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(54) **Eljárás bakteriális poliszacharidok hordozófehérjékhez történő konjugálására**

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(54) **CONJUGATION PROCESS OF BACTERIAL POLYSACCHARIDES TO CARRIER PROTEINS**

Konjugationsverfahren für bakterielle Polysaccharide an Trägerproteine

Procédé de conjugaison de polysaccharides bactériens à protéines porteuses

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WO-A2-2007/071707 US-A- 4 356 170
US-A1- 2007 184 071

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Description

[0001] The present invention relates to a process for conjugation. In particular, it relates to the conjugation of saccharides and proteins using reductive amination.

BACKGROUND

[0002] Bacterial capsular polysaccharides have been widely used in immunology for many years for the prevention of bacterial disease. A problem with such a use, however, is the T-independent nature of the immune response. These antigens are thus poorly immunogenic in young children. This problem has been overcome through conjugating the polysaccharide antigens to a carrier protein (a source of T-helper epitopes) which may then be used to elicit a T-dependent immune response, even in the first year of life.

[0003] Various conjugation techniques are known in the art. Conjugates can be prepared by direct reductive amination methods as described in, US200710184072 (Hausdorff) US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508. The conjugation method may alternatively rely on activation of hydroxyl groups of the saccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. Such conjugates are described in PCT published application WO 93/15760 Uniformed Services University and WO 95/08348 and WO 96/29094. See also Chu C. et al Infect. Immunity, 1983 245 256.

[0004] Reductive amination involves two steps, (1) oxidation of the antigen, (2) reduction of the antigen and a carrier protein to form a conjugate. The oxidation step may involve reaction with periodate, however oxidation by periodate may lead to size reduction (WO94/05325).

SUMMARY OF INVENTION

[0005] The inventors have surprisingly found that using lower concentrations of periodate in the presence of low phosphate may lead to retention of size and/or the retention of epitopes.

[0006] In a first aspect of the invention there is provided a process for conjugating a bacterial saccharide(s) comprising the steps of

a) reacting the bacterial saccharide with 0.001-0.7, 0.005-0.5, 0.01-0.5, 0.1-0.5 or 0.1-0.2, molar equivalents of periodate to form an activated bacterial saccharide;
b) mixing the activated bacterial saccharide with a carrier protein;
c) reacting the activated bacterial saccharide and the carrier protein with a reducing agent to form a conjugate;

or

a) reacting the bacterial saccharide with 0.001-0.7, 0.005-0.5, 0.01-0.5, 0.1-0.5 or 0.1-0.2 molar equivalents of periodate to form an activated bacterial saccharide;
b) mixing the activated bacterial saccharide with a linker;
c') reacting the activated bacterial saccharide with the linker using a reducing agent to form a bacterial saccharide-linker;
d) reacting the bacterial saccharide-linker with a carrier protein to form a conjugate;

wherein step a) occurs in a buffer which does not contain an amine group, and the buffer has a concentration between 1-100mM.

[0007] There is disclosed a conjugate obtainable by the process of the invention.

[0008] There is disclosed a conjugate obtained by the process of the invention.

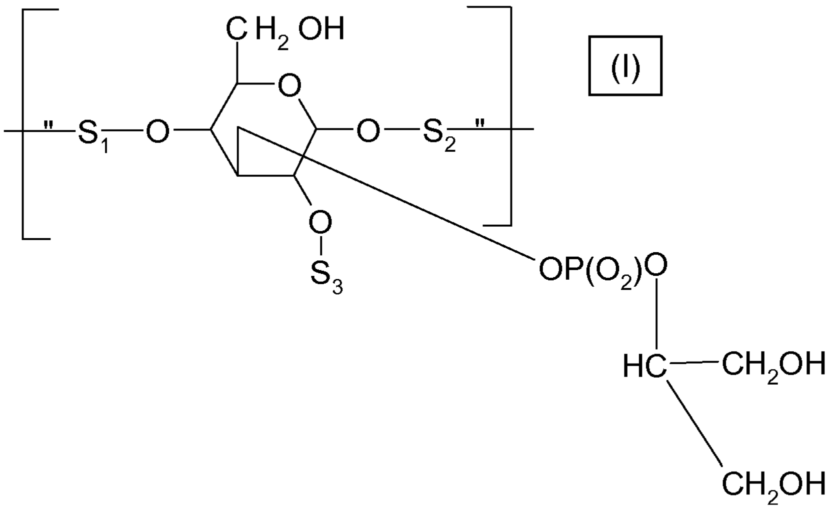
[0009] There is disclosed an immunogenic composition comprising the conjugate of the invention and a pharmaceutically acceptable excipient.

[0010] There is disclosed a vaccine comprising the immunogenic composition of the invention.

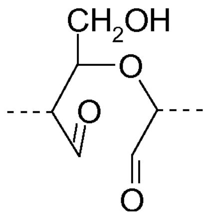
[0011] There is disclosed a use of the immunogenic composition of the invention or the vaccine of the invention in the prevention or treatment of bacterial disease

[0012] There is disclosed a use of the immunogenic composition of the invention or the vaccine of the invention in the preparation of a medicament for the prevention or treatment of bacterial disease. There is disclosed a method of preventing or treating bacterial infection comprising administration of the immunogenic composition of the invention or the vaccine of the invention to a patient.There is disclosed an activated bacterial saccharide, wherein the activated bacterial saccharide comprises a repeat unit of formula (I):

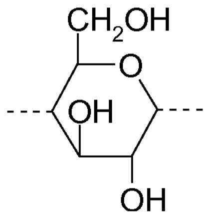
(I)



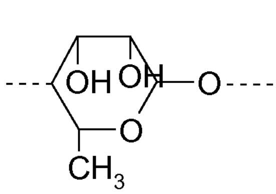
wherein the activated bacterial saccharide comprises n repeat units and n is between 2 and 2400, between 500 and 2000, between 750 and 1500, between 1000 and 2000 or between 1500 and 2300.
wherein at least 0.001 %, 0.01 %, 0.1 %, 0.5%, 1%, 2%, 5%, 10% or 30% but less than 0.001%, 0.01%, 0.1%, 0.5%, 1%, 2%, 5%, 10%, 30% or 50% of S1 is



and the remainder is



wherein S2 is either



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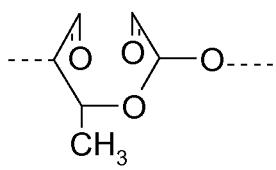
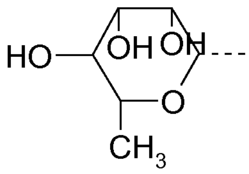


and wherein S3 is either



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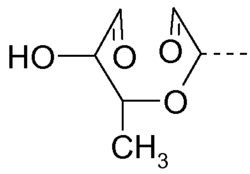


DESCRIPTION OF FIGURES

[0013]

Figure 1. Size of 23F and 6B polysaccharides following periodate treatment. The line marked with triangles shows the size of 6B in 10mM phosphate buffer, the line marked with diamonds shows the size of 23F in 10mM phosphate buffer and the line marked with squares shows the size of 23F in 100mM phosphate buffer.

Figure 2. Comparison of immunogenicity of 23F conjugates using either CDAP or reductive amination conjugation. Graph a) describes the results of an ELISA assay. Graph b) describes the results of an opsonophagocytosis assay.

Figure 3 Evaluation of the immunogenicity of PS06B-CRM conjugated using the conjugation methods described in example 4 in a Balb/c mouse model.

Figure 4 Evaluation of the immunogenicity of PS06B-CRM conjugated using the conjugation methods described in example 4 in a guinea pig model.

DETAILED DESCRIPTION OF THE INVENTION

[0014] The invention relates to an improved process for conjugating an antigen to a carrier protein. In particular, the invention provides a process for conjugating a bacterial saccharide(s) comprising the steps of

a) reacting the bacterial saccharide with 0.001-0.7, 0.005-0.5, 0.01-0.5, 0.1-0.5 or 0.1-0.2 molar equivalents of periodate to form an activated bacterial saccharide;
b) mixing the activated bacterial saccharide with a carrier protein;
c) reacting the activated bacterial saccharide and the carrier protein with a reducing agent to form a conjugate;

or

a) reacting the bacterial saccharide with 0.001-0.7, 0.005-0.5, 0.01-0.5, 0.1-0.5 or 0.1-0.2 molar equivalents of periodate to form an activated bacterial saccharide;
b) mixing the activated bacterial saccharide with a linker;
c') reacting the activated bacterial saccharide with the linker using a reducing agent to form a bacterial saccharide-linker;
d) reacting the bacterial saccharide-linker with a carrier protein to form a conjugate;

wherein step a) occurs in a buffer which does not contain an amine group, and the buffer has a concentration between 1-100mM.
The term 'periodate' includes both periodate and periodic acid. This term also includes both metaperiodate (IO_4^-) and orthoperiodate (IO_6^{5-}), however in one particular embodiment the periodate used in the method of the invention is metaperiodate. The term 'periodate' also includes the various salts of periodate including sodium periodate and potassium periodate. In one embodiment the periodate used is sodium metaperiodate. When an antigen 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 an antigen with periodate' includes oxidation of vicinal hydroxyl groups by periodate.
[0015] For the purposes of the invention an 'activated bacterial saccharide' is a bacterial saccharide which has been activated by step a) of the process of the invention.
[0016] For the purposes of the invention the term 'conjugate' indicates a bacterial saccharide linked covalently to a carrier protein. In one embodiment a bacterial saccharide is linked directly to a carrier protein. In a second embodiment a bacterial saccharide is linked to a protein through a spacer/linker.
[0017] The buffer used in step a) is a buffer which does not contain an amine group. In one embodiment the buffer is selected from the list consisting of phosphate buffer, borate buffer, acetate buffer, carbonate buffer, maleate buffer and

citrate buffer. In a second embodiment the buffer is an inorganic buffer. The term inorganic buffer includes any buffer solution wherein the buffering capacity is due to the presence of a compound which does not contain carbon. Inorganic buffers of the invention include phosphate buffer and borate buffer. In one embodiment the buffer is phosphate buffer.

[0018] In one embodiment the buffer has a concentration between 1-100mM, 5-80mM, 1-50mM, 1-25mM, 10-40mM, 1-10mM, 5-15mM, 8-12mM, 10-20mM, 5-20mM, 10-50mM, around 10mM or around 20mM. In a further embodiment the pH in step a) is pH 2.5-8.0, pH5.0-7.0, pH 5.5-6.5, pH 5.8-6.3, or around pH 6.0.

[0019] The term "saccharide" throughout this specification may indicate polysaccharide, techoic acid or oligosaccharide and includes all three. It may indicate lipopolysaccharide (LPS) or lipooliogosaccharide (LOS). Before use Polysaccharides may be isolated from a source strain or isolated from the source strain and sized to some degree by known methods (see for example EP497524 and EP497525; Shousun Chen Szu et al. - Carbohydrate Research Vol 152 p7-20 (1986)) for instance by microfluidisation. Oligosaccharides have a low number of repeat units (typically 5-30 repeat units) and are typically hydrolysed polysaccharides.The bacterial saccharide may be a bacterial capsular saccharide. Disclosed are bacterial saccharides originating from Group B Streptococcus, *Vibrio cholera*, *Streptococus pneumoniae* (*S.pneumoniae)*, *Haemophilus influenzae (H.influenzae)*, *Neisseria meningitidis (N.meningitidis)*, *Staphylococcus aureus (S.aureus)*, enterococci, Salmonella Vi, or *Staphylococcus epidermidis (S.epidermidis).* Disclosed are bacterial saccharides originating from *S.pneumoniae*, *H.influenzae*, *N.meningitidis*, *S.aureus*, enterococci, Salmonella Vi, or *S.epidermidis.* The bacterial saccharide may be a bacterial capsular saccharide selected from a list consisting of: *N. meningitidis* serogroup A (MenA), B (MenB), C (MenC), W135 (MenW) or Y (MenY), Group B Streptococcus group Ia, Ib, II, III, IV, V, VI, or VII, *Staphylococcus aureus* type 5, *Staphylococcus aureus* type 8, *Salmonella typhi* (Vi saccharide), *Vibrio cholerae*, or *H. influenzae* type b. In one embodiment the bacterial saccharide is a capsular saccharide from *Streptococcus pneumoniae* serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F or 33F. The bacterial saccharide may be an *S.pneumoniae* capsular saccharide selected from the group consisting of 5, 6B, 6A, 7F, 9V, 14, or 23F. The bacterial saccharide may be an *S.pneumoniae* capsular saccharide 23F, 6B or 6A. The bacterial saccharide of the invention is an *S.pneumoniae* capsular saccharide 23F. Also disclosed is Haemophilus influenzae b (Hib) polysaccharide or oligosaccharide. The bacterial saccharide may contain vicinal anti diols.

[0020] The bacterial saccharide may be either a native polysaccharide or may have been reduced in size by a factor of no more than x2, x4, x6, x8, x10 or x20 (for instance by microfluidization [e.g. by Emulsiflex C-50 apparatus] or other known technique [for instance heat, chemical, oxidation, sonication methods]). In one embodiment the bacterial saccharide is microfluidised before step a). Oligosaccharides may have been reduced in size substantially further [for instance by known heat, chemical, or oxidation methods].

[0021] For the purposes of the invention, "native polysaccharide" refers to a bacterial saccharide that has not been subjected to a process, the purpose of which is to reduce the size of the saccharide. A polysaccharide can become slightly reduced in size during normal purification procedures. Such a saccharide is still native. Only if the polysaccharide has been subjected to techniques which reduce a saccharide in size would the polysaccharide not be considered native.

[0022] The weight-average molecular weight of a bacterial saccharide suitable for conjugation by the process of the invention may be between 20kDa and 2000kDa, between 30kDa and 1000kDa, between 40kDa and 500kDa, between 50kDa and 400kDa, between 75kDa and 300kDa or between 1000kDa and 2000kDa. In the case of the native 23F capsular saccharide from *S.pneumoniae,* the average molecular weight of the native polysaccharide is between 750-1500 kDa or 1200-1300kDa. In the case of the native Hib saccharide, the average molecular weight of the native polysaccharide is between 100 and 250kDa. The molecular weight or average molecular weight of a saccharide herein refers to the weight-average molecular weight (Mw) of the bacterial saccharide measured prior to conjugation and is measured by MALLS. The MALLS technique is well known in the art. For MALLS analysis of saccharides, two columns (TSKG6000 and 5000PWxl) may be used in combination and the saccharides are eluted in water. Saccharides are detected using a light scattering detector (for instance Wyatt Dawn DSP equipped with a 10mW argon laser at 488nm) and an inferometric refractometer (for instance Wyatt Otilab DSP equipped with a P100 cell and a red filter at 498nm). MALLS analyses may be carried out using a TSKGMPwxl and 50 mM Na/K PO4, 200 mM NaCl pH 7.0 as elution buffer with 0.75 ml/min using RI/DAWN-EOS detector. In an embodiment, the polydispersity of the saccharide is 1-1.5, 1-1.3, 1-1.2, 1-1.1 or 1-1.05 and after conjugation to a carrier protein, the polydispersity of the conjugate is 1.0-2.5, 1.0-2.0. 1.0-1.5, 1.0-1.2, 1.5-2.5, 1.7-2.2 or 1.5-2.0. All polydispersity measurements are generated by MALLS.

[0023] Treatment with periodate may lead to a reduction in the size of the bacterial saccharide (sizing effect). In one embodiment the process of the invention reduces this sizing effect. This is seen for the 23F bacterial saccharide from Streptococcus pneumoniae (as in example 1). For this reason, in one embodiment the average molecular weight of a bacterial saccharide of the invention is between 1-1100kDa, 100-470kDa, 200-300kDa, 600-1100kDa or 800-1000kDa after step a) (measured by MALLS as described above). In one embodiment the average molecular weight of the 23F saccharide is between 100-470kDa or 200-300kDa after step a). In one embodiment the average molecular weight of the Hib bacterial saccharide is between 1 and 50kDa or between 5 and 10kDa after step a).

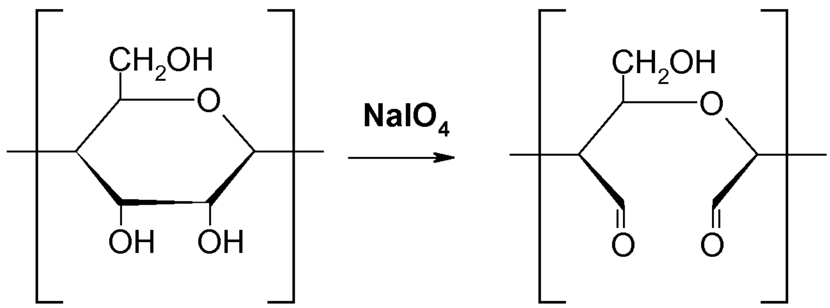
[0024] The term "carrier protein" is intended to cover both small peptides and large polypeptides (>10 kDa). The carrier protein may be any peptide or protein. It may comprise one or more T-helper epitopes. The carrier protein may be tetanus toxoid (TT), tetanus toxoid fragment C, non-toxic mutants of tetanus toxin [note all such variants of TT are considered to be the same type of carrier protein for the purposes of this invention], polypeptides comprising tetanus toxin T-cell epitopes such as N19 (WO2006/067632), diphtheria toxoid (DT), CRM197, other non-toxic mutants of diphtheria toxin [such as CRM176, CRM 197, CRM228, CRM 45 (Uchida et al J. Biol. Chem. 218; 3838-3844, 1973); CRM 9, CRM 45, CRM102, CRM 103 and CRM107 and other mutations described by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc, 1992; deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala 158 to Gly and other mutations disclosed in US 4709017 or US 4950740; mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in US 5917017 or US 6455673; or fragment disclosed in US 5843711] (note all such variants of DT are considered to be the same type of carrier protein for the purposes of this invention), pneumococcal pneumolysin (Kuo et al (1995) Infect Immun 63; 2706-13), OMPC (meningococcal outer membrane protein - usually extracted from N. meningitidis serogroup B - EP0372501), synthetic peptides (EP0378881, EP0427347), heat shock proteins (WO 93/17712, WO 94/03208), pertussis proteins (WO 98/58668, EP0471177), cytokines, lymphokines, growth factors or hormones (WO 91/01146), artificial proteins comprising multiple human CD4+ T cell epitopes from various pathogen derived antigens (Falugi et al (2001) Eur J Immunol 31; 3816-3824) such as N19 protein (Baraldoi et al (2004) Infect Immun 72; 4884-7) pneumococcal surface protein PspA (WO 02/091998), iron uptake proteins (WO 01/72337), toxin A or B of C. difficile (WO 00/61761), H. influenzae Protein D (EP594610 and WO 00/56360), pneumococcal PhtA (WO 98/18930, also referred to Sp36), pneumococcal PhtD (disclosed in WO 00/37105, and is also referred to Sp036D), pneumococcal PhtB (disclosed in WO 00/37105, and is also referred to Sp036B), or PhtE (disclosed in WO00/30299 and is referred to as BVH-3).

[0025] In one embodiment of the invention the carrier protein is selected from the group consisting of: tetanus toxoid (TT), fragment C of tetanus toxoid, diphtheria toxoid (DT), CRM197, Pneumolysin (Ply), protein D, PhtD, PhtDE and N19. In a further embodiment the carrier protein is CRM197. In a still further embodiment the carrier protein is tetanus toxoid (TT).

[0026] In one embodiment step a) is carried out in the dark.

[0027] When an antigen reacts with periodate, periodate oxidises vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. The oxidation step (step

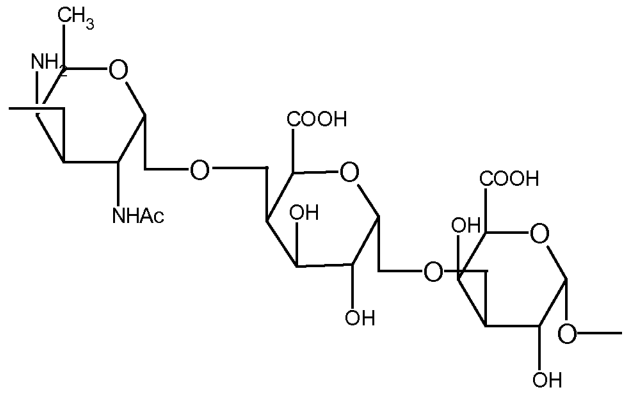
a)) may occur as described below:



[0028] When low concentrations of buffer, in particular phosphate buffer and low amounts of periodate are used, this may reduce the sizing effect described above.

[0029] *Streptococcus pneumoniae* capsular saccharides contain vicinal hydroxyl groups which are capable of being oxidised by periodate as can be seen from the structures of the repeated regions shown below:

PS1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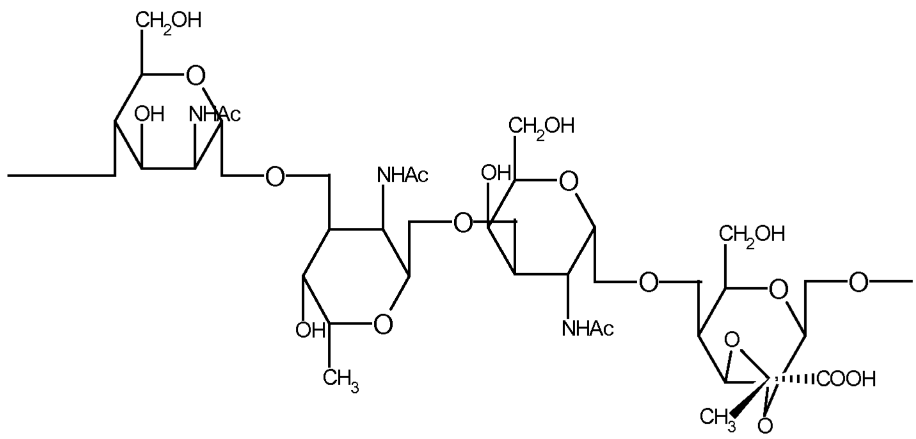
PS4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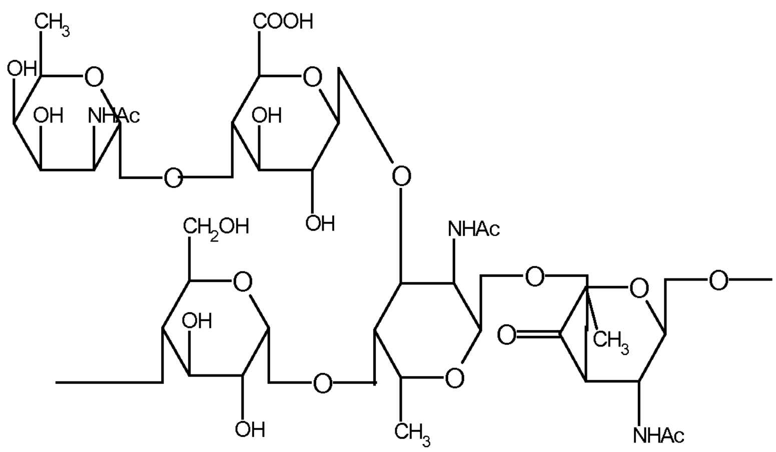
PS5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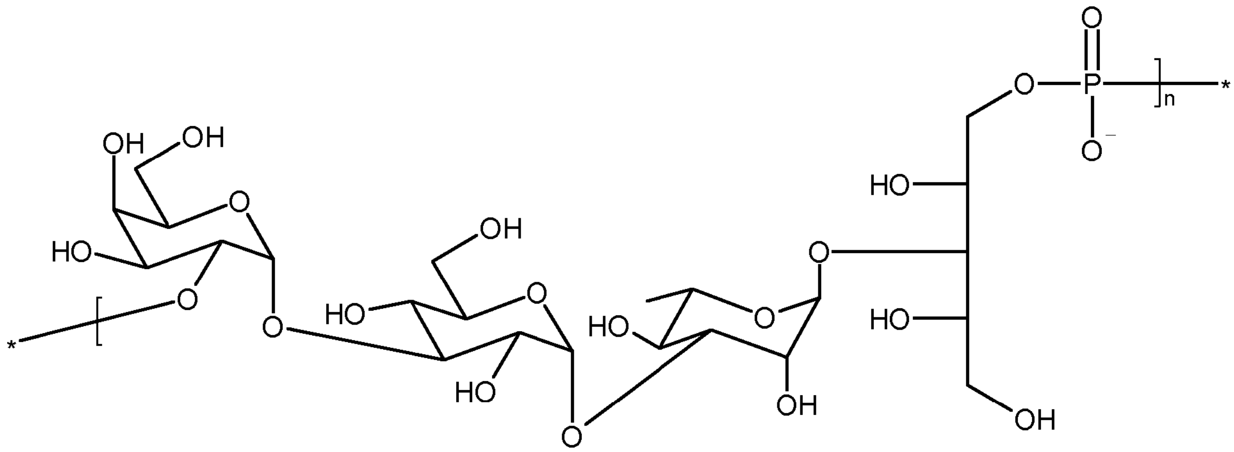

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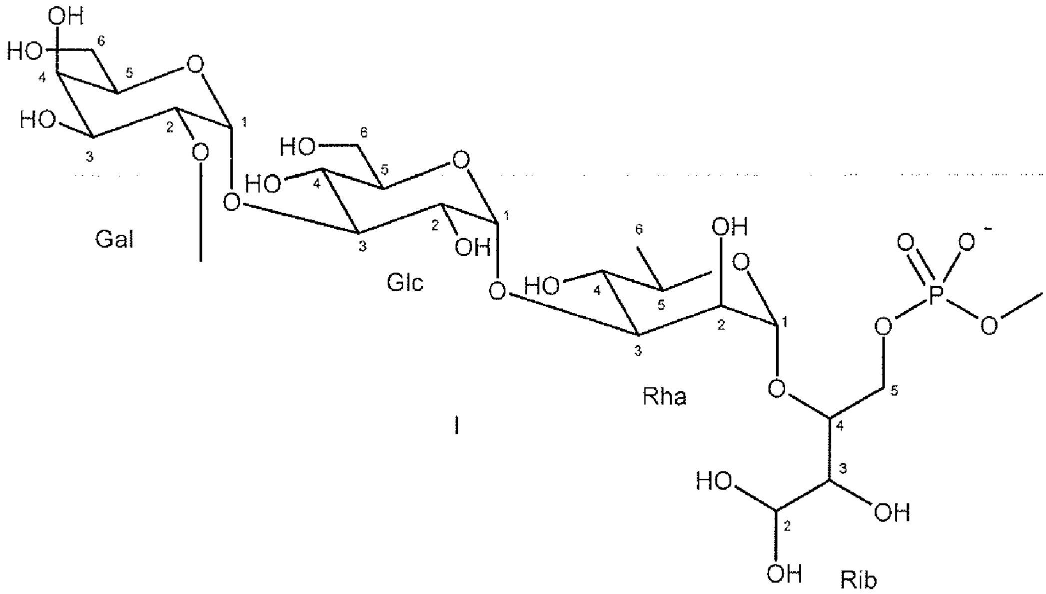

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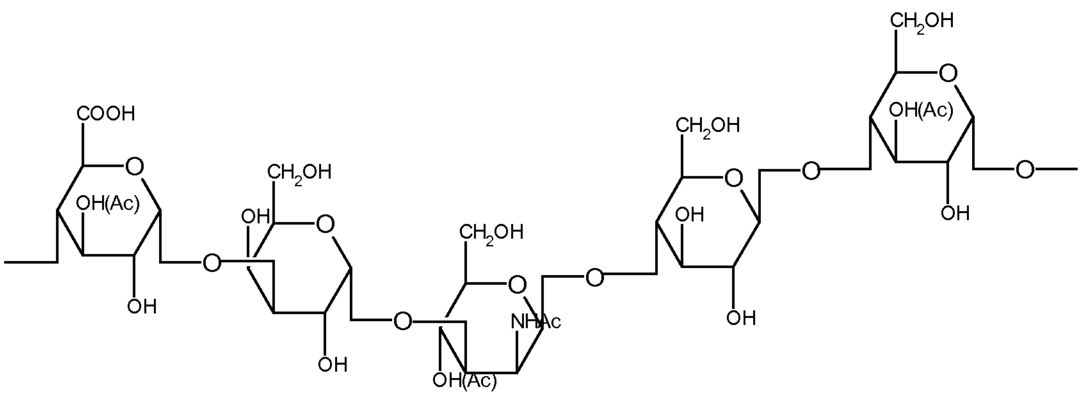
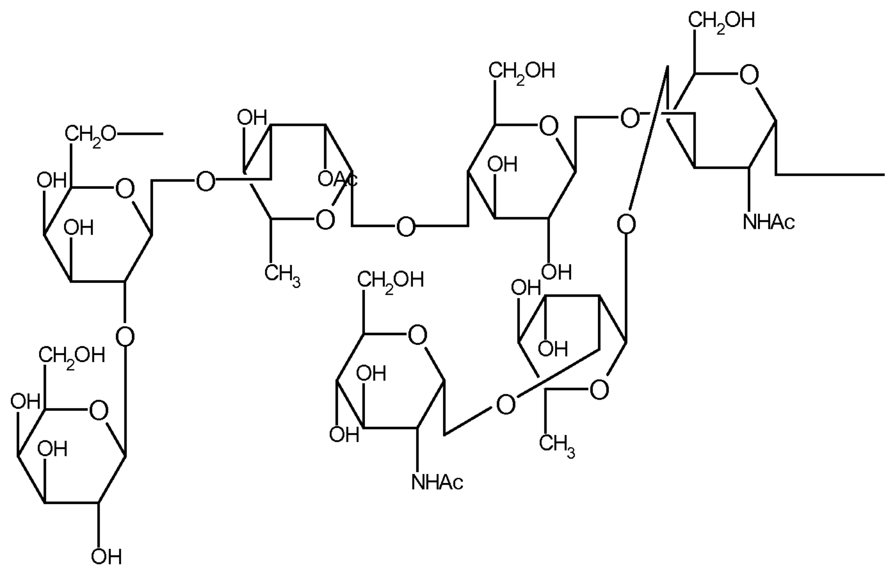


PS7F



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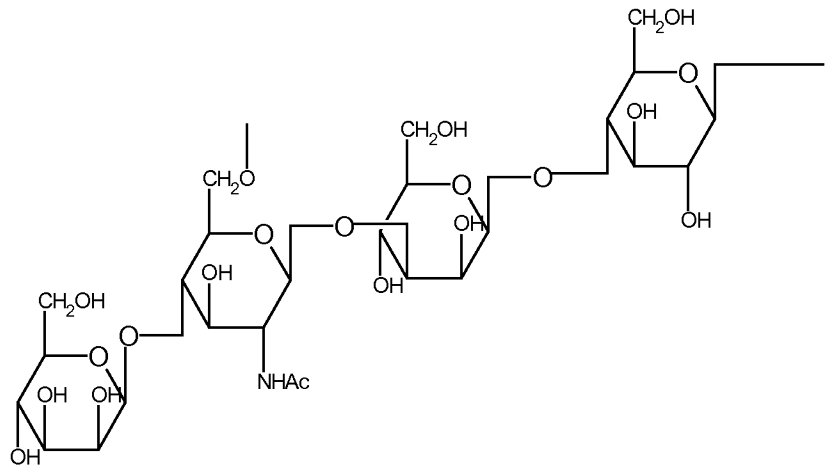
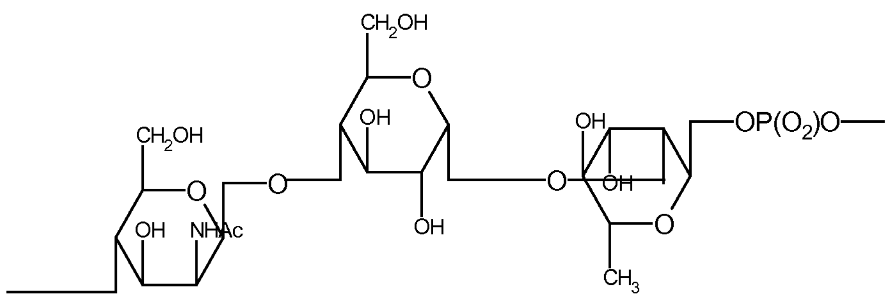
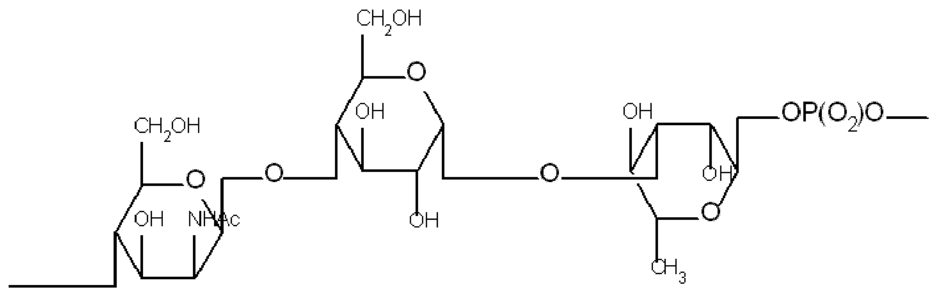


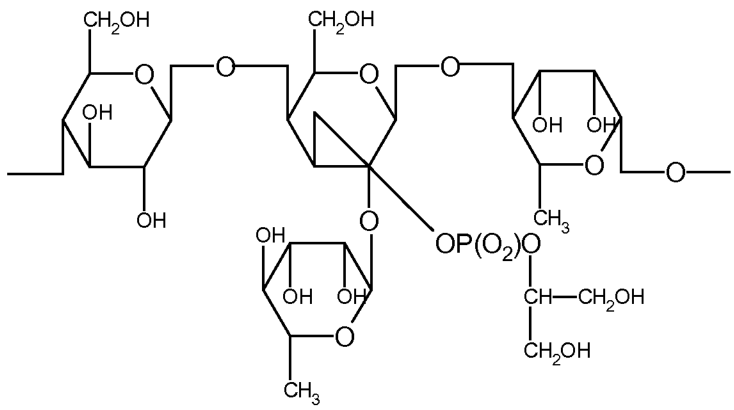
PS19F



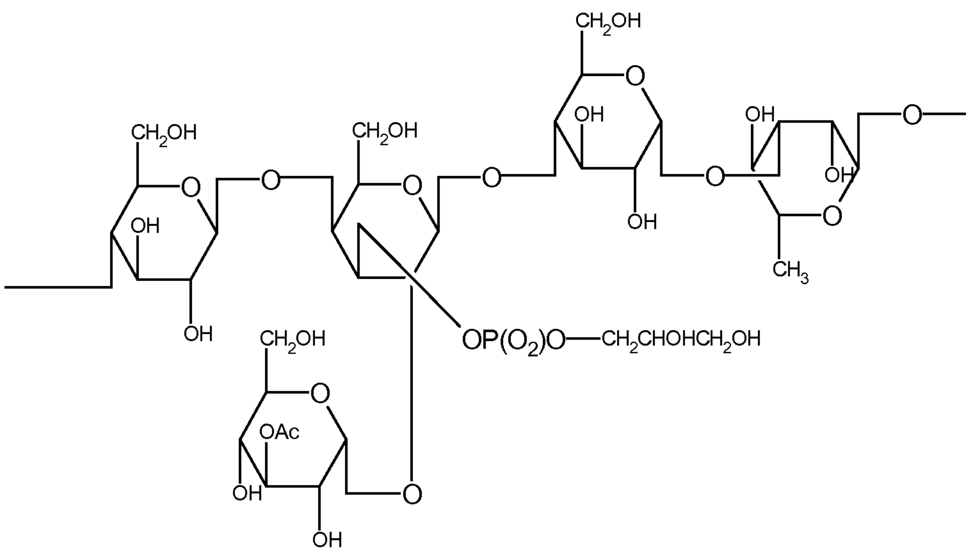
PS19A



PS23F

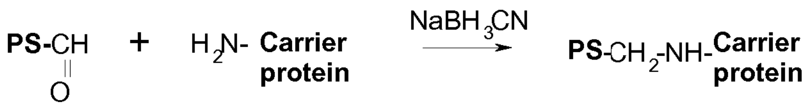


PS18C



[0030] In one embodiment less than 0.001%, 0.01%, 0.1%, 0.5%, 1%, 2%, 5%, 10%, 30% or 50% of the vicinal diols of the bacterial saccharide become oxidised during step a).

[0031] In one embodiment the carbonyl group produced in step a) reacts with an amine group on the carrier protein in step c). This may occur according to the following reaction scheme:



[0032] In one embodiment the bacterial saccharide is present at a concentration of between 0.2g/l and 14g/l 8g/l and 12g/l, 10 g/l and 12g/l, 1g/l and 4g/l, 0.2g/l and 1g/l or between 0.4g/l and 0.6g/l or around 11g/l or around 0.5g/l in step a). In one embodiment the initial concentration of carrier protein in step b) is between 0.5g/l and 35g/l, 25g/l and 35g/l, 0.5g/l and 5g/l or between 0.8g/l and 2g/l or around 32g/l or 1g/l. In a further embodiment the initial concentration of activated bacterial saccharide in step b) is between 0.2g/l and 20g/l, 10g/l and 28g/l, or 0.2g/l and 4g/l or between 1 g/l and 2g/l or around 15g/l or 1.6 g/l. In a further embodiment the initial ratio of activated bacterial saccharide to carrier protein in step b) is 2.0:1 to 0.1:1, 1.8:1 to 0.4:1, 1.4:1 to 1.6:1, 1:1 to 1.4:1, 1.8:1 to 1.6:1, 0.8:1 to 0.4:1, 0.7:1 to 0.5:1, or 0.7:1 to 0.6:1 (w/w). In a further embodiment the final ratio of carrier protein to bacterial saccharide after step c) or c') is 0.5:1 to 4:1, 0.8:1 to 3.2:1, 0.5:1 to 1.8:1, 1.4:1 to 1.8:1, 1:1 to 1.2:1 or 2.5:1 to 3.5:1.

[0033] In one embodiment the temperature of the reaction in step a) is 4-40°C, 10-32°C, 17-30°C or 22-27°C. Typically this temperature is maintained through step a). The reaction temperature during step c) is 4-40°C, 10-32°C, 17-30°C or 22-27°C. Typically this temperature is maintained through step c).

[0034] In one embodiment step a) of the process of the invention takes place in less than 30 hours, between 5 and 25 hours, between 15 and 25 hours, between 30 minutes and 25 hours, between 1 hour and 35 hours, between 10 and 20 hours, or between 15 and 20 hours around 18 hours or around 1 hour. In one embodiment step c) of the process of the invention takes place in between 10-60 hours, 10-20 hours, 20-60 hours, between 30-50 hours, or between 35-45 hours.

[0035] Conjugation may also occur through the addition of a hetero-or homo-bifunctional linker using the chemistry of the invention. One end of the linker will react with the activated antigen by reductive amination, however the other end of the linker may react with the carrier protein using any type of chemistry. For this reason the linker will contain at least one reactive amino group, if the linker is homo-bifunctional it will contain two reactive amino groups, if the linker is hetero-bifunctional it will contain one reactive amino group and a different reactive group, in one embodiment this second reactive group is a reactive carbonyl group. In one embodiment the linker is between 1 and 20 Angstroms in length. In a further embodiment the linker has between 4 and 20, 4 and 12, or 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).

[0036] In general the following types of chemical groups on the carrier protein can be used for coupling / conjugation as the second reactive group:

A) Carboxyl (for instance via aspartic acid or glutamic acid). In one embodiment this group is linked to an amino group on a linker with carbodiimide chemistry e.g. with EDAC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide)). Note: instead of EDAC above, any suitable carbodiimide may be used.

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

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

D) The protein could be modified to contain an alkynyl or azide group , this could be conjugated to the linker using the 'click' chemistry (described in Tetrahedron letters (June 2005) 46:4479-4482).

[0037] Note: instead of EDAC above, any suitable carbodiimide may be used.

[0038] Reducing agents which are suitable for use in the process of the invention include the cyanoborohydrides, such as sodium cyanoborohydride, borane-pyridine, or borohydride exchange resin. In one embodiment the reducing agent is sodium cyanoborohydride. In one embodiment between 0.5 and 2, 0.6 and 1.5 or 0.8 and 1.2 or around 1.0 molar equivalent of sodium cyanoborohydride is used in step c). In a further embodiment the reducing agent comprises sodium triacetoxyborohydride, in a further embodiment between 2 and 10 or between 3 and 9 molar equivalent or around 2.5 molar equivalent of sodium triacetoxyborohydride is used in step c).

[0039] Before step c) the activated bacterial saccharide and the carrier protein may be lyophilised. In one embodiment the activated bacterial saccharide and the carrier protein are lyophilised together. This can occur before step b), or after step b). In one embodiment the lyophilisation 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 a further embodiment the non-reducing sugar is selected from the group consisting of sucrose, trehalose or mannitol.

[0040] In one embodiment steps b) and/or c) are carried out in DMSO (dimethylsulfoxide) solvent. In a further embodiment steps b) and/or c) are carried out in DMF (dimethylformamide) solvent. The DMSO or DMF solvent may be used to reconstitute the activated bacterial saccharide and carrier protein which has been lyophilised.

[0041] At the end of step c) there may be unreacted carbonyl groups remaining in the conjugates, these may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride ($NaBH_4$), for example the product of step c) may be reacted with sodium borohydride for for 15 mins-15hrs, 15mins-45mins, 2-10hrs or 3-5hrs, around 30 mins or around 4 hrs. In a further embodiment capping is achieved by mixing the product of step c) with around 2 molar equivalents or between 1.5 and 10 molar equivalents of $NaBH_4$.

[0042] The invention also provides a further step e) of purifying the conjugate, step e) may comprise diafilitration, for example diafiltration with a cut-off of 100kDa. In addition or alternatively step e) may comprise ion exchange chromatography. In a further embodiment step e) may comprise size exclusion chromatography. In one embodiment the process of embodiments 1-51 comprises a further step f), wherein the conjugate is sterile filtered.

[0043] The conjugate may also be mixed with further antigens. In one embodiment the further antigens comprise at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 *S.pneumoniae* saccharides selected from the group consisting of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. In one embodiment the further antigens comprise *S.pneumoniae* saccharides 4, 6B, 9V, 14, 18C and 19F. In one embodiment the further antigens comprise *S.pneumoniae* saccharides 4, 9V, 14, 18C, 19F and 23F. In one embodiment the further antigens comprise *S.pneumoniae* saccharides1, 4, 5, 6B, 7F, 9V, 14, 18C, and 19F. In one embodiment the further antigens comprise *S.pneumoniae* saccharides1, 4, 5, 7F, 9V, 14, 18C, 19F and 23F. In one embodiment the further antigens comprise *S.pneumoniae* saccharides1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A and 19F. In one embodiment the further antigens comprise *S.pneumoniae* saccharides1, 3, 4, 5, 6A, 7F, 9V, 14, 18C, 19A, 19F and 23F. In one embodiment the further antigens comprise *S.pneumoniae* saccharides1, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A and 19F. In one embodiment the further antigens comprise *S.pneumoniae* saccharides1, 4, 5, 6A, 7F, 9V, 14, 18C, 19A, 19F and 23F.

[0044] Any of the saccharides listed as 'further antigens' are optionally conjugated to a carrier protein either by the process of the invention or by a different process. Optionally these further antigens are conjugated to the carrier proteins listed above.

[0045] In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 1 conjugated to protein D or CRM197. In an embodiment, the further antigens comprise S. *pneumoniae* capsular saccharide 3 conjugated to protein D, CRM197, pneumolysin or PhtD or fragment or fusion protein thereof. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 4 conjugated to protein D or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 5 conjugated to protein D or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 6B conjugated to protein D or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 7F conjugated to protein D or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 9V conjugated to protein D or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 14 conjugated to protein D or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 23F conjugated to protein D or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 18C conjugated to tetanus toxoid or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 19A conjugated to pneumolysin or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 22F conjugated to CRM197 or PhtD or fragment of fusion protein thereof. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 6A conjugated to pneumolysin or a *H. influenzae* protein, optionally protein D or PhtD or fusion protein thereof or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 6C conjugated to pneumolysin or a *H. influenzae* protein, optionally protein D or PhtD or fusion protein thereof or CRM197. In an embodiment, the further antigens comprise *S. pneumoniae* capsular saccharide 19F conjugated to Diphtheria toxoid (DT).

[0046] The further antigens may also comprise *Streptococcus pneumoniae* proteins. In one embodiment the further antigens comprise at least 1 protein selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133.

[0047] The further antigens may also comprise antigens from further bacterial species. Also disclosed is the vaccine or immunogenic composition comprises antigens originating from *S.pneumoniae (S. pneumoniae), Haemophilus influenzae (H. Influenzae), Neisseria meningitidis (N. Meningitidis),Escherichia coli (E.col)i, Moraxella cattharlis (M.cattarhalis),* tetanus, diphtheria, pertussis, Staphylococcus epidermidis (*S.epidermidis)*, enterococci, Pseudomonas or *Staphylococcus aureus (S. aureus).*Also disclosed are further antigens comprising *M.cattarhalis* antigens, preferred *M.cattarhalis* antigens are: OMP106 [WO 97/41731 (Antex) & WO 96/34960 (PMC)]; OMP21; LbpA & LbpB [WO 98/55606

(PMC)]; TbpA & TbpB [WO 97/13785 & WO 97/32980 (PMC)]; CopB [Helminen ME, et al. (1993) Infect. Immun. 61:2003-2010]; UspA1/2 [WO 93/03761 (University of Texas)]; and OmpCD. Examples of non-typeable Haemophilus influenzae antigens which can be included in a combination vaccine (especially for the prevention of otitis media) include: Fimbrin protein [(US 5766608 - Ohio State Research Foundation)] and fusions comprising peptides therefrom [eg LB1(f) peptide fusions; US 5843464 (OSU) or WO 99/64067]; OMP26 [WO 97/01638 (Cortecs)]; P6 [EP 281673 (State University of New York)]; TbpA and TbpB; Hia; Hmw1,2; Hap; and D15.Also disclosed are further antigens comprising Diphtheria toxoid (DT), tetanus toxoid (TT), and pertussis components [typically detoxified Pertussis toxoid (PT) and filamentous haemagglutinin (FHA) with optional pertactin (PRN) and/or agglutinin 1+2], for example the marketed vaccine INFANRIX-DTPaTM (SmithKlineBeecham Biologicals) which contains DT, TT, PT, FHA and PRN antigens, or with a whole cell pertussis component for example as marketed by SmithKlineBeecham Biologicals s.a., as TritanrixTM. In a further embodiment the further antigens comprise Hepatitis B surface antigen (HepB).Also disclosed are further antigens comprising the PRP capsular saccharide of *H.influenzae* (Hib). Also disclosed are further antigens comprising at least one capsular saccharide from *N.meningitidis* A, C, W or Y. In a further embodiment the further antigens comprise at least one conjugate of a capsular saccharide from *N.meningitidis* A, C, W or Y.

[0048] The conjugate may also be mixed with an adjuvant. Suitable adjuvants include, but are not limited to, aluminium salts (aluminium phosphate or aluminium hydroxide), monophosphoryl lipid A (for example 3D-MPL), saponins (for example QS21), oil in water emulsions, blebs or outer membrane vesicle preparations from Gram negative bacterial strains (such as those taught by WO02/09746), lipid A or derivatives thereof, alkyl glucosamide phosphates or combinations of two or more of these adjuvants.

[0049] In a further embodiment the conjugate of the invention is mixed with a pharmaceutically acceptable excipient.Also disclosed is a conjugate obtainable by the process of the invention. In a further aspect of the invention there is provided a conjugate obtained by the process of the invention. The invention also provides an immunogenic composition comprising the conjugate of the invention and a pharmaceutically acceptable excipient. In one embodiment the pharmaceutical acceptable excipient does not contain a chloride salt, in a further embodiment the pharmaceutical excipient does not contain sodium chloride. In one embodiment the pharmaceutical excipient comprises a buffer selected from the group consisting of maleate, tris, or citrate. In a further embodiment the buffer is maleate buffer. The immunogenic composition of the invention may comprise further antigens, in particular those described as 'further antigens' above. The immunogenic composition may comprise an adjuvant, particularly those described above.Also disclosed is a vaccine comprising the immunogenic.

[0050] The vaccine preparations containing immunogenic compositions may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Intranasal administration of vaccines for the treatment of pneumonia or otitis media is possible (as nasopharyngeal carriage of pneumococci can be more effectively prevented, thus attenuating infection at its earliest stage). Although the vaccine may be administered as a single dose, components thereof may also be co-administered together at the same time or at different times (for instance pneumococcal saccharide conjugates could be administered separately, at the same time or 1-2 weeks after the administration of the any bacterial protein component of the vaccine for optimal coordination of the immune responses with respect to each other). In addition to a single route of administration, 2 different routes of administration may be used. For example, saccharides or saccharide conjugates may be administered IM (or ID) and bacterial proteins may be administered IN (or ID). In addition, the vaccines may be administered IM for priming doses and IN for booster doses.

[0051] The content of protein antigens in the vaccine will typically be in the range 1-100μg, optionally 5-50μg, most typically in the range 5 - 25μg. Following an initial vaccination, subjects may receive one or several booster immunizations adequately spaced.

[0052] Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

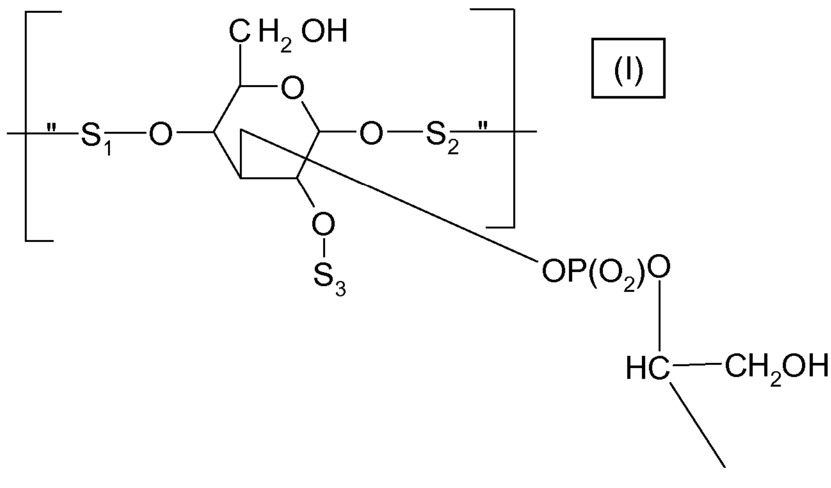
[0053] Although the vaccines may be administered by any route, administration of the described vaccines into the skin (ID) forms one embodiment of the present invention. Human skin comprises an outer "horny" cuticle, called the stratum corneum, which overlays the epidermis. Underneath this epidermis is a layer called the dermis, which in turn overlays the subcutaneous tissue. Researchers have shown that injection of a vaccine into the skin, and in particular the dermis, stimulates an immune response, which may also be associated with a number of additional advantages. Intradermal vaccination with the vaccines described herein forms an optional feature. The conventional technique of intradermal injection, the "mantoux procedure", comprises steps of cleaning the skin, and then stretching with one hand, and with the bevel of a narrow gauge needle (26-31 gauge) facing upwards the needle is inserted at an angle of between 10-15°. Once the bevel of the needle is inserted, the barrel of the needle is lowered and further advanced whilst providing a slight pressure to elevate it under the skin. The liquid is then injected very slowly thereby forming a bleb or bump on the skin surface, followed by slow withdrawal of the needle.

[0054] More recently, devices that are specifically designed to administer liquid agents into or across the skin have been described, for example the devices described in WO 99/34850 and EP 1092444, also the jet injection devices described for example in WO 01/13977; US 5,480,381, US 5,599,302, US 5,334,144, US 5,993,412, US 5,649,912, US 5,569,189, US 5,704,911, US 5,383,851, US 5,893,397, US 5,466,220, US 5,339,163, US 5,312,335, US 5,503,627, US 5,064,413, US 5,520, 639, US 4,596,556, US 4,790,824, US 4,941,880, US 4,940,460, WO 97/37705 and WO 97/13537. Alternative methods of intradermal administration of the vaccine preparations may include conventional syringes and needles, or devices designed for ballistic delivery of solid vaccines (WO 99/27961), or transdermal patches (WO 97/48440; WO 98/28037); or applied to the surface of the skin (transdermal or transcutaneous delivery WO 98/20734 ; WO 98/28037).

[0055] When the vaccines are to be administered to the skin, or more specifically into the dermis, the vaccine is in a low liquid volume, particularly a volume of between about 0.05 ml and 0.2 ml.

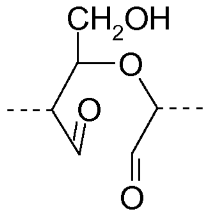
[0056] The content of antigens in the skin or intradermal vaccines of the present invention may be similar to conventional doses as found in intramuscular vaccines (see above). However, it is a feature of skin or intradermal vaccines that the formulations may be "low dose". Accordingly the protein antigens in "low dose" vaccines are optionally present in as little as 0.1 to 10μg or 0.1 to 5 μg per dose; and the saccharide (optionally conjugated) antigens may be present in the range of 0.01-1 μg, or between 0.01 to 0.5 μg of saccharide per dose.

[0057] As used herein, the term "intradermal delivery" means delivery of the vaccine to the region of the dermis in the skin. However, the vaccine will not necessarily be located exclusively in the dermis. The dermis is the layer in the skin located between about 1.0 and about 2.0 mm from the surface in human skin, but there is a certain amount of variation between individuals and in different parts of the body. In general, it can be expected to reach the dermis by going 1.5 mm below the surface of the skin. The dermis is located between the stratum corneum and the epidermis at the surface and the subcutaneous layer below. Depending on the mode of delivery, the vaccine may ultimately be located solely or primarily within the dermis, or it may ultimately be distributed within the epidermis and the dermis.Also disclosed is a vaccine kit, comprising a vial containing an immunogenic composition, optionally in lyophilised form, and further comprising a vial containing an adjuvant as described herein. It is envisioned that in this aspect of the invention, the adjuvant will be used to reconstitute the lyophilised immunogenic composition.Also discloses is a method of immunising a human host against bacterial disease infection comprising administering to the host an immunoprotective dose of the immunogenic composition or vaccine or. Also disclosed is a method of immunising a human host against infection caused by *S. pneumoniae* and/or *Haemophilus influenzae* comprising administering to the host an immunoprotective dose of the immunogenic composition or vaccine or kit. A further aspect is an immunogenic composition for use in the treatment or prevention of bacterial disease. A further aspect is an immunogenic composition for use in the treatment or prevention of disease caused by *S.pneumoniae* and/or *Haemophilus influenzae* infection Also disclosed is an use of the immunogenic composition or vaccine or kit in the manufacture of a medicament for the treatment or prevention of bacterial diseases. Also disclosed is an use of the immunogenic composition or vaccine or kit in the manufacture of a medicament for the treatment or prevention of diseases caused by *S. pneumoniae* and/or *Haemophilus influenzae* infection.Also disclosed is an activated bacterial saccharide, wherein the activated bacterial saccharide comprises a repeat unit of formula (I):

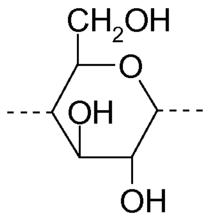


(I)

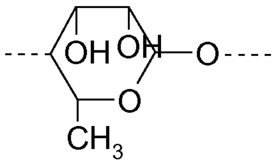
wherein the activated bacterial saccharide comprises n repeat units and n is between 2 and 2400, between 20 and 2000, between 50 and 1500, between 1000 and 2000, between 1000 and 2500 or between 1500 and 2300.
wherein at least 0.001%, 0.01%, 0.1%, 0.5%, 1%, 2%, 5%, 10% or 30% but less than 0.001%, 0.01%, 0.1%, 0.5%, 1%, 2%, 5%, 10%, 30% or 50% of S1 is



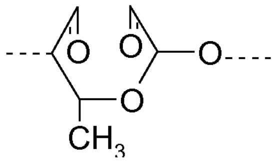
and the remainder is



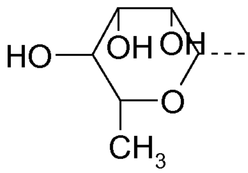
wherein S2 is either



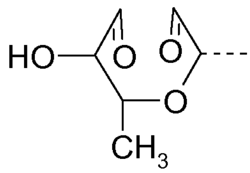
or



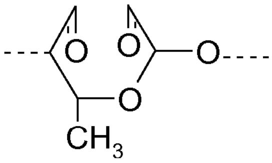
and wherein S3 is either



or



Alternatively less than 0.001%, 0.1%, 0.5% 1%, 2%, 3%, 5%, 10%, 30% or 50% of S2 is



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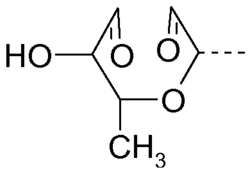
Alternatively less than 0.1%, 0.5%, 1%, 2%, 3%, 5%, 10%, 30% or 50% of S3 is



[0058] "Around" or "approximately" are defined as within 10% more or less of the given figure for the purposes of the invention.

[0059] The terms "comprising", "comprise" and "comprises" herein are intended by the inventors to be optionally substitutable with the terms "consisting of", "consist of" and "consists of", respectively, in every instance.

[0060] Embodiments herein relating to "vaccine compositions" of the invention are also applicable to embodiments relating to "immunogenic compositions" of the invention, and vice versa.

[0061] All references or patent applications cited within this patent specification are incorporated by reference herein.

[0062] In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

[0063] The patent application also discloses the following paragraphs:

1. A process for conjugating a bacterial saccharide comprising the steps of

a) reacting the bacterial saccharide with 0.001-0.7, 0.005-0.5, 0.01-0.5, 0.1-0.5 or 0.1-0.2 molar equivalents of periodate to form an activated bacterial saccharide;
b) mixing the activated bacterial saccharide with a carrier protein;
c) reacting the activated bacterial saccharide and the carrier protein with a reducing agent to form a conjugate;

or

a) reacting the bacterial saccharide with 0.001-0.7, 0.005-0.5, 0.01-0.5, 0.1-0.5 or 0.1-0.2, 0.5-0.8, 0.1-0.8, 0.3-1.0 or 0.4-0.9 molar equivalents of periodate to form an activated bacterial saccharide;
b) mixing the activated bacterial saccharide with a linker;
c') reacting the activated bacterial saccharide with the linker using a reducing agent to form a bacterial saccharide-linker;
d) reacting the bacterial saccharide-linker with a carrier protein to form a conjugate;

wherein step a) occurs in a buffer which does not contain an amine group, and the buffer has a concentration between 1-100mM and wherein the bacterial saccharide is *S.pneumoniae* capsular saccharide 23F.

2. The process of paragraph 1 wherein the buffer is selected from the group consisting of phosphate buffer, borate buffer, acetate buffer, carbonate buffer and citrate buffer.

3. The process of any one of paragraphs 1-2 wherein the buffer is an inorganic buffer.

4. The process of any one of paragraphs1-3 wherein the buffer is phosphate buffer.

5. The process of any one of paragraphs 1-4 wherein the buffer has a concentration between 1-50mM, 1-25mM, 1-10mM, 5-15mM, 8-12mM, or 10-50mM or around 10mM.

6. The process of any one of paragraphs 1-5 wherein the pH in step a) is pH 3.5-8.0 5.0-7.0, or pH 5.5-6.5 or around pH 6.0.

7. The process of any one of paragraphs 1-6 wherein the bacterial saccharide is either a native polysaccharide or is reduced in size by a factor of no more than x20 (for instance by microfluidization).

8. The process of any one of paragraphs 1-7 wherein the bacterial saccharide is microfluidised before step a).

9. The process of any one of paragraphs 8-9 wherein the average molecular weight of the native polysaccharide is between 20kDa and 2000kDa or between 30kDa and 1000kDa.

10. The process of paragraph 9 wherein the average molecular weight of the native polysaccharide is between 750-1500 kDa.

11. The process of paragraph 9 wherein the average molecular weight of the native polysaccharide is between 100 and 250 kDa.

12. The process of any one of paragraphs 1-11 wherein the average molecular weight of the bacterial saccharide is between 1-1100kDa, 100-470kDa, 200-300kDa, 600-1100kDa or 800-1000kDa after step a).

13. The process of paragraph 1 wherein the average molecular weight of the 23F saccharide is between 100-470kDa or 200-300kDa after step a).

14. The process of any one of paragraphs 1-13 wherein the carrier protein is selected from the group consisting of tetanus toxoid, fragment C of tetanus toxoid, diphtheria toxoid, CRM197, Pneumolysin, protein D, PhtD, PhtDE and N19.

15. The process of any one of paragraphs 1-14 wherein step a) is carried out in the dark.

16. The process of any one of paragraphs 1-15 wherein the bacterial saccharide is present at a concentration of between 0.2g/l and 14 g/l, 8g/l and 12g/l, 10g/l and 12g/l, 1g/l and 4g/l 0.2g/l and 1g/l or between 0.4g/l and 0.6g/l in step a).

17. The process of any one of paragraphs 1-16 wherein the initial concentration of carrier protein in step b) is between 05.g/l and 35 g/l, 25g/l and 35g/l, or 0.5g/l and 5g/l.

18. The process of any one of paragraphs 1-17 wherein the initial concentration of activated bacterial saccharide in step b) is between 0.2g/l and 20g/l, 10g/l and 28g/l, or between 0.2g/l and 4g/l.

19. The process of any one of paragraphs 1-18 wherein the initial ratio of activated bacterial saccharide to carrier protein in step b) is 2.0:1 to 0.1:1, 1.8:1 to 0.4:1, 1:1 to 1.4:1, 1.8:1 to 1.6:1, 1.4:1 to 1.6:1, 0.8:1 to 0.4:1, 0.7:1 to 0.5:1, or 0.7:1 to 0.6:1 (wt/wt).

20. The process of any one of paragraphs 1-19 wherein the final ratio of carrier protein to bacterial saccharide after step c) or c') is 0.5:1 to 4:1, 0.8:1 to 3.2:1, 0.5:1 to 1.8:1, 1.4:1 to 1.8:1, 1:1 to 1.2:1, 2:1 to 2.4:1, or 2.5:1 to 3.5:1.

21. The process of any one of paragraphs 1-20 wherein the temperature of the reaction in step a) is maintained at 4-40°C, 10-32°C, 17-30°C or 22-27°C.

22. The process of any one of paragraphs 1-21 wherein the temperature of the reaction in step c) is maintained at 4-40°C, 10-32°C, 17-30°C or 22-27°C.

23. The process of any one of paragraphs 1-22 wherein step a) has a duration of less than 30 hours or between 15 and 25 hours, or 10 and 20 hours, between 30 minutes and 25 hours, between 1 hour and 35 hours.

24. The process of any one of paragraphs 1-23 wherein step c) has a duration of between 10-60, or 20-60 hours.

25. The process of any one of paragraphs 1-24 wherein the activated bacterial saccharide is conjugated directly to the carrier protein.

26. The process of any one of paragraphs 1-25 wherein the activated bacterial saccharide is conjugated to the carrier protein through a linker.

27. The process of paragraph 26 wherein the linker is between 1 and 20 Angstroms in length.

28. The process of any one of paragraphs 26 or 27 wherein the linker comprises an ADH linker.

29. The process of any one of paragraphs 1-28 wherein the reducing agent comprises sodium cyanoborohydride.

30. The process of any one of paragraphs 1-28 wherein the reducing agent comprises sodium triacetoxyborohydride.

31. The process of paragraph 30 wherein between 0.5 and 2 or 0.8 and 1.2 molar equivalent of reducing agent is used in step c).

32. The process of paragraph 31 wherein between 2 and 10 molar equivalent of reducing agent is used in step c).

33. The process of any one of paragraphs 1-32 wherein any unreacted carbonyl groups are capped by reaction with sodium borohydride ($NaBH_4$).

34. The process of paragraph 33 wherein around 2M equivalents of sodium borohydride are used.

35. The process of any one of paragraphs 33 or 34 wherein the product of step c) is reacted with sodium borohydride for 15 mins-15 hrs, 15 mins- 45 mins, 2-10hrs, or 3-5hrs.

36. The process of any one of paragraphs 1-35 wherein the activated bacterial saccharide and the carrier protein are lyophilised before step b).

37. The process of any one of paragraphs 1-36 wherein the activated bacterial saccharide and the carrier protein are lyophilised before step c).

38. The process of any one of paragraphs 36-37 wherein the activated bacterial saccharide and the carrier protein are lyophilised in the presence of a non-reducing sugar.

39. The process of any one of paragraphs 36-38 wherein the non-reducing sugar is selected from the group consisting of sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit.

40. The process of any one of paragraphs 1-39 steps b) and/or c) are carried out in DMSO solvent.

41. The process of any one of paragraphs 1-39 wherein step b) and/or step c) are carried out in DMF solvent.

42. The process of any one of paragraphs 1-41 comprising a further step e) of purifying the conjugate.

43. The process of paragraph 42 wherein step e) comprises purifying the conjugate using diafiltration.

44. The process of any one of paragraphs 42-43 wherein step e) comprises purifying the conjugate using ion exchange chromatography.

45. The process of paragraph 44 wherein step e) comprises purifying the conjugate using size exclusion chromatography.

46. The process of any one of paragraphs 1-45 comprising a further step f), wherein the conjugate is sterile filtered.

47. The process of any one of paragraphs 1-46 containing a further step of mixing the conjugate with further antigens.

48. The process of paragraph 47 wherein the further antigens comprises at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 *S.pneumoniae* saccharides selected from the group consisting of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

49. The process of any one of paragraphs 47-48 wherein the further antigens comprise *S.pneumoniae* saccharides 4, 9V, 14, 18C, and 19F.

50. The process of any one of paragraphs 47-49 wherein the further antigens comprise *S.pneumoniae* saccharide 19A.

51. The process of any one of paragraphs 47-50 wherein the further antigens comprise *S.pneumoniae* saccharide 6A.

52. The process of any one of paragraphs 47-51 wherein the further antigens comprise *S.pneumoniae* saccharide 1.

53. The process of any one of paragraphs 47-52 wherein the further antigens comprise *S.pneumoniae* saccharide 5.

54. The process of any one of paragraphs 47-53 wherein the further antigens comprise *S.pneumoniae* saccharide 7F.

55. The process of any one of paragraphs 47-54 wherein the further antigens comprise *S.pneumoniae* saccharide 3.

56. The process of any one of paragraphs 47-55 wherein the further antigens comprise *S.pneumoniae* saccharide 6B.

57. The process of any one of paragraphs 47-56 wherein the further antigens comprise *S.pneumoniae* saccharide 6B.

58. The process of any one of paragraphs 47-57 wherein the further antigens comprise one or more *S.pneumoniae* proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp13 25 and Sp133.

59. The process of any one of paragraphs 48-58 wherein the further antigens comprise Diphtheria Toxoid (DT), Tetanus Toxoid (TT), and either killed whole-cell *Bordetella pertussis* (Pw), or two or more acellular pertussis components (Pa).

60. The process of any one of paragraphs 48-59 wherein the further antigens comprise Hepatitis B surface antigen (HepB).

61. The process of any one of paragraphs 48-60 wherein the further antigens comprise *Haemophilus influenzae* b (Hib) polysaccharide or oligosaccharide.

62. The process of any one of paragraphs 48-61 wherein the further antigens comprise one or more conjugates of a carrier protein and a capsular polysaccharide of a bacterium selected from the group consisting of *N.meningitidis* type A (MenA), *N.meningitidis* type C (MenC), *N.meningitidis* type W (MenW) and *N.meningitidis* type Y (MenY).

63. The process of any one of paragraphs 1-62 wherein the conjugate is mixed with an adjuvant.

64. The process of paragraph 63 wherein the adjuvant is an aluminium salt.

65. The process of any one of paragraphs 1-64 wherein the conjugate is mixed with a pharmaceutically acceptable excipient.

66. A conjugate obtainable by the process of any one of paragraphs 1-65.

67. A conjugate obtained by the process of any one of paragraphs 1-65.

68. An immunogenic composition comprising the conjugate of any one of paragraphs 66-67 and a pharmaceutically acceptable excipient.

69. The immunogenic composition of paragraph 68 wherein the pharmaceutical excipient does not contain a chloride salt.

70. The immunogenic composition of any one of paragraphs 68-69 wherein the pharmaceutical excipient comprises a buffer.

71. The immunogenic composition of paragraph 70wherein the buffer is maleate buffer.

72. The immunogenic composition of paragraph 71 which comprises at least one further antigen.

73. The immunogenic composition of paragraph 72 wherein the further antigens comprise *S.pneumoniae* saccharides selected from the group consisting of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

74. The immunogenic composition of any one of paragraphs 72-73 wherein the further antigens comprise *S.pneumoniae* saccharides 4, 9V, 14, 18C, and 19F.

75. The immunogenic composition of any one of paragraphs 72-74 wherein the further antigens comprise *S.pneumoniae* saccharide 19A.

76. The immunogenic composition of any one of paragraphs 72-75 wherein the further antigens comprise *S.pneumoniae* saccharide 6A.

77. The immunogenic composition of any one of paragraphs 72-76 wherein the further antigens comprise *S.pneumoniae* saccharide 1.

78. The immunogenic composition of any one of paragraphs 72-77 wherein the further antigens comprise *S.pneumoniae* saccharide 5.

79. The immunogenic composition of any one of paragraphs 72-78 wherein the further antigens comprise *S.pneumoniae* saccharide 7F.

80. The immunogenic composition of any one of paragraphs 72-79 wherein the further antigens comprise *S.pneumoniae* saccharide 3.

81. The immunogenic composition of any one of paragraphs 72-80 wherein the further antigens comprise *S.pneumoniae* saccharide 6B.

82. The immunogenic composition of any one of paragraphs 72-81 wherein the further antigens comprise *S.pneumoniae* saccharide 6C.

83. The immunogenic composition of any one of paragraphs 72-82 wherein the further antigens comprise one or more *S.pneumoniae* proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133.

84. The immunogenic composition of any one of paragraphs 72-83 wherein the further antigens comprise diphtheria toxoid (DT), tetanus toxoid (TT), and either killed whole-cell *Bordetella pertussis* (Pw), or two or more acellular pertussis components (Pa).

85. The immunogenic composition of any one of paragraphs 72-84 wherein the further antigens comprise *Haemophilus influenzae* b (Hib) polysaccharide or oligosaccharide.

86. The immunogenic composition of any one of paragraphs 72-85 wherein the further antigens comprise Hepatitis B surface antigen (HepB)

87. The immunogenic composition of any one of paragraphs 72-86 wherein the further antigens comprise one or more conjugates of a carrier protein and a capsular polysaccharide of a bacterium selected from the group *N.meningitidis* type A (MenA), *N.meningitidis* type C (MenC), *N.meningitidis* type W (MenW) and *N.meningitidis* type Y (MenY).

88. The immunogenic composition of any one of paragraphs 72-87 further comprising an adjuvant.

89. The immunogenic composition of paragraph 88 wherein the adjuvant is an aluminium salt.

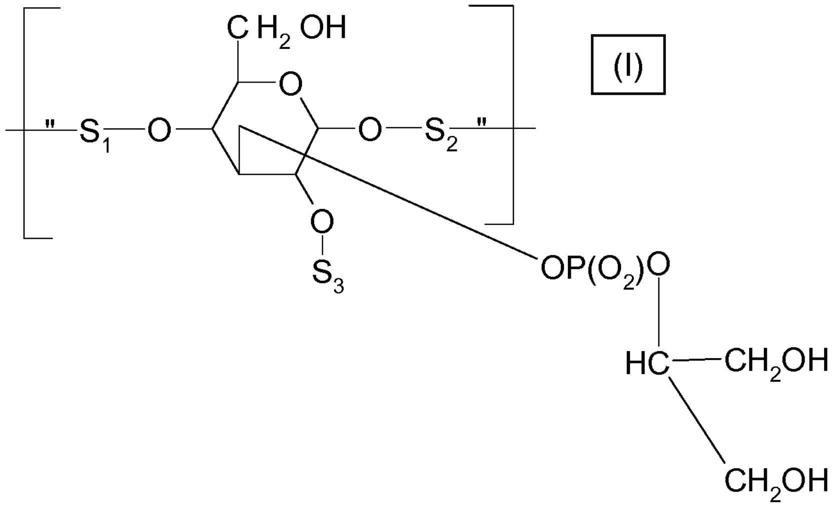
90. A vaccine comprising the immunogenic composition of any one of paragraphs 68-89.

91. A use of the immunogenic composition of any one of paragraphs 68-89 or the vaccine of paragraph 90 in the prevention or treatment of bacterial disease.

92. A use of the immunogenic composition of any one of paragraphs 68-89 or the vaccine of paragraph 90 in the preparation of a medicament for the prevention or treatment of bacterial disease.

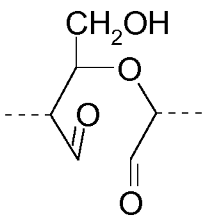
93. A method of preventing or treating bacterial infection comprising administering the immunogenic composition of paragraphs 68-89 or the vaccine of paragraph 90 to a patient.

94. An activated bacterial saccharide, wherein the activated bacterial saccharide comprises a repeat unit of formula (I):

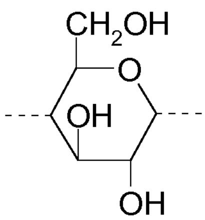


(I)

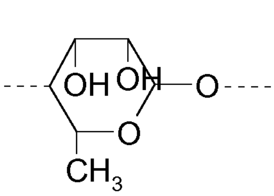
wherein the activated bacterial saccharide comprises n repeat units and n is between 2 and 2400, between 500 and 2000, between 750 and 1500, between 1000 and 2000 or between 1500 and 2300.
wherein at least 0.001% but less than 50% of S1 is



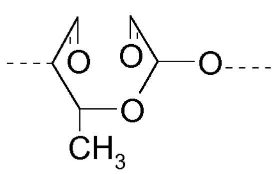
and the remainder is



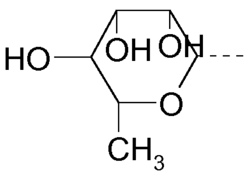
wherein S2 is either



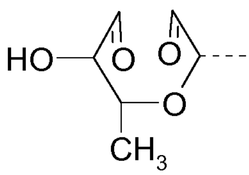
or



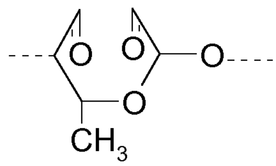
and wherein S3 is either



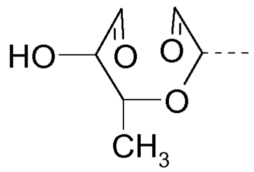
or



95. The compound of paragraph 108 wherein less than 0.001%, 0.1 %, 0.5% 1%, 2%, 3%, 5%, 10%, 30% or 50% of S2 is



96. The compound of paragraphs 108 or 109 wherein less than 0.1 %, 0.5%, 1%, 2%, 3%, 5%, 10%, 30% or 50% of S3 is



EXAMPLES

Example 1 - Oxidation of 23F and 6B using periodate

[0064] Polysaccharides (PS) 23F or 6B were dissolved in 100mM KH_2PO_4 (pH 7.4), 10mM KH_2PO_4 or WFI, to form solutions of 2mg PS/ml. The solution was incubated for 2 hours under agitation at room temperature. After this time the pH was adjusted to pH 6.0 with 1NHCl. Periodate was added as a powder or in liquid form (10mg/ml in WFI) in various amounts to achieve a range of molar ratios (table 1). The solutions were incubated for 17 hours at room temperature (20-25°C), after which time the samples were dialyzed or diafiltered against WFI.

[0065] High performance gel filtration chromatography coupled with refractive index and multiangle laser lights scattering (MALLS-) detectors was used to measure the molecular weight. Size exclusion media (TSK5000PWXL-Tosoh) was used to profile the molecular size distribution of the polysaccharide (elution 0.5ml/min in NaCl 0.2M-NaN3 0.02%).

[0066] Table 1 and figure 1 describe the results of these experiments. These demonstrate that for the 23F saccharide substantial sizing occurs on oxidation using high molar equivalents of periodate in 100mM phosphate buffer. This sizing effect can be reduced by reducing the concentration of phosphate buffer or the molar equivalents of periodate used.

Table 1:

23F				6B			
Sample	**molar equivalent of periodate**	**Buffer**	**Size (KDa)**	**Sample**	**molar equivalent of periodate**	**buffer**	**Size (KDa)**
23F native	0	Water	861	6B	0	10mM phosphate	1022
23F native	0	10mM phosphate	847	6B	0.1	10mM phosphate	975
23F native	0	100mM phosphate	860	6B	0.2	10mM phosphate	990
23F ATCC native	0	100mM phosphate	1655	6B	0.3	10mM phosphate	961
23F	1	100mM phosphate	<1	6B	0.75	10mM phosphate	868
23F	1	Water	36				
23F	1.2	100mM phosphate	<1				

(continued)

23F				6B			
Sample	molar equivalent of periodate	Buffer	Size (KDa)	Sample	molar equivalent of periodate	buffer	Size (KDa)
23FATCC	1	100mM phosphate	2				
23FATCC	0.125	100mM phosphate	39				
23F	0.1	10mM phosphate	466.9				
23F	0.15	10mM phosphate	398.5				
23F	0.2	10mM phosphate	336				
23F	0.5	10mM phosphate	179.1				

Example 2 - conjugation of 23F to CRM197 using reductive amination and CDAP chemistry

Reductive amination

[0067] 1g of PS23F was dissolved in 500ml of 10mM KH_2PO_4 pH 7.15. This solution was incubated at room temperature for two hours. The pH was adjusted to 6.0N with 1M HCl. 111 mg of periodate ($NaIO_4$, 0.4 molar equivalents of periodate) was added to the PS23F solution, and the solution was incubated for 17 hours in the dark at room temperature to oxidise PS23F. The solution was then diafiltered against WFI.

[0068] The activated PS23F was lyophilised with the CRM197 protein (at a CRM/PS ratio (w/w): 0.625) in the presence of a stabilising agent.

[0069] 900mg of the lyophilised PS23F/CRM197 mixture was solubilised by addition of 350ml of DMSO solvent and incubating for 2 hours at room temperature. To reduce the PS23F/CRM197 mixture 1 molar equivalent of $NaBH_3CN$ was added (735μl of a solution of 100mg/ml in WFI). The solution was incubated for a further 40 hours room temperature (15°C-25°C) under agitation. After this time 2 molar equivalent of $NaBH_4$ (100mg/ml in WFI) was added and the solution incubated for 4 hours at room temperature. 2200ml of 150mM NaCl was added before diafiltration (cut-off 100kDa) and purification by DEAE. The fractions of interest were pooled and filtered through a 0.22μm filter.

CDAP

[0070] 200mg of microfluidized PS23F was dissolved in water until a concentration of 10mg/ml was obtained. NaCl was added to this solution at a final concentration of 2M.

[0071] Sufficient CDAP solution (100mg/ml freshly prepared in 5/50 v/v acetonitrile/WFI) was added to reach a CDAP:PS ratio of 0.75mg/mg PS.

[0072] After 90 seconds, the pH was raised to pH 9.5 by addition of 0.1 N NaOH. 3 minutes later sufficient CRM197 (10mg/ml in 0.15M NaCl) was added to reach a ratio of 1.5 (CRM197:PS (w/w)), the pH was maintained at pH 9.5. This solution was incubated for 1 hour at pH 9.5.

[0073] After this coupling step, 10ml of 2M glycine solution was added to the mixture and the pH was adjusted to pH9.0 (the quenching pH). The solution was stirred for 30 minutes at room temperature. The conjugate was purified using a 5μm filter followed by Sephacryl S400HR (XK50/100) which removes small molecules and unconjugated polysaccharides and protein. The flow rate was fixed at 150ml/hour. Elution was achieved using 150mM NaCl. The fractions of interest were pooled and filtered using Milipack 20. The resulting conjugate had a final CRM197/PS ratio (w/w) of 1.35/w.

Example 3 - Immunogenicity of 23F-CRM197 conjugates made by reductive amination and CDAP chemistry

[0074] Conjugates were made using the methods described in example 2. Female guinea pigs were immunized intramuscularly three times (at days 0, 14 and 28) with 0.25μg of the PS23F-CRM197 conjugates. Animals were bled on day 42 and the antibody response directed against PS23F was measured by ELISA and OPA.

ELISA

[0075] Microplates were coated with purified pneumococcal polysaccharide in PBS buffer. The plates were washed four times with 0.9% NaCl and 0.05% Tween 20. Sera were incubated for 1 hour at 37°C with CPS (V/V) in PBS 0.05 % Tween 20. Sera were added to the microwells and serially diluted (two-fold dilution step) in PBS- 0.05% Tween. The plates were incubated under agitation for 30 minutes at room temperature. The plates were washed as above and an anti-guinea pig IgG antibodies peroxydase conjugate was added, the plates were then incubated for 30 minutes at RT. After washing, the substrate (4 mg of OPDA in 10 ml of citrate 0.1 M pH 4.5 and 5 μl of H_2O_2) was added to each well for 15 minutes. The reaction was stopped by addition of HCl 1 N. Absorbance was read at 490-620 nm using a spectrophotometer. The colour developed is directly proportional to the amount of antibody present in the serum. The level of anti-PS IgG present in the sera is determined by comparison to the reference curve serum added on each plate and expressed in μg/ml.

[0076] Results were analysed statistically after assuming homogeneity of variance (checked by Cochrans's C test) and normality (checked using the Shapiro-Wilk test). All statistics were carried out using Anova (Tukey-HSD) on log transformation concentration IgG.

Opsonophagocytosis

[0077] Serum samples were heated for 45 min at 56 °C to inactivate any remaining endogenous complement. Twenty-five microlitre aliquots of each 1:2 diluted serum sample were serially diluted (two fold) in 25 μl OPA buffer (HBSS-14.4% inactivated FBS) per well of a 96-well round bottom microtitre plate. Subsequently, 25 μl of a mixture of activated HL-60 cells (1 × 107 cells/ml), freshly thawed pneumococcal working seed and freshly thawed baby rabbit complement in an e.g. 4/2/1 ratio (v/v/v) was added to the diluted sera to yield a final volume of 50 μl. The assay plate was incubated for 2 h at 37 °C with orbital shaking (210 rpm) to promote the phagocytic process. The reaction was stopped by laying the microplate on ice for at least 1 min. A 20 μl aliquot of each well of the plate was then transferred into the corresponding well of a 96-well flat bottom microplate and 50 μl of Todd-Hewitt Broth-0.9% agar was added to each well. After overnight incubation at 37 °C and 5% CO2, pneumococcal colonies appearing in the agar were counted using an automated image analysis system (KS 400, Zeiss, Oberkochen, Germany). Eight wells without serum sample were used as bacterial controls to determine the number of pneumococci per well. The mean number of CFU of the control wells was determined and used for the calculation of the killing activity for each serum sample. The OPA titre for the serum samples was determined by the reciprocal dilution of serum able to facilitate 50% killing of the pneumococci. The opsonophagocytic titre was calculated by using a 4-parameter curve fit analysis.

[0078] Results were analysed statistically after assuming homogeneity of variance (checked by Cochrans's C test) and normality (checked using the Shapiro-Wilk test). All statistics were performed by Anova (Tukey-HSD) on log transformation concentration IgG for ELISA and Kruskal-Wallis on log dilution for OPA.

[0079] A significantly higher antibody response was induced in the guinea pigs after immunisation with PS23F-CRM197 conjugated by reductive amination than PS23F-CRM197 conjugated by CDAP chemistry as seen in figure 2.

Table 2

Assay	23F-CRM197 made by reductive amination	23F-CRM197 made by CDAP
ELISA Titer (μg/ml)	213.3	40.5
OPA (50% killing)	9232	591

Example 4 - a further example of reductive amination of 23F

23 F-C RM-RA-116

[0080] 150 mg of native PS23F (PS23FP114) was dissolved at a concentration of 2 mg/ml in 10 mM phosphate buffer (pH 7.2) for 4 hours. After dissolution, pH was adjusted to pH 6.0 with 1N HCl. Then 0.4 molar equivalent of periodate (Na IO_4) was added to the PS solution and incubated for 17 hrs in the dark at 25°C. The solution is then diafiltered (cut off 30 kDa) against WFI and the oxidised PS was filtered on 0.22 μm membrane.

[0081] 50 mg of oxidised PS and 75 mg of CRM197 were lyophilized together (CRM/PS ratio (w/w): 1.5/1) in the presence of a stabilising agent. Lyophilized PS + CRM197 was solubilised with 20 ml of DMSO for 2 hrs at room temperature (15-25°C). 1 molar equivalent of TAB (Sodium triacetoxyborohydride) was then added (13.7 mg) and after

17 hrs under agitation, 2 molar equivalent of $NaBH_4$ (100 mg/ml in 0.1 M NaOH) was added followed by an incubation at room temperature for 30 minutes. The solution was diluted 5x by addition of WFI followed by a diafiltration (cut-off 30 kDa) against 10 mM phosphate buffer, 150 mM NaCl pH 7.2. The conjugate was then loaded onto DEAE resin and eluted in 10 mM phosphate buffer, 500 mM NaCl pH 7.2. The conjugate was finally filtered on 0.22 μm. The resulting conjugate has a final CRM/PS ratio (w/w) of 2.3 /1.

[0082] For further conjugates, a second diafiltration step was added after DEAE column in order to change the buffer (150 mM NaCl as final buffer).

Example 5 - conjugation of 6B to CRM197 using reductive amination (with different protein:saccharide ratios and different sized microfluidised 6B saccharides) and CDAP chemistry

6B-CRM-RA-122

[0083] 200 mg of microfluidized PS6B (84 kDa, 11.7 mg/ml) was diluted at 2 mg/ml in 10 mM phosphate buffer (pH 7.2). pH was adjusted to pH 6.0 with 1N HCl. Then 0.1 molar equivalent of periodate (Na IO_4) was added to the PS solution and incubated for 17 hrs in the dark at room temperature. The solution is then diafiltered (cut off 30 kDa) against WFI. 50 mg of PS and 30 mg of CRM197 were lyophilized together (CRM/PS ratio (w/w): 0.6/1) in the presence of a stabilising agent. Lyophilized PS + CRM197 were solubilised with 20 ml of DMSO for 3 hrs at room temperature. Then 2.5 molar equivalent of TAB (Sodium triacetoxyborohydride) was added (38.7 mg) and after 16 hrs under agitation, 2 molar equivalent of $NaBH_4$ (100 mg/ml in 0.1 M NaOH) was added followed by an incubation at room temperature for 30 minutes. The solution was diluted 4x by addition of WFI followed by a diafiltration (cut-off 100 kDa). The conjugate was then filtered on 0.22 μm. The resulting conjugate has a final CRM/PS ratio (w/w) of 1.1/1.

6B-CRM-RA-123:

[0084] Microfluidized PS6B (84 kDa) was conjugated to CRM197 as described for 6B-CRM-RA-122 except the freeze-drying step was carried out using an initial CRM197/PS ratio (w/w) of 2/1 and 30ml of DMSO was used for the dissolution in DMSO step (instead of 20 ml). The resulting conjugate had a final CRM/PS ratio (w/w) of 3.0/1.

6B-CRM-RA-124:

[0085] 200 mg of microfluidized PS6B (350 kDa, 11.7 mg/ml) having a molecular weight of 350 kDa was diluted to 2 mg/ml in 10 mM phosphate buffer (pH 7.2). pH was adjusted to pH 6.0 with 1N HCl. Then 0.1 molar equivalent of periodate (Na IO_4) was added to the PS solution and incubated for 17 hrs in the dark at room temperature. The solution is then diafiltered (cut off 100 kDa) against WFI. 50 mg of PS and 60 mg of CRM197 were lyophilized together (CRM/PS ratio (w/w): 1.2/1) in the presence of a stabilising agent. Lyophilized PS + CRM197 were solubilised with 20 ml of DMSO for 5 hrs at room temperature. 2.5 molar equivalent of TAB (Sodium triacetoxyborohydride) was then added (38.7 mg) and after 16 hrs under agitation, 2 molar equivalent of NaBH4 (100 mg/ml in 0.1 M NaOH) were added followed by incubation for 30 min at room temperature. The solution was diluted 4x by addition of WFI followed by a diafiltration (cut-off 100 kDa). The conjugate was then filtered on 0.22 μm. The resulting conjugate has a final CRM/PS ratio (w/w) of 1.6/1.

6B-CRM-RA-125:

[0086] Microfluidized PS6B (350 kDa) was conjugated to CRM197 as described for 6B-CRM-RA-124 except the freeze-drying step was carried out using an initial CRM197/PS ratio (w/w) of 2/1 and the dissolution in DMSO was carried out using 33 ml (instead of 20 ml). The resulting conjugate had a final CRM/PS ratio (w/w) of 2.9/1.

6B-CRM-003:

[0087] 50 mg of microfluidized PS6B were diluted at 10 mg/ml in water (10 mg/ml). NaCl in solid form was added to reach a final concentration of 2M. CDAP solution (100 mg/ml freshly prepared in 50/50 v/v acetonitrile/WFI) was added to reach the appropriate CDAP/PS ratio (1.5 mg/mg PS). After 1.5 minutes, the pH was raised to the activation pH 9.5 by addition of 0.1 N NaOH and was stabilised at this pH until addition of CRM197. After 3 minutes, CRM197 (10 mg/ml in 0.15 M NaCl) was added to reach a ratio CRM197 /PS (w/w) of 2; the pH was maintained at the coupling pH 9.5. The solution was left for 2 hrs under pH regulation.

[0088] After the coupling step, 2.5 ml of 2M glycine solution was added to the mixture. The pH was adjusted to the quenching pH (pH 9.0). The solution was stirred for 30 min at room temperature. Then the conjugate was filtered using a 5 μm filter and injected on Sephacryl S400HR (XK26/100) column to remove small molecules (including DMAP) and unconjugated PS and protein. Flow rate was fixed at 30 ml/h. Elution was carried out in 150 mM NaCl. Interesting fractions were pooled and filtered on Millipack 20. The resulting conjugate had a final CRM197/PS ratio (w/w) of 1.5/1.

6B-CRM-RA-144

[0089] 1 g of microfluidized PS6B (245 kDa, 9.47 mg/ml) was diluted to 2 mg/ml in 10 mM phosphate buffer (pH 7.2). The pH was adjusted to pH 6.0 with 1N HCl. 0.1 molar equivalent of periodate ($NaIO_4$) was then added to the PS solution and incubated for 18 hrs in the dark at room temperature. The solution was then diafiltered against WFI (Sartocon Slice200 Hydrosart 100kDa). 200 mg of oxidized PS and 240 mg of CRM197 were lyophilized together (CRM/PS ratio (w/w): 1.2/1) in the presence of a stabilising agent. Lyophilized PS + CRM197 were solubilised with 80 ml of DMSO for 6 hrs at 25°C. Then 2.5 molar equivalent of TAB (Sodium triacetoxyborohydride) was added (154.9 mg) and after 16 hrs under agitation at 25°C, 2 molar equivalent of $NaBH_4$ (100 mg/ml in 0.1 M NaOH) was added and incubated for 30 min. The solution was diluted 5x in WFI and after 30 min was diafiltered 10x with 150 mM NaCl and then 5x with PO_4 (K/K_2) 10mM pH7.2+ 150 mM NaCl (Sartorius Sartocon Slice 200 Hydrosart 100 kDa). Then the retentate was loaded onto a DEAE column (XK26/40). The column was washed with PO_4($K/_K2$) 10mM pH7.2 /NaCl 150mM buffer. The conjugate was eluted with PO_4(K/K_2) 10mM pH7.2 /NaCl 500 mM buffer. The eluate was concentrated and diafiltered with 5 volumes of 150 mM NaCl and then filtered on 0.22 μm filter. The resulting conjugate has a final CRM/PS ratio (w/w) of 1.6/1.

Example 6 - Immunogenicity of 6B-CRM197 conjugates made by reductive amination and CDAP chemistry

[0090] Groups of 40 female Balb/c mice (4 weeks-old) were immunized intramuscularly three times at days 0, 14 and 28 with 0.1 μg of PS6B conjugates produced by reductive amination or CDAP chemistry formulated on $AlPO_4$. PS6B-PD was used as benchmark. Mice were bled on day 42 and the antibody response directed against each antigen was measured by ELISA and OPA.

[0091] Groups of 20 female guinea pig (150 gr from Hartley) were immunized intramuscularly three times at days 0, 14 and 28 with 0.25 μg of PS6B conjugates produced by reductive amination or CDAP chemistry adjuvanted with $AlPO_4$. PS6B-PD was used as benchmark. Guinea pigs were bled on day 42 and the antibody response directed against each antigen was measured by ELISA and OPA.

Mouse and Guinea pig OPA

[0092] Serum samples were heated for 45 min at 56 °C to inactivate any remaining endogenous complement. Twenty-five microlitre aliquots of each 1:2 diluted serum sample was two-fold serially diluted in 25 μl OPA buffer (HBSS-14.4% inactivated FBS) per well of a 96-well round bottom microtitre plate. Subsequently, 25 μl of a mixture of activated HL-60 cells (1×10^7 cells/ml), freshly thawed pneumococcal working seed and freshly thawed baby rabbit complement in an e.g. 4/2/1 ratio (v/v/v) were added to the diluted sera to yield a final volume of 50 μl. The assay plate was incubated for 2 h at 37 °C with orbital shaking (210 rpm) to promote the phagocytic process. The reaction was stopped by laying the microplate on ice for at least 1 min. A 20 μl aliquot of each well of the plate was then transferred into the corresponding well of a 96-well flat bottom microplate and 50 μl of Todd-Hewitt Broth-0.9% agar was added to each well. After overnight incubation at 37 °C and 5% CO_2, pneumococcal colonies appearing in the agar were counted using an automated image analysis system (KS 400, Zeiss, Oberkochen, Germany). Eight wells without serum sample were used as bacterial controls to determine the number of pneumococci per well. The mean number of CFU of the control wells was determined and used for the calculation of the killing activity for each serum sample. The OPA titre for the serum samples was determined by the reciprocal dilution of serum able to facilitate 50% killing of the pneumococci. The opsonophagocytic titre was calculated by using a 4-parameter curve fit analysis.

[0093] Table 3 describes the GMC levels obtained by immunisation of balb/c mice with the conjugates made using the methods of example 4.

Table 3

	G1	G2	G3	G4	G5	G6
Subject/Result	PS06B-CRM122 (R:1/1,PS 84	PS06B-CRM123 (R:3/1,PS 84 kDa)	PS06B-CRM124 (R:1.5/1,PS 350 kDa)	PS06B-CRM125 (R:2.9/1,PS 350 kDa)	PS06B-CRM003 (CDAP)	PS06B-PD
GMC (UG-ML)	0.83	0.37	1.18	0.64	0.31	0.10
Responders(%)	31/40	26/40	33/40	29/40	29/40	15/40

[0094] The immunogenicity of these conjugates in balb/c mice is described in figure 3. Together figure 3, and table 3 demonstrate that in the mouse model the conjugates produced by reductive amination were comparable with those produced using CDAP chemistry. In particular figure 3 demonstrates that the immunogenicities of the conjugates produced using reductive amination was higher than the immunogenicity of the conjugate made using CDAP chemistry.

[0095] Table 4 describes the GMC levels obtained by immunisation of guinea pigs with the conjugates made using the methods of example 4.

Table 4

	G1	G2	G3	G4	G5	G6
Subject/Result	PS06B-CRM122 (R:1/1,PS 84 kDa)	PS06B-CRM123 (R:3/1,PS84 kDa)	PS06B-CRM124 (R:1.5/1,PS0350 kDa)	PS06B-CRM125 (R:2.9/1,PS 350 kDa)	PS06B-CRM003 (CDAP)	PS06B-PD
GMC (UG-ML)	3.51	7.70	2.84	19.93	3.70	1.55
Responders(%)	20/20	20/20	20/20	20/20	20/20	20/20

[0096] The immunogenicity of these conjugates in guinea pigs is described in figure 4. Similar to the experiments carried out in the mouse model, the results in table 4 and figure 4 show that the conjugates produced by reductive amination were comparable with those produced using CDAP chemistry, in particular PS06B-CRM125 demonstrated significantly higher GMC levels and immunogenicities than the conjugate produced using CDAP.

Example 7 Conjugation of Hib to tetanus toxoid using reductive amination

Hib-IO4-LS080

[0097] 2.9 g of PS (orcinol dosage, AHIBCPA007 lot) were dissolved in 260 ml of 10 mM phosphate buffer (Na/K_2) pH 6.2 for 4h30 at room temperature and then overnight at +4°C. The viscosity follow-up was done during the dissolution. After 4 hours dissolution the viscosity seemed to be stable. PS was diluted at 10 mg/ml with phosphate buffer and then oxidised in the dark with 0.07 molar equivalent of $NaIO_4$ during 60 minutes. Oxidised PS was diafiltered (Sartorius Hydrosart 2 kDa) against 3.5 volumes of phosphate buffer and then filtered on a 0.22μm filter. The number of repeating units obtained after oxidation was estimated by ^{1}H-NMRD and was found to be around 21.

Hib-TT-LS210, 212 and 213

[0098] 200 mg of oxidised PS (14.56 mg/ml) were mixed with 300 mg of TT (31.18 mg/ml, TT/PS ratio (w/w): 1.5/1) and diluted to 4 mg/ml with 36.64 ml of 10 mM phosphate buffer (Na/K_2) pH 6.2. The solution was lyophilized in the presence of a stabilising agent. Lyophilized PS + TT was solubilised with 20 ml of DMSO for 6 hrs at 25°C. Then 10 Meq of TAB (Sodium triacetoxyborohydride) were added (38.7 mg) and after 16 hrs under agitation, 2 molar equivalent of $NaBH_4$ (100 mg/ml in 0.1 M NaOH) was added followed by an incubation for 30 min at room temperature. The solution was diluted 3x by addition of WFI followed by a diafiltration step (5 volumes of WFI followed by 5 volumes of 10 mM acetate buffer 150 mM NaCl pH 6.2, 100 kDa MWCO). The sample was then loaded on Sephacryl S300HR resin. Elution was carried out in 10 mM acetate buffer using 150 mM NaCl (pH 6.2). Interesting fractions were pooled and filtered on a 0.22μm filter. The resulting conjugates had a final TT/PS ratio (w/w) of 2.1/1.

Claims

1. A process for conjugating a bacterial saccharide comprising the steps of

   a) reacting the bacterial saccharide with 0.001-0.7 molar equivalents of periodate to form an activated bacterial saccharide;
   b) mixing the activated bacterial saccharide with a carrier protein;
   c) reacting the activated bacterial saccharide and the carrier protein with a reducing agent to form a conjugate;

   or

   a) reacting the bacterial saccharide with 0.001-0.7 molar equivalents of periodate to form an activated bacterial saccharide;
   b) mixing the activated bacterial saccharide with a linker;
   c') reacting the activated bacterial saccharide with the linker using a reducing agent to form a bacterial saccharide-linker;
   d) reacting the bacterial saccharide-linker with a carrier protein to form a conjugate;

   wherein step a) occurs in a buffer which does not contain an amine group, and the buffer has a concentration between 1-100mM and wherein the bacterial saccharide is S. *pneumoniae* capsular saccharide 23F.

2. The process of claim 1 wherein the buffer is selected from the group consisting of phosphate buffer, borate buffer, acetate buffer, carbonate buffer and citrate buffer optionally wherein the buffer has a concentration between 1-50mM, 1-25mM, 1-10mM, 5-15mM, 8-12mM, or 10-50mM or around 10mM.

3. The process of any one of claims 1-2 wherein the pH in step a) is pH 3.5-8.0 5.0-7.0, or pH 5.5-6.5 or around pH 6.0.

4. The process of any one of claims 1-3 wherein the average molecular weight of the bacterial saccharide is between 1-1100kDa, 100-470kDa, 200-300kDa, 600-1100kDa or 800-1000kDa after step a).

5. The process of any one of claims 1-4 wherein the average molecular weight of the 23F saccharide is between 100-470kDa or 200-300kDa after step a).

6. The process of any one of claims 1-5 wherein the carrier protein is selected from the group consisting of tetanus toxoid, fragment C of tetanus toxoid, diphtheria toxoid, CRM197, Pneumolysin, protein D, PhtD, PhtDE and N19.

7. The process of any one of claims 1-6 wherein the reducing agent comprises sodium cyanoborohydride or sodium triacetoxyborohydride.

8. The process of any one of claims 1-7 comprising a further step e) of purifying the conjugate.

9. The process of any one of claims 1-8 containing a further step of mixing the conjugate with further antigens optionally wherein the further antigens comprises at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 *S.pneumoniae* saccharides selected from the group consisting of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F and 33F.

10. The process of claim 9 wherein the further antigens comprise one or more *S.pneumoniae* proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133.

11. The process of claim 9 or claim 10 wherein the further antigens comprise *S. pneumoniae* saccharides 1, 4, 5, 6B, 7F, 9V, 14, 18C and 19F.

12. The process of any one of claims 1-11 which comprises the step of reacting the bacterial saccharide with 0.1-0.5 molar equivalents of periodate to form an activated bacterial saccharide.

13. The process of any one of claims 1-12 which reduces the sizing effect on the bacterial saccharide.

14. The process of any one of claims 1-13 wherein the conjugate is mixed with an adjuvant or a pharmaceutically acceptable excipient.

Patentansprüche

1. Verfahren zur Konjugation eines bakteriellen Saccharids, umfassend die folgenden Schritte:

   a) Umsetzen des bakteriellen Saccharids mit 0,001-0,7 molaren Äquivalenten von Perjodat, um ein aktiviertes, bakterielles Saccharid zu bilden;
   b) Vermischen des aktivierten, bakteriellen Saccharids mit einem Trägerprotein;
   c) Umsetzen des aktivierten, bakteriellen Saccharids und des Trägerproteins mit einem Reduktionsmittel, um ein Konjugat zu bilden;

   oder

   a) Umsetzen des bakteriellen Saccharids mit 0,001-0,7 molaren Äquivalenten von Perjodat, um ein aktiviertes, bakterielles Saccharid zu bilden;
   b) Vermischen des aktivierten, bakteriellen Saccharids mit einem Linker;
   c') Umsetzen des aktivierten, bakteriellen Saccharids mit dem Linker unter Verwendung eines Reduktionsmittels, um einen bakteriellen Saccharid-Linker zu bilden;
   d) Umsetzen des bakteriellen Saccharid-Linkers mit einem Trägerprotein, um ein Konjugat zu bilden;

   wobei Schritt a) in einem Puffer erfolgt, der keine Aminogruppe enthält, und der Puffer eine Konzentration zwischen 1-100 mM aufweist und wobei das bakterielle Saccharid S. pneumoniae-Kapselsaccharid 23F ist.

2. Verfahren gemäß Anspruch 1, wobei der Puffer aus der Gruppe, bestehend aus Phosphatpuffer, Boratpuffer, Acetatpuffer, Carbonatpuffer und Zitratpuffer, ausgewählt ist, wobei der Puffer gegebenenfalls eine Konzentration zwischen 1-50 mM, 1-25 mM, 1-10 mM, 5-15 mM, 8-12 mM oder 10-50 mM oder ungefähr 10 mM aufweist.

3. Verfahren gemäß einem der Ansprüche 1-2, wobei der pH in Schritt a) pH 3,5-8,0, 5,0-7,0 oder pH 5,5-6,5 oder ungefähr pH 6,0 ist.

4. Verfahren gemäß einem der Ansprüche 1-3, wobei das mittlere Molekulargewicht des bakteriellen Saccharids zwischen 1-1.100 kDa, 100-470 kDa, 200-300 kDa, 600-1.100 kDa oder 800-1.000 kDa nach Schritt a) liegt.

5. Verfahren gemäß einem der Ansprüche 1-4, wobei das mittlere Molekulargewicht des 23F-Saccharids zwischen 100-470 kDa oder 200-300 kDa nach Schritt a) liegt.

6. Verfahren gemäß einem der Ansprüche 1-5, wobei das Trägerprotein aus der Gruppe, bestehend aus Tetanustoxoid, Fragment C des Tetanustoxoids, Diphtherietoxoid, CRM197, Pneumolysin, Protein D, PhtD, PhtDE und N19, ausgewählt ist.

7. Verfahren gemäß einem der Ansprüche 1-6, wobei das Reduktionsmittel Natriumcyanoborhydrid oder Natriumtriacetoxyborhydrid umfasst.

8. Verfahren gemäß einem der Ansprüche 1-7, umfassend einen weiteren Schritt e) zur Aufreinigung des Konjugats.

9. Verfahren gemäß einem der Ansprüche 1-8, enthaltend einen weiteren Schritt zur Vermischung des Konjugats mit weiteren Antigenen, wobei die weiteren Antigene gegebenenfalls zumindest 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 oder 20 S. pneumoniae-Saccharide, ausgewählt aus der Gruppe, bestehend aus 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F und 33F, umfassen.

10. Verfahren gemäß Anspruch 9, wobei die weiteren Antigene ein oder mehrere S. pneumoniae-Proteine, ausgewählt aus der Gruppe, bestehend aus der Poly-Histidin-Triad-Familie (PhtX), Cholin-bindenden Protein-Familie (CbpX), trunkiertem CbpX, LytX-Familie, trunkiertem LytX, trunkiertem CbpX-trunkiertem LytX chimären Proteinen (oder Fusionen), Pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 und Sp133, umfassen.

11. Verfahren gemäß Anspruch 9 oder Anspruch 10, wobei die weiteren Antigene S. pneumoniae-Saccharide 1, 4, 5, 6B, 7F, 9V, 14, 18C und 19F umfassen.

12. Verfahren gemäß einem der Ansprüche 1-11, das den Schritt der Umsetzung des bakteriellen Saccharids mit 0,1-0,5 molaren Äquivalenten von Perjodat umfasst, um ein aktiviertes, bakterielles Saccharid zu bilden.

13. Verfahren gemäß einem der Ansprüche 1-12, das die Größen-bestimmende Wirkung auf das bakterielle Saccharid verringert.

14. Verfahren gemäß einem der Ansprüche 1-13, wobei das Konjugat mit einem Adjuvans oder einem pharmazeutisch akzeptablen Exzipienten vermischt wird.

Revendications

1. Procédé de conjugaison d'un saccharide bactérien comprenant les étapes de

a) réaction du saccharide bactérien avec 0,001 à 0,7 équivalent molaire de periodate pour former un saccharide bactérien activé ;
b) mélange du saccharide bactérien activé avec une protéine de transport ;
c) réaction du saccharide bactérien activé et de la protéine de transport avec un agent réducteur pour former un conjugué ;

ou

a) réaction du saccharide bactérien avec 0,001 à 0,7 équivalent molaire de periodate pour former un saccharide bactérien activé ;
b) mélange du saccharide bactérien activé avec un lieur ;
c') réaction du saccharide bactérien activé avec le lieur en utilisant un agent réducteur pour former un saccharide bactérien-lieur ;

d) réaction du saccharide bactérien-lieur avec une protéine de transport pour former un conjugué ;

dans lequel l'étape a) se produit dans un tampon qui ne contient pas de groupe amine, et le tampon a une concentration entre 1 et 100 mM et dans lequel le saccharide bactérien est le saccharide capsulaire 23F de *S. pneumoniae.*

2. Procédé selon la revendication 1, dans lequel le tampon est sélectionné dans le groupe constitué des tampon phosphate, tampon borate, tampon acétate, tampon carbonate et tampon citrate, facultativement dans lequel le tampon a une concentration comprise entre 1 et 50 mM, 1 et 25 mM, 1 et 10 mM, 5 et 15 mM, 8 et 12 mM, ou 10 et 50 mM ou d'environ 10 mM.

3. Procédé selon l'une quelconque des revendications 1 et 2, dans lequel le pH dans l'étape a) est pH 3,5 à 8,0, 5,0 à 7,0, ou pH 5,5 à 6,5 ou d'environ pH 6,0.

4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel la masse moléculaire moyenne du saccharide bactérien est comprise entre 1 et 1 100 kDa, 100 et 470 kDa, 200 et 300 kDa, 600 et 1 100 kDa ou 800 et 1 000 kDa après l'étape a).

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel la masse moléculaire moyenne du saccharide 23F est comprise entre 100 et 470 kDa ou 200 et 300 kDa après l'étape a).

6. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel la protéine de transport est sélectionnée dans le groupe constitué de l'anatoxine tétanique, du fragment C de l'anatoxine tétanique, de l'anatoxine diphtérique, de CRM197, de la pneumolysine, de la protéine D, de PhtD, de PhtDE et de N19.

7. Procédé selon l'une quelconque des revendications 1 à 6, dans lequel l'agent réducteur comprend du cyanoborohydrure de sodium ou du triacétoxyborohydrure de sodium.

8. Procédé selon l'une quelconque des revendications 1 à 7, comprenant une autre étape e) de purification du conjugué.

9. Procédé selon l'une quelconque des revendications 1 à 8, contenant une autre étape de mélange du conjugué avec d'autres antigènes facultativement dans lequel les autres antigènes comprennent au moins 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 ou 20 saccharides de *S. pneumoniae* sélectionnés dans le groupe constitué de 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F et 33F.

10. Procédé selon la revendication 9, dans lequel les autres antigènes comprennent une ou plusieurs protéines de *S. pneumoniae* sélectionnées dans le groupe constitué de la famille de la triade polyhistidine (PhtX), de la famille des protéines de liaison de la choline (CbpX), des produits tronqués de CbpX, de la famille LytX, des produits tronqués de LytX, des protéines chimères (ou des fusions) produit tronqué de CbpX-produit tronqué de LytX, de la pneumolysine (Ply), de PspA, de PsaA, de Sp128, de Sp101, de Sp130, de Sp125 et de Sp133.

11. Procédé selon la revendication 9 ou la revendication 10, dans lequel les autres antigènes comprennent les saccharides de *S. pneumoniae* 1, 4, 5, 6B, 7F, 9V, 14, 18C et 19F.

12. Procédé selon l'une quelconque des revendications 1 à 11, qui comprend l'étape de réaction du saccharide bactérien avec 0,1 à 0,5 équivalent molaire de periodate pour former un saccharide bactérien activé.

13. Procédé selon l'une quelconque des revendications 1 à 12, qui réduit l'effet de réduction de taille sur le saccharide bactérien.

14. Procédé selon l'une quelconque des revendications 1 à 13, dans lequel le conjugué est mélangé avec un adjuvant ou un excipient pharmaceutiquement acceptable.

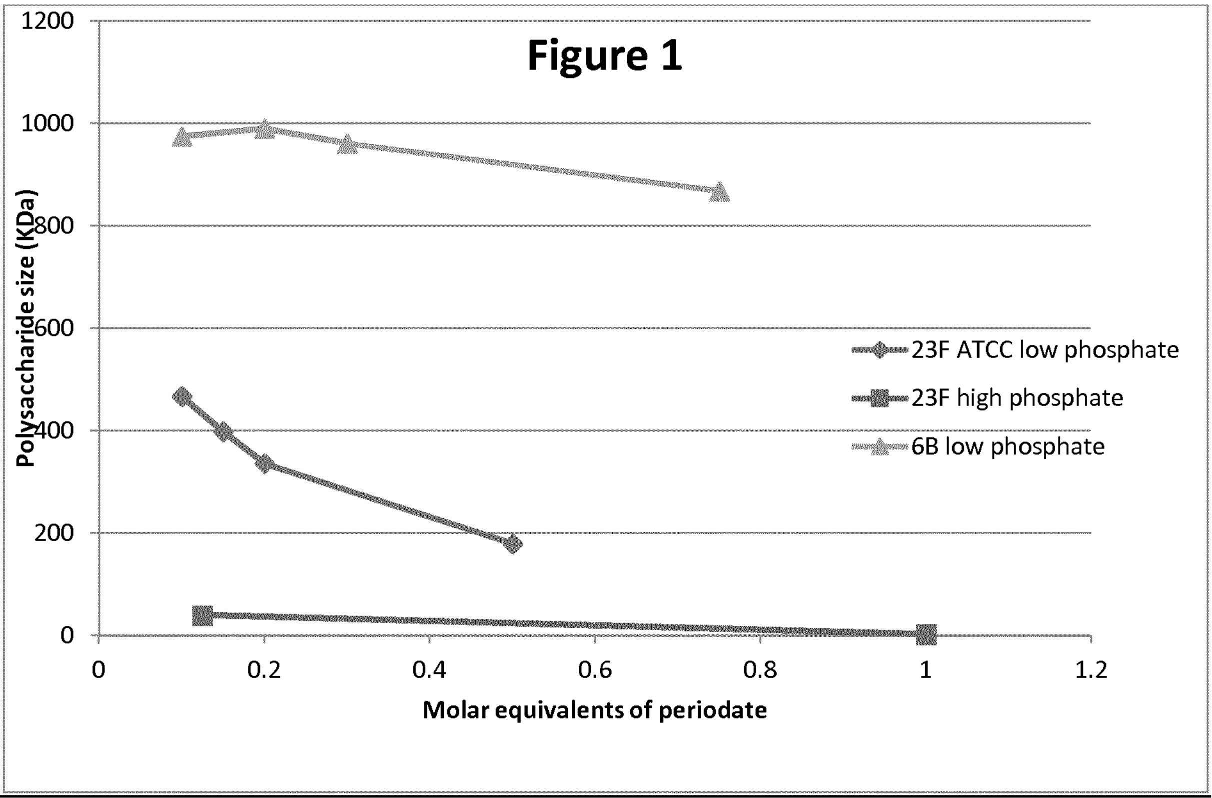
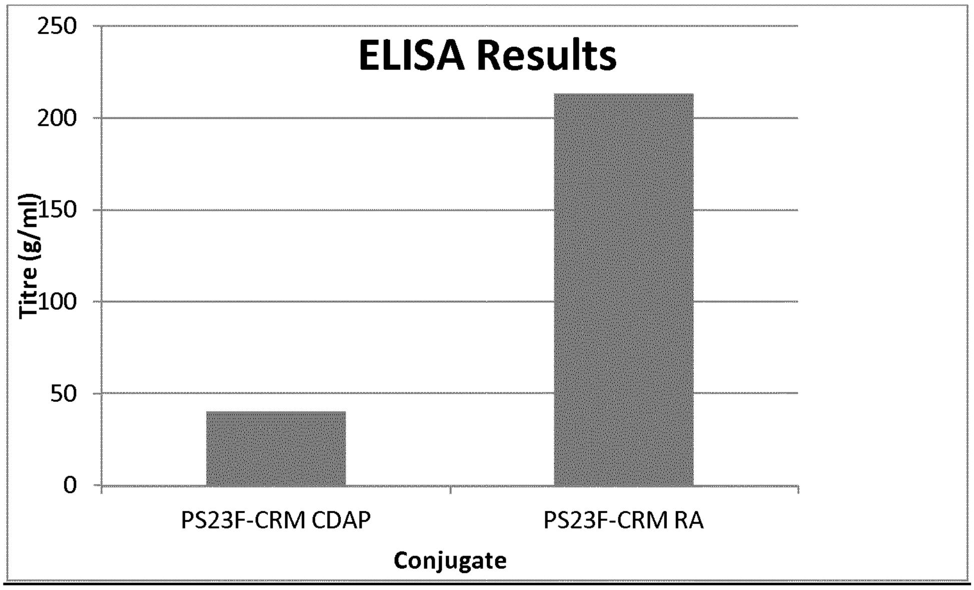


Figure 1
Polysaccharide size (KDa)
1200
1000
800
600
400
200
0
0
0.2
0.4
0.6
0.8
1
1.2
Molar equivalents of periodate
23F ATCC low phosphate
23F high phosphate
6B low phosphate

a)

Figure 2


ELISA Results
250
200
150
100
50
0
Titre (g/ml)
PS23F-CRM CDAP
PS23F-CRM RA
Conjugate

b)

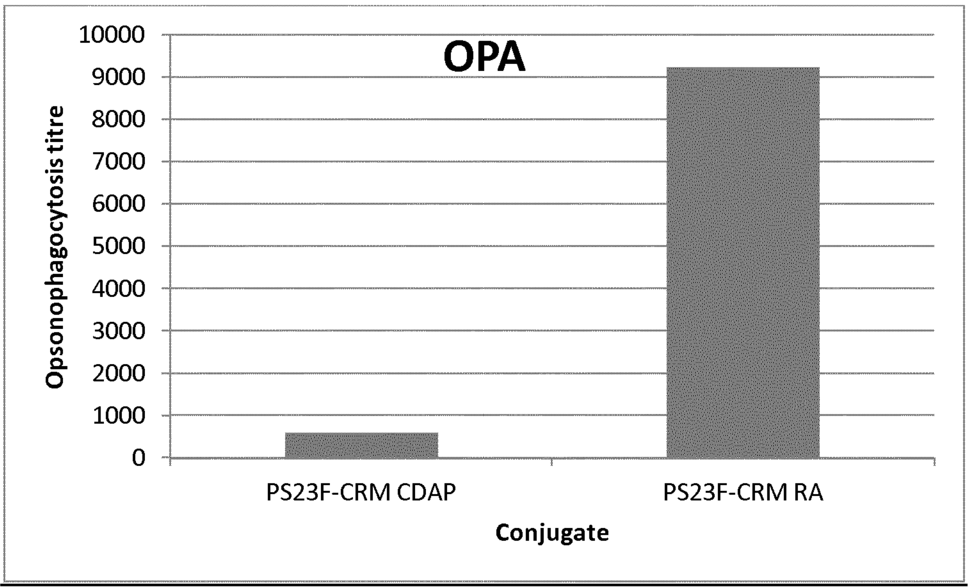

OPA
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Opsonophagocytosis titre
PS23F-CRM CDAP
PS23F-CRM RA
Conjugate

Figure 3

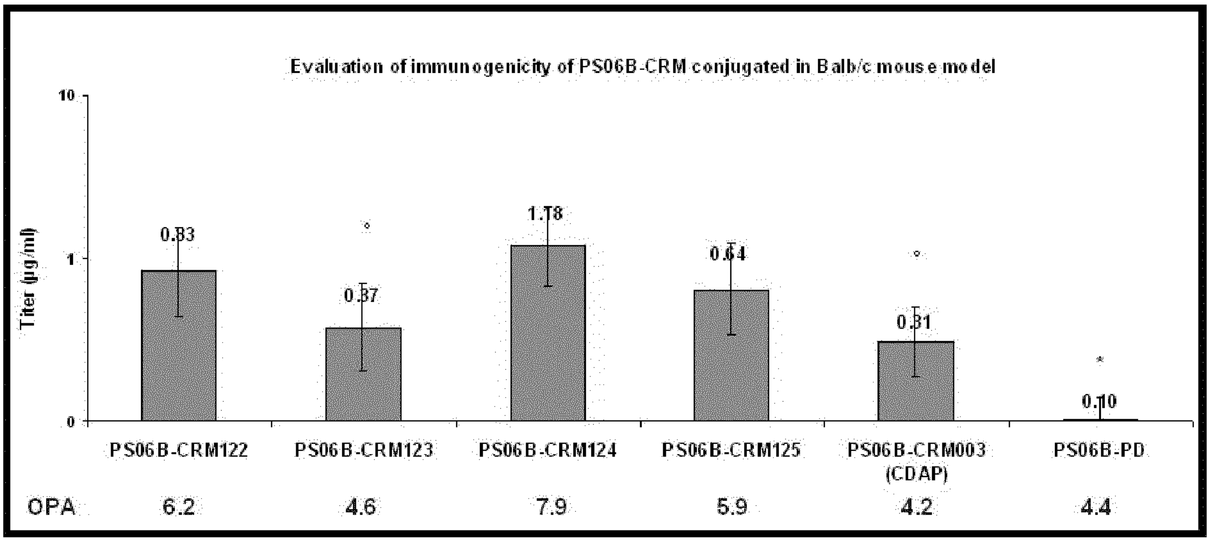
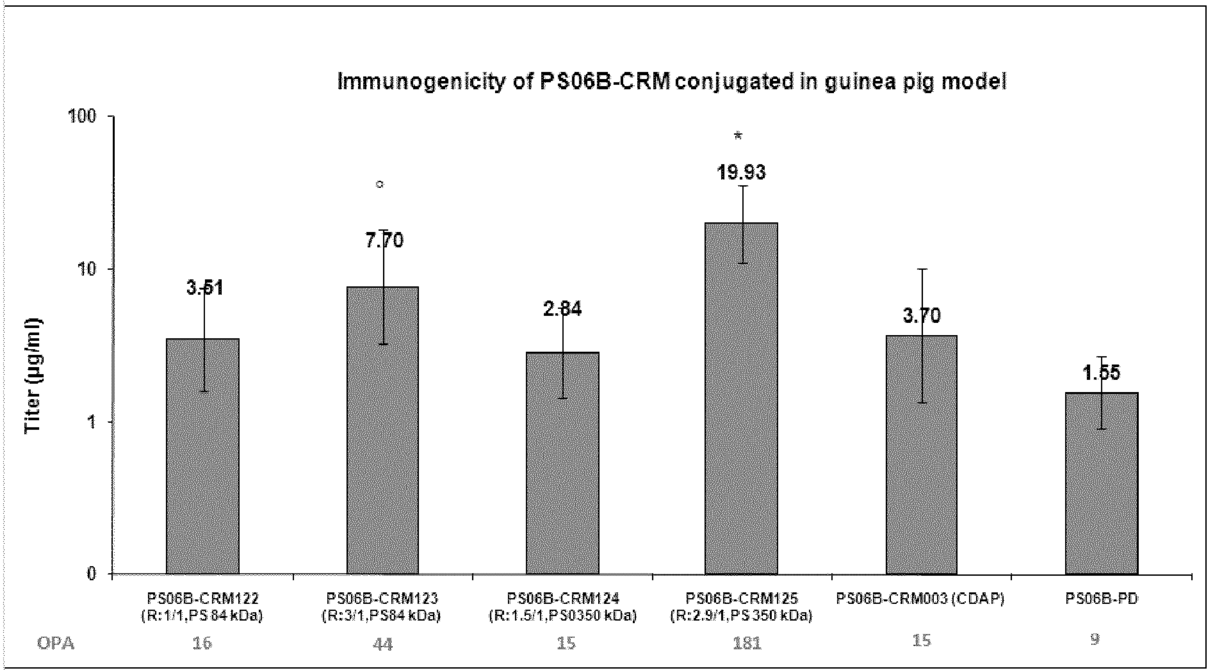

Evaluation of immunogenicity of PS06B-CRM conjugated in Balb/c mouse model
Titer (µg/ml)
10
1
0
0.83
0.37
1.78
0.64
0.31
0.10
PS06B-CRM122
PS06B-CRM123
PS06B-CRM124
PS06B-CRM125
PS06B-CRM003 (CDAP)
PS06B-PD
OPA
6.2
4.6
7.9
5.9
4.2
4.4

Figure 4


Immunogenicity of PS06B-CRM conjugated in guinea pig model
100
10
1
0
Titer (µg/ml)
3.51
7.70
2.84
19.93
3.70
1.55
PS06B-CRM122 (R:1/1,PS 84 kDa)
PS06B-CRM123 (R:3/1,PS84 kDa)
PS06B-CRM124 (R:1.5/1,PS0350 kDa)
PS06B-CRM125 (R:2.9/1,PS 350 kDa)
PS06B-CRM003 (CDAP)
PS06B-PD
OPA
16
44
15
181
15
9

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Szabadalmi igénypontok

1. Eljárás bakteriális szacharid konjugálására, amely magában foglalja a következő lépéseket:

a) a bakteriális szacharid reagáltatása 0,001-0,7 molekvivalens perjodáttal, ezáltal aktivált bakteriális szacharid képzése,

b) az aktivált bakteriális szacharid összekeverése hordozófehérjével,

c) az aktivált bakteriális szacharid és a hordozófehérje reagáltatása redukáló hatóanyaggal, ezáltal konjugátum képzése,

vagy

a) a bakteriális szacharid reagáltatása 0,001-0,7 molekvivalens perjodáttal, ezáltal aktivált bakteriális szacharid képzése,

b) az aktivált bakteriális szacharid összekeverése kötőcsoporttal,

c') az aktivált bakteriális szacharid reagáltatása a kötőcsoporttal redukáló hatóanyag alkalmazásával, ezáltal bakteriális szacharid-kötőcsoport képzése,

d) a bakteriális szacharid-kötőcsoport reagáltatása hordozófehérjével, ezáltal konjugátum képzése,

ahol az a) lépés amincsoportot nem tartalmazó, 1-100 mM koncentrációjú pufferben valósul meg, és ahol a bakteriális szacharid *S. pneumoniae* 23F kapszuláris szacharid.

2. Az 1. igénypont szerinti eljárás, ahol a puffer a foszfátpuffer, borátpuffer, acetátpuffer, karbonátpuffer és citrátpuffer által alkotott csoportból van kiválasztva, adott esetben ahol a puffer koncentrációja 1-50 mM, 1-25 mM, 1-10 mM, 5-15 mM, 8-12mM vagy 10-50mM vagy mintegy 10 mM.

3. Az 1. vagy 2. igénypont szerinti eljárás, ahol az a) lépésben a pH 3,5-8,0; 5,0-7,0 vagy 5,5-6,5 vagy mintegy 6,0.

4. Az 1-3. igénypontok bármelyike szerinti eljárás, ahol a bakteriális szacharid átlagos molekulatömege az a) lépés után 1-1100 kDa, 100-470 kDa, 200-300 kDa, 600-1100 kDa vagy 800-1000 kDa.

5. Az 1-4. igénypontok bármelyike szerinti eljárás, ahol a 23F szacharid átlagos molekulatömege az a) lépés után 100-470 kDa vagy 200-300 kDa.

6. Az 1-5. igénypontok bármelyike szerinti eljárás, ahol a hordozófehérje a tetanusz toxoid, a tetanusz toxoid C fragmense, a diftéria toxoid, a CRM197, a pneumolizin, a D, a PhtD, a PhtDE és az N19 fehérje által alkotott csoportból van kiválasztva.

7. Az 1-6. igénypontok bármelyike szerinti eljárás, ahol redukáló hatóanyag tartalmaz nátrium-ciano-bórhidridet vagy nátrium-triacetoxi-bórhidridet.

8. Az 1-7. igénypontok bármelyike szerinti eljárás, amely tartalmaz egy további lépést is: e) a konjugátum tisztítása.

9. Az 1-8. igénypontok bármelyike szerinti eljárás, amely tartalmaz egy további lépést: a konjugátum összekeverése további antigénekkel; adott esetben ahol a további antigén tartalmaz legalább 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 vagy 20 *S. pneumoniae* szacharidot, amely a következők által alkotott csoportból van kiválasztva: 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F és 33F.

10. A 9. igénypont szerinti eljárás, ahol a további antigének tartalmaznak egy vagy több *S. pneumoniae* fehérjét, amely(ek) a következők által alkotott csoportból van(nak) kiválasztva: poli-hisztidin-triád család (PhtX), kolinkötő-fehérje család (CbpX), csonkolt CbpX, LytX-család, csonkolt LytX, csonkolt CbpX- csonkolt

LytX kiméra fehérjék (vagy fúziós fehérjék), pneumolizin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 és Sp133.

11. A 9. vagy 10. igénypont szerinti eljárás, ahol a további antigének tartalmaznak *S. pneumoniae* 1, 4, 5, 6B, 7F, 9V, 14, 18C és 19F szacharidot.

12. Az 1-11. igénypontok bármelyike szerinti eljárás, amely magában foglalja a következő lépést: a bakteriális szacharid reagáltatása 0,1-0,5 molekvivalens perjodáttal, ezáltal aktivált bakteriális szacharid előállítása.

13. Az 1-12. igénypontok bármelyike szerinti eljárás, amely csökkenti a méretcsökkentő hatást a bakteriális szacharidon.

14. Az 1-13. igénypontok bármelyike szerinti eljárás, ahol a konjugátum össze van keverve adjuvánssal vagy gyógyászatilag elfogadható segédanyaggal.