aspect, the composition comprises about 5 to about 15 mg/mL of cholesterol.

30 In one particular aspect, the composition comprises about 11 mg/mL of cholesterol.

In one aspect, the immunogenic composition of the invention comprises about 0.5 to about 20 mg/mL or higher of DMPC, including about 0.5 mg/mL, about 1.0 mg/mL, about 2.0 mg/mL, about 3.0 mg/mL, about 4.0 mg/mL, about 5.0 mg/mL, about 6.0 mg/mL, about 7.0 mg/mL, about 8.0 mg/mL, about 9.0 mg/mL, about 10.0 mg/mL, about 11.0 mg/mL, about 12.0 mg/mL, about 13.0 mg/mL, about 14.0 mg/mL, about 15.0 mg/mL, about 16.0 mg/mL, about 17.0 mg/mL, about 18.0 mg/mL, about 19.0 mg/mL, about 20.0 mg/mL or higher of DMPC.

In one aspect, the composition comprises about 5 to about 15 mg/mL of DMPC.

In one aspect, the composition comprises about 14 mg/mL of DMPC.

In one aspect, the immunogenic composition of the invention comprises about 0.5 to about 3.0 mg/mL or higher of DMPG, including about 0.5 mg/mL, about 0.6 mg/mL, about 0.7 mg/mL, about 0.8 mg/mL, about 0.9 mg/mL, about 1.0 mg/mL, about 1.1 mg/mL, about 1.2 mg/mL, about 1.3 mg/mL, about 1.4 mg/mL, about 1.5 mg/mL, about 1.6 mg/mL, about 1.7 mg/mL, about 1.8 mg/mL, about 1.9 mg/mL, about 2.0 mg/mL or higher, or at least about 2.5 mg/mL, at least about 3.0 mg/mL or higher of DMPG.

In one aspect, the composition comprises about 1.0 to about 2.0 mg/mL of DMPG.

In one particular aspect, the composition comprises about 1.6 mg/ml of DMPG.

In a preferred aspect, the immunogenic composition comprising a saponin containing liposomal adjuvant comprises Monophosphoryl 3-Deacyl Lipid A (also known as 3D-PHAD) at about 0.4 mg/mL, QS-21 at about 0.2 mg/mL, DMPC at about 14 mg/mL, DMPG about 1.6 mg/ml and cholesterol at about 11 mg/mL.

In another aspect, the immunogenic composition comprising a saponin containing liposomal adjuvant comprises Monophosphoryl 3-Deacyl Lipid A (also known as 3D-PHAD) at about 0.8 mg/mL, QS-21 at about 0.4 mg/mL, DMPC at about 28 mg/mL, DMPG about 3.2 mg/ml and cholesterol at about 22 mg/mL.

In another aspect, the immunogenic composition comprising a saponin containing liposomal adjuvant comprises Monophosphoryl 3-Deacyl Lipid A (also known as 3D-PHAD) at about 0.2 mg/mL, QS-21 at about 0.1 mg/mL, DMPC at about 7 mg/mL, DMPG about 0.8 mg/ml and cholesterol at about 5.5 mg/mL.

The saponin containing liposomal adjuvant of the invention may be homogeneous or heterogeneous. As used herein, the term "homogeneous" shall mean a final adjuvant formulation comprising liposomes having a size range of 30 – 400 nm, as determined by methods known in the art including, but not limited to, Dynamic light scattering (DLS), Transmission electron cryomicroscopy (e.g. cryo-TEM or cryo-EM), Nanoparticle Tracking Analysis (NTA, e.g. ViewSizer). A "homogeneous" adjuvant formulation may also mean a final adjuvant formulation comprising liposomes having a polydispersity index (PDI) of between about 0.05 to 0.5 or between about 0.05 to about 0.3, preferably about 0.3. As used herein, the term "heterogeneous" shall mean a final adjuvant formulation comprising liposomes having varying sizes ranging from 30 nm to over 10 micrometers, about 30 nm to 4 micrometers, about 30 nm to 1400 nm, preferably about 30 nm to 1000 nm as determined by methods known in the art including, but not limited to, Dynamic light scattering (DLS), Transmission electron microscopy (e.g. cryo-TEM or cryo-EM), Nanoparticle Tracking Analysis (NTA, e.g. ViewSizer). A "heterogeneous" adjuvant formulation may also mean a final adjuvant formulation comprising liposomes having a polydispersity index (PDI) > 0.5. The term "about" as used herein refers to $\pm 5\%$ of the referenced value.

In an embodiment, the saponin containing liposomal adjuvant of the invention is homogeneous.

In an embodiment, the saponin containing liposomal adjuvant of the invention is heterogeneous.

Unless noted otherwise, the saponin containing liposomal adjuvant used in the Examples of the present application is a heterogeneous saponin containing liposomal adjuvant.

In one aspect, the saponin containing liposomal adjuvant of the invention is produced according to the process and methods described in PCT Application No. PCT/IB2023/052255, which is incorporated by reference herein in its entirety. Multiple processes are described to prepare heterogeneous and homogeneous liposomal adjuvant formulations scalable for manufacturing.

In the present invention, the saponin containing liposomal adjuvant may be produced by a first method for producing a homogeneous adjuvant formulation comprising a liposome bilayer comprising (a) monophosphoryl lipid A (MPLA), (b) a saponin, and (c) a liposome composition comprising (i) at least one phospholipid selected from phosphatidylcholine (PC) and/or phosphatidylglycerol (PG), wherein the phospholipid is selected from the group consisting of dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG), dipalmitoyl phosphatidylglycerol (DPPG), and distearyl phosphatidylglycerol (DSPG), and a combination thereof, and (ii) cholesterol, wherein the mole percent concentration of the cholesterol in the liposome composition is greater than 50% (mol/mol), or the molar ratio of cholesterol to phospholipid is greater than 1, said method comprising the steps of:

- (i) dissolving the phospholipids, cholesterol and MPLA in an organic solvent to form an organic phase;
- (ii) injecting the organic phase of step (i) into an aqueous phase at a specific flowrate and at a specific ratio of the organic phase to the aqueous phase to form a liposome;
- (iii) stirring the liposome of step (ii) to form an intermediate liposome;
- (iv) removing the organic phase of the intermediate liposome of step (iii);
- (v) concentrating the intermediate liposome of step (iv); and
- (vi) compounding the intermediate liposome of step (v) with a saponin to form a final adjuvant formulation having a size range of about 30-400 nm with a polydispersity of 0.05 to 0.5, thereby producing the homogeneous adjuvant formulation.

In one aspect of the first method for producing a homogeneous adjuvant composition, in step (i) the phospholipids, cholesterol and MPLA are dissolved in the organic solvent by sonication, heat or a combination thereof, preferably by heating. In one aspect, the organic solvent is ethanol or isopropyl alcohol. In another aspect, the organic phase is heated to a temperature between 45°C to 65°C.

In another aspect of the first method for producing a homogeneous adjuvant composition, the aqueous phase comprises water, and optionally, a buffer. In a preferred aspect, the buffer

comprises 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In a preferred aspect, the aqueous phase is 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In another aspect, the aqueous phase is at a temperature between 20°C to 60°C. In another aspect, the flowrate of step (ii) is 0.5 mL/min to 400 mL/min or quick addition. In another aspect, the flowrate of step (ii) is 0.5 mL/min to 400 mL/min. In another aspect, the injection of step (ii) is conducted by rapid injection of the organic phase of step (i).

In another aspect of the first method for producing a homogeneous adjuvant composition, the intermediate liposome of step (iii) is stirred at a rate of 700 rpm to 900 rpm.

In another aspect of the first method for producing a homogeneous adjuvant composition, the ratio of organic phase to aqueous phase of step (ii) ranges from 1:4 to 1:16. In a preferred aspect, the ratio is 1:8.

In another aspect of the first method for producing a homogeneous adjuvant composition, the size of the intermediate liposome in step (iv) is downsized by using a high pressure extruder or a microfluid homogenizer. In one aspect, the size of the liposome is downsized in step (iv) by using membrane sizes ranging from 50 nm to 120 nm or homogenization pressures between 17000 PSI and 24000 PSI, or a combination of both.

In another aspect of the first method for producing a homogeneous adjuvant composition, the organic solvent is removed before downsizing of step (iv) or after downsizing of step (iv). In one aspect, removing the organic phase of the intermediate liposome of step (iv) is by Tangential Flow Filtration (TFF). In another aspect, the TFF is TFF diafiltration. In another aspect, the TFF comprises membranes having a molecular weight cut-off (MWCO) ranging from 100-500 kDa.

In another aspect of the first method for producing a homogeneous adjuvant composition, the concentrating of step (v) is by Ultrafiltration. In one aspect, the Ultrafiltration comprises a bioburden reduction filter and a sterile filter.

In another aspect of the first method for producing a homogeneous adjuvant composition, the compounding of step (vi) is at a mixing speed of 300 rpm. In one aspect, the compounding of step (vi) ranges from 1 hour to 24 hours. In another aspect, the compounding of step (vi) occurs at room temperature or from 2-8°C.

In another aspect of the first method for producing a homogeneous adjuvant composition, the final adjuvant formulation has a size range of about 30 nm - 400 nm.

In another aspect of the first method for producing a homogeneous adjuvant composition, the final adjuvant formulation has a polydispersity of 0.05 to 0.5.

In a further aspect of the first method for producing a homogeneous adjuvant composition, the injecting of step (ii) is by pump or syringe injection. In a preferred aspect, the injecting of step (ii) is by pump.

In one aspect, the saponin containing liposomal adjuvant of the invention may be produced by a second method for producing a homogeneous adjuvant formulation comprising a liposome bilayer comprising (a) monophosphoryl lipid A (MPLA), (b) a saponin, and (c) a liposome

composition comprising (i) at least one phospholipid selected from phosphatidylcholine (PC) and/or phosphatidylglycerol (PG), wherein the phospholipid is selected from the group consisting of dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG), dipalmitoyl phosphatidylglycerol (DPPG), distearyl phosphatidylglycerol (DSPG) and a combination thereof, and (ii) cholesterol, wherein the mole percent concentration of the cholesterol in the liposome composition is greater than 50% (mol/mol), or the molar ratio of cholesterol to phospholipid is greater than 1, said method comprising the steps of:

- (i) dissolving the phospholipids, cholesterol and MPLA in an organic solvent or a mixture of organic solvents to form an organic phase;
- (ii) injecting the organic phase of step (i) into an aqueous phase at a specific flowrate and at a specific ratio of the organic phase to the aqueous phase to form an intermediate liposome;
- (iii) concentrating the intermediate liposome of step (ii);
- (iv) removing the organic phase of the intermediate liposome of step (iii);
- (v) filtering the intermediate liposome of step (iv); and
- (vi) compounding the intermediate liposome of step (v) with a saponin to form a final adjuvant formulation having a size range of about 30-400 nm with a polydispersity of 0.05 to 0.5, thereby producing the homogeneous adjuvant formulation.

In another aspect of the second method for producing a homogeneous adjuvant composition, in step (i) the phospholipids, cholesterol and MPLA are dissolved in the organic solvent by sonication, heat, stirring or a combination thereof. In one aspect, the organic solvent is ethanol or isopropyl alcohol or other organic solvents. In one aspect, the organic solvent is ethanol or isopropyl alcohol. In another aspect, the organic phase is heated to a temperature between 45°C to 65°C. Preferably, between 50°C to 65°C or between 45°C to 55°C.

In another aspect of the second method for producing a homogeneous adjuvant composition, the aqueous phase comprises water, and optionally, a buffer. In one aspect, the buffer comprises 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In a preferred aspect, the aqueous phase is 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In another aspect, the aqueous phase is at a temperature between 20 °C to 65°C. In another aspect, the flowrate of step (ii) is 5 mL/min to 15 mL/min. In another aspect, the flowrate of step (ii) is 12 mL/min. In another aspect, the intermediate liposome of step (ii) is stirred at a rate of 100 rpm to 1000 rpm. In another aspect, the ratio of organic phase to aqueous phase of step (ii) ranges from 1:2 to 1:16. In a preferred aspect, the ratio is 4:7.

In another aspect of the second method for producing a homogeneous adjuvant composition, the concentrating of step (iii) is by Ultrafiltration.

In another aspect of the second method for producing a homogeneous adjuvant composition, removing the organic phase of the intermediate liposome of step (iv) is by Tangential

Flow Filtration (TFF). In one aspect, the TFF is TFF diafiltration. In another aspect, the TFF comprises membranes having a molecular weight cut-off (MWCO) ranging from 100-500 kDa.

In another aspect of the second method for producing a homogeneous adjuvant composition, the filtering of step (v) comprises a bioburden reduction filter and a sterile filter.

5 In another aspect of the second method for producing a homogeneous adjuvant composition, the compounding of step (vi) is at a mixing speed of 350 rpm for 1 hour at RT.

In another aspect of the second method for producing a homogeneous adjuvant composition, the final adjuvant formulation has a size range of about 50-200 nm. Preferably, the final adjuvant formulation has a size of about 100 nm.

10 In another aspect of the second method for producing a homogeneous adjuvant composition, the final adjuvant formulation has a polydispersity of 0.05 to 0.5. Preferably, the final adjuvant formulation has a PDI of <0.2

In a further aspect of the second method for producing a homogeneous adjuvant composition, the injecting of step (ii) is by pump or syringe injection. In a preferred aspect, the
15 injecting of step (ii) is by pump.

In another aspect, the saponin containing liposomal adjuvant of the invention may be produced by a first method for producing a heterogeneous adjuvant formulation comprising a liposome bilayer comprising (a) monophosphoryl lipid A (MPLA), (b) a saponin, and (c) a liposome composition comprising (i) at least one phospholipid selected from phosphatidylcholine (PC) and/or phosphatidylglycerol (PG), wherein the phospholipid is selected from the group consisting of dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG), dipalmitoyl phosphatidylglycerol (DPPG), distearyl phosphatidylglycerol (DSPG), and a combination thereof, and (ii) cholesterol, wherein the mole percent concentration of the cholesterol in the liposome
20 composition is greater than 50% (mol/mol), or the molar ratio of cholesterol to phospholipid is greater than 1, said method comprising the steps of:

(i) dissolving the phospholipids, cholesterol and MPLA in an organic solvent to form a lipid solution as an organic phase;

(ii) simultaneously injecting the organic phase of step (i) and an aqueous phase together
30 at specific flowrates and at a specific ratio of the organic solvent phase to the aqueous phase to form a liposome;

(iii) concentrating the intermediate liposome of step (ii);

(iv) removing the organic phase of the intermediate liposome of step (iii);

(v) treating the intermediate liposome of step (iv) by microfluidizer for 10 passes at ~17500
35 psi;

(vi) filtering the intermediate liposome of step (v) using 0.22 um membrane; and

(vii) compounding the intermediate liposome of step (vi) with a saponin,

thereby producing the heterogeneous adjuvant formulation.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, in step (i) the phospholipids, cholesterol and MPLA are dissolved in the organic solvent by sonication, heat, stirring or a combination thereof. In one aspect, the organic solvent is ethanol or isopropyl alcohol or their mixture. In another aspect, the lipid formulation organic phase is heated to a temperature of 45- 65°C.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, the aqueous phase comprises water, and optionally, a buffer. In one aspect, the buffer comprises 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In a preferred aspect, the aqueous phase is 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In another aspect, the aqueous phase is at a temperature between 45-60°C.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, in step (ii) the organic phase has a flowrate of 0.5 to 2 ml/min and the aqueous phase has a flowrate of 5 to 15 ml/min. In another aspect of the third method, in step (ii) the organic phase has a flowrate of 1.333 ml/min and the aqueous phase has a flowrate of 10.667 ml/min.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, the intermediate liposome of step (iii) is stirred at a rate of 100 rpm to 900 rpm.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, the ratio of organic phase to aqueous phase of step (ii) ranges from 1:4 to 1:8. In a preferred aspect, the ratio is 1:8.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, removing the organic phase of the intermediate liposome of step (iii) and step (iv) is by Tangential Flow Filtration (TFF). In one aspect, the TFF is TFF ultrafiltration and diafiltration. In another aspect, the TFF comprises membranes having a molecular weight cut-off (MWCO) ranging from 100-500 kDa.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, the concentrating of step (iii) is by Ultrafiltration.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, the size of the intermediate liposome in step (iv) is treated by using a microfluidizer.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, the organic solvent is removed before microfluidizing of step (v).

In another aspect of the first method for producing a heterogeneous adjuvant formulation, a buffer is added to the compounding of step (vii). In one aspect, the compounding of step (vii) is at a mixing speed of 350 rpm for 1 hour to 48 at RT or agitated for 1hr. In another aspect, following the compounding of step (vii) the intermediate liposome is stored at RT without stirring for up to 48 hours.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, the final adjuvant formulation has a size of about 300 nm to 1000 nm.

In another aspect of the first method for producing a heterogeneous adjuvant formulation, the final adjuvant formulation has a polydispersity of 0.4-1.0.

In a further aspect of the first method for producing a heterogeneous adjuvant formulation, the injecting of step (ii) is by Nanoassemblr or pump or syringe injection.

5 In another aspect, the saponin containing liposomal adjuvant of the invention may be produced by a second method for producing a heterogeneous adjuvant formulation comprising a liposome bilayer comprising (a) monophosphoryl lipid A (MPLA), (b) a saponin, and (c) a liposome composition comprising (i) at least one phospholipid selected from phosphatidylcholine (PC) and/or phosphatidylglycerol (PG), wherein the phospholipid is selected from the group consisting
10 of dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG), dipalmitoyl phosphatidylglycerol (DPPG), distearyl phosphatidylglycerol (DSPG), and a combination thereof, and (ii) cholesterol, wherein the mole percent concentration of the cholesterol in the liposome composition is greater than 50% (mol/mol), or the molar ratio of cholesterol to phospholipid is
15 greater than 1, said method comprising the steps of:

(i) dissolving the phospholipids, cholesterol and MPLA in an organic solvent to form a lipid formulation an organic phase;

(ii) injecting the organic phase of step (i) into an aqueous phase at a specific flowrate and at a specific ratio of the organic phase to the aqueous phase to form a liposome;

20 (iii) stirring the liposome of step (ii) to form an intermediate liposome;

(iv) concentrating the intermediate liposome of step (iii);

(v) removing the organic phase of the intermediate liposome of step (iv); and

(vi) compounding the intermediate liposome of step (vi) with a saponin, thereby producing the heterogeneous adjuvant formulation.

25 In another aspect of the second method for producing a heterogeneous adjuvant formulation, in step (i) the phospholipids, cholesterol and MPLA are dissolved in the organic solvent by sonication, heat, stirring or a combination thereof. In one aspect, the organic solvent comprises ethyl acetate and isopropyl alcohol. In another aspect, the lipid formulation organic phase is heated to a temperature from 50°C to 65°C.

30 In another aspect of the second method for producing a heterogeneous adjuvant formulation, the aqueous phase comprises water, and optionally, a buffer. In one aspect, the buffer comprises 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In a preferred aspect, the aqueous phase is 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In another aspect, the aqueous phase is at a temperature of 20-25°C. In a further aspect, the flowrate of step (ii) is
35 10 to 30 mL/min. In a further aspect, the flowrate of step (ii) is 20 mL/min.

In another aspect of the second method for producing a heterogeneous adjuvant formulation, the intermediate liposome of step (iii) is stirred at a rate of 100 rpm to 900 rpm.

In another aspect of the second method for producing a heterogeneous adjuvant formulation, the ratio of organic phase to aqueous phase of step (ii) ranges from 1:4 to 1:8. In a preferred aspect, the ratio is 1:8.

5 In another aspect of the second method for producing a heterogeneous adjuvant formulation, the organic solvent is removed before compounding of step (vi).

In another aspect of the second method for producing a heterogeneous adjuvant formulation, removing the organic phase of the intermediate liposome of step (iv) and step (v) is by Tangential Flow Filtration (TFF). In one aspect, the TFF is TFF ultrafiltration and diafiltration. In another aspect, the TFF comprises membranes having a molecular weight cut-off (MWCO)
10 ranging from 100-500 kDa.

In another aspect of the second method for producing a heterogeneous adjuvant formulation, the concentrating of step (vi) is by Ultrafiltration.

In another aspect of the second method for producing a heterogeneous adjuvant formulation, a buffer is added to the compounding of step (vi).

15 In another aspect of the second method for producing a heterogeneous adjuvant formulation, the compounding of step (vi) is at a mixing speed of 300 rpm for 1 hour at RT. In one aspect, following the compounding of step (vii) the intermediate liposome is stored at RT without stirring for 24 hours.

In another aspect of the second method for producing a heterogeneous adjuvant
20 formulation, the final adjuvant formulation has a size range of 300 nm to 1000 nm.

In another aspect of the second method for producing a heterogeneous adjuvant formulation, the final adjuvant formulation has a polydispersity of 0.4 to 1.0.

In another aspect of the second method for producing a heterogeneous adjuvant formulation, the injecting of step (ii) is by pipette, pump or syringe injection.

25 In another aspect, the saponin containing liposomal adjuvant of the invention may be produced by a third method for producing a heterogeneous adjuvant formulation comprising a liposome bilayer comprising (a) monophosphoryl lipid A (MPLA), (b) a saponin, and (c) a liposome composition comprising (i) at least one phospholipid selected from phosphatidylcholine (PC) and/or phosphatidylglycerol (PG), wherein the phospholipid is selected from the group consisting
30 of dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG), dipalmitoyl phosphatidylglycerol (DPPG), distearyl phosphatidylglycerol (DSPG), and a combination thereof, and (ii) cholesterol, wherein the mole percent concentration of the cholesterol in the liposome composition is greater than 50% (mol/mol), or the molar ratio of cholesterol to phospholipid is
35 greater than 1, said method comprising the steps of:

(i) preparing a lyophilized organic phase comprising the phospholipids, cholesterol and MPLA;

(ii) rehydrating the lyophilized organic phase of step (i) with an aqueous phase to form an intermediate liposome;

(iii) downsizing of the intermediate liposome of step (ii) using microfluidizer;

(iv) compounding the intermediate liposome of step (iii) with a saponin,

5 thereby producing the heterogeneous adjuvant formulation.

In another aspect of the third method for producing a heterogeneous adjuvant formulation, in step (i) the phospholipids, cholesterol and MPLA are dissolved in the organic solvent by sonication, heat, stirring or a combination thereof. In one aspect, the organic solvent comprises tert-butyl alcohol (TBA) or its mixture. In another aspect, the lipid organic phase is heated to a
10 temperature from 25°C to 65°C. In another aspect, the organic phase solution is lyophilized.

In another aspect of the third method for producing a heterogeneous adjuvant formulation, the aqueous phase comprises water or a buffer. In one aspect, the buffer comprises 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In a preferred aspect, the aqueous phase is 10 mM phosphate at pH 6.2 containing 150 mM NaCl. In another aspect, the aqueous phase is at a
15 temperature from 20°C to 70°C.

In another aspect of the third method for producing a heterogeneous adjuvant formulation, the intermediate liposome of step (ii) is stirred at a rate of 100-1000 rpm.

In another aspect of the third method for producing a heterogeneous adjuvant formulation, the size of the intermediate liposome in step (iii) is downsized with a microfluidizer and pressure
20 at or about 18640 PSI.

In another aspect of the third method for producing a heterogeneous adjuvant formulation, the organic solvent is removed before rehydration of step (ii). In one aspect, removing the organic solvent is by lyophilization.

In another aspect of the third method for producing a heterogeneous adjuvant formulation,
25 a buffer is added to the compounding of step (iv).

In another aspect of the third method for producing a heterogeneous adjuvant formulation, the compounding of step (iv) is at a mixing speed of 300 rpm or by agitation for 1 hour at RT.

In another aspect of the third method for producing a heterogeneous adjuvant formulation, the compounding of step (iv) the intermediate liposome is stored at RT without stirring for 24
30 hours.

In another aspect of the third method for producing a heterogeneous adjuvant formulation, the final adjuvant formulation has a size > 300nm.

In another aspect of the third method for producing a heterogeneous adjuvant formulation, the final adjuvant formulation has a polydispersity >0.4.

35 In one aspect of the methods for producing a homogeneous or heterogeneous adjuvant formulation, the saponin is selected from the group consisting of QS-7, QS- 18, QS-21, or a mixture thereof. In a preferred aspect, the saponin is QS-21.

