(54) **MENINGOCOCCUS SEROGROUP X CONJUGATE**

MENINGOKOKKEN-SEROGRUPPE-X-KONJUGAT

CONJUGUÉ DE SÉROGROUPE X DE MÉNINGOCOQUE

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Description

TECHNICAL FIELD

5   **[0001]** This invention is in the field of bacterial capsular saccharides, particularly *Neisseria meningitidis* serogroup X capsular polysaccharides. The polysaccharides may be conjugated to carriers in order to form conjugates. The polysaccharides and conjugates are useful for immunisation, particularly in aqueous formulations.

BACKGROUND ART

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[0002] The capsular saccharides of bacteria have been used for many years in vaccines against capsulated bacteria. As saccharides are T-independent antigens, however, they are poorly immunogenic. Conjugation to a carrier can convert T-independent antigens into T-dependent antigens, thereby enhancing memory responses and allowing protective immunity to develop. The most effective saccharide vaccines are therefore based on glycoconjugates, and the prototype
15   conjugate vaccine was against *Haemophilus influenzae* type b ('Hib') [*e.g.* see chapter 14 of ref. 86].
[0003] Based on the organism's capsular polysaccharide, twelve serogroups of *N.meningitidis* have been identified (A, B, C, H, I, K, L, 29E, W135, X, Y and Z). Group A is the pathogen most often implicated in epidemic disease in sub-Saharan Africa. Serogroups B and C are responsible for the vast majority of cases in USA and in most developed countries. Serogroups W135 and Y are responsible for the remaining cases in USA and developed countries. A tetravalent
20   vaccine of capsular polysaccharides from serogroups A, C, Y and W135 has been known for many years [1,2]. Although effective in adolescents and adults, it induces a poor immune response and short duration of protection and cannot be used in infants [*e.g.* ref. 3] because polysaccharides are T cell-independent antigens that induce a weak immune response which cannot be boosted. The polysaccharides in this vaccine are not conjugated [4]. Conjugate vaccines against serogroup C have been approved for human use, and include Menjugate™ [5], Meningitec™ and NeisVac-C™. Mixtures
25   of conjugates from serogroups A+C are known [6-8] and mixtures of conjugates from serogroups A+C+W135+Y have been reported [9-13].
[0004] The structure of the group X capsular polysaccharide has been known since the 1970s [14] and this serogroup has been associated with a number of outbreaks of meningococcal disease, *e.g.* in sub-Saharan Africa and China [15,16]. Serogroup X is known to have a significantly higher attack rate than serogroup A among children below 5 years
30   of age. Although the need for a vaccine against this serogroup has been recognised for many years [17], no effective vaccine has been developed. Conjugate vaccines against serogroup X have been proposed [17,18], but it remains unknown whether such conjugates would be immunogenic or protective against this serogroup.
[0005] Accordingly, there remains a need for conjugates of serogroup X capsular polysaccharides. Moreover, there remains a need for conjugates that can be used for vaccination against diseases caused by this serogroup.
35   **[0006]** The structure of the group X capsular polysaccharide consists of N-acetylglucosamine-4-phosphate residues held together by α1-4 phosphodiester bonds without O-acetyl groups [19]: {→4)-D-Glc*p*NAc-α-(1→OPO3→} (Figure 1). Based on the similarity between their structures, a biosynthetic relationship between MenA and MenX capsular polysaccharides has been postulated [14]. MenA capsular polysaccharide tends to hydrolyse significantly in aqueous solution [20]. This instability is thought to be caused by the presence of a phosphodiester linkage involving the anomeric position
40   and of the N-Acetyl group in position 2 of mannosamine, which can assist departure of a phosphomonoester group [21]. Another possibility is that the hydroxyl groups at position 4 of the N-acetylmannosamine subunits interact with the phosphodiester groups facilitating hydrolysis via an internal participation mechanism, as seen in the capsular polysaccharide of type 6A pneumococcus [22] and *Haemophilus influenzae* type b [23]. The similarity in the structures of the MenX and MenA capsular polysaccharides, particularly their common anomeric phosphodiester linkage, means that the
45   MenX polysaccharide may suffer from similar stability problems when in aqueous solution. The intrinsic instability of the MenA capsular polysaccharide in aqueous solution means that it is often presented in a lyophilized form when contained in vaccines (*e.g.* in the polysaccharide vaccine Mencevax™ and the conjugate vaccines MenAfriVac™, Menveo™ and Nimenrix™). Although the MenX capsular polysaccharide could similarly be presented in a lyophilised form to improve its stability, an aqueous formulation would be more convenient. The only vaccine containing a MenA capsular polysaccharide conjugate in an aqueous formulation is Menactra™, but this vaccine requires storage at low temperatures. Such
50   cold storage is expensive and presents practical difficulties in many of the countries where MenA and MenX outbreaks are common (*e.g.* sub-Saharan Africa).
[0007] Accordingly, there is a need for aqueous formulations of serogroup X capsular polysaccharides and conjugates thereof, particularly aqueous formulations that do not require refrigeration.
55   **[0008]** The development of a vaccine against MenX requires a method for polysaccharide quantification that can be used as an in-process assay and/or for the characterization of the final vaccine. The presence of phosphate groups in the MenX capsular polysaccharide means that the polysaccharide can be quantified by a colorimetric method that measures total phosphorus content [24]. However, this method lacks selectivity and therefore would not be suitable for

certain in-process applications, *e.g.* for the analysis of polysaccharide in phosphate buffers or in the presence of phosphate-containing impurities. A more selective method would be NMR, which has been proposed already for MenX polysaccharide quantification [25]. However, this approach requires pure samples and a large amount of material. Reference 26 demonstrates an alternative approach, where the MenX polysaccharide is quantified by HPAEC-PAD, which is more sensitive than NMR and more selective than measuring phosphate content. The authors of ref. 26 quantified the MenX polysaccharide by hydrolysing the sample to make glucosamine, and comparing the amount of glucosamine released against a calibration curve derived from an N-acetyl-glucosamine-6-phosphate quantitative standard. However, glucosamine may be present because of contamination, leading to inaccurate results.

[0009]   Accordingly, there is a need for alternative or improved methods for assaying the MenX polysaccharide, and in particular for methods that are more selective for MenX.

DISCLOSURE OF THE INVENTION

[0010]   The invention is based in part on methods for conjugating a serogroup X capsular polysaccharide to a carrier molecule. The inventors have found that the resulting conjugates are immunogenic and capable of inducing a bactericidal antibody response. Serogroup X conjugates are therefore useful in immunogenic compositions, and in particular in vaccines. The inventors have also discovered that it is possible to combine serogroup X capsular polysaccharide antigens, *e.g.* serogroup X conjugates, with other antigens without losing the immune response to serogroup X. In particular, serogroup X conjugates may be combined with other conjugates, *e.g.* conjugates comprising other bacterial capsular saccharide antigens. Serogroup X conjugates are particularly suitable for combination with conjugates comprising capsular saccharide antigens from other *N.meningitidis* serogroups, *e.g.* serogroups A, C, W135 and Y. In these combinations, not only does the serogroup X conjugate retain its immunogenicity, but the serogroup A, C, W135 and/or Y conjugates also retain their immunogenicity. Furthermore, the inventors have also found that despite its structural similarity to the serogroup A capsular polysaccharide, the capsular polysaccharide from serogroup X is surprisingly stable in solution. Serogroup X capsular polysaccharides and conjugates thereof may therefore be particularly suitable for use in aqueous formulations.

[0011]   The invention provides an immunogenic composition comprising a conjugate of a *Neisseria meningitidis* serogroup X capsular polysaccharide and a carrier molecule, wherein coupling of the capsular polysaccharide to the carrier protein is direct. The inventors have found that particularly stable conjugates of a serogroup X capsular polysaccharide and a carrier molecule may be made using the process of the first process described below. For example, the conjugates may contain less than 50% free saccharide after 28 days at 37 °C. The % free saccharide may be determined as described in *Stability study (2)* below. Accordingly, the invention provides an immunogenic composition comprising a conjugate of a serogroup X capsular polysaccharide and a carrier molecule which comprises less than 50% free saccharide after 28 days at 37 °C. The immunogenic composition may in particular comprise less than 25% free saccharide, particularly less than 20% free saccharide and more particularly less than 15% free saccharide, e.g. about 10% free saccharide.

[0012]   The conjugate is typically obtained or obtainable by one of the four processes described below. However, the conjugate may alternatively be made by any suitable method.

[0013]   A first process for preparing a conjugate of a serogroup X capsular polysaccharide and a carrier molecule, comprises the steps of: (a) oxidising a primary hydroxyl group in the capsular polysaccharide, to give an oxidised polysaccharide with an aldehyde group; and (b) coupling the oxidised polysaccharide to a carrier molecule via the aldehyde group, thereby giving the conjugate. This process is thought to be particularly suitable (*e.g.* in terms of yield) for relatively long polysaccharides, *e.g.* those with a degree of polymerisation (DP) of between 20 and 200, particularly between 60 and 100 (*e.g.* between 70 and 90, particularly around 80). This process also contains relatively few steps, making it easier to scale-up to an industrial setting. The resulting conjugates may also be more stable, particularly compared to conjugates in which the polysaccharide is linked to the carrier via its reducing terminus. The coupling in step (b) is *e.g.* by reductive amination between the aldehyde group and a primary amine group on the carrier molecule.

[0014]   A second process for preparing a conjugate of a serogroup X capsular polysaccharide and a carrier molecule, comprises the steps of: (a) reductive amination of the reducing terminus of the capsular polysaccharide, to give a modified polysaccharide with a primary amine group bonded to the C-1 atom of the terminal subunit by a covalent bond; and (b) coupling the modified polysaccharide to a carrier molecule via the primary amine group, thereby giving the conjugate. This process is particularly suitable for relatively short polysaccharides *e.g.* polysaccharides with a DP between 5 and 50, particularly between 10 and 20, *e.g.* about 15.

[0015]   A third process for preparing a conjugate of a serogroup X capsular polysaccharide and a carrier molecule, comprises the steps of: (a) reduction of the reducing terminus of the capsular polysaccharide, to give a modified polysaccharide with two vicinal hydroxyl groups at that terminus; (b) oxidative cleavage of the vicinal hydroxyl groups, to give a further modified polysaccharide with an aldehyde group at the terminus; (c) reductive amination of the aldehyde group, to give a further modified polysaccharide with a primary amine group at the terminus and (d) coupling the further modified

polysaccharide to a carrier molecule via the primary amine group, thereby giving the conjugate. This process may provide a better yield than the first and second processes, particularly for relatively short polysaccharides *e.g.* polysaccharides with a DP between 5 and 50, particularly between 10 and 20, *e.g.* about 15.

[0016] A fourth process for preparing a conjugate of a serogroup X capsular polysaccharide and a carrier molecule, comprises the steps of: (a) reduction of the reducing terminus of the capsular polysaccharide, to give a modified polysaccharide with two vicinal hydroxyl groups at that terminus; (b) oxidative cleavage of the vicinal hydroxyl groups, to give a further modified polysaccharide with an aldehyde group at the terminus; (c) direct coupling of the further modified polysaccharide to the carrier molecule by reductive amination of the aldehyde group with a primary amine group on the carrier molecule, thereby giving the conjugate.

The capsular polysaccharide

[0017] The invention involves the capsular polysaccharide of *N.meningitidis* serogroup X. The structure of the group X capsular polysaccharide consists of N-acetylglucosamine-4-phosphate residues held together by α1-4 phosphodiester bonds without O-acetyl groups [19]: {$\rightarrow$4)-D-Glc*p*NAc-α-(1$\rightarrow$OPO3$\rightarrow$} (Figure 1).

[0018] The capsular polysaccharide can be purified by known techniques, for example by the method described in ref. 19. In general, meningococcal capsular polysaccharides are prepared by a process comprising the steps of polysaccharide precipitation (*e.g.* using a cationic detergent), ethanol fractionation, cold phenol extraction (to remove protein) and ultracentrifugation (to remove LPS) [*e.g.* ref. 27]. However, a preferred process for the serogroup X capsular polysaccharide is described in ref. 10. This process involves polysaccharide precipitation followed by solubilisation of the precipitated polysaccharide using a lower alcohol. Precipitation can be achieved using a cationic detergent such as tetrabutylammonium and cetyltrimethylammonium salts (*e.g.* the bromide salts), or hexadimethrine bromide and myristyltrimethylammonium salts. Cetyltrimethylammonium bromide ('CTAB') is typically used [28]. Solubilisation of the precipitated material can be achieved using a lower alcohol such as methanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol, 2-methyl-propan-1-ol, 2-methyl-propan-2-ol, diols, *etc.,* but ethanol is particularly suitable for solubilising CTAB-polysaccharide complexes. Ethanol is preferably added to the precipitated polysaccharide to give a final ethanol concentration (based on total content of ethanol and water) of between 50% and 95%.

[0019] After re-solubilisation, the polysaccharide may be further treated to remove contaminants. This is particularly important in situations where even minor contamination is not acceptable (*e.g.* for human vaccine production). This will typically involve one or more steps of filtration *e.g.* depth filtration, filtration through activated carbon may be used, size filtration and/or ultrafiltration.

[0020] Once filtered to remove contaminants, the polysaccharide may be precipitated for further treatment and/or processing. This can be conveniently achieved by exchanging cations (*e.g.* by the addition of calcium or sodium salts).

[0021] The invention is not limited to polysaccharides purified from natural sources, however, and the polysaccharides may be obtained by other methods, such as total or partial synthesis, *e.g.* by the enzymatic synthesis described in ref. 29.

[0022] The polysaccharide may be chemically modified relative to the capsular polysaccharide as found in nature. For example, the polysaccharide may be de-N-acetylated (partially or fully), N-propionated (partially or fully), *etc.* De-acetylation may occur before, during or after conjugation, but typically occurs before conjugation. The degree of N-acetylation of the serogroup X capsular polysaccharide used in the invention may be 0-100%, 50-100%, 75-100%, 80-100%, 90-100%, or 95-100%. Typically, the degree of N-acetylation is 100%. The degree of N-acetylation of the polysaccharide can be determined by any method known in the art, for example, by proton NMR (*e.g.* as described in references 30 and 31.

[0023] Capsular polysaccharides will generally be used in the form of oligosaccharides. These are conveniently formed by fragmentation of purified capsular polysaccharide (*e.g.* by hydrolysis), which will usually be followed by purification of the fragments of the desired size.

[0024] Fragmentation of polysaccharides is preferably performed to give a final average degree of polymerisation (DP) in the oligosaccharide of between 20 and 200, particularly between 60 and 100 (*e.g.* between 70 and 90, particularly around 80). The inventors have found that polysaccharides of this length are particularly suitable for use in the first process described above. However, the inventors have found that shorter polysaccharides may also be used, *e.g.* polysaccharides with a DP between 5 and 50, particularly between 10 and 20, *e.g.* about 15. The inventors have found that polysaccharides of this length are particularly suitable for use in the second and third processes described above. DP can conveniently be measured by ion exchange chromatography, NMR or by colorimetric assays [32].

[0025] The polysaccharide may be sized in order to obtain a desired range of polysaccharide sizes [33]. This can be achieved in various ways, such as ultrafiltration followed by ion-exchange chromatography.

The carrier molecule

[0026] The invention involves the use of carrier proteins. In general, covalent conjugation of saccharides to carriers enhances the immunogenicity of saccharides as it converts them from T-independent antigens to T-dependent antigens,

thus allowing priming for immunological memory. Conjugation is particularly useful for paediatric vaccines [e.g. ref. 34] and is a well known technique [e.g. reviewed in refs. 35 to 43].

[0027] Preferred carrier proteins are bacterial toxins, such as diphtheria or tetanus toxins, or toxoids or mutants thereof, particularly diphtheria toxoid or tetanus toxoid. The inventors have found that the CRM197 diphtheria toxin mutant [44] is particularly suitable. Protein D from *H.influenzae* [45-47] may also be used.

[0028] Other suitable carrier proteins include the *N.meningitidis* outer membrane protein complex [48], synthetic peptides [49,50], heat shock proteins [51,52], pertussis proteins [53,54], cytokines [55], lymphokines [55], hormones [55], growth factors [55], human serum albumin (typically recombinant), artificial proteins comprising multiple human CD4^{+} T cell epitopes from various pathogen-derived antigens [56] such as N19 [57], pneumococcal surface protein PspA [58], pneumolysin [59] or its non-toxic derivatives [60], iron-uptake proteins [61], toxin A or B from *C.difficile* [62], a GBS protein [63], a GAS protein [64] *etc.*

[0029] Other suitable carrier proteins are described in ref. 65, in particular the carrier protein of SEQ ID NO: 9 in that document. These carrier proteins are also described in ref. 66, and further details are provided in the section *"Exemplary carrier proteins"* below.

Oxidation

[0030] In step (a) of the first process described above, a primary hydroxyl group in the capsular polysaccharide is oxidised to give an aldehyde group. The primary hydroxyl group is bonded to the C-6 atom of a MenX capsular polysaccharide subunit by a covalent bond, such that the step proceeds as follows:
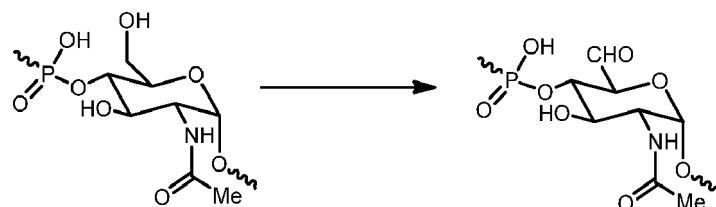
[0031] This step may involve oxidation of more than one such primary hydroxyl group, resulting in the introduction of more than one aldehyde group along the polysaccharide chain. For example, the inventors have found that suitable conjugates can be prepared by oxidising the primary hydroxyl group on between 1-50%, particularly 1-20% and more particularly 1-10%, e.g. about 4-8%, of residues within the serogroup X polysaccharide. Hydroxyl groups may be converted to aldehydes by various oxidation reactions (*e.g.* Swern oxidation, Dess-Martin oxidation, Cr[VI] oxidations, *etc.*). However the inventors have found that the TEMPO (2,2,6,6-tetramethylpiperidinyloxy radical)-mediated oxidation is particularly suitable. TEMPO-mediated oxidation is described in of ref. 67. To prevent oxidation of the aldehyde group to a carboxyl group, TEMPO-mediated oxidation is preferably carried out in non-aqueous conditions, *e.g.* using a DMF solvent as described in ref. 68. The skilled person would be capable of identifying suitable conditions for oxidation. For example, the inventors have found that treatment of polysaccharide with TEMPO (0.06eq relative to the MenX repeating subunit), $NaHCO_3$ (9eq relative to the MenX repeating subunit) and TCC (trichloroisocyanuric acid, 2eq relative to the MenX repeating subunit) at 0°C overnight is suitable.

Oxidative cleavage

[0032] In step (b) of the the third and fourth processes described above, two vicinal hydroxyl groups in the capsular polysaccharide undergo oxidative cleavage to give an aldehyde group:
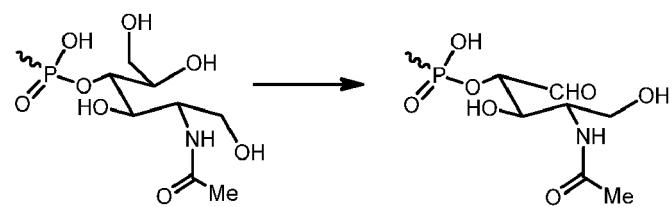
[0033] Oxidative cleavage (*e.g.* using $NaIO_4$, $Pb(OAc)_4$, *etc.*) is well known in the art. The inventors have found that reacting the polysaccharide at 6-8 mg/ml in 10mM NaPi buffer at pH 7.2 with $NaIO_4$ (10eq relative to the molecular weight of MenX, solid) for 1.5 hours at room temperature is suitable.

Reduction

[0034]   In step (a) of the third and fourth processes described above, the reducing terminus of the capsular polysaccharide is reduced to give a modified polysaccharide with two vicinal hydroxyl groups at the terminus:
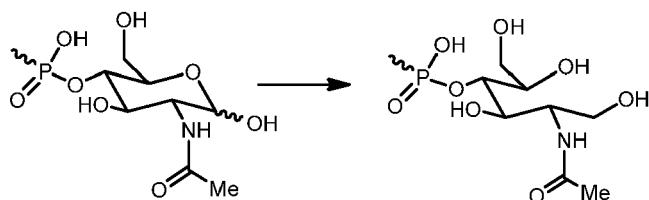
[0035]   Reduction of polysaccharides (*e.g.* using $NaBH_4$, *etc.*) is well known in the art. The inventors have found that reacting the polysaccharide at 15 mg/ml in 10mM NaPi buffer at pH 8 with $NaBH_4$ (12eq relative to the molecular weight of MenX, solid) for 1.5 hours at room temperature is suitable.

Reductive amination

[0036]   In step (a) of the second process described above, the reducing terminus of the capsular polysaccharide is subjected to reductive amination to give a modified polysaccharide with a primary amine group bonded to the C-1 atom of the terminal subunit by a covalent bond:
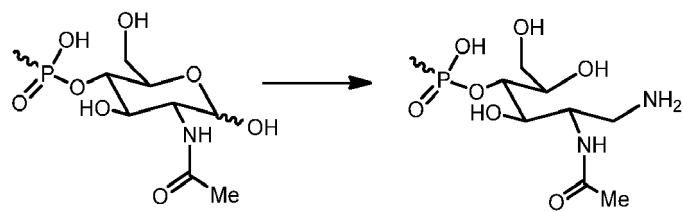
[0037]   Reductive amination is a standard technique in organic chemistry. For example, the aldehyde group at the reducing terminus may be converted into a primary amine group using ammonia. This can conveniently be achieved using an ammonium salt (*e.g.* ammonium chloride or ammonium acetate) in combination with an appropriate reducing agent (*e.g.* cyanoborohydrides, such as sodium cyanoborohydride $NaBH_3CN$; borane-pyridine; sodium triacetoxyborohydride; borohydride exchange resin). The skilled person would be capable of identifying suitable conditions for reductive amination.

[0038]   Reductive amination is also carried out in step (c) of the third process described above, to give a modified polysaccharide with a primary amine group at the terminus. For example, the aldehyde group may be converted into a primary amine group as described above. The reductive amination may therefore result in a modified polysaccharide with a primary amine group bonded to the C-5 atom of the terminal subunit by a covalent bond:
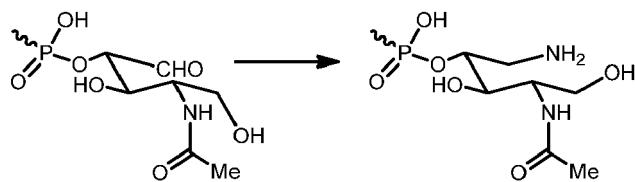
[0039]   Reductive amination is also carried out in step (c) of the fourth process described above, to give the conjugate. The reductive amination is between the aldehyde group of the further modified polysaccharide and a primary amine group on the carrier molecule.

Coupling to a carrier molecule

[0040]   The coupling of the oxidised polysaccharide to the carrier molecule via the aldehyde group in step (b) of the first process described above is direct. The coupling of the modified polysaccharide to the carrier molecule via the primary amine group in step (b) of the second process above is also direct. The coupling of the modified polysaccharide to the

carrier molecule via the primary amine group in step (d) of the third process described above is also direct. For all three steps, any suitable conjugation reaction can be used.

[0041]   Attachment of the polysaccharide to the carrier is typically via a primary amine ($-NH_2$) group e.g. in the side chain of a lysine or residue in a carrier protein, or of an arginine residue. Attachment to the carrier may also be via a sulphydryl (-SH) group *e.g.* in the side chain of a cysteine residue.

[0042]   For the first process described above, the inventors have found that direct coupling may be conveniently achieved by reacting the aldehyde group in the oxidised polysaccharide with an amine group in the carrier by reductive amination. Direct coupling of this nature is therefore preferred. As discussed above, reductive amination is a standard technique, and has been used extensively in the production of conjugates of capsular polysaccharides for vaccine use. In one approach, an aldehyde group in the oxidised polysaccharide reacts with an amine group in the carrier. This can conveniently be achieved by combining the polysaccharide with the carrier in the presence of an appropriate reducing agent (*e.g.* cyanoborohydrides, such as sodium cyanoborohydride $NaBH_3CN$; borane-pyridine; sodium triacetoxyboro-hydride; borohydride exchange resin; *etc.*). The skilled person would be capable of identifying suitable conditions for reductive amination. For example, the inventors have found that treatment of oxidised polysaccharide with 10mg/ml CRM197 at a 4:1 w/w ratio and $NaBH_3CN$ at a 1:1 w/w ratio in a NaPi 10mM pH 7.2 buffer is suitable. This mixture may be left for 72 hours with slow stirring at 37°C to effect the reductive amination.

[0043]   Conjugates with a polysaccharide:protein ratio (w/w) of between 1:20 (*i.e.* excess protein) and 20:1 (*i.e.* excess polysaccharide) are of interest. Ratios of 1:10 to 1:1 are preferred, particularly ratios between 1:2 and 1:1 and, most preferably, about 1:1.5. For conjugates made by the first process, a typical ratio is between 0.1 and 1.0, more particularly between 0.2 and 0.4, *e.g.* about 0.35. For conjugates made by the second process, a typical ratio is between 0.1 and 1.0, more particularly between 0.1 and 0.3, *e.g.* about 0.22. For conjugates made by third process, a typical ratio is between is between 0.1 and 1.0, more particularly between 0.1 and 0.3, *e.g.* about 0.21.

[0044]   Compositions may include a small amount of free carrier [72]. When a given carrier protein is present in both free and conjugated form in a composition of the invention, the unconjugated form is preferably no more than 5% of the total amount of the carrier protein in the composition as a whole, and more preferably present at less than 2% by weight.

[0045]   After conjugation, free and conjugated polysaccharides can be separated. There are many suitable methods, including hydrophobic chromatography, tangential ultrafiltration, diafiltration *etc.* [see also refs. 73 & 74, *etc.*].

Combinations of conjugates and other antigens

[0046]   As well as providing individual conjugates as described above, an immunogenic composition may comprise one or more further antigens.

[0047]   The further antigen(s) may comprise further conjugates. In these embodiments, it is possible to use more than one carrier for the different conjugates in the composition, *e.g.* to reduce the risk of carrier suppression. Typically, the same carrier is used for all conjugates. However, the inventors have found that the use of a different carrier for the *N.meningitidis* serogroup X conjugate may reduce immune interference when the *N.meningitidis* serogroup X conjugate is combined with further conjugate(s). Accordingly, in some embodiments, the *N.meningitidis* serogroup X conjugate uses one carrier (particularly tetanus toxoid or SEQ ID NO: 9 of ref. 65 and ref. 66), while the further conjugate(s) use a different carrier (particularly CRM197).

[0048]   A single carrier protein might carry more than one polysaccharide antigen [75,76]. To achieve this goal, different polysaccharides can be mixed prior to the conjugation process. Typically, however, there are separate conjugates for each polysaccharide, with the different polysaccharides being mixed after conjugation. The separate conjugates are typically based on the same carrier, as discussed above.

[0049]   The further antigen(s) may in particular be selected from the group consisting of serogroup A capsular polysaccharide, serogroup C capsular polysaccharide, serogroup Y capsular polysaccharide and serogroup W135 capsular polysaccharide. Typically, the further antigen(s) selected from this group are each separately conjugated to a carrier protein. Preferred carrier proteins are bacterial toxins, such as diphtheria or tetanus toxins, or toxoids or mutants thereof, particularly diphtheria toxoid or tetanus toxoid. The inventors have found that the CRM197 diphtheria toxin mutant is particularly suitable. Protein D from *H.influenzae* may also be used. Typically, the same carrier protein is used for all of the conjugates, optionally including the *N.meningitidis* serogroup X conjugate. The inventors have found that the CRM197 diphtheria toxin mutant is particularly suitable, although diphtheria toxoid and tetanus toxoid may also be used. As noted above, the inventors have found that the use of a different carrier for the *N.meningitidis* serogroup X conjugate may reduce immune interference when the conjugate is combined with further conjugate(s). Accordingly, in some embodiments, the *N.meningitidis* serogroup X conjugate uses one carrier (particularly tetanus toxoid or SEQ ID NO: 9 of ref. 65 and ref. 66), while the further conjugate(s) uses a different carrier (particularly CRM197).

[0050]   The following combinations are specifically envisaged for use in the invention:

1) a *N.meningitidis* serogroup X conjugate and a conjugate of a serogroup A capsular polysaccharide and a carrier

protein;

2) a *N.meningitidis* serogroup X conjugate and a conjugate of a serogroup W135 capsular polysaccharide and a carrier protein;

3) a *N.meningitidis* serogroup X conjugate, a conjugate of a serogroup A capsular polysaccharide and a carrier protein, and a conjugate of a serogroup W135 capsular polysaccharide and a carrier protein; and

4) a *N.meningitidis* serogroup X conjugate, a conjugate of a serogroup A capsular polysaccharide and a carrier protein, a conjugate of a serogroup C capsular polysaccharide and a carrier protein, a conjugate of a serogroup W135 capsular polysaccharide and a carrier protein, and a conjugate of a serogroup Y capsular polysaccharide and a carrier protein.

[0051]   By including antigens from serogroup X and serogroup A and/or serogroup W135, compositions comprising combinations 1)-3) can provide protection against the serogroups that cause the majority of *N.meningitidis* disease in Africa. Such combinations are therefore particularly preferred. Although the addition of further antigens, *e.g.* the antigens from serogroups C and Y included in combination 4), may provide additional protection, the benefit of this additional protection may not outweigh the additional costs involved. Accordingly, in some embodiments of the invention, the composition does not contain an antigen from serogroup C, particularly a conjugate of a serogroup C capsular polysaccharide and a carrier protein. Similarly, in the same or other embodiments of the invention, the composition does not contain an antigen from serogroup Y, particularly a conjugate of a serogroup Y capsular polysaccharide and a carrier protein.

[0052]   The further antigen(s) may comprise additional bacterial, viral or parasitic antigens. These may be selected from the following:

- a saccharide antigen from *Streptococcus pneumoniae* [*e.g.* refs. 77-79; chapters 22 & 23 of ref. 86].
- an antigen from hepatitis A virus, such as inactivated virus [*e.g.* 80, 81; chapter 15 of ref. 86].
- an antigen from hepatitis B virus, such as the surface and/or core antigens [*e.g.* 81,82; chpater 16 of ref. 86].
- an antigen from hepatitis C virus [*e.g.* 83].
- an antigen from *Bordetella pertussis,* such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis,* optionally also in combination with pertactin and/or agglutinogens 2 and 3 [*e.g.* refs. 84 & 85; chapter 21 of ref. 86].
- a diphtheria antigen, such as a diphtheria toxoid [*e.g.* chapter 13 of ref. 86].
- a tetanus antigen, such as a tetanus toxoid [*e.g.* chapter 27 of ref. 86].
- a saccharide antigen from *Haemophilus influenzae* B [*e.g.* chapter 14 of ref. 86]
- an antigen from *Chlamydia pneumoniae* [*e.g.* 87, 88, 89, 90, 91, 92, 93].
- an antigen from *Chlamydia trachomatis* [*e.g.* 94].
- an antigen from *Porphyromonas gingivalis* [*e.g.* 95].
- polio antigen(s) [*e.g.* 96, 97; chapter 24 of ref. 86] such as IPV.
- rabies antigen(s) [*e.g.* 98] such as lyophilised inactivated virus [*e.g.* 99, RabAvert™].
- measles, mumps and/or rubella antigens [*e.g.* chapters 19, 20 and 26 of ref. 86].
- influenza antigen(s) [*e.g.* chapters 17 & 18 of ref. 86], such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* [*e.g.* 100].
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [*e.g.* 101, 102, 103].
- an antigen from *Streptococcus agalactiae* (group B streptococcus) [*e.g.* 64, 104-106].
- an antigen from *S.epidermidis* [*e.g.* type I, II and/or III capsular polysaccharide obtainable from strains ATCC-31432, SE-360 and SE-10 as described in refs. 107, 108 and 109.

[0053]   Where a saccharide or carbohydrate antigen is used, it is typically conjugated to a carrier in order to enhance immunogenicity. Conjugation of *H.influenzae* B, meningococcal and pneumococcal saccharide antigens is well known.

[0054]   Toxic protein antigens may be detoxified where necessary (*e.g.* detoxification of pertussis toxin by chemical and/or genetic means [85]).

[0055]   Where a diphtheria antigen is included in the composition it is typical also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is typical also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is typical also to include diphtheria and tetanus antigens.

[0056]   Antigens may be adsorbed to an aluminium salt.

[0057]   Antigens in the composition will typically be present at a concentration of at least 1µg/ml each. In general, the

concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

[0058] As an alternative to using proteins antigens in the composition of the invention, nucleic acid encoding the antigen may be used [*e.g.* refs. 110 to 118]. Protein components of the compositions of the invention may thus be replaced by nucleic acid (usually DNA *e.g.* in the form of a plasmid) that encodes the protein.

[0059] In practical terms, there may be an upper limit to the number of antigens included in compositions of the invention. The number of antigens in a composition of the invention may be less than 20, less than 19, less than 18, less than 17, less than 16, less than 15, less than 14, less than 13, less than 12, less than 11, less than 10, less than 9, less than 8, less than 7, less than 6, less than 5, less than 4, or less than 3.

Pharmaceutical compositions and methods

[0060] Pharmaceutical composition comprise (a) a *N.meningitidis* serogroup X conjugate, and (b) a pharmaceutically acceptable carrier. Typical 'pharmaceutically acceptable carriers' include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, sucrose [119], trehalose [120], lactose, and lipid aggregates (such as oil droplets or liposomes). Such carriers are well known to those of ordinary skill in the art. The vaccines may also contain diluents, such as water, saline, glycerol, *etc.* Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present. Sterile pyrogen-free, phosphate-buffered physiologic saline is a typical carrier. A thorough discussion of pharmaceutically acceptable excipients is available in reference 121.

[0061] Compositions of the invention may be in an aqueous formulation (*i.e.* solutions or suspensions) or in a dried form (*e.g.* lyophilised). Aqueous formulations are preferred because the inventors have found that the serogroup X capsular polysaccharide is surprisingly stable in an aqueous environment. If a dried vaccine is used then it will be reconstituted into an aqueous formulation prior to injection. Lyophilisation of conjugate vaccines is known in the art *e.g.* the Menjugate™ product is presented in lyophilised form, whereas NeisVac-C™ and Meningitec™ are presented in aqueous form. To stabilise conjugates during lyophilisation, it may be typical to include a sugar alcohol (*e.g.* mannitol) or a disaccharide (*e.g.* sucrose or trehalose) *e.g.* at between 1mg/ml and 30mg/ml (*e.g.* about 25 mg/ml) in the composition. After reconstitution, these stabilisers may be present in the aqueous formulation.

[0062] Compositions may be presented in vials, or they may be presented in ready-filled syringes. The syringes may be supplied with or without needles. A syringe will include a single dose of the composition, whereas a vial may include a single dose or multiple doses.

[0063] Aqueous formulations of the invention are also suitable for reconstituting other vaccines from a lyophilised form. Where a composition of the invention is to be used for such extemporaneous reconstitution, the invention provides a kit, which may comprise two vials, or may comprise one ready-filled syringe and one vial, with the contents of the syringe being used to reactivate the contents of the vial prior to injection.

[0064] Compositions of the invention may be packaged in unit dose form or in multiple dose form. For multiple dose forms, vials are preferred to pre-filled syringes. Effective dosage volumes can be routinely established, but a typical human dose of the composition has a volume of 0.5ml *e.g.* for intramuscular injection.

[0065] The pH of the composition is typically between 6 and 8, *e.g.* about 7. Stable pH may be maintained by the use of a buffer. Typical buffers, *e.g.* for use in the aqueous formulations of the invention, are phosphate salts. For example, a mixture of anhydrous dibasic sodium phosphate and monobasic sodium phosphate is typical. A suitable concentration is 10 mM anhydrous dibasic sodium phosphate and 10 mM monobasic sodium phosphate. If a composition comprises an aluminium hydroxide salt, it is typical to use a histidine buffer [122]. The composition may be sterile and/or pyrogen-free. Compositions of the invention may be isotonic with respect to humans.

[0066] Compositions of the invention are immunogenic, and are more preferably vaccine compositions. Vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat infection), but will typically be prophylactic. Immunogenic compositions used as vaccines comprise an immunologically effective amount of antigen(s), as well as any other components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

[0067] Within each dose, the quantity of an individual saccharide antigen will generally be between 1-50 μg (measured as mass of saccharide) *e.g.* about 1μg, about 2.5μg, about 4μg, about 5μg, or about 10μg.

[0068] *N.meningitidis* affects various areas of the body and so the compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectables, either as aqueous solutions or suspen-

sions. The composition may be prepared for pulmonary administration *e.g.* as an inhaler, using a fine powder or a spray. The composition may be prepared as a suppository or pessary. The composition may be prepared for nasal, aural or ocular administration e.g. as spray, drops, gel or powder [*e.g.* refs 123 & 124]. Success with nasal administration of pneumococcal saccharides [125,126],Hib saccharides [127], MenC saccharides [128], and mixtures of Hib and MenC saccharide conjugates [129] has been reported.

[0069] Compositions of the invention may include an antimicrobial, particularly when packaged in multiple dose format.

[0070] Compositions of the invention may comprise detergent *e.g.* a Tween (polysorbate), such as Tween 80. Detergents are generally present at low levels *e.g.* <0.01%.

[0071] Compositions of the invention may include sodium salts (*e.g.* sodium chloride) to give tonicity. A concentration of 2-20mg/ml, e.g. about 10+2mg/ml or about 5±1mg/ml (particularly about 4.25mg) NaCl is typical.

[0072] Compositions of the invention will generally include a buffer. A phosphate buffer is typical.

[0073] Compositions of the invention will generally be administered in conjunction with other immunoregulatory agents. In particular, compositions will usually include one or more adjuvants. Such adjuvants include, but are not limited to:

A. Mineral-containing compositions

[0074] Mineral containing compositions suitable for use as adjuvants in the invention include mineral salts, such as aluminium salts and calcium salts. The invention includes mineral salts such as hydroxides (*e.g.* oxyhydroxides), phosphates (*e.g.* hydroxyphosphates, orthophosphates), sulphates, *etc.* [*e.g.* see chapters 8 & 9 of ref. 130], or mixtures of different mineral compounds (*e.g.* a mixture of a phosphate and a hydroxide adjuvant, optionally with an excess of the phosphate), with the compounds taking any suitable form (*e.g.* gel, crystalline, amorphous, *etc.*), and with adsorption to the salt(s) being typical. The mineral containing compositions may also be formulated as a particle of metal salt [131].

[0075] Aluminum salts may be included in vaccines of the invention such that the dose of Al^{3+} is between 0.2 and 1.0 mg per dose.

[0076] A typical aluminium phosphate adjuvant is amorphous aluminium hydroxyphosphate with PO_4/Al molar ratio between 0.84 and 0.92, included at 0.6mg Al^{3+}/ml. Adsorption with a low dose of aluminium phosphate may be used *e.g.* between 50 and 100μg Al^{3+} per conjugate per dose. Where an aluminium phosphate it used and it is desired not to adsorb an antigen to the adjuvant, this is favoured by including free phosphate ions in solution (*e.g.* by the use of a phosphate buffer).

B. Oil Emulsions

[0077] Oil emulsion compositions suitable for use as adjuvants in the invention include squalene-water emulsions, such as MF59 (5% Squalene, 0.5% Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer) [Chapter 10 of ref. 130; see also refs. 132-134]. MF59 is used as the adjuvant in the FLUAD™ influenza virus trivalent subunit vaccine.

[0078] Particularly useful adjuvants for use in the compositions are submicron oil-in-water emulsions. Preferred submicron oil-in-water emulsions for use herein are squalene/water emulsions optionally containing varying amounts of MTP-PE, such as a submicron oil-in-water emulsion containing 4-5% w/v squalene, 0.25-1.0% w/v Tween 80 (polyoxyelthylenesorbitan monooleate), and/or 0.25-1.0% Span 85 (sorbitan trioleate), and, optionally, N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphophoryloxy)-ethylamine (MTP-PE). Submicron oil-in-water emulsions, methods of making the same and immunostimulating agents, such as muramyl peptides, for use in the compositions, are described in detail in references 132 & 135-136.

[0079] Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) may also be used as adjuvants in the invention.

C. Saponin formulations [chapter 22 of ref. 130]

[0080] Saponin formulations may also be used as adjuvants in the invention. Saponins are a heterologous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponins isolated from the bark of the *Quillaia saponaria* Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from *Smilax ornata* (sarsaprilla), *Gypsophilla paniculata* (brides veil), and *Saponaria officianalis* (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs.

[0081] Saponin compositions have been purified using HPLC and RP-HPLC. Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in ref. 137. Saponin formulations may also comprise a sterol, such as cholesterol [138].

[0082] Combinations of saponins and cholesterols can be used to form unique particles called immunostimulating complexes (ISCOMs) [chapter 23 of ref. 130]. ISCOMs typically also include a phospholipid such as phosphatidyleth-anolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of QuilA, QHA and QHC. ISCOMs are further described in refs. 138-140. Optionally, the ISCOMS may be devoid of additional detergent(s) [141].

[0083] A review of the development of saponin based adjuvants can be found in refs. 142 & 143.

D. Virosomes and virus-like particles

[0084] Virosomes and virus-like particles (VLPs) can also be used as adjuvants in the invention. These structures generally contain one or more proteins from a virus optionally combined or formulated with a phospholipid. They are generally non-pathogenic, non-replicating and generally do not contain any of the native viral genome. The viral proteins may be recombinantly produced or isolated from whole viruses. These viral proteins suitable for use in virosomes or VLPs include proteins derived from influenza virus (such as HA or NA), Hepatitis B virus (such as core or capsid proteins), Hepatitis E virus, measles virus, Sindbis virus, Rotavirus, Foot-and-Mouth Disease virus, Retrovirus, Norwalk virus, human Papilloma virus, HIV, RNA-phages, Qβ-phage (such as coat proteins), GA-phage, fr-phage, AP205 phage, and Ty (such as retrotransposon Ty protein p1). VLPs are discussed further in refs. 144-149. Virosomes are discussed further in, for example, ref. 150

E. Bacterial or microbial derivatives

[0085] Adjuvants suitable for use in the invention include bacterial or microbial derivatives such as non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), Lipid A derivatives, immunostimulatory oligonucleotides and ADP-ribosylat-ing toxins and detoxified derivatives thereof.

[0086] Non-toxic derivatives of LPS include monophosphoryl lipid A (MPL) and 3-O-deacylated MPL (3dMPL). 3dMPL is a mixture of 3 de-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred "small particle" form of 3 De-O-acylated monophosphoryl lipid A is disclosed in ref. 151. Such "small particles" of 3dMPL are small enough to be sterile filtered through a 0.22μm membrane [151]. Other non-toxic LPS derivatives include monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives *e.g.* RC-529 [152,153].

[0087] Lipid A derivatives include derivatives of lipid A from *Escherichia coli* such as OM-174. OM-174 is described for example in refs. 154 & 155.

[0088] Immunostimulatory oligonucleotides suitable for use as adjuvants in the invention include nucleotide sequences containing a CpG motif (a dinucleotide sequence containing an unmethylated cytosine linked by a phosphate bond to a guanosine). Double-stranded RNAs and oligonucleotides containing palindromic or poly(dG) sequences have also been shown to be immunostimulatory.

[0089] The CpG's can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or single-stranded. References 156, 157 and 158 disclose possible analog substitutions *e.g.* replacement of guanosine with 2'-deoxy-7-deazaguanosine. The adjuvant effect of CpG oligonucleotides is further discussed in refs. 159-164.

[0090] The CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT [165]. The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN, or it may be more specific for inducing a B cell response, such a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. 166-168. Preferably, the CpG is a CpG-A ODN.

[0091] Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form "immunomers". See, for example, refs. 165 & 169-171.

[0092] Bacterial ADP-ribosylating toxins and detoxified derivatives thereof may be used as adjuvants in the invention. Preferably, the protein is derived from *E. coli* (*E. coli* heat labile enterotoxin "LT"), cholera ("CT"), or pertussis ("PT"). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 172 and as parenteral adjuvants in ref. 173. The toxin or toxoid is preferably in the form of a holotoxin, comprising both A and B subunits. Preferably, the A subunit contains a detoxifying mutation; preferably the B subunit is not mutated. Preferably, the adjuvant is a detoxified LT mutant such as LT-K63, LT-R72, and LT-G192. The use of ADP-ribosylating toxins and detoxified derivaties thereof, particularly LT-K63 and LT-R72, as adjuvants can be found in refs. 174-181. Numerical reference for amino acid sub-stitutions is preferably based on the alignments of the A and B subunits of ADP-ribosylating toxins set forth in ref. 182, specifically incorporated herein by reference in its entirety.

F. Human immunomodulators

[0093] Human immunomodulators suitable for use as adjuvants in the invention include cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 [183], *etc.*) [184], interferons (*e.g.* interferon-y), macrophage colony stimulating factor, and tumor necrosis factor.

G. Bioadhesives and Mucoadhesives

[0094] Bioadhesives and mucoadhesives may also be used as adjuvants in the invention. Suitable bioadhesives include esterified hyaluronic acid microspheres [185] or mucoadhesives such as cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrollidone, polysaccharides and carboxymethylcellulose. Chitosan and derivatives thereof may also be used as adjuvants in the invention [186].

H. Microparticles

[0095] Microparticles may also be used as adjuvants in the invention. Microparticles (*i.e.* a particle of ~100nm to ~150μm in diameter, more preferably ~200nm to ~30μm in diameter, and most preferably ~500nm to ~10μm in diameter) formed from materials that are biodegradable and non-toxic (*e.g.* a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, *etc.*), with poly(lactide-co-glycolide) are preferred, optionally treated to have a negatively-charged surface (*e.g.* with SDS) or a positively-charged surface (*e.g.* with a cationic detergent, such as CTAB).

I. Liposomes (Chapters 13 & 14 of ref. 130)

[0096] Examples of liposome formulations suitable for use as adjuvants are described in refs. 187-189.

J. Polyoxyethylene ether and polyoxyethylene ester formulations

[0097] Adjuvants suitable for use in the invention include polyoxyethylene ethers and polyoxyethylene esters [190]. Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol [191] as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol [192]. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-steoryl ether, polyoxytheylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

K. Polyphosphazene (PCPP)

[0098] PCPP formulations are described, for example, in refs. 193 and 194.

L. Muramyl peptides

[0099] Examples of muramyl peptides suitable for use as adjuvants in the invention include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), and N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE).

M. Imidazoquinolone Compounds.

[0100] Examples of imidazoquinolone compounds suitable for use adjuvants in the invention include Imiquamod and its homologues (*e,g.* "Resiquimod 3M"), described further in refs. 195 and 196.

N. Thiosemicarbazone Compounds.

[0101] Examples of thiosemicarbazone compounds, as well as methods of formulating, manufacturing, and screening for compounds all suitable for use as adjuvants in the invention include those described in ref. 197. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.

O. Tryptanthrin Compounds.

[0102] Examples of tryptanthrin compounds, as well as methods of formulating, manufacturing, and screening for compounds all suitable for use as adjuvants in the invention include those described in ref. 198. The tryptanthrin compounds are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.

[0103] The immunogenic compositions may also comprise combinations of one or more of the adjuvants identified above. For example, the following combinations may be used as adjuvant compositions in the invention: (1) a saponin and an oil-in-water emulsion [199]; (2) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) [200]; (3) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) + a cholesterol; (4) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) [201]; (5) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions [202]; (6) SAF, containing 10% squalane, 0.4% Tween 80™, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion. (7) Ribi™ adjuvant system (RAS), (Ribi Immunochem) containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); and (8) one or more mineral salts (such as an aluminium salt) + a non-toxic derivative of LPS (such as 3dMPL).

[0104] Other substances that act as immunostimulating agents are disclosed in chapter 7 of ref. 130.

[0105] The use of aluminium salt adjuvants is particularly useful, and antigens are generally adsorbed to such salts. The Menjugate™ and NeisVac™ conjugates use a hydroxide adjuvant, whereas Meningitec™ uses a phosphate adjuvant. It is possible in compositions of the invention to adsorb some antigens to an aluminium hydroxide but to have other antigens in association with an aluminium phosphate. Typically, however, only a single salt is used, *e.g.* a hydroxide or a phosphate, but not both. Not all conjugates need to be adsorbed *i.e.* some or all can be free in solution.

Methods of treatment

[0106] The invention discloses a method for raising an immune response in a mammal, comprising administering a pharmaceutical composition of the invention to the mammal. The immune response is preferably protective and preferably involves antibodies. The method may raise a booster response.

[0107] The mammal is preferably a human. Where the vaccine is for prophylactic use, the human is preferably a child (*e.g.* a toddler or infant) or a teenager; where the vaccine is for therapeutic use, the human is preferably an adult. A vaccine intended for children may also be administered to adults e.g. to assess safety, dosage, immunogenicity, *etc.*

[0108] The invention also provides an immunogenic composition of the invention for use as a medicament. The medicament is preferably able to raise an immune response in a mammal (*i.e.* it is an immunogenic composition) and is more preferably a vaccine.

[0109] Preferred compositions of the invention can confer an antibody titre in a patient that is superior to the criterion for seroprotection for each antigenic component for an acceptable percentage of human subjects. Antigens with an associated antibody titre above which a host is considered to be seroconverted against the antigen are well known, and such titres are published by organisations such as WHO. Preferably more than 80% of a statistically significant sample of subjects is seroconverted, more preferably more than 90%, still more preferably more than 93% and most preferably 96-100%.

[0110] Compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (*e.g.* subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or by rectal, oral, vaginal, topical, transdermal, intranasal, ocular, aural, pulmonary or other mucosal administration. Intramuscular administration to the thigh or the upper arm is preferred. Injection may be via a needle (*e.g.* a hypodermic needle), but needle-free injection may alternatively be used. A typical intramuscular dose is 0.5 ml.

[0111] The invention may be used to elicit systemic and/or mucosal immunity.

[0112] Dosage treatment can be a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. A primary dose schedule may be followed by a booster dose schedule. Suitable timing between priming doses (*e.g.* between 4-16 weeks), and between priming and boosting, can be routinely determined.

Exemplary carrier proteins

[0113] As discussed above, the inventors have found that the carrier proteins described in ref. 65 and ref. 66 are particularly suitable for use as a carrier molecule in the invention, especially the protein of SEQ ID NO: 9 in those documents (which is also SEQ ID NO: 9 herein).

[0114] These carrier molecules comprise a spr0096 antigen and a spr2021 antigen. Typically, the carrier molecule comprises the spr0096 antigen and the spr2021 antigen as a single polypeptide chain (a "hybrid" polypeptide). The spr0096 antigen, spr2021 antigen and the nature of the hybrid polypeptide are described in more detail below.

spr0096 antigen

[0115] The original 'spr0096' polypeptide sequence was annotated in reference 203 as 'hypothetical protein' (see GI: 15902140). For reference purposes, the amino acid sequence of full length spr0096 as found in the R6 strain is given as SEQ ID NO: 1 herein.

[0116] The spr0096 antigen of the invention comprises at least one CD4+ T cell epitope. CD4+ T cells help B lymphocytes to produce antibodies against antigens [204]. T-cell epitopes can be identified empirically (*e.g.* using PEPSCAN [205,206] or similar methods), or they can be predicted (*e.g.* using the Jameson-Wolf antigenic index [207], matrix-based approaches [208], TEPITOPE [209], neural networks [210], OptiMer & EpiMer [211,212], ADEPT [213], Tsites [214], hydrophilicity [215], antigenic index [216] or the methods disclosed in reference 217, *etc.*).

[0117] Preferred spr0096 antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 1; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 1, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These spr0096 polypeptides include variants of SEQ ID NO: 1 (*e.g.* SEQ ID NO: 2; see below). Preferred fragments of (b) comprise at least one CD4+ T cell epitope from SEQ ID NO: 1. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 1 while retaining at least one CD4+ T cell epitope of SEQ ID NO: 1. Other fragments omit one or more protein domains. One suitable fragment is SEQ ID NO: 14, which omits the natural leader peptide sequence. The spr0096 antigen may consist of a single CD4+ T cell epitope from SEQ ID NO: 1.

[0118] A variant form of spr0096, with an insert near its C-terminus relative to SEQ ID NO: 1, is SEQ ID NO: 2 herein. The use of this variant for immunisation is reported in reference 218 (SEQ ID NO: 150 therein), where it is annotated as a LysM domain protein. Thus a spr0096 antigen for use with the invention may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 2; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 2, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These polypeptides include variants of SEQ ID NO: 2. Preferred fragments of (b) comprise at least one CD4+ T cell epitope from SEQ ID NO: 2. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 2 while retaining at least one CD4+ T cell epitope of SEQ ID NO: 2. Other fragments omit one or more protein domains. One suitable fragment is SEQ ID NO: 15, which omits the natural leader peptide sequence. Immunogenic fragments of SEQ ID NO: 2 are identified in table 1 of reference 218. The spr0096 antigen may consist of a single CD4+ T cell epitope from SEQ ID NO: 2.

[0119] A spr0096 antigen may be used in the form of a dimer *e.g.* a homodimer.

spr2021 antigen

[0120] The original 'spr2021' polypeptide sequence was annotated in reference 203 as 'General stress protein GSP-781' (see GI: 15904062). For reference purposes, the amino acid sequence of full length spr2021 as found in the R6 strain is given as SEQ ID NO: 3 herein.

[0121] The spr2021 antigen of the invention comprises at least one CD4+ T cell epitope.

[0122] Preferred spr2021 antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 3; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 3, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These spr2021 polypeptides include variants of SEQ ID NO: 3. Preferred fragments of (b) comprise at least one CD4+ T cell epitope from SEQ ID NO: 3. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 3 while retaining at least one CD4+ T cell epitope of SEQ ID NO: 3. Other fragments omit one or more protein domains. One suitable fragment is SEQ ID NO: 4, which omits the natural leader peptide sequence. The spr0096 antigen may consist of a single CD4+ T cell epitope from SEQ ID NO: 3.

[0123] Reference 218 annotates spr2021 as a secreted 45kDa protein with homology to GbpB and discloses its use as an immunogen (SEQ ID NO: 243 therein; SP2216). Immunogenic fragments of spr2021 are identified in table 1 of

reference 218 (page 73). Another useful fragment of spr2021 is disclosed as SEQ ID NO: 1 of reference 219 (amino acids 28-278 of SEQ ID NO: 3 herein).

Hybrid polypeptide

[0124] Typically, the spr0096 antigen and spr2021 antigen are expressed as a single polypeptide chain (a 'hybrid' polypeptide). Hybrid polypeptides can be represented by the formula $NH_2-A-\{-X-L-\}_n-B-COOH$, wherein: A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; n is an integer of 2 or more (*e.g.* 2, 3, 4, 5, 6, *etc.*); each X is an amino acid sequence of an spr0096 antigen or an spr2021 antigen (as described above), wherein at least one X is an spr0096 antigen and at least one X is an spr2021 antigen; and L is an optional linker amino acid sequence. Usually n is 2. When n is 2, X_1 is usually an spr0096 antigen and X_2 is usually an spr2021 antigen. When n is more than 2, each spr0096 antigen (when more than one is present) may be the same or different and each spr2021 antigen (when more than one is present) may be the same or different.

[0125] The spr0096 antigen or spr2021 antigen that is the amino acid sequence of each X is as defined above. Where these antigens are defined in terms of (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to a given sequence; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of a given sequence, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more), the level of identity in (a) and the value of 'n' in (b) may be the same for each X.

[0126] The leader peptide sequence in the wild-type form of each -X- moiety may be included or omitted in the hybrid protein. In some embodiments, the leader peptides will be deleted except for that of the -X- moiety located at the N-terminus of the hybrid protein i.e. the leader peptide of X_1 will be retained, but the leader peptides of $X_2 \ldots X_n$ will be omitted. This is equivalent to deleting all leader peptides and using the leader peptide of X_1 as moiety -A-.

[0127] For each n instances of {-X-L-}, linker amino acid sequence -L- may be present or absent. For instance, when n=2 the hybrid may be $NH_2-X_1-L_1-X_2-L_2-COOH$, $NH_2-X_1-X_2-COOH$, $NH_2-X_1-L_1-X_2-COOH$, $NH_2-X_1-X_2-L_2-COOH$, *etc.* Linker amino acid sequence(s) -L- will typically be short *(e.g.* 20 or fewer amino acids *i.e.* 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples comprise short peptide sequences which facilitate cloning, poly-glycine linkers (i.e. comprising Gly_n where n = 2, 3, 4, 5, 6, 7, 8, 9, 10 or more), and histidine tags (*i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable linker amino acid sequences will be apparent to those skilled in the art. A useful linker is GSGGGG (SEQ ID NO:5) or GSGSGGGG (SEQ ID NO:6), with the Gly-Ser dipeptide being formed from a *Bam*HI restriction site, thus aiding cloning and manipulation, and the $(Gly)_4$ tetrapeptide being a typical poly-glycine linker. Other suitable linkers, particularly for use as the final L_n are a Leu-Glu dipeptide or SEQ ID NO: 7.

[0128] -A- is an optional N-terminal amino acid sequence. This will typically be short *(e.g.* 40 or fewer amino acids *i.e.* 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include leader sequences to direct protein trafficking, or short peptide sequences which facilitate cloning or purification (*e.g.* histidine tags *i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable N-terminal amino acid sequences will be apparent to those skilled in the art. If X_1 lacks its own N-terminus methionine, -A-is preferably an oligopeptide (*e.g.* with 1, 2, 3, 4, 5, 6, 7 or 8 amino acids) which provides a N-terminus methionine *e.g.* Met-Ala-Ser, or a single Met residue.

[0129] -B- is an optional C-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include sequences to direct protein trafficking, short peptide sequences which facilitate cloning or purification (*e.g.* comprising histidine tags *i.e.* His_n where n = 3, 4, 5, 6, 7, 8, 9, 10 or more, such as SEQ ID NO: 8), or sequences which enhance protein stability. Other suitable C-terminal amino acid sequences will be apparent to those skilled in the art.

[0130] Examples of hybrids include polypeptides that comprise an amino acid sequence of spr0096-spr2021 (e.g. SEQ ID NO: 9) or spr2021-spr0096 (e.g. SEQ ID NO: 10). The hybrid may also comprise an amino acid sequence having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 9 or 10. Typically, the hybrid comprises an amino acid sequence of SEQ ID NO: 9. The hybrid may also comprise an amino acid sequence having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 9.

[0131] In particular embodiments, the carrier molecule comprises (a) one or more (*e.g.* 1, 2, 3, 4, 5, *etc.*) CD4$^+$ T cell epitopes from SEQ ID NO: 2; and (b) one or more (*e.g.* 1, 2, 3, 4, 5, *etc.*) CD4$^+$ T cell epitopes from SEQ ID NO: 3.

Method for assaying a sample

[0132] A method for assaying a sample suspected to contain serogroup X capsular polysaccharide is disclosed, comprising the steps of: (i) hydrolysing any serogroup X capsular polysaccharide in the sample, to give a hydrolysate;

(ii) subjecting the hydrolysate to liquid chromatography; and (iii) detecting any glucosamine-4-phosphate separated in step (ii).

[0133] The method can be used for quantifying serogroup X capsular polysaccharide in the sample. In this way, it is possible to determine the concentration of the polysaccharide in the sample. Typically, the quantification involves comparison with a N-acetylglucosamine-4-phosphate standard. However, other standards may be used, including glucosamine-6-phosphate.

[0134] Although the method has been developed for serogroup X capsular polysaccharide, it is suitable for any substance with glucosamine-4-phosphate in its structure, *e.g.* bacterial lipid A. Accordingly, also disclosed is a method for assaying a sample suspected to contain a substance with glucosamine-4-phosphate in its structure, comprising the steps of: (i) hydrolysing any substance with glucosamine-4-phosphate in its structure in the sample, to give a hydrolysate; (ii) subjecting the hydrolysate to liquid chromatography; and (iii) detecting any glucosamine-4-phosphate separated in step (ii).

Sample

[0135] The sample is typically a vaccine, *e.g.* when the method is used for polysaccharide quantification in the characterization of a vaccine product. However, the method can also be used as an in-process assay during vaccine manufacture. In these embodiments, the sample will be a process intermediate from the manufacturing process. The method is capable of quantifying very low concentrations of serogroup X capsular polysaccharide (≥ 0.5 μg/ml) and is therefore suitable for assaying serogroup X capsular polysaccharide in small samples, *e.g.* taken in-process during a manufacturing process. The method is also specific for serogroup X capsular polysaccharide, even when impurities are present. The sample may therefore be a fermentation broth, or a supernatant taken from a fermentation broth.

[0136] The sample may contain free (unconjugated) serogroup X capsular polysaccharide and/or conjugated serogroup X capsular polysaccharide. Thus the method may be used to assay polysaccharide prepared from a bacterium, polysaccharide after purification, polysaccharide prior to conjugation, and/or polysaccharide after conjugation.

[0137] In a sample containing conjugated serogroup X capsular polysaccharide, a comparison of levels of free polysaccharide to the total polysaccharide in a sample (*i.e.* the ratio of unconjugated polysaccharide : (unconjugated + conjugated) polysaccharide) can be used to determine stability. High levels of unconjugated polysaccharide are undesirable. A time-series of such assays can reveal if a conjugate is stable e.g. during storage. The level of free polysaccharide can also be used to check if a conjugation reaction has gone to completion.

[0138] The sample will typically be aqueous, but may have been reconstituted into aqueous form from a dried form e.g. from a lyophilisate. Thus the sample may contain lyophilisation stabilizers. These stabilizers include substances such as sugar alcohols (*e.g.* mannitol, *etc.*), disaccharides (*e.g.* sucrose, trehalose, *etc.*), and other simple saccharides. An advantage of the methods is that they can assay serogroup X capsular polysaccharide against a background of impurities, without requiring any pre-separation of the polysaccharide and the impurities.

[0139] The sample may be diluted prior to analysis. After analysis, the level of polysaccharide in the sample can then be related to the level in the original undiluted material. Dilution is useful, for example, to ensure that analysis of a sample gives a result within a desired portion of a calibration curve.

[0140] In addition to serogroup X capsular polysaccharide, the sample may contain other bacterial capsular saccharides *e.g.* from *Haemophilus influenzae* type B, from other meningococcal serogroups (*e.g.* A, C, W135 and/or Y), from *Streptococcus pneumoniae, etc.*

[0141] Samples may also include other components, such as non-antigen components often found in vaccines. For example, these may include carriers, adjuvants, excipients, buffers, *etc.,* as described above.

[0142] In some situations, it is useful to spike the sample with a known amount of the analyte in question *e.g.* to add a known quantity of serogroup X capsular polysaccharide, either in conjugated or unconjugated form. Spiking studies can be useful for calibration, and for studying sensitivity, variability, recovery, *etc.*

Hydrolysis

[0143] The method involves hydrolysis of the serogroup X capsular polysaccharide. Typical hydrolysis methods involve acid hydrolysis e.g. using trifluoroacetic acid (TFA). The inventors have found that particularly effective conditions are treatment with 2M TFA for between 2 and 3 (*e.g.* 2.5) hours at 100°C. These conditions allow good release of the polysaccharide's monomer subunits, without their degradation. However, shorter or longer treatments, *e.g.* for between 1 and 6 hours, are also possible.

[0144] Total serogroup X capsular polysaccharide can be prepared from a sample including conjugated polysaccharide by subjecting the whole sample to hydrolysis, as described above. If measurement of only conjugated or unconjugated serogroup X capsular polysaccharide is desired, however, then conjugated and unconjugated polysaccharide should be separated from each other prior to hydrolysis. Suitable separation techniques include selective precipitation, size-

based methods, solid-phase extraction [220], *etc.*

Liquid chromatography

[0145] The results of serogroup X capsular polysaccharide hydrolysis are analysed by liquid chromatography. Thus the methods will typically utilize a liquid chromatography column, and will involve analysing the output of such a column.
[0146] Various liquid chromatography columns can be used, but the method will typically be used with high performance liquid chromatography (HPLC). The method is particularly useful for analysing the results of separation by high performance anion exchange chromatography (HPAEC) or by high performance cation exchange chromatography (HPCEC). HPAEC is a common technique used for saccharide characterisation, often in combination with pulsed amperometric detection (PAD) [221,222] to detect and quantify the polysaccharide. Suitable HPAEC-PAD systems are provided by Dionex™ Corporation (Sunnyvale, CA) *e.g.* the BioLC™ system. In these systems, the eluate from a HPAEC column is analysed using PAD *i.e.* based on electrical current. At suitable (high) pH, carbohydrates can be electrocatalytically oxidised at the surface of electrodes by applying a positive potential. The current generated is this way is proportional to the carbohydrate concentration, allowing detection and quantification of the carbohydrate by amperometry. Compared with simple amperometric detection, PAD intersperses short pulses of a cleaning and regeneration potential with the standard detecting potential, thereby avoiding difficulties that arise when oxidation products of analytes foul the electrodes.
[0147] Non-amperometric methods can be combined with PAD for analyzing eluates *e.g.* see ref. 223.
[0148] Thus the hydrolysed serogroup X capsular polysaccharide can be subjected to HPAEC for separation and the separated materials can be detected and, optionally, quantified by PAD. As shown in the examples below, HPAEC-PAD can separate hydrolysed glucosamine-4-phosphate residues from other background materials in a sample.
[0149] Preferred columns are those that spontaneously retain saccharides such that they have to be eluted from the column. Elution from the chromatography column can be an isocratic elution or a gradient elution. Eluents including hydroxide and/or acetate salts are typical eluents used during HPAEC-PAD analysis of saccharides. It is also possible, however, to use anions such as nitrate, chloride, *etc.* Sodium salts are typically used. For eluting analytes from AEC columns then the eluent will generally be basic *e.g.* the pH will be >8, >9, >10, >11, >12, >13, *etc.* Hydroxide salts (*e.g.* NaOH) can be used to achieve the desired pH.
[0150] Eluates may be subjected to chemical suppression of hydroxide ions, particularly where the ions interfere with an analytical detection technique that is being used. A micromembrane suppressor can conveniently be used, such as the MMS products from Dionex™. The 'MMS III' product uses continuous chemical suppression to enhance analyte conductivities while decreasing eluent conductivity, and enables direct conductivity detection with ion-exchange applications using isocratic or gradient elution over wide concentration ranges.
[0151] Suitable HPAEC columns for use with the methods are the "CarboPac" columns marketed by Dionex, such as the PA1 [10 μm diameter polystyrene substrate 2% crosslinked with divinylbenzene, agglomerated with 500 nm MicroBead quaternary ammonium functionalized latex (5% crosslinked)], PA100, PA20, PA10 [10 μm diameter ethylvinylbenzene substrate 55% crosslinked with divinylbenzene, agglomerated with 460 nm MicroBead difunctional quaternary ammonium ion (5% crosslinked)], PA200 or MA1 columns.
[0152] Analytical HPAEC columns can be used in conjunction with pre-columns and/or trap columns. For instance, a PA10 analytical column can be used in conjunction with an inline PA10 guard column, and/or an inline trap (pre-treatment) column. Such columns can remove materials that would otherwise interfere with analyses *e.g.* an "AminoTrap" column can remove amino acids prior to saccharide analysis. Borate traps can also be used. TYPICAL "AminoTrap" resin has a 10 μm diameter substrate (ethylvinylbenzene 55% crosslinked with divinylbenzene) grafted with difunctional quaternary ammonium anion exchange sites, whereas a typical "BorateTrap "has a 20 μm diameter high capacity resin with very high selectivity for borate.
[0153] The PA1 and PA10 columns are both anion-exchange columns designed to be used with PAD to deliver high resolution separations of mono- and disaccharides, and the resins in both are 10 μm diameter nonporous beads covered with a fine latex of functionalized MicroBeads. Their pellicular resin structure permits excellent mass transfer, resulting in high resolution chromatography and rapid re-equilibration. Whereas PA1 is an all-purpose column suitable for determining monosaccharides and disaccharides in a variety of matrices, and is the column of choice for high resolution separations of linear polysaccharides, PA10 is optimized to determine the amino, neutral, and acidic monosaccharides that are found in the carbohydrate moieties of mammalian glycoproteins. The main difference between the PA1 and PA10 columns is that the resin in PA1 is polystyrene 2% crosslinked with divinylbenzene, but in PA10 it is ethylvinylbenzene 55% crosslinked with divinylbenzene.
[0154] To date, the most preferred HPAEC separation method for serogroup X capsular polysaccharide involves a CarboPac PA1 column (4x250mm) combined with a Guard PA1 pre-column (4x50mm).
**[0155]** After elution and detection, the method may include the further step of determining a characteristic of any serogroup X capsular polysaccharide that was identified in the sample *e.g.* its DP (typically an average DP), its molecular

weight, its purity, *etc.*

### *Preparation of N-acetylglucosamine-4-phosphate*

[0156] Processes and reagents useful for preparing *N*-acetylglucosamine-4-phosphate are disclosed. As discussed above, this compound can be used as an analytical standard in the method for assaying serogroup X capsular polysaccharide.

[0157] The process may comprise *N*-deprotecting a compound of formula A and *N*-acylating the deprotected compound to give a compound of formula B;
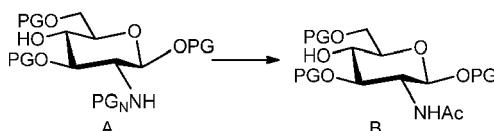

wherein PG is an oxygen protecting group; PG_N is a nitrogen protecting group and all the PG groups are the same. PG and PG_N may be any suitable protecting group, for example as described in reference 224.

[0158] The process may comprise introducing an organophosphate group into a compound of formula B to give a compound of formula C:
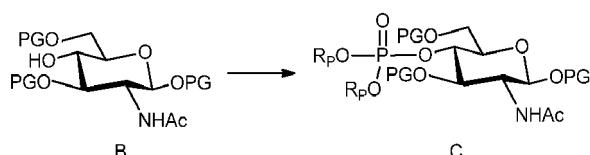

wherein both R_P groups are the same and R_P is either H or an arylmethyl phosphate protecting group, for example as described in reference 224; or the R_P groups are joined together to form a single arylmethyl protecting group, for example *o*-xylenyl. PG is as defined above and all the PG groups are the same. Where R_P is H, the compound of formula B is typically reacted with a phosphorylating reagent and an oxidising agent. The phosphorylating agent may be salicyl chlorophosphite, typically in the presence of pyridine and pivaloyl chloride. The phosphorylating agent may also be PCl_3 followed by water or aqueous $NaHCO_3$. The oxidising agent may be I_2 or *m*CPBA, typically I_2. Where the R_P groups are joined together to form a single protecting group, the compound of formula B is typically reacted with an *o*-xylene containing organophosphorus reagent and then an oxidising agent. The o-xylene containing organophosphorus reagent is typically a phosphoramidite such as *N*-diethyl-1,5-dihydro-3*H*-2,3,4-benzodioaxaphosphine-3-amine. The oxidising agent may be I_2 or *m*CPBA, typically *m*CPBA. Where the R_P groups are not joined together to form a single protecting group, the compound of formula B is typically reacted with a pyrophosphate reagent containing the R_P groups. A suitable pyrophosphate reagent is tetrabenzylpyrophospate, in which case R_P is a benzyl group.

[0159] The process may comprise deprotecting a compound of formula C to give *N*-acetylglucosamine-4-phosphate:
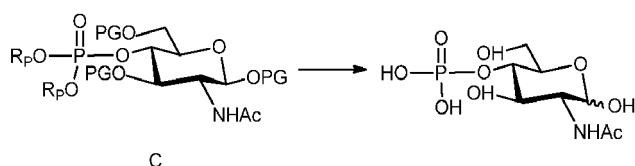

[0160] PG and R_P are as defined above and all PG groups are the same. Typically, when R_p is a phosphate protecting group, all the PG groups and the R_P group are removed in the same step, for example by hydrogenolysis.

[0161] A process for making a compound of formula A above, may comprise reducing a compound of formula Z to give a compound of formula A':
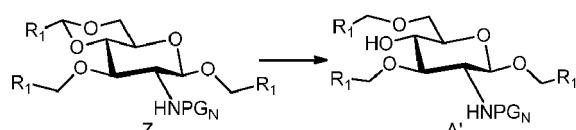
wherein Ri is phenyl or phenyl substituted with one or more groups selected from alkyl, such as methyl or ethyl; O-alkyl, such as O-methyl; and nitro, such that the R_1CH_2- moiety in formula A' is a substituted or unsubstituted benzyl ether oxygen protecting group. The protecting group may for example be any suitable protecting group described in reference 224. In particular, the protecting group may be benzyl, p-methoxybenzyl, 3,4-dimethoxybenzyl, 2,6-dimethoxybenzyl, o-nitrobenzyl, p-nitrobenzyl, and is typically benzyl. PG_N is a nitrogen protecting group as defined above. Typical PG_N groups are phthalimide; carbamates such as Boc and Fmoc; nosyl, tosyl and mesyl. Particularly, PG_N is phthalimide. Typically, the reaction comprises reacting a compound of formula Z, wherein Ri is phenyl and PG_N is a pthalimide group, with a boron containing compound and a Lewis acid in an organic solvent to give a compound of formula A'. The boron containing compound may be a borohydirde reagent such as trialkylaminoborane, particularly triethylaminoborane or trimethylaminoborane, and is typically trimethylaminoborane. The Lewis acid may be $AlCl_3$ or a boron containing Lewis acid, such as a boron trifluoride complex, and is typically $BF_3{\cdot}Et_2O$. The organic solvent may be a chlorinated organic solvent, for example $CHCl_3$ or CH_2Cl_2 or a non-chlorinated solvent, and is typically acetonitrile. The reaction may be carried out in a temperature range of from about -10 to about 10°C, from about -5 to about 5°C, typically at about 0°C.

[0162] An example of the process comprises step i) *N*-deprotecting a compound of formula A' and step ii) *N*-acylating the deprotected compound to give a compound of formula B':
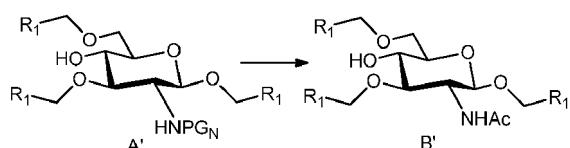

wherein Ri and PG_N are as defined above. Typically, the reaction comprises reacting a compound of formula A' wherein PG_N is phthalimide in step i) with 1,2-diaminoethane in an alcoholic solvent such as MeOH or EtOH, typically EtOH. Step i) is usually carried out between room temperature and the reflux point of the solvent, for example at a temperature of about 25°C or more, from about 25 to about 80°C, from about 35 to about 80°C, from about 45 to about 80°C, from about 55 to about 80°C, from about 65 to about 80°C, from about 75 to about 80°C typically about 80°C or about the refluxing point of the solvent. Step ii) includes adding an acylating agent. The acylating agent may be any suitable acylating agent, typically Ac_2O with an amine base such as pyridine or triethylamine or imidazole. Alternatively the acylating agent may be about a 4:1 mixture of $EtOH:Ac_2O$. Step ii) may be carried out at about room temperature, for example at about 25°C. Typically there is no purification step between steps i) and ii), for example only solvent from step i) is removed before step ii) performed.

[0163] An example of the process comprises introducing an organophosphate group into a compound of formula B' to give a compound of formula C':
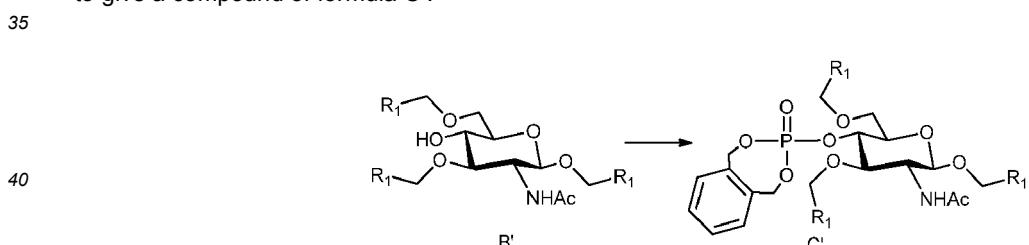
wherein Ri is as defined above. Typically, the compound of formula B' is first reacted with an *o*-xylene containing organophosphorus reagent, *e.g.* a phosphoramidite such as N-diethyl-1,5-dihydro-3*H*-2,3,4-benzodloaxaphosphine-3-amine, in the presence of an amine base, typically 1*H*-tetrazole, in an organic solvent such as THF or CH_2Cl_2, typically CH_2Cl_2. An oxidising agent, such as I_2 in pyridine and water or *m*CPBA, is then added. The *m*CPBA is usually used at a temperature of from about -20°C to about 20°C, from about -15°C to about 15°C, from about -10°C to about 10°C, from about -5°C to about 5°C, and typically about 0°C. The *m*CPBA is usually added to the same reaction solvent as was used in the first step. The completion of the first step may be detected, for example by TLC analysis, before the *m*CPBA is added.

[0164] A further example of the process comprises introducing an organophosphate group into a compound of formula B' to give a compound of formula C":
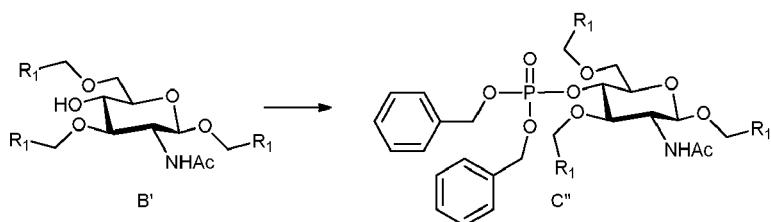
wherein Ri is as defined above. Typically, the compound of formula B' is reacted with a pyrophosphate reagent, *e.g.* tetrabenzylpyrophosphate. The reaction is carried out in the presence of a base, suitable bases are lithium amides such as LDA or LiHMDS. The reaction is carried out in an organic solvent, for example a chlorinated solvent such as CH_2Cl_2 or an ether solvent, typically diethyl ether or THF. Typically, the reaction is carried out below about 0°C, for example about -10°C, about -20°C, about -30°C, about -40°C, about -50°C, about -60°C, about -70°C, suitably about -80°C then about -30°C.

[0165] A further example of the process comprises introducing an organophosphate group into a compound of formula B' to give a compound of formula C''':
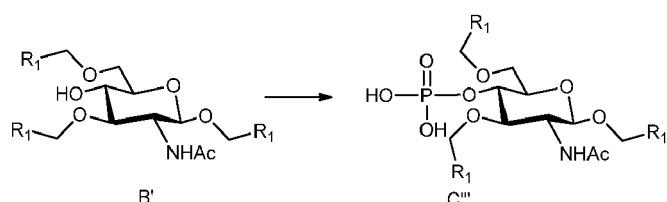

wherein Ri is as defined above. Typically, the compound of formula B' is phosphorylated with a suitable phosphorylating reagent and oxidising agent. Typical phosphorylating reagents are salicyl chlorophosphite or PCl_3 and water or aqueous $NaHCO_3$. When the phosphorylating agent is salicyl chlorophosphite, the reaction is typically carried out in a pyridine solvent and pivaloyl chloride is typically present. The reaction is usually carried out at room temperature, for example about 25°C. An oxidising agent such as I_2 in pyridine and water, or *m*CPBA is then added, typically I_2. The reaction is typically cooled to below about 0°C before addition of the oxidant, for example to about -10°C, about -20°C, about -30°C, about -40°C, about -50°C, typically about -40°C. Oxidation is typically completed at a temperature of about 0°C. The I_2 is typically added as a solution in pyridine/water. The concentration of the solution is typically about 0.1 to 1 M, about 0.2 to 0.9 M, about 0.3 to 0.8 M, about 0.4 to 0.7 M, suitably about 0.5M. The ratio of pyridine to water is typically about 10:1 to 30:1, about 12:1 to about 28:1, about 14:1 to about 26:1 about 16:1 to about 24:1, about 18:1 to about 22:1, suitably about 19:1. The oxidant is usually added to the same reaction solvent as was used in the first step. The completion of the first step may be detected, for example by TLC analysis, before the oxidant is added. The compound of formula C''' is usually isolated as a salt, typically the di-triethylammonium salt.

[0166] When the phosphorylating agent is PCl_3 and water or aqueous $NaHCO_3$, the reaction is typically carried out in an organic solvent such as MeCN. Water or aqueous $NaHCO_3$ is added after PCl_3, suitably at room temperature. An oxidising agent such as I_2 or *m*CPBA is then added, typically I_2. Usually, the solvents are removed before the addition of the oxidant and a new solvent is added. The new solvent is typically a mixture of pyridine and triethylamine. The pyridine to triethylamine ratio is typically about 2:1 to about 8:1, suitably about 4:1. I_2 is typically added as a solution in pyridine water. The concentration of the solution is typically about 0.1 to 1 M, about 0.2 to 0.9M, about 0.3 to 0.8 M, suitably about 0.4M. The compound of formula C''' is usually isolated as a salt, typically the di-triethylammonium salt.

[0167] An example of the process comprises deprotecting, typically by hydrogenolysis, a compound of formula C' to give *N*-acetylglucosamine-4-phosphate:

wherein Ri is as defined above. Typically, the compound of formula C' is reacted with H_2 in the presence of a palladium catalyst such as 10% Pd/C or Pearlman's catalyst $PdOH_2$/C, in an alcoholic solvent, for example MeOH or EtOH, and

typically MeOH. The reaction is usually carried out at a pressure of from about 1 to about 5 atm, from about 1 to about 4 atm, from about 1 to about 3 atm, from about to about 2 atm, typically about 1 atm.

[0168] A further example of the process comprises deprotecting, typically by hydrogenolysis, a compound of formula C" to give *N*-acetylglucosamine-4-phosphate:

wherein Ri is as defined above. Typically, the compound of formula C" is reacted with H_2 in the presence of a palladium catalyst such as 10% Pd/C or Pearlman's catalyst $Pd(OH)_2$/C, in an alcoholic solvent, for example MeOH or EtOH, and typically MeOH. The reaction is usually carried out at a pressure of from about 1 to about 5 atm, from about 1 to about 4 atm, from about 1 to about 3 atm, from about to about 2 atm, typically about 1 atm.

[0169] A further example of the process comprises deprotecting, typically by hydrogenolysis, a compound of formula C''', or the di-triethylammonium salt thereof, to give *N*-acetylglucosamine-4-phosphate:
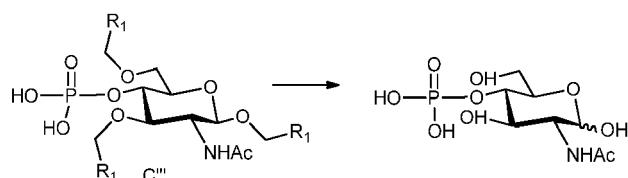
wherein Ri is as defined above. Typically, the compound of formula C''' is reacted with H_2 in the presence of a palladium catalyst such as 10% Pd/C or Pearlman's catalyst $Pd(OH)_2$/C, in an alcoholic solvent, for example MeOH or EtOH, and typically MeOH. The reaction is usually carried out at a pressure of from about 1 to about 5 atm, from about 1 to about 4 atm, from about 1 to about 3 atm, from about to about 2 atm, typically about 1 atm.

[0170] The *N*-acetylglucosamine-4-phosphate may be purified, for example by crystallisation, or more typically chromatography, particularly chromotography using a hydrophobically modified silica stationary phase.

[0171] The process may be as follows:
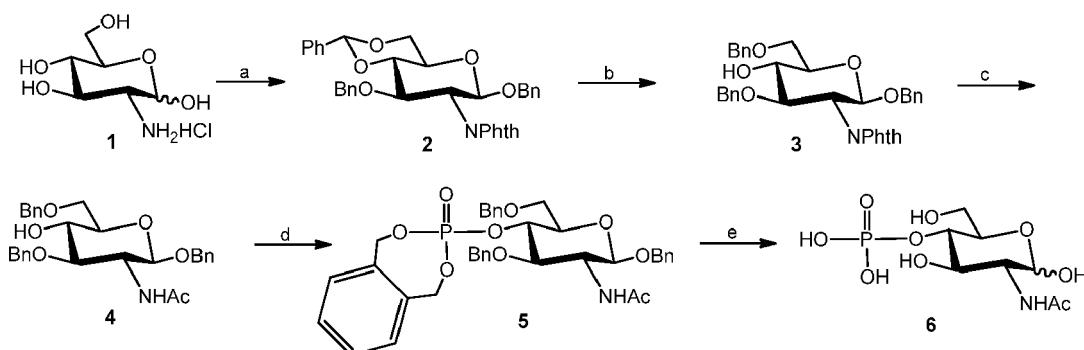
[0172] Suitable reaction conditions for this process are provided in the section *"Modes for Carrying out the Invention"* below *(e.g.* in Scheme 1).

General

[0173] The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.,* references 225-232, *etc.*

[0174] "GI" numbering is used above. A GI number, or "GenInfo Identifier", is a series of digits assigned consecutively

to each sequence record processed by NCBI when sequences are added to its databases. The GI number bears no resemblance to the accession number of the sequence record. When a sequence is updated (*e.g.* for correction, or to add more annotation or information) then it receives a new GI number. Thus the sequence associated with a given GI number is never changed.

[0175] Where an antigen "domain" is omitted, this may involve omission of a signal peptide, of a cytoplasmic domain, of a transmembrane domain, of an extracellular domain, *etc.*

[0176] The term "comprising" encompasses "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

[0177] The term "about" in relation to a numerical value x means, for example, x±10%.

[0178] The word "substantially" does not exclude "completely" *e.g.* a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

[0179] It will be appreciated that sugar rings can exist in open and closed form and that, whilst closed forms are shown in structural formulae herein, open forms are also encompassed by the invention. Similarly, it will be appreciated that sugars can exist in pyranose and furanose forms and that, whilst pyranose forms are shown in structural formulae herein, furanose forms are also encompassed. Different anomeric forms of sugars are also encompassed.

[0180] References to a percentage sequence identity between two amino acid sequences means that, when aligned, that percentage of amino acids are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in section 7.7.18 of ref. 233. A preferred alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is disclosed in ref. 234.

[0181] Particular embodiments of the invention include:

1. An immunogenic composition comprising a conjugate of a *Neisseria meningitidis* serogroup X capsular polysaccharide and a carrier molecule, wherein the coupling of the capsular polysaccharide is direct.

2. The immunogenic composition comprising a conjugate of embodiment 1, wherein the conjugate is obtainable by a process comprising the steps of: (a) oxidising a primary hydroxyl group in the capsular polysaccharide, to give an oxidised polysaccharide with an aldehyde group; and (b) coupling the oxidised polysaccharide to a carrier molecule via the aldehyde group, thereby giving the conjugate.

3. The immunogenic composition comprising a conjugate of embodiment 2, where the oxidation in step (a) is of the primary hydroxyl group on between 1-10% of the residues in the capsular polysaccharide.

4. The immunogenic composition comprising a conjugate of embodiment 3, where the oxidation is of the primary hydroxyl group on between 4-8% of the residues in the capsular polysaccharide.

5. The immunogenic composition comprising a conjugate of any of embodiments 2-4, where the oxidation in step (a) is TEMPO-mediated oxidation.

6. The immunogenic composition comprising a conjugate of any of embodiments 2-5, wherein the coupling in step (b) is by reductive amination between the aldehyde group and a primary amine group on the carrier molecule.

7. The immunogenic composition comprising a conjugate of embodiment 1, wherein the conjugate is obtainable by a process comprising the steps of: (a) reductive amination of the reducing terminus of the capsular polysaccharide, to give a modified polysaccharide with a primary amine group bonded to the C-1 atom of the terminal subunit by a covalent bond; and (b) coupling the modified polysaccharide to a carrier molecule via the primary amine group, thereby giving the conjugate.

8. The immunogenic composition comprising a conjugate of embodiment 1, wherein the conjugate is obtainable by a process comprising the steps of: (a) reduction of the reducing terminus of the capsular polysaccharide, to give a modified polysaccharide with two vicinal hydroxyl groups at that terminus; (b) oxidative cleavage of the vicinal hydroxyl groups, to give a further modified polysaccharide with an aldehyde group at the terminus; (c) reductive amination of the aldehyde group, to give a further modified polysaccharide with a primary amine group at the terminus and (d) coupling the further modified polysaccharide to a carrier molecule via the primary amine group, thereby giving the conjugate.

9. The immunogenic composition comprising a conjugate of embodiment 8, wherein the primary amine group is bonded to the C-5 atom of the terminal subunit by a covalent bond.

10. The immunogenic composition comprising a conjugate of any of embodiments 2-9, wherein the capsular polysaccharide is an oligosaccharide.

11. The immunogenic composition comprising a conjugate of embodiment 10, wherein the oligosaccharide has a degree of polymerisation between 60 and 100 or between 10 and 20.

12. The immunogenic composition comprising a conjugate of any of embodiments 1-11, wherein the carrier molecule is a diphtheria or tetanus toxoid, CRM197 or protein D.

13. The immunogenic composition comprising a conjugate of any of embodiments 1-11, wherein the carrier molecule comprises a spr0096 antigen and a spr2021 antigen.

14. The immunogenic composition comprising a conjugate of embodiment 13, wherein the spr0096 antigen comprises an amino acid sequence having 50% or more identity to SEQ ID NO: 1 or SEQ ID NO: 2.

15. The immunogenic composition comprising a conjugate according to embodiment 13 or embodiment 24, wherein the spr2021 antigen comprises an amino acid sequence having 50% or more identity to SEQ ID NO: 3.

16. The immunogenic composition comprising a conjugate according to any of embodiments 13-15, wherein the carrier molecule comprises the spr0096 antigen and the spr2021 antigen as a single polypeptide chain.

17. The immunogenic composition comprising a conjugate according to embodiment 16, wherein the polypeptide chain is of the formula NH_2-A-{-X-L-}$_n$-B-COOH, wherein: A is an optional N terminal amino acid sequence; B is an optional C terminal amino acid sequence; n is an integer of 2 or more; each X is an amino acid sequence of an spr0096 antigen or an spr2021 antigen, wherein at least one X is an spr0096 antigen and at least one X is an spr2021 antigen; and L is an optional linker amino acid sequence.

18. The immunogenic composition comprising a conjugate according to embodiment 17, wherein *n* is 2.

19. The immunogenic composition comprising a conjugate according to embodiment 18, wherein X_1 is an spr0096 antigen and X_2 is an spr2021 antigen.

20. The immunogenic composition comprising a conjugate according to embodiment 19, wherein the polypeptide chain comprises an amino acid sequence having 50% or more identity to SEQ ID NO: 9, particularly an amino acid sequence of SEQ ID NO: 9.

21. The immunogenic composition of any of embodiments 1-20, further comprising one or more further antigens.

22. The immunogenic composition of embodiment 21, further comprising a serogroup A capsular polysaccharide.

23. The immunogenic composition of embodiment 22, wherein the serogroup A capsular polysaccharide is conjugated to a carrier molecule.

24. The immunogenic composition of any of embodiments 21-23, further comprising a serogroup W135 capsular polysaccharide.

25. The immunogenic composition of embodiment 24, wherein the composition comprises a serogroup A capsular polysaccharide conjugated to a carrier molecule.

26. The immunogenic composition of embodiment 24 or embodiment 25, wherein the serogroup W135 capsular polysaccharide is conjugated to a carrier molecule.

27. The immunogenic composition of embodiment 23, 25 or 26, wherein the carrier molecule is as defined in embodiment 12.

28. The immunogenic composition of any of embodiments 21-27, further comprising a serogroup C capsular polysaccharide.

29. The immunogenic composition of any of embodiments 21-28, further comprising a serogroup Y capsular polysaccharide.

30. The immunogenic composition of embodiment 28 or embodiment 29, wherein the capsular polysaccharide is conjugated to a carrier molecule.

31. The composition of embodiment 30, wherein the carrier molecule is as defined in embodiment 12.

32. The immunogenic composition of any of embodiments 1-31, wherein the composition is in an aqueous formulation.

33. A vaccine comprising the immunogenic composition of any of embodiments 1-32.

BRIEF DESCRIPTION OF DRAWINGS

[0182]

Figure 1 shows the repeat unit of the serogroup X capsular polysaccharide.

Figure 2 shows an ultra performance liquid chromatogram for native and hydrolysed serogroup X capsular polysaccharide.

Figure 3 shows a scheme for the conjugation of a serogroup X capsular polysaccharide to CRM197 by TEMPO oxidation followed by reductive amination, and an SDS PAGE analysis of the resultant conjugate.

Figure 4 shows a chromatogram of conjugation mixture run on a Sephacryl S300 column with phosphate buffered saline.

Figure 5 shows a scheme for the conjugation of a serogroup X capsular polysaccharide to CRM197 via SIDEA linker, and an SDS PAGE analysis of the resultant conjugate.

Figure 6 shows a scheme for the conjugation of a serogroup X capsular polysaccharide to CRM197 via SIDEA linker using a different method, and an SDS PAGE analysis of the resultant conjugate.

Figure 7 shows an SDS PAGE analysis of a serogroup X capsular polysaccharide-CRM197 conjugate made using a different linker.

Figure 8 shows IgG antibody titres against serogroup X capsular polysaccharide and serum bactericidal antibody titres against serogroup X following immunisation with a variety of *N.meningitidis* conjugates.

Figure 9 shows IgG antibody titres against serogroup A capsular polysaccharide and serum bactericidal antibody titres against serogroup A from the same experiment.

Figure 10 shows IgG antibody titres against serogroup C capsular polysaccharide and serum bactericidal antibody titres against serogroup C from the same experiment.

Figure 11 shows IgG antibody titres against serogroup W135 capsular polysaccharide and serum bactericidal antibody titres against serogroup W135 from the same experiment.

Figure 12 shows IgG antibody titres against serogroup Y capsular polysaccharide and serum bactericidal antibody titres against serogroup Y from the same experiment.

Figure 13 shows 2D 1H-31P HMBC NMR spectrum recorded at 400 MHz and $25 \pm 0.1°C$ on MenA (a) and MenX (b) oligosaccharide generated by acidic hydrolysis. Peaks assignments are labelled.

Figure 14 shows a) avDP and b) pH as a function of time collected for MenA and MenX capsular polysaccharides

and C) O-acetyl status for MenA capsular polysaccharide only at 37°C and 45°C.

Figure 15 shows profiles of avDP as a function of time, collected for the stability study at 37°C and 45°C for a) MenA and b) MenX capsular polysaccharide at time points (a) 0, (b) 7, (c) 10, (d) 14, (e) 21 days at 45°C and at (f) 7, (g) 14, (h) 21, (i) 28 days at 37°C. In addition a profile of experimentally degraded MenX capsular polysaccharide, obtained by acidic treatment (sodium acetate pH 4.0, at 80°C for ∼4 hrs), is shown in b) (l).

Figure 16 shows IgG antibody titres against serogroup X capsular polysaccharide and serum bactericidal antibody titres against serogroup X following immunisation with a variety of *N.meningitidis* conjugates.

Figure 17 shows a scheme for the conjugation of a serogroup X capsular polysaccharide to CRM197 using a further method, and an SDS PAGE analysis of the resultant conjugate.

Figure 18 shows IgG antibody titres against serogroup A capsular polysaccharide and serum bactericidal antibody titres against serogroup A following immunisation with a variety of *N.meningitidis* conjugates.

Figure 19 shows IgG antibody titres against serogroup C capsular polysaccharide and serum bactericidal antibody titres against serogroup C following immunisation with a variety of *N.meningitidis* conjugates.

Figure 20 shows IgG antibody titres against serogroup W135 capsular polysaccharide and serum bactericidal antibody titres against serogroup W135 following immunisation with a variety of *N.meningitidis* conjugates.

Figure 21 shows IgG antibody titres against serogroup Y capsular polysaccharide following immunisation with a variety of *N. meningitidis* conjugates.

Figure 22 shows high affinity IgG antibody titres against serogroup X capsular polysaccharide following immunisation with a variety of *N.meningitidis* conjugates.

MODES FOR CARRYING OUT THE INVENTION

Bacterial growth for serogroup X capsular polysaccharide production

[0183]   In order to identify optimal bacterial growth conditions for production and release of serogroup X capsular polysaccharide in the supernatant, three different media were tested using the MenX 5967 (ST 750) strain. Different growths were performed in flasks and monitored by Proton Nuclear Magnetic Resonance spectroscopy (1H NMR). Culture supernatants were analyzed by NMR sequence with a diffusion filter to cut off signals deriving from lower molecular weight (MW) species and highlight the signals of higher MW serogroup X capsular polysaccharide. Further analysis of the corresponding pellets by 1H High-Resolution Magic Angle Spinning NMR (HR-MAS NMR) in solid state did not show serogroup X capsular polysaccharide signals, indicating that the majority of the polysaccharide was released in the supernatant (considering the limit of detection of this assay, the maximum amount of polysaccharide remaining on the bacteria should be 1/8 of the starting amount). Similar results were obtained with the three media.
[0184]   In addition to the NMR methodology, a more accurate method for serogroup X capsular polysaccharide quantification in the clarified culture broth was developed (see below) using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). As shown in the table below, medium #3 resulted in the highest amount of polysaccharide. It was therefore selected for a higher scale (18 L) fermentation which yielded 356 μg/mL of serogroup X capsular polysaccharide in the supernatant.

MenX 5967 (ST 750) strain growth in different media and relative polysaccharide production

[0185]

Growth medium*	OD(600nm)	Saccharide (μg/mL)	μg saccharide/OD
#1	2	22.55	13.3
#2	6	42.73	7.1

(continued)

Growth medium*	OD(600nm)	Saccharide (μg/mL)	μg saccharide/OD
#3	2.8	62.6	22.4

*1. modified Catlin v.6: casaminoacids 10 g/L, NaCl 5.8 g/L, glucose 10 g/L, K_2HPO_4 4 g/L, NH_4Cl 1 g/L, K_2SO_4 1 g/L, $MgCl_2 \cdot 6\ H_2O$ 0.4 g/L, $CaCl_2 \cdot 2\ H_2O$ 0.03 g/L, Fe(III) citrate 0.5 mg/L, pH 7.2; 2. MCDM1: glucose 10 g/L, soy peptone 15 g/L, NaCl 5.80 g/L, K_2SO_4 1 g/L, K_2HPO_4 4 g/L, L-glutamic acid 5 g/L, L-arginine 0.3 g/L, L-serine 0.5 g/L, L-cysteine 0.23 g/L, $MgCl_2$ 0.19 g/L, $CaCl_2$ 0.021 g/L, $FeSO_4$ 0.002 g/L; 3. modified Frantz: L-Glutamic acid 1.6 g/L, $Na_2HPO_4 \cdot 2H_2O$ 15.5 g/L, KCl 0.09 g/L, NH_4Cl 1.25 g/L, pH 7.6, supplemented with: glucose 50 g/L, $MgSO_4 \cdot 7H_2O$ 30 g/L, 25 g/L ultrafiltered yeast extract, L-cysteine 1.5 g/L.

Purification of serogroup X capsular polysaccharide

[0186]   The process for purifying serogroup X capsular polysaccharide was purified by a method adapted from reference 235.

Conjugate production and characterisation

[0187]   Conjugates were made using polysaccharides of different chain lengths and different conjugation chemistries.

Conjugation by oxidation and reductive amination (method A):

[0188]   Purified serogroup X capsular polysaccharide was hydrolysed in 50 mM sodium acetate at pH 4.7, 100 °C for 1 hour (Figure 2). The average degree of polymerisation of the resulting oligosaccharide was determined to be 80, corresponding to a molecular weight of 25-30kDa, by NMR The polysaccharide depolymerization was monitored in process by Ultra Performance Liquid Chromatography-Size Exclusion Chromatography (UPLC-SEC) and phosphorus ($_{31}$P) NMR spectroscopy, and it was quenched by neutralization when the desired avDP was reached. The buffer was exchanged with tetrabutyl ammonium bromide to allow dissolution of the saccharide in dimethylformamide solvent. The saccharide was then oxidized with TEMPO (0.06eq relative to the MenX repeating subunit), $NaHCO_3$ (9eq relative to the MenX repeating subunit), TCC (2eq relative to the MenX repeating subunit) at 0°C overnight. This oxidation generates an aldehyde group at the C-6 position of individual subunits (Figure 3). The oxidised saccharide was purified by precipitation with acetone/NaCl and gel filtration using a Sephadex G15 column. The saccharide was quantified using HPAEC-PAD and its structural identity confirmed using NMR. There were approximately 4.5 oxidized groups per chain, corresponding to a degree of oxidation of approximately 6 % along the *circa* 80 residue chain. The molecular weight distribution of the oxidized saccharide was measured by UPLC-SEC.

[0189]   The aldehyde group was used for conjugation to carrier protein CRM197 by reductive amination (Figure 3). Briefly, the saccharide was mixed with 10mg/ml CRM197 at a 4:1 w/w ratio and $NaBH_3CN$ at a 1:1 w/w ratio in a NaPi 10mM pH 7.2 buffer. The mixture was left for 72 hours with slow stirring at 37°C. Conjugates were purified on a Sephacryl S300 column with phosphate buffered saline and fractions collected into pools (Figure 4). Conjugation was verified by SDS PAGE (Figure 3). The properties of the purified conjugates (pool 1) are given below:

MenX (µg/mL)	CRM197 (µg/mL)	Total mass MenX (mg)	Total mass CRM197 (mg)	MenX/CRM197 (w/w)	MenX yield (%)	CRM197 yield (%)	MenX/CRM197 (mol/mol)	Kd (SEC)	EU/µg (LAL)
104.8	151.3	1.26	1.82	0.69	12.6	72.6	1.7	0.7	1.6

[0190] Conjugates were also made by this method in which the polysaccharides were not hydrolysed with sodium acetate and therefore had a native average degree of polymerisation. Further conjugates were made containing polysaccharides with an average degree of polymerisation of 130.

[0191] Conjugates were also made by this method in which the carrier protein was tetanus toxoid (TT) or SEQ ID NO: 9 (SEQ9). The polysaccharides in these conjugates had an average degree of polymerisation of 130. Other characteristics of these conjugates are given below:

Carrier	MenX (µg/mL)	Carrier (µg/mL)	MenX/carrier (w/w)	MenX/carrier (mol/mol)	EU/µg (LAL)
TT	197.6	660.80	0.3	0.4	18.81
SEQ9	159.7	757.70	0.21	0.3	6.57

Conjugation by reductive amination followed by reaction with SIDEA linker (method B):

[0192] Purified serogroup X capsular polysaccharide was hydrolysed in 50 mM sodium acetate at pH 4.7, 100 °C for 2 hours (Figure 2). The average degree of polymerisation of the resulting oligosaccharide was determined to be 15, corresponding to a molecular weight of 5kDa, by NMR The saccharide was then solubilised at 5 mg/ml in 5mM sodium acetate buffer at pH 6.5 with 300 mg/ml NH_4OAc and 49 mg/ml $NaBH_3CN$ for 5 days at 37°C. This step resulted in reductive amination of the terminal aldehyde group to generate a primary amine group (Figure 5). The reaction mixture was then purified by tangential flow filtration with a 200 cm^2 Hydrosart (cellulose) 2kDa-cut off membrane against 1M NaCl and water. The primary amine group was then used for activation with SIDEA and subsequent conjugation to carrier protein CRM197 (Figure 5). Briefly, the modified saccharide was dissolved in DMSO/water at 9:1 (v/v) with NEt_3 (at a molar ratio of NEt_3:total NH2 groups of 5:1) at a mol SIDEA:total mol NH_2 groups of 12:1 for 3 hours at room temperature. The reaction mixture was then purified by precipitation with 90% dioxane (v/v). The SIDEA-modified saccharide was then reacted with 25 mg/ml CRM197 at a ratio of 13:1 (molar ratio active ester groups:CRM197) in a 25mM NaPi buffer at pH 7.2. The mixture was left for 5 hours with slow stirring at room temperature. The conjugates were purified by precipitation with $(NH_4)_2SO_4$. Conjugation was verified by SDS PAGE (Figure 5). The properties of one lot of these conjugates are given below:

MenX (µg/mL)	CRM197 (µg/mL)	Total mass MenX (mg)	Total mass CRM197 (mg)	MenX/CRM197 (w/w)	MenX/CRM197 (mol/mol)	MenX yield (%)	Kd (SEC)	EU/ µg (LAL)
167.3	514.4	0.17	0.51	0.33	4.22	12.6	0.32	0.8

Conjugation by reduction, oxidation and reductive amination followed by reaction with SIDEA linker (method C):

[0193] Purified serogroup X capsular saccharide was reacted at 15 mg/ml in 10mM NaPi buffer at pH 8 with $NaBH_4$ (12eq relative to the molecular weight of MenX, solid) for 1.5 hours at room temperature. This step resulted in reduction of the saccharide. The reduced saccharide was then reacted at 6-8 mg/ml in 10mM NaPi buffer at pH 7.2 with $NaIO_4$ (10eq relative to the molecular weight of MenX, solid) for 1.5 hours at room temperature. The combined effect of these two steps is the generation of an aldehyde group at the reducing terminus of the saccharide (Figure 6). The modified saccharide is then subjected to reductive amination to provide a primary amine group that can be used for activation with SIDEA and subsequent conjugation to carrier protein CRM197 (Figure 6). Briefly, the modified saccharide solubilised at 4-5 mg/ml in 10mM NaPi buffer at pH 7 with 300 mg/ml NH_4OAc and 49 mg/ml $NaBH_3CN$ for 5 days at 37°C. The modified saccharide was then dissolved in DMSO/water at 9:1 (v/v) with NEt_3 (at a molar ratio of NEt_3:total NH2 groups of 5:1) at a mol SIDEA:total mol NH_2 groups of 12:1 for 3 hours at room temperature. The reaction mixture was then purified by precipitation with 80% acetone (v/v). The resulting SIDEA-modified saccharide was then reacted with 25 mg/ml CRM197 at a ratio of 13:1 (molar ratio active ester groups:CRM197) in a 100mM NaPi buffer at pH 7.2. The mixture was left overnight with slow stirring at room temperature. The conjugates were purified by precipitation with $(NH_4)_2SO_4$. Conjugation was verified by SDS PAGE (Figure 6). The properties of one lot of these conjugates are given below:

MenX (μg/mL)	CRM197 (μg/mL)	MenX/CRM197 (w/w)	MenX/CRM197 (mol/mol)	Free saccharide (%)	EU/μg (LAL)
129.60	628.70	0.21	2.1	<6	0.01

Conjugation via alternative linker (method D):

[0194]   The purified serogroup X capsular polysaccharide with an average degree of polymerisation of 15, as described above, was also conjugated to CRM197 using a different linker according to the method of Figure 7 in US 61/534,751. Conjugation was verified by SDS PAGE (Figure 7 herein).

Conjugation by reduction, oxidation and reductive amination with the carrier (method E):

[0195]   Purified serogroup X capsular saccharide was reacted at 15 mg/ml in 10mM NaPi buffer at pH 8 with $NaBH_4$ (12eq relative to the molecular weight of MenX, solid) for 2 hours at room temperature. This step resulted in reduction of the saccharide. The reduced saccharide was then reacted at 6-8 mg/ml in 10mM NaPi buffer at pH 7.2 with $NaIO_4$ (10eq relative to the molecular weight of MenX, solid) for 1.5 hours at room temperature. The combined effect of these two steps is the generation of an aldehyde group at the reducing terminus of the saccharide (Figure 17). The modified saccharide is then subjected to reductive amination with carrier protein CRM197. Briefly, the modified saccharide at 2mg/ml was dissolved in 300mM NaPi buffer at pH 8 (at a weight ratio of saccharide:CRM197 of 8:1) and $NaBH_3CN$ (at a weight ratio of saccharide: $NaBH_3CN$ of 4:1) for 4 days at 37°C. Conjugation was verified by SDS PAGE (Figure 17).

Immunisation study (1)

[0196]   *General assay protocol:* Balb/c mice were immunized by subcutaneous injection according to the schedule described below. The injection volume was 200 μl and the injection contained alum phosphate adjuvant (120 μg per dose). Injections were carried out on days 1, 14 and 28, with bleeds taken at day 0 (for preimmune sera), 28 (post second immunisation sera) and 42 (post third sera).

Grp	Mice per group	Immunogen	Antigen dose
1	8	PBS	-
2	16	MenX-CRM197 (method A)	1μg
3	16	MenX-CRM197 (method D)	1μg
4	16	MenX-CRM197 (method B)	1μg
5	16	MenX-CRM197 (method A) + MenACWY	1μg + 2,1,1, 1μg
6	16	MenX-CRM197 (method D)+ MenACWY	1μg + 2,1,1, 1μg
7	16	MenX-CRM197 (method B)+ MenACWY	1μg + 2,1,1, 1μg
8	16	MenACWY	2,1,1, 1ug
9	16	MenX-CRM197 (method D)	0.1μg
MenACWY = mixture of MenA-CRM197, MenC-CRM197, MenW135-CRM197 and MenY-CRM197 prepared according to ref. 10.			

[0197]   The post third immunisation IgG antibody titre against serogroup X capsular polysaccharide and serum bactericidal antibody titre against serogroup X strain Z9615 are shown in Figure 8. The serogroup X conjugates were immunogenic and induced bactericidal antibodies. The response was not diminished when the dose was reduced ten-fold (to 0.1μg). Responses were slightly reduced when the conjugates were combined with conjugates derived from serogroups A, C, W135 and Y, but still well above controls. Accordingly, immune interference between these conjugates and the serogroup X conjugates appears to be relatively small.

[0198]   The post third immunisation IgG antibody titres against serogroups A, C, W135 and Y capsular polysaccharides and serum bactericidal antibody titres against these serogroups (using strains F8238, 11, 240070 and 860800 respectively) were also measured for groups 5, 6, 7 and 8. Results are shown in Figures 9-12. The responses to the serogroup A, C, W135 and Y conjugates were generally not diminished when combined with the serogroup X conjugates. Once

again, these results suggest that there is little immune interference between these conjugates and the serogroup X conjugates.

[0199] Anti-serogroup X capsular polysaccharide IgM ELISA units were found to be low for all the conjugates, as expected for conjugate vaccines due to effective isotype switching from IgM to IgG.

[0200] A modified ELISA was used to measure higher avidity IgG antibodies only (Figure 22). The modified ELISA uses a chaotropic salt to select and detect higher avidity IgG antibodies only. Anti-serogroup X capsular polysaccharide IgG ELISA units were low for all the conjugates both after the second and the third dose compared to the units by standard ELISA, but a statistically significant booster effect was observed after the third dose for all the conjugates (P from 0.0006 to <0.0001).

[0201] The following table summarises the rabbit complement serum bactericidal antibody titres against the various strains from the pooled post third immunisation sera.

Antigen Name	Antigen Dose (μg)	MenX Z9615	MenA F8238	MenC 11	MenW 240070	MenY 860800
PBS + AlumPhosphate	-	<4	<16	<16	<16	32
MenX-CRM197 (A)	1	4096	<16	<16	<16	32
MenX-CRM197 (D)	1	4096	n/a	n/a	n/a	n/a
MenX-CRM197 (B)	1	4096	n/a	n/a	n/a	n/a
MenX-CRM197 (A) + MenACWY	1 + 2,1,1,1	256	4096	4096	512	1024
MenX-CRM197 (D) + MenACWY	1 + 2,1,1,1	1024	2048	4096	1024	1024
MenX-CRM197 (B) + MenACWY	1 + 2,1,1,1	1024	2048	4096	1024	1024
MenACWY	2,1,1,1	<4	2048	8192	1024	2048
MenX-CRM197 (D)	0.1	4096	n/a	n/a	n/a	n/a

Immunisation study (2)

[0202] *General assay protocol:* Balb/c mice were immunized by subcutaneous injection according to the schedule described below. The injection volume was 200 μl and the injection contained alum phosphate adjuvant.

Group	Mice per group	Immunogen	Antigen dose
1	1-8	PBS	1μg
2	9-16	MenX-CRM197 (method A, native avDP)	1μg
3	17-24	MenX-CRM197 (method A, 80 avDP)	1μg
4	25-32	MenX-CRM197 (method A, 130 avDP)	1μg
5	33-40	MenX-TT (method A, 130 avDP)	1μg
6	41-48	MenX-SEQ9 (method A, 130 avDP)	1μg
7	49-56	MenX-CRM197 (method A, native avDP) + MenACWY	1μg + 2,1,1, 1μg
8	57-64	MenX-CRM197 (method A, 80 avDP) + MenACWY	1μg + 2,1,1, 1μg
9	65-72	MenX-CRM197 (method A, 130 avDP) + MenACWY	1μg + 2,1,1, 1μg
10	73-80	MenX-TT (method A, 130 avDP) + MenACWY	1μg + 2,1,1, 1μg
11	81-88	MenX-SEQ9 (method A, 130 avDP) + MenACWY	1μg + 2,1,1, 1μg
MenACWY = mixture of MenA-CRM197, MenC-CRM197, MenW135-CRM197 and MenY-CRM197 prepared according to ref. 10.			

[0203] The post third immunisation IgG antibody titre against serogroup X capsular polysaccharide and serum bactericidal antibody titre against serogroup X strain Z9615 are shown in Figure 16. The serogroup X conjugates were immunogenic and induced bactericidal antibodies. Responses were slightly reduced when the MenX-CRM197 conjugates were combined with conjugates derived from serogroups A, C, W135 and Y, but still well above controls. In contrast, little or no reduction was seen when MenX-TT or MenX-SEQ9 conjugates were combined with these conjugates. Accordingly, the use of a different carrier protein for the MenX polysaccharide may help to reduce any immune interference between the serogroup X conjugate and these conjugates.

[0204] The post third immunisation IgG antibody titre against serogroup A capsular polysaccharide and serum bactericidal antibody titre against serogroup A strain F8238 when the MenX conjugates were combined with conjugates derived from serogroups A, C, W135 and Y are shown in Figure 18. Corresponding data for serogroups C, W135 and Y are shown in Figures 19-21.

Stability study (1)

[0205] *Materials:* Purified MenA and MenX polysaccharides were obtained according to the method of ref. 10. The purity of the polysaccharide preparation was assessed by estimation of residual protein and nucleic acids contents, which were lower than 1% w/w of saccharide.

[0206] *NMR analyses:* 1H, 13C and 31P NMR experiments were recorded on Bruker Avance III 400 MHz spectrometer, equipped with a high precision temperature controller, and using 5-mm broadband probe (Bruker). For data acquisition and processing, TopSpin version 2.6 software (Bruker) was used. 1H NMR spectra were collected at 25 ± 0.1 °C with 32k data points over a 10 ppm spectral width, accumulating 128 scans. The spectra were weighted with 0.2 Hz line broadening and Fourier-transformed. The transmitter was set at the water frequency which was used as the reference signal (4.79 ppm). 13C NMR spectra were recorded at 100.6 MHz and 37 ± 0.1°C, with 32k data points over a 200 ppm spectral width, accumulating 4k scans. The spectra were weighted with 0.2 Hz line broadening and Fourier-transformed. The transmitter was set at the acetone frequency which was used as the reference signal (30.89 ppm). 31P NMR spectra were recorded at 161.9 MHz at 25 ± 0.1°C, with 32k data points over a 20 ppm spectral width, accumulating approximately 1k of scans. The spectra were weighted with 3.0 Hz line broadening and Fourier-transformed. 85% phosphoric acid in deuterium oxide was used as an external standard (0 ppm). All the 1H and 31P NMR spectra were obtained in quantitative manner using a total recycle time to ensure a full recovery of each signal (5 x Longitudinal Relaxation Time T1). To confirm the degradation mechanism of MenA and MenX capsular polysaccharides and consequently to assign the 31P NMR peaks, bidimensional 1H-31P Heteronuclear Multiple-Bond Correlation (HMBC) experiments were acquired on MenA and MenX oligosaccaride samples, previously generated by acidic hydrolysis in 50 mM sodium acetate pH 4.8 (saccharide concentration of ~10 mg/mL) at 73°C for ~2.5 hrs and pH 4.0 at 80°C for ~5.5 hrs (saccharide concentration of ~2.5 mg/mL) respectively. The average degree of polymerization (avDP) of MenA and MenX oligosaccharides was ~12 and ~10 respectively, as estimated by 31P NMR analysis (see paragraph *Stability experiments* below). These NMR analytical samples were prepared by solubilizing approximately 10 mg of dried saccharide in 0.75 mL of deuterium oxide (99.9% atom D - Aldrich), with a standard pulse-program. 4096 and 512 data points were collected in F2 and F1 dimension respectively. 64 scans were accumulated prior to Fourier transformation to yield a digital resolution of 0.2 Hz and 5.0 Hz per point in F2 and F1 respectively.

[0207] *HPLC analyses:* HPLC analyses were conducted using CarboPac PA200 column (4 mm x 250 mm; Dionex) with guard column (4 mm x 50 mm; Dionex) connected to an ICS 3000 Dionex system equipped with a Pulsed Amperometric Detector. 100 mM NaOH + 10 mM sodium nitrate buffer was used for column equilibration and a three-step gradient with increasing amount of sodium nitrate (100 mM NaOH + 10 mM, 250 mM, 500 mM sodium nitrate for 80, 15 and 3 min respectively) was used for elution. A flow rate of 0.4 mL/min was used for the entire run of 120 min. 20 μL samples were injected at a concentration of approximately 1 mg/mL. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A quadruple-potential waveform for carbohydrates was applied. The resulting chromatographic data were processed using Chromeleon software 6.8 (Dionex).

[0208] *Stability experiments:* MenA and MenX polysaccharide solutions at a concentration of approximately 1 mg/mL in 100 mM potassium phosphate buffer pH 7.0 prepared with deuterated water were incubated at 37°C and 45°C respectively. At different time points, samples were withdrawn and analysed by NMR and HPLC. pH was also monitored at each time point. The avDP of MenA and MenX was monitored for polysaccharide stability. avDP values were calculated by the integration of 31P NMR spectra and expressed as [(Pde/Pme) +1], where Pde is molar concentration of the phosphodiester in chain groups and Pme the molar concentration of phosphomonoester end groups. The HPLC profiles were also evaluated semi-quantitatively in order to confirm the more accurate stability evaluation collected by 31P NMR assay

[0209] *Degradation mechanism of MenA and MenX polysaccharides* The NMR ^{1}H-^{31}P HMBC data on the MenA oligosaccharide, generated by mild acidic hydrolysis, are reported in Figure 13(a). Due to the presence of O-acetyl

groups at C_3 and C_4 of mannosamine residues, several spin systems were detected and assigned: (i) proton at C1 of 3- or 4-O-acetylated residues $(H_1\text{-}P_{de})^{3/4OAc}$; (ii) proton at C_1 of de-O-acetylated residues $(H_1\text{-}P_{de})^{deOAc}$; (iii) proton at C_3 and C_4 geminal of O-acetyl groups $(H_3/H_4\text{-}P_{de})^{3/4OAc}$; (iv) proton at C_2 of 3-O-acetylated residues $(H_2\text{-}P_{de})^{3OAc}$; (v) proton at C_2 of 4-O-acetylated residues $(H_2\text{-}P_{de})^{4OAc}$; (vi) proton at C_2 of de-O-acetylated residues $(H_2\text{-}P_{de})^{deOAc}$; (vii) protons at C_5 and C_6 of 3- or 4-O-acetylated residues $(H_{5/6}\text{-}P_{de})^{3/4OAc}$; (viii) protons at C_3, C_4, C_5 and C_6 of de-O-acetylated residues $(H_{3/4/5/6}\text{-}P_{de})^{deOAc}$. The attachment of phosphate at C_6, confirmed by the cross peaks of phospho-monoester to proton at C_6 of 3- or 4-O-acetylated residues $(H_6\text{-}P_{me})^{3/4OAc}$ and to proton at C_6 of de-O-acetylated residues $(H_6\text{-}P_{me})^{deOAc}$, indicated that during hydrolysis the phosphodiester bond is cleaved leaving a phosphate group attached to the non-reducing terminus, which is consistent with the lower stability of the phosphate-Ci linkage. Because no other [1]H-^{31}P scalar correlation was detected, no phosphate migration involving free hydroxyl groups at C4 or C3 occurred during hydrolysis. ^{1}H-^{31}P HMBC on the MenX oligosaccharides (Figure 13(b)) also indicated that the phosphate-Ci linkage is less stable and in this case the non-reducing terminus has a phosphate group attached at C_4: the monoester phosphate shows cross-correlation only with proton at C_4. Also for MenX, no phosphate migration involving free hydroxyl groups at C3 or C6 occurred during hydrolysis. All the ^{31}P spin systems were assigned, the phosphodiester and phos-phomonoester signals at -1.40 and 4.65 ppm respectively. The proton NMR profile was assigned also by collecting the ^{31}P-decoupled spectrum which reduces the peaks structure due to this scalar coupling. All the spectra assignments were in agreement with the published results mainly based on ^{13}C NMR analysis (ref. 30). 13C NMR chemical shifts of MenX capsular polysaccharide were in agreement with published data (ref. 14), as shown in Table 1 below:

Table 1: 13C NMR chemical shifts of MenX capsular polysaccharide.

	C_1	C_2	C_3	C_4	C_5	C_6	$CH_3{}^{NAc}$	CO^{NAc}
Chemical shift (ppm)	95.2	54.8	71.1	75.1	73.2	61.3	23.2	175.6

[0210] *Thermal stability of MenA and MenX polysaccharides.* Degradation of MenA and MenX capsular polysaccha-rides, as the consequence of hydrolysis at phosphodiester bonds, results in fragments of lower avDP which expose newly-formed phosphomonoester end groups. In NMR experiments, these phosphomonester groups generate a ^{31}P resonance signal at higher fields than that originated by the internal phosphodiester groups thus allowing the avDP calculation as described in *Stability experiments* above. The variation of avDP during storage is an indicator of the polysaccharide stability and so the avDP of samples of MenA and MenX capsular polysaccharides, taken at different time points during exposure at 37°C and 45°C, was measured by ^{31}P NMR (Table 2 and Figure 14(a)):

Table 2: avDP estimated by 31P NMR analysis and pH values detected on MenA and MenX samples at different time points and temperatures of 37°C and 45°C. O-acetylation status of MenA capsular polysaccharide, expressed as mol O-Acetyl groups per mol of repeating unit, is also reported.

Temperature (°C)	Time (days)	MenA PS			MenX PS	
		pH	avDP	OAc (mol/mol)	pH	avDP
37	0	6.97	>100	0.932	6.96	>100
	7	6.91	88.1	0.916	6.91	>100
	14	6.91	68.2	0.916	6.89	>100
	21	7.00	46.1	0.897	6.93	>100
	28	6.96	22.9	0.883	6.91	>100
45	0	6.97	>100	0.932	6.96	>100
	7	6.95	22.4	0.891	6.94	>100
	10	6.93	15.1	0.886	6.86	>100
	14	6.91	10.5	0.851	6.85	>100
	21	6.90	5.1	0.826	6.87	>100

[0211] At the sample concentration used, the sensitivity of the technique did not allow measurement of the avDP at time zero for both polysaccharides, when the avDP is higher than 100. For each time point sample the pH was maintained in the range of 7.0 $\pm$ 0.1 (Figure 14(b)).

[0212] At 37°C MenA capsular polysaccharide degraded to an avDP of 22.9 after 28 days of incubation, while at 45°C degradation was accelerated with an avDP of 5.1 after 21 days. Under the same conditions, MenX capsular polysaccharide did not show degradation (avDP>100 for all time points at both incubation temperatures; based on the assay sensitivity, 100 is the maximum avDP value detectable). HPLC profiles of MenA capsular polysaccharide incubated at 37°C and 45°C (Figure 15) progressively showed increased intensity peaks of shorter oligosaccharides indicating de-polymerisation of chains. In comparison, HPLC profiles collected on all MenX samples remain practically unmodified with a broad peak due to long chain polysaccharides at approximately 87 min which additionally demonstrates the higher stability of this carbohydrate. ^{1}H NMR analysis confirmed that the incubation of MenA and MenX capsular polysaccharides at 37°C and 45°C did not alter the structure of the polysaccharide repeating units. Only a limited decreasing of O-acetylation level (O-acetyl groups are present in MenA capsular polysaccharide only), from 0.932 to 0.883 and 0.826 mol/mol repeating unit at 37°C and 45°C was respectively observed (Table 2 and Figure 14(c)). Taken together, these NMR and HPLC data confirm the higher stability of MenX as compared to MenA capsular polysaccharide in aqueous solution.

Stability study (2)

[0213] *Materials:* MenX-CRM197 conjugates were prepared according to methods A, B and C above.

[0214] The conjugates prepared according to method A contained polysaccharides with an average degree of polymerisation of 100. Other characteristics of this conjugate lot are given below:

MenX (μg/mL)	CRM197 (μg/mL)	MenX/CRM197 (w/w)	MenX/CRM197 (mol/mol)	Free saccharide (%)
477.3	1378	0.35	0.7	<2.3

[0215] The conjugates prepared according to method B had the following characteristics:

MenX (μg)	CRM197 (mg)	MenX/CRM197 (w/w)	Free saccharide (%)
383	1.71	0.22	5.8

[0216] The conjugates prepared according to method C contained polysaccharides with an average degree of polymerisation of 19. Other characteristics of this conjugate lot are given below:

MenX (μg/mL)	CRM197 (μg/mL)	MenX/CRM197 (w/w)	MenX/CRM197 (mol/mol)	Free saccharide (%)
129.6	628.7	0.21	2.1	<6

[0217] Accelerated stability studies were performed to provide preliminary information on the stability of these conjugates. Stability studies of were performed at 37°C for 28 days, the time points for measurement were every 7 days (0, 7, 14, 21, 28 days). Samples were monitored by measuring the free saccharide released from the conjugates. The separation of free saccharide was performed by SPE-C4 cartridge using as elution buffer ACN 10-20% + TFA 0,05%. The total and free saccharide was quantified by HPAEC-PAD analysis, allowing a % free saccharide to be calculated. Values for the three lots of conjugate are given below:

Time (days)	% free saccharide		
	Method A conjugates	Method B conjugates	Method C conjugates
0	<2.3	5.8	<2.1
7	4.2	29.9	24.9
14	6.2	51.2	31.5
21	8.7	48.7	34.8
28	10.0	56.1	42.8

[0218] The conjugates made using method A were more stable than the conjugates made by methods B and C.

Analytical study

[0219] *Materials:* MenX polysaccharide was produced by bacterial growth of the *Neisseria meningitidis* X5967 strain (ST 750) and purified by a method adapted from reference 235. The purity of the polysaccharide preparation was assessed by estimation of residual protein and nucleic acid content using colorimetric assays (both were present at <1% w/w of saccharide), and endotoxin content using the LAL assay (<10 EU/μg of saccharide). Sodium acetate salt (Thermo Scientific Dionex), Sodium hydroxide 50% solution (J.T. Baker), Trifluoroacetic acid (Sigma), Water MilliQ grade (Millipore) were of pro analysis quality.

[0220] *General methods:* Total phosphorus content was measured according to the method of reference 24.

[0221] Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F254 (Sigma Aldrich); after exam under UV light, compounds were visualized by heating with 10% (v/v) ethanolic H_2SO_4. Column chromatography was performed using pre-packed silica cartridges RediSep (Teledyne-Isco, 0.040-0.063 nm). Unless otherwise specified, a gradient 0 → 100% of the elution mixture was applied in a Combiflash Rf (Teledyne-Isco) instrument.

^{1}H, ^{13}C and ^{31}P NMR experiments were recorded on Bruker Avance III 400 MHz spectrometer, equipped with a high precision temperature controller, and using 5-mm broadband probe (Bruker). For data acquisition and processing, Top-Spin version 2.6 software (Bruker) was used.

$_1$H NMR spectra were collected at 25$\pm$0.1 °C with 32k data points over a 10 ppm spectral width. The spectra were weighted with 0.2 Hz line broadening and Fouriertransformed. Chemical shift values were reported in ppm, relative to internal Me$_4$Si (0.00 ppm, CDCl$_3$) or the solvent signal (4.79 ppm, D2O). ^{13}C NMR spectra were recorded at 100.6 MHz and 37$\pm$0.1 °C, with 32k data points over a 200 ppm spectral width. The spectra were weighted with 0.2 Hz line broadening and Fourier-transformed. Chemical shift values were reported in ppm relative to the signal of CDCl$_3$ (77.0 ppm, CDCl$_3$). ^{31}P NMR spectra were recorded at 161.9 MHz at 25$\pm$0.1 °C, with 32k data points over a 20 ppm spectral width. The spectra were weighted with 3.0 Hz line broadening and Fourier-transformed. 85% phosphoric acid in deuterium oxide was used as an external standard (0 ppm).

[0222] Exact masses were measured by electron spray ionization cut-off spectroscopy, using a Q-Tof micro Macromass (Waters) instrument. Optical rotation was measured with a P-2000 Jasco polarimeter.*Benzyl 3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside 3.* The starting material **2** (ref. 236) (1.8 g, 3.1 mmol) was dissolved in acetonitrile (200 ml) under nitrogen, and treated with trimethylamineborane (1.4 g, 18.4 mmol) and BF$_3$·Et$_2$O (2.6 ml, 18.4 mmol) at 0°C. After stirring for 1 h at 0°C, the mixture was allowed to reach ambient temperature, at which time the reaction was complete (TLC, 7:3 cyclohexane-EtOAc). MeOH (3 ml) and triethylamine (3 ml) were added, and the mixture was concentrated. The residue was partitioned with aq NaHCO$_3$, and combined organic layers were concentrated and purified on silica gel (cyclohexane-EtOAc) to afford 1.5 g of product **3** (83%). [α]$_D^{24}$= +1.9 (c 0.5, CHCl$_3$). ^{1}H NMR (CDCl$_3$, 400 MHz): δ = 7.80-6.95 (m, 19 H, Ph), 5.15 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.78, 4.47 (2 d, 2 H, ^2J 12.2 Hz, CH_2Ph), 4.72, 4.51 (2 d, 2 H, ^2J 12.0 Hz, CH_2Ph), 4.67, 4.59 (2 d, 2 H, ^2J 12.0 Hz, CH_2Ph), 4.26-4.18 (m, 2 H, H-2,3), 3.87-3.88 (m, 3 H, H-4,6), 3.66-3.62 (m, 1 H, H-5), 2.89 (d, 1 H, $J_{2,OH}$ 2.3 Hz, OH-4). ^{13}C NMR (CDCl$_3$, 100 MHz): δ = 167.81 (CO), 138.15, 137.59, 137.10, 133.67, 131.61, 128.12, 127.91, 127.86, 127.81, 127.58, 127.40 (Ar), 97.35 (C-1), 78.49 (C-3), 74.37, 74.24(CH$_2$Ph), 73.78 (C-5), 73.45 (C-4), 70.80 (CH$_2$Ph), 70.69 (C-6), 55.37 (C-2). ESI HR-MS (C$_{35}$H$_{33}$NO$_7$): m/z = ([*M*+Na]$^+$ found 597.2547; calc 597.2601); ([*M*+Na]$^+$ found 618.1895; calc 618.1894).

[0223] *Benzyl 2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranoside 4.* A mixture of N-phthalimido compound **3** (1 g, 1.7 mmol) in EtOH (20 ml), containing 1.2 ml of ethylenediamine, was refluxed overnight. After TLC (toluene-EtOAc 4:1) showed the reaction was complete, the mixture was concentrated and re-dissolved in 4:1 EtOH-Ac$_2$O (25 ml). The mixture was stirred for 3 h, then concentrated. Chromatography of the residue (cyclohexane-EtOAc) gave 740 mg of monosaccharide **4,** whose NMR data were identical with those recently reported in literature [237].

[0224] *Benzyl 2-acetamido-3,6-di-O-benzyl-4-(1,5-dihydro-3-oxo-3λ^5-3H-2,4,3-benzodioxaphosphepin-3-yl)-β-D-glucopyranoside 5.* N,N-diethyl-1,5-dihydro-3*H*-2,3,4-benzodioxaphosphepin-3-amine (717 mg, 3 mmol) was added to a solution of the monosaccharide (500 mg, 1 mmol) in CH$_2$Cl$_2$ (9 ml) and 0.45 M 1*H*-tetrazole in acetonitrile (9 ml) at 0°C. After 10 min the iced bath was removed and stirring was continued. After stirring further 3 h the reaction went to completion (TLC, 1:1 toluene-EtOAc). The mixture was cooled to -20°C and *m*-CPBA was added. After 20 min some aq NaHCO$_3$ was added to quench it. The mixture was diluted with CH$_2$Cl$_2$ and extracted in a separatory funnel with aq NaHCO$_3$. Combined organic layers were concentrated and the residue was purified on silica gel (cyclohexane-EtOAc) to furnish 630 mg of product (92%). White crystals from EtOAc, m.p. 159-160°C. = [α]$_D^{24}$= +34.7 (c 0.1, CHCl$_3$). ^{1}H NMR (CDCl$_3$, 400 MHz): δ = 7.41-7.12 (m, 18 H, Ph), 5.90 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 5.17-5.12 (m, 2 H, 2 C*H*Ph), 5.00-4.78 (m, 4 H, 4 C*H*Ph), 4.65-4.58 (m, 5 H, 4 C*H*Ph, H-4), 4.32 (t, 1 H, *J* 9.0 Hz, H-3), 3.89 (d, 1 H, $J_{6a,5}$ 9.0 Hz, H-6a), 3.76-3.69 (m, 2 H, H-5,6b), 3.46-3.42 (m, 1 H, H-2), 1.80 (s, 3 H, C*H*$_3$CO). ^{13}C NMR (CDCl$_3$, 100 MHz): δ = 170.61 (CO), 138.26, 137.36, 134.98, 128.94, 128.35, 127.95, 127.98, 127.80, 127.71, 127.57, 127.50 (Ar), 98.85 (C-1), 78.71 (C-3), 76.72 (C-4), 73.97 (C-5), 73.76, 73.42, 70.09 (CH$_2$Ph), 69.04 (C-6), 68.30, 60.25 (CH$_2$Ph), 56.95 (C-2), 23.40 (CH$_3$CO). ^{31}P NMR (CDCl$_3$, 162 MHz): δ = 0.32. ESI HR-MS (C$_{37}$H$_{40}$NO$_9$P): m/z = ([*M*+H]$^+$ found 674.2476; calc 674.2519) .

[0225] *2-Acetamido-2-deoxy-β-D-glucopyranosyl phosphate* 6. The protected monosaccharide **5** (100 mg, 0.15 mmol) was dissolved in MeOH (10 ml) and hydrogenated over 10% Pd/C (30 mg). The mixture was stirred for 1 d, then it was filtered through a celite pad. The solvent was evaporated and the recovered crude material was purified on a C-18 Isolute SPE cartridge. Fractions containing the sugar were freeze-dried to give 42 mg of foamy product **6** (95%), whose NMR data were in agreement with those reported in literature [238].

[0226] *High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) for MenX quantification:* MenX samples were treated with TFA at a final concentration of 2 M diluted to a total volume of 600 μL in the range 0.5-8 μg/mL. Samples were heated at 100°C for 2.5 hours in a closed screw-cap test tube, then chilled at 2-8°C for about 30 minutes, added of 700 μL NaOH 2 M and filtered with 0.45 μm Acrodisc (PALL) filters before analysis. A pure preparation of MenX PS or the synthetic monomer *4-GlcNAc-4P*, titered through the colorimetric method for total phosphorus content, were used for building the calibration curve, set up with standards in the range of 0.5-8 μg/mL. HPAEC-PAD was performed with a Dionex ICS3000 equipped with a CarboPac PA1 column (4 x 250 mm; Dionex) coupled with PA1 guard column (4 x 50 mm; Dionex). Samples were run with a flow rate of 1 mL/min, using a gradient in 10 minutes from 100 mM to 500 mM AcONa in 100 mM NaOH. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A quadruple-potential waveform for carbohydrates was applied. The resulting chromatographic data were processed using Chromeleon software 6.8.

[0227] *Acid hydrolysis of MenX polysaccharide and GlcNAc-4P and NMR characterization:* A large scale acid hydrolysis was conducted on MenX polysaccharide and on the synthetic monomer (10 mg). Both the samples were dissolved in 2 mL 2 M TFA and hydrolysed at 100°C for 2.5 hours. The samples were dried and exchanged with D_2O for three times before analysis.

[0228] *Selection of hydrolysis conditions:* To identify the optimal conditions for MenX hydrolysis able to completely release the monomer subunits and minimize their degradation, different reaction times for the hydrolysis of MenX polysaccharide (from 1 to 6 hours) performing the hydrolysis in 2 M TFA at 100°C were explored. A pure preparation of MenX polysaccharide, titered through the colorimetric method for total phosphorus content, was used at two different concentrations (0.5 and 2 μg/mL). One prevalent peak was detected by HPAEC-PAD analysis. The area of the peak increased over time, with a maximum among two and three hours, before decreasing for longer times. Eventually 2.5 hours was selected as optimal time of hydrolysis

[0229] The linearity of the method was verified in the range 0.5-8 μg/mL (R^2 = 99.807). The method was successfully applied to purification process intermediates, including fermentation broths, and in order to determine the accuracy of the method a recovery study was conducted. Known amounts of polysaccharide were added to standard samples that were subjected to the analysis. The recovery was calculated based on the difference between the total concentration determined for the spiked samples and the concentration found in the un-spiked samples. The mean recovery ranged from 98 to 102%, indicating a high grade of accuracy. Repeatability inter-analysis was performed by analyzing the same sample four times with a CV of 1% and a corresponding average CV of 0.5%.

[0230] *Synthesis of 4P-GlcNAc:* As shown in Scheme 1, the synthesis of target compound **6** commenced from the regioselective ring opening of protected GlcN **2** (92% yield), which was prepared from galactosamine hydrochloride as described in reference 236. Removal of the *N*-phthalimido protection by means of ethylenediamine, followed by selective *N*-acetylation provided known compound **4** in 87% yield [237]. Reaction of **4** with *N,N*-diethyl-1,5-dihydro-3*H*-2,3,4-benzodioxaphosphepin-3 amine and 1*H*-tetrazole and subsequent oxidation with *m*-chloroperbenzoic acid (*m*-CPBA) enabled the phosphate group introduction in significantly higher yield than previously reported with other methods [238] and furnished crystalline shelf stable compound **5** (m. p. 159-160°C). A phosphomonoester peak at 0.32 ppm in the ^{31}P NMR spectrum, which correlated with the H-4 signal at 4.58 ppm and two couples of C*H* systems (5.13, 4.98 and 5.14, 4.99 ppm respectively) in the ^{1}H-^{31}P HMBC NMR spectrum allowed to assess the structure of **5.** Finally hydrogenolysis over 10% Pd-C provided the target 4P-GlcNAc **6** in excellent yield (95%) respect to 50% yield attained when unprotected phosphate was present. NMR data of the final product were in good agreement with those reported in literature [239].
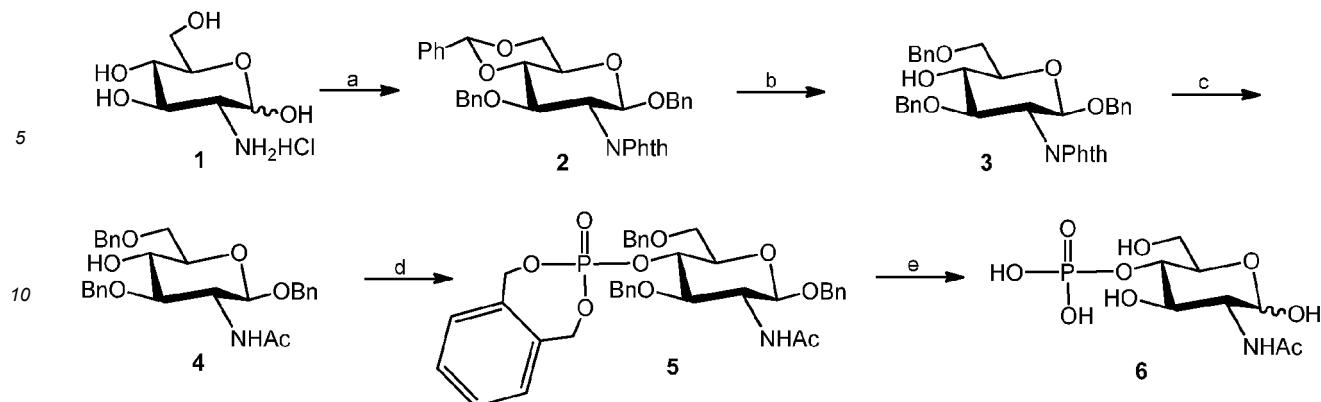
[0231] *NMR characterization of the products formed by acid hydrolysis of MenX polysaccharide or 4P-GlcNAc:* MenX polysaccharide and the synthetic monomer **6** were hydrolyzed at larger scale according to the procedure optimized for the HPAEC-PAD analysis in order to confirm the structure of the resulting species by NMR analysis. In both cases 4P-GlcNH was assessed as the prevalent species.

^{1}H NMR spectrum of 4P-GlcNAc **6** showed the α/β anomeric peaks at 5.19 and 4.72 ppm respectively, and the proton signals in the range 4.00-3.69 ppm. H-2α and H-2β were assigned at 3.91 ppm and 3.72 ppm signals by homo-nuclear COSY NMR correlation. One single peak for phosphate monoester at 0.58 ppm was detected at the ^{31}P NMR

[0232] After hydrolysis of both the standard **6** and the native MenX PS, ^{1}H NMR analysis of the attained 4P-GlcN showed two major anomeric signals corresponding at α/β mixtures in the ratio of 5.5:4.5 and 6.7:3.3 at 5.40 and 4.92 ppm, respectively. Remaining ring proton signals fell between 4.08 and 3.44 ppm, while H-2α (dd, *J* 3.7 and 10.3 Hz, at 3.91 ppm) and H-2β (dd, *J* 8.5 and 10.5 Hz, at 3.06 ppm) were shifted up-field due to the loss of the acetyl group. Furthermore, no *N*-acetyl CH_3 signals were detected indicating that hydrolysis resulted in total de-*N*-acetylation.

[0233] Bidimensional ^{1}H-^{31}P HMBC NMR evidenced two overlapping cross peaks, assigned to the phosphate monoester signals at 0.68 and 0.14 ppm of the ^{31}P NMR spectrum, correlating with H-4α and H-4β at 3.94 and 3.96 ppm, respectively, in the ^{1}H NMR

[0234] *Use of 4P-GlcNAc as standard for MenX quantification by HPAEC-PAD:* The synthetic monomer **6** was quantified by the colorimetric method for total phosphorus content and then used for building a calibration curve (in the range 0.5-8 μg/mL) in comparison to the native MenX polysaccharide. After subjecting the synthetic monomer and the native polysaccharide to the same hydrolysis conditions optimized for MenX polysaccharide samples, the same peak was detected by HPAEC-PAD and the curves obtained perfectly overlapped. The concentration of unknown samples and intermediates of the polysaccharide purification process was consistent independently from the curve used for the quantification (the difference in saccharide concentration values was < 2% for all the tested samples). Mixtures of hydrolyzed MenX polysaccharide and synthetic monomer were also analyzed by HPAEC-PAD on a CarboPac PA1 column, eluting with 10 mM sodium hydroxide, to verify the eventual formation of GlcN in the hydrolysis conditions used [26]. Formation of GlcN was less than 5% in moles both for native MenX and synthetic monomer samples.

[0235] We also verified the possibility to use the commercially available glucosamine-6-phosphate (6P-GlcN) as standard for the analysis, using the same hydrolysis conditions optimized for MenX. The resulting calibration curve overlapped those obtained with native MenX and its synthetic monomer, but the elution time of the resulting peak detected by HPAEC-PAD was different (8.97 min against 9.88 min for MenX), demonstrating that utilization of 4P-GlcNAc is more straightforward.

[0236] This method for MenX polysaccharide quantification is a crucial analytical tool for monitoring the saccharide content of purification process intermediates and a final conjugate vaccine. In addition to allowing the process yield to be calculated, the quantification allows calculation of the saccharide/protein ratio of the conjugate and the % of free saccharide, both of which are important parameters for verifying the quality and consistency of a final vaccine formulation.

[0237] The use of a synthetic monomer means that there is no need for the standardization of a batch of polysaccharide for the analysis. The overall method is rapid, permits detection of very low concentrations of sugar ($\geq$ 0.5 μg/mL of polysaccharide), with minimal sample clean-up and has been verified to work well for the characterization of purification process intermediates, including fermentation broths. The method may be suitable for the quantification of intermediates of conjugation processes and for the characterization of the final vaccine formulations.

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1               5               10              15

Ala Leu Phe Ala Val Phe Ala Pro Ser Phe Val Ser Ala Gln Glu Ser
            20              25              30

Ser Thr Tyr Thr Val Lys Glu Gly Asp Thr Leu Ser Glu Ile Ala Glu
        35              40              45

Thr His Asn Thr Thr Val Glu Lys Leu Ala Glu Asn Asn His Ile Asp
    50              55              60

Asn Ile His Leu Ile Tyr Val Asp Gln Glu Leu Val Ile Asp Gly Pro
65              70              75              80

Val Ala Pro Val Ala Thr Pro Ala Pro Ala Thr Tyr Ala Ala Pro Ala
            85              90              95

Ala Gln Asp Glu Thr Val Ser Ala Pro Val Ala Glu Thr Pro Val Val
            100             105             110

Ser Glu Thr Val Val Ser Thr Val Ser Gly Ser Glu Ala Glu Ala Lys
        115             120             125

Glu Trp Ile Ala Gln Lys Glu Ser Gly Gly Ser Tyr Thr Ala Thr Asn
    130             135             140

Gly Arg Tyr Ile Gly Arg Tyr Gly Ser Trp Thr Ala Ala Lys Asn Phe
145             150             155             160

Trp Leu Asn Asn Gly Trp Tyr
                165
```

<210> 2
<211> 195
<212> PRT
<213> Streptococcus pneumoniae

<400> 2

```
Met Lys Ser Ile Thr Lys Lys Ile Lys Ala Thr Leu Ala Gly Val Ala
1               5               10                  15

Ala Leu Phe Ala Val Phe Ala Pro Ser Phe Val Ser Ala Gln Glu Ser
        20              25                  30

Ser Thr Tyr Thr Val Lys Glu Gly Asp Thr Leu Ser Glu Ile Ala Glu
        35              40                  45

Thr His Asn Thr Thr Val Glu Lys Leu Ala Glu Asn Asn His Ile Asp
    50              55                  60

Asn Ile His Leu Ile Tyr Val Asp Gln Glu Leu Val Ile Asp Gly Pro
65                  70              75                  80

Val Ala Pro Val Ala Thr Pro Ala Pro Ala Thr Tyr Ala Ala Pro Ala
            85              90                  95

Ala Gln Asp Glu Thr Val Ser Ala Pro Val Ala Glu Thr Pro Val Val
            100             105                 110

Ser Glu Thr Val Val Ser Thr Val Ser Gly Ser Glu Ala Glu Ala Lys
        115             120                 125

Glu Trp Ile Ala Gln Lys Glu Ser Gly Gly Ser Tyr Thr Ala Thr Asn
    130             135                 140

Gly Arg Tyr Ile Gly Arg Tyr Gln Leu Thr Asp Ser Tyr Leu Asn Gly
145                 150             155                 160

Asp Tyr Ser Ala Glu Asn Gln Glu Arg Val Ala Asp Ala Tyr Val Ala
            165             170                 175

Gly Arg Tyr Gly Ser Trp Thr Ala Ala Lys Asn Phe Trp Leu Asn Asn
        180             185                 190

Gly Trp Tyr
        195
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<210> 3
<211> 392
<212> PRT
<213> Streptococcus pneumoniae

<400> 3

```
Met Lys Lys Lys Ile Leu Ala Ser Leu Leu Leu Ser Thr Val Met Val
1               5               10                  15

Ser Gln Val Ala Val Leu Thr Thr Ala His Ala Glu Thr Thr Asp Asp
        20              25                  30

Lys Ile Ala Ala Gln Asp Asn Lys Ile Ser Asn Leu Thr Ala Gln Gln
        35              40                  45

Gln Glu Ala Gln Lys Gln Val Asp Gln Ile Gln Glu Gln Val Ser Ala
        50              55                  60
```

44

```
Ile Gln Ala Glu Gln Ser Asn Leu Gln Ala Glu Asn Asp Arg Leu Gln
65              70              75              80

Ala Glu Ser Lys Lys Leu Glu Gly Glu Ile Thr Glu Leu Ser Lys Asn
                85              90              95

Ile Val Ser Arg Asn Gln Ser Leu Glu Lys Gln Ala Arg Ser Ala Gln
            100             105             110

Thr Asn Gly Ala Val Thr Ser Tyr Ile Asn Thr Ile Val Asn Ser Lys
            115             120             125

Ser Ile Thr Glu Ala Ile Ser Arg Val Ala Ala Met Ser Glu Ile Val
    130             135             140

Ser Ala Asn Asn Lys Met Leu Glu Gln Gln Lys Ala Asp Lys Lys Ala
145             150             155             160

Ile Ser Glu Lys Gln Val Ala Asn Asn Asp Ala Ile Asn Thr Val Ile
            165             170             175

Ala Asn Gln Gln Lys Leu Ala Asp Asp Ala Gln Ala Leu Thr Thr Lys
            180             185             190

Gln Ala Glu Leu Lys Ala Ala Glu Leu Ser Leu Ala Ala Glu Lys Ala
    195             200             205

Thr Ala Glu Gly Glu Lys Ala Ser Leu Leu Glu Gln Lys Ala Ala Ala
    210             215             220

Glu Ala Glu Ala Arg Ala Ala Ala Val Ala Glu Ala Ala Tyr Lys Glu
225             230             235             240

Lys Arg Ala Ser Gln Gln Gln Ser Val Leu Ala Ser Ala Asn Thr Asn
            245             250             255

Leu Thr Ala Gln Val Gln Ala Val Ser Glu Ser Ala Ala Ala Pro Val
            260             265             270

Arg Ala Lys Val Arg Pro Thr Tyr Ser Thr Asn Ala Ser Ser Tyr Pro
    275             280             285

Ile Gly Glu Cys Thr Trp Gly Val Lys Thr Leu Ala Pro Trp Ala Gly
    290             295             300

Asp Tyr Trp Gly Asn Gly Ala Gln Trp Ala Thr Ser Ala Ala Ala Ala
305             310             315             320

Gly Phe Arg Thr Gly Ser Thr Pro Gln Val Gly Ala Ile Ala Cys Trp
            325             330             335

Asn Asp Gly Gly Tyr Gly His Val Ala Val Val Thr Ala Val Glu Ser
            340             345             350

Thr Thr Arg Ile Gln Val Ser Glu Ser Asn Tyr Ala Gly Asn Arg Thr
    355             360             365

Ile Gly Asn His Arg Gly Trp Phe Asn Pro Thr Thr Thr Ser Glu Gly
    370             375             380

Phe Val Thr Tyr Ile Tyr Ala Asp
385             390
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<210> 4
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<212> **PRT**
<213> Streptococcus pneumoniae

<400> 4

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1               5               10              15

Asn Leu Thr Ala Gln Gln Gln Glu Ala Gln Lys Gln Val Asp Gln Ile
            20              25              30

Gln Glu Gln Val Ser Ala Ile Gln Ala Glu Gln Ser Asn Leu Gln Ala
        35              40              45

Glu Asn Asp Arg Leu Gln Ala Glu Ser Lys Lys Leu Glu Gly Glu Ile
        50              55              60

Thr Glu Leu Ser Lys Asn Ile Val Ser Arg Asn Gln Ser Leu Glu Lys
65              70              75              80

Gln Ala Arg Ser Ala Gln Thr Asn Gly Ala Val Thr Ser Tyr Ile Asn
            85              90              95

Thr Ile Val Asn Ser Lys Ser Ile Thr Glu Ala Ile Ser Arg Val Ala
            100             105             110

Ala Met Ser Glu Ile Val Ser Ala Asn Asn Lys Met Leu Glu Gln Gln
        115             120             125

Lys Ala Asp Lys Lys Ala Ile Ser Glu Lys Gln Val Ala Asn Asn Asp
        130             135             140

Ala Ile Asn Thr Val Ile Ala Asn Gln Gln Lys Leu Ala Asp Asp Ala
145             150             155             160

Gln Ala Leu Thr Thr Lys Gln Ala Glu Leu Lys Ala Ala Glu Leu Ser
            165             170             175

Leu Ala Ala Glu Lys Ala Thr Ala Glu Gly Glu Lys Ala Ser Leu Leu
        180             185             190

Glu Gln Lys Ala Ala Ala Glu Ala Glu Ala Arg Ala Ala Ala Val Ala
        195             200             205

Glu Ala Ala Tyr Lys Glu Lys Arg Ala Ser Gln Gln Gln Ser Val Leu
        210             215             220

Ala Ser Ala Asn Thr Asn Leu Thr Ala Gln Val Gln Ala Val Ser Glu
225             230             235             240

Ser Ala Ala Ala Pro Val Arg Ala Lys Val Arg Pro Thr Tyr Ser Thr
            245             250             255

Asn Ala Ser Ser Tyr Pro Ile Gly Glu Cys Thr Trp Gly Val Lys Thr
            260             265             270

Leu Ala Pro Trp Ala Gly Asp Tyr Trp Gly Asn Gly Ala Gln Trp Ala
            275             280             285

Thr Ser Ala Ala Ala Ala Gly Phe Arg Thr Gly Ser Thr Pro Gln Val
            290             295             300
```

```
Gly Ala Ile Ala Cys Trp Asn Asp Gly Gly Tyr Gly His Val Ala Val
305                 310             315                 320

Val Thr Ala Val Glu Ser Thr Thr Arg Ile Gln Val Ser Glu Ser Asn
                325             330                 335

Tyr Ala Gly Asn Arg Thr Ile Gly Asn His Arg Gly Trp Phe Asn Pro
                340             345             350

Thr Thr Thr Ser Glu Gly Phe Val Thr Tyr Ile Tyr Ala Asp
                355             360             365
```

<210> 5
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<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic linker

<400> 5

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                    1                   5
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<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic linker

<400> 6

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                    1                   5
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<210> 7
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic linker

<400> 7

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                    1                   5
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<210> 8
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<212> PRT
<213> Artificial Sequence

<220>
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<400> 8

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                              1                 5
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<210> 9
<211> 555
<212> PRT
<213> Streptococcus pneumoniae

<400> 9

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                              His His His His His His
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Met Val Ser Ala Gln Glu Ser Ser Thr Tyr Thr Val Lys Glu Gly Asp
1               5               10              15

Thr Leu Ser Glu Ile Ala Glu Thr His Asn Thr Thr Val Glu Lys Leu
            20              25              30

Ala Glu Asn Asn His Ile Asp Asn Ile His Leu Ile Tyr Val Asp Gln
        35              40              45

Glu Leu Val Ile Asp Gly Pro Val Ala Pro Val Ala Thr Pro Ala Pro
    50              55              60

Ala Thr Tyr Ala Ala Pro Ala Ala Gln Asp Glu Thr Val Ser Ala Pro
65              70              75              80

Val Ala Glu Thr Pro Val Val Ser Glu Thr Val Val Ser Thr Val Ser
            85              90              95

Gly Ser Glu Ala Glu Ala Lys Glu Trp Ile Ala Gln Lys Glu Ser Gly
        100             105             110

Gly Ser Tyr Thr Ala Thr Asn Gly Arg Tyr Ile Gly Arg Tyr Gln Leu
        115             120             125

Thr Asp Ser Tyr Leu Asn Gly Asp Tyr Ser Ala Glu Asn Gln Glu Arg
    130             135             140

Val Ala Asp Ala Tyr Val Ala Gly Arg Tyr Gly Ser Trp Thr Ala Ala
145             150             155             160

Lys Asn Phe Trp Leu Asn Asn Gly Trp Tyr Gly Ser Gly Ser Gly Gly
            165             170             175

Gly Gly Ala Glu Thr Thr Asp Asp Lys Ile Ala Ala Gln Asp Asn Lys
        180             185             190

Ile Ser Asn Leu Thr Ala Gln Gln Gln Glu Ala Gln Lys Gln Val Asp
        195             200             205

Gln Ile Gln Glu Gln Val Ser Ala Ile Gln Ala Glu Gln Ser Asn Leu
    210             215             220

Gln Ala Glu Asn Asp Arg Leu Gln Ala Glu Ser Lys Lys Leu Glu Gly
225             230             235             240

Glu Ile Thr Glu Leu Ser Lys Asn Ile Val Ser Arg Asn Gln Ser Leu
            245             250             255

Glu Lys Gln Ala Arg Ser Ala Gln Thr Asn Gly Ala Val Thr Ser Tyr
        260             265             270

Ile Asn Thr Ile Val Asn Ser Lys Ser Ile Thr Glu Ala Ile Ser Arg
        275             280             285

Val Ala Ala Met Ser Glu Ile Val Ser Ala Asn Asn Lys Met Leu Glu
```

```
              290                    295                    300

        Gln Gln Lys Ala Asp Lys Lys Ala Ile Ser Glu Lys Gln Val Ala Asn
        305                 310                 315                 320

        Asn Asp Ala Ile Asn Thr Val Ile Ala Asn Gln Gln Lys Leu Ala Asp
                        325                 330                 335

        Asp Ala Gln Ala Leu Thr Thr Lys Gln Ala Glu Leu Lys Ala Ala Glu
                        340                 345                 350

        Leu Ser Leu Ala Ala Glu Lys Ala Thr Ala Glu Gly Glu Lys Ala Ser
                        355                 360                 365

        Leu Leu Glu Gln Lys Ala Ala Ala Glu Ala Glu Ala Arg Ala Ala Ala
                        370                 375                 380

        Val Ala Glu Ala Ala Tyr Lys Glu Lys Arg Ala Ser Gln Gln Gln Ser
        385                 390                 395                 400

        Val Leu Ala Ser Ala Asn Thr Asn Leu Thr Ala Gln Val Gln Ala Val
                        405                 410                 415

        Ser Glu Ser Ala Ala Ala Pro Val Arg Ala Lys Val Arg Pro Thr Tyr
                        420                 425                 430

        Ser Thr Asn Ala Ser Ser Tyr Pro Ile Gly Glu Cys Thr Trp Gly Val
                        435                 440                 445

        Lys Thr Leu Ala Pro Trp Ala Gly Asp Tyr Trp Gly Asn Gly Ala Gln
                        450                 455                 460

        Trp Ala Thr Ser Ala Ala Ala Ala Gly Phe Arg Thr Gly Ser Thr Pro
        465                 470                 475                 480

        Gln Val Gly Ala Ile Ala Cys Trp Asn Asp Gly Gly Tyr Gly His Val
                        485                 490                 495

        Ala Val Val Thr Ala Val Glu Ser Thr Thr Arg Ile Gln Val Ser Glu
                        500                 505                 510

        Ser Asn Tyr Ala Gly Asn Arg Thr Ile Gly Asn His Arg Gly Trp Phe
                        515                 520                 525

        Asn Pro Thr Thr Thr Ser Glu Gly Phe Val Thr Tyr Ile Tyr Ala Asp
                        530                 535                 540

        Ala Ala Ala Leu Glu His His His His His His
        545                 550                 555
```

<210> 10
<211> 552
<212> PRT
<213> Streptococcus pneumoniae

<400> 10

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        Met Ala Glu Thr Thr Asp Asp Lys Ile Ala Ala Gln Asp Asn Lys Ile
        1               5                   10                  15

        Ser Asn Leu Thr Ala Gln Gln Gln Glu Ala Gln Lys Gln Val Asp Gln
                        20                  25                  30
```

```
              Ile Gln Glu Gln Val Ser Ala Ile Gln Ala Glu Gln Ser Asn Leu Gln
                      35              40              45

        5     Ala Glu Asn Asp Arg Leu Gln Ala Glu Ser Lys Lys Leu Glu Gly Glu
                      50              55              60

              Ile Thr Glu Leu Ser Lys Asn Ile Val Ser Arg Asn Gln Ser Leu Glu
              65              70              75              80

       10     Lys Gln Ala Arg Ser Ala Gln Thr Asn Gly Ala Val Thr Ser Tyr Ile
                          85              90              95

              Asn Thr Ile Val Asn Ser Lys Ser Ile Thr Glu Ala Ile Ser Arg Val
                      100             105             110

       15     Ala Ala Met Ser Glu Ile Val Ser Ala Asn Asn Lys Met Leu Glu Gln
                      115             120             125

              Gln Lys Ala Asp Lys Lys Ala Ile Ser Glu Lys Gln Val Ala Asn Asn
                      130             135             140

       20     Asp Ala Ile Asn Thr Val Ile Ala Asn Gln Gln Lys Leu Ala Asp Asp
              145             150             155             160

              Ala Gln Ala Leu Thr Thr Lys Gln Ala Glu Leu Lys Ala Ala Glu Leu
                          165             170             175

       25     Ser Leu Ala Ala Glu Lys Ala Thr Ala Glu Gly Glu Lys Ala Ser Leu
                      180             185             190

              Leu Glu Gln Lys Ala Ala Ala Glu Ala Glu Ala Arg Ala Ala Ala Val
                      195             200             205

       30     Ala Glu Ala Ala Tyr Lys Glu Lys Arg Ala Ser Gln Gln Gln Ser Val
                      210             215             220

              Leu Ala Ser Ala Asn Thr Asn Leu Thr Ala Gln Val Gln Ala Val Ser
              225             230             235             240

       35     Glu Ser Ala Ala Ala Pro Val Arg Ala Lys Val Arg Pro Thr Tyr Ser
                          245             250             255

              Thr Asn Ala Ser Ser Tyr Pro Ile Gly Glu Cys Thr Trp Gly Val Lys
       40                 260             265             270

              Thr Leu Ala Pro Trp Ala Gly Asp Tyr Trp Gly Asn Gly Ala Gln Trp
                      275             280             285

       45     Ala Thr Ser Ala Ala Ala Gly Phe Arg Thr Gly Ser Thr Pro Gln
                      290             295             300

              Val Gly Ala Ile Ala Cys Trp Asn Asp Gly Gly Tyr Gly His Val Ala
              305             310             315             320

       50     Val Val Thr Ala Val Glu Ser Thr Thr Arg Ile Gln Val Ser Glu Ser
                          325             330             335

              Asn Tyr Ala Gly Asn Arg Thr Ile Gly Asn His Arg Gly Trp Phe Asn
                      340             345             350

       55     Pro Thr Thr Thr Ser Glu Gly Phe Val Thr Tyr Ile Tyr Ala Asp Gly
                      355             360             365
```

```
Ser Gly Ser Gly Gly Gly Gly Val Ser Ala Gln Glu Ser Ser Thr Tyr
    370             375             380

Thr Val Lys Glu Gly Asp Thr Leu Ser Glu Ile Ala Glu Thr His Asn
385             390             395                     400

Thr Thr Val Glu Lys Leu Ala Glu Asn Asn His Ile Asp Asn Ile His
                405             410                     415

Leu Ile Tyr Val Asp Gln Glu Leu Val Ile Asp Gly Pro Val Ala Pro
                420             425             430

Val Ala Thr Pro Ala Pro Ala Thr Tyr Ala Ala Pro Ala Ala Gln Asp
            435             440             445

Glu Thr Val Ser Ala Pro Val Ala Glu Thr Pro Val Val Ser Glu Thr
    450             455             460

Val Val Ser Thr Val Ser Gly Ser Glu Ala Glu Ala Lys Glu Trp Ile
465             470             475                     480

Ala Gln Lys Glu Ser Gly Gly Ser Tyr Thr Ala Thr Asn Gly Arg Tyr
            485             490             495

Ile Gly Arg Tyr Gln Leu Thr Asp Ser Tyr Leu Asn Gly Asp Tyr Ser
            500             505             510

Ala Glu Asn Gln Glu Arg Val Ala Asp Ala Tyr Val Ala Gly Arg Tyr
    515             520             525

Gly Ser Trp Thr Ala Ala Lys Asn Phe Trp Leu Asn Asn Gly Trp Tyr
    530             535             540

Leu Glu His His His His His His
545             550
```

1. An immunogenic composition comprising a conjugate of a *Neisseria meningitidis* serogroup X capsular polysaccharide and a carrier protein, wherein coupling of the capsular polysaccharide to the carrier protein is direct.

2. The immunogenic composition of claim 1, wherein the capsular polysaccharide is an oligosaccharide and wherein the final average degree of polymerization (DP) in the oligosaccharide is between 20 and 200.

3. The immunogenic composition of claim 1 or 2, wherein the carrier protein is a diphtheria or tetanus toxoid, CRM197 or protein D.

4. The immunogenic composition of claim 1 or 2, wherein the carrier protein comprises a spr0096 antigen and a spr2021 antigen.

5. The immunogenic composition of any preceding claim, further comprising one or more further antigens.

6. The immunogenic composition of claim 5, further comprising a *Neisseria meningitidis* serogroup A capsular polysaccharide optionally conjugated to a carrier molecule.

7. The immunogenic composition of any one of claims 1 to 6, wherein the composition is in an aqueous formulation.

8. The immunogenic composition any one of claims 1 to 7, wherein the conjugate is obtainable by a process comprising the steps of: (a) oxidising a primary hydroxyl group in the capsular polysaccharide, to give an oxidised polysaccharide with an aldehyde group; and (b) coupling the oxidised polysaccharide to the carrier protein via the aldehyde group,

thereby giving the conjugate.

9. The immunogenic composition of claim 8, where the oxidation in step (a) is of the primary hydroxyl group on between 1-10% of the residues in the capsular polysaccharide.

10. The immunogenic composition of any one of claims 1 to 7, wherein the conjugate is obtainable by a process comprising the steps of: (a) reductive amination of the reducing terminus of the capsular polysaccharide, to give a modified polysaccharide with a primary amine group bonded to the C-1 atom of the terminal subunit by a covalent bond; and (b) coupling the modified polysaccharide to the carrier protein via the primary amine group, thereby giving the conjugate.

11. The immunogenic composition of any one of claim 1 to 7, wherein the conjugate is obtainable by a process comprising the steps of: (a) reduction of the reducing terminus of the capsular polysaccharide, to give a modified polysaccharide with two vicinal hydroxyl groups at that terminus; (b) oxidative cleavage of the vicinal hydroxyl groups, to give a further modified polysaccharide with an aldehyde group at the terminus; (c) reductive amination of the aldehyde group, to give a further modified polysaccharide with a primary amine group at the terminus and (d) coupling the further modified polysaccharide to the carrier protein via the primary amine group, thereby giving the conjugate.

12. A vaccine comprising the immunogenic composition of any preceding claim.

13. The immunogenic composition of any one of claims 1 to 11 or the vaccine of claim 12 for use in a method of raising an immune response in a mammal comprising administering to the mammal the immunogenic composition.

Patentansprüche

1. Immunogene Zusammensetzung, umfassend ein Konjugat eines *Neisseria* meningitidis-Serogruppe X-Kapselpolysaccharids und eines Trägerproteins, wobei das Koppeln des Kapselpolysaccharids an das Trägerprotein direkt erfolgt.

2. Immunogene Zusammensetzung nach Anspruch 1, wobei das Kapselpolysaccharid ein Oligosaccharid ist und wobei der durchschnittliche Endgrad der Polymerisation (degree of polymerization; DP) in dem Oligosaccharid zwischen 20 und 200 beträgt.

3. Immunogene Zusammensetzung nach Anspruch 1 oder 2, wobei das Trägerprotein ein Diphtherie- oder Tetanustoxoid, CRM197 oder Protein D ist.

4. Immunogene Zusammensetzung nach Anspruch 1 oder 2, wobei das Trägerprotein ein spr0096-Antigen und ein spr2021-Antigen umfasst.

5. Immunogene Zusammensetzung nach irgendeinem vorangegangenen Anspruch, welche weiterhin ein oder mehrere weitere Antigene umfasst.

6. Immunogene Zusammensetzung nach Anspruch 5, weiterhin umfassend ein *Neisseria meningitidis*-Serogruppe A-Kapselpolysaccharid, welches optional an ein Trägermolekül konjugiert ist.

7. Immunogene Zusammensetzung nach irgendeinem der Ansprüche 1 bis 6, wobei die Zusammensetzung in einer wässrigen Formulierung vorliegt.

8. Immunogene Zusammensetzung nach irgendeinem der Ansprüche 1 bis 7, wobei das Konjugat durch ein Verfahren erhalten wird, welches die Schritte umfasst: (a) Oxidieren einer primären Hydroxylgruppe in dem Kapselpolysaccharid unter Erhalt eines oxidierten Polysaccharids mit einer Aldehydgruppe; und (b) Koppeln des oxidierten Polysaccharids an das Trägerprotein über die Aldehydgruppe, wodurch sich das Konjugat ergibt.

9. Immunogene Zusammensetzung nach Anspruch 8, wobei die Oxidation in Schritt (a) an der primären Hydroxylgruppe an zwischen 1-10% der Reste in dem Kapselpolysaccharid erfolgt.

10. Immunogene Zusammensetzung nach irgendeinem der Ansprüche 1 bis 7, wobei das Konjugat durch ein Verfahren

erhältlich ist, welches die Schritte umfasst: (a) reduktive Aminierung des reduzierenden Terminus des Kapselpolysaccharids unter Erhalt eines modifizierten Polysaccharids mit einer primären Aminogruppe, die mit dem C-1-Atom der terminalen Untereinheit durch eine kovalente Bindung verbunden ist; und (b) Koppeln des modifizierten Polysaccharids an das Trägerprotein über die primäre Amingruppe, wodurch sich das Konjugat ergibt.

11. Immunogene Zusammensetzung nach irgendeinem der Ansprüche 1 bis 7, wobei das Konjugat durch ein Verfahren erhältlich ist, welches die Schritte umfasst: (a) Reduzieren des reduzierenden Terminus des Kapselpolysaccharids unter Erhalt eines modifizierten Polysaccharids mit zwei vicinalen Hydroxylgruppen an diesem Terminus; (b) oxidative Spaltung der vicinalen Hydroxylgruppen unter Erhalt eines weiteren modifizierten Polysaccharids mit einer Aldehydgruppe am Terminus; (c) reduktive Aminierung der Aldehydgruppe unter Erhalt eines weiteren modifizierten Polysaccharids mit einer primären Amingruppe am Terminus und (d) Koppeln des weiteren modifizierten Polysaccharids an das Trägerprotein über die primäre Amingruppe, wodurch sich das Konjugat ergibt.

12. Impfstoff, welcher die immunogene Zusammensetzung nach irgendeinem vorangegangenen Anspruch umfasst.

13. Immunogene Zusammensetzung nach irgendeinem der Ansprüche 1 bis 11 oder Impfstoff nach Anspruch 12 zur Verwendung in einem Verfahren zum Hervorrufen einer Immunantwort in einem Säugetier, umfassend das Verabreichen der immunogenen Zusammensetzung an das Säugetier.

Revendications

1. Composition immunogène comprenant un conjugué d'un polysaccharide capsulaire de sérogroupe X *Neisseria meningitidis* et une protéine porteuse, dans laquelle le couplage du polysaccharide capsulaire avec la protéine porteuse est direct.

2. Composition immunogène selon la revendication 1, dans laquelle le polysaccharide capsulaire est un oligosaccharide et dans laquelle le degré moyen final de polymérisation (DP) dans l'oligosaccharide se situe entre 20 et 200.

3. Composition immunogène selon la revendication 1 ou 2, dans laquelle la protéine porteuse est une anatoxine diphtérique ou tétanique, du CRM197 ou une protéine D.

4. Composition immunogène selon la revendication 1 ou 2, dans laquelle la protéine porteuse comprend un antigène spr0096 et un antigène spr2021.

5. Composition immunogène selon l'une quelconque des revendications précédentes, comprenant en outre un ou plusieurs autres antigènes.

6. Composition immunogène selon la revendication 5, comprenant en outre un polysaccharide capsulaire de sérogroupe A *Neisseria meningitidis* éventuellement conjugué avec une molécule porteuse.

7. Composition immunogène selon l'une quelconque des revendications 1 à 6, dans laquelle la composition se trouve dans une formulation aqueuse.

8. Composition immunogène selon l'une quelconque des revendications 1 à 7, dans laquelle le conjugué peut être obtenu par un processus comprenant les étapes consistant : (a) à oxyder un groupement hydroxyle primaire dans le polysaccharide capsulaire pour donner un polysaccharide oxydé avec un groupement aldéhyde ; et (b) à coupler le polysaccharide oxydé avec la protéine porteuse via le groupement aldéhyde, en donnant ainsi le conjugué.

9. Composition immunogène selon la revendication 8, dans laquelle l'oxydation à l'étape (a) est celle du groupement hydroxyle primaire sur une portion comprise entre 1 et 10 % des résidus dans le polysaccharide capsulaire.

10. Composition immunogène selon l'une quelconque des revendications 1 à 7, dans laquelle le conjugué peut être obtenu par un processus comprenant les étapes : (a) d'amination réductrice du terminal réducteur du polysaccharide capsulaire pour donner un polysaccharide modifié avec un groupement amine primaire lié à l'atome C-1 de la sous-unité terminale par une liaison covalente ; et (b) de couplage du polysaccharide modifié avec la protéine porteuse via le groupement amine primaire, en donnant ainsi le conjugué.

11. Composition immunogène selon l'une quelconque des revendications 1 à 7, dans laquelle le conjugué peut être obtenu par un processus comprenant les étapes : (a) de réduction du terminal réducteur du polysaccharide capsulaire pour donner un polysaccharide modifié avec deux groupements hydroxyle vicinaux sur ce terminal ; (b) de clivage oxydant des groupements hydroxyle vicinaux pour donner un autre polysaccharide modifié avec un groupement aldéhyde au terminal ; (c) d'amination réductrice du groupement aldéhyde pour donner un autre polysaccharide modifié avec un groupement amine primaire au terminal et (d) de couplage de l'autre polysaccharide modifié avec la protéine porteuse via le groupement amine primaire, en donnant ainsi le conjugué.

12. Vaccin comprenant la composition immunogène selon l'une quelconque des revendications précédentes.

13. Composition immunogène selon l'une quelconque des revendications 1 à 11 ou vaccin selon la revendication 12 pour utilisation dans un procédé permettant d'obtenir une réponse immunitaire chez un mammifère, comprenant l'administration au mammifère de la composition immunogène.

FIGURE 1

FIGURE 2

Native MenX PS
MenX avDP 80 after 1h hydrolysis
MenX avDP 15 after 2h hydrolysis
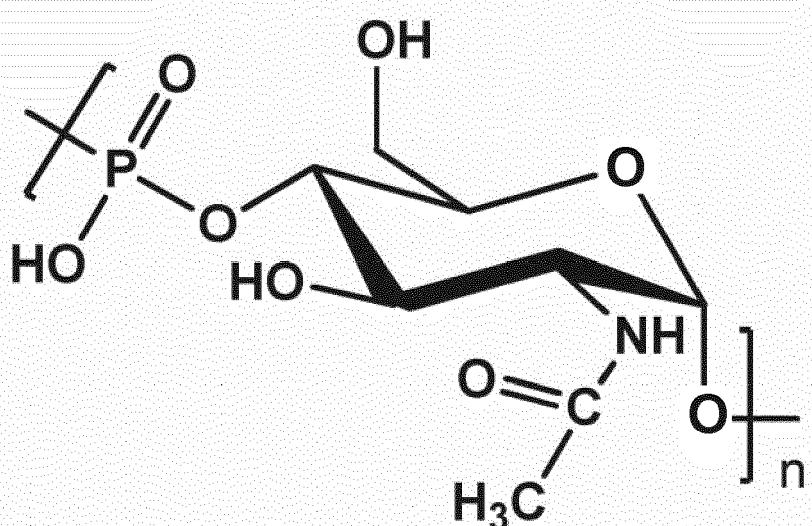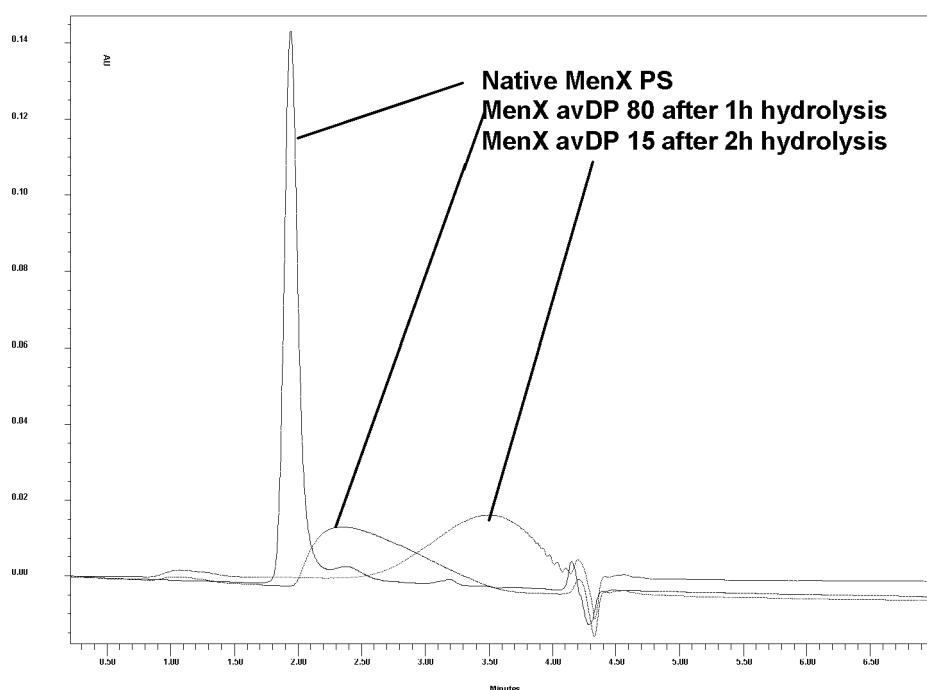
Acquity UPLC BEH200 1.7 μm (4.6 x 150 mm), 214 nm detection,
100mM NaCl 100mM NaPi 30% CH_3CN pH 7.2; Vtot 4.343 min

FIGURE 3

1. Marker
2. MenX-CRM197
3. CRM197

FIGURE 4

Pool 1 = fr 14-19
Pool 2 = fr 20-22
Pool 3 = fr 23-36

FIGURE 5
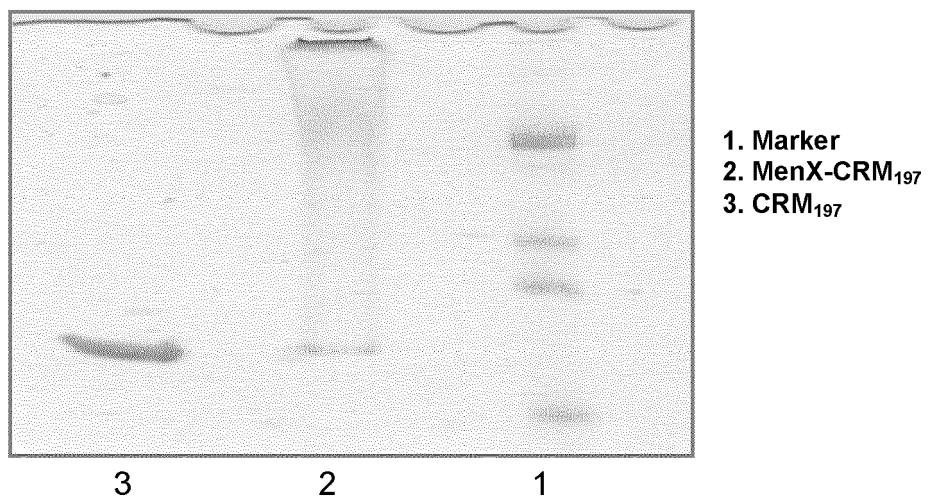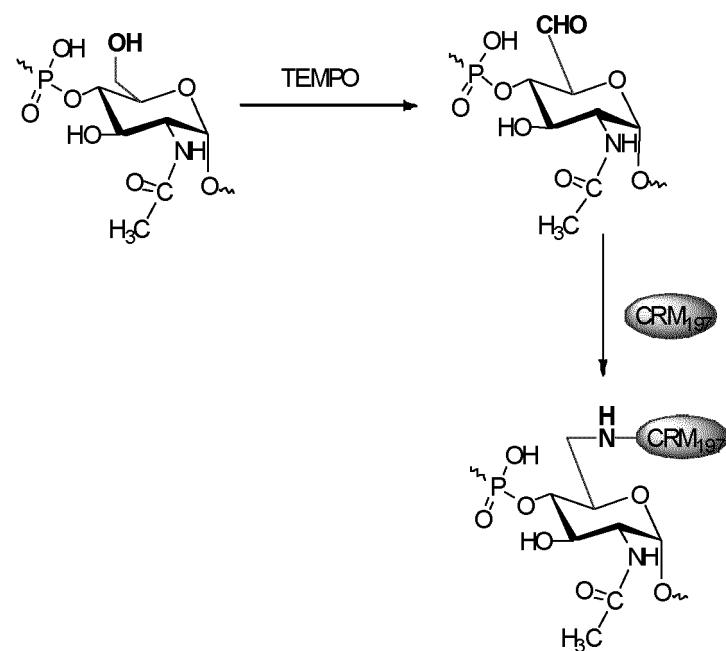
1. Marker
2. CRM197
3. MenX-CRM197
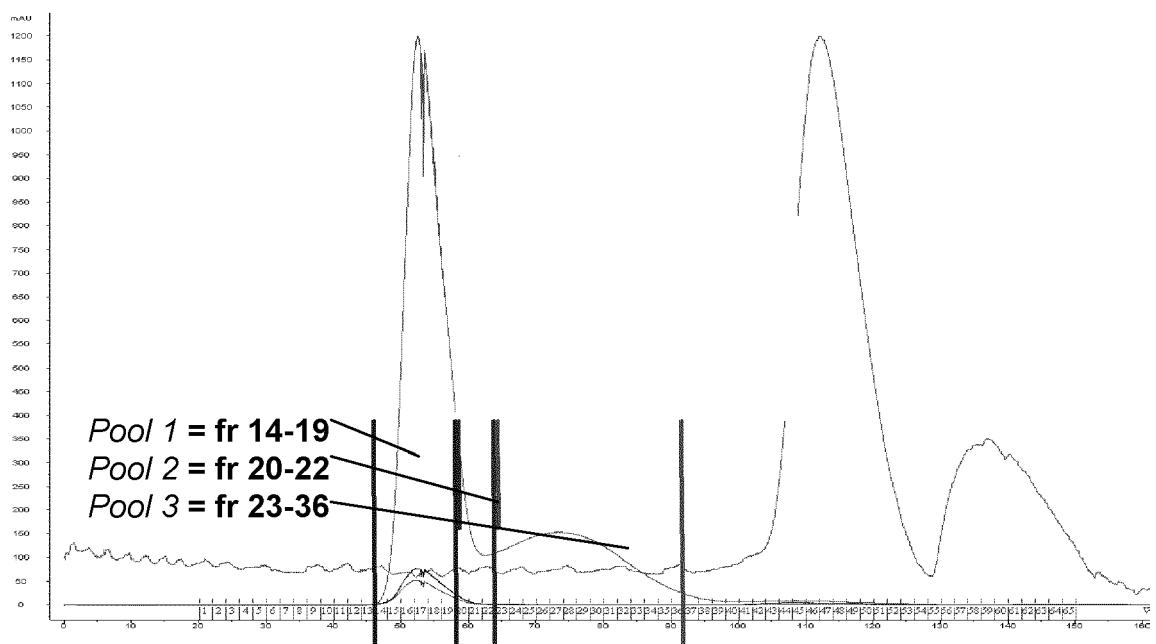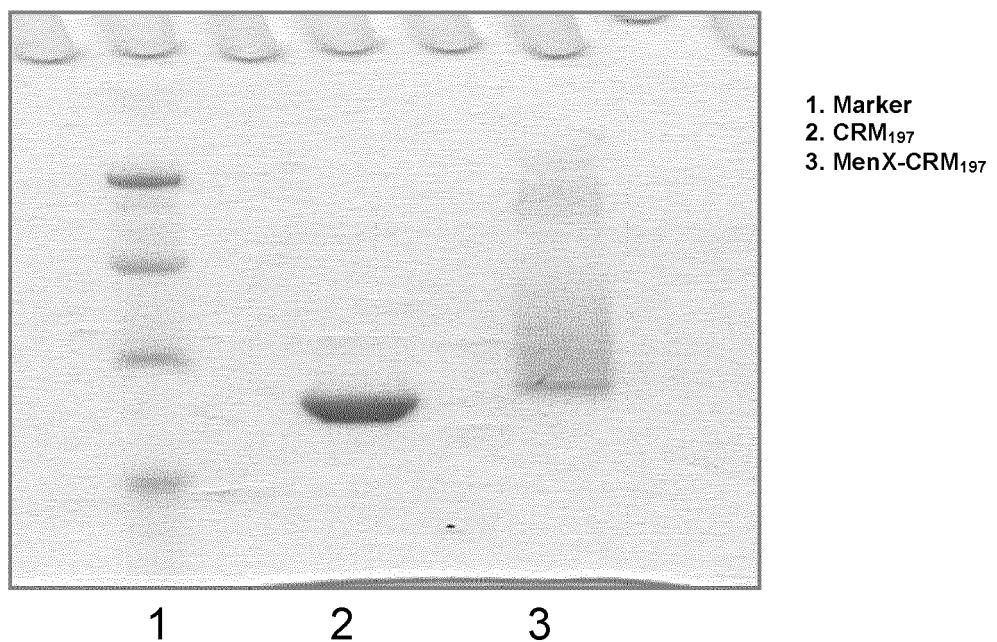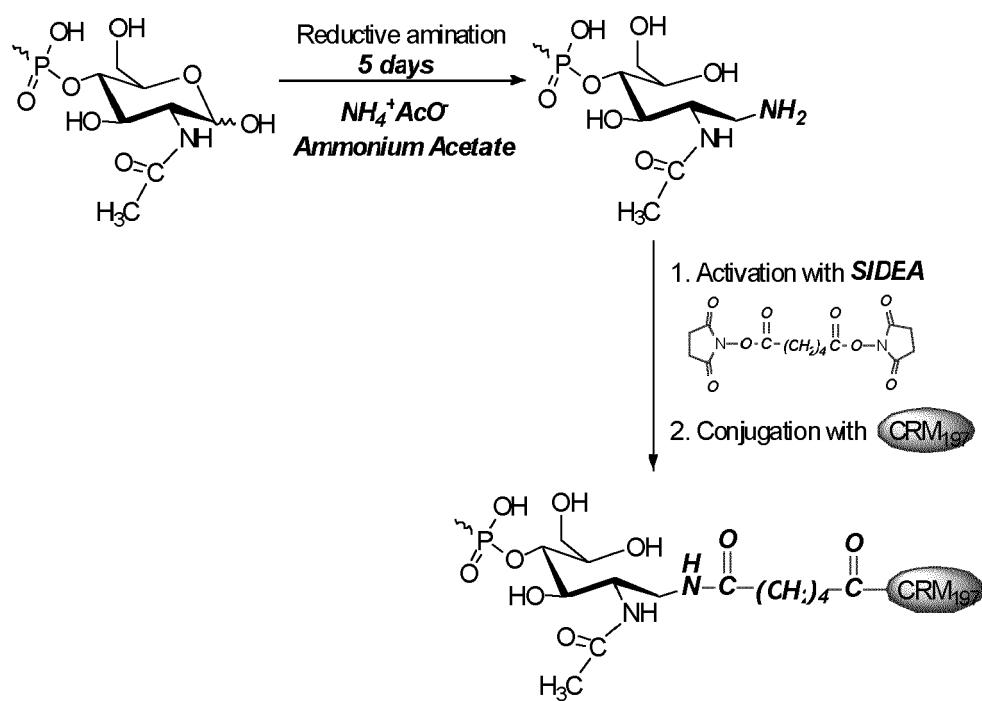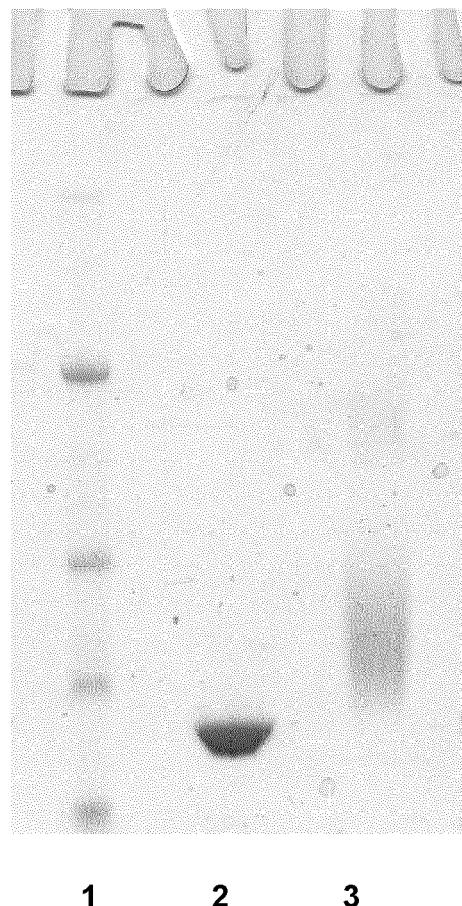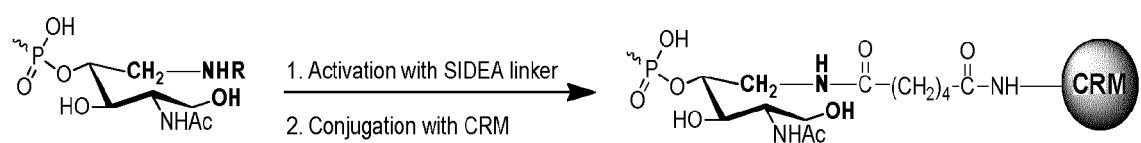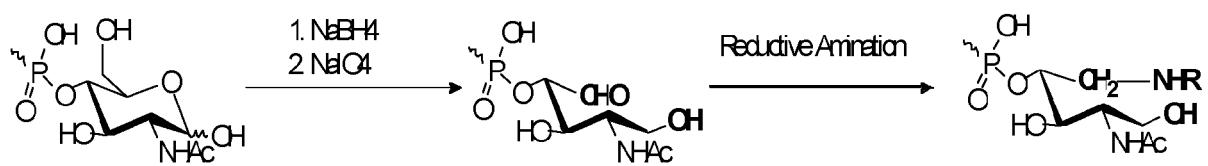
FIGURE 6

SDS-Page 7%TA
1. Marker
2. CRM$_{197}$
3. MenX-CRM$_{197}$

1   2   3

FIGURE 7

1. Marker
2. CRM$_{197}$
3. MenX-CRM$_{197}$

1   2   3

FIGURE 8

rSBA titer								
<4	4096	4096	4096	256	1024	1024	<4	4096

FIGURE 9

rSBA titer			
4096	2048	2048	2048

FIGURE 10

rSBA titer			
4096	4096	4096	8192

FIGURE 11

	rSBA titer			
	512	1024	1024	1024

FIGURE 12

rSBA titer			
1024	1024	1024	2048
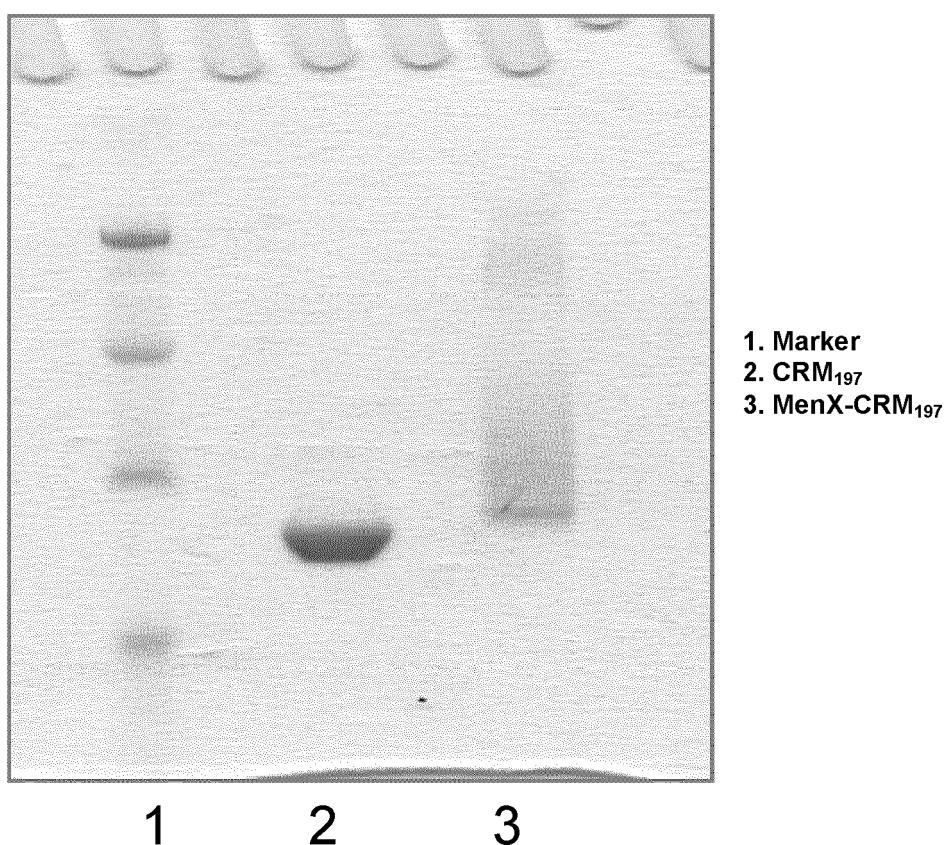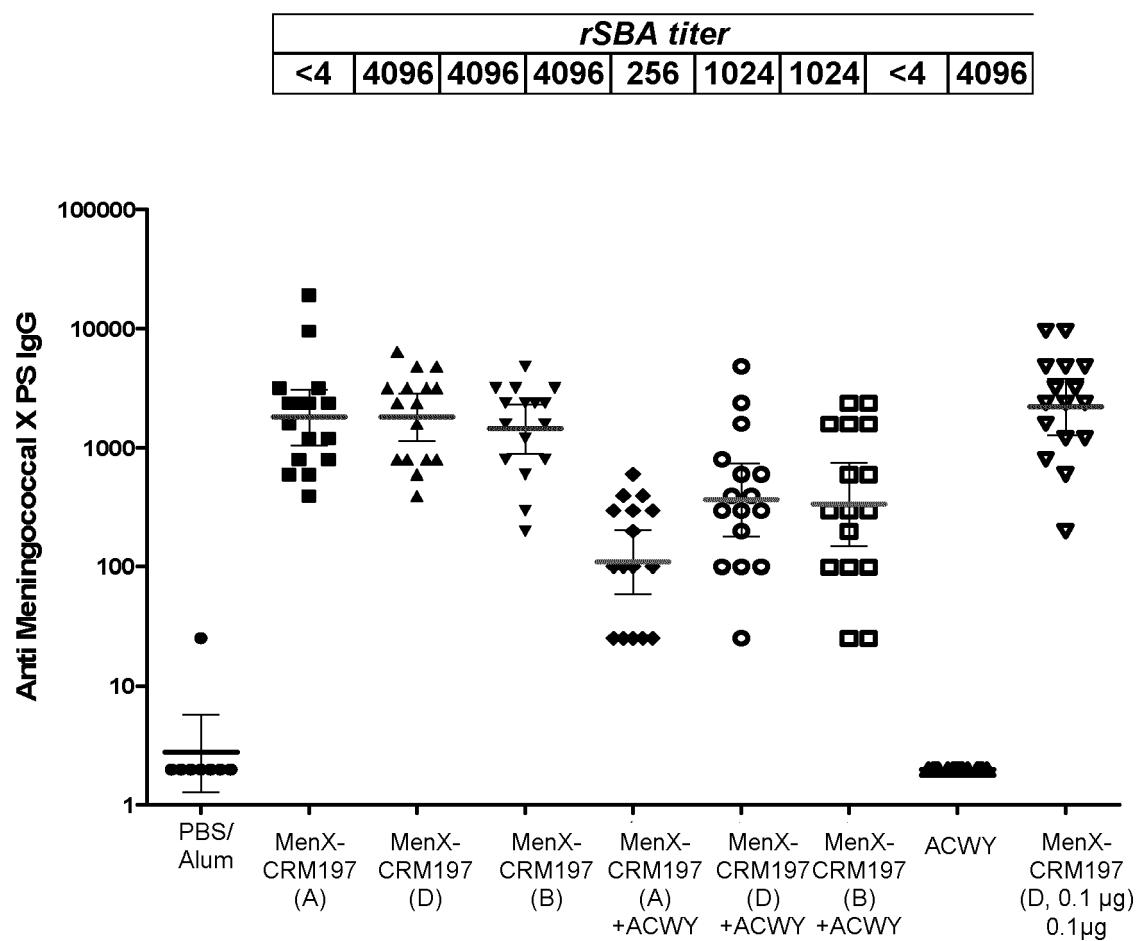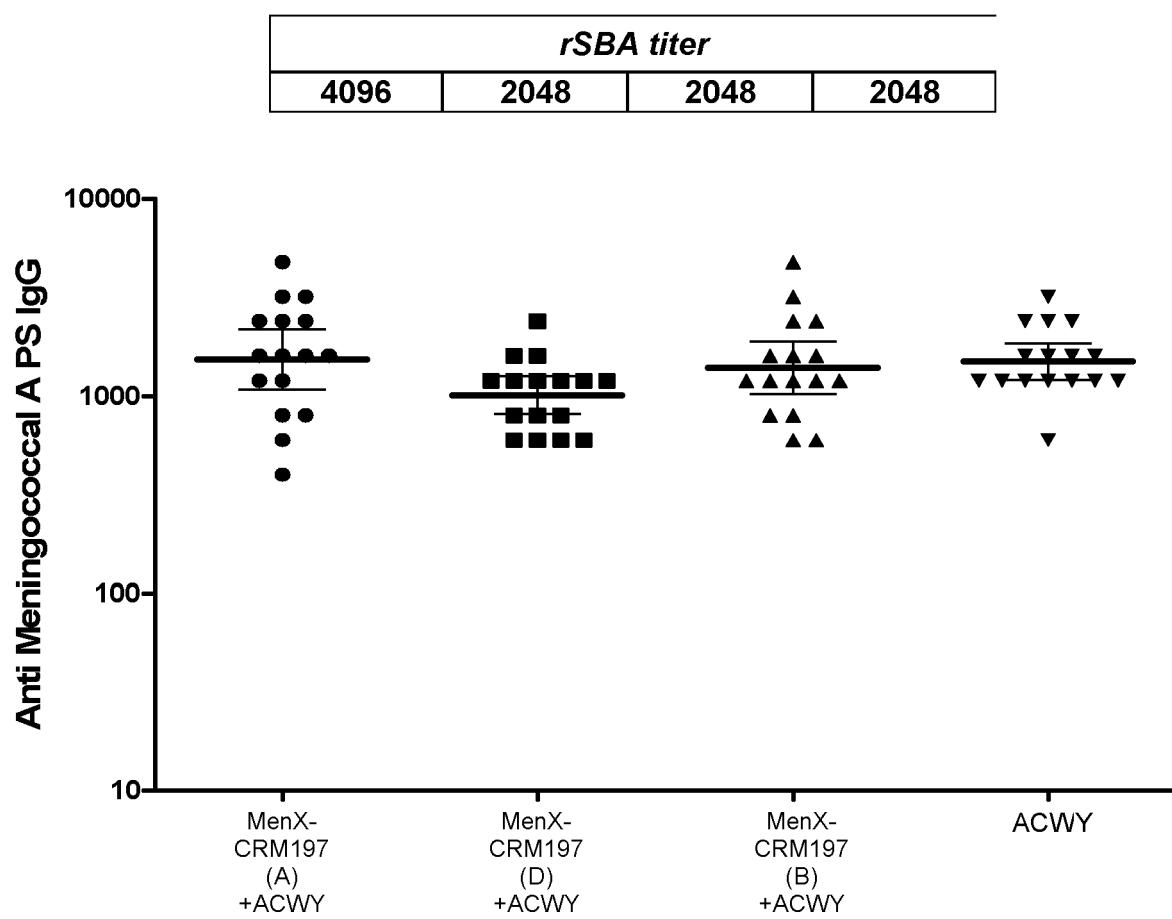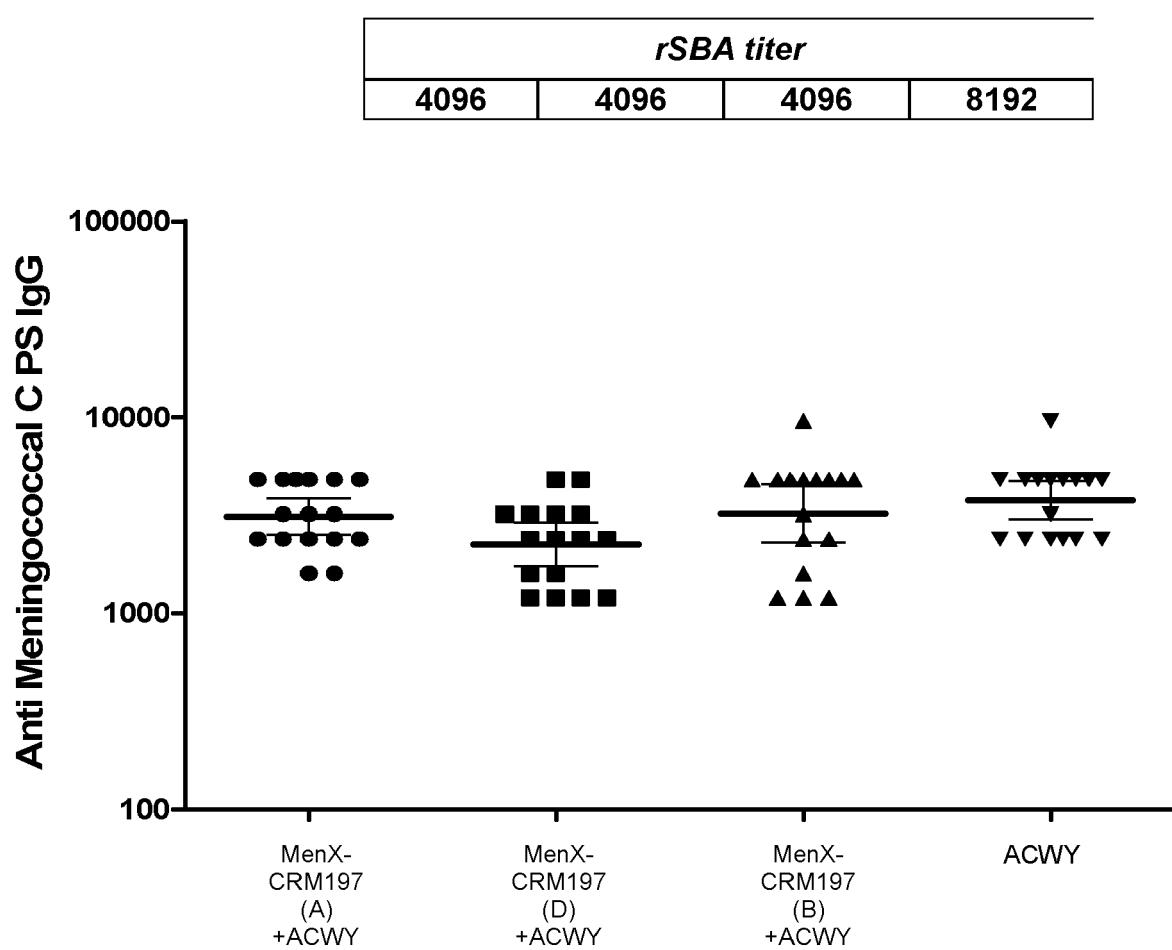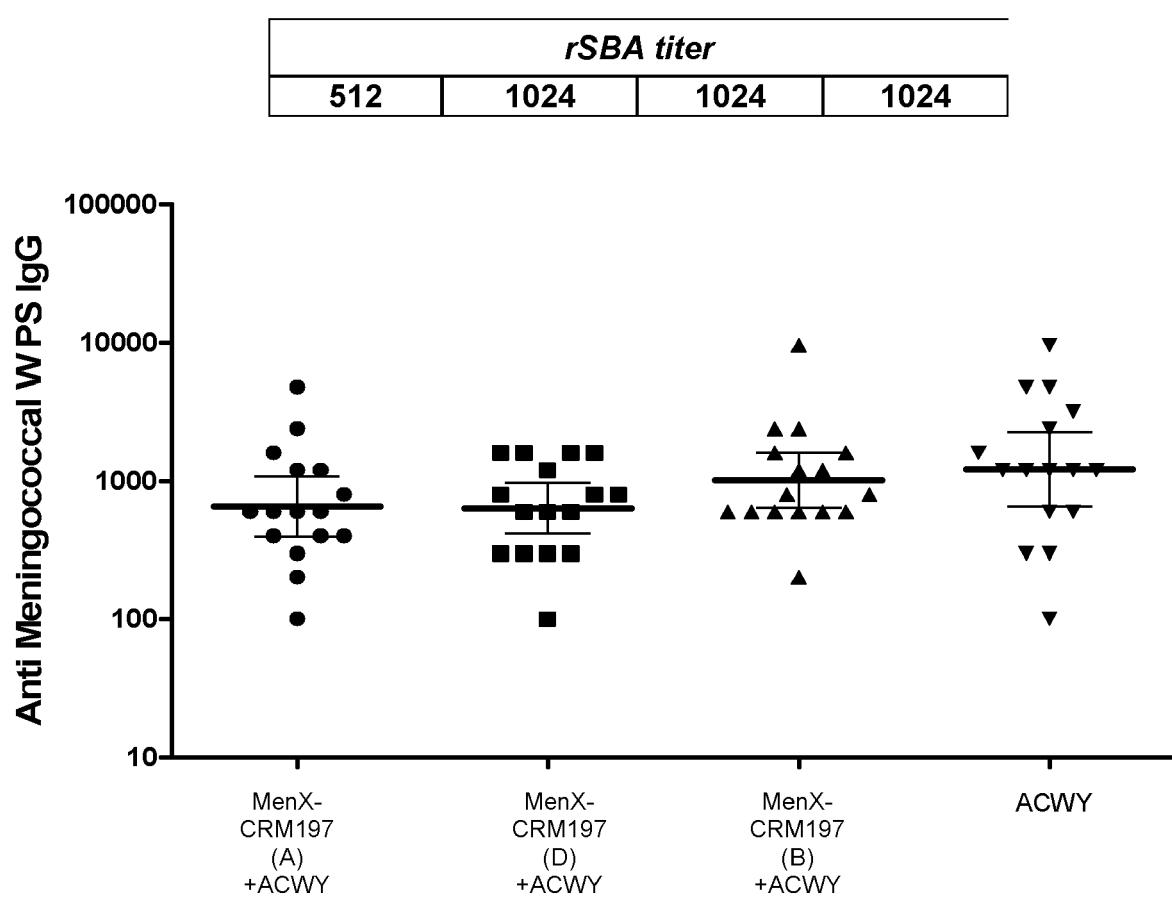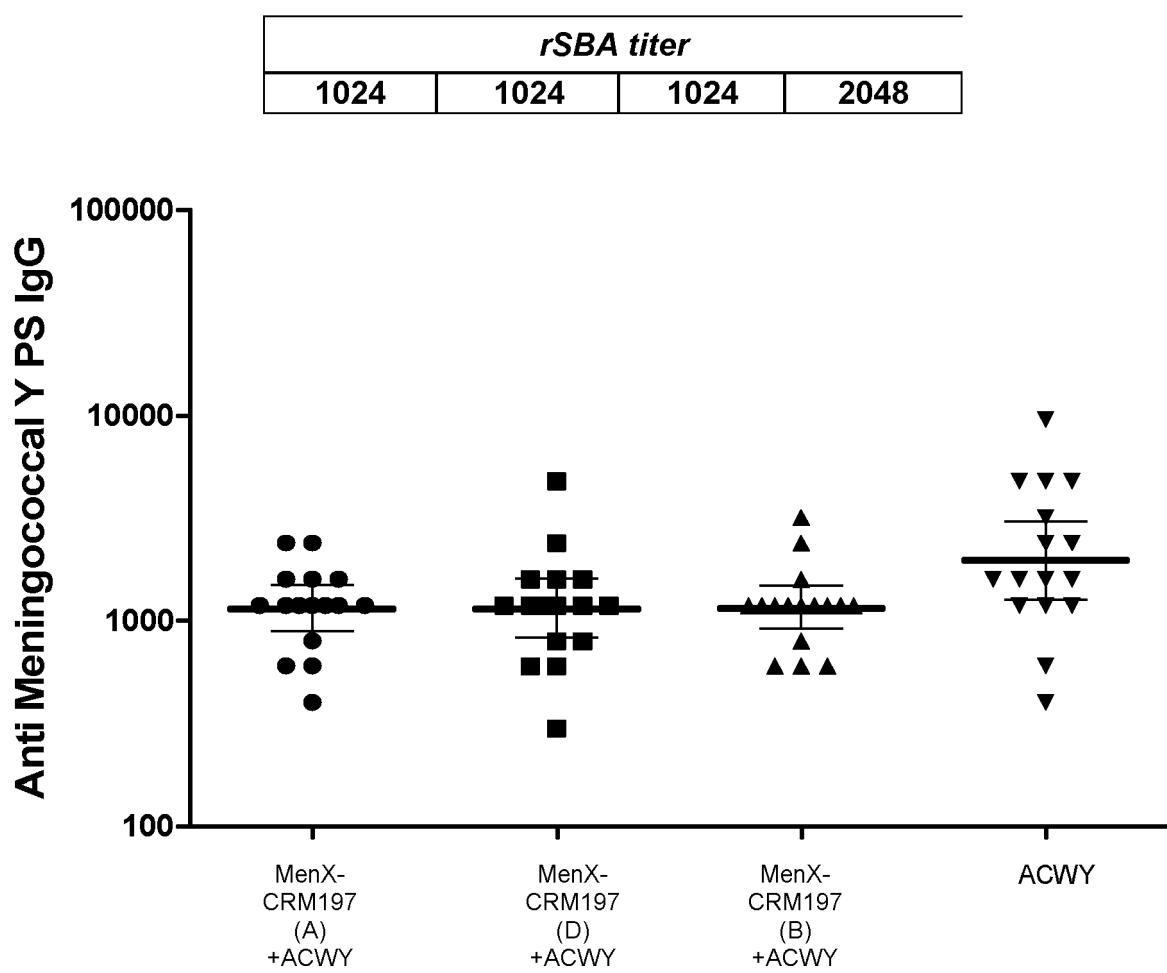
FIGURE 13

A)
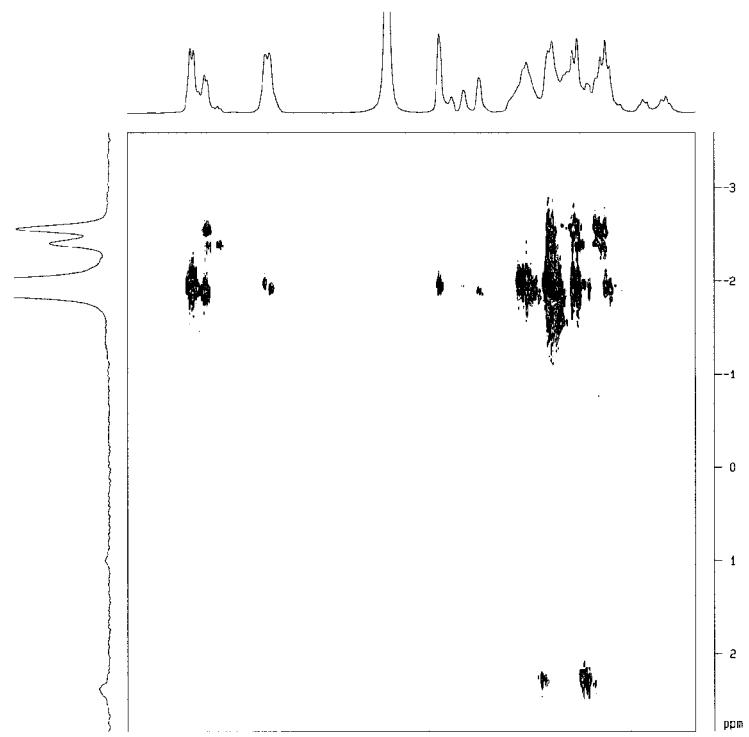
B)
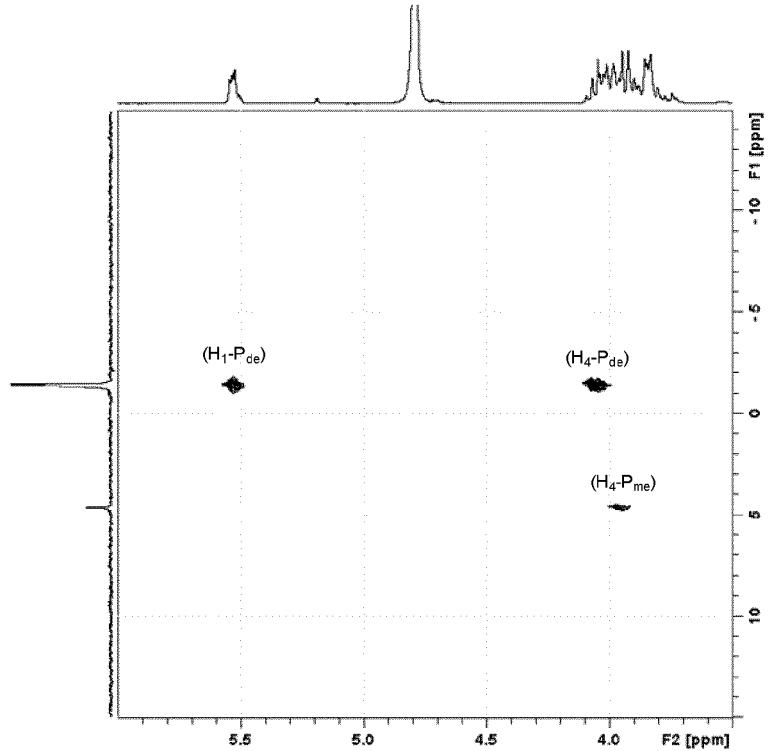
FIGURE 14

A)
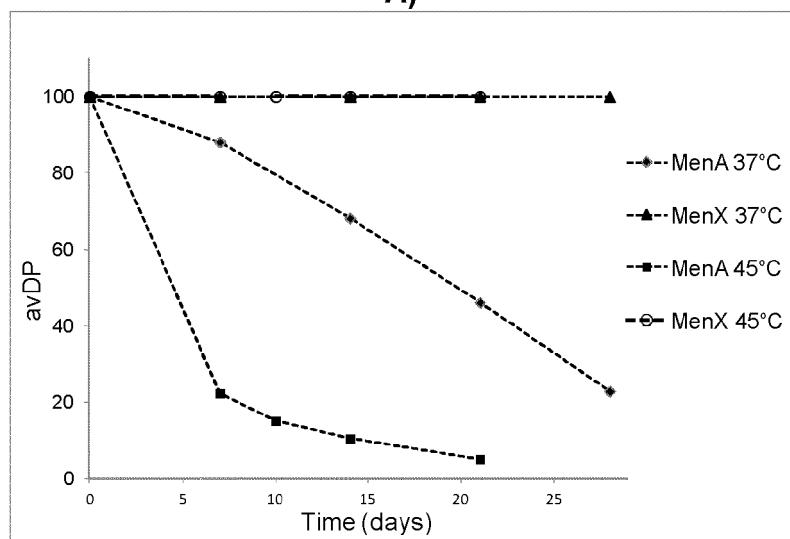
B)
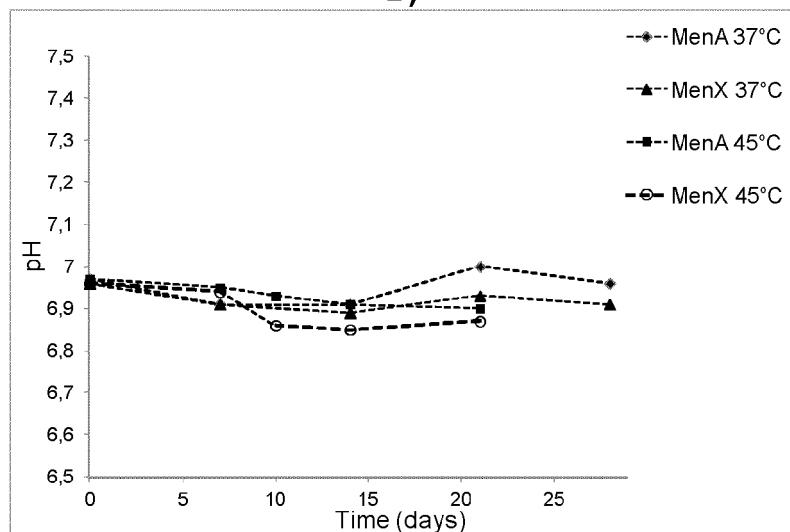
C)
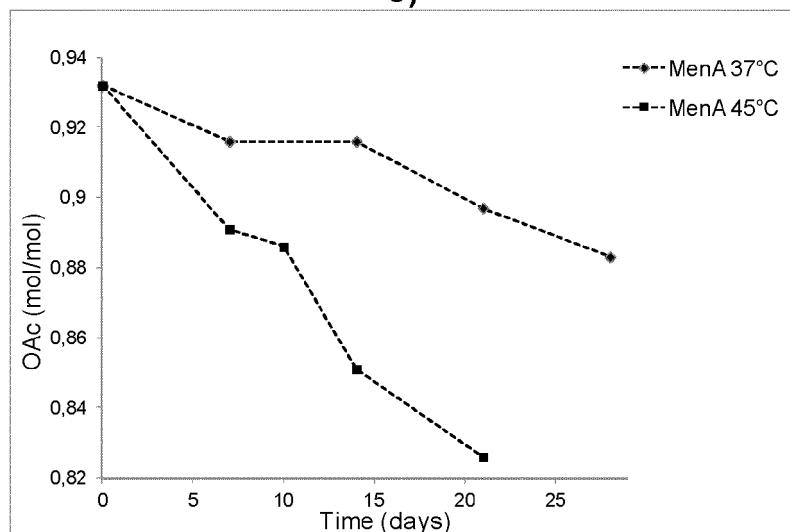
FIGURE 15

A)
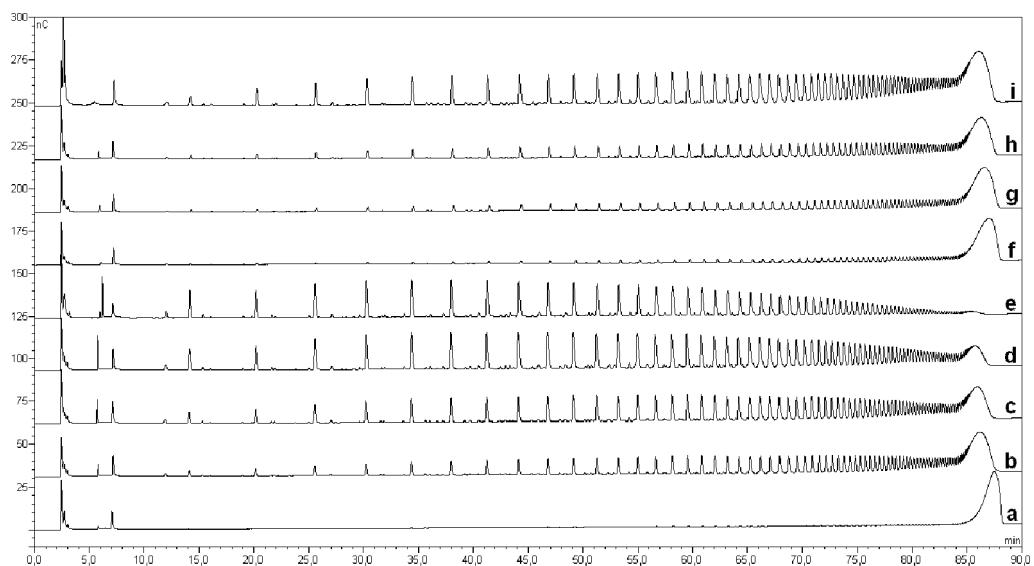
B)
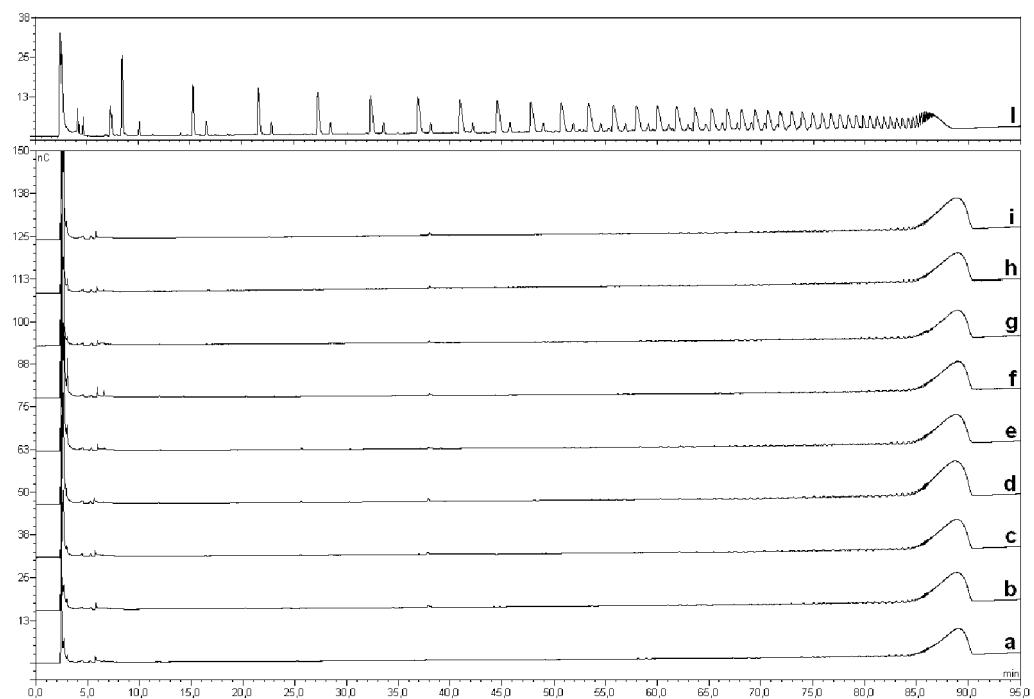
FIGURE 16
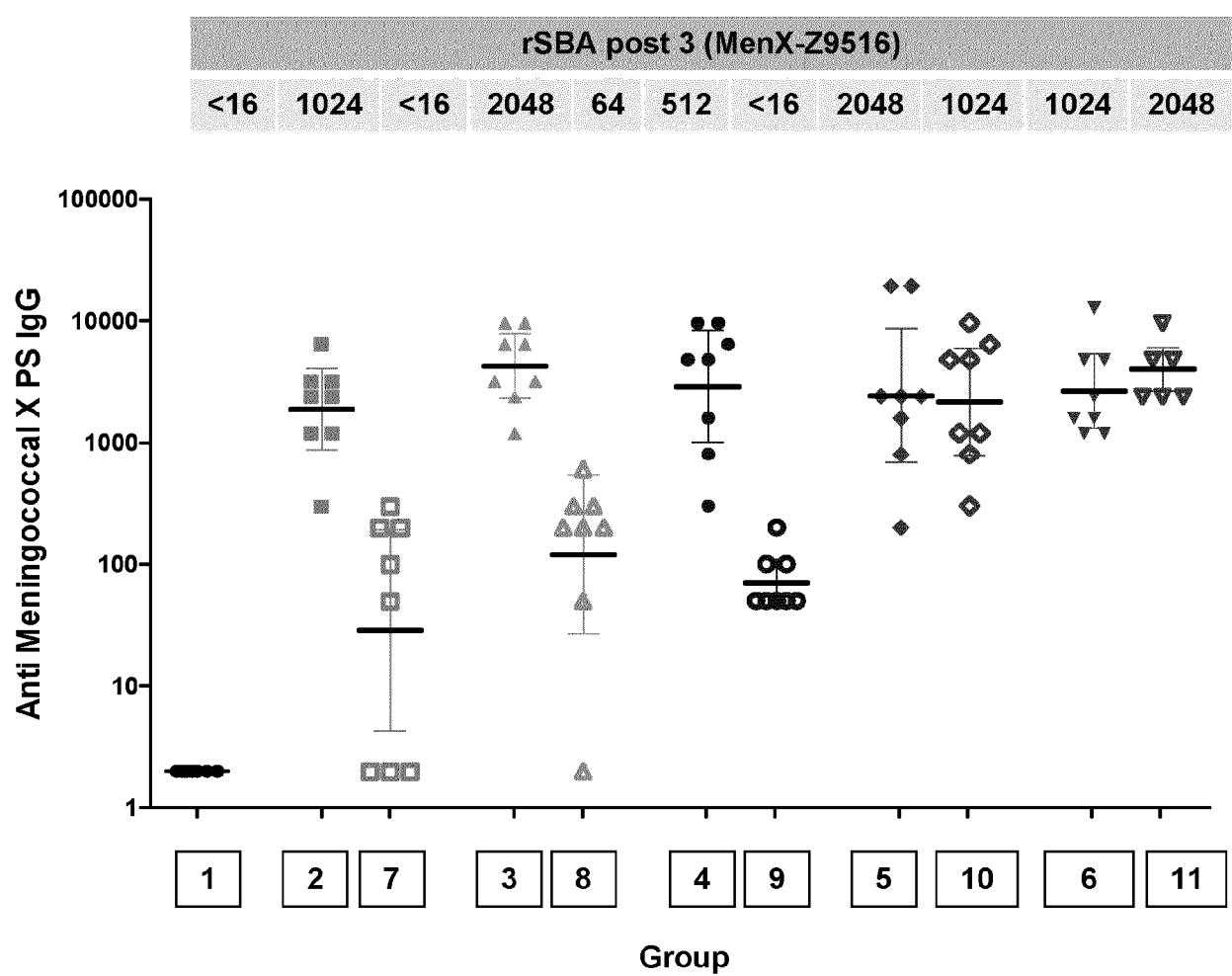
FIGURE 17
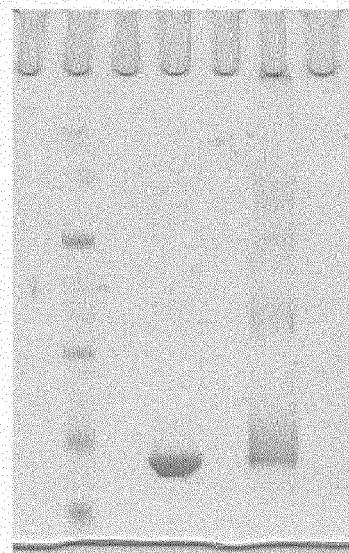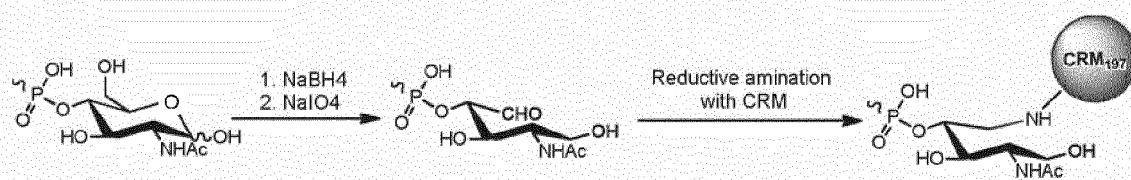
SDS-Page 3-8%TA
1. Marker
2. CRM_{197}
3. $MenX-CRM_{197}$

1   2   3

FIGURE 18

FIGURE 19
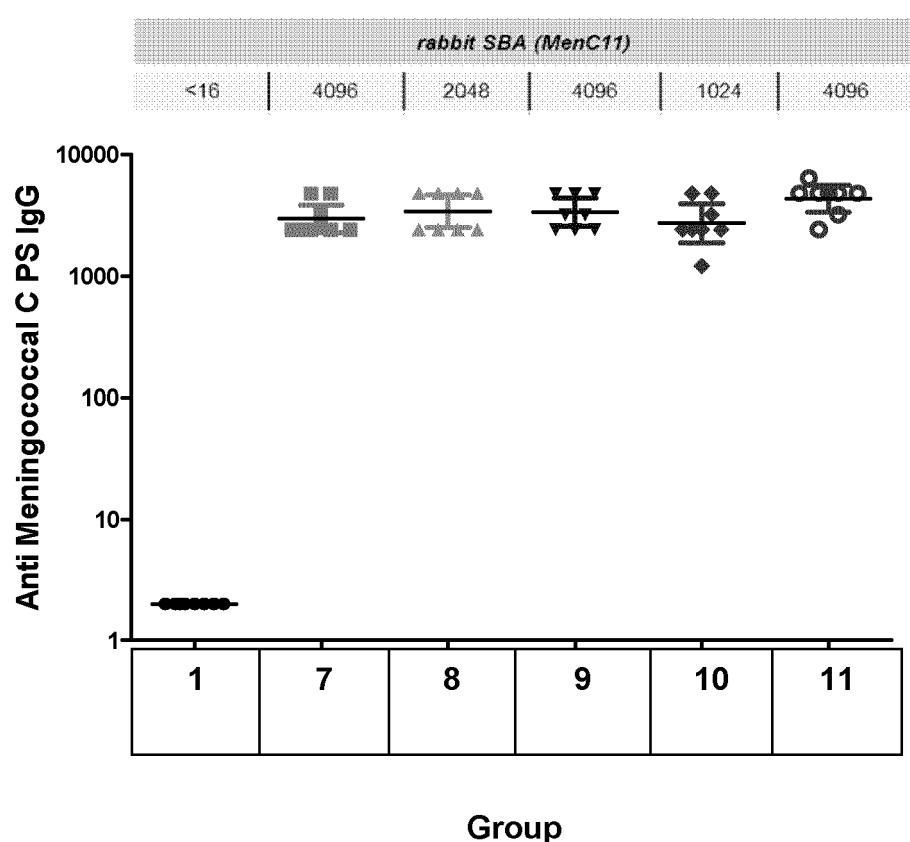
## FIGURE 20
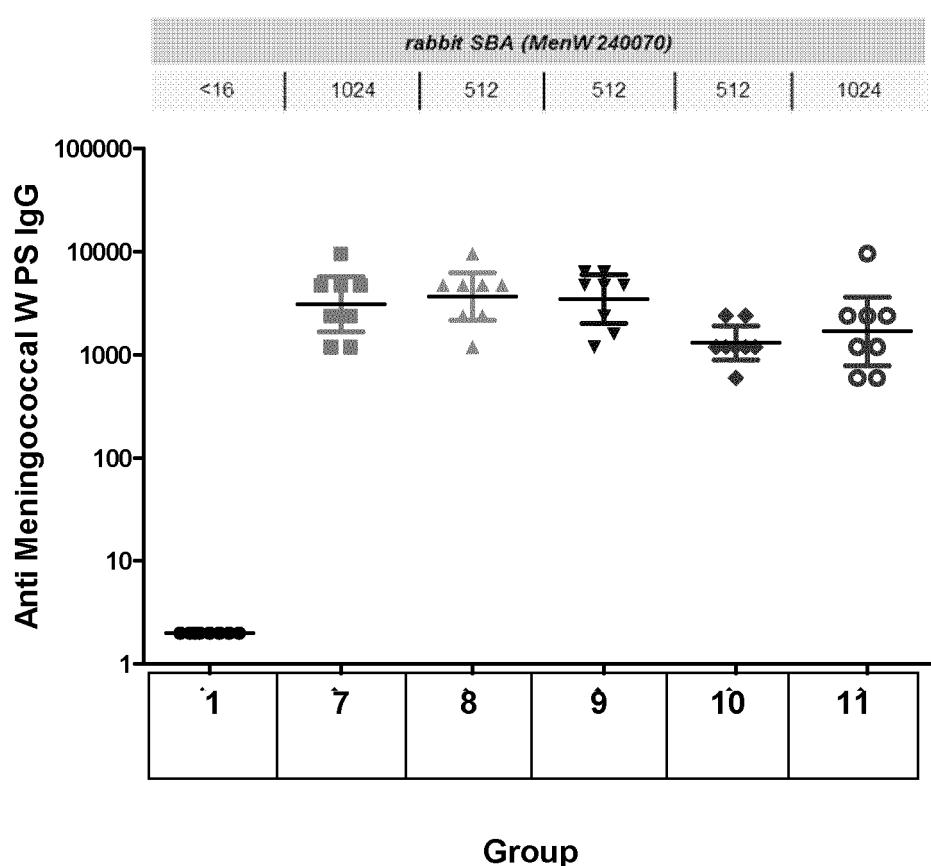
FIGURE 21
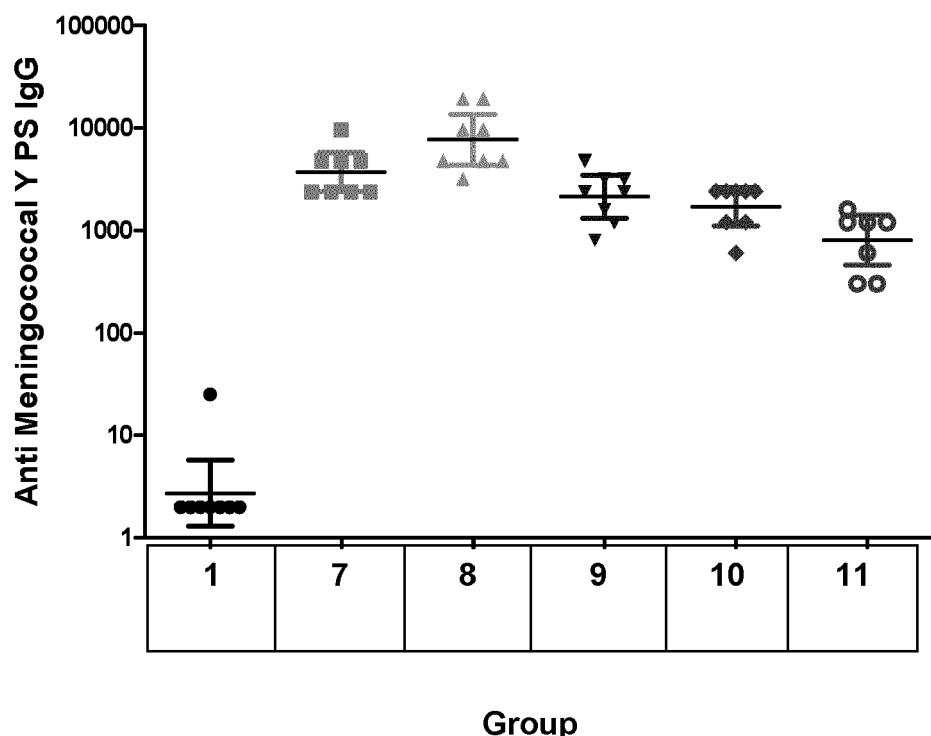
FIGURE 22
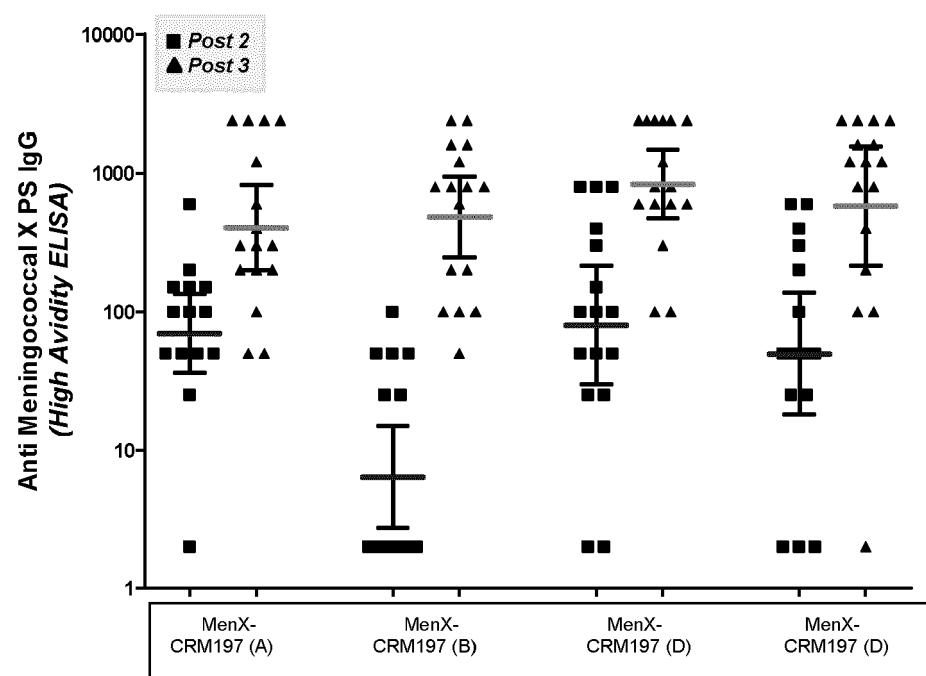
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