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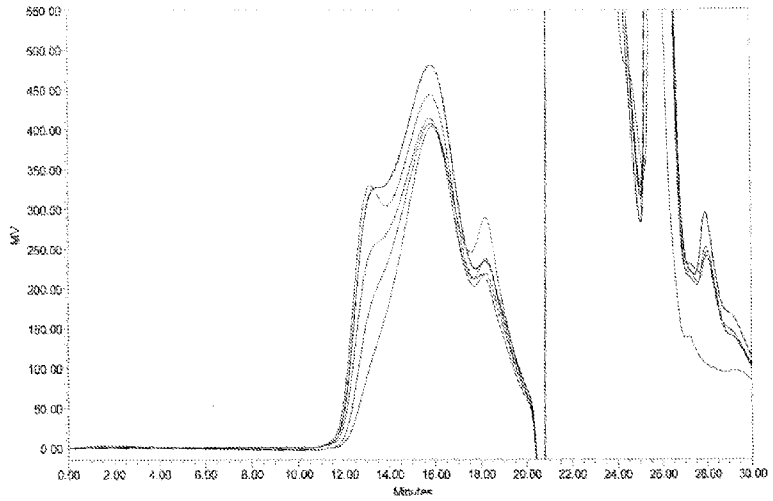
(54) Title: IMMUNOGENIC COMPOSITION

Figure 1



(57) Abstract: The invention provides immunogenic polysaccharide protein conjugates comprising capsular polysaccharides from N. Meningitidis serogroup X and methods for preparation thereof. The present invention relates to N. meningitidis X saccharide-carrier protein conjugates prepared by a conjugation reaction. Accordingly, the instant invention relates to multivalent meningococcal polysaccharide protein conjugate composition comprising capsular saccharide from serogroups X and at least one capsular saccharide from A, C, W135 and Y wherein, i) polysaccharides A C W135 X are sized mechanically whereas polysaccharide Y is sized chemically, ii) all saccharide are conjugated to carrier protein via a linker with a cyanylation conjugation chemistry iii) all saccharide to protein ratios in final conjugates are between 0.2 - 0.6 and iv) at least two different carrier proteins selected from the group consisting of TT, DT and CRM197 are utilized.

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IMMUNOGENIC COMPOSITION

BACKGROUND OF THE INVENTION

Neisseria meningitidis (meningococcus) is a Gram negative human pathogen. It colonizes the pharynx, causing meningitis and, occasionally, septicemia in the absence of meningitis. It is closely related to *N. gonorrhoeae*, although one feature that clearly differentiates meningococcus is the presence of a polysaccharide capsule that is present in all pathogenic meningococci.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).

Serogroup A ('MenA') is most common cause of epidemic disease in sub-Saharan Africa. Serogroups B & C are responsible for the majority of cases in developed countries, with the remaining cases being caused by serogroups W135 & Y.

The vaccine utilizing the polysaccharide (PS)alone have relatively low immunogenicity. To overcome the relatively low immunogenicity of polysaccharide, PS vaccines are conjugated to protein carriers to increase immunogenicity and provide long-term protection in young children. Many meningococcal conjugate vaccines are already approved and marketed throughout the world. Examples of such vaccines, known as "Neisseria meningitidis conjugates" are monovalent meningococcal A conjugate (MenAfriVac),monovalent meningococcal C conjugate (Meningitec) and quadrivalent A C Y W meningococcal conjugates(Menveo & Menactra).

As well as being used for classification, the capsular polysaccharide has been used for vaccination. An injectable tetravalent vaccine of capsular polysaccharides from serogroups A, C, Y & W135 has been known for many years and is licensed for human use. Although effective in adolescents and adults, it induces a poor immune response and short duration of protection and cannot be used in infants.Mencevax ACWY™ and Menomune™ both contain 50µg of each purified polysaccharide once reconstituted from their lyophilised forms. The capsular saccharides of serogroups A, C, W135 & Y have also been combined in the form of conjugates to give tetravalent vaccines *e.g.*the unadjuvanted Menactra™ product. Also conjugated serogroup A polysaccharide have been approved for human use as MenAfriVac™, serogroup C oligosaccharides have been approved for human use as Menjugate™ , Meningitec™ and NeisVac-C™.

N. meningitidis serogroup X strains were first described in the 1960s and have been isolated from a few cases of invasive meningococcal diseases in North America, Europe, Australia, and China. Outbreaks of N. meningitidis serogroup X strains have been reported in Niger, western Kenya, and northern Ghana. N.

meningitidis serogroup X strains were reported to be very efficient in colonization among military recruits in the United Kingdom.Refer Abdullah Kilic et al ;Neisseria meningitidis Serogroup X Sequence Type 767 in Turkey;Journal Of Clinical Microbiology, Nov. 2010, p. 4340–4341;Vol. 48, No. 11

It was reported that repeated mass vaccination in many African countries might have contributed to colonization by and meningococcal diseases due to serogroup X strains and might result in a changed profile of meningococcal disease Refer Gagneux, S. P et al ;Prospective study of a serogroup X Neisseria meningitidis outbreak in northern Ghana.J. Infect. Dis. 185:618–626;2002.

The capsular polysaccharides of serogroup B, C, Y, and W135 meningococci are composed of sialic acid derivatives. Serogroup B and C meningococci express (α 2-8)- and(α 2-9 239)-linked polysialic acid, respectively, while alternating sequences of D-glucose or D-galactose and sialic acid are expressed by serogroup Y and W135 N. meningitidis. In contrast,the capsule of serogroup A meningococci is composed of(α 1-6)-linked N-acetylmannosamine 6-phosphate , while N. meningitidis serogroup X synthesizes capsular polymers of(α 1-4)-linked N-acetylglucosamine 1-phosphate.Refer Yih-Ling Tzeng et al ;Genetic Basis for Biosynthesis of the (134)-Linked N-Acetyl-D-Glucosamine 1-Phosphate Capsule of Neisseria meningitidis Serogroup X; Infection And Immunity, Dec. 2003, p. 6712–6720 ;Vol. 71, No. 12

The existing meningococcal conjugate vaccines are based on A C Y W135 polysaccharides.The increase in incidence of MenX disease in African Meningitis Belt in the last 5 years [1,4] warrants development and introduction of a MenX polysaccharide conjugate vaccine in selected areas of the region to prevent and control future epidemics.Though has been reported earlier.Inspite of availability of comprehensive seroprevalence and structural data for meningococcal X , a commercially viable conjugate vaccine including X polysaccharide is yet to be developed due to extremely limited success on purification,conjugation and formulation stability aspects for the same. This provides an additional challenge for successfully addressing and controlling various parameters,especially when employing a scalable conjugation process for the large-scale manufacture of Neisseria meningitidis conjugates containing Neisseria meningitidis X polysaccharide.

The present invention arises from the surprising discovery that it is possible to prepare a monovalent or multivalent immunogenic composition based on conjugates of meningococcal polysaccharide from serogroup X by utilizing a scalable and efficient conjugation process.

SUMMARY OF THE INVENTION

The present invention relates to N.meningitidis X saccharide-carrier protein conjugates prepared by a conjugation reaction comprising of i)sizing of polysaccharide ii)CPPT based activation of sized polysaccharide having average molecular weight between 100-150 Kda, at a pH between 9 to 9.5 iii)ADH addition after a duration of about 2 to 5 minutes followed by incubation period of 4-20 hrs and iv)reacting ADH activated polysaccharide with purified non-activated carrier protein in a ratio between 0.75 - 1.5 in presence of MES buffer and EDAC followed by incubation period of 3 -4 hrs,characterized in that the conjugation reaction is carried at 2-8°C resulting in a conjugate yield from 20% to about 30% and having saccharide to protein ratio from 0.2 to about 0.6 in final conjugate.

Alternatively,N.meningitidis X saccharide-carrier protein conjugates can also be prepared by a conjugation reaction comprising of i)sizing of polysaccharide ii)CPPT based activation of sized polysaccharide having average molecular weight between 100-150 Kda, at a pH between 9 to 9.5 iii)addition of ADH activated carrier protein in a saccharide:protein ratio between 0.5 - 2 after 2-3 minutes followed by incubation period of 2 to 20 hrs characterized in that the conjugation reaction is carried at 22°C to 25°C resulting in a conjugate yield from 5% to about 10%.

Accordingly,the instant invention relates to multivalent meningococcal polysaccharide protein conjugate composition comprising capsular saccharide from serogroups X and atleast one capsular saccharide from A, C, W135 and Y wherein,i)polysaccharides A C W135 X are sized mechanically whereas polysaccharide Y is sized chemically,ii)all saccharide are conjugated to carrier protein via a linker with a cyanylation conjugation chemistry iii)all saccharide to protein ratios in final conjugates are between 0.2 - 0.6 and iv) atleast two different carrier proteins selected from the group consisting of TT, DT and CRM197 are utilized.

DESCRIPTION OF THE DRAWINGS

Figure 1: Overlay of conjugation reaction when Men X Ps (215 KDa) conjugated to Hydrazine derivatized TT

Figure 2: Purified Conjugate when Men X Ps (215 KDa) conjugated to Hydrazine derivatized TT

Figure 3: Overlay of conjugation reaction when Men X Ps(326 KDa) conjugated to ADH derivatized TT

Figure 4: Purified Conjugate when Men X Ps(326 KDa) conjugated to ADH derivatized TT

Figure 5: Overlay of conjugation reaction when Men X Ps (120 KDa)conjugated to ADH derivatized TT

Figure 6: Purified Conjugate when Men X Ps(120 KDa) conjugated to ADH derivatized TT

Figure 7: Chromatogram of Native Meningococcal X polysaccharide.

Figure 8: Overlay of conjugation reaction when Men X Ps(510 KDa) activated with ADH and conjugated to purified TT.

Figure 9: Purified Conjugate when Men X Ps(510 KDa) activated with ADH and conjugated to purified TT.

Figure 10: Overlay of conjugation reaction when Men X Ps(250 KDa) activated with ADH and conjugated to purified TT.

Figure 11: Purified Conjugate when Men X Ps(250 KDa) activated with ADH and conjugated to purified TT.

DETAILED DESCRIPTION OF THE INVENTION

"Multivalent immunogenic compositions" refer to :

Composition I comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii) tetanus toxoid; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) diphtheria toxoid;(d) a conjugate of (i) capsular saccharide of serogroup W135 N.meningitidis and (ii) tetanus toxoid; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii) tetanus toxoid.

Composition II comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii)CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) tetanus toxoid;(d) a conjugate of (i) capsular saccharide of serogroup W135 N.meningitidis and (ii)CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii)CRM197.

Composition III comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii)CRM 197; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii)CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) tetanus toxoid;(d) a conjugate of (i) capsular saccharide of serogroup W135 N.meningitidis and (ii)CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii)tetanus toxoid charachterized in that conjugates

containing tetanus toxoid as carrier protein are found to enhance immunogenicity of conjugates containing CRM 197 as carrier protein.

composition IV comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii)tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii)CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197;(d) a conjugate of (i) capsular saccharide of serogroup W135 N.meningitidis and (ii)CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii)tetanus toxoid.

Composition V comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii)CRM 197; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii)CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197;(d) a conjugate of (i) capsular saccharide of serogroup W135 N.meningitidis and (ii)CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii)tetanus toxoid.

Accordingly in a first embodiment, the composition can comprise of serogroup A,C,Y,W135 and X saccharide at an amount of 0.5-10µg ,0.5-5µg or 0.5-2µg per 0.5 ml dose.

Another aspect of first embodiment is that said composition can comprise of 10 µg of serogroup A saccharide, 5µg of serogroup C saccharide, 5µg of serogroup W135 saccharide, 5µg of serogroup Y saccharide and 5µg of serogroup X saccharide.

Alternatively said multivalent immunogenic composition can comprise of 5 µg of serogroup A saccharide, 5µg of serogroup C saccharide, 5µg of serogroup W135 saccharide, 5µg of serogroup Y saccharide and 5µg of serogroup X saccharide.

Accordingly in a second embodiment,said one or more N.meningitidis saccharide conjugates can optionally be adsorbed onto aluminium hydroxide, aluminium phosphate or a mixture of both or unadsorbed onto adjuvant.

One aspect of second embodiment is that aluminium salt adjuvant can be added at an amount of 20-300µg,20-200µg,25-150µg of Al^{+++} per 0.5 ml dose.

Another aspect of second embodiment is that aluminium salt adjuvant can be added at an amount of 25-125µg of Al^{+++} per 0.5 ml.

A third embodiment of the instant invention is that said composition can comprise of a preservative selected from thiomersal and 2-phenoxyethanol.

One aspect of third embodiment is that said can further comprise of sodium phosphate, sodium chloride or combination thereof.

A fourth embodiment of the instant invention is that said multivalent immunogenic composition can be in a buffered liquid form or in a lyophilized form.

One aspect of fourth embodiment is that said lyophilized immunogenic composition can comprise of a stabilizer combination selected from a) 2 to 5% (w/v) Trehalose, 0.25 to 0.75% sodium citrate;b) 2 to 5% (w/v) Sucrose and 0.25 to 0.75% sodium citrate;c) 2 to 5% (w/v) Sucrose,2 to 5% (w/v) Lactose and 0.25 to 0.75% sodium citrate;and d) 2 to 5% (w/v) Trehalose ,2 to 5% (w/v) Lactose and 0.25 to 0.75% sodium citrate.

Another aspect of the fourth embodiment is that said lyophilized immunogenic composition can further comprise a buffer selected from Tris and phosphate.

Accordingly in a fifth embodiment,said polysaccharides A C W and X can be mechanically sized to have an average molecular weight between 100-600 Kda,100-400 Kda,preferably 100-200 Kda,most preferably 100-150 Kda. Mechanical sizing methods like homogenization, microfluidization and high pressure cell disruption are preferred.

In another aspect of fifth embodiment, said polysaccharide Y can be sized to have an average molecular weight between 90-110 KDa, by a method selected from acid hydrolysis, alkaline degradation, oxidation by periodate, ozonolysis , enzymatic hydrolysis, sonication , electron beam fragmentation.Preferably, chemical sizing is by using sodium acetate at a temperature from 60 to 80°C.

In a sixth embodiment,each of the N. meningitidis saccharides is conjugated to the carrier protein via a hetero or homo-bifunctional linker with cyanylation conjugation chemistry.

In one aspect of sixth embodiment,said sized polysaccharide is activated by utilizing a cyanylation reagent selected from but not limited to 1-cyano-4- (dimethylamino)- pyridinium tetrafluoroborate ('CDAP'), p-nitrophenylcyanate and N-cyanotriethylammonium tetrafluoroborate ('CTEA').In a preferred conjugation process, cyanylating reagent is other than CDAP and can be selected from a group of l-cyano- 4-

pyrrolidinopyridinium tetrafluoroborate (CPPT), 1- cyano- imidazole (1-CI), 1-cyanobenzotriazole (1-CBT), or 2- cyanopyridazine -3(2H)one (2-CPO), or a functional derivative or modification thereof.

In another aspect of sixth embodiment,said activated polysaccharide or carrier protein,particularly polysaccharide is reacted with hydrazine, carbohydrazide, hydrazine chloride, a dihydrazide ,a mixture thereof ,preferably with adipic acid dihydrazide.

Hydrazide groups can be introduced into proteins through the carboxyl groups of aspartic acid and glutamic acid residues on the protein using a carbodiimide reaction, for example, by reaction with hydrazine, carbohydrazide, succinyl dihydrazide, adipic acid dihydrazide, hydrazine chloride (e.g., hydrazine dihydrochloride) or any other dihydrazides in the presence of EDC. EDC is employed as a catalyst to activate and modify the protein reactant with hydrazine or the dihydrazide. Any water-soluble carbodiimide including EDC can be used as a catalyst. EDC-catalyzed proteins generally have a tendency to polymerize and precipitate. See Schneerson et al., Infect. Immun. 1986, 52:519-528; Shafer et al., Vaccine 2000; 18(13): 1273-1281; and Inman et al., Biochemistry 1969; 8:4074-4082.

In a seventh embodiment,said multivalent meningococcal polysaccharide protein conjugate composition contains polysaccharides from A, B, C, H, I, K, L, 29E, W135, Y and Z conjugated individually to two or more different types of carrier proteins. The capsular saccharides are chosen from meningococcal serogroups A, C, W135 Y and X, such that the compositions include saccharides from 1, 2, 3, 4,or 5 of these five serogroups. Specific compositions comprise saccharides from: serogroups A & X; serogroups X & W135; serogroups X & Y; serogroups C & X;serogroups A Y & X; serogroups C, X & W135; serogroups X, Y & W135; serogroups A,C & X;serogroups Y,C & X;serogroups A,W & X;serogroups Y &W135 & C & X;serogroups Y & W135 & A & X;serogroups C & W135 & A & X;serogroups Y & C & A & X; serogroups A & C & Y & W135 & X.Compositions including at least serogroup X are preferred , and compositions including saccharides from all five serogroups are most preferred.

In an aspect of seventh embodiment, said carrier protein can be selected from a group of but not limited to CRM 197,diphtheria toxoid,tetanus toxoid, pertussis toxoid, E. coli LT, E: coli ST, and exotoxin A from Pseudomonas aeruginosa, outer membrane complex c (OMPC), porins, transferrin binding proteins, pneumolysin, pneumococcal surface protein A (PspA) , pneumococcal adhesin protein (PsaA),pneumococcal surface proteins BVH-3 and BVH-11 , protective antigen (PA) of Bacillus anthracis and detoxified edema factor (EF) and lethal factor (LF) of Bacillus anthracis, ovalbumin, keyhole limpet hemocyanin (KLH), human serum albumin, bovine serum albumin (BSA) and purified protein derivative of

tuberculin (PPD).Preferably, combinations of carrier proteins to be utilized comprise tetanus toxoid & diphtheria toxoid,CRM197 & tetanus toxoid.

In another aspect of third embodiment, conjugation reaction utilizes linkers selected from the group consisting of adipic acid dihydrazide, ε-aminohexanoic acid, chlorohexanol dimethyl acetal, D-glucuronolactone, cystamine and p-nitrophenylethyl amine.

After conjugation,conjugates can be purified from unreacted protein and polysaccharide by any standard techniques including, inter alia, size exclusion chromatography, density gradient centrifugation,ultrafiltration, hydrophobic interaction chromatography or ammonium sulfate fractionation. See, e.g., P. W. Anderson, et. al. (1986). J. Immunol. 137: 1181-1186. See also H. J. Jennings and C. Lugowski (1981) J. Immunol. 127: 1011-1018.

In an eighth embodiment, said immunogenic composition of the instant invention can further comprise of an additional non-meningococcal polysaccharide protein conjugate,wherein said polysaccharides and oligosaccharides for use can be selected from but not limited to pneumococcal polysaccharides of serogroups 1, 2, 3, 4,5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F; Haemophilus influenzae type b polysaccharide polyribosylribitol phosphate, group B streptococcal polysaccharides of serotypes III and V and Salmonella typhi Vi polysaccharide. Other polysaccharides of pneumococcal and group B streptococcal serotypes are also suitable for use herein, as are other T-independent polysaccharide and oligosaccharide antigens, for example, polysaccharides or oligosaccharides derived from group A streptococcus, Staphylococci, Enterococci, Klebsiella pneumoniae, E. coli, Pseudomonas aeruginosa, and Bacillus anthracis. While bacterial polysaccharides and oligosaccharides are particularly preferred, gram (-) bacterial lipopolysaccharides and lipooligosaccharides and their polysaccharide and oligosaccharide derivatives, and viral polysaccharides and oligosaccharides can also be employed.

Compositions of the invention may be presented and packaged in various ways.The 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. Injectable compositions will usually be liquid solutions or suspensions. Alternatively, they may be presented in solid form (*e.g.* freeze-dried) for solution or suspension in liquid vehicles prior to injection.

Examples:

Example 1:

Preparation of Meningococcal X polysaccharide

a) Fermentation and Purification of Meningococcal X Polysaccharide

Meningococcal X polysaccharides are obtained from N.meningitidis strains (8210 & 9601) by utilizing a suitable fermentation medium in a continuous fed-batch fermentation mode under optimal fermentor conditions.Further Meningococcal X capsular polysaccharides are typically prepared by a process comprising the steps of CTAB based precipitation , Ethanol(96%)treatment followed by depth filtration , carbon filtration , $CaCl_2$ precipitation, Ethanol (96%)treatment and ultrafiltration.

b) Sizing of Meningococcal X Polysaccharide

Purified Meningococcal X polysaccharides were subjected to 1-2 passes of mechanical sizing(Constant systems cell disruptor)in WFI at a pressure of about 30-40 kpsi.

Example 2:

Conjugation of Meningococcal X polysaccharide to carrier protein.

a) Meningococcal X Polysaccharide of varying average molecular weight conjugated to hydrazine derivatized tetanus toxoid(TT)

Firstly homogenized Polysaccharide of X (Strain 9601), Average molecular weight 215kD on SEC HPLC, (30 mg/ml) 45 mg was activated with 90 mg CDAP (dissolved 100mg/ml in acetonitrile), pH of mixture was adjusted to 9.5 with 1M NaOH,.Then after 3 min hydrazine activated TT (30mg/ml in 1M NaCl) 67.5 was added to the reaction. The reaction was monitored on HPLC and continued upto 18 hrs. After 18 hrs reaction was quenched by addition of glycine and crude conjugate was purified by diafiltration 300kD TFF membrane in Tris 10mM pH 7.2. Shodex columns SB-804 HQ and SB-805 HQ were used sequentially with PBS as mobile phase at 1 ml/min flow rate. Polysaccharide concentration and protein concentration were determined by phosphorous assay and modified Lowry assay respectively.

Secondly Polysaccharide of X (Strain 8210), average molecular weight 326 kD on SEC HPLC, (24 mg/ml in 2M NaCl) 60 mg was activated with 150 mg CPPT (dissolved 114mg/ml in acetonitrile)and pH of mixture was adjusted to 9.5 with 2.5M NaOH,.Then after 3 min ADH activated TT (37mg/ml in 2M NaCl) 37.5mg was added to the reaction and the reaction was monitored on HPLC and continued upto 5 hrs. After 5 hrs

reaction was quenched by addition of glycine and crude conjugate was purified by diafiltration with 500kD TFF membrane in 10mM PBS followed by Tris 10mM pH 7.2.

Further Polysaccharide of X (Strain 8210), having average molecular weight 120 kD on SEC HPLC, (20 mg/ml in 1M NaCl) 200 mg was activated with 400 mg CPIP (dissolved 114mg/ml in acetonitrile), and pH of mixture was adjusted to 9.5 with 1M NaOH.Then after 3 min ADH activated TT (30mg/ml) 150 mg was added to the reaction. The reaction was monitored on HPLC and continued upto 4 hrs. After 4 hrs reaction was quenched by addition of glycine and crude conjugate was purified by diafiltration with 300kD TFF membrane in 10mM PBS followed by Tris 10mM pH 7.2.

b) Meningococcal X Polysaccharide of varying average molecular weight activated with ADH and conjugated to purified non-activated tetanus toxoid(TT)

Firstly Polysaccharide of X (Strain 8210) having average molecular weight 510kD on SEC HPLC (27 mg/ml in 2M NaCl) 200 mg was activated with 400 mg CPPT (dissolved 114mg/ml in acetonitrile) and pH of mixture was adjusted to 9.5 with 2.5M NaOH. Then after 3 min , ADH 1.5g (100mg/ml in carbonate buffer) was added and reaction was continued upto 4 hrs. After 4 hrs glycine was added and reaction mixture was diafiltered on 8kD TFF membrane. Further ADH-Men X polysaccharide was concentrated. To 44 mg of this(7.5mg/ml), purified TT (37.5 mg/ml in 0.9% NaCl) and MES pH 6.0 buffer were added so that final buffer strength of MES was 100mM, followed by addition of 37.5 mg EDAC (dissolved in 100mM MES, pH 6.0). The reaction was continued for 4 hrs and monitored on HPLC. Unbound polysaccharide was removed by Gel filtration Chromatography using Toyopearl HW65 resin on Akta Chromatography System.(GE Amersham).The fractions were collected and pooled based on peak profile and saccharide-protein ratio.

Secondly,Polysaccharide of X (Strain 8210)having average molecular weight 250kD on SEC HPLC was concentrated to 18 mg/ml in 2M NaCl. A quantity of 200 mg was activated with 296 mg CPPT (dissolved 114mg/ml in acetonitrile) and pH of mixture was adjusted to 9.5 with 2.5M NaOH. Then after 3 min ADH 1.12g (100mg/ml in carbonate buffer) was added and reaction was continued upto 4 hrs. After 4 hrs glycine was added and reaction mixture was diafiltered on 8kD TFF membrane. ADH-Men X polysaccharide was then concentrated.Further to 200 mg of this (7.5mg/ml), purified TT (36.7 mg/ml in 0.9% NaCl) and MES pH 6.0 buffer were added so that final buffer strength of MES was 100mM, followed by addition of 200 mg EDAC (dissolved in 100mM MES, pH 6.0). The reaction was continued for 4 hrs and monitored on HPLC. The crude conjugate was purified by diafiltration 500kD TFF membrane in 10mM PBS followed by Tris 10mM pH 7.2.

Table 1 Meningococcal X polysaccharide-protein Conjugation

Avg Mw of Meningococcal X polysaccharide(KDa)	ADH activation	Saccharide/ Protein ratio	Polysaccharide Titer (mg/ml)	Protein Titer (mg/ml)
215	TT (ADH activated)	0.23	0.211	0.921
326	TT (ADH activated)	0.57	0.25	0.435
120	Meningococcal X polysaccharide (ADH activated); TT non-activated	0.59	0.20	0.34
510	Meningococcal X polysaccharide (ADH activated); TT non-activated	0.53	0.180	0.34
250	Meningococcal X polysaccharide (ADH activated); TT non-activated	0.43	0.130	0.30

Above data indicates that final conjugate yield of about 20 to 30% can be obtained by utilizing i) Meningococcal X polysaccharide of Avg Mw of about 100 to 200 kDa ,ii)ADH activated Meningococcal X polysaccharide iii) non-activated TT iv)saccharide:protein ratio between 0.5 to 2 during conjugation reaction v) CPPT as cyanylation reagent and vi) conjugation reaction incubation at 2 to 8°C vi)saccharide:protein ratio between 0.2 to 0.6 in final conjugate.

Example 3:

Conjugation of Meningococcal A,C,Y,W135 polysaccharide to carrier protein CRM197.

Purified Meningococcal polysaccharides A C Y W135 having average Mw between 100 to 200 were conjugated to CRM197 in a saccharide:protein ratio of less than 1 by utilizing a suitable cyanylation reagent(CDAP or CPPT).The conjugates were further purified by diafiltration on 300kD TFF with 50 volumes of 10mM PBS and 50 volume of 10mM Tris.

Example 4:

Lyophilization & Formulation of Men A C Y W135 & X conjugate containing two different carrier proteins

Lyophilized formulations containing N.meningitidis conjugates, sodium citrate and Tris buffer in various combinations with trehalose,sucrose and lactose were prepared wherein free polysaccharide content was within limits and moisture content was less than 2%.Said stabilizer combination was selected from a) 2 to 5% (w/v) Trehalose, 0.25 to 0.75% sodium citrate; b) 2 to 5% (w/v) Sucrose and 0.25 to 0.75% sodium citrate; c) 2 to 5% (w/v) Sucrose,2 to 5% (w/v) Lactose and 0.25 to 0.75% sodium citrate;and d) 2 to 5% (w/v) Trehalose ,2 to 5% (w/v) Lactose and 0.25 to 0.75% sodium citrate.

Table 2

Liquid formulation containing Monovalent Men X—tetanus toxoid conjugate

Formulation Code	Strain	Composition	Amount per 0.5ml
IRCXLT1	9601	Men X-TT conjugate + Saline + Thiomersal	0.5µg
IRCXLTA1	9601	Men X-TT conjugate + Saline + Thiomersal + AlPO4	0.5µg with 125µg Al^{+++}
IRCXNT1	9601	Men X-TT conjugate+Saline + Thiomersal	1µ
IRCXNAT1	9601	Men X-TT conjugate+Saline + Thiomersal+AlPO4	1µg with 125µg Al^{+++}
IRCX2LT1	8210	Men X-TT conjugate+Saline + Thiomersal	0.5µg
IRCX2LAT1	8210	Men X-TT conjugate+Saline + Thiomersal	0.5µg with 125µg Al^{+++}
IRCX2NT1	8210	Men X-TT conjugate+Saline + Thiomersal	1µg
IRCX2NATA1	8210	Men X-TT conjugate+Saline + Thiomersal	1µg with 125µg Al^{+++}

Table 3

Multivalent Liquid formulation containing Men X—tetanus toxoid conjugate

Formulation Code	Strain	Composition amount per 0.5ml (µ)					Excipients
		A-CRM 197	C-CRM 197	Y-CRM 197	W-CRM 197	X -TT	
IRCPT1	9601	1	1	1	1	1	Sodium Chloride and Thiomersal
IRCPTA1	9601	1	1	1	1	1	Sodium Chloride Thiomersal +125µg Al^{+++}
IRCP2T1	8210	1	1	1	1	1	Sodium Chloride

							and Thiomersal
IRCP2TA1	8210	1	1	1	1	1	Sodium Chloride Thiomersal + 125µg Al^{+++}

Table 4

Multivalent Liquid formulation containing Men X—TT conjugate (Strain 8210)

Formulation Code	Composition amount per 0.5ml (µ)					Excipients
	A- CRM 197	C- CRM 197	Y- CRM 197	W- CRM 197	X- TT	
IRCP4T1	1	1	1	1	1	Sodium Chloride and Thiomersal
IRCP4TA1	1	1	1	1	1	Sodium Chloride Thiomersal +25µg Al^{+++}

Table 5

Multivalent Liquid formulation containing Men X—TT conjugate (Strain 8210)

Formulation Code	Composition amount per 0.5ml (µ)					Excipients
	A- CRM 197	C- CRM 197	Y- TT	W- CRM 197	X- TT	
IRCP5T1	1	1	1	1	1	Sodium Chloride and Thiomersal
IRCP5TA1	1	1	1	1	1	Sodium Chloride Thiomersal + 25µg Al^{+++}

Table 6

Lyophilized multivalent formulation containing Men X—TT conjugate (Strain 8210)

Formulation Code	Composition amount per Vial					Excipients Quantity in a vial
	A- CRM 197	C- CRM 197	Y- TT	W- CRM 197	X- TT	

IRCLPS3Sc	25	25	25	25	25	Tris 0.6 mg, Sucrose 15mg, Sodium citrate 2.5 mg
IRCLPT3Sc	25	25	25	25	25	Tris, Trehalose 15mg, Sodium citrate 2.5 mg
IRCLPS3L2Sc	25	25	25	25	25	Tris, Sucrose 15mg, Lactose 10mg, Sodium citrate 2.5 mg

Example 5:

Biological activity of Meningococcal monovalent and multivalent conjugate composition containing Men X saccharide conjugate.

Each formulation was immunized into six female Swiss Albino Mice of 16-20 g body weight. Mice were immunized subcutaneously on Day 0, 14 and 28. Each mouse was bled after 1 & 2 week post second immunization (Day 21 and Day 35).

Titration of antibody was done by bead based assay and SBA. Pre-immunization serum samples from all six mice were mixed to prepare a single pool serum for each formulation from study 4 onwards and also postimmunization serum sample from six mice all belonging to Swiss Albino strain for each formulation were mixed to prepare pool1, 2 & 3 using serum from Mouse1+2, 3+4 and 5+6, respectively. Each of the pools was analyzed for total IgG titers (Multiplexed bead based assay) and functional antibody titers (SBA).

Table 7

Formulation Code	Ig G			SBA		
	Day 21	Day 28	Day 35	Day 21	Day 28	Day 35
IRCXLT1	504	800	12800	8	20	128
IRCXLTA1	12800	32254	51200	256	323	323
IRCXNT1	2540	2540	8063	81	51	406
IRCXNAT1	6400	12800	32254	203	323	406

Table 8

Formulation Code	Ig G		SBA	
	Day 28	Day 35	Day 28	Day 35
IRCX2LT1	79	3200	2	20
IRCX2LAT1	4032	40637	20	128
IRCX2NT1	1270	51200	5	256
IRCX2NATA1	16127	51200	5	256

Table 9

Formulation Code	Men A						Men C					
	Ig G			SBA			Ig G			SBA		
	Day 21	Day 28	Day 35	Day 21	Day 28	Day 35	Day 21	Day 28	Day 35	Day 21	Day 28	Day 35
IRCPT1	200	200	2016	2	2	13	635	800	8063	2	2	16
IRCPTA1	20319	25600	32254	13	13	51	16127	16127	20319	25	32	102

Table 10

Formulation Code	Men W135						Men Y					
	Ig G			SBA			Ig G			SBA		
	Day 21	Day 28	Day 35	Day 21	Day 28	Day 35	Day 21	Day 28	Day 35	Day 21	Day 28	Day 35
IRCPT1	3200	3200	16127	20	20	81	252	200	1270	2	16	5
IRCPTA1	1600	2016	12800	40	3	25	504	635	1270	10	6	8

Table 11

Formulation Code	Men X					
	Ig G			SBA		
	Day 21	Day 28	Day 35	Day 21	Day 28	Day 35
IRCPT1	1600	2016	32254	64	40	256
IRCPTA1	5080	10159	51200	81	102	406

Table 12

Formulation Code	Men A				Men C				Men W			
	Ig G		SBA		Ig G		SBA		Ig G		SBA	
	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35
IRCP2T1	79	252	2	5	400	6400	3	20	800	10159	6	81
IRCP2TA1	3200	10159	5	16	3200	25600	10	25	3200	32254	2	20

Table 13

Formulation Code	Men Y				Men X			
	Ig G		SBA		Ig G		SBA	
	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35
IRCP2T1	504	5080	25	64	2540	20319	16	81
IRCP2TA1	1600	10159	3	81	6400	32254	40	102

Table 14

Formulation Code	Men A	Men C	Men W135	Men Y	Men X
	SBA	SBA	SBA	SBA	SBA
	Day 35	Day 35	Day 35	Day 35	Day 35
IRCP4T1	13	10	20	8	512
IRCP4TA1	16	25	6	5	512
IRCP5T1	13	25	10	256	406
IRCP5TA1	203	203	102	1625	512

Table 15

Formulation Code	Men A				Men C				Men W135			
	Ig G		SBA		Ig G		SBA		Ig G		SBA	
	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35
IRCLPS3Sc	635	4032	2	5	3200	6400	2	25	3200	10159	6	40
IRCLPS3DSc+ AlPO4	1600	6400	3	25	4032	25600	20	323	4032	12800	13	64
IRCLPT3Sc	200	1270	2	6	317	2540	4	25	2016	4032	10	64
IRCLPT3Sc+ AlPO4	1600	6400	5	51	3200	10159	25	102	2540	16127	20	102
IRCLPS3L2Sc	317	1270	2	2	800	1600	5	13	1600	4032	64	203
IRCLPS3L2Sc+ AlPO4	635	1270	5	8	5080	10159	13	64	3200	8063	8	25

Table 16

Formulation Code	Men Y				Men X			
	Ig G		SBA		Ig G		SBA	
	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35	Day 28	Day 35
IRCLPS3Sc	800	1008	8	5	1008	16127	32	323
IRCLPS3DSc+ AlPO4	2540	16127	25	323	3200	51200	40	406
IRCLPT3Sc	504	4032	6	32	252	12800	16	323
IRCLPT3Sc+ AlPO4	504	4032	4	256	1270	16127	8	256
IRCLPS3L2Sc	504	2016	10	162	317	12800	10	406
IRCLPS3L2Sc+ AlPO4	635	1600	5	8	400	2016	64	102

Above mice immunogenicity data indicates that liquid and lyophilized compositions of monovalent X-tetanus toxoid conjugate and multivalent conjugates containing X-tetanus toxoid conjugate are found to be immunogenic.Further monovalent liquid composition containing 1µg of X-tetanus toxoid conjugate,sodium chloride,thiomersal and 125 µg Al^{+++} gives optimal immunogenic response.Also liquid multivalent composition of 0.5 ml containing A-CRM197,C-CRM197,Y-tetanus toxoid,W-CRM197 and X-tetanus toxoid conjugates with 1µg each of all 5 saccharides,sodium chloride,thiomersal and 25µg Al^{+++} gives optimal immunogenic response.Thus in this pentavalent conjugate composition, conjugates containing tetanus toxoid as carrier protein are found to enhance immunogenicity of conjugates containing CRM 197 as carrier protein.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and

should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We Claim:

1. An immunogenic composition comprising a N.meningitidis X polysaccharide-protein conjugate,wherein said capsular X saccharide is derived from N.meningitidis X strain selected from 8210 and 9601.

2. An immunogenic composition of claim 1,wherein atleast one additional saccharide conjugate(s) comprises a N. meningitidis capsular saccharide derived from serogroups A, B, C, W135 and Y.

3. An immunogenic composition of claim 1, wherein the N. meningitidis X strain is selected from 8210,9601,9592,9554 and 2526.

4. An immunogenic composition of claim 2, wherein the composition comprises each of capsular polysaccharide of N. meningitidis serogroups A, C, W-135,Y & X by utilizing atleast 2 different carrier proteins for conjugating all 5 polysaccharides.

5. An immunogenic composition of claim 1 or 4, wherein each N. meningitidis saccharide(s) are conjugated to a carrier protein selected from the group consisting of TT, DT, CRM197, fragment C of TT, protein D, OMPC and pneumolysin.

6. An immunogenic composition of claim 5, wherein each N. meningitidis saccharide(s) are conjugated to a carrier protein selected from the group consisting of TT, DT and CRM197.

7. An immunogenic composition of claim 6, wherein each N. meningitidis saccharide is conjugated to a carrier protein selected from the group consisting of TT and DT.

8. An immunogenic composition of claim 4,wherein the composition comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii) tetanus toxoid; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) diphtheria toxoid;(d) a conjugate of (i) capsular saccharide of serogroup W135 N.meningitidis and (ii) tetanus toxoid; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii) tetanus toxoid.

9. An immunogenic composition of claim 4,wherein the composition comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii)CRM 197; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii)CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) tetanus toxoid;(d) a conjugate of (i) capsular saccharide of

serogroup W135 N.meningitidis and (ii)CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii)tetanus toxoid; charachterized in that conjugates containing tetanus toxoid as carrier protein are found to enhance immunogenicity of conjugates containing CRM 197 as carrier protein.

10. An immunogenic composition of claim 4,wherein the composition comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii) tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii)CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) tetanus toxoid;(d) a conjugate of (i) capsular saccharide of serogroup W135 N.meningitidis and (ii)CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii)CRM197.

11. An immunogenic composition of claim 4,wherein the composition comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii)tetanus toxoid; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii)CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197;(d) a conjugate of (i) capsular saccharide of serogroup W135 N.meningitidis and (ii)CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii)tetanus toxoid.

12. An immunogenic composition of claim 4,wherein the composition comprises (a)a conjugate of (i) the capsular saccharide of serogroup A N meningitidis and (ii)CRM 197; (b) a conjugate of (i) capsular saccharide of serogroup C N meningitidis and (ii)CRM197; (c) a conjugate of (i) capsular saccharide of serogroup Y N. meningitidis and (ii) CRM197;(d) a conjugate of (i) capsular saccharide of serogroup W135 N.meningitidis and (ii)CRM197; and (d) a conjugate of (i) capsular saccharide of serogroup X N meningitidis and (ii)tetanus toxoid.

13. An immunogenic composition of claim 4 ,wherein each capsular polysaccharide has an average size of between 100 and 600 KDa.

14. An immunogenic composition of claim 13 ,wherein each capsular polysaccharide has an average size of between 100 and 300 KDa.

15. An immunogenic composition of claim 14 ,wherein each capsular polysaccharide has an average size of between 100 and 200 KDa.

16. An immunogenic composition of claim 4 ,wherein atleast 3 N.meningitidis polysaccharides from serogroups A, C, W135 and X post-sizing have an average size of between 100 and 150 KDa.

17. An immunogenic composition of claim 16 ,wherein the sizing is by using high pressure cell disruption system.

18. An immunogenic composition of claim 4 ,wherein N.meningitidis polysaccharide from serogroups Y post-chemical sizing have an average size of between 90 and 110 KDa.

19. An immunogenic composition of claim 18 ,wherein the chemical sizing is by using sodium acetate at a temperature from 60 to 80°C.

20. An immunogenic composition of claim 4, wherein each of the N. meningitidis saccharides is conjugated to the carrier protein via a hetero or homo-bifunctional linker with cyanylation conjugation chemistry.

21. An immunogenic composition of claim 20, wherein the linker is ADH.

22.An immunogenic composition of any preceding claim, wherein said cyanylation reagent is selected from a group of l-cyano- 4- pyrrolidinopyridinium tetrafluoroborate (CPPT), 1- cyano- imidazole (1-CI), 1-cyanobenzotriazole (1-CBT), or 2- cyanopyridazine -3(2H)one (2-CPO), or a functional derivative or modification thereof.

23. An immunogenic composition of claim 20, wherein the N.meningitidis X saccharide-tetanus toxoid conjugates are prepared by a conjugation reaction comprising of i)sizing of polysaccharide ii)CPPT based activation of sized polysaccharide having average molecular weight between 100-150 Kda, at a pH between 9 to 9.5 iii)ADH addition after a duration of about 2 to 3 minutes followed by incubation period of 4-20 hrs,iv)diafiltration to remove unreacted ADH and v)reacting ADH activated polysaccharide with purified non-activated carrier protein in a ratio between 0.75 - 1.5 in presence of MES buffer and EDAC followed by incubation period of 3 -4 hrs, characterized in that the conjugation reaction is carried at a temperature between 2-8°C and ratio of saccharide to protein in final conjugate is between 0.2 to 0.6.

24. An immunogenic composition of claim 20, wherein the N.meningitidis X saccharide- tetanus toxoid conjugates are prepared by a conjugation reaction comprising of i)sizing of polysaccharide ii)CPPT based activation of sized polysaccharide having average molecular weight between 100-150 Kda, at a pH between 9 to 9.5 iii)addition of ADH activated carrier protein in a saccharide:protein ratio between

0.5 - 2 after 2-3 minutes followed by incubation period of 2 to 20 hrs characterized in that the conjugation reaction is carried at 22°C to about 25°C and ratio of saccharide to protein in final conjugate is between 0.2 to 0.6.

25. An immunogenic composition of claim 1 or 2, comprising serogroup A,C,Y,W135 and X saccharide at an amount of 0.5-10µg ,0.5-5µg or 0.5-2µg per 0.5 ml dose.

26. An immunogenic composition of claim 25, comprising 10 µg of serogroup A saccharide, 5µg of serogroup C saccharide, 5µg of serogroup W135 saccharide, 5µg of serogroup Y saccharide and 5µg of serogroup X saccharide.

27. An immunogenic composition of claim 25, comprising 5 µg of serogroup A saccharide, 5µg of serogroup C saccharide, 5µg of serogroup W135 saccharide, 5µg of serogroup Y saccharide and 5µg of serogroup X saccharide.

28. An immunogenic composition according to claim 25, wherein one or more N.meningitidis saccharide conjugates are optionally adsorbed onto aluminium hydroxide, aluminium phosphate or a mixture of both or unadsorbed onto adjuvant.

29. An immunogenic composition according to claim 28, comprising a step of adding aluminium salt adjuvant at an amount of 20-300µg,20-200µg,25-150µg of Al^{+++} per 0.5 ml dose.

30. An immunogenic composition according to claim 29,comprising a step of adding aluminium salt adjuvant at an amount of 25-125µg of Al^{+++} per 0.5 ml.

31. An immunogenic composition of claim 28,further comprising a preservative selected from thiomersal and 2-phenoxyethanol.

32. An immunogenic composition of claim 31,wherein the composition comprises sodium phosphate, sodium chloride or combination thereof.

33. An immunogenic composition according to any preceding claim wherein the saccharide conjugates are in a buffered liquid form.

34. An immunogenic composition according to any preceding claim wherein the saccharide conjugates are in lyophilized form.

35. A lyophilized immunogenic composition according to claim 34,wherein the stabilizer combination is selected from a) 2 to 5% (w/v) Trehalose, 0.25 to 0.75% sodium citrate;b) 2 to 5% (w/v) Sucrose and 0.25 to 0.75% sodium citrate;c) 2 to 5% (w/v) Sucrose,2 to 5% (w/v) Lactose and 0.25 to 0.75% sodium citrate;and d) 2 to 5% (w/v) Trehalose ,2 to 5% (w/v) Lactose and 0.25 to 0.75% sodium citrate.

36. A lyophilized immunogenic composition according to claim 35 ,further comprising a buffer selected from Tris and phosphate.

37. A method of immunizing a human host against disease caused by Neisseria meningitidis infection comprising administering to the host an immunoprotective dose of the immunogenic composition or vaccine of claims 1-36.

Figure 1

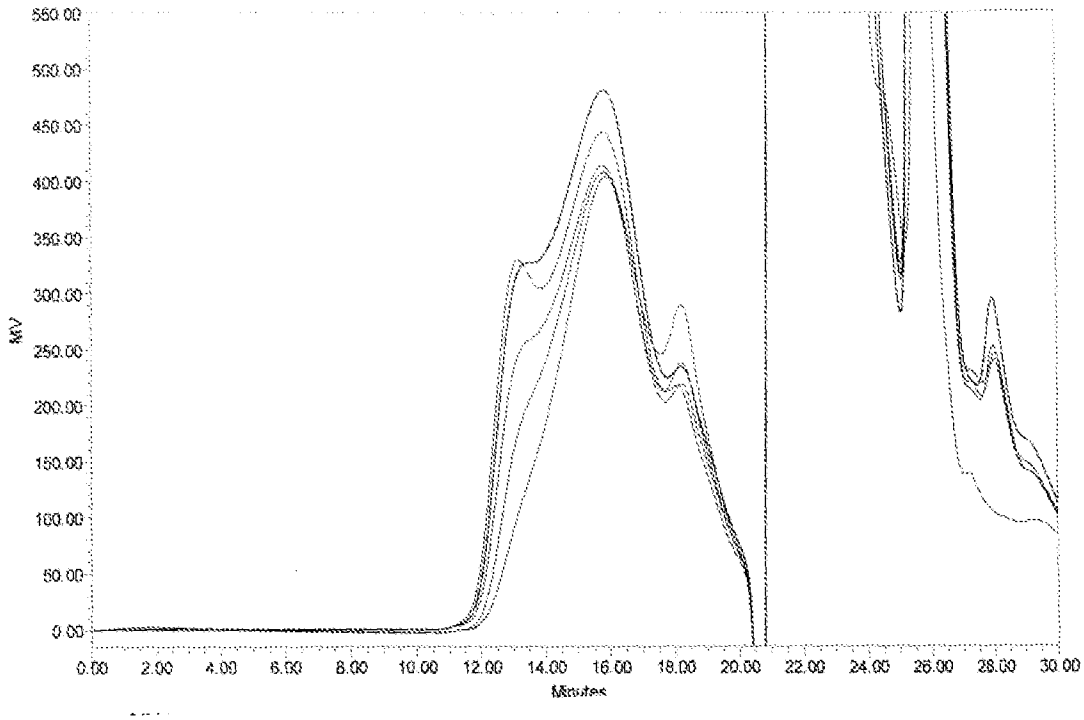

Figure 2

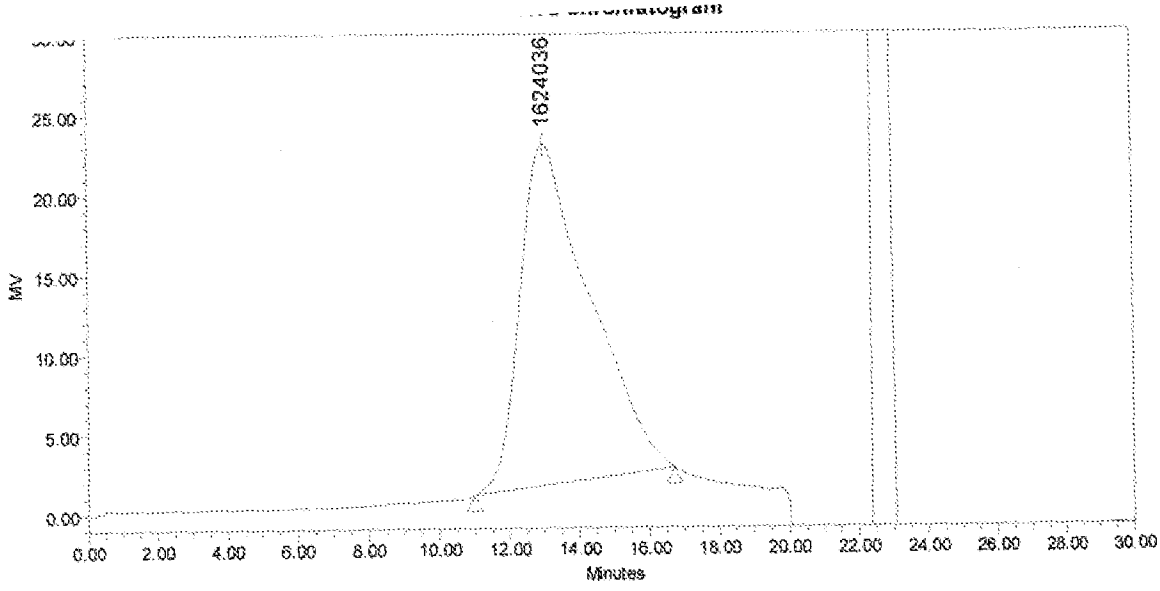

Figure 3

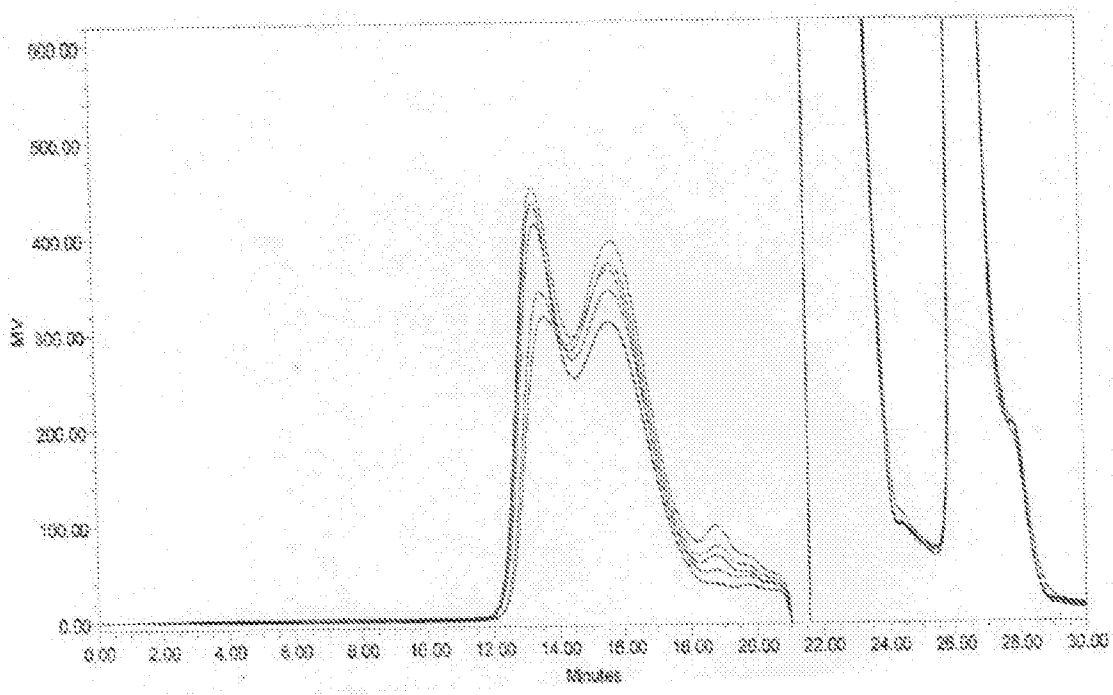

Figure 4

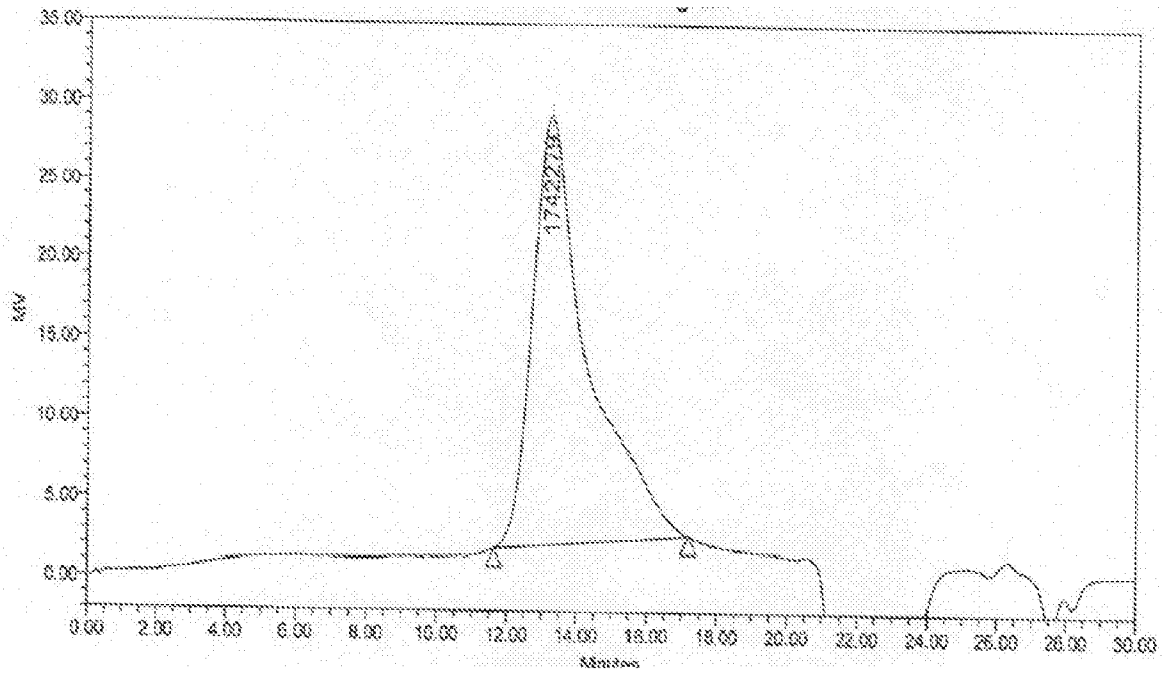

Figure 5

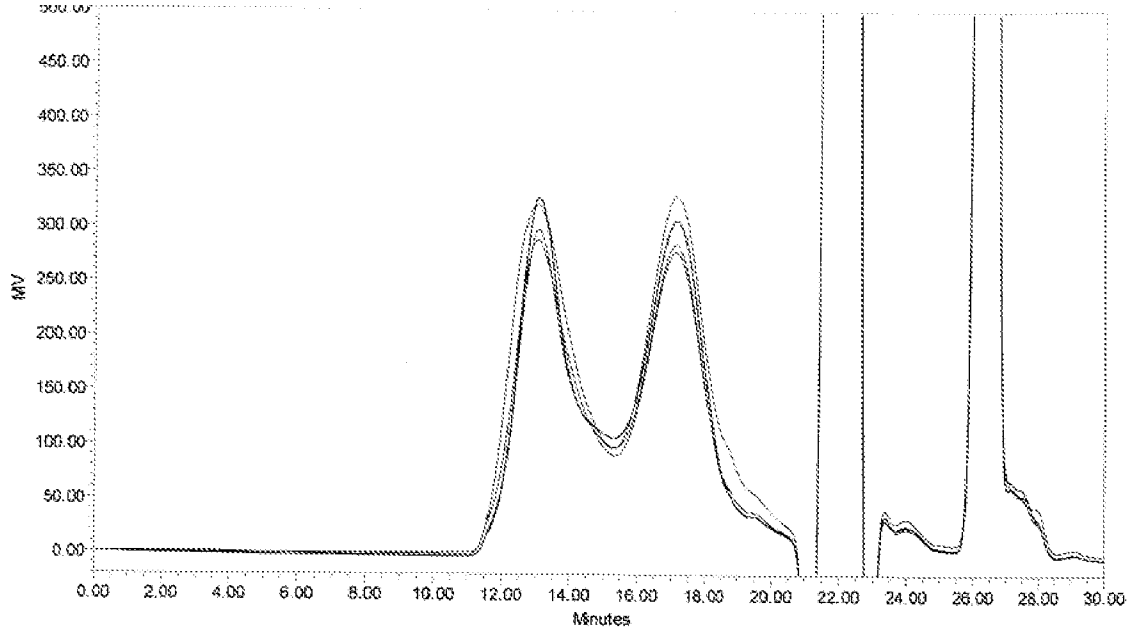

Figure 6

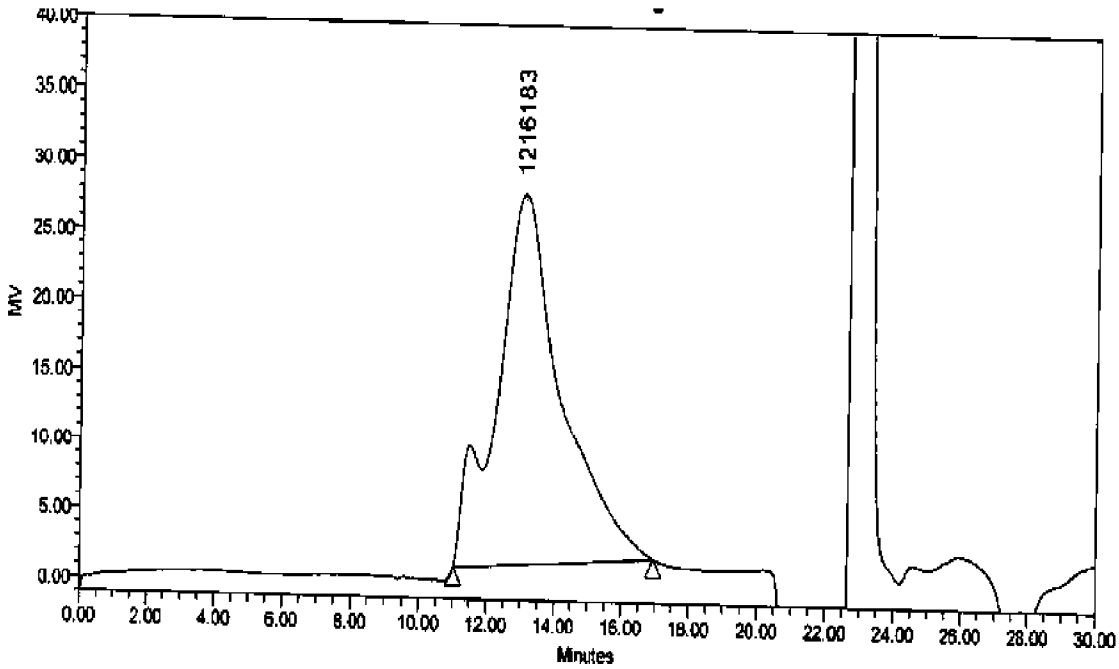

Figure 7

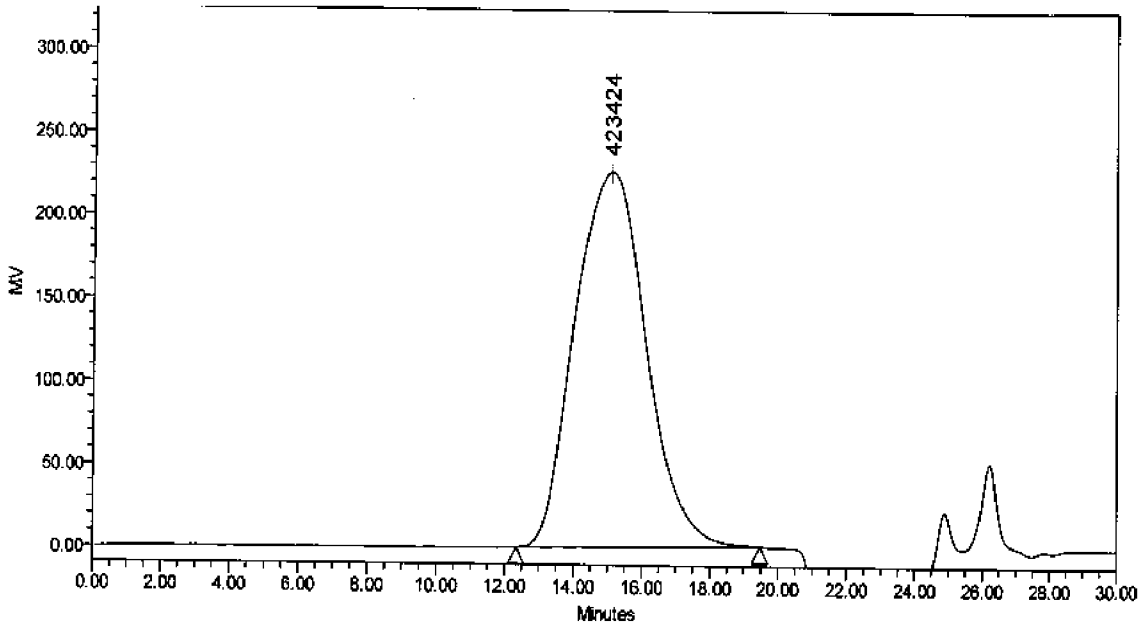
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Figure 8

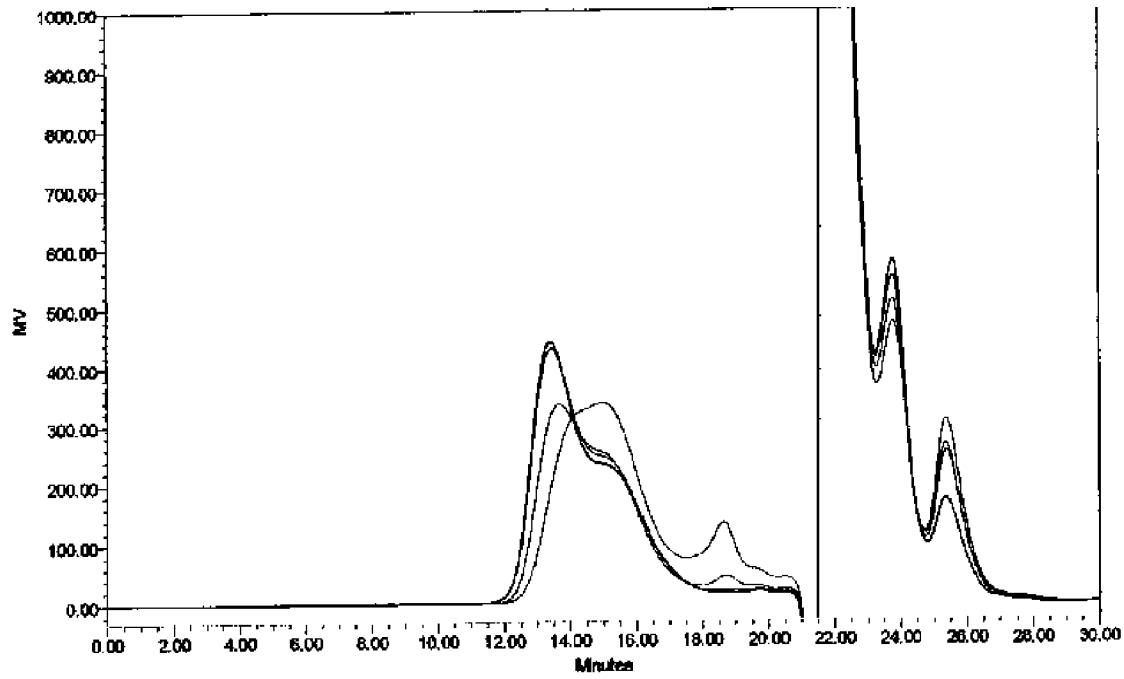
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Figure 9

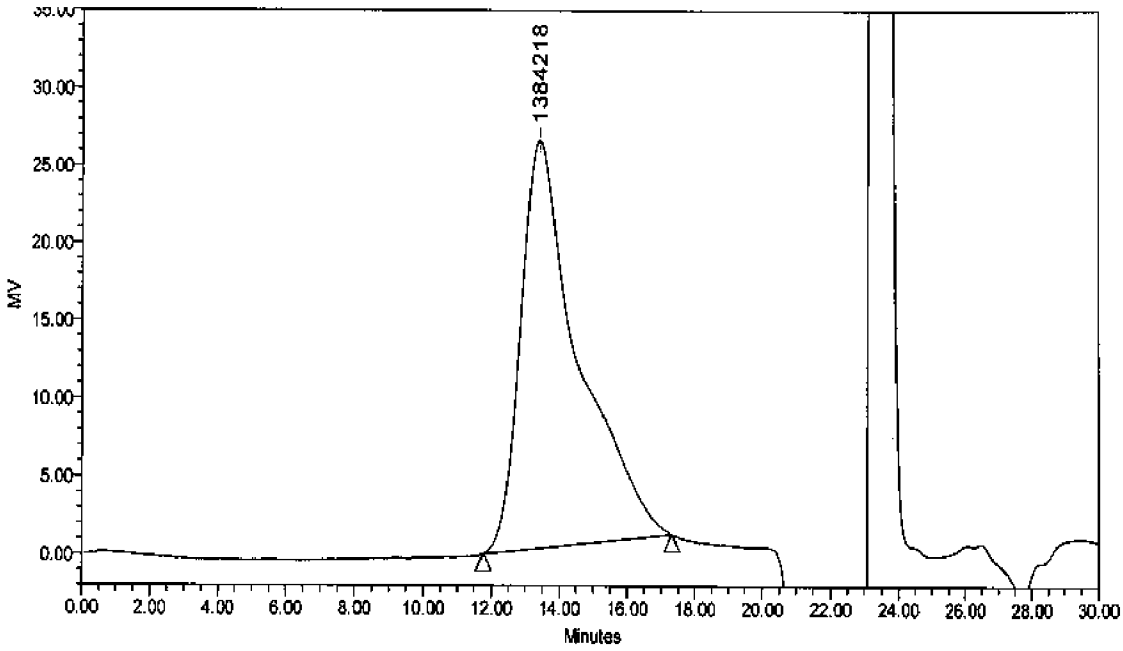


Figure 10

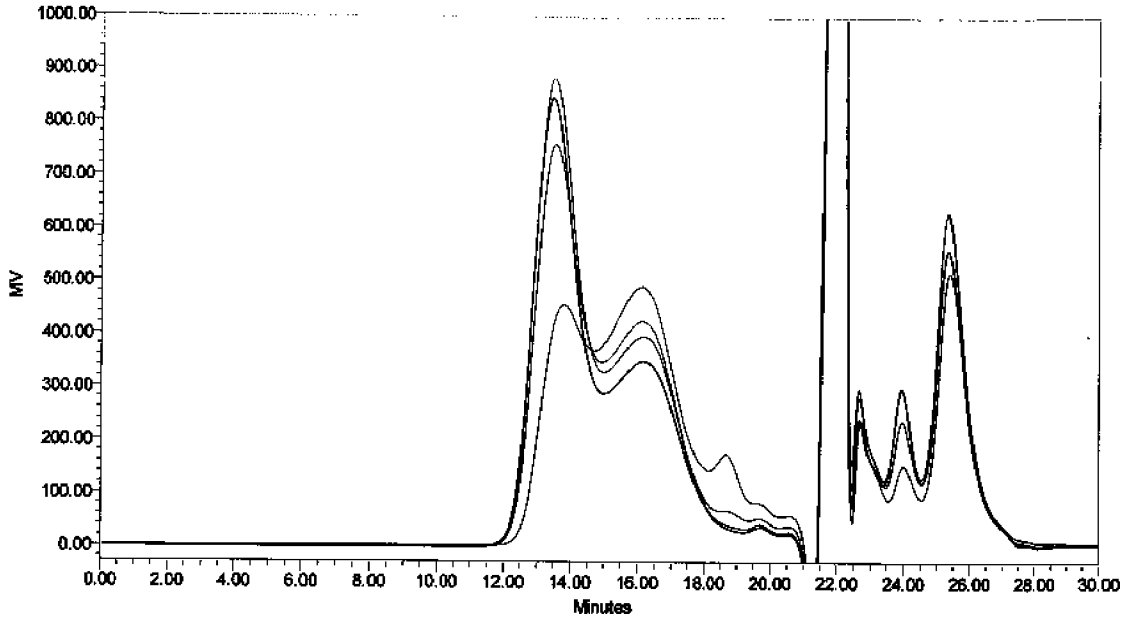
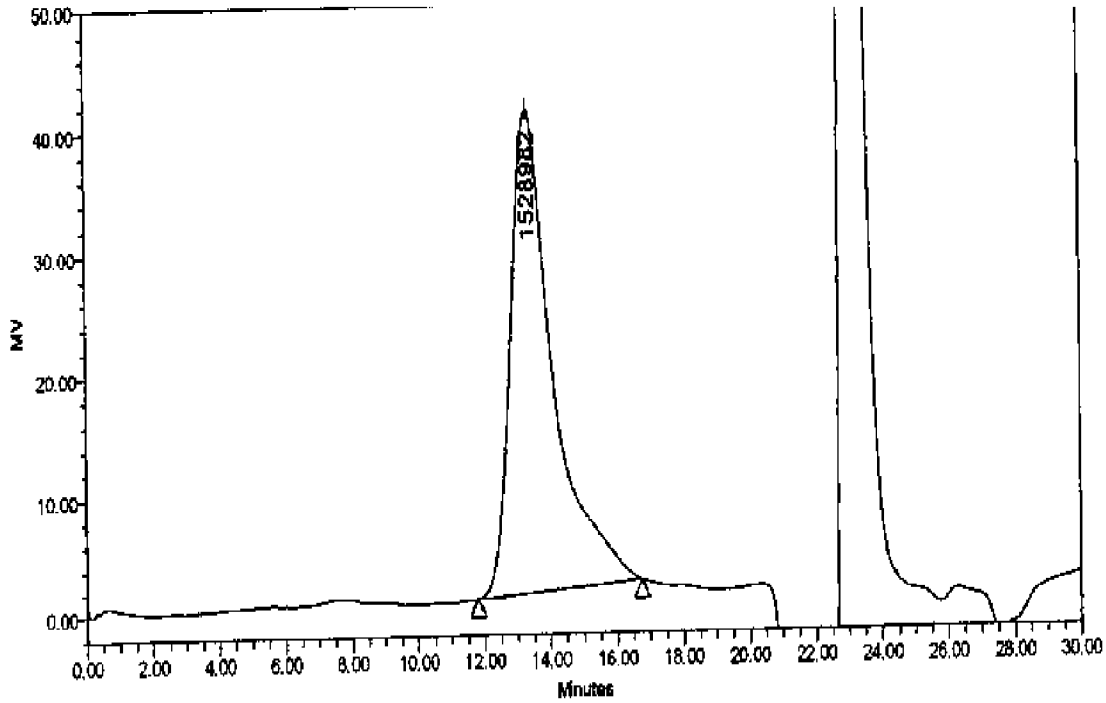


Figure 11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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2013/050739

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/116 (2013.01)
USPC - 424/197.11
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 39/095, 39/116, 39/385 (2013.01)
USPC - 424/190.1, 184.1, 197.11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 39/095, 39/116, 39/385 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patent, USPTO, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2008/102173 A1 (KAPRE et al.) 28 August 2008 (28.08.2008) entire document	1-21, 23-32
Y	MOTHERSHED et al. "Use of Real-Time PCR to Resolve Slide Agglutination Discrepancies in Serogroup Identification of Neisseria meningitidis," Journal of Clinical Microbiology, 01 January 2004 (01.01.2004), Vol. 42, No. 1, Pgs. 320-328. entire document	1-21, 23-32
Y	WO 2003/051392 A2 (LAFERRIERE et al) 26 June 2003 (26.06.2003) entire document	4, 8-21, 23, 24
Y	US 2008/0193476 A1 (BIEMENS et al.) 14 August 2008 (14.08.2008) entire document	13-21, 23, 24
Y	EP 1741442 A2 (CONSTANTINO) 10 January 2007 (10.01.2007) entire document	19
Y	EP 2308504 A2 (BORKOWSKI) 13 April 2011 (13.04.2011) entire document	31, 32

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 June 2013	28 JUN 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2013/050739

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 22, 33-37
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.