In one aspect of the methods for producing a homogeneous or heterogeneous adjuvant formulation, said at least one phospholipid is a mixture of dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG).

5 In one aspect, the liposome composition of the adjuvant formulation may comprise cholesterol at a mole percent concentration of over 50% (mol/mol), of about 55% to about 71% (mol/mol), or preferably about 55% (mol/mol).

In one aspect, the saponin containing liposomal adjuvant of the invention is a monophosphoryl lipid A (MPLA)-containing liposome composition comprising at least one saponin (e.g., QS-21) as described in US Patent No. 10,434,167 (e.g. ALFQ), which is hereby
10 incorporated by reference in its entirety.

4. Further adjuvants

The immunogenic compositions disclosed herein may further comprise an additional adjuvant. In some embodiments, the immunogenic compositions disclosed herein may further
15 comprise at least an additional adjuvant.

The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. Antigens may act primarily as a delivery system, primarily as an immune modulator or have strong features of both. Suitable adjuvants include those suitable for use in mammals, including humans.

20 In an embodiment, the immunogenic compositions disclosed herein further comprise aluminum salts (alum) as adjuvant (e.g., aluminum phosphate, aluminum sulfate or aluminum hydroxide).

In a preferred embodiment, the immunogenic compositions disclosed herein further comprise aluminum phosphate or aluminum hydroxide as adjuvant. In an even preferred
25 embodiment, the immunogenic compositions disclosed herein comprise aluminum phosphate as adjuvant.

In an embodiment of the present invention, the immunogenic compositions as disclosed herein further comprise a CpG Oligonucleotide as adjuvant. A CpG oligonucleotide as used herein refers to an immunostimulatory CpG oligodeoxynucleotide (CpG ODN), and accordingly these
30 terms are used interchangeably unless otherwise indicated. Immunostimulatory CpG oligodeoxynucleotides contain one or more immunostimulatory CpG motifs that are unmethylated cytosine-guanine dinucleotides, optionally within certain preferred base contexts. The methylation status of the CpG immunostimulatory motif generally refers to the cytosine residue in the dinucleotide. An immunostimulatory oligonucleotide containing at least one unmethylated CpG
35 dinucleotide is an oligonucleotide which contains a 5' unmethylated cytosine linked by a phosphate bond to a 3' guanine, and which activates the immune system through binding to Toll-like receptor 9 (TLR-9). In another embodiment the immunostimulatory oligonucleotide may contain one or more methylated CpG dinucleotides, which will activate the immune system

through TLR9 but not as strongly as if the CpG motif(s) was/were unmethylated. CpG immunostimulatory oligonucleotides may comprise one or more palindromes that in turn may encompass the CpG dinucleotide. CpG oligonucleotides have been described in a number of issued patents, published patent applications, and other publications, including U.S. Patent Nos. 5 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068.

In an embodiment of the present invention, the immunogenic compositions as disclosed herein comprise any of the CpG Oligonucleotide described at page 3, line 22, to page 12, line 36, of WO 2010/125480.

Different classes of CpG immunostimulatory oligonucleotides have been identified. These 10 are referred to as A, B, C and P class, and are described in greater detail at page 3, line 22, to page 12, line 36, of WO 2010/125480. Methods of the invention embrace the use of these different classes of CpG immunostimulatory oligonucleotides.

5. Further antigens

15 Immunogenic compositions of the invention comprise conjugated *S. pneumoniae* saccharide antigen(s) (glycoconjugate(s)). They may also further include antigen(s) from other pathogen(s), particularly from bacteria and/or viruses. Preferred further antigens are selected from: a diphtheria toxoid (D), a tetanus toxoid (T), a pertussis antigen (P), which is typically acellular (Pa), a hepatitis B virus (HBV) surface antigen (HBsAg), a hepatitis A virus (HAV) 20 antigen, a conjugated *Haemophilus influenzae* type b capsular saccharide (Hib), inactivated poliovirus vaccine (IPV).

In an embodiment, the immunogenic compositions of the invention comprise D-T-Pa. In an embodiment, the immunogenic compositions of the invention comprise D-T-Pa-Hib, D-T-Pa-IPV or D-T-Pa-HBsAg. In an embodiment, the immunogenic compositions of the invention 25 comprise D-T-Pa-HBsAg-IPV or D-T-Pa-HBsAg-Hib. In an embodiment, the immunogenic compositions of the invention comprise D-T-Pa-HBsAg-IPV-Hib.

Pertussis antigens: *Bordetella pertussis* causes whooping cough. Pertussis antigens in vaccines are either cellular (whole cell, in the form of inactivated *B. pertussis* cells) or acellular. Preparation of cellular pertussis antigens is well documented (e.g., it may be obtained by heat 30 inactivation of phase I culture of *B. pertussis*). Preferably, however, the invention uses acellular antigens. Where acellular antigens are used, it is preferred to use one, two or (preferably) three of the following antigens: (1) detoxified pertussis toxin (pertussis toxoid, or PT); (2) filamentous hemagglutinin (FHA); (3) pertactin (also known as the 69 kilodalton outer membrane protein). FHA and pertactin may be treated with formaldehyde prior to use according to the invention. PT 35 is preferably detoxified by treatment with formaldehyde and/or glutaraldehyde. Acellular pertussis antigens are preferably adsorbed onto one or more aluminum salt adjuvants. As an alternative, they may be added in an unadsorbed state. Where pertactin is added then it is preferably already adsorbed onto an aluminum hydroxide adjuvant. PT and FHA may be adsorbed onto an aluminum

hydroxide adjuvant or an aluminum phosphate. Adsorption of all of PT, FHA and pertactin to aluminum hydroxide is most preferred.

Inactivated poliovirus vaccine: Poliovirus causes poliomyelitis. Rather than use oral poliovirus vaccine, preferred embodiments of the invention use IPV. Prior to administration to patients, polioviruses must be inactivated, and this can be achieved by treatment with formaldehyde. Poliomyelitis can be caused by one of three types of poliovirus. The three types are similar and cause identical symptoms, but they are antigenically different and infection by one type does not protect against infection by others. It is therefore preferred to use three poliovirus antigens in the invention: poliovirus Type 1 (e.g., Mahoney strain), poliovirus Type 2 (e.g., MEF-1 strain), and poliovirus Type 3 (e.g., Saukett strain). The viruses are preferably grown, purified and inactivated individually, and are then combined to give a bulk trivalent mixture for use with the invention.

Diphtheria toxoid: *Corynebacterium diphtheriae* causes diphtheria. Diphtheria toxin can be treated (e.g., using formalin or formaldehyde) to remove toxicity while retaining the ability to induce specific anti-toxin antibodies after injection. These diphtheria toxoids are used in diphtheria vaccines. Preferred diphtheria toxoids are those prepared by formaldehyde treatment. The diphtheria toxoid can be obtained by growing *C. diphtheriae* in growth medium, followed by formaldehyde treatment, ultrafiltration and precipitation. The toxoided material may then be treated by a process comprising sterile filtration and/or dialysis. The diphtheria toxoid is preferably adsorbed onto an aluminum hydroxide adjuvant.

Tetanus toxoid: *Clostridium tetani* causes tetanus. Tetanus toxin can be treated to give a protective toxoid. The toxoids are used in tetanus vaccines. Preferred tetanus toxoids are those prepared by formaldehyde treatment. The tetanus toxoid can be obtained by growing *C. tetani* in growth medium, followed by formaldehyde treatment, ultrafiltration and precipitation. The material may then be treated by a process comprising sterile filtration and/or dialysis.

Hepatitis A virus antigens: Hepatitis A virus (HAV) is one of the known agents which causes viral hepatitis. A preferred HAV component is based on inactivated virus, and inactivation can be achieved by formalin treatment.

Hepatitis B virus (HBV) is one of the known agents which causes viral hepatitis. The major component of the capsid is a protein known as HBV surface antigen or, more commonly, HBsAg, which is typically a 226-amino acid polypeptide with a molecular weight of ~24 kDa. All existing hepatitis B vaccines contain HBsAg, and when this antigen is administered to a normal vaccinee it stimulates the production of anti-HBsAg antibodies which protect against HBV infection. For vaccine manufacture, HBsAg has been made in two ways: purification of the antigen in particulate form from the plasma of chronic hepatitis B carriers or expression of the protein by recombinant DNA methods (e.g., recombinant expression in yeast cells). Unlike native HBsAg (i.e., as in the plasma-purified product), yeast-expressed HBsAg is generally non-glycosylated, and this is the most preferred form of HBsAg for use with the invention.

Conjugated *Haemophilus influenzae* type b antigens: *Haemophilus influenzae* type b (Hib) causes bacterial meningitis. Hib vaccines are typically based on the capsular saccharide antigen, the preparation of which is well documented. The Hib saccharide can be conjugated to a carrier protein in order to enhance its immunogenicity, especially in children. Typical carrier proteins are

5 tetanus toxoid, diphtheria toxoid, CRM₁₉₇, *H. influenzae* protein D, and an outer membrane protein complex from serogroup B meningococcus. The saccharide moiety of the conjugate may comprise full-length polyribosylribitol phosphate (PRP) as prepared from Hib bacteria, and/or fragments of full-length PRP. Hib conjugates may or may not be adsorbed to an aluminum salt adjuvant.

10 In an embodiment the immunogenic compositions of the invention further include a conjugated *N. meningitidis* serogroup Y capsular saccharide (MenY), and/or a conjugated *N. meningitidis* serogroup C capsular saccharide (MenC).

In an embodiment the immunogenic compositions of the invention further include a conjugated *N. meningitidis* serogroup A capsular saccharide (MenA), a conjugated *N.*

15 *meningitidis* serogroup W135 capsular saccharide (MenW135), a conjugated *N. meningitidis* serogroup Y capsular saccharide (MenY), and/or a conjugated *N. meningitidis* serogroup C capsular saccharide (MenC).

In an embodiment the immunogenic compositions of the invention further include a conjugated *N. meningitidis* serogroup W135 capsular saccharide (MenW135), a conjugated *N.*

20 *meningitidis* serogroup Y capsular saccharide (MenY), and/or a conjugated *N. meningitidis* serogroup C capsular saccharide (MenC).

6. Formulation

The immunogenic compositions of the invention may be formulated in liquid form (i.e.,

25 solutions or suspensions) or in a lyophilized form. In an embodiment, the immunogenic composition of the invention is formulated in a liquid form. In an embodiment, the immunogenic composition of the invention is formulated in a lyophilized form. Liquid formulations may advantageously be administered directly from their packaged form and are thus ideal for injection without the need for reconstitution in aqueous medium as otherwise required for lyophilized

30 compositions of the invention.

Formulation of the immunogenic composition of the present disclosure can be accomplished using art-recognized methods. For instance, the individual polysaccharides and/or conjugates can be formulated with a physiologically acceptable vehicle to prepare the composition. Examples of such vehicles include, but are not limited to, water, buffered saline,

35 polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions.

The present disclosure provides an immunogenic composition comprising any of combination of glycoconjugates disclosed herein and a pharmaceutically acceptable excipient, carrier, or diluent.

In an embodiment, the immunogenic composition of the disclosure is in liquid form, preferably in aqueous liquid form.

Immunogenic compositions of the disclosure may comprise one or more of a buffer, a salt, a divalent cation, a non-ionic detergent, a cryoprotectant such as a sugar, and an anti-oxidant
5 such as a free radical scavenger or chelating agent, or any multiple combinations thereof.

In an embodiment, the immunogenic compositions of the disclosure comprise a buffer. In an embodiment, said buffer has a pKa of about 3.5 to about 7.5. In some embodiments, the buffer is phosphate, succinate, histidine or citrate. In some embodiments, the buffer is succinate. In some embodiments, the buffer is histidine. In certain embodiments, the buffer is succinate at a
10 final concentration of 1 mM to 10 mM. In one particular embodiment, the final concentration of the succinate buffer is about 5 mM.

In an embodiment, the immunogenic compositions of the disclosure comprise a salt. In some embodiments, the salt is selected from the groups consisting of magnesium chloride, potassium chloride, sodium chloride and a combination thereof. In one particular embodiment,
15 the salt is sodium chloride. In one particular embodiment, the immunogenic compositions of the invention comprise sodium chloride at 150 mM.

In an embodiment, the immunogenic compositions of the disclosure comprise a surfactant. In an embodiment, the surfactant is selected from the group consisting of polysorbate 20 (TWEENTM20), polysorbate 40 (TWEENTM40), polysorbate 60 (TWEENTM60), polysorbate 65
20 (TWEENTM65), polysorbate 80 (TWEENTM80), polysorbate 85 (TWEENTM85), TRITONTM N-101, TRITONTM X-100, octoxynol 40, nonoxynol-9, triethanolamine, triethanolamine polypeptide oleate, polyoxyethylene-660 hydroxystearate (PEG-15, Solutol H 15), polyoxyethylene-35-ricinoleate (CREMOPHOR[®] EL), soy lecithin and a poloxamer.

In certain embodiments, the immunogenic composition of the disclosure has a pH of 5.5
25 to 7.5, more preferably a pH of 5.6 to 7.0.

In one embodiment, the present disclosure provides a container filled with any of the immunogenic compositions disclosed herein. In one embodiment, the container is selected from the group consisting of a vial, a syringe, a flask, a fermentor, a bioreactor, a bag, a jar, an ampoule, a cartridge and a disposable pen. In certain embodiments, the container is siliconized.

30 In an embodiment, the container of the present disclosure is made of glass, metals (e.g., steel, stainless steel, aluminium, etc.) and/or polymers (e.g., thermoplastics, elastomers, thermoplastic-elastomers). In an embodiment, the container of the present disclosure is made of glass.

In one embodiment, the present disclosure provides a syringe filled with any of the
35 immunogenic compositions disclosed herein. In certain embodiments, the syringe is siliconized and/or is made of glass.

A typical dose of the immunogenic composition of the invention for injection has a volume of 0.1 mL to 2 mL. In an embodiment, the immunogenic composition of the invention for injection has a volume of 0.2 mL to 1 mL, even more preferably a volume of about 0.5 mL.

5 **7. Uses of the immunogenic compositions of the invention**

The glycoconjugates disclosed herein may be use as antigens. For example, they may be part of a vaccine.

Therefore, in an embodiment, the immunogenic compositions of the invention are for use as a medicament.

10 In an embodiment, the immunogenic compositions of the invention are for use as a vaccine.

Therefore, in an embodiment, the immunogenic compositions described herein are for use in generating an immune response in a subject. In one aspect, the subject is a mammal, such as a human, non-human primate, cat, sheep, pig, horse, bovine or dog. Preferably, the subject is a
15 human.

The immunogenic compositions described herein may be used in therapeutic or prophylactic methods for preventing, treating or ameliorating a bacterial infection, disease or condition in a subject. In particular, immunogenic compositions described herein may be used to prevent, treat or ameliorate a *S. pneumoniae* infection, disease or condition in a subject.
20 Preferably, the immunogenic compositions described herein may be used to prevent, treat or ameliorate *S. pneumoniae* infection, disease or condition by the serotypes contained in the composition in a subject.

Thus, in one aspect, the disclosure provides a method of preventing, treating or ameliorating an infection, disease or condition associated with the *S. pneumoniae* serotypes
25 present in the composition in a subject, comprising administering to the subject an immunologically effective amount of an immunogenic composition of the disclosure.

In an aspect, the disclosure with the *S. pneumoniae* serotypes present in the composition in a subject, comprising administering to the subject an immunologically effective amount of an immunogenic composition of the disclosure.

30 In some such embodiments, the infection, disease or condition is selected from the group consisting of pneumonia, sinusitis, otitis media, acute otitis media, meningitis, bacteremia, sepsis, pleural empyema, conjunctivitis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, mastoiditis, cellulitis, soft tissue infection and brain abscess.

In an embodiment, the disclosure provides a method of inducing an immune response to
35 the *S. pneumoniae* serotypes present in the composition in a subject comprising administering to the subject an immunologically effective amount of an immunogenic composition of the invention. In one aspect, the subject is a mammal, such as a human, cat, sheep, pig, horse, bovine or dog. Preferably the subject is a human.

In an embodiment, the disclosure provides a method of inducing an immune response to the *S. pneumoniae* serotypes present in the composition in a subject comprising administering to the subject an immunologically effective amount of an immunogenic composition of the invention. In one aspect, the subject is a mammal, such as a human, cat, sheep, pig, horse, bovine or dog.

5 Preferably the subject is a human.

In an embodiment, the immunogenic compositions disclosed herein are for use as a vaccine. In such embodiments the immunogenic compositions described herein may be used to prevent infection by the *S. pneumoniae* serotypes present in the composition in a subject. Thus, in one aspect, the invention provides a method of preventing an infection by the *S. pneumoniae* serotypes present in the composition in a subject comprising administering to the subject an immunologically effective amount of an immunogenic composition of the disclosure. In some such
10 embodiments, the infection is selected from the group consisting of pneumonia, sinusitis, otitis media, acute otitis media, meningitis, bacteremia, sepsis, pleural empyema, conjunctivitis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, mastoiditis, cellulitis, soft tissue
15 infection and brain abscess. In one aspect, the subject is a mammal, such as a human, cat, sheep, pig, horse, bovine or dog. Preferably the subject is a human.

In one aspect, the invention provides a method of preventing an infection by the *S. pneumoniae* serotypes present in the composition in a subject comprising administering to the subject an immunologically effective amount of an immunogenic composition of the disclosure. In
20 some such embodiments, the infection is selected from the group consisting of pneumonia, sinusitis, otitis media, acute otitis media, meningitis, bacteremia, sepsis, pleural empyema, conjunctivitis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, mastoiditis, cellulitis, soft tissue infection and brain abscess. In one aspect, the subject is a mammal, such as
a human, cat, sheep, pig, horse, bovine or dog. Preferably the subject is a human.

25 The immunogenic composition of the present disclosure can be used to protect or treat a human susceptible to infection by a *S. pneumoniae* serotype present in the composition, by means of administering the immunogenic composition via a systemic or mucosal route. In an embodiment, the immunogenic composition of the invention is administered by intramuscular, intraperitoneal, intradermal or subcutaneous routes. . In an embodiment, the immunogenic
30 composition of the invention is administered by intramuscular, intraperitoneal, intradermal or subcutaneous injection. In an embodiment, the immunogenic composition of the invention is administered by intramuscular or subcutaneous injection. In an embodiment, the immunogenic composition of the invention is administered by intramuscular injection. In an embodiment, the immunogenic composition of the invention is administered by subcutaneous injection.

35 The immunogenic composition of the present disclosure can be used to protect or treat a human susceptible to infection by a *S. pneumoniae* serotype present in composition, by means of administering the immunogenic composition via a systemic or mucosal route. In an embodiment, the immunogenic composition of the invention is administered by intramuscular,

intraperitoneal, intradermal or subcutaneous routes. . In an embodiment, the immunogenic composition of the invention is administered by intramuscular, intraperitoneal, intradermal or subcutaneous injection. In an embodiment, the immunogenic composition of the invention is administered by intramuscular or subcutaneous injection. In an embodiment, the immunogenic composition of the invention is administered by intramuscular injection. In an embodiment, the immunogenic composition of the invention is administered by subcutaneous injection.

8. Subject to be treated with the immunogenic compositions of the invention

As disclosed herein, the immunogenic compositions described herein may be used in various therapeutic or prophylactic methods for preventing, treating or ameliorating a bacterial infection, disease or condition in a subject.

In a preferred embodiment, said subject is a human. In a most preferred embodiment, said subject is a new-born (i.e., under three months of age), an infant (i.e., from 3 months to one year of age) or a toddler (i.e., from one year to four years of age). In an embodiment, the immunogenic compositions disclosed herein are for use as a vaccine.

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

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

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

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

In an embodiment, said disease is a primary immunodeficiency disorder. Preferably, said primary immunodeficiency disorder is selected from the group consisting of: combined T- and B-cell immunodeficiencies, antibody deficiencies, well-defined syndromes, immune dysregulation diseases, phagocyte disorders, innate immunity deficiencies, autoinflammatory disorders, and

complement deficiencies. In an embodiment, said primary immunodeficiency disorder is selected from the one disclosed on page 24, line 11, to page 25, line 19, of WO 2010/125480.

In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated suffers from a disease selected from the groups consisting of: HIV-infection, acquired immunodeficiency syndrome (AIDS), cancer, chronic heart or lung disorders, congestive heart failure, diabetes mellitus, chronic liver disease, alcoholism, cirrhosis, spinal fluid leaks, cardiomyopathy, chronic bronchitis, emphysema, chronic obstructive pulmonary disease (COPD), spleen dysfunction (such as sickle cell disease), lack of spleen function (asplenia), blood malignancy, leukaemia, multiple myeloma, Hodgkin's disease, lymphoma, kidney failure, nephrotic syndrome and asthma.

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

In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated is taking a drug or treatment that lowers the body's resistance to infection. In an embodiment, said drug is selected from the one disclosed on page 26, line 33, to page 26, line 4, of WO 2010/125480.

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

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

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

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

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

In a particular embodiment of the present invention, the immunocompromised subject to be vaccinated has a CD4+ cell count below 500/mm³, or CD4+ cell count below 300/mm³, or CD4+ cell count below 200/mm³, CD4+ cell count below 100/mm³, CD4+ cell count below 75/mm³, or CD4+ cell count below 50/mm³.

CD4 cell tests are normally reported as the number of cells in mm³. Normal CD4 counts are between 500 and 1,600, and CD8 counts are between 375 and 1,100. CD4 counts drop dramatically in people with HIV.

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

9. Regimen

In some cases, as little as one dose of the immunogenic composition according to the invention is needed, but under some circumstances, such as conditions of greater immune deficiency, a second, third or fourth dose may be given. Following an initial vaccination, subjects can receive one or several booster immunizations adequately spaced.

In an embodiment, the schedule of vaccination of the immunogenic composition according to the invention is a single dose. In a particular embodiment, said single dose schedule is for healthy persons being at least 2 years of age.

In an embodiment, the schedule of vaccination of the immunogenic composition according to the invention is a multiple dose schedule. In a particular embodiment, said multiple dose schedule consists of a series of 2 doses separated by an interval of about 1 month to about 2 months. In a particular embodiment, said multiple dose schedule consists of a series of 2 doses separated by an interval of about 1 month, or a series of 2 doses separated by an interval of about 2 months.

In another embodiment, said multiple dose schedule consists of a series of 3 doses separated by an interval of about 1 month to about 2 months. In another embodiment, said multiple dose schedule consists of a series of 3 doses separated by an interval of about 1 month, or a series of 3 doses separated by an interval of about 2 months.

In another embodiment, said multiple dose schedule consists of a series of 3 doses separated by an interval of about 1 month to about 2 months followed by a fourth dose about 10 months to about 13 months after the first dose. In another embodiment, said multiple dose schedule consists of a series of 3 doses separated by an interval of about 1 month followed by a fourth dose about 10 months to about 13 months after the first dose, or a series of 3 doses

separated by an interval of about 2 months followed by a fourth dose about 10 months to about 13 months after the first dose.

In an embodiment, the multiple dose schedule consists of at least one dose (e.g., 1, 2 or 3 doses) in the first year of age followed by at least one toddler dose.

5 In an embodiment, the multiple dose schedule consists of a series of 2 or 3 doses separated by an interval of about 1 month to about 2 months (for example 28-56 days between doses), starting at 2 months of age, and followed by a toddler dose at 12-18 months of age. In an embodiment, said multiple dose schedule consists of a series of 3 doses separated by an interval of about 1 month to about 2 months (for example 28-56 days between doses), starting at 2 months
10 of age, and followed by a toddler dose at 12-15 months of age. In another embodiment, said multiple dose schedule consists of a series of 2 doses separated by an interval of about 2 months, starting at 2 months of age, and followed by a toddler dose at 12-18 months of age.

In an embodiment, the multiple dose schedule consists of a 4-dose series of vaccine at 2, 4, 6, and 12-15 months of age.

15 In an embodiment, a prime dose is given at day 0 and one or more boosts are given at intervals that range from about 2 to about 24 weeks, preferably with a dosing interval of 4-8 weeks.

In an embodiment, a prime dose is given at day 0 and a boost is given about 3 months later.

20 **10 Particular embodiments of the invention are set forth in the following numbered paragraphs 1 to 220:**

1. An immunogenic composition comprising glycoconjugates from different serotypes of *S. pneumoniae* and a saponin containing liposomal adjuvant.
- 25 2. The immunogenic composition of paragraph 1 wherein the saponin containing liposomal adjuvant is a monophosphoryl lipid A (MPLA)-containing liposome composition comprising at least one saponin.
3. The immunogenic composition of paragraph 1 wherein the saponin containing liposomal adjuvant comprises a monophosphoryl lipid A (MPLA)-containing liposome composition and
30 at least one saponin, wherein the liposome composition comprises i) a lipid bilayer comprising phospholipids and ii) cholesterol.
4. The immunogenic composition of paragraph 3 wherein the saponin is selected from QS-7, QS-18, QS-21, or a mixture thereof.
5. The immunogenic composition of any one of paragraphs 3-5 wherein the saponin is QS-21.
- 35 6. The immunogenic composition of any one of paragraphs 3-6 wherein the phospholipid is selected from dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG), dipalmitoyl phosphatidylglycerol (DPPG), and distearyl phosphatidylglycerol (DSPG).

7. The immunogenic composition of any one of paragraphs 3-6 wherein the phospholipids are DMPC and DMPG.
8. The immunogenic composition of paragraph 1 wherein the saponin containing liposomal adjuvant comprises Monophosphoryl Lipid A (MPLA), QS-21, DMPC, DMPG and cholesterol.
- 5 9. The immunogenic composition of paragraph 1 wherein the saponin containing liposomal adjuvant comprises Monophosphoryl Lipid A (MPLA), QS-21, DMPC, DMPG and cholesterol in a phosphate buffer.
- 10 10. The immunogenic composition of any one of paragraphs 2-9 wherein MPLA is Monophosphoryl 3-Deacyl Lipid A.
- 11 11. The immunogenic composition of any one of paragraphs 2-10 comprising about 0.1 to about 1.0 mg/mL or higher of MPLA.
- 12 12. The immunogenic composition of any one of paragraphs 2-10 comprising about 0.2 to about 0.6 mg/mL of MPLA.
- 13 13. The immunogenic composition of any one of paragraphs 2-10 comprising about 0.3 to about 0.5 mg/mL of MPLA.
- 14 14. The immunogenic composition of any one of paragraphs 2-10 comprising about 0.4 mg/mL of MPLA.
- 15 15. The immunogenic composition of any one of paragraphs 1-14 comprising about 0.05 to about 1.0 mg/mL or higher of QS-21.
- 16 16. The immunogenic composition of any one of paragraphs 1-14 comprising about 0.1 to about 0.4 mg/mL of QS-21.
- 17 17. The immunogenic composition of any one of paragraphs 1-14 comprising about 0.2 mg/mL of QS-21.
- 18 18. The immunogenic composition of any one of paragraphs 1-17 comprising about 0.5 to about 20 mg/mL or higher of cholesterol.
- 19 19. The immunogenic composition of any one of paragraphs 1-17 comprising about 5 to about 15 mg/mL of cholesterol.
- 20 20. The immunogenic composition of any one of paragraphs 1-17 comprising about 11 mg/mL of cholesterol.
- 21 21. The immunogenic composition of any one of paragraphs 1-20 comprising about 0.5 to about 20 mg/mL or higher of DMPC.
- 22 22. The immunogenic composition of any one of paragraphs 1-20 comprising about 5 to about 15 mg/mL of DMPC.
- 23 23. The immunogenic composition of any one of paragraphs 1-20 comprising about 14 mg/mL of DMPC.
- 24 24. The immunogenic composition of any one of paragraphs 1-23 comprising about 0.5 to about 3.0 mg/mL or higher of DMPG.

25. The immunogenic composition of any one of paragraphs 1-24 comprising about 1.0 to about 2.0 mg/mL of DMPG.
26. The immunogenic composition of any one of paragraphs 1-24 comprising about 1.6 mg/ml of DMPG.
- 5 27. The immunogenic composition of paragraph 1 wherein said saponin containing liposomal adjuvant comprises Monophosphoryl 3-Deacyl Lipid A at about 0.4 mg/mL, QS-21 at about 0.2 mg/mL, DMPC at about 14 mg/mL, DMPG about 1.6 mg/ml and cholesterol at about 11 mg/mL.
- 10 28. The immunogenic composition of paragraph 1 wherein said saponin containing liposomal adjuvant comprises Monophosphoryl 3-Deacyl Lipid A at about 0.8 mg/mL, QS-21 at about 0.4 mg/mL, DMPC at about 28 mg/mL, DMPG about 3.2 mg/ml and cholesterol at about 22 mg/mL.
- 15 29. The immunogenic composition of paragraph 1 wherein said saponin containing liposomal adjuvant comprises Monophosphoryl 3-Deacyl Lipid A at about 0.2 mg/mL, QS-21 at about 0.1 mg/mL, DMPC at about 7 mg/mL, DMPG about 0.8 mg/ml and cholesterol at about 5.5 mg/mL.
30. The immunogenic composition of any one of paragraphs 1-29 wherein said saponin containing liposomal adjuvant is homogeneous.
- 20 31. The immunogenic composition of any one of paragraphs 1-29 wherein said saponin containing liposomal adjuvant is heterogeneous.
32. The immunogenic composition of any one of paragraphs 1-31 comprising at least 20 glycoconjugates from different serotypes of *S. pneumoniae*.
33. The immunogenic composition of any one of paragraphs 1-31 comprising between 20 and 45 glycoconjugates from different serotypes of *S. pneumoniae*.
- 25 34. The immunogenic composition of any one of paragraphs 1-31 comprising 25 glycoconjugates from different serotypes of *S. pneumoniae*.
35. The immunogenic composition of any one of paragraphs 1-31 comprising 35 glycoconjugates from different serotypes of *S. pneumoniae*.
- 30 36. The immunogenic composition of any one of paragraphs 1-31 comprising 45 glycoconjugates from different serotypes of *S. pneumoniae*.
37. The immunogenic composition of any one of paragraphs 1-36 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.
- 35 38. The immunogenic composition of any one of paragraphs 1-36 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.

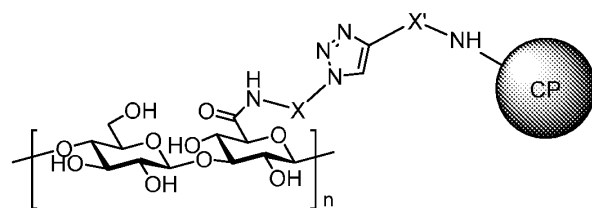
39. The immunogenic composition of any one of paragraphs 1-36 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B.
- 5 40. The immunogenic composition of any one of paragraphs 1-33 comprising from 21 to 25 pneumococcal glycoconjugates from different serotypes of *S. pneumoniae*, wherein said serotypes are selected from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B.
- 10 41. The immunogenic composition of any one of paragraphs 1-36 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B.
42. The immunogenic composition of paragraph 41 wherein said immunogenic composition is a 25-valent pneumococcal conjugate composition.
- 15 43. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least one glycoconjugate from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.
- 20 44. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least two glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.
- 25 45. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least three glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.
- 30 46. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least four glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.
- 35 47. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least five glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.

48. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least ten glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.
49. The immunogenic composition of paragraph 48 which is a 35-valent pneumococcal conjugate composition.
50. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least eleven glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.
51. The immunogenic composition of paragraph 50 which is a 36-valent pneumococcal conjugate composition.
52. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least fifteen glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.
53. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least twenty glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.
54. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least five glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38.
55. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least nine glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38.
56. The immunogenic composition of paragraph 55 which is a 34-valent pneumococcal conjugate composition.
57. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B,

- 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least ten glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38.
58. The immunogenic composition of paragraph 57 which is a 35-valent pneumococcal conjugate composition.
59. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 35F and 38 wherein said immunogenic composition is a 35-valent pneumococcal conjugate composition.
60. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 34, 35F and 38 wherein said immunogenic composition is a 35-valent pneumococcal conjugate composition.
61. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 21, 27, 31, 34, 35F and 38 wherein said immunogenic composition is a 35-valent pneumococcal conjugate composition.
62. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least eleven glycoconjugates from *S. pneumoniae* serotypes 7C, 9N, 16F, 17F, 20B, 21, 27, 31, 34, 35F and 38 wherein said immunogenic composition is a 36-valent pneumococcal conjugate composition.
63. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugates from *S. pneumoniae* are conjugated to CRM₁₉₇.
64. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to CRM₁₉₇.
65. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP.
66. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugates from *S. pneumoniae* are all individually conjugated to CRM₁₉₇.

67. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
- 5 68. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least one other glycoconjugate is conjugated to TT and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
69. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least one other glycoconjugate is conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
- 10 70. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, one other glycoconjugate is conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
71. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least two other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
- 15 72. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, two other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
73. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least three other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
- 20 74. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, three other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
- 25 75. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least four other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
76. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, four other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
- 30 77. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, at least five other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
- 35 78. The immunogenic composition of any one of paragraphs 1-62 wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP, five other glycoconjugates are conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.

79. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B, wherein the glycoconjugate from *S. pneumoniae* serotype 3 is conjugated to SCP and the other glycoconjugates are all individually conjugated to CRM₁₉₇.
80. The immunogenic composition of paragraph 79 wherein said immunogenic composition is a 25-valent pneumococcal conjugate composition.
81. The immunogenic composition of any one of paragraphs 1-33 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B, wherein the glycoconjugates are all individually conjugated to CRM₁₉₇.
82. The immunogenic composition of paragraph 81 wherein said immunogenic composition is a 25-valent pneumococcal conjugate composition.
83. The immunogenic composition of any one of paragraphs 37 to 82 wherein said glycoconjugate from *S. pneumoniae* serotype 3 comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII):

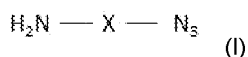


(VII),

- wherein X is selected from the group consisting of $\text{CH}_2(\text{CH}_2)_{n'}$, $(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{NHCO}(\text{CH}_2)_{n'}$, $\text{NHCO}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{OCH}_2(\text{CH}_2)_{n'}$ and $\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$; where n' is selected from 1 to 10 and m is selected from 1 to 4,
- and wherein X' is selected from the group consisting of $\text{CH}_2\text{O}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$, $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_{m'}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$, where n'' is selected from 0 to 10 and m' is selected from 0 to 4.
84. The immunogenic composition of any one of paragraphs 37 to 82 wherein said glycoconjugate from *S. pneumoniae* serotype 3 comprises a serotype 3 saccharide covalently conjugated to a carrier protein (CP) through a spacer and have the general formula (VII), wherein X is $\text{CH}_2(\text{CH}_2)_{n'}$, where n' is 2 and wherein X' is $\text{CH}_2\text{O}(\text{CH}_2)_{n''}\text{CH}_2\text{C}=\text{O}$ where n'' is 1.
85. The immunogenic composition of any one of paragraphs 37 to 82 wherein said glycoconjugate from *S. pneumoniae* serotype 3 is produced according to a method, comprising the steps of:
- (a) reacting an isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide with a carbonic acid derivative and an azido linker in an aprotic solvent to produce an activated azido polysaccharide,

- (b) reacting a carrier protein with an agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group where the NHS moiety reacts with the amino groups to form an amide linkage thereby obtaining an alkyne functionalized carrier protein,
- (c) reacting the activated azido polysaccharide of step (a) with the activated alkyne-carrier protein of step (b) by Cu^{+1} mediated azide-alkyne cycloaddition reaction to form a glycoconjugate.

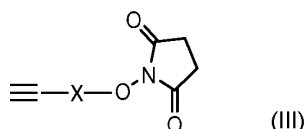
86. The immunogenic composition of paragraph 85 wherein the isolated *Streptococcus pneumoniae* serotype 3 polysaccharide is sized before the activation step (a).
87. The immunogenic composition of paragraph 85 wherein the isolated *Streptococcus pneumoniae* serotype 3 polysaccharide is sized to a weight average molecular weight between 100 kDa and 200 kDa.
88. The immunogenic composition of any one of paragraphs 85-87 wherein said carbonic acid derivative is selected from the group consisting of 1,1'-carbonyldiimidazole (CDI), 1,1'-carbonyl-di-(1,2,4-triazole) (CDT), disuccinimidyl carbonate (DSC) and N-hydroxysuccinimidyl chloroformate.
89. The immunogenic composition of any one of paragraphs 85-87 wherein said carbonic acid derivative is 1,1'-carbonyldiimidazole (CDI).
90. The immunogenic composition of any one of paragraphs 85-87 wherein said carbonic acid derivative is 1,1'-Carbonyl-di-(1,2,4-triazole) (CDT).
91. The immunogenic composition of any one of paragraphs 85-90 wherein said azido linker is a compound of formula (I),



- wherein X is selected from the group consisting of $\text{CH}_2(\text{CH}_2)_n$, $(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{NHCO}(\text{CH}_2)_n$, $\text{NHCO}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$, $\text{OCH}_2(\text{CH}_2)_n$ and $\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2$;
- where n is selected from 1 to 10 and m is selected from 1 to 4.
92. The immunogenic composition of any one of paragraphs 85-90 wherein said azido linker is a compound of formula (II),



93. The immunogenic composition of any one of paragraphs 85-92 wherein said agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group is an agent bearing an N-Hydroxysuccinimide (NHS) moiety and a terminal alkyne.
94. The immunogenic composition of any one of paragraphs 85-92 wherein said agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group is an agent bearing an N-Hydroxysuccinimide (NHS) moiety and a cycloalkyne.
95. The immunogenic composition of any one of paragraphs 85-92 wherein said agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group is a compound of formula (III),

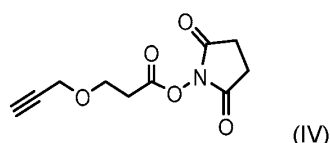


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where X is selected from the group consisting of $\text{CH}_2\text{O}(\text{CH}_2)_n\text{CH}_2\text{C}=\text{O}$ and $\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_m(\text{CH}_2)_n\text{CH}_2\text{C}=\text{O}$, where n is selected from 0 to 10 and m is selected from 0 to 4.

96. The immunogenic composition of any one of paragraphs 85-92 wherein said agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group is a compound of formula (IV):

10



97. The immunogenic composition of any one of paragraphs 85-96 wherein step a) comprises reacting the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide with a carbonic acid derivative followed by reacting the carbonic acid derivative-activated polysaccharide with an azido linker in an aprotic solvent to produce an activated azido polysaccharide.
98. The immunogenic composition of any one of paragraphs 85-97 wherein at step a) the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide is reacted with said carbonic acid derivative in an aprotic solvent.
99. The immunogenic composition of any one of paragraphs 85-98 wherein at step a) the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide is reacted with a carbonic acid derivative in a solution consisting essentially of dimethylsulphoxide (DMSO).
100. The immunogenic composition of any one of paragraphs 85-99 wherein at step a) the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide is reacted with CDI in an aprotic solvent comprising 0.1% to 1% (v/v) water.
101. The immunogenic composition of any one of paragraphs 85-99 wherein at step a) the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide is reacted with CDI in DMSO comprising 0.1% to 1% (v/v) water.
102. The immunogenic composition of any one of paragraphs 85-101 wherein at step a) carbonic acid derivative activation is followed by the addition of water.
103. The immunogenic composition of any paragraph 102 wherein water is added to bring the total water content in the mixture to between about 1% to about 10% (v/v).
104. The immunogenic composition of any one of paragraphs 85-103 wherein step a) further comprises reacting the carbonic acid derivative-activated polysaccharide with an amount of

30

azido linker that is between 0.01-10 molar equivalent to the amount of polysaccharide

Repeat Unit of the activated polysaccharide.

105. The immunogenic composition of any one of paragraphs 85-104 wherein the degree of activation of the activated polysaccharide following step a) is between 0.5 to 50%.
- 5 106. The immunogenic composition of any one of paragraphs 85-105 wherein step b) comprises reacting the carrier protein with an amount of agent bearing an N-Hydroxysuccinimide (NHS) moiety and an alkyne group that is 0.1-10 molar equivalents to the lysines on the carrier.
107. The immunogenic composition of any one of paragraphs 85-106 wherein the degree of activation of the activated carrier following step b) is between 1 and 50.
108. The immunogenic composition of any one of paragraphs 85-107 wherein the conjugation reaction c) is carried out in aqueous buffer in the presence of copper (I) as catalyst.
109. The immunogenic composition of any one of paragraphs 85-107 wherein the conjugation reaction c) is carried out in aqueous buffer in the presence an oxidant and of copper (I) as catalyst.
- 15 110. The immunogenic composition of any one of paragraphs 85-107 wherein the conjugation reaction c) is carried out in aqueous buffer in the presence of copper (I) as catalyst and ascorbate as oxidant, wherein the reaction mixture further comprises THPTA (tris(3-hydroxypropyltriazolylmethyl)amine) and aminoguanidine.
- 20 111. The immunogenic composition of any one of paragraphs 85-110 wherein the initial input ratio (weight by weight) of activated azido polysaccharide to activated alkyne-carrier at step c) is between 0.1 and 3.
112. The immunogenic composition of any one of paragraphs 85-111 wherein following step c), the method further comprises a step of capping the unreacted azido groups remained in the conjugate with an azido group capping agent.
- 25 113. The immunogenic composition of paragraph 112 wherein, said azido group capping agent is a compound of formula (V),

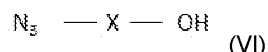
$$\text{≡—X—OH} \quad (\text{V})$$

wherein X is (CH₂)_n wherein n is selected from 1 to 15.
- 30 114. The immunogenic composition of paragraph 112 wherein said azido group capping agent is propargyl alcohol.
115. The immunogenic composition of any one of paragraphs 112-114 wherein the capping of the unreacted azido groups is performed with an amount of capping agent that is between 0.05 to 20 molar equivalents to the amount of polysaccharide repeat unit of the activated polysaccharide.
- 35

116. The immunogenic composition of any one of paragraphs 85-115 wherein following step c), the method further comprises a step of capping the unreacted alkyne groups remained in the conjugate with an alkyne group capping agent.

117. The immunogenic composition of paragraph 116 wherein said alkyne group capping agent is an agent bearing an azido group.

118. The immunogenic composition of paragraph 117 wherein said alkyne group capping agent is a compound of formula (VI),



wherein X is $(\text{CH}_2)_n$ wherein n is selected from 1 to 15.

119. The immunogenic composition of paragraphs 117 wherein said alkyne group capping agent is 3-azido-1-propanol.

120. The immunogenic composition of any one of paragraphs 116-119 wherein the capping of the unreacted alkyne groups is performed with an amount of capping agent that is between 0.05 to 20 molar equivalents to the amount of polysaccharide repeat unit of the activated polysaccharide.

121. The immunogenic composition of any one of paragraphs 37 to 82 wherein said glycoconjugate from *S. pneumoniae* serotype 3 is prepared using reductive amination chemistry.

122. The immunogenic composition of any one of paragraphs 37 to 82 wherein said glycoconjugate from *S. pneumoniae* serotype 3 is produced according to a method, comprising the steps of:

(a) reacting an isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide with an oxidizing agent;

(b) compounding the activated polysaccharide of step (a) with a carrier protein; and

(c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

123. The immunogenic composition of paragraph 122 wherein, the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide is sized before the activation step (a).

124. The immunogenic composition of paragraph 122 wherein, the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide is sized before the activation step (a) to a weight average molecular weight between 100 kDa and 200 kDa.

125. The immunogenic composition of any one of paragraphs 37 to 82 wherein said glycoconjugate from *S. pneumoniae* serotype 3 is produced according to a method, comprising the steps of:

(a) reacting an isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide with an oxidizing agent;

(a') quenching the oxidation reaction by addition of a quenching agent;

(b) compounding the activated polysaccharide of step (a) or (a') with a carrier protein; and
 (c) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a glycoconjugate.

126. The immunogenic composition of paragraph 125 wherein, the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide is sized before the activation step (a).
127. The immunogenic composition of paragraph 125 wherein, the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide is sized before the activation step (a) to a weight average molecular weight between 100 kDa and 200 kDa.
128. The immunogenic composition of any one of paragraphs 122 to 127 wherein said oxidizing agent is periodate.
129. The immunogenic composition of any one of paragraphs 122 to 127 wherein said oxidizing agent is periodic acid.
130. The immunogenic composition of any one of paragraphs 122 to 129 wherein step a) comprises reacting the isolated *Streptococcus pneumoniae* serotype 3 capsular polysaccharide with 0.01-2 molar equivalents of periodate.
131. The immunogenic composition of any one of paragraphs 122 to 130 wherein the degree of oxidation of the activated serotype 3 polysaccharide is between 2 and 8.
132. The immunogenic composition of any one of paragraphs 122 to 130 wherein the degree of oxidation of the activated serotype 3 polysaccharide is between 11 to 19.
133. The immunogenic composition of any one of paragraphs 122 to 130 wherein the degree of oxidation of the activated serotype 3 polysaccharide is about 15.
134. The immunogenic composition of any one of paragraphs 122 to 133 wherein the initial input ratio (weight by weight) of activated serotype 3 capsular polysaccharide to carrier protein at step b) is between 4:1 and 0.1:1.
135. The immunogenic composition of any one of paragraphs 122 to 133 wherein the initial input ratio (weight by weight) of activated serotype 3 capsular polysaccharide to carrier protein is between 1.5:1 and 0.5:1.
136. The immunogenic composition of any one of paragraphs 122 to 135 wherein the reduction reaction (c) is carried out in aqueous solvent.
137. The immunogenic composition of any one of paragraphs 122 to 135 wherein the reduction reaction (c) is carried out in aprotic solvent.
138. The immunogenic composition of any one of paragraphs 122 to 135 wherein the reduction reaction (c) is carried out in a solution consisting essentially of dimethylsulphoxide (DMSO).
139. The immunogenic composition of any one of paragraphs 122 to 138 wherein the reducing agent is sodium cyanoborohydride, sodium triacetoxymethylborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMe₂PrN-BH₃, benzylamine-BH₃ or 5-ethyl-2-methylpyridine borane (PEMB).

140. The immunogenic composition of any one of paragraphs 122 to 138 wherein the reducing agent is sodium cyanoborohydride.
141. The immunogenic composition of any one of paragraphs 122 to 139 wherein between 0.2 and 20 molar equivalents of reducing agent is used in step c).
- 5 142. The immunogenic composition of any one of paragraphs 122 to 139 wherein between 0.5 and 3 molar equivalents of reducing agent is used in step c).
143. The immunogenic composition of any one of paragraphs 122 to 142 wherein, the product of step c) is reacted with 1 to 20 molar equivalents of sodium borohydride for 15 mins-15hrs.
- 10 144. The immunogenic composition of any one of paragraphs 37 to 143 wherein the glycoconjugate from *S. pneumoniae* serotype 3 comprises a serotype 3 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide is between 50 kDa and 1,000 kDa.
145. The immunogenic composition of any one of paragraphs 37 to 143 wherein the glycoconjugate from *S. pneumoniae* serotype 3 comprises a serotype 3 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide is between 100 kDa and 300 kDa.
- 15 146. The immunogenic composition of any one of paragraphs 37 to 143 wherein the glycoconjugate from *S. pneumoniae* serotype 3 comprises a serotype 3 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide is between 100 kDa and 200 kDa.
- 20 147. The immunogenic composition of any one of paragraphs 37 to 143 wherein the glycoconjugate from *S. pneumoniae* serotype 3 comprises a serotype 3 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide is between 200 kDa and 300 kDa.
- 25 148. The immunogenic composition of any one of paragraphs 37 to 143 wherein the glycoconjugate from *S. pneumoniae* serotype 3 comprises a serotype 3 capsular polysaccharide wherein the weight average molecular weight (Mw) of said polysaccharide is between 100 kDa and 300 kDa.
- 30 149. The immunogenic composition of any one of paragraphs 37 to 148 wherein the glycoconjugate from *S. pneumoniae* serotype 3 has a weight average molecular weight (Mw) of between 250 kDa and 20,000 kDa.
150. The immunogenic composition of any one of paragraphs 37 to 148 wherein the glycoconjugate from *S. pneumoniae* serotype 3 has a weight average molecular weight (Mw) of between 500 kDa and 15,000 kDa.
- 35 151. The immunogenic composition of any one of paragraphs 37 to 148 wherein the glycoconjugate from *S. pneumoniae* serotype 3 has a weight average molecular weight (Mw) of between 500 kDa and 10,000 kDa.

152. The immunogenic composition of any one of paragraphs 37 to 148 wherein the glycoconjugate from *S. pneumoniae* serotype 3 has a weight average molecular weight (Mw) of between 500 kDa and 5,000 kDa.
- 5 153. The immunogenic composition of any one of paragraphs 37 to 148 wherein the glycoconjugate from *S. pneumoniae* serotype 3 has a weight average molecular weight (Mw) of between 600 kDa and 3,000 kDa.
154. The immunogenic composition of any one of paragraphs 37 to 153 wherein the degree of conjugation of the serotype 3 glycoconjugate is between 2 and 15.
155. The immunogenic composition of any one of paragraphs 37 to 153 wherein the degree of
10 conjugation of the serotype 3 glycoconjugate is between 4 and 7.
156. The immunogenic composition of any one of paragraphs 37 to 155 wherein the ratio of serotype 3 polysaccharide to carrier protein in the serotype 3 glycoconjugate is between 0.5 and 3.0 (w/w).
157. The immunogenic composition of any one of paragraphs 37 to 155 wherein the ratio of
15 serotype 3 polysaccharide to carrier protein in the serotype 3 glycoconjugate is between 0.5 and 1.5 (w/w).
158. The immunogenic composition of any one of paragraphs 37 to 155 wherein the ratio of serotype 3 polysaccharide to carrier protein in the serotype 3 glycoconjugate is between 0.9 and 1.1 (w/w).
- 20 159. The immunogenic composition of any one of paragraphs 37 to 158 wherein said serotype 3 glycoconjugate comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 4 saccharide repeat units of the polysaccharide.
160. The immunogenic composition of any one of paragraphs 37 to 158 wherein said serotype 3 glycoconjugate comprises at least one covalent linkage between the carrier protein and the
25 polysaccharide for every 10 saccharide repeat units of the polysaccharide.
161. The immunogenic composition of any one of paragraphs 37 to 158 wherein said serotype 3 glycoconjugate comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 15 saccharide repeat units of the polysaccharide.
162. The immunogenic composition of any one of paragraphs 37 to 158 wherein said serotype
30 3 glycoconjugate comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 25 saccharide repeat units of the polysaccharide.
163. The immunogenic composition of any one of paragraphs 37 to 158 wherein said serotype 3 glycoconjugate comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 50 saccharide repeat units of the polysaccharide.
- 35 164. The immunogenic composition of any one of paragraphs 37 to 158 wherein said serotype 3 glycoconjugate comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 100 saccharide repeat units of the polysaccharide.

165. The immunogenic composition of any one of paragraphs 37 to 158 wherein said serotype 3 glycoconjugate comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 5 to 10 saccharide repeat units of the polysaccharide.
- 5 166. The immunogenic composition of any one of paragraphs 37 to 158 wherein said serotype 3 glycoconjugate comprises at least one covalent linkage between the carrier protein and the polysaccharide for every 10 to 20 saccharide repeat units of the polysaccharide.
167. The immunogenic composition of any one of paragraphs 37 to 166 comprising less than about 50% of free serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide.
- 10 168. The immunogenic composition of any one of paragraphs 37 to 166 comprising less than about 40% of free serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide.
169. The immunogenic composition of any one of paragraphs 37 to 166 comprising less than about 25% of free serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide.
- 15 170. The immunogenic composition of any one of paragraphs 37 to 166 comprising less than about 20% of free serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide.
171. The immunogenic composition of any one of paragraphs 37 to 166 comprising less than about 15% of free serotype 3 polysaccharide compared to the total amount of serotype 3 polysaccharide.
- 20 172. The immunogenic composition of any one of paragraphs 37 to 171 wherein at least 30% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.
173. The immunogenic composition of any one of paragraphs 37 to 171 wherein at least 40% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.
- 25 174. The immunogenic composition of any one of paragraphs 37 to 171 wherein at least 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.
175. The immunogenic composition of any one of paragraphs 37 to 171 wherein at least 60% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.
- 30 176. The immunogenic composition of any one of paragraphs 37 to 171 wherein between 50% and 80% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.
177. The immunogenic composition of any one of paragraphs 37 to 171 wherein between 65% and 80% of the serotype 3 glycoconjugate has a K_d below or equal to 0.3 in a CL-4B column.
- 35 178. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose comprises about 0.1 μ g to about 5 μ g of saccharide for a given serotype.
179. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose comprises about 0.2 μ g to about 4 μ g of saccharide for a given serotype.

180. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose comprises about 0.5 µg to about 1.1 µg of saccharide for each particular glycoconjugate.
181. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose comprises about 0.5 µg, 0.55 µg, about 1.0 µg, 1.1 µg, about 1.5 µg, about 2.0 µg, about 2.2 µg, about 2.5 µg, about 3.0 µg, about 3.5 µg, about 4.0 µg, about 4.5 µg, or about 5.0 µg of saccharide for each particular glycoconjugate.
182. The immunogenic composition of any one of paragraphs 1 to 181 wherein the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates.
183. The immunogenic composition of any one of paragraphs 1 to 181 wherein the amount of saccharide for glycoconjugates from *S. pneumoniae* serotype 3 and 6B is about two times the dose of the other glycoconjugates.
184. The immunogenic composition of any one of paragraphs 1 to 181 wherein the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates and the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 3 is about three times the dose of the other glycoconjugates.
185. The immunogenic composition of any one of paragraphs 1 to 181 wherein the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates and the amount of saccharide for glycoconjugate from *S. pneumoniae* serotype 3 is about four times the dose of the other glycoconjugates.
186. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate is about 0.5 µg to 1.1 µg except for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B which is about 1.0 µg to 2.2 µg.
187. The immunogenic composition of paragraph 186 wherein the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates.
188. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 1.0 µg.
189. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.55 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 1.1 µg.
190. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.0

µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.0 µg.

- 5 191. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.1 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.2 µg.
- 10 192. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg to 1.1 µg except for the dose of saccharide for the glycoconjugates from *S. pneumoniae* serotypes 3 and 6B which is about 1.0 µg to 2.2 µg.
193. The immunogenic composition of paragraph 192 wherein the dose of saccharide for glycoconjugates from *S. pneumoniae* serotypes 3 and 6B is about two times the dose of the other glycoconjugates.
- 15 194. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg and the dose of saccharide for the glycoconjugates from *S. pneumoniae* serotypes 3 and 6B is about 1.0 µg.
- 20 195. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.55 µg and the dose of saccharide for the glycoconjugates from *S. pneumoniae* serotypes 3 and 6B is about 1.1 µg.
- 25 196. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.0 µg and the dose of saccharide for the glycoconjugates from *S. pneumoniae* serotypes 3 and 6B is about 2.0 µg.
197. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.1 µg and the amount of saccharide for the glycoconjugates from *S. pneumoniae* serotypes 3 and 6B is about 2.2 µg.
- 30 198. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg to 1.1 µg except for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B which is about 1.0 µg to 2.2 µg and for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 which is about 1.5 µg to 3.3 µg.
- 35 199. The immunogenic composition of paragraph 198 wherein the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates and the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 3 is about three times the dose of the other glycoconjugates.

200. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 1.0 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 1.5 µg.
201. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.55 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 1.1 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 1.65 µg.
202. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.0 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.0 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 3.0 µg.
203. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.1 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.2 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 3.3 µg.
204. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg to 1.1 µg except for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B which is about 1.0 µg to 2.2 µg and for the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 which is about 2.0 µg to 4.4 µg.
205. The immunogenic composition of paragraph 204 wherein the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 6B is about two times the dose of the other glycoconjugates and the dose of saccharide for glycoconjugate from *S. pneumoniae* serotype 3 is about four times the dose of the other glycoconjugates.
206. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.5 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 1.0 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 2.0 µg.
207. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 0.55 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about

1.1 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 2.2 µg.

208. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.0 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.0 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 4.0 µg.

209. The immunogenic composition of any one of paragraphs 1 to 177 wherein each dose of saccharide for each particular glycoconjugate of the immunogenic composition is about 1.1 µg, the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 6B is about 2.2 µg and the dose of saccharide for the glycoconjugate from *S. pneumoniae* serotype 3 is about 4.4 µg.

210. The immunogenic composition of any one of paragraphs 1 to 209 wherein each dose comprises 10 µg to 150 µg of carrier protein.

211. The immunogenic composition of any one of paragraphs 1 to 209 wherein each dose comprises 15 µg to 100 µg of carrier protein.

212. The immunogenic composition of any one of paragraphs 1 to 209 wherein each dose comprises 25 µg to 100 µg of carrier protein.

213. The immunogenic composition of any one of paragraphs 1 to 209 wherein each dose comprises 25 µg to 75 µg of carrier protein.

214. The immunogenic composition of any one of paragraphs 1 to 209 wherein each dose comprises about 25 µg, about 30 µg, about 35 µg, about 40 µg, about 45 µg, about 50 µg, about 60 µg or about 70 µg of carrier protein.

215. The immunogenic composition of any one of paragraphs 1 to 209 wherein each dose comprises from about 30 µg to about 70 µg of carrier protein.

216. The immunogenic composition of any one of paragraphs 1 to 215 wherein the dose for injection has a volume of 0.1 mL to 2 mL.

217. The immunogenic composition of any one of paragraphs 1 to 215 wherein the dose for injection has a volume of 0.2 mL to 1 mL.

218. The immunogenic composition of any one of paragraphs 1 to 215 wherein the dose for injection has a volume of about 0.5 mL.

219. The immunogenic composition of any one of paragraphs 1 to 218 for use as a medicament.

220. The immunogenic composition of any one of paragraphs 1 to 218 for use as a vaccine.

35

As used herein, the term "about" means within a statistically meaningful range of a value, such as a stated concentration range, time frame, molecular weight, temperature or pH. Such a range can be within an order of magnitude, typically within 20%, more typically within 10%, and even

more typically within 5% or within 1% of a given value or range. Sometimes, such a range can be within the experimental error typical of standard methods used for the measurement and/or determination of a given value or range. The allowable variation encompassed by the term "about" will depend upon the particular system under study, and can be readily appreciated by one of ordinary skill in the art. Whenever a range is recited within this application, every whole number integer within the range is also contemplated as an embodiment of the disclosure.

The terms "comprising", "comprise" and "comprises" herein are intended by the inventors to be optionally substitutable with the terms "consisting essentially of", "consist essentially of", "consists essentially of", "consisting of", "consist of" and "consists of", respectively, in every instance.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All references or patent applications cited within this patent specification are incorporated by reference herein.

The invention is illustrated in the accompanying examples. The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

EXAMPLE

Example 1: QS-21 containing liposomal adjuvant induces significantly higher serotype-specific OPA titers

The objective of this study was to evaluate whether QS-21 containing liposomal adjuvant had any antigen dose sparing effect when combined with low dose of a 20-valent pneumococcal conjugate vaccine (PCV20) in infant rhesus macaques.

Infant Rhesus monkey studies

A total of 28 age and sex matched infant rhesus macaques (3-6 months old) were randomly divided into 4 groups (Table 1). Infants were vaccinated intramuscular on both limbs with a dose of 0.25 ml/limb under sedation.

The serotypes included in PCV20 vaccine are 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F (all conjugated to CRM₁₉₇).

Control group received the full human clinical dose (2.2 µg/dose (4.4 µg/dose for 6B)) of PCV20 which also contain 125 µg/dose of AlPO₄.

Dose sparing groups received either quarter dose of PCV20 (0.55 µg (1.1 µg for 6B)) mixed with full dose of AlPO₄ (125 µg/dose) and QS-21 containing liposomal adjuvant (100 µg of 3D-PHAD and 50 µg of QS-21) or quarter dose of PCV20 (0.55 µg (1.1 µg for 6B)) conjugates independently reconstituted mixed with only QS-21 containing liposomal adjuvant (100 µg of 3D-PHAD and 50 µg of QS-21).

Merck's 15-valent PCV vaccine (Vaxneuvance®) that contain AlPO₄ (125 µg/dose) was included as additional control (Table 1).

Pre-bleeds to assess baseline Serotype 3-specific sera titers were collected 1 week (wk= -1) before primary vaccination (D0). Two repeat vaccinations were administered at week 8 and week 16 following primary vaccination for groups that did not include QS-21 containing liposomal adjuvant. Whole blood for sera was collected at 4- and 8-weeks post-dose 1 (PD1); 1, 4 and 8 weeks after PD2 and 1, 4, 16 and 36 weeks after PD3. Sera was collected from whole blood and divided in to 500 µl aliquots and stored at -80°C. Frozen sera received were thawed, barcoded, heat inactivated and transferred to respective assay teams to determine serotype specific IgG and OPA titers.

Opsonophagocytosis assay

Streptococcus pneumoniae (Pn) becomes susceptible to uptake and destruction by polymorphonucleocytes and macrophages when opsonized with antibody and/or complement. This process is called opsonophagocytosis.

Microcolony opsonophagocytic assay (mcOPA) was performed as described previously (see e.g. WO2022/249106).

For the Pn3 mcOPA, reaction mixtures composed of target bacterial cells and heat-inactivated test serum are incubated for 30 minutes at 25°C in an environmental shaker. Differentiated HL-60 tissue culture cells (effector cells) and baby rabbit complement are then added to the reaction mixture, and incubated for 45 minutes at 37°C in an environmental shaker. Functional anti-S. *pneumoniae* antibody titers are determined by measuring bacterial survival in mcOPA reactions containing the test serum. The assay mixture is plated and grown overnight. On day 2, the number of non-phagocytosed live bacteria is determined. The mcOPA antibody titer is the reciprocal of the serum dilution resulting in 50% reduction in the number of bacterial colonies when compared to the bacteria-effector cell-complement control wells that do not contain serum.

Direct Luminex immunoassays for quantitation of sera IgG

Both *Streptococcus pneumoniae* direct Luminex immunoassays (dLIAs) were used to quantify IgG antibody concentrations in serum for the following pneumo polysaccharides: 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F, and 33F (Pavliakova, Danko, et al., *Msphere* 3.4 (2018): 10-1128). Signals expressed by Luminex reader as median fluorescent intensities (MFIs) were used to calculate antigen specific IgG concentrations against the established IgG concentrations of the reference standard.

Table 1

Vaccine	Antigen dose (µg)	AIPO ₄ dose (µg)	QS-21 containing liposomal adjuvant dose	#IRM
PCV20	2.2 (6B: 4.4 µg)	125	--	7
PCV20	0.55 (6B: 1.1 µg)	125	100 µg 3D-PHAD/ 50 µg QS-21	7
PCV20	0.55 (6B: 1.1 µg)	--	100 µg 3D-PHAD/ 50 µg QS-21	7
PCV15	2.0 (6B: 4.0 µg)	125	--	7

Results

5 Quarter dose of PCV20 with QS-21 containing liposomal adjuvant (QS-21 adi) induced significantly improved serotype 3 OPA and IgG titers than full dose PCV20 with AIPO₄.

A quarter dose of PCV20 when combined with QS-21 containing liposomal adjuvant was evaluated to determine if this adjuvant/dose level could generate equivalent functional antibody responses to animals who received a full dose PCV20 containing AIPO₄ in infant rhesus macaques. Since large volumes of blood cannot be collected from infant rhesus macaques, only serotype 3 (ST3) OPA titers were measured. Interestingly, infant macaques vaccinated with quarter dose of PCV20 with QS-21 containing liposomal adjuvant with or without AIPO₄ generated ST3 specific OPA titers that significantly exceeded OPA titers measured from infant macaques who received full dose PCV20 vaccinated (post dose 1 week 4; Fig. 1). ST3 specific OPA geomean titers in QS-21 containing liposomal adjuvanted groups were 4-6 fold higher than those who received full dose PCV20 (Fig.1). The presence or absence of AIPO₄ did not significantly influence QS-21 containing liposomal adjuvant induced OPA titers from quarter dose PCV20 vaccinated infant monkeys (Fig. 1) indicating AIPO₄ could be dispensable in generating antigen dose sparing response. Full dose PCV15 vaccinated infant macaques generated 1.5 - 2 fold lower than quarter dose PCV20 – QS-21 containing liposomal adjuvant vaccinated infant macaques (Fig. 1).

When serotype 3 specific IgG response from infant macaques were measured, a similar pattern to OPA response emerged (Fig. 2). As observed with ST3-OPA titers, animal receiving a quarter dose PCV20 with QS-21 containing liposomal adjuvant in the presence or absence of AIPO₄ induced >5 fold increase in ST3 specific IgG titers compared to animals administered a full dose PCV20 with AIPO₄ adjuvant (Fig. 2).

Quarter dose of PCV20 with QS-21 containing liposomal adjuvant generated higher IgG titers for all serotypes included in PCV20 compared to full dose PCV20 with AIPO₄

IgG titers against all serotypes included in PCV20 were measured using sera obtained at week 4 post dose 1 (using serotype specific multiplex Luminex IgG assays). As observed with serotype 3 specific IgG titers, all other serotypes included in quarter dose PCV20 adjuvanted with QS-21 containing liposomal adjuvant (±AIPO₄) generated higher IgG titers than full dose PCV20 with AIPO₄ adjuvant (Table 2). Depending on the serotypes, QS-21 containing liposomal adjuvant

adjuvanted quarter dose PCV20 induced titers that were 2-33 fold higher compared to full dose PCV20 with AlPO₄ (see Table 2). Higher antibody titers were measured with LiNA-2B groups in the presence or absence of AlPO₄ (Table 2)

Table 1. Infant NHP (N=7) Post dose 1 week 4 IgG geomean titers (µg/ml) - all PCV20 serotypes									
PCV20 serotypes	Vaccines/Conjugate dose								
	PCV20 (AlPO₄) 2.2 µg (6B - 4.4 µg)			PCV20(AlPO₄ + LiNA-2B) 0.55 µg (6B - 1.1 µg)			PCV20 (LiNA-2B) 0.55 µg (6B - 1.1 µg)		
	Geomean titer	Lower 95% CI	Upper 95% CI	Geomean titer	Lower (95%) CI	Upper (95%) CI	Geomean titer	Lower (95%) CI	Upper (95%) CI
PN003	0.63	0.30	1.3	3.69	2.0	6.6	3.88	1.9	7.7
PN06B	0.037	0.01	0.13	1.05	0.25	4.4	1.2	0.3	4.4
PN19A	0.48	0.16	1.5	4.4	2.1	8.9	2.13	1.0	4.5
PN19F	0.60	0.34	1.1	7.4	3.4	16	3.2	0.22	47
PN23F	BLQ	--	--	0.07	0.042	0.13	0.021	0.007	0.064
PN001	0.20	0.075	0.52	2.39	0.80	7.2	4.78	0.93	25
PN004	1.13	0.37	3.5	10.85	5.8	20	12	4.8	28
PN005	0.033	0.010	0.10	1.66	0.58	4.8	0.49	0.19	1.3
PN014	0.033	0.006	0.18	0.14	0.022	0.85	0.099	0.014	0.68
PN06A	0.028	0.005	0.15	0.15	0.018	1.2	0.28	0.025	3.2
PN07F	2.80	0.08	9.8	18	8.4	37	11	8.3	13
PN09V	0.072	0.019	0.28	0.58	0.17	2.0	0.69	0.17	2.9
PN18C	5.31	2.3	12	11.15	4.3	29	10.20	7.5	14
PN008	2.62	0.98	7.0	17	11	29	14	8.6	23
PN10A	2.4	0.70	8.0	14	8.6	23	5.7	1.4	23
PN11A	4.11	1.4	12	7.9	0.44	141	5.8	0.38	88
PN12F	0.019	0.006	0.057	0.15	0.045	0.49	0.32	0.080	1.3
PN15B	2.05	0.37	11	42.10	14	126	34.79	9.4	129
PN22F	6.51	3.2	13	29	15	56	27	12	60
PN33F	0.42	0.083	2.1	7.60	2.1	27	3.62	0.86	15

5

The study results indicate that QS-21 containing liposomal adjuvant in the presence or absence of AlPO₄ has a dose sparing effect on PCV20 in infant rhesus macaques. At the 4 week post dose 1 timepoint, infant rhesus macaques that received a quarter dose of QS-21 containing liposomal adjuvant adjuvanted PCV20 (0.55 µg/dose (6B - 1.1 µg)) induced several fold increase in serotype specific IgG and OPA (only serotype 3 tested) titers compared to PCV20 administered at the full human clinical dose (2.2 µg/dose (6B - 4.4 µg)).

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Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, certain changes and modifications may be practiced within the scope of the appended claims.

15

Claims

1. An immunogenic composition comprising glycoconjugates from different serotypes of *S. pneumoniae* and a saponin containing liposomal adjuvant comprising a monophosphoryl lipid A (MPLA)-containing liposome composition and at least one saponin, wherein the liposome composition comprises i) a lipid bilayer comprising phospholipids and ii) cholesterol.
2. The immunogenic composition of claim 1 wherein the saponin is QS-21.
3. The immunogenic composition of any one of claims 1-2 wherein the phospholipids are DMPC and DMPG.
4. The immunogenic composition of claim 1 wherein the saponin containing liposomal adjuvant comprises Monophosphoryl Lipid A (MPLA), QS-21, DMPC, DMPG and cholesterol in a phosphate buffer.
5. The immunogenic composition of any one of claims 1-4 wherein MPLA is Monophosphoryl 3-Deacyl Lipid A.
6. The immunogenic composition of any one of claims 1-5 comprising about 0.1 to about 1.0 mg/mL or higher of MPLA.
7. The immunogenic composition of any one of claims 1-6 comprising about 0.05 to about 1.0 mg/mL or higher of QS-21.
8. The immunogenic composition of any one of claims 1-7 comprising about 0.5 to about 20 mg/mL or higher of cholesterol.
9. The immunogenic composition of any one of claims 1-8 comprising about 0.5 to about 20 mg/mL or higher of DMPC.
10. The immunogenic composition of any one of claims 1-9 comprising about 0.5 to about 3.0 mg/mL or higher of DMPG.
11. The immunogenic composition of claim 1 wherein said saponin containing liposomal adjuvant comprises Monophosphoryl 3-Deacyl Lipid A at about 0.4 mg/mL, QS-21 at about 0.2 mg/mL, DMPC at about 14 mg/mL, DMPG about 1.6 mg/ml and cholesterol at about 11 mg/mL.
12. The immunogenic composition of claim 1 wherein said saponin containing liposomal adjuvant comprises Monophosphoryl 3-Deacyl Lipid A at about 0.8 mg/mL, QS-21 at about 0.4 mg/mL, DMPC at about 28 mg/mL, DMPG about 3.2 mg/ml and cholesterol at about 22 mg/mL.
13. The immunogenic composition of claim 1 wherein said saponin containing liposomal adjuvant comprises Monophosphoryl 3-Deacyl Lipid A at about 0.2 mg/mL, QS-21 at about 0.1 mg/mL, DMPC at about 7 mg/mL, DMPG about 0.8 mg/ml and cholesterol at about 5.5 mg/mL.
14. The immunogenic composition of any one of claims 1-13 wherein said saponin containing liposomal adjuvant is homogeneous.
15. The immunogenic composition of any one of claims 1-13 wherein said saponin containing liposomal adjuvant is heterogeneous.
16. The immunogenic composition of any one of claims 1-15 comprising at least 20 glycoconjugates from different serotypes of *S. pneumoniae*.

17. The immunogenic composition of any one of claims 1-16 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F.
18. The immunogenic composition of any one of claims 1-16 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B.
19. The immunogenic composition of any one of claims 1-18 comprising glycoconjugates from *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15A, 15B, 18C, 19A, 19F, 22F, 23A, 23B, 23F, 24F, 33F and 35B and additionally comprising at least ten glycoconjugates from *S. pneumoniae* serotypes 2, 6C, 7C, 7F, 9N, 10B, 15C, 16F, 17F, 20A, 20B, 21, 22A, 24B, 27, 29, 31, 33B, 34, 35F, 38, 72 and 73.
20. The immunogenic composition of claim 19 which is a 35-valent pneumococcal conjugate composition.
21. The immunogenic composition of any one of claims 1 to 20 for use as a medicament.
22. The immunogenic composition of any one of claims 1 to 20 for use as a vaccine.