(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
03 March 2022 (03.03.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/043855 A1

(51) International Patent Classification:
A61K 39/09 (2006.01) *A61K 39/00* (2006.01)
A61P 31/04 (2006.01)

(21) International Application Number:
PCT/IB2021/057714

(22) International Filing Date:
23 August 2021 (23.08.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/070,410 26 August 2020 (26.08.2020) US
63/229,359 04 August 2021 (04.08.2021) US

(71) Applicant: **PFIZER INC.** [US/US]; 235 East 42nd Street, New York, New York 10017 (US).

(72) Inventors: **ANDERSON, Annaliesa Sybil**; c/o Pfizer Inc., 401 N. Middletown Road, Pearl River, New York 10965 (US). **DONALD, Robert George Konrad**; c/o Pfizer Inc., 401 N. Middletown Road, Pearl River, New York 10965 (US). **HAWKINS, Julio Cesar**; c/o Pfizer Inc., 401 N. Middletown Road, Pearl River, New York 10965 (US). **KODALI, Srinivas**; c/o Pfizer Inc., 401 N. Middletown Road, Pearl River, New York 10965 (US). **SIMON, Raphael**; c/o Pfizer Inc., 401 N. Middletown Road, Pearl River, New York 10965 (US).

(74) Agent: **ZIELINSKI, Bryan C.**; PFIZER INC., 235 East 42nd Street, MS 235/9/86, New York, New York 10017 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

WO 2022/043855 A1

(54) Title: GROUP B STREPTOCOCCUS POLYSACCHARIDE-PROTEIN CONJUGATES, METHODS FOR PRODUCING CONJUGATES, IMMUNOGENIC COMPOSITIONS COMPRISING CONJUGATES, AND USES THEREOF

(57) Abstract: The invention relates to immunogenic polysaccharide-protein conjugates comprising a capsular polysaccharide (CP) from Streptococcus agalactiae, commonly referred to as group B streptococcus (GBS), and a carrier protein, wherein the CP is selected from the group consisting of serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX, and wherein the CP has a sialic acid level of greater than about 60%. The invention also relates to methods of making the conjugates and immunogenic compositions comprising the conjugates. The invention further relates to methods for inducing an immune response in subjects against GBS and/or for reducing or preventing invasive GBS disease in subjects using the compositions disclosed herein. The resulting antibodies can be used to treat or prevent GBS infection via passive immunotherapy.

GROUP B STREPTOCOCCUS POLYSACCHARIDE-PROTEIN CONJUGATES, METHODS FOR PRODUCING CONJUGATES, IMMUNOGENIC COMPOSITIONS COMPRISING CONJUGATES, AND USES THEREOF

FIELD OF THE INVENTION

The invention relates to immunogenic polysaccharide-protein conjugates comprising a capsular polysaccharide (CP) from *Streptococcus agalactiae*, commonly referred to as group B streptococcus (GBS), and a carrier protein, wherein the CP is selected from the group consisting of serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX, and wherein the CP has a sialic acid level of greater than about 60%. The invention also relates to methods of making the conjugates and immunogenic compositions comprising the conjugates. The invention also relates to immunogenic compositions comprising polysaccharide-protein conjugates, wherein the conjugates comprise a CP from at least one GBS serotype selected from serotypes VI, VII, VIII, and IX, and optionally one or more additional GBS serotype(s). The invention further relates to methods for inducing an immune response in subjects against GBS and/or for reducing or preventing invasive GBS disease in subjects using the compositions disclosed herein. The resulting antibodies can be used to treat or prevent GBS infection via passive immunotherapy, or to immunize mothers for protection of offspring via maternal antibody transfer.

BACKGROUND OF THE INVENTION

Streptococcus agalactiae are Gram positive polysaccharide encapsulated organisms that are also known as group B streptococcus (GBS). They are a common commensal of the human gastrointestinal and genital tract and also a cause of serious disease in infants and older adults (Baker, C.J., Vaccine, 31(Suppl. 4):D3-D6 (2013)). The main risk factor for GBS infection in infants is maternal colonization (Dillon, H.C., et al., J. Pediatr., 110(1):31-36 (1987)). As many as one in four women carry GBS recto-vaginally, which can infect the amniotic fluid or baby before or during delivery causing sepsis, pneumonia, and meningitis (Baker 2013; Heath, P.T., et al., BMJ Clin. Evid. (Online), pii:0323 (2014)). It is estimated that at least twenty five percent of infants who survive GBS meningitis suffer from neurologic impairment with 19% experiencing cognitive delay, cerebral palsy, blindness, and hearing loss (Libster, R., et al., Pediatrics, 130(1):e8-152012 (2012)). GBS is also linked to miscarriages, preterm deliveries and stillbirths (McDonald, H.M., et al., Infectious Diseases in Obstetrics and Gynecology, 8(5-6):220-227 (2000); Randis, T.M., et al., The Journal of Infectious Diseases, 210(2):265-273 (2014): Kessous, R., et al., J. Matern. Fetal Neonatal Med., 25(10):1983-1986 (2012)). Very low birth weight infants are at much higher risk of infection, with up to 3% infected and mortality rates of up to 30%, even with immediate antibiotic treatment (Heath 2014).

The introduction in the late 1990's of GBS screening and intrapartum antibiotic prophylaxis (IAP) in the U.S. reduced rates of neonatal disease occurring within the first week of

life (early onset disease [EOD]), but has had no measurable impact on rates of late onset disease (LOD) appearing thereafter within the first 3 months of life. U.S. rates of EOD and LOD cases are currently 0.25 and 0.27 per 1,000 births respectively (Centers for Disease Control and Prevention (CDC), Active Bacterial Core (ABC) Surveillance Report (2013) available at http://www.cdc.gov/abcs/reports-findings/survreports/gbs13.pdf). Following introduction of pneumococcal conjugate vaccines for prevention of invasive pneumococcal disease, including bacteremia and meningitis, and in spite of IAP for prevention of GBS disease, GBS has become the single most common cause of neonatal sepsis (EOD) and meningitis in infants (< 2 mo) in the U.S. (Verani, J.R., et al., MMWR, 59(RR10):1-32 (2010); Thigpen, M.C., et al., New England Journal of Medicine, 364(21):2016-2025 (2011)). Unlike in the U.S., the introduction of prevention guidelines for invasive GBS disease and IAP has not reduced the incidence of EOD in the Netherlands or the U.K. (Bekker, V., et al., The Lancet Infectious Diseases, 14(11):1083-1089 (2014); Lamagni, T.L., et al., Clin. Infect. Dis., 57(5):682-688 (2013)). This lack of effect may be due to the lack of universal screening and restricting IAP to mothers in the highest risk groups (e.g., fever, prolonged ruptured membranes). Rates of EOD are significantly higher in countries that do not use IAP, with a mean incidence reported of 0.75 per 1,000 live births (95% CI 0.58-0.89) (Edmond, K.M, et al., Lancet, 379(9815):547-556 (2012)).

Another population at risk for GBS disease is the elderly. Risk factors include chronic medical problems such diabetes mellitus, cancer, heart failure, neurologic, and urologic conditions. According to CDC ABC surveillance data, the annual U.S. incidence of invasive GBS in 2013 was 0.28/1,000 adults or 12,400 cases/year in adults ≥ 65 years of age. This rate approaches the incidence of invasive pneumococcal disease in the elderly (vs. 0.30/1,000 for >65). These rates are expected to continue to increase in both the U.S. and in Europe (CDC 2013; Lamagni 2013).

One approach to prevent GBS disease among infants and the elderly is the use of a capsule polysaccharide-based vaccine. The implementation of a maternal GBS prophylactic vaccine has the potential to prevent GBS disease among infants in the U.S., regardless of whether IAP is used. Although polysaccharides can be immunogenic on their own, conjugation of polysaccharides to protein carriers has been used to improve immunogenicity, particularly in infants and the elderly. Polysaccharide-protein conjugate vaccines are made using polysaccharides, generally from the surface coat of bacteria, linked to protein carriers. The chemical bonding of the polysaccharide and protein carrier induces an immune response against bacteria displaying the polysaccharide contained within the vaccine on their surface, thus preventing disease. Accordingly, vaccination using polysaccharides from pathogenic bacteria is a potential strategy for boosting host immunity.

The structure of the polysaccharides that cover bacteria vary greatly, even within a single species of bacteria. For example, in GBS there are ten different serotypes (i.e., serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII and IX) due to variation in the bacterial polysaccharide capsule.

Therefore, it is desirable for polysaccharide-based vaccines to consist of a panel of polysaccharides to ensure breadth of coverage against different circulating strains.

The carrier protein can be either a related protein antigen from the target pathogen, boosting the specific immune response to that pathogen, or a generally immunogenic protein that serves more as an adjuvant or general immune response stimulant.

Individual monovalent polysaccharide-protein conjugates of GBS serotypes Ia, Ib, II, III, and V have been evaluated in phase 1 and 2 clinical trials in non-pregnant adults (Brigtsen, A.K., et al., Journal of Infectious Diseases, 185(9):1277-1284 (2002); Baker, C.J., et al., J. Infect. Dis., 188(1):66-73 (2003); Baker, C.J., et al., J. Infect. Dis., 189(6):1103-1112 (2004); Baker, C.J., et al., Vaccine, 25(1):55-63 (2007)). Bivalent II-TT and III-TT glycoconjugate vaccines and a trivalent vaccine comprising Ia-CRM_{197}, Ib-CRM_{197} and III-CRM_{197} glycoconjugates have also been studied (Baker JID 2003; Clicaltrials.gov NCT01193920, NCT01412801, and NCT01446289). However, no GBS vaccines have yet to be approved.

Moreover, while the tri-valent vaccine covers >90% of invasive strains causing neonatal disease in South Africa (Madzivhandila, M., et al., PloS One, 6(3):e17861 (2011)), these same serotypes represent only 62% and 66% of invasive isolates in North America and Europe, respectively, based on surveillance of recent neonatal isolates from a global collection of 901 samples collected between 2004-2013 from the Tigecycline Evaluation and Surveillance Trial (T.E.S.T., http://www.testsurveillance.com/).

Analysis of the strains obtained from the T.E.S.T. samples showed that 95% of the strains collected belonged to one of the five documented major serotypes (Ia, Ib, II, III, and V) and a further 3% were serotype IV. A series of publications have also confirmed the appearance of serotype IV over the last decade in the Americas and in Europe (Diedrick, M.J., et al., J. Clin. Microbiol., 48(9):3100-3104 (2010); Teatero (2014); Meehan, M. et al., European Journal of Clinical Microbiology & Infectious Diseases, 33(7):1155-1162 (2014); Florindo, C., et al., Euro Surveillance: Bulletin European sur les Maladies Transmissibles (European Communicable Disease Bulletin), 19(23) (2014); Palmiero, J.K., et al., Journal of Clinical Microbiology, 48(12):4397-4403 (2010)). A study surveying recto/vaginal carriage in adults, which is a risk factor for transmission of GBS to the infant, also found 97% of isolates belonging to one of these six serotypes, with serotype IV representing a frequency of ~4%. The study was designed to monitor carriage of beta-hemolytic streptococci (which includes GBS), *Clostridium difficile*, and *Staphylococcus aureus* in healthy U.S. adults (*see* Matson, M.A., et al, ICAAC, Abstract I-306 (Washington, DC, Sep. 5-9, 2014)).

Similarly, analysis of T.E.S.T. samples showed 98% of U.S. blood isolates from older adults ≥ 65 years of age belong to the same six predominant serogroups. The most noticeable difference between the elderly isolates and the other populations is the serogroup distribution. For the isolates from elderly patients, serotype V strains constitute the largest group (34% *vs.* 18% for neonatal or 18% for adult carriage strains).

Studies of GBS epidemiology have found that there is a geographic variance of serotype prevalence and indicate that the prevalence of non-GBS6 serotypes depends on a number of factors including geography, age of patient and whether the surveillance is based on colonization or invasive disease.

For instance, serotype VI and VIII isolates have been shown to be predominant colonizers of healthy pregnant women in Japan (Lachenauer, C.S., et al., JID 179(4):1030-1033 (1999). Rates of non-GBS6 serotype colonization are significantly higher in Asia than in Western countries. In a Japanese study of 73 pregnant women, rates of serotype VI and VIII carriage were 35.6% and 24.7%, respectively. In contrast, these serotypes are rarely observed among pregnant women in the US [1, 2]. However, these serotypes do not cause correspondingly high rates of disease in infants. In a large study of GBS epidemiology in Japan in which neonatal-disease-causing isolates were collected in 2011-2015 (n= 132), the prevalence of non-GBS6 serotypes was negligible (1.1% for VI in EOD only; 1.1% of serotype IX, for late onset disease only) [3]. These results are in line with an earlier neonatal invasive isolate study (n=60) in which a similar serotype prevalence rates were found: III (48.3%), Ia (30.0%), and Ib (10.0%) [4]. This trend for low rates of non-GBS6 neonatal disease seems to be similar in China, although studies published to date have involved smaller numbers of isolates (< 50) [5-7].

Significantly, the rate of invasive disease caused by non-GBS6 serotypes is much higher in elderly patients. In a large Japanese study based on isolates obtained in 2010-2013 (n=443), the prevalence of invasive serotype VI was 9.5% [8]. Similar trends are observed in Taiwan where high rates of invasive serotype VI disease are seen in the elderly but not in infants [9].

Based on these surveillance studies serotype VI appears to be an emerging threat that encourages inclusion in a second-generation vaccine, especially one aimed at the elderly demographic. The incidence of invasive disease caused by serotypes VII, VIII and IX is currently rare, but may become more important should serotype replacement occur following introduction of a GBS6 vaccine.

Accordingly, a need exists for polysaccharide-protein conjugate vaccines or monoclonal antibodies to confer passive immunity as a means to prevent or treat GBS diseases, including those caused by emerging serotypes VI, VII, VIII and IX, among broad populations worldwide.

SUMMARY OF THE INVENTION

In an embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a group B streptococcus (GBS) capsular polysaccharide and a carrier protein, wherein the capsular polysaccharide has a sialic acid level of greater than about 60%, greater than about 95%, or about 100%.In another embodiment, the invention comprises an immunogenic composition as described herein, wherein the capsular polysaccharide is selected from the group consisting of serotypes VI, VII, VIII, and IX.

In still another embodiment, the invention comprises an immunogenic composition as described herein, wherein the capsular polysaccharide has at least about 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, or 0.95 mM sialic acid per mM of polysaccharide.

In yet another embodiment, the invention comprises an immunogenic composition as described herein, wherein the capsular polysaccharide has a molecular weight of between about 5 kDa and about 1,000 kDa, between about 25 kDa and about 750 kDa, between about 25 kDa and about 400 kDa, between about 25 kDa and about 200 kDa, or between about 100 kDa and about 400 kDa.

In another embodiment, the invention comprises an immunogenic composition as described herein, wherein the molecular weight of the conjugate is between about 300 kDa and about 20,000 kDa, between about 1,000 kDa and about 15,000 kDa, or between about 1,000 kDa and about 10,000 kDa.

In a further embodiment, the invention comprises an immunogenic composition as described, wherein the capsular polysaccharide is less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% O-acetylated.

In another embodiment, the invention comprises an immunogenic composition as described, wherein the capsular polysaccharide has at least about 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.3, 0.35 or about 0.4 mM O-acetate per mM saccharide repeating unit.

In an embodiment, the invention comprises an immunogenic composition as described herein, wherein the carrier protein is selected from CRM_{197}, Diphtheria toxoid (DT), tetanus toxoid (TT), and Streptococcal C5a peptidase (SCP).

In another embodiment, the invention comprises a method of isolating a capsular polysaccharide comprising reacting an organic reagent with a cell broth comprising a capsular polysaccharide producing bacterium.

In another embodiment, the invention comprises a method of isolating a capsular polysaccharide as described, wherein the bacterium is not lysed, and/or wherein the bacterium is heat killed.

In another embodiment, the invention comprises a method of isolating a capsular polysaccharide as described, wherein the method further comprises the step of centrifuging to provide a cell paste.

In yet another embodiment, the invention comprises a method of isolating a capsular polysaccharide as described, wherein the method further comprises the step of filtering, optionally including a filtering step that is a diafiltration.

In still another embodiment, the invention comprises a method of isolating a capsular polysaccharide as described, wherein the capsular polysaccharide producing bacterium comprises *Streptococcus agalactiae.*

In another embodiment, the invention comprises a method of isolating a capsular polysaccharide as described, wherein the pH of the reaction is about 5.5 to about 9.5.

In still another embodiment, the invention comprises a method of isolating a capsular polysaccharide as described, wherein the reaction takes place at a temperature of about 20°C to about 85°C.

In yet another embodiment, the invention comprises a method of isolating a capsular polysaccharide as described, wherein the reaction time is about 10 hours to about 90 hours.

In another embodiment, the invention comprises a method of making the immunogenic composition as described herein, wherein the capsular polysaccharide is isolated according to a method as described herein.

In an embodiment, the invention comprises an immunogenic composition comprising a capsular polysaccharide-protein conjugate prepared by a method as described herein.

In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate as described herein, wherein the conjugate comprises a capsular polysaccharide from group B streptococcus (GBS) serotype VI and a carrier protein, and at least one additional serotype selected from the group consisting of Ia, Ib, II, III, IV, V, VII, VIII, and IX.

In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS capsular polysaccharide serotype VI and a carrier protein. In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS capsular polysaccharide serotype VI, and further comprises at least one additional serotype selected from Ia, Ib, II, III, IV, V, VII, VIII, and IX.

In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS serotype VI capsular polysaccharide, and further comprises at least one additional serotype selected from Ia, Ib, II, III, IV, and V. In a further embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS serotype VI capsular polysaccharide, and further comprises at least one additional serotype selected from VII, VIII, and IX.

In still another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate as described herein, wherein the conjugate comprises a capsular polysaccharide from group B streptococcus (GBS) serotype VI and a carrier protein, wherein the capsular polysaccharide has a sialic acid level of greater than about 60%, greater than about 95%, or about 100%.

In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS capsular polysaccharide serotype VII and a carrier protein. In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS

capsular polysaccharide serotype VII, and further comprises at least one additional serotype selected from Ia, Ib, II, III, IV, V, VI, VIII, and IX.

In yet another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate as described herein, wherein the conjugate comprises a capsular polysaccharide from group B streptococcus (GBS) serotype VII capsular polysaccharide and a carrier protein, wherein the capsular polysaccharide has a sialic acid level of greater than about 60%, greater than about 95%, or about 100%.

In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS capsular polysaccharide serotype VIII and a carrier protein. In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS capsular polysaccharide serotype VIII, and further comprises at least one additional serotype selected from Ia, Ib, II, III, IV, V, VI, VII, and IX.

In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate as described herein, wherein the conjugate comprises a capsular polysaccharide from group B streptococcus (GBS) serotype VIII capsular polysaccharide and a carrier protein, wherein the capsular polysaccharide has a sialic acid level of greater than about 60%, greater than about 95%, or about 100%.

In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS capsular polysaccharide serotype IX and a carrier protein. In another embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate comprising a GBS capsular polysaccharide serotype IX, and further comprises at least one additional serotype selected from Ia, Ib, II, III, IV, V, VI, VII, and VIII.

In a further embodiment, the invention comprises an immunogenic composition comprising a polysaccharide-protein conjugate as described herein, wherein the conjugate comprises a capsular polysaccharide from group B streptococcus (GBS) serotype IX capsular polysaccharide and a carrier protein, wherein the capsular polysaccharide has a sialic acid level of greater than about 60%, greater than about 95%, or about 100%.

In still another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VI and VII, and a carrier protein.

In yet another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VI and VIII, and a carrier protein.

In another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VI and IX, and a carrier protein.

In still another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VII and VIII, and a carrier protein.

In an embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VII and IX, and a carrier protein.

In another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VIII and IX, and a carrier protein.

In yet another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VI, VII and VIII, and a carrier protein.

In still another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VI, VII and IX, and a carrier protein.

In an embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VI, VIII and IX, and a carrier protein.

In another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes VI, VII, VIII and IX, and a carrier protein.

In yet another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes Ia, Ib, II, III, IV, V, and VI, and a carrier protein.

In yet another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes Ia, Ib, II, III, IV, V, VI and VII, and a carrier protein.

In still another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes Ia, Ib, II, III, IV, V, VI, VII and VIII, and a carrier protein.

In an embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII and IX, and a carrier protein.

In an embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates, wherein the conjugates comprise capsular polysaccharides from GBS serotypes Ia and VI, and a carrier protein.

In still another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes Ib and VI, and a carrier protein.

In yet another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes II and VI, and a carrier protein.

In another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes III and VI, and a carrier protein.

In a further embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes IV and VI, and a carrier protein.

In another embodiment, the invention comprises an immunogenic composition comprising polysaccharide-protein conjugates as described herein, wherein the conjugates comprise capsular polysaccharides from GBS serotypes V and VI, and a carrier protein.

In another embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition further comprises a pharmaceutically acceptable excipient, buffer, stabilizer, adjuvant, a cryoprotectant, a salt, a divalent cation, a non-ionic detergent, an inhibitor of free radical oxidation, a carrier, or a mixture thereof.

In still another embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition further comprises a buffer selected from the group consisting of HEPES, PIPES, MES, Tris (trimethamine), phosphate, acetate, borate, citrate, glycine, histidine and succinate.

In yet another embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition further comprises a surfactant selected from the group consisting of polyoxyethylene sorbitan fatty acid esters, polysorbate-80, polysorbate-60, polysorbate-40, polysorbate-20, and polyoxyethylene alkyl ethers.

In an embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition further comprises an excipient selected from the group consisting of starch, glucose, lactose, sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol, palatinit, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, glycine, arginine, lysine, sodium chloride (NaCl), dried skim milk, glycerol, propylene glycol, water, and ethanol.

In another embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition further comprises an adjuvant selected from

Streptococcal C5a peptidase (SCP), an aluminum-based adjuvant, or QS-21, and wherein the aluminum-based adjuvant is selected from the group consisting of aluminum phosphate, aluminum hydroxyl phosphate, and aluminum hydroxide.

In an embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition comprises a buffer, a surfactant, an excipient, and optionally an adjuvant, wherein the composition is buffered to a pH of about 6.0 to about 7.0.

In yet another embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition comprises histidine, polysorbate-80, sodium chloride, and optionally aluminum phosphate, wherein the composition is buffered to a pH of about 6.0 to about 7.0.

In still another embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition comprises about 10 mM to about 25 mM of histidine, about 0.01% to about 0.03% (v/w) of polysorbate-80, about 10 mM to about 250 mM of sodium chloride, and optionally about 0.25 mg/ml to about 0.75 mg/ml of aluminum as aluminum phosphate.

In a further embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition comprises a dose of about 5 mcg/ml to about 50 mcg/ml.

In an embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition is lyophilized, optionally in the presence of at least one excipient, wherein the at least one excipient is selected from the group consisting of starch, glucose, lactose, sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol, palatinit, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, glycine, arginine, lysine, sodium chloride (NaCl), dried skim milk, glycerol, propylene glycol, water, and ethanol.

In yet another embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition comprises about 1% (w/v) to about 10% (w/v) of the at least one excipient.

In another embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition further comprises an additional excipient selected from mannitol or glycine, wherein the composition comprises about 1% (w/v) to about 10% (w/v) of the additional excipient.

In an embodiment, the invention comprises an immunogenic composition as described herein, wherein the composition is reconstituted with water, water for injection (WFI), an adjuvant suspension, or saline.

In another embodiment, the invention comprises an immunogenic composition as described herein for use as a medicament.

In still another embodiment, the invention comprises an immunogenic composition as described herein for use in a method of inducing an immune response against GBS in a subject.

In yet another embodiment, the invention comprises an immunogenic composition as described herein, wherein the subject is a female planning to become pregnant or a pregnant female, wherein the female is optionally in her second half of pregnancy, at least at 20 weeks gestation, or at 27 weeks to 36 weeks gestation.

In another embodiment, the invention comprises an immunogenic composition as described herein, wherein the subject is an adult 50 years of age or older, 65 years of age or older, or 85 years of age or older.

In an embodiment, the invention comprises an immunogenic composition as described herein, wherein the subject is immunocompromised, and/or wherein the subject has a medical condition selected from the group consisting of obesity, diabetes, HIV infection, cancer, cardiovascular disease, or liver disease.

In still another embodiment, the invention comprises an immunogenic composition as described herein, wherein the group B streptococcus is *Streptococcus agalactiae*.

In yet another embodiment, the invention comprises a method of inducing an immune response against group B streptococcus comprising administering to a subject an effective amount of the immunogenic composition as described herein.

In another embodiment, the invention comprises a method of preventing or reducing a disease or condition associated with group B streptococcus in a subject comprising administering to a subject an effective amount of the immunogenic composition as described herein.

In still another embodiment, the invention comprises a method of preventing or reducing a disease or condition associated with group B streptococcus in a subject comprising administering to a subject an effective amount of the immunogenic composition as described herein, wherein the subject is a female planning to become pregnant or a pregnant female, optionally wherein the female is in her second half of pregnancy, at least at 20 weeks gestation, or at 27 weeks to 36 weeks gestation.

In another embodiment, the invention comprises a method of preventing or reducing a disease or condition associated with group B streptococcus in a subject comprising administering to a subject an effective amount of the immunogenic composition as described herein, wherein the subject is an adult 50 years of age or older, 65 years of age or older, or 85 years of age or older, and/or wherein the subject is immunocompromised, and optionally wherein the subject has a medical condition selected from the group consisting of obesity, diabetes, HIV infection, cancer, cardiovascular disease, or liver disease.

In an embodiment, the invention comprises a method of preventing or reducing a disease or condition associated with group B streptococcus in a subject comprising

administering to a subject an effective amount of the immunogenic composition as described herein, wherein the group B streptococcus is *Streptococcus agalactiae*.

In another embodiment, the invention comprises a method of inducing an immune response against group B streptococcus serotype V, or serotype VI, or serotype VII, or serotype VIII, or serotype IX, comprising administering to a subject an immunogenic composition as described herein.

In still another embodiment, the invention comprises an antibody that binds to a capsular polysaccharide in an immunogenic conjugate as described herein.

In a further embodiment, the invention comprises a composition comprising an antibody as described herein, or a method of producing an antibody comprising administering an immunogenic composition as described herein to a subject.

In an embodiment, the invention comprises a method of conferring passive immunity to a subject comprising the steps of generating an antibody preparation using an immunogenic composition as described herein, and administering the antibody preparation to the subject to confer passive immunity.

In another embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein comprising the steps of (a) reacting the GBS capsular polysaccharide with an oxidizing agent resulting in an activated polysaccharide, and (b) reacting the activated polysaccharide with the carrier protein resulting in a polysaccharide-protein conjugate, and optionally wherein step (b) is carried out in a polar aprotic solvent selected from the group consisting of dimethylsulfoxide (DMSO), sulfolane, dimethylformamide (DMF), and hexamethylphosporamide (HMPA).

In an embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein the polysaccharide is reacted with 0.01 to 10.0 molar equivalents of the oxidizing agent, wherein the oxidizing agent is a periodate, optionally including sodium periodate.

In another embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein the oxidation reaction of step (a) is between 1 hour and 50 hours, and optionally wherein the temperature of the oxidation reaction is maintained between about 2°C and about 25°C, optionally wherein the oxidation reaction is carried out in a buffer selected from the group consisting of sodium phosphate, potassium phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), and Bis-Tris, and further optionally wherein the buffer has a concentration of between about 1 mM and about 500 mM.

In yet another embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein the oxidation reaction is carried out at a pH between about 4.0 and about 8.0, optionally wherein the oxidizing agent is 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), and optionally wherein N-

chlorosuccinimide (NCS) is a cooxidant, and/or wherein step (a) further comprises quenching the oxidation reaction by addition of a quenching agent.

In another embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein the concentration of polysaccharide is between about 0.1 mg/mL and about 10.0 mg/mL, and optionally wherein the degree of oxidation of the activated polysaccharide is between 5 and 25.

In still another embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein the method further comprises the step of lyophilizing the activated polysaccharide in the presence of a saccharide selected from the group consisting of sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit.

In a further embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein step (b) comprises compounding the activated polysaccharide with a carrier protein, and reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a GBS capsular polysaccharide-carrier protein conjugate; optionally wherein the concentration of activated polysaccharide in step (b) is between about 0.1 mg/mL and about 10.0 mg/mL; and/or optionally wherein the initial ratio (weight by weight) of activated polysaccharide to carrier protein is between 5:1 and 0.1:1.

In still a further embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein the reducing agent is selected from the group consisting of sodium cyanoborohydride, sodium triacetoxyborohydride, sodium and zinc borohydride in the presence of Bronsted or Lewis acids, pyridine borane, 2-picoline borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMe^{i}PrN-BH_3, benzylamine-BH_3 or 5-ethyl-2-methylpyridine borane (PEMB), and/or wherein the quantity of reducing agent is between about 0.1 and about 10.0 molar equivalents.

In an embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein the duration of reduction reaction of step (2) is between 1 hour and 60 hours, and/or wherein the temperature of the reduction reaction is maintained between 10°C and 40°C.

In a further embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein the method further comprises a step (step (c)) of capping unreacted aldehyde by addition of a borohydride, and optionally wherein the quantity of borohydride is between about 0.1 and about 10.0 molar equivalents, and wherein the borohydride is selected from the group consisting of sodium borohydride ($NaBH_4$), sodium cyanoborohydride, lithium borohydride, potassium borohydride, tetrabutylammonium borohydride, calcium borohydride, and magnesium borohydride, optionally

wherein the duration of capping step is between 0.1 hours and 10 hours, and/or wherein the temperature of the capping step is maintained between about 15°C and about 45°C.

In another embodiment, the invention comprises a method of making an immunogenic polysaccharide-protein conjugate as described herein, wherein the polysaccharide-protein conjugate comprises less than about 40% of free polysaccharide compared to the total amount of polysaccharide.

In yet another embodiment, the invention comprises a method of making a polysaccharide-protein conjugate as described herein, wherein the ratio (weight by weight) of polysaccharide to carrier protein in the conjugate is between about 0.5 and about 3.0, and/or wherein the degree of conjugation of the conjugate is between 2 and 15.

In still another embodiment, the invention comprises a method of making a polysaccharide-protein conjugate as described herein, comprising the steps of (a) reacting isolated GBS capsular polysaccharide with an oxidizing agent; (b) quenching the oxidation reaction of step (a) by addition of a quenching agent resulting in an activated GBS capsular polysaccharide; (c) compounding the activated GBS capsular polysaccharide with a carrier protein; (d) reacting the compounded activated GBS capsular polysaccharide and carrier protein with a reducing agent to form a GBS capsular polysaccharide-carrier protein conjugate; and (e) capping unreacted aldehyde by addition of sodium borohydride ($NaBH_4$), wherein steps (c) and (d) are carried out in DMSO.

In an embodiment, the invention comprises a method of making a polysaccharide-protein conjugate as described herein, comprising the steps of (a) reacting isolated GBS capsular polysaccharide with an oxidizing agent; (b) quenching the oxidation reaction of step (a) by addition of a quenching agent resulting in an activated GBS capsular polysaccharide; (c) compounding the activated GBS capsular polysaccharide with a carrier protein; (d) reacting the compounded activated GBS capsular polysaccharide and carrier protein with a reducing agent to form a GBS capsular polysaccharide-carrier protein conjugate; (e) capping unreacted aldehyde by addition of sodium borohydride ($NaBH_4$), wherein steps (c) and (d) are carried out in DMSO; and (f) purifying the polysaccharide-protein conjugate.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the immunogenicity and cross-reactivity of GBS capsular polysaccharide serotype VI conjugate.

FIG. 2A–2C show immunogenicity of A) GBS capsular polysaccharide serotype VII conjugate, B) GBS capsular polysaccharide serotype VIII conjugate, and C) GBS capsular polysaccharide serotype IX conjugate.

FIG. 3A–3B show the immunogenicity and cross-reactivity of A) GBS capsular polysaccharide serotype VII, and B) GBS capsular polysaccharide serotype IX conjugate.

FIG. 4 shows the immunogenicity of a multivalent GBS conjugate vaccine.

DETAILED DESCRIPTION OF THE INVENTION

This invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting.

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated by reference in their entirety.

The terms used herein have the meanings recognized and known to those of skill in the art, however, for convenience and completeness, particular terms and their meanings are set forth below and throughout the specification.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

The term "about" or "approximately" means within a statistically meaningful range of a value. Such a range can be within an order of magnitude, typically within 20%, more typically still within 10%, and even more typically within 5% of a given value or range. The allowable variation encompassed by the term "about" or "approximately" depends on the particular system under study, and can be readily appreciated by one of ordinary skill in the art. Whenever a range is recited within this application, every whole number integer within the range is also contemplated as an embodiment of the invention.

It is noted that in this disclosure, terms such as "comprises", "comprised", "comprising", "contains", "containing" and the like can have the meaning attributed to them in U.S. patent law; *e.g.*, they can mean "includes", "included", "including" and the like. Such terms refer to the inclusion of a particular ingredients or set of ingredients without excluding any other ingredients. Terms such as "consisting essentially of" and "consists essentially of" have the meaning attributed to them in U.S. patent law, *e.g.*, they allow for the inclusion of additional ingredients or steps that do not detract from the novel or basic characteristics of the invention, *i.e.*, they exclude additional unrecited ingredients or steps that detract from novel or basic characteristics of the invention, and they exclude ingredients or steps of the prior art, such as documents in the art that are cited herein or are incorporated by reference herein, especially as it is a goal of this document to define embodiments that are patentable, *e.g.*, novel, non-obvious, inventive, over the prior art, *e.g.*, over documents cited herein or incorporated by reference herein. And, the terms "consists of" and "consisting of" have the meaning ascribed to them in U.S. patent law; namely, that these terms are close-ended. Accordingly, these terms refer to the inclusion of a particular ingredient or set of ingredients and the exclusion of all other ingredients.

The term "antigen" generally refers to a biological molecule, usually a protein, peptide, polysaccharide, lipid or conjugate which contains at least one epitope to which a cognate antibody can selectively bind; or in some instances, to an immunogenic substance that can stimulate the production of antibodies or T-cell responses, or both, in an animal, including compositions that are injected or absorbed into an animal. The immune response may be generated to the whole molecule, or to one or more various portions of the molecule (*e.g.*, an epitope or hapten). The term may be used to refer to an individual molecule or to a homogeneous or heterogeneous population of antigenic molecules. An antigen is recognized by antibodies, T cell receptors or other elements of specific humoral and/or cellular immunity. The term "antigen" includes all related antigenic epitopes. Epitopes of a given antigen can be identified using any number of epitope mapping techniques, well known in the art (*see, e.g.*, Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, NJ). For example, linear epitopes may be determined by, *e.g.*, concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, *e.g.*, U.S. Patent No. 4,708,871; Geysen, H.M., et al., Proc. Natl. Acad. Sci. USA, 81:3998-4002 (1984); Geysen, H.M., et al., Molec. Immunol., 23(7):709-715 (1986), all incorporated herein by reference in their entireties. Similarly, conformational epitopes may be identified by determining spatial conformation of amino acids such as by, *e.g.*, x-ray crystallography and 2-dimensional nuclear magnetic resonance (*see, e.g.*, Epitope Mapping Protocols, *supra*). Furthermore, for purposes of the present invention, an "antigen" may also be used to refer to a protein that includes modifications, such as deletions, additions and substitutions (generally conservative in nature, but they may be non-conservative), to the native sequence, as long as the protein maintains the ability to elicit an immunological response. These modifications may be deliberate, as through site-directed mutagenesis, or through particular synthetic procedures, or through a genetic engineering approach, or may be accidental, such as through mutations of hosts, which produce the antigens. Furthermore, the antigen can be derived, obtained, or isolated from a microbe, *e.g.*, a bacterium, or can be a whole organism. Similarly, an oligonucleotide or polynucleotide, which expresses an antigen, such as in nucleic acid immunization applications, is also included in the definition. Synthetic antigens are also included, for example, polyepitopes, flanking epitopes, and other recombinant or synthetically derived antigens (Bergmann, C., et al., Eur. J. Immunol., 23(11):2777-2781(1993); Bergmann, C.C., et al., J. Immunol., 157(8):3242-3249(1996); Suhrbier, A., Immunol. and Cell Biol., 75(4):402-408 (1997)).

The terms "vaccine" or "vaccine composition", which are used interchangeably, refer to pharmaceutical compositions comprising at least one immunogenic composition that induces an immune response in an animal.

Capsular Polysaccharides

As used herein, the term "saccharide" refers to a single sugar moiety or monosaccharide unit as well as combinations of two or more single sugar moieties or monosaccharide units covalently linked to form disaccharides, oligosaccharides, and polysaccharides. The term "saccharide" may be used interchangeably with the term "carbohydrate." The polysaccharide may be linear or branched.

A “monosaccharide” as used herein refers to a single sugar residue in an oligosaccharide. The term "disaccharide" as used herein refers to a polysaccharide composed of two monosaccharide units or moieties linked together by a glycosidic bond.

In one embodiment, the polysaccharide is an oligosaccharide (OS). An “oligosaccharide” as used herein refers to a compound containing two or more monosaccharide units or moieties. Within the context of an oligosaccharide, an individual monomer unit or moiety is a monosaccharide which is, or can be, bound through a hydroxyl group to another monosaccharide unit or moiety. Oligosaccharides can be prepared by either chemical synthesis from protected single residue sugars or by chemical degradation of biologically produced polysaccharides. Alternatively, oligosaccharides may be prepared by *in vitro* enzymatic methods.

In a preferred embodiment, the polysaccharide is a polysaccharide (PS), which refers to a linear or branched polymer of at least 5 monosaccharide units or moieties. For clarity, larger number of repeating units, wherein *n* is greater than about 5, such as greater than about 10, will be referred to herein as a polysaccharide.

In one embodiment, the polysaccharide is a cell surface polysaccharide. A cell surface polysaccharide refers to a polysaccharide having at least a portion located on the outermost bacterial cell membrane or bacterial cell surface, including the peptidoglycan layer, cell wall, and capsule. Typically, a cell surface polysaccharide is associated with inducing an immune response *in vivo*. A cell surface polysaccharide may be a “cell wall polysaccharide” or a “capsular polysaccharide.” A cell wall polysaccharide typically forms a discontinuous layer on the bacterial surface.

In one embodiment, the polysaccharide is a capsular polysaccharide. A capsular polysaccharide refers to a glycopolymer that includes repeating units of one or more monosaccharides joined by glycosidic linkages. A capsular polysaccharide typically forms a capsule-like layer around a bacterial cell. "Capsular polysaccharide" or "capsule polysaccharide" refers to the polysaccharide capsule that is external to the cell wall of most isolates of streptococci. For example, all GBS capsular polysaccharides have a branched repeat structure with a terminal α2–3-linked sialic acid that is required for bacterial virulence. Capsule-associated sialic acid (quantified by HPLC assay) has been detected in > 94% of invasive neonatal isolates from T.E.S.T. cultured *in vitro*.

The present inventors have discovered that the sialic acid level of GBS capsular polysaccharides is an important characteristic for producing an immune response. Prior disclosures have only provided conflicting information regarding sialic acid levels for serotype V, finding that desialylated serotype V was preferred (Int'l Patent Appl. Pub. No. WO 2012/035519) and that sialic acid content >50% for serotype V could be used (Int'l Patent Appl. Pub. No. WO 2014/053612). However, nothing in these references describe the importance of sialic acid levels for at least a majority of GBS polysaccharides on immunogenicity. The present inventors have surprisingly found that GBS capsular polysaccharides require about 60% or more sialic acid prior to conjugation to provide an immune response comparable to those polysaccharides having native sialic acid levels (i.e. 100% or greater than about 95%). Sialic acid levels of even 58%, which is within the prior range disclosed for serotype V, negatively impacted immunogenicity.

Accordingly, in one embodiment of the invention, the capsular polysaccharides comprise their natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), and up to about 5% (sialylation level greater than about 95%).

It should be noted that 100% sialic acid level corresponds to about 1.0 mM sialic acid per mM of polysaccharide. Therefore, the capsular polysaccharides may have about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide.

The terminal sialic residues of some capsular polysaccharide (CP) serotypes are partially O-acetylated (OAc) (Lewis, A.L., et al., Proceedings of the National Academy of Sciences USA, 101(30):11123-8 (2004)). Serotypes Ib, III, IV, V, VI, and IX are partially O-acetylated (up to ~40%), whereas serotypes Ia, II, and VII have little or no O-acetylation (less than about 5%) (Lewis 2004). In one embodiment of the invention, the capsular polysaccharides comprise their natural O-acetylation level (about 0% to about 40%). In another embodiment, the capsular polysaccharides may be de-O-acetylated (less than about 5%). The degree of O-acetylation of the polysaccharide or oligosaccharide can be determined by any

method known in the art, for example, by proton NMR (Lemercinier, X., et al., Carbohydrate Research, 296:83-96 (1996); Jones, C., et al., Journal of Pharmaceutical and Biomedical Analysis, 30:1233-1247 (2002); Int'l Patent Appl. Pub. Nos. WO 2005/033148 and WO 00/56357). Another commonly used method is described by Hestrin, S., J. Biol. Chem., 180:249-261 (1949).

Also, 100% O-acetate corresponds to about 1.0 mM O-acetate per mM of saccharide repeating unit. Accordingly, partially O-acetylated polysaccharides comprise at least about 0.1, 0.2, 0.3, 0.35 or about 0.4 mM O-acetate per mM saccharide repeating unit. A de-O-acetylated polysaccharide comprises less than about 0.01, 0.02, 0.03, 0.04, or 0.05 mM O-acetate per mM saccharide repeating unit.

Streptococcal microorganisms capable of causing invasive disease generally also are capable of producing a CP that encapsulates the bacterium and enhances its resistance to clearance by host innate immune system. The CP serves to cloak the bacterial cell in a protective capsule that renders the bacteria resistant to phagocytosis and intracellular killing. Bacteria lacking a capsule are more susceptible to phagocytosis. Capsular polysaccharides are frequently an important virulence factor for many bacterial pathogens, including *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Staphylococcus aureus*.

The capsule polysaccharide can be used to serotype a particular species of bacteria. Typing is usually accomplished by reaction with a specific antiserum or monoclonal antibody generated to a specific structure or unique epitope characteristic of the capsule polysaccharide. There are ten GBS serotypes: Ia, Ib, and II–IX (Ferrieri, P., et al., Emerg. Infect. Dis. [Internet], 19(4) (2013), *available at* http://wwwnc.cdc.gov/eid/article/19/4/12-1572_article.

In one embodiment of the invention, the polysaccharide is isolated from *Streptococcus agalactiae*. The polysaccharide may be isolated from any encapsulated strain of *S. agalactiae*, such as 090, A909 (ATCC Accession No. BAA-1138), 515 (ATCC Accession No. BAA-1177), B523, CJB524, MB 4052 (ATCC Accession No. 31574), H36B (ATCC Accession No. 12401), S40, S42, MB 4053 (ATCC Accession No. 31575), M709, 133, 7357, PFEGBST0267, MB 4055 (ATCC Accession No. 31576), 18RS21 (ATCC Accession No. BAA-1175), S16, S20, V8 (ATCC Accession No. 12973), DK21, DK23, UAB, 5401, PFEGBST0708, MB 4082 (ATCC Accession No. 31577), M132, 110, M781 (ATCC Accession No. BAA-22), D136C(3) (ATCC Accession No. 12403), M782, S23, 120, MB 4316 (M-732; ATCC Accession No. 31475), M132, K79, COH1 (ATCC Accession No. BAA-1176), PFEGBST0563, 3139 (ATCC Accession No. 49446), CZ-NI-016, PFEGBST0961, 1169-NT1, CJB111(ATCC Accession No. BAA-23), CJB112, 2603 V/R (ATCC Accession No. BAA-611), NCTC 10/81, CJ11, PFEGBST0837, 118754, 114852, 114862, 114866, 118775, B 4589, B 4645, SS1214, CZ-PW-119, 7271, CZ-PW-045, JM9130013, JM9130672, IT-NI-016, IT-PW-62, and IT-PW-64.

The polysaccharides described herein may be isolated by methods known in the art, including, for example, methods described herein. As used herein, "isolated" refers to being

obtained from and separated from a particular source. The term “isolated” further refers to not being in its respective naturally occurring form, state, and/or environment. For example, “isolated from streptococcus” refers to a matter that was obtained from and separated from a streptococcus cell. The isolated polysaccharide is not naturally occurring. The term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring or from its host organism if it is a recombinant entity, or taken from one environment to a different environment). For example, an "isolated" capsule polysaccharide, protein or peptide is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized, or otherwise present in a mixture as part of a chemical reaction. In the present invention, the proteins or polysaccharides may be isolated from the bacterial cell or from cellular debris, so that they are provided in a form useful in the manufacture of an immunogenic composition. The term "isolated" or "isolating" may include purifying, or purification, including methods for purifying an isolated polysaccharide known in the art and/or methods described herein. The language "substantially free of cellular material" includes preparations of a polypeptide/protein in which the polypeptide/protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, a capsule polysaccharide, protein or peptide that is substantially free of cellular material includes preparations of the capsule polysaccharide, protein or peptide having less than about 30%, 20%, 10%, 5%, 2.5%, or 1% (by dry weight) of contaminating protein or polysaccharide or other cellular material. When the polypeptide/protein is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, 10%, or 5% of the volume of the protein preparation. When polypeptide/protein or polysaccharide is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, *i.e.*, it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein or polysaccharide. Accordingly, such preparations of the polypeptide/protein or polysaccharide have less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or compounds other than polypeptide/protein or polysaccharide fragment of interest.

In one embodiment of the invention, the polysaccharide is isolated from a bacterium. In another embodiment of the invention, the polysaccharide is produced recombinantly. In further embodiment, the polysaccharide is synthetic or chemically synthesized according to conventional methods. In yet another embodiment of the invention, the polysaccharide is prepared by expression in a surrogate host after cloning and expressing a biosynthetic pathway to produce the polysaccharide. In one embodiment, the polysaccharide is immunogenic. For example, the inventors discovered that each polysaccharide described herein is capable of inducing or eliciting an immune response. The term “immunogenic” refers to an ability to initiate, trigger, cause, enhance, improve, and/or augment a humoral and/or cell-mediated immune

response in a mammal. In one embodiment, the mammal is a human, primate, rabbit, pig, mouse, etc.

The molecular weight of the capsular polysaccharide is a consideration for use in immunogenic compositions. High molecular weight capsular polysaccharides are able to induce certain antibody immune responses due to a higher valence of the epitopes present on the antigenic surface. The isolation and purification of high molecular weight capsular polysaccharides is contemplated for use in the conjugates, compositions and methods of the present invention.

However, in one embodiment, the polysaccharide may be sized to a molecular weight (MW) range that is lower than the molecular weight of the native capsular polysaccharide prior to conjugation to a carrier protein. The size of the purified capsular polysaccharide is reduced in order to generate conjugates with advantageous filterability characteristics and/or yields.

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

In one embodiment, the polysaccharide described herein is capable of inducing opsonic activity. In another embodiment, the polysaccharide described herein is capable of inducing opsonic and phagocytic activity (*e.g.*, opsonophagocytic activity).

Opsonic activity or opsonization refers to a process by which an opsonin (for example, an antibody or a complement factor) binds to an antigen (*e.g.*, an isolated polysaccharide described herein), which facilitates attachment of the antigen to a phagocyte or phagocytic cell (*e.g.*, a macrophage, dendritic cell, and polymorphonuclear leukocyte (PMNL). Some bacteria, such as, for example, encapsulated bacteria that are not typically phagocytosed due to the presence of the capsule, become more likely to be recognized by phagocytes when coated with an opsonic antibody. In one embodiment, the polysaccharide induces an immune response, such as, *e.g.*, an antibody, that is opsonic. In one embodiment, the opsonic activity is against a Gram positive coccus, preferably against a *Streptococcus* species, more preferably against at least one strain of *S. agalactiae*.

In yet another embodiment, the polysaccharide described herein is capable of inducing a bactericidal immune response. In one embodiment, the bactericidal activity is against a Gram positive coccus, preferably against a *Streptococcus* species, more preferably against at least one strain of *S. agalactiae*.

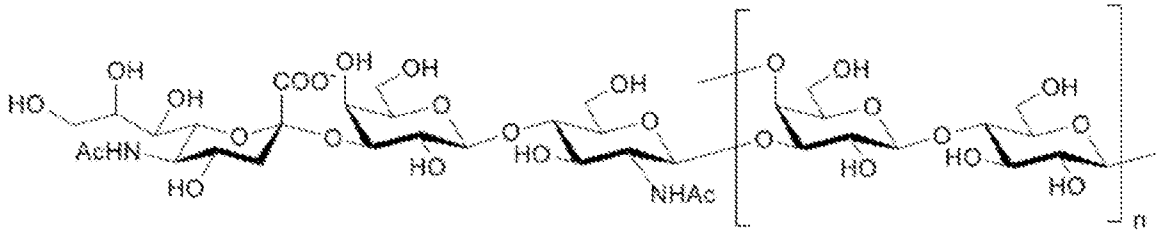
Methods for measuring opsonization, phagocytosis, and/or bactericidal activity are known in the art, such as, for example, by measuring reduction in bacterial load *in vivo* (*e.g.*, by measuring bacteremia levels in mammals challenged with a *Streptococcus* species) and/or by measuring bacterial cell killing *in vitro* (*e.g.*, an *in vitro* opsonophagocytic assay). In one

embodiment, the polysaccharide is capable of inducing opsonic, phagocytic, and/or bactericidal activity as compared to an appropriate control, such as, for example, as compared to antisera raised against a heat-killed Gram positive coccus.

Serotype Ia

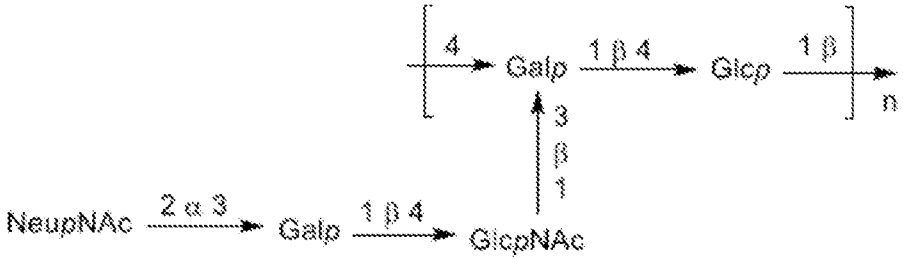
One embodiment includes a serotype Ia GBS capsular polysaccharide. The structure of serotype Ia can be depicted as follows:

a)



or

b)



The molecular weight of serotype Ia capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 25 kDa and about 750 kDa, between about 25 kDa and about 500 kDa, between about 25 kDa and about 450 kDa, between about 25 kDa and about 400 kDa, between about 25 kDa and about 350 kDa, between about 25 kDa and about 300 kDa, between about 25 kDa and about 250 kDa, between about 25 kDa and about 200 kDa, between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about 550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa,

between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. In one preferred embodiment, the molecular weight of the capsular polysaccharide prior to conjugation is between about 25 kDa and about 200 kDa. In another preferred embodiment, the molecular weight of the capsular polysaccharide prior to conjugation is between about 100 kDa and about 400 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In a particular embodiment, a high pressure homogenization process is used to reduce the size of native GBS capsular polysaccharide serotype Ia while preserving the structural features, such as sialic acid, of the polysaccharide.

In one embodiment of the invention, the serotype Ia capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95%) prior to conjugation.

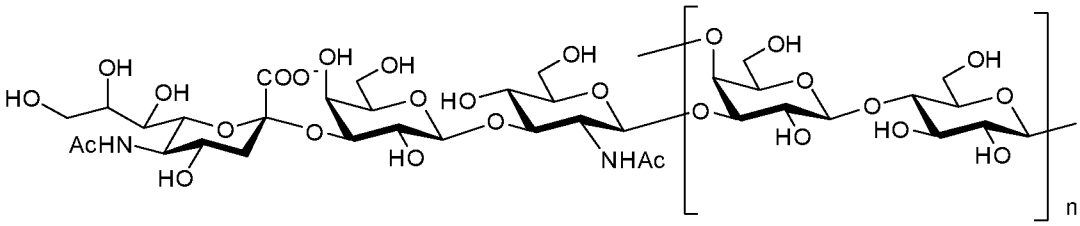
In another embodiment, the serotype Ia capsular polysaccharide has about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

Serotype Ia capsular polysaccharides are less than about 5% O-acetylated. Some exemplary strains of serotype Ia capsular polysaccharides of the invention include 090, A909 (ATCC Accession No. BAA-1138), 515 (ATCC Accession No. BAA-1177), B523, CJB524, and MB 4052 (ATCC Accession No. 31574).

Serotype Ib

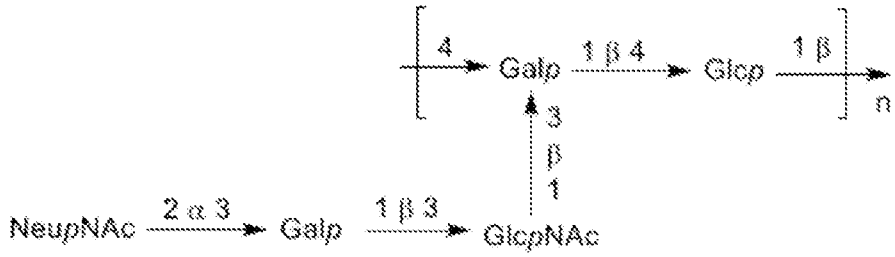
One embodiment includes a serotype Ib GBS capsular polysaccharide. The structure of serotype Ib can be depicted as follows:

a)



or

b)



The molecular weight of serotype Ib capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 25 kDa and about 750 kDa, between about 25 kDa and about 500 kDa, between about 25 kDa and about 450 kDa, between about 25 kDa and about 400 kDa, between about 25 kDa and about 350 kDa, between about 25 kDa and about 300 kDa, between about 25 kDa and about 250 kDa, between about 25 kDa and about 200 kDa, between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about

550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. In one preferred embodiment, the molecular weight of the capsular polysaccharide prior to conjugation is between about 25 kDa and about 400 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In one embodiment of the invention, the serotype Ib capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95%) prior to conjugation.

In another embodiment, the serotype Ib capsular polysaccharide has about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

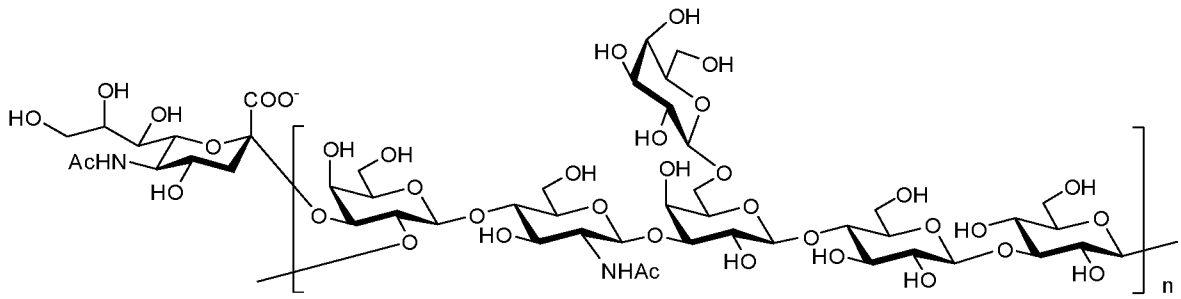
Serotype Ib capsular polysaccharides are between about 0% and about 40% O-acetylated. In one embodiment of the invention, the polysaccharide is de-O-acetylated (i.e., less than about 5% O-acetylated). Some exemplary strains of serotype Ib capsular polysaccharides

of the invention include H36B (ATCC Accession No. 12401), S40, S42, MB 4053 (ATCC Accession No. 31575), M709, 133, 7357, and PFEGBST0267.

Serotype II

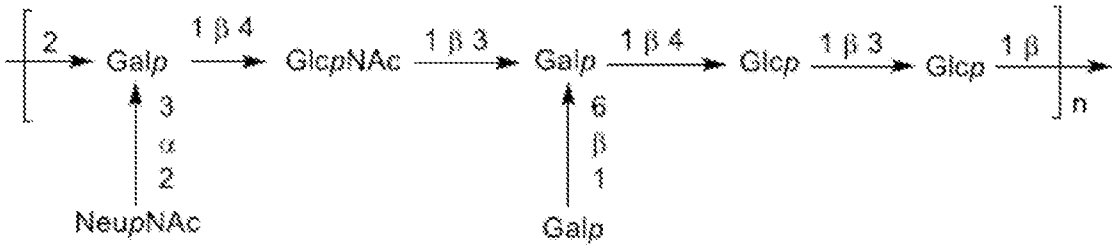
One embodiment includes a serotype II GBS capsular polysaccharide. The structure of serotype II can be depicted as follows:

a)



or

b)



The molecular weight of serotype II capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 25 kDa and about 750 kDa, between about 25 kDa and about 500 kDa, between about 25 kDa and about 450 kDa, between about 25 kDa and about 400 kDa, between about 25 kDa and about 350 kDa, between about 25 kDa and about 300 kDa, between about 25 kDa and about 250 kDa, between about 25 kDa and about 200 kDa, between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about

550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. In one preferred embodiment, the molecular weight of the capsular polysaccharide prior to conjugation is between about 25 kDa and about 400 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In one embodiment of the invention, the serotype II capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95%) prior to conjugation.

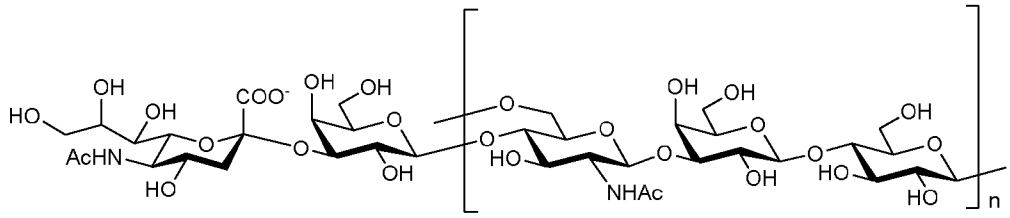
In another embodiment, the serotype II capsular polysaccharide has about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

Serotype II capsular polysaccharides are less than about 5% O-acetylated. Some exemplary strains of serotype II capsular polysaccharides of the invention include MB 4055 (ATCC Accession No. 31576), 18RS21 (ATCC Accession No. BAA-1175), S16, S20, V8 (ATCC Accession No. 12973), DK21, DK23, UAB, 5401, and PFEGBST0708.

Serotype III

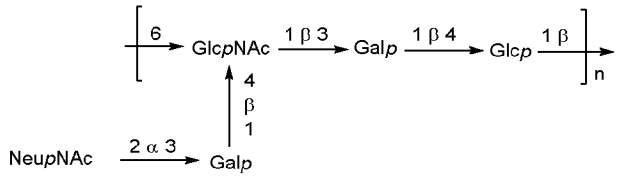
One embodiment includes a serotype III GBS capsular polysaccharide. The structure of serotype III can be depicted as follows:

a)



or

b)



The molecular weight of serotype III capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 25 kDa and about 750 kDa, between about 25 kDa and about 500 kDa, between about 25 kDa and about 450 kDa, between about 25 kDa and about 400 kDa, between about 25 kDa and about 350 kDa, between about 25 kDa and about 300 kDa, between about 25 kDa and about 250 kDa, between about 25 kDa and about 200 kDa, between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about 550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about

600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. In one preferred embodiment, the molecular weight of the capsular polysaccharide prior to conjugation is between about 25 kDa and about 200 kDa. In another preferred embodiment, the molecular weight of the capsular polysaccharide prior to conjugation is between about 100 kDa and about 400 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In a particular embodiment, a high pressure homogenization process is used to reduce the size of native GBS capsular polysaccharide serotype III while preserving the structural features, such as sialic acid, of the polysaccharide.

In one embodiment of the invention, the serotype III capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95%) prior to conjugation.

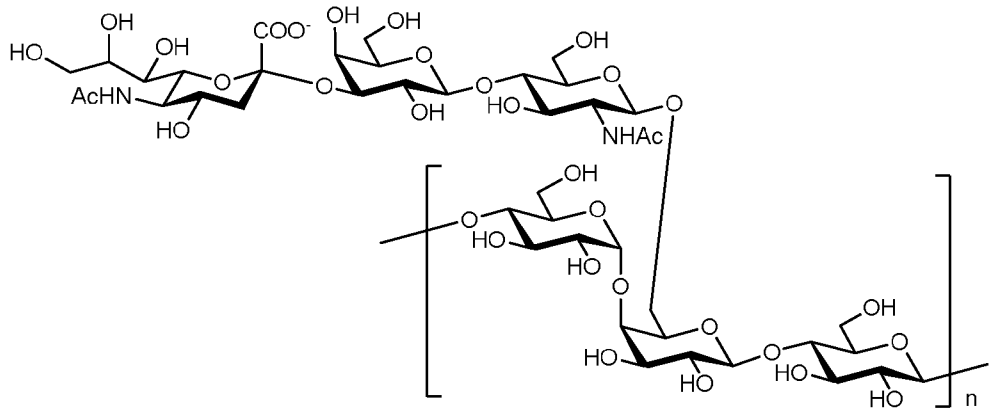
In another embodiment, the serotype III capsular polysaccharide has about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

Serotype III capsular polysaccharides are between about 0% and about 40% O-acetylated. In one embodiment of the invention, the polysaccharide is de-O-acetylated (i.e., less than about 5% O-acetylated). Some exemplary strains of serotype III capsular polysaccharides of the invention include MB 4082 (ATCC Accession No. 31577), M132, 110, M781 (ATCC Accession No. BAA-22), D136C(3) (ATCC Accession No. 12403), M782, S23, 120, MB 4316 (M-732; ATCC Accession No. 31475), M132, K79, COH1 (ATCC Accession No. BAA-1176), and PFEGBST0563.

Serotype IV

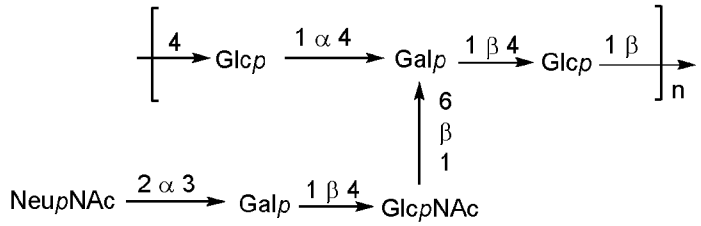
One embodiment includes a serotype IV GBS capsular polysaccharide. The structure of serotype IV can be depicted as follows:

a)



or

b)



The molecular weight of serotype IV capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 25 kDa and about 750 kDa, between about 25 kDa and about 500 kDa, between about 25 kDa and about 450 kDa, between about 25 kDa and about 400 kDa, between about 25 kDa and about 350 kDa, between about 25 kDa and about 300 kDa, between about 25 kDa and about 250 kDa, between about 25 kDa and about 200 kDa, between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about 550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450

kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. In one preferred embodiment, the molecular weight of the capsular polysaccharide prior to conjugation is between about 25 kDa and about 400 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In one embodiment of the invention, the serotype IV capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95%) prior to conjugation.

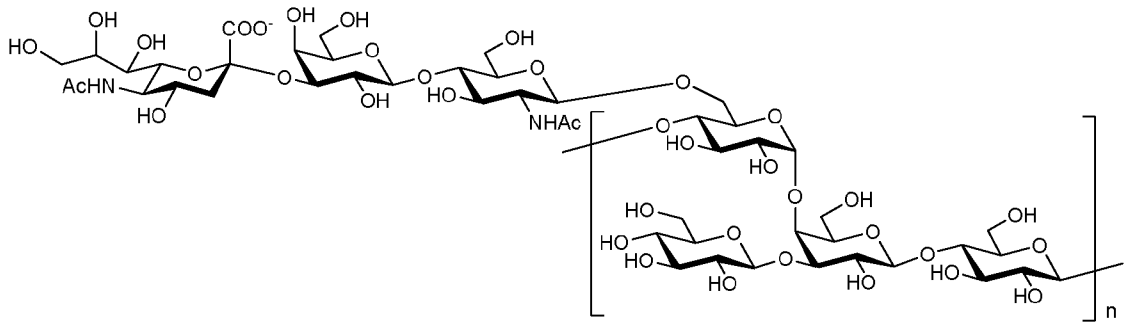
In another embodiment, the serotype IV capsular polysaccharide has about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

Serotype IV capsular polysaccharides are between about 0% and about 40% O-acetylated. In one embodiment of the invention, the polysaccharide is de-O-acetylated (i.e., less than about 5% O-acetylated). Some exemplary strains of serotype IV capsular polysaccharides of the invention include 3139 (ATCC Accession No. 49446), CZ-NI-016, and PFEGBST0961.

Serotype V

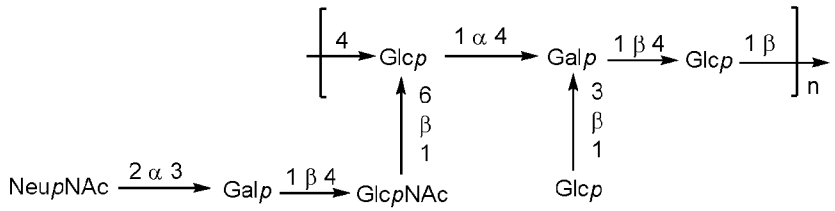
One embodiment includes a serotype V GBS capsular polysaccharide. The structure of serotype V can be depicted as follows:

a)



or

b)



The molecular weight of serotype V capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 25 kDa and about 750 kDa, between about 25 kDa and about 500 kDa, between about 25 kDa and about 450 kDa, between about 25 kDa and about 400 kDa, between about 25 kDa and about 350 kDa, between about 25 kDa and about 300 kDa, between about 25 kDa and about 250 kDa, between about 25 kDa and about 200 kDa, between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about 550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about

200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. In one preferred embodiment, the molecular weight of the capsular polysaccharide prior to conjugation is between about 25 kDa and about 400 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In one embodiment of the invention, the serotype V capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95%) prior to conjugation.

In another embodiment, the serotype V capsular polysaccharide has about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

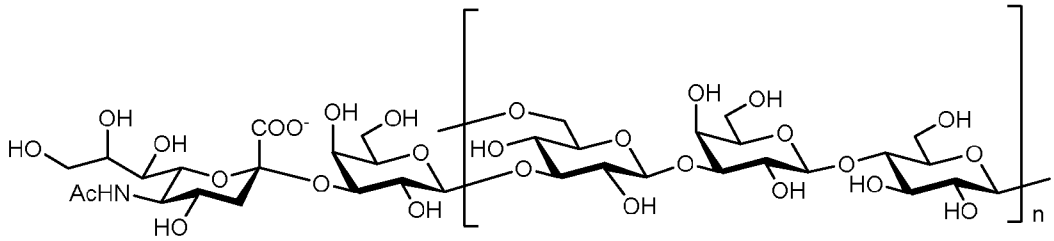
Serotype V capsular polysaccharides are between about 0% and about 40% O-acetylated. In one embodiment of the invention, the polysaccharide is de-O-acetylated (i.e., less than about 5% O-acetylated). Some exemplary strains of serotype V capsular polysaccharides of the invention include 1169-NT1, CJB111 (ATCC Accession No. BAA-23), CJB112, 2603 V/R (ATCC Accession No. BAA-611), NCTC 10/81, CJ11, and PFEGBST0837.

Serotype VI

GBS Serotype VI capsular polysaccharides are described by von Hunolstein, C., et al., Infection and Immunity, 6194):1272-1280 (1993), the disclosures of which are hereby

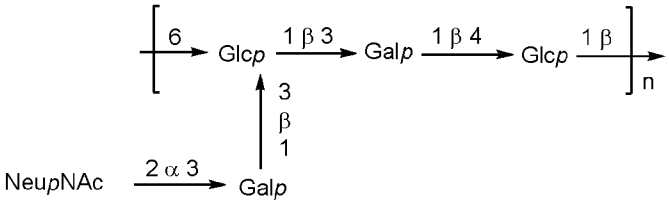
incorporated by reference in their entirety. The structure of serotype VI can be depicted as follows:

a)



or

b)



The molecular weight of serotype VI capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about 550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa,

between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

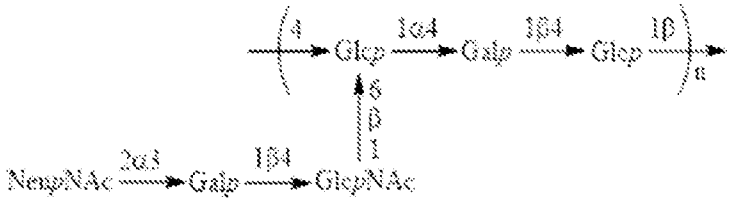
In one embodiment of the invention, the serotype VI capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95poly%) prior to conjugation.

In another embodiment, the serotype VI capsular polysaccharide has about 1.0 mM sialic acid per mM of saccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

Serotype VI capsular polysaccharides are between about 0% and about 40% O-acetylated. In one embodiment of the invention, the polysaccharide is de-O-acetylated (i.e., less than about 5% O-acetylated). Some exemplary strains of serotype IIII capsular polysaccharides of the invention include 118754, 114852, 114862, 114866, 118775, B 4589, B 4645, SS1214, and CZ-PW-119.

Serotype VII

GBS Serotype VII capsular polysaccharides are described by Kogan, G., et al., Carbohydrate Research, 277(1):1-9 (1995), the disclosures of which are hereby incorporated by reference in their entirety. The repeating unit of serotype VII is as follows:



The molecular weight of serotype VII capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 50 kDa and about 750 kDa,

between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about 550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In one embodiment of the invention, the serotype VII capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95%) prior to conjugation.

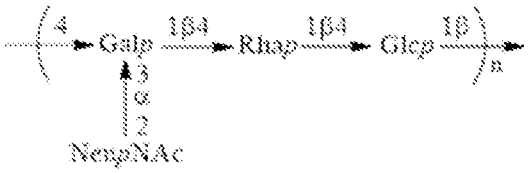
In another embodiment, the serotype VII capsular polysaccharide has about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of

polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

Serotype VII capsular polysaccharides are less than about 5% O-acetylated. Some exemplary strains of serotype VII capsular polysaccharides of the invention include 7271 and CZ-PW-045.

Serotype VIII

GBS Serotype VIII capsular polysaccharides are described by Kogan, G., et al., The Journal of Biological Chemistry, 271(15):8786-8790 (1996), the disclosures of which are hereby incorporated by reference in their entirety. The repeating unit of serotype VIII is as follows:



The molecular weight of serotype VIII capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about 550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa,

between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

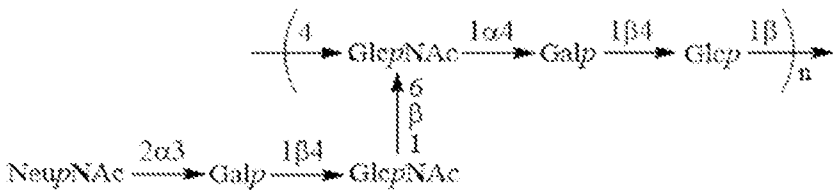
In one embodiment of the invention, the serotype VIII capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95%) prior to conjugation.

In another embodiment, the serotype VIII capsular polysaccharide has about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

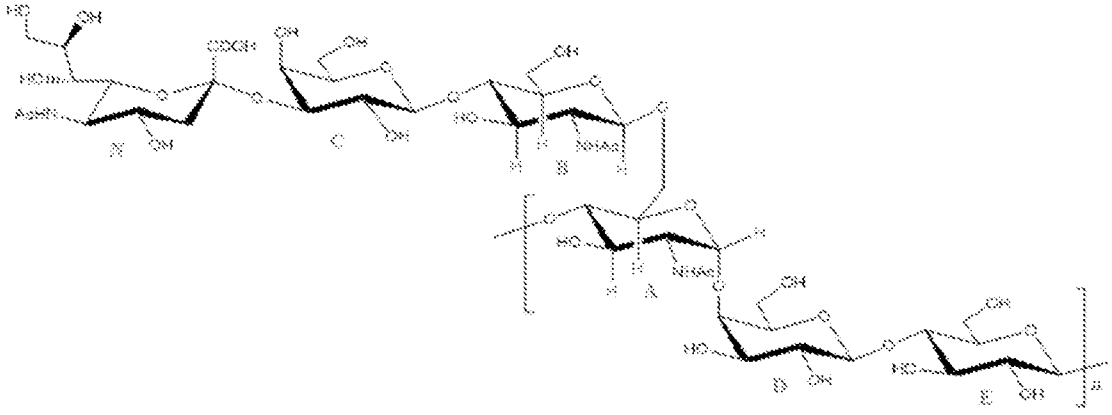
Serotype VIII capsular polysaccharides are between about 0% and about 40% O-acetylated. In one embodiment of the invention, the polysaccharide is de-O-acetylated (i.e., less than about 5% O-acetylated). Some exemplary strains of serotype VIII capsular polysaccharides of the invention include JM9130013 and JM9130672.

Serotype IX

GBS Serotype IX capsular polysaccharides have previously been described by Berti, F., et al., The Journal of Biological Chemistry, 289(34):23437-2348 (2014), and others. However, the configuration of the Glc*p*NAc in the backbone of GBS serotype IX polysaccharide is alpha (α), which is different from previously published structural details that have proposed this linkage to be in the β configuration. The structure of serotype IX can be more accurately depicted as follows:



This corresponding structure for GBS serotype IX may also be represented as follows:



The molecular weight of serotype IX capsular polysaccharides prior to conjugation are between about 5 kDa and about 1,000 kDa, such as between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa, between about 100 kDa and about 600 kDa, between about 100 kDa and about 550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In one embodiment of the invention, the serotype IX capsular polysaccharide comprises its natural sialic acid level, such as about 100% or greater than about 95%. In another embodiment, the capsular polysaccharides may be desialylated up to about 40% (sialylation level greater than about 60%), such as up to about 35% (sialylation level greater than about 65%), up to about 30% (sialylation level greater than about 70%), up to about 25% (sialylation level greater than about 75%), up to about 20% (sialylation level greater than about 80%), up to about 15% (sialylation level greater than about 85%), up to about 10% (sialylation level greater than about 90%), or up to about 5% (sialylation level greater than about 95%) prior to conjugation.

In another embodiment, the serotype IX capsular polysaccharide has about 1.0 mM sialic acid per mM of polysaccharide, such as at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation. In a further embodiment, the capsular polysaccharide may have at least about 0.6 mM sialic acid per mM of polysaccharide, such as at least about 0.65 mM sialic acid per mM of polysaccharide, at least about 0.7 mM sialic acid per mM of polysaccharide, at least about 0.75 mM sialic acid per mM of polysaccharide, at least about 0.8 mM sialic acid per mM of polysaccharide, at least about 0.85 mM sialic acid per mM of polysaccharide, at least about 0.9 mM sialic acid per mM of polysaccharide, or at least about 0.95 mM sialic acid per mM of polysaccharide prior to conjugation.

Serotype IX capsular polysaccharides are between about 0% and about 40% O-acetylated. In one embodiment of the invention, the polysaccharide is de-O-acetylated (i.e., less than about 5% O-acetylated). Some exemplary strains of serotype IX capsular polysaccharides of the invention include IT-NI-016, IT-PW-62, and IT-PW-64.

Polysaccharide-Protein Conjugates

As used herein, "conjugates" comprise a capsule polysaccharide usually of a desired range of molecular weight and a carrier protein, wherein the capsule polysaccharide is conjugated to the carrier protein. Conjugates may or may not contain some amount of free capsule polysaccharide. As used herein, "free capsule polysaccharide" refers to capsule polysaccharide that is non-covalently associated with (*i.e.*, non-covalently bound to, adsorbed to or entrapped in or with) the conjugated capsular polysaccharide-carrier protein. The terms "free capsule polysaccharide," "free polysaccharide" and "free saccharide" may be used interchangeably and are intended to convey the same meaning. Regardless of the nature of the carrier molecule, it can be conjugated to the capsular polysaccharide either directly or through a linker. As used herein, "to conjugate", "conjugated" and "conjugating" refers to a process whereby a bacterial capsular polysaccharide is covalently attached to the carrier molecule. Conjugation enhances the immunogenicity of the bacterial capsular polysaccharide. The conjugation can be performed according to the methods described below or by processes known in the art.

A “conjugate immunogenic composition,” as used herein, refers to an immunogenic composition wherein the immunogenic material includes an antigenic polysaccharide that is covalently linked to a carrier protein to produce a polysaccharide-protein conjugate. In one embodiment, a polysaccharide-protein conjugate of the invention may be formulated as a multivalent immunogenic composition.

As used herein, the term “molecular weight” of polysaccharide or of carrier protein-polysaccharide conjugate refers to molecular weight calculated by size exclusion chromatography (SEC) combined with multiangle laser light scattering detector (MALLS).

As used herein, a “polysaccharide-protein conjugate” refers to a polysaccharide molecule conjugated to a protein carrier molecule through one or more covalent bonds. It may be desirable to conjugate the polysaccharide to a protein from another species known to be immunogenic in the target host. Accordingly, in one embodiment, the carrier molecule is a carrier protein. As defined herein, such a foreign protein is referred to as a “carrier protein.” Carrier proteins serve to enhance the antigenicity and immunogenicity of the polysaccharide. As used herein, the term “carrier effect” refers to the process where the antigenicity and immunogenicity of a weakly immunogenic or non-immunogenic molecule is enhanced, by being attached to a more immunogenic molecule as carrier (*e.g.*, a heterologous protein). In this case, the polysaccharide in the combined polysaccharide-protein conjugate becomes more immunogenic than if it were presented alone. Carrier proteins contain T cell epitopes for stimulating T-cell help for producing antibody responses.

"Carrier protein" or "protein carrier" as used herein, refers to any protein molecule that may be conjugated to an antigen (such as the capsular polysaccharides) against which an immune response is desired. Conjugation of an antigen such as a polysaccharide to a carrier protein can render the antigen immunogenic. Carrier proteins are preferably proteins that are non-toxic and non-reactogenic and obtainable in sufficient amount and purity. Examples of carrier proteins are toxins, toxoids or any mutant cross-reactive material (CRM_{197}) of the toxin from tetanus, diphtheria, pertussis, *Pseudomonas* species, *E. coli*, *Staphylococcus* species, and *Streptococcus* species. Carrier proteins should be amenable to standard conjugation procedures. In an embodiment, the carrier protein is Streptococcal C5a peptidase (SCP). In another embodiment of the present invention, CRM_{197} is used as the carrier protein.

Cross-reacting materials or CRMs are especially useful for some embodiments of the present invention. One may produce genetically altered proteins, which are antigenically similar to the certain bacterial toxins, yet non-toxic. These are called “cross reacting materials”, or CRMs. CRM_{197} (Wyeth/Pfizer Inc., Sanford, NC) is noteworthy since it has a single amino acid change from the native diphtheria toxin and is immunologically indistinguishable from it. *See* Pappenheimer, A.M., et al., Immunochem., 9(9):891-906 (1972); U.S. Pat. No. 5,614,382, the disclosures of which are hereby incorporated by reference in their entirety. CRM_{197} is a non-toxic variant (*i.e.*, toxoid) of diphtheria toxin isolated from cultures of *Corynebacterium*

diphtheriae strain C7 (β197) grown in casamino acids and yeast extract-based medium. CRM_{197} is purified through ultra-filtration, ammonium sulfate precipitation, and ion-exchange chromatography. A culture of *C. diphtheriae* strain C7 (β197), which produces CRM_{197} protein, has been deposited with the American Type Culture Collection, Rockville, Maryland and has been assigned accession number ATCC 53281. Other diphtheria toxoids are also suitable for use as carrier proteins. CRM3201 is a genetically manipulated variant of pertussis toxin. *See* Black, W.J., et al., Science, 240(4852):656-659 (1988), the disclosure of which is hereby incorporated by reference in its entirety.

Streptococcal C5a peptidase (SCP) is a cell wall anchored virulence protein encoded by members of the beta hemolytic streptococcus genus that proteolytically inactivates the alpha fragment of complement component 5 (C5a) that is responsible for polymorphonuclear cell recruitment to the site of infection (2005. PNAS. 102(51):18391.) It is a target of protective antibodies, wherein IgG antibodies directed against SCP can mediate opsonophagocytosis. Additionally, SCP could serve as a carrier protein to enhance the immune response to conjugated GBS CPS polysaccharide haptens.

In addition to a diphtheria toxoid (DT), CRM_{197}, SCP, and a pertussis toxoid, further examples of carrier proteins include a tetanus toxoid (TT), a cholera toxoid (*e.g.*, as described in Int'l Patent Appl. Pub. No. WO 2004/083251), an *E. coli* heat labile toxoid (LT), an *E. coli* heat stable toxoid (ST), pneumolysin from *S. pneumonia* (wild-type or mutant with reduced toxicity), pneumococcal surface protein A (PspA), pneumococcal adhesin protein A (PsaA), a C5a peptidase from *Streptococcus*, hemolysin from *Staphylococcal aureus ,* Nontypeable *Haemophilus influenzae* (NTHi) proteins, *Haemophilus influenzae* protein D, *Clostridium perfringens* exotoxins/toxoid, hepatitis B surface antigen, hepatitis B core antigen, rotavirus VP 7 protein, and respiratory syncytial virus F and G protein, ovalbumin, keyhole limpet haemocyanin (KLH), bovine serum albumin (BSA), purified protein derivative of tuberculin (PPD), and a *Pseudomonas* exotoxin or its derivatives, including a recombinantly-produced non-toxic mutant *Pseudomonas aeruginosa* Exotoxin A. Bacterial outer membrane proteins such as outer membrane protein complex c (OMPC), porins, transferrin binding proteins, or *C. difficile* enterotoxin (toxin A) and cytotoxin (toxin B) can also be used. Other proteins, such as ovalbumin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or purified protein derivative of tuberculin (PPD) can also be used as carrier proteins. In a preferred embodiment, the carrier protein is a diphtheria toxoid. More preferably, the carrier protein is CRM_{197}. In another embodiment of the invention, the carrier protein is tetanus toxoid.

For the synthesis of a multivalent conjugate immunogenic composition, polysaccharide-protein conjugates may be produced by conjugating a mixture of polysaccharides purified from bacteria of two different species to a carrier protein. Alternatively, a multivalent conjugate immunogenic composition may be produced by combining polysaccharides purified from bacteria of two or more different serotypes of the same bacteria and conjugating them as a

mixture to a carrier protein. Alternatively, polysaccharide-protein conjugates produced by reacting a single type of polysaccharide with carrier protein in separate reactions using different polysaccharides, may be mixed. Thus, a multivalent immunogenic composition may include a carrier protein bearing a homogeneous or a heterogeneous population of linked polysaccharides.

After conjugation of the capsular polysaccharide to the carrier protein, the polysaccharide-protein conjugates are purified (enriched with respect to the amount of polysaccharide-protein conjugate) by a variety of techniques. These techniques include, *e.g.*, concentration/diafiltration operations, precipitation/elution, column chromatography, and depth filtration.

As described above, the present invention relates to conjugates comprising GBS capsular polysaccharides conjugated to carrier proteins. One embodiment of the invention provides conjugates comprising a GBS serotype VI capsular polysaccharide conjugated to a carrier protein and at least one additional conjugate comprising a GBS serotype Ia capsular polysaccharide conjugated to a carrier protein, a GBS serotype Ib capsular polysaccharide conjugated to a carrier protein, a GBS serotype II capsular polysaccharide conjugated to a carrier protein, a GBS serotype IIII capsular polysaccharide conjugated to a carrier protein, a GBS serotype V capsular polysaccharide conjugated to a carrier protein, a GBS serotype VII capsular polysaccharide conjugated to a carrier protein, a GBS serotype VIII capsular polysaccharide conjugated to a carrier protein, or a GBS serotype IX capsular polysaccharide conjugated to a carrier protein. In one aspect of the invention, the polysaccharides have a molecular weight of between about 5 kDa and 1,000 kDa; the conjugates have molecular weights of between about 300 kDa and about 20,000 kDa; and the conjugates comprise less than about 40% free polysaccharide relative to total polysaccharide. In one embodiment, the conjugates comprise less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, or less than about 5% free polysaccharide relative to total polysaccharide.

In one embodiment, the serotype Ia, Ib, II, III, IV, V, VI, VII, VIII, and/or IX polysaccharide has a molecular weight before conjugation of between about 5 kDa and about 1,000 kDa, such as between about 50 kDa and about 750 kDa, between about 50 kDa and about 500 kDa, between about 50 kDa and about 450 kDa, between about 50 kDa and about 400 kDa, between about 50 kDa and about 350 kDa, between about 50 kDa and about 300 kDa, between about 50 kDa and about 250 kDa, between about 50 kDa and about 200 kDa, between about 75 kDa and about 750 kDa, between about 75 kDa and about 500 kDa, between about 75 kDa and about 450 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 350 kDa, between about 75 kDa and about 300 kDa, between about 75 kDa and about 250 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 750 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 650 kDa,

between about 100 kDa and about 600 kDa, between about 100 kDa and about 550 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 450 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 350 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and 750 kDa, between about 200 kDa and about 700 kDa, between about 200 kDa and about 650 kDa, between about 200 kDa and about 600 kDa, between about 200 kDa and about 550 kDa, between about 200 kDa and about 500 kDa, between about 200 kDa and about 450 kDa, between about 200 kDa and about 400 kDa, , between about 250 kDa and about 750 kDa, between about 250 kDa and about 700 kDa, between about 250 kDa and about 650 kDa, between about 250 kDa and about 600 kDa, between about 250 kDa and about 550 kDa, between about 250 kDa and about 500 kDa, between about 250 kDa and about 450 kDa, between about 250 kDa and about 400 kDa, between about 300 kDa and 750 kDa, between about 300 kDa and about 700 kDa, between about 300 kDa and about 650 kDa, between about 300 kDa and about 600 kDa, between about 300 kDa and about 550 kDa, or between about 300 kDa and about 500 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the disclosure.

In one embodiment, the conjugate has a molecular weight of between about 300 kDa and about 20,000 kDa, such as between about 300 kDa and about 15,000 kDa, between about 300 kDa and about 10,000 kDa, between about 300 kDa and about 9,000 kDa, between about 300 kDa and about 8,000 kDa, between about 300 kDa and about 7,000 kDa, between about 300 kDa and about 6,000 kDa, between about 300 kDa and about 5,000 kDa, between about 300 kDa and about 4,000 kDa, between about 300 kDa and about 3,000 kDa, between about 300 kDa and about 2,000 kDa, between about 300 kDa and about 1,000 kDa, between about 500 kDa and about 20,000 kDa, between about 500 kDa and about 15,000 kDa, between about 500 kDa and about 10,000 kDa, between about 500 kDa and about 9,000 kDa, between about 500 kDa and about 8,000 kDa, between about 500 kDa and about 7,000 kDa, between about 500 kDa and about 6,000 kDa, between about 500 kDa and about 5,000 kDa, between about 500 kDa and about 4,000 kDa, between about 500 kDa and about 3,000 kDa, between about 500 kDa and about 2,000 kDa, between about 500 kDa and about 1,000 kDa, between about 1,000 kDa and about 20,000 kDa, between about 1,000 kDa and about 15,000 kDa, between about 1,000 kDa and about 10,000 kDa, between about 1,000 kDa and about 9,000 kDa, between about 1,000 kDa and about 8,000 kDa, between about 1,000 kDa and about 7,000 kDa, between about 1,000 kDa and about 6,000 kDa, between about 1,000 kDa and about 5,000 kDa, between about 1,500 kDa and about 20,000 kDa, between about 1,500 kDa and about 15,000 kDa, between about 1,500 kDa and about 10,000 kDa, between about 1,500 kDa and about 9,000 kDa, between about 1,500 kDa and about 8,000 kDa, between about 1,500 kDa and about 7,000 kDa, between about 1,500 kDa and about 6,000 kDa, between about 1,500 kDa and about 5,000 kDa, between about 2,000 kDa and about 20,000 kDa, between about 2,000 kDa and about 15,000 kDa, between about 2,000 kDa and about 10,000 kDa,

between about 2,000 kDa and about 9,000 kDa, between about 2,000 kDa and about 8,000 kDa, between about 2,000 kDa and about 7,000 kDa, between about 2,000 kDa and about 6,000 kDa, between about 2,500 kDa and about 20,000 kDa, between about 2,500 kDa and about 15,000 kDa, between about 2,500 kDa and about 10,000 kDa, between about 2,500 kDa and about 9,000 kDa, between about 2,500 kDa and about 8,000 kDa, between about 2,500 kDa and about 7,000 kDa, between about 2,500 kDa and about 6,000 kDa, between about 3,000 kDa and about 20,000 kDa, between about 3,000 kDa and about 15,000 kDa, between about 3,000 kDa and about 10,000 kDa, between about 3,000 kDa and about 9,000 kDa, between about 3,000 kDa and about 8,000 kDa, between about 3,000 kDa and about 7,000 kDa, or between about 3,000 kDa and about 6,000 kDa.

In an embodiment, a GBS serotype VI capsular polysaccharide conjugate has a molecular weight of any of the above ranges.

In an embodiment, a GBS serotype Ia capsular polysaccharide conjugate has a molecular weight of any of the above ranges.

In an embodiment, a GBS serotype Ib capsular polysaccharide conjugate has a molecular weight of any of the above ranges.

In an embodiment, a GBS serotype II capsular polysaccharide conjugate has a molecular weight of any of the above ranges.

In an embodiment, a GBS serotype III capsular polysaccharide conjugate has a molecular weight of any of the above ranges.

In an embodiment, a GBS serotype V capsular polysaccharide conjugate has a molecular weight of any of the above ranges.

In an embodiment, a GBS serotype VII capsular polysaccharide conjugate has a molecular weight of any of the above ranges.

In an embodiment, a GBS serotype VIII capsular polysaccharide conjugate has a molecular weight of any of the above ranges.

In an embodiment, a GBS serotype IX capsular polysaccharide conjugate has a molecular weight of any of the above ranges.

In one embodiment, the conjugates of the invention have at least about 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 0.97 or 0.98 mM sialic acid per mM polysaccharide. In a preferred embodiment, the conjugates have at least about 0.9 or 0.95 mM sialic acid per mM polysaccharide.

In an embodiment, a GBS serotype VI capsular polysaccharide conjugate has a sialic acid content of at least any of the above value.

In an embodiment, a GBS serotype Ia capsular polysaccharide conjugate has a sialic acid content of at least any of the above value.

In an embodiment, a GBS serotype Ib capsular polysaccharide conjugate has a sialic acid content of at least any of the above value.

In an embodiment, a GBS serotype II capsular polysaccharide conjugate has a sialic acid content of at least any of the above value.

In an embodiment, a GBS serotype III capsular polysaccharide conjugate has a sialic acid content of at least any of the above value.

In an embodiment, a GBS serotype V capsular polysaccharide conjugate has a sialic acid content of at least any of the above value.

In an embodiment, a GBS serotype VII capsular polysaccharide conjugate has a sialic acid content of at least any of the above value.

In an embodiment, a GBS serotype VIII capsular polysaccharide conjugate has a sialic acid content of at least any of the above value.

In an embodiment GBS, a serotype IX capsular polysaccharide conjugate has a sialic acid content of at least any of the above value.

In an embodiment, the conjugate of the invention comprises less than about 0.01, 0.02, 0.03, 0.04, or 0.05 mM O-acetate per mM saccharide repeating unit. In another embodiment, the conjugate comprises at least about 0.1, 0.2, 0.3, 0.35 or about 0.4 mM O-acetate per mM saccharide repeating unit.

In an embodiment, a GBS serotype VI capsular polysaccharide conjugate has an O-acetate content of any of the above value.

In an embodiment, a GBS serotype Ia capsular polysaccharide conjugate has an O-acetate content of any of the above value.

In an embodiment, a GBS serotype Ib capsular polysaccharide conjugate has an O-acetate content of any of the above value.

In an embodiment, a GBS serotype II capsular polysaccharide conjugate has an O-acetate content of any of the above value.

In an embodiment, a GBS serotype III capsular polysaccharide conjugate has an O-acetate content of any of the above value.

In an embodiment, a GBS serotype V capsular polysaccharide conjugate has an O-acetate content of any of the above value.

In an embodiment, a GBS serotype VII capsular polysaccharide conjugate has an O-acetate content of any of the above value.

In an embodiment, a GBS serotype VIII capsular polysaccharide conjugate has an O-acetate content of any of the above value.

In an embodiment GBS, a serotype IX capsular polysaccharide conjugate has an O-acetate content of any of the above value.

In a further embodiment, the immunogenic conjugate comprises less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, or less than about 5% of free GBS capsular polysaccharide compared to the total amount of GBS capsular polysaccharide. In a preferred

embodiment the immunogenic conjugate comprises less than about 5% of unreacted free saccharide compared to the total amount of GBS capsular polysaccharide.

In yet another embodiment, the ratio (weight by weight) of GBS capsular polysaccharide to carrier protein in the conjugate is between about 0.5 and about 3.0. In one aspect, the ratio of GBS capsular polysaccharide to carrier protein in the conjugate is between about 0.5 and about 2.0, between about 0.5 and about 1.5, between about 0.5 and about 1.0, between about 1.0 and about 1.5, or between about 1.0 and about 2.0. In a preferred embodiment, the ratio of GBS capsular polysaccharide to carrier protein in the conjugate is between about 0.8 and about 1.0.

In another embodiment, the degree of conjugation of the conjugate is between 2 and 15, between 2 and 13, between 2 and 10, between 2 and 8, between 2 and 6, between 2 and 5, between 2 and 4, between 3 and 15, between 3 and 13, between 3 and 10, between 3 and 8, between 3 and 6, between 3 and 5, between 3 and 4, between 5 and 15, between 5 and 10, between 8 and 15, between 8 and 12, between 10 and 15, or between 10 and 12. In a preferred embodiment, the degree of conjugation of the conjugate is between 2 and 5.

Conjugation

Conjugation may be direct, where the atoms from the polysaccharide are covalently bonded to atoms from the protein surface. Alternatively, conjugation may be through a linker molecule, which reacts with both the polysaccharide and the protein and connects the two, tethering the carbohydrate to the protein.

Where a carrier and one or more antigens such as a polysaccharide are conjugated (*i.e.*, covalently associated), conjugation may be by any chemical method, process or genetic technique known in the art. For example, a carrier polypeptide and one or more antigens selected from a group comprising a carbohydrate, an oligosaccharide, a lipid, a lipooligosaccharide, a polysaccharide, an oligosaccharide-protein conjugate, a polysaccharide-protein conjugate, a peptide-protein conjugate, an oligosaccharide-peptide conjugate, a polysaccharide-peptide conjugate, a protein-protein conjugate, a lipooligosaccharide-protein conjugate, a polysaccharide-protein conjugate, or any combination thereof, may be conjugated by techniques, including, but not limited to: (1) direct coupling via protein functional groups (*e.g.*, thiol-thiol linkage, amine-carboxyl linkage, amine-aldehyde linkage; enzyme direct coupling); (2) homobifunctional coupling of amines (*e.g.*, using bis-aldehydes); (3) homobifunctional coupling of thiols (*e.g.*, using bis-maleimides); (4) homobifunctional coupling via photoactivated reagents (5) heterobifunctional coupling of amines to thiols (*e.g.*, using maleimides); (6) heterobifunctional coupling via photoactivated reagents (*e.g.*, the β-carbonyidiazo family); (7) introducing amine-reactive groups into a poly- or oligosaccharide via cyanogen bromide activation or carboxymethylation; (8) introducing thiol-reactive groups into a poly- or oligosaccharide via a heterobifunctional compound such as maleimido-hydrazide; (9) protein-lipid conjugation via introducing a hydrophobic group into the protein and (10) protein-lipid

conjugation via incorporating a reactive group into the lipid. Also, contemplated are heterobifunctional "non-covalent coupling" techniques such the Biotin-Avidin interaction. Other methods well known in the art for effecting conjugation of oligosaccharides and polysaccharides to immunogenic carrier proteins are also within the scope of some embodiments of the invention.

In an embodiment, the GBS capsular polysaccharide-protein conjugates are obtained by activating polysaccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide may be coupled directly or via a spacer (linker) group to an amino group on the carrier protein. For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using GMBS) or a haloacetylated carrier protein (for example using iodoacetimide, SIB, SIAB, sulfo-SIAB, SIA, or SBAP).

In one aspect, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein using carbodiimide (e.g., EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described for example in Int'l Patent Appl. Pub. Nos. WO 93/15760, WO 95/08348, and WO 96/29094.

Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, and TSTU. Many are described in Int'l Patent Appl. Pub. No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with 1,1 carbonyldiimidazole (CDI) or 1,1 carboyl di 1,2,4 triazole (CDT)_(See Bethell, et al., J. Biol. Chem., 254:2572-2574 (1979); Hearn, et al., J. Chromatogr., 218:509-518 (1981)) followed by reaction with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI/CDT to form a CDI/CDT carbamate intermediate, and coupling the CDI/CDT carbamate intermediate with an amino group on a protein.

In preferred embodiments, the GBS capsular polysaccharide-protein conjugates of the invention are prepared using reductive amination. Reductive amination involves two steps: (1) oxidation of the polysaccharide to generate aldehyde functionalities from vicinal diols in individual hexasaccharide unit and (2) reduction of the activated polysaccharide and a carrier protein to form a conjugate.

In an embodiment, GBS capsular polysaccharide is activated (oxidized) by a process comprising the steps of:

(a) reacting isolated GBS capsular polysaccharide with an oxidizing agent; and

(b) quenching the oxidation reaction by addition of a quenching agent resulting in an activated GBS capsular polysaccharide.

In an aspect of the invention, the concentration of the isolated capsular polysaccharide is between about 0.1 mg/mL and about 10.0 mg/mL, such as between about 0.5 mg/mL and about 5.0 mg/mL mg/mL, between about 1.0 mg/mL and about 3.0 mg/mL, or about 2.0 mg/mL.

In a particular embodiment, the oxidizing agent is periodate. The periodate oxidizes vicinal hydroxyl groups to form carbonyl or aldehyde groups and causes cleavage of a C-C bond. The term 'periodate' includes both periodate and periodic acid. This term also includes both metaperiodate (IO_4^-) and orthoperiodate (IO_6^{5-}). The term 'periodate' also includes the various salts of periodate including sodium periodate and potassium periodate. In a preferred embodiment, the oxidizing agent is sodium periodate. In a preferred embodiment, the periodate used for the oxidation of GBS capsular polysaccharides is metaperiodate. In a preferred embodiment the periodate used for the oxidation of serotype capsular polysaccharide is sodium metaperiodate.

In another embodiment, the polysaccharide is reacted with 0.01 to 10.0, 0.05 to 5.0, 0.1 to 1.0, 0.5 to 1.0, 0.7 to 0.8, 0.05 to 0.5, or 0.1 to 0.3 molar equivalents of oxidizing agent. In a particular embodiment, the polysaccharide is reacted with about 0.05, about 0.1, about 0.15, about 0.2, about 0.25, about 0.3, about 0.35, about 0.4, about 0.45, about 0.5, about 0.55, about 0.6, about 0.65, about 0.7, about 0.75, about 0.8, about 0.85, about 0.9, or about 0.95 molar equivalents of oxidizing agent. In a further embodiment, the polysaccharide is reacted with about 0.1 molar equivalents of oxidizing agent. In a further embodiment, the polysaccharide is reacted with about 0.15 molar equivalents of oxidizing agent. In an additional embodiment, the polysaccharide is reacted with about 0.25 molar equivalents of oxidizing agent. In yet another embodiment, the polysaccharide is reacted with about 0.5 molar equivalents of oxidizing agent. In an alternative embodiment, the polysaccharide is reacted with about 0.6 molar equivalents of oxidizing agent. In a further embodiment, the polysaccharide is reacted with about 0.7 molar equivalents of oxidizing agent.

In one aspect of the invention, the duration of the oxidation reaction is between about 1 hour and about 50 hours, between about 10 hours and about 30 hours, between about 15 hours and about 20 hours, between about 15 hours and about 17 hours, or about 16 hours.

In another aspect of the invention, the temperature of the oxidation reaction is maintained between about 2°C and about 25°C, between about 2°C and about 8°C, or between about 20°C and about 25°C. In one preferred embodiment, the temperature of the reaction is maintained at about 23°C. In another preferred embodiment, the temperature of the reaction is maintained at about 5°C.

In a further aspect, the oxidation reaction is carried out in a buffer selected from the group consisting of sodium phosphate, potassium phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), and Bis-Tris. In a preferred embodiment, the buffer is potassium phosphate.

In an additional aspect, the buffer has a concentration of between about 1 mM and about 500 mM, between about 1 mM and about 300mM, or between about 50 mM and about 200mM. In a preferred embodiment the buffer has a concentration of about 100mM.

In one aspect, the oxidation reaction is carried out at a pH between about 4.0 and about 8.0, between about 5.0 and about 7.0, or between about 5.5 and about 6.5. In a preferred embodiment, the pH is about 6.0.

In one embodiment, the activated GBS capsular polysaccharide is obtained by reacting about 0.5 mg/L to about 5.0 mg/mL of isolated capsular polysaccharide with about 0.05 to about 0.3 molar equivalents periodate at a temperature between about 20°C and 25°C.

In another embodiment, the activated GBS capsular polysaccharide is obtained by reacting about 0.5 mg/L to about 5.0 mg/mL of isolated capsular polysaccharide with about 0.05 to about 0.3 molar equivalents periodate at a temperature between about 2°C and about 8°C.

In another embodiment, the activated GBS capsular polysaccharide is purified according to methods known to one skilled in the art, such as gel permeation chromatography (GPC), dialysis, or ultrafiltration/diafiltration. For example, the activated capsular polysaccharide is purified by concentration and diafiltration using an ultrafiltration device.

In one embodiment, the degree of oxidation of the activated GBS capsular polysaccharide is between 5 and 25, such as between 5 and 15, between 5 and 10, between 10 and 25, between 10 and 20, between 10 and 15. In a preferred embodiment the degree of oxidation of the activated GBS capsular polysaccharide is between 10 and 20, between 11 and 19, between 12 and 18, between 13 and 17, or between 14 and 16.

In another embodiment, the activated GBS capsular polysaccharide has a molecular weight between about 5 kDa and about 1,000 kDa, such as between about 50 kDa and about 300 kDa, between about 75 kDa and about 400 kDa, between about 75 kDa and about 200 kDa, between about 100 kDa and about 700 kDa, between about 100 kDa and about 500 kDa, between about 100 kDa and about 400 kDa, between about 100 kDa and about 300 kDa, between about 200 kDa and about 400 kDa, an between about 300 kDa and about 700 kDa. In a preferred embodiment, the activated GBS capsular polysaccharide has a molecular weight of between about 75 kDa and about 400 kDa

In an embodiment, the activated GBS capsular polysaccharide is lyophilized, optionally in the presence of saccharide. In a preferred embodiment, the saccharide is selected from sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit. In a preferred embodiment, the saccharide is sucrose. The lyophilized activated capsular polysaccharide can then be compounded with a solution comprising the carrier protein.

In another embodiment, the activated GBS capsular polysaccharide is compounded with the carrier protein and lyophilized, optionally in the presence of a saccharide. In one aspect, the saccharide is selected from sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit. In a preferred embodiment, the saccharide is sucrose. The co-

lyophilized polysaccharide and carrier protein can then be resuspended in solution and reacted with a reducing agent.

The activated GBS capsular polysaccharide can be conjugated to a carrier protein by a process comprising the step of:

(a) compounding the activated GBS capsular polysaccharide with a carrier protein, and

(b) reacting the compounded activated GBS capsular polysaccharide and carrier protein with a reducing agent to form a GBS capsular polysaccharide-carrier protein conjugate.

The conjugation of activated GBS capsular polysaccharide with a protein carrier by reductive amination in a polar aprotic solvent is suitable to maintain low levels of the free polysaccharide as compared, for example, to reductive amination in aqueous solution where the level of unreacted (free) polysaccharide is significantly elevated. In a preferred embodiment, step (a) and step (b) are carried out in a polar aprotic solvent.

In one embodiment, step (a) comprises dissolving lyophilized GBS capsular polysaccharide in a solution comprising a carrier protein and a polar aprotic solvent. In another embodiment, step (a) comprises dissolving co-lyophilized GBS capsular polysaccharide and carrier protein in a polar aprotic solvent.

In one embodiment, the polar aprotic solvent is selected from the group consisting of dimethylsulfoxide (DMSO), sulfolane, dimethylformamide (DMF), and hexamethylphosporamide (HMPA). In a preferred embodiment, the polar aprotic solvent is DMSO.

When steps (a) and (b) are carried out in aqueous solution, steps (a) and (b) are carried out in a buffer in an aqueous medium, preferably selected from PBS, MES, HEPES, Bis-tris, ADA, PIPES, MOPSO, BES, MOPS, DIPSO, MOBS, HEPPSO, POPSO, TEA, EPPS, Bicine or HEPB at a pH between about 6.0 and about 8.5, between about 7.0 and about 8.0, or between about 7.0 and about 7.5. In a preferred embodiment the buffer is PBS. In a preferred embodiment the pH is about 7.3.

In one embodiment, the concentration of activated GBS capsular polysaccharide in step (b) is between about 0.1 mg/mL and about 10.0 mg/mL, between about 0.5 mg/mL and about 5.0 mg/mL, or between about 0.5 mg/mL and about 2.0 mg/mL. In a preferred embodiment, the concentration of activated serotype GBS capsular polysaccharide in step (b) is about 0.1 mg/mL, about 0.2 mg/mL, about 0.3 mg/mL, about 0.4 mg/mL, about 0.5 mg/mL, about 0.6 mg/mL, about 0.7 mg/mL, about 0.8 mg/mL, about 0.9 mg/mL, about 1.0 mg/mL, about 1.1 mg/mL, about 1.2 mg/mL, about 1.3 mg/mL, about 1.4 mg/mL, about 1.5 mg/mL, about 1.6 mg/mL, about 1.7 mg/mL, about 1.8 mg/mL, about 1.9 mg/mL, about 2.0 mg/mL, about 2.1 mg/mL, about 2.2, about 2.3 mg/mL, about 2.4 mg/mL, about 2.5 mg/mL, about 2.6 mg/mL, about 2.7 mg/mL, about 2.8 mg/mL, about 2.9 mg/mL, or about 3.0 mg/mL.

In another embodiment the initial ratio (weight by weight) of activated serotype GBS capsular polysaccharide to carrier protein is between 5:1 and 0.1:1, 2:1 and 0.1:1, 2:1 and 1:1, 1.5:1 and 1:1, 0.1:1 and 1:1, 0.3:1 and 1:1, 0.6:1 and 1:1. In a preferred embodiment the initial

ratio of activated serotype GBS capsular polysaccharide to carrier protein is about 0.4:1, 0.5:1, 0.6:1, 0.7:1, 0.8:1, 0.9:1, 1:1, 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, 1.9:1, 2:1.

In an embodiment, the reducing agent is sodium cyanoborohydride, sodium triacetoxyborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, amine boranes such as pyridine borane, 2-picoline borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMe^{i}PrN-BH_3, benzylamine-BH_3 or 5-ethyl-2-methylpyridine borane (PEMB). In a preferred embodiment, the reducing agent is sodium cyanoborohydride.

In another embodiment, the quantity of reducing agent used in step (b) is between about 0.1 and about 10.0 molar equivalents, between about 0.5 and about 5.0 molar equivalents, or between about 1.0 and about 2.0 molar equivalents. In a preferred embodiment, the quantity of reducing agent used in step (b) is about 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2.0 molar equivalents.

In still another embodiment, the duration of step (b) is between 1 hour and 60 hours, between 10 hours and 50 hours, between 40 hours and 50 hours, or between 42 hours and 46 hours. In a preferred embodiment, the duration of step (b) is about 44 hours.

In a further embodiment, the temperature of the reaction in step (b) is maintained between 10°C and 40°C, between 15°C and 30°C, or between 20°C and 26°C. In a preferred embodiment, the temperature of the reaction in step (b) is maintained at about 23°C.

In an additional embodiment, the process for the preparation of an immunogenic conjugate comprising GBS capsular polysaccharide covalently linked to a carrier protein further comprises a step (step (c)) of capping unreacted aldehydes (quenching) by addition of a borohydride.

In one embodiment, the capping reagent is a borohydride selected from the group consisting of sodium borohydride ($NaBH_4$), sodium cyanoborohydride, lithium borohydride, potassium borohydride, tetrabutylammonium borohydride, calcium borohydride, and magnesium borohydride. In a preferred embodiment, the capping reagent is sodium borohydride.

In yet another embodiment, the quantity of borohydride used in step (c) is between about 0.1 and about 10.0 molar equivalents, between about 0.5 and about 5.0 molar equivalents, or between about 1.0 and 3.0 molar equivalents. In a preferred embodiment, the quantity of borohydride used in step (c) is about 2.0 molar equivalents.

In one embodiment, the borohydride used in step (c) is $NaBH_4$ in a concentration of about 2.0 molar equivalents.

In one embodiment, the duration of step (c) is between 0.1 hours and 10 hours, between 0.5 hours and 5 hours, between 2 hours and 4 hours. In a preferred embodiment, the duration of step (c) is about 3 hours.

In another embodiment, the temperature of the reaction in step (c) is maintained between about 15°C and about 45°C, between about 15°C and about 30°C, or between about

20°C and about 26°C. In a preferred embodiment, the temperature of the reaction in step (c) is maintained at about 23°C.

After conjugation of the GBS capsular polysaccharide to the carrier protein and capping, the polysaccharide-protein conjugate can be purified (enriched with respect to the amount of polysaccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration, precipitation/elution, column chromatography (DEAE or hydrophobic interaction chromatography), and depth filtration.

In a further embodiment, the immunogenic conjugate comprises less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, or less than about 5% of free GBS capsular polysaccharide compared to the total amount of GBS capsular polysaccharide. In a preferred embodiment the immunogenic conjugate comprises less than about 5% of unreacted free saccharide compared to the total amount of GBS capsular polysaccharide.

In another embodiment, the GBS polysaccharide-protein conjugate has a molecular weight between about 300 kDa and about 20,000 kDa, such as between about 1,000 kDa and about 15,000 kDa or between about 1,000 kDa and about 10,000 kDa.

In yet another embodiment, the ratio (weight by weight) of GBS capsular polysaccharide to carrier protein in the conjugate is between about 0.5 and about 3.0. In one aspect, the ratio of GBS capsular polysaccharide to carrier protein in the conjugate is between about 0.5 and about 2.0, between about 0.5 and about 1.5, between about 0.5 and about 1.0, between about 1.0 and about 1.5, or between about 1.0 and about 2.0. In a preferred embodiment, the ratio of GBS capsular polysaccharide to carrier protein in the conjugate is between about 0.8 and about 1.0.

In another embodiment, the degree of conjugation of the conjugate is between 2 and 15, between 2 and 13, between 2 and 10, between 2 and 8, between 2 and 6, between 2 and 5, between 2 and 4, between 3 and 15, between 3 and 13, between 3 and 10, between 3 and 8, between 3 and 6, between 3 and 5, between 3 and 4, between 5 and 15, between 5 and 10, between 8 and 15, between 8 and 12, between 10 and 15, or between 10 and 12. In a preferred embodiment, the degree of conjugation of the conjugate is between 2 and 5.

In one aspect of the invention, GBS capsular polysaccharide-protein conjugates are obtained by reductive amination method described above. For example, in one aspect the present disclosure provides a GBS capsular polysaccharide-protein conjugates comprising a polysaccharide conjugated to a carrier protein that is produced or obtainable by the method comprising the steps of:

(a) reacting isolated GBS capsular polysaccharide with an oxidizing agent;

(b) quenching the oxidation reaction by addition of a quenching agent resulting in an activated GBS capsular polysaccharide;

(c) compounding the activated GBS capsular polysaccharide with a carrier protein,

(d) reacting the compounded activated GBS capsular polysaccharide and carrier protein with a reducing agent to form a GBS capsular polysaccharide-carrier protein conjugate, and optionally

(e) capping unreacted aldehyde by addition of sodium borohydride ($NaBH_4$).

In a preferred embodiment, steps (c) and (d) are carried out in DMSO.

In another aspect of the invention, the GBS capsular polysaccharide-protein conjugates of the invention are prepared using reductive amination as described above, but with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) free radical and N-chlorosuccinimide (NCS) as the cooxidant in the activation/oxidization step. See Int'l Patent Appl. Pub. No. WO 2014/097099, which is incorporated herein by reference in its entirety. In such an embodiment, the glycoconjugates from GBS capsular polysaccharides are prepared using TEMPO free radical to oxidize primary alcohols of the saccharide to aldehydes using NCS as the cooxidant (hereinafter "TEMPO/NCS oxidation"), such as described at Example 7 and of Int'l Patent Appl. Pub. No. WO 2014/097099. Therefore in one aspect, conjugates of GBS capsular polysaccharides are obtainable by a method comprising the steps of: a) reacting a GBS capsular polysaccharide with TEMPO and NCS in an solvent to produce an activated saccharide; and b) reacting the activated saccharide with a carrier protein comprising one or more amine groups (hereinafter "TEMPO/NCS-reductive amination"). In one embodiment, the solvent may be an aqueous solvent or DMSO.

In one aspect, GBS capsular polysaccharide-protein conjugates are obtained by said method. For example, in one aspect the present disclosure provides a GBS capsular polysaccharide-protein conjugate comprising a polysaccharide conjugated to a carrier protein that is produced or obtainable by the method comprising the steps of: a) reacting a saccharide with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and N-chlorosuccinimide (NCS) in an solvent to produce an activated saccharide; and b) reacting the activated saccharide with a carrier protein comprising one or more amine groups. In one embodiment, the solvent may be an aqueous solvent or DMSO.

Immunogenic Compositions

After the individual conjugates are purified, they may be combined to formulate an immunogenic composition of the present invention, which may be used, for example, in a vaccine. Formulation of the immunogenic composition of the present invention can be accomplished using art-recognized methods.

An "immune response" to an immunogenic composition is the development in a subject of a humoral and/or a cell-mediated immune response to molecules present in the composition of interest (for example, an antigen, such as a protein or polysaccharide). For purposes of the present invention, a "humoral immune response" is an antibody-mediated immune response and involves the generation of antibodies with affinity for the antigens present in the

immunogenic compositions of the invention, while a "cell-mediated immune response" is one mediated by T-lymphocytes and/or other white blood cells. A "cell-mediated immune response" is elicited by the presentation of antigenic epitopes in association with Class I or Class II molecules of the major histocompatibility complex (MHC). This activates antigen-specific CD4+ T helper cells or CD8+ cytotoxic T lymphocyte cells (CTLs). CTLs have specificity for peptide or lipid antigens that are presented in association with proteins encoded by the MHC or CD1 and expressed on the surfaces of cells. CTLs help induce and promote the intracellular destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with classical or nonclassical MHC molecules on their surface. A "cell-mediated immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells. The ability of a particular antigen or composition to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, by assaying for T-lymphocytes specific for the antigen in a sensitized subject, or by measurement of cytokine production by T cells in response to restimulation with antigen. Such assays are well known in the art. *See, e.g.*, Erickson, A.L., et al., J. Immunol., 151(8):4189-4199 (1993); Doe, B., et al., Eur. J. Immunol. 24(10):2369-2376 (1994).

The term "immunogenic" refers to the ability of an antigen or a vaccine to elicit an immune response, either humoral or cell-mediated, or both.

An "immunogenic amount", or an "immunologically effective amount" or "dose", each of which is used interchangeably herein, generally refers to the amount of antigen or immunogenic composition sufficient to elicit an immune response, either a cellular (T cell) or humoral (B cell or antibody) response, or both, as measured by standard assays known to one skilled in the art.

“Immune interference” or “significant immune interference” as used herein refers to a statistically significant decrease in immune response to an individual antigen in a multivalent or multicomponent vaccine compared to the immune response to the same antigen when administered in a monovalent vaccine.

A "protective" immune response refers to the ability of an immunogenic composition to elicit an immune response, either humoral or cell mediated, which serves to protect the subject from an infection. The protection provided need not be absolute, *i.e.*, the infection need not be totally prevented or eradicated, if there is a statistically significant improvement compared with a control population of subjects, *e.g.*, infected animals not administered the vaccine or immunogenic composition. Protection may be limited to mitigating the severity or rapidity of onset of symptoms of the infection. Several assays are known in the art to determine whether an immune response is indicative of a "protective immune response." For instance, an increase

in antibody levels may be measured by a binding assay, such as a whole cell ELISA assay described further below. Other assays include measuring functional antibody responses, such as the facilitation of bacterial killing, which can be tested with an opsonophagocytosis assay (OPA) as described below. In particular situations, a "protective immune response" could include the induction of a two-fold increase in antibody levels or a four-fold increase in antibody levels specific for a particular antigen in at least 50% of subjects. In another situation, a “protective immune response” could include a decrease in bacterial count of at least 10%, 25%, 50%, 65%, 75%, 80%, 85%, 90%, 95% or more.

The amount of a particular conjugate in a composition is generally calculated based on total polysaccharide, conjugated and non-conjugated, for that conjugate. For example, a GBS capsular polysaccharide conjugate with 20% free polysaccharide will have about 80 mcg/ml of conjugated GBS capsular polysaccharide and about 20 mcg/ml of non-conjugated GBS capsular polysaccharide in a 100 mcg/ml GBS capsular polysaccharide dose. The protein carrier contribution to the conjugate is usually not considered when calculating the dose of a conjugate. The amount of conjugate can vary depending upon the streptococcal serotype. Generally, each dose will comprise about 0.01 mg/ml to about 100 mcg/ml of each polysaccharide, particularly about 1 mcg/ml to about 70 mcg/ml, and more particularly about 5 mcg/ml to about 50 mcg/ml. The "immunogenic amount" of the different polysaccharide components in the immunogenic composition, may diverge and each may comprise about 0.01 mcg/ml, about 0.1 mcg/ml, about 0.25 mcg/ml, about 0.5 mcg/ml, about 1 mcg/ml, about 2 mcg/ml, about 3 mcg/ml, about 4 mcg/ml, about 5 mcg/ml, about 6 mcg/ml, about 7 mcg/ml, about 8 mcg/ml, about 9 mcg/ml, about 10 mcg/ml, about 15 mcg/ml, about 20 mcg/ml, about 25 mcg/ml, about 30 mcg/ml, about 40 mcg/ml, about 50 mcg/ml, about 60 mcg/ml, about 70 mcg/ml, about 80 mcg/ml, about 90 mcg/ml, or about 100 mcg/ml of any particular polysaccharide antigen. A dose or immunogenic amount of a multivalent immunogenic composition would indicate the dose of each polysaccharide unless indicated otherwise. For example, a 10 mcg/ml dose of a hexavalent immunogenic composition would contain 10 mcg/ml of each of the six polysaccharides.

The effectiveness of an antigen as an immunogen can be measured by measuring the levels of B cell activity by measuring the levels of circulating antibodies specific for the antigen in serum using immunoassays, immunoprecipitation assays, functional antibody assays, such as *in vitro* opsonic assay and many other assays known in the art. Another measure of effectiveness of an antigen as a T-cell immunogen can be measured by either by proliferation assays, by cytolytic assays, such as chromium release assays to measure the ability of a T cell to lyse its specific target cell. Furthermore, in the present invention, an "immunogenic amount" may also be defined by measuring the serum levels of antigen specific antibody induced following administration of the antigen or by measuring the ability of the antibodies so induced to enhance the opsonophagocytic ability of particular white blood cells as described herein. The

level of protection of the immune response may be measured by challenging the immunized host with the antigen that has been injected. For example, if the antigen to which an immune response is desired is a bacterium, the level of protection induced by the "immunogenic amount" of the antigen can be measured by detecting the percent survival or the percent mortality after challenge of the animals with the bacterial cells. In one embodiment, the amount of protection may be measured by measuring at least one symptom associated with the bacterial infection, for example, a fever associated with the infection. The amount of each of the antigens in the multi-antigen or multi-component vaccine or immunogenic compositions will vary with respect to each of the other components and can be determined by methods known to the skilled artisan. Such methods would include, for example, procedures for measuring immunogenicity and/or *in vivo* efficacy.

The term "immunogenic composition" relates to any pharmaceutical composition containing an antigen, *e.g.*, a microorganism, or a component thereof, which composition can be used to elicit an immune response in a subject. The immunogenic compositions of the present invention can be used to treat a human susceptible to GBS infection, by means of administering the immunogenic compositions via a systemic transdermal or mucosal route. These administrations can include injection via the intramuscular (i.m.), intraperitoneal (i.p.), intradermal (i.d.) or subcutaneous routes; application by a patch or other transdermal delivery device; or via mucosal administration to the oral/alimentary, respiratory or genitourinary tracts. In one embodiment, the immunogenic composition may be used in the manufacture of a vaccine or in the elicitation of a polyclonal or monoclonal antibodies that could be used to passively protect or treat an animal.

In one aspect, the present invention relates to immunogenic compositions that include an effective amount of at least one polysaccharide, oligosaccharide, polysaccharide-protein conjugate, or biological equivalent thereof, as described herein. For example, in one embodiment, the immunogenic composition includes polysaccharide-protein conjugates, wherein the capsular polysaccharide is selected from the group consisting of group B streptococcus serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII and IX and wherein the capsular polysaccharide has a sialic acid level of greater than about 60%. In another example, the immunogenic composition includes polysaccharide-protein conjugates, wherein the conjugates comprise capsular polysaccharides from group B streptococcus serotype VI and at least one additional serotype selected from the group consisting of serotypes Ia, Ib, II, III, IV, V, VII, VIII and IX. In another embodiment, the immunogenic composition comprises polysaccharide-protein conjugates, wherein the conjugates comprise capsular polysaccharides from group B streptococcus serotype VI and at least two additional serotypes selected from the group consisting of serotypes Ia, Ib, II, III, IV, V, VII, VIII and IX. In yet another embodiment, the immunogenic composition comprises polysaccharide-protein conjugates, wherein the conjugates comprise capsular polysaccharides from group B streptococcus serotype VI and at

least three additional serotypes selected from the group consisting of serotypes Ia, Ib, II, III, IV, V, VII, VIII and IX. In a further embodiment, the immunogenic composition comprises polysaccharide-protein conjugates, wