Conjugation Process

DESCRIPTION

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

BACKGROUND

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.

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.

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~~

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.

In a first aspect, there is provided 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 sachharide with a linker;

c) reacting the activated bacterial saccharide with the linker using a reducing agent to form a bacterial sachharide-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 6B.

In ~~a first aspect of the invention~~one embodiment, 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, 0.1-1.2, 0.1-0.5, 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 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, 0.1-1.2, 0.1-0.5, 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.

In ~~a second aspect of the invention~~one embodiment, there is provided a conjugate obtainable by the process ~~of the invention~~disclosed herein.

In a ~~third~~ second aspect ~~, of the invention~~ there is provided a conjugate obtained by the process of ~~the invention~~disclosed herein.

In a ~~fourth~~ third aspect ~~of the invention~~, there is provided an immunogenic composition comprising the conjugate ~~of the invention~~ disclosed herein and a pharmaceutically acceptable excipient.

In ~~a fifth aspect of the invention~~one embodiment, there is provided a vaccine comprising the immunogenic composition ~~of the invention~~disclosed herein.

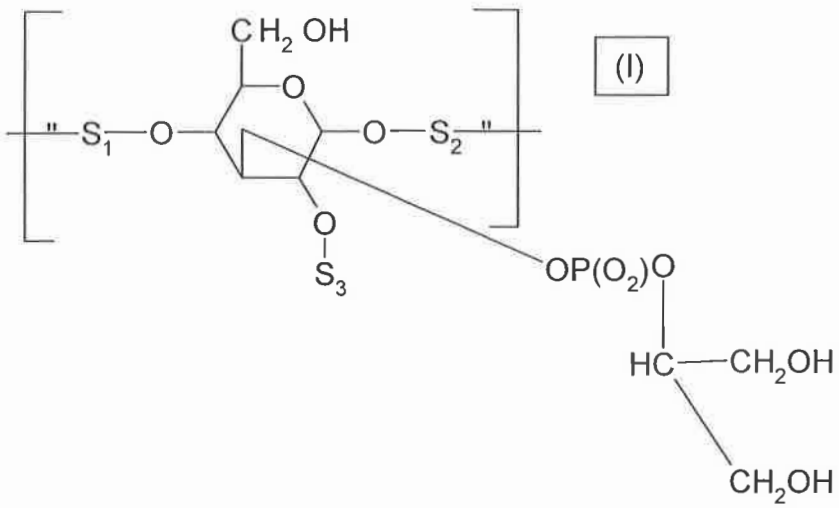
In ~~a sixth aspect of the invention~~one embodiment, there is provided a use of the immunogenic composition ~~of the invention~~disclosed herein or the vaccine ~~of the invention~~disclosed herein in the prevention or treatment of bacterial disease

In ~~a seventh aspect of the invention~~one embodiment, there is provided a use of the immunogenic composition ~~of the invention~~disclosed herein or the vaccine ~~of the~~

~~invention~~disclosed herein in the preparation of a medicament for the prevention or treatment of bacterial disease.

In ~~a eighth aspect of the invention~~ one embodiment, there is provided a method of preventing or treating bacterial infection comprising administration of the immunogenic composition ~~of the invention~~disclosed herein or the vaccine ~~of the invention~~disclosed herein to a patient.

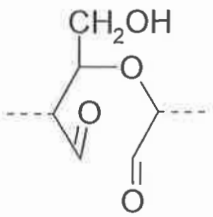
In ~~an ninth aspect of the invention~~one embodiment, there is provided an activated bacterial saccharide, wherein the activated bacterial saccharide comprises a repeat unit of formula (I):



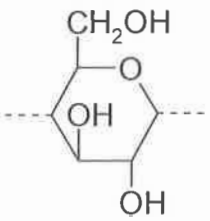
(I)

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



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, 6A or 6B.

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.

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

4. The process of any one of claims 1-3 wherein the buffer is phosphate buffer.

5. The process of any one of claims 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 claims 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 claims 1-6 wherein step a) is reacting the bacterial saccharide with 0.1-0.5 molar equivalents of periodate to form an activated bacterial saccharide.

8. The process of any one of claims 1-7 wherein the bacterial saccharide contains vicinal diols.

9. The process of any one of claims 1-8 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).

10. The process of any one of claims 1-9 wherein the bacterial saccharide is microfluidised before step a).

11. The process of any one of claims 9-10 wherein the average molecular weight of the native polysaccharide is between 20kDa and 2000kDa or between 30kDa and 1000kDa.

12. The process of claim 11 wherein the average molecular weight of the native polysaccharide is between 750-1500 kDa.

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

14. The process of any one of claims 1-13 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).

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

16. The process of any one of claims 1-15 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.

17. The process of any one of claims 1-16 wherein step a) is carried out in the dark.

18. The process of claim 8 wherein less than 0.001%, 0.01%, 0.1%, 0.5%, 1%, 2%, or 5% of the vicinal diols of the bacterial saccharide become oxidised during step a).

19. The process of claim 18 wherein during step c) the carbonyl group reacts with an amine group on the carrier protein.

20. The process of any one of claims 1-19 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).

21. The process of any one of claims 1-20 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.

22. The process of any one of claims 1-21 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.

23. The process of any one of claims 1-22 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).

24. The process of any one of claims 1-23 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.

25. The process of any one of claims 1-24 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.

26. The process of any one of claims 1-25 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.

27. The process of any one of claims 1-26 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.

28. The process of any one of claims 1-27 wherein step c) has a duration of between 10-60, or 20-60 hours.

29. The process of any one of claims 1-28 wherein the activated bacterial saccharide is conjugated directly to the carrier protein.

30. The process of any one of claims 1-28 wherein the activated bacterial saccharide is conjugated to the carrier protein through a linker.

31. The process of claim 30 wherein the linker is between 1 and 20 Angstroms in length.

32. The process of any one of claims 30 or 31 wherein the linker comprises an ADH linker.

33. The process of any one of claims 1-32 wherein the reducing agent comprises sodium cyanoborohydride.

34. The process of any one of claims 1-32 wherein the reducing agent comprises sodium triacetoxyborohydride.

35. The process of claim 34 wherein between 0.5 and 2 or 0.8 and 1.2 molar equivalent of reducing agent is used in step c).

36. The process of claim 35 wherein between 2 and 10 molar equivalent of reducing agent is used in step c).

37. The process of any one of claims 1-36 wherein any unreacted carbonyl groups are capped by reaction with sodium borohydride ($NaBH_4$).

38. The process of claim 37 wherein around 2M equivalents of sodium borohydride are used.

39. The process of any one of claims 37 or 38 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.

40. The process of any one of claims 1-39 wherein the activated bacterial saccharide and the carrier protein are lyophilised before step b).

41. The process of any one of claims 1-40 wherein the activated bacterial saccharide and the carrier protein are lyophilised before step c).

42. The process of any one of claims 40-41 wherein the activated bacterial saccharide and the carrier protein are lyophilised in the presence of a non-reducing sugar.

43. The process of any one of claims 40-42 wherein the non-reducing sugar is selected from the group consisting of sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit.

44. The process of any one of claims 1-43 steps b) and/or c) are carried out in DMSO solvent.

45. The process of any one of claims 1-43 wherein step b) and/or step c) are carried out in DMF solvent.

46. The process of any one of claims 1-45 comprising a further step e) of purifying the conjugate.

47. The process of claim 46 wherein step e) comprises purifying the conjugate using diafiltration.

48. The process of any one of claims 46-47 wherein step e) comprises purifying the conjugate using ion exchange chromatography.

49. The process of claim 48 wherein step e) comprises purifying the conjugate using size exclusion chromatography.

50. The process of any one of claims 1-49 comprising a further step f), wherein the conjugate is sterile filtered.

51. The process of any one of claims 1-50 containing a further step of mixing the conjugate with further antigens.

52. The process of claim 51 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.

53. The process of any one of claims 51-52 wherein the further antigens comprise *S.pneumoniae* saccharides 4, 9V, 14, 18C, and 19F.

54. The process of any one of claims 51-53 wherein the further antigens comprise *S.pneumoniae* saccharide 19A.

55. The process of any one of claims 51-54 wherein the further antigens comprise *S.pneumoniae* saccharide 6A.

56. The process of any one of claims 51-55 wherein the further antigens comprise *S.pneumoniae* saccharide 1.

57. The process of any one of claims 51-56 wherein the further antigens comprise *S.pneumoniae* saccharide 5.

58. The process of any one of claims 51-57 wherein the further antigens comprise *S.pneumoniae* saccharide 7F.

59. The process of any one of claims 51-58 wherein the further antigens comprise *S.pneumoniae* saccharide 3.

60. The process of any one of claims 51-59 wherein the further antigens comprise *S.pneumoniae* saccharide 23F.

61. The process of any one of claims 51-60 wherein the further antigens comprise *S.pneumoniae* saccharide 6B.

62. The process of any one of claims 51-61 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.

63. The process of any one of claims 52-62 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).

64. The process of any one of claims 52-63 wherein the further antigens comprise Hepatitis B surface antigen (HepB).

65. The process of any one of claims 50-63 wherein the further antigens comprise *Haemophilus influenzae* b (Hib) polysaccharide or oligosaccharide.

66. The process of any one of claims 50-64 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).

67. The process of any one of claims 1-66 wherein the conjugate is mixed with an adjuvant.

68. The process of claim 67 wherein the adjuvant is an aluminium salt.

69. The process of any one of claims 1-68 wherein the conjugate is mixed with a pharmaceutically acceptable excipient.

70. A conjugate obtainable by the process of any one of claims 1-69.

71. A conjugate obtained by the process of any one of claims 1-69.

72. An immunogenic composition comprising the conjugate of any one of claims 70-71 and a pharmaceutically acceptable excipient.

73. The immunogenic composition of claim 72 wherein the pharmaceutical excipient does not contain a chloride salt.

74. The immunogenic composition of any one of claims 72-73 wherein the pharmaceutical excipient comprises a buffer.

75. The immunogenic composition of claim 74 wherein the buffer is maleate buffer.

76. The immunogenic composition of claim 75 which comprises at least one further antigen.

77. The Immunogenic composition of claim 76 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.

78. The immunogenic composition of any one of claims 76-77 wherein the further antigens comprise *S.pneumoniae* saccharides 4, 9V, 14, 18C, and 19F.

79. The immunogenic composition of any one of claims 76-78 wherein the further antigens comprise *S.pneumoniae* saccharide 19A.

80. The immunogenic composition of any one of claims 76-79 wherein the further antigens comprise *S.pneumoniae* saccharide 6A.

81. The immunogenic composition of any one of claims 76-80 wherein the further antigens comprise *S.pneumoniae* saccharide 1.

82. The immunogenic composition of any one of claims 76-81 wherein the further antigens comprise *S.pneumoniae* saccharide 5.

83. The immunogenic composition of any one of claims 76-82 wherein the further antigens comprise *S.pneumoniae* saccharide 7F.

84. The immunogenic composition of any one of claims 76-83 wherein the further antigens comprise *S.pneumoniae* saccharide 3.

85. The immunogenic composition of any one of claims 76-84 wherein the further antigens comprise *S.pneumoniae* saccharide 23F.

86. The immunogenic composition of any one of claims 76-85 wherein the further antigens comprise *S.pneumoniae* saccharide 6B.

87. The immunogenic composition of any one of claims 76-86 wherein the further antigens comprise *S.pneumoniae* saccharide 6C.

88. The immunogenic composition of any one of claims 76-87 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.

89. The immunogenic composition of any one of claims 76-88 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).

90. The immunogenic composition of any one of claims 76-89 wherein the further antigens comprise *Haemophilus influenzae* b (Hib) polysaccharide or oligosaccharide.

91. The immunogenic composition of any one of claims 76-90 wherein the further antigens comprise Hepatitis B surface antigen (HepB)

92. The immunogenic composition of any one of claims 76-91 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).

93. The immunogenic composition of any one of claims 76-92 further comprising an adjuvant.

94. The immunogenic composition of claim 93 wherein the adjuvant is an aluminium salt.

95. A vaccine comprising the immunogenic composition of any one of claims 72-94.

96. A use of the immunogenic composition of any one of claims 72-94 or the vaccine of claim 95 in the preparation of a medicament for the prevention or treatment of bacterial disease.

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 6B.

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, 23F 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 any one of claims 1-10 wherein the conjugate is mixed with an adjuvant or a pharmaceutically acceptable excipient.

12. A conjugate obtained by the process of any one of claims 1-11.

13. An immunogenic composition comprising the conjugate of claim 12 and a pharmaceutically acceptable excipient.

14. An immunogenic composition according to claim 13 wherein the pharmaceutical excipient comprises a maleate buffer.

15. The immunogenic composition of claim 13 or 14 when used for the prevention or treatment of bacterial disease.

16. A process for conjugating a bacterial saccharide according to claim 1, substantially as hereinbefore described with reference to any of the Examples and/or Figures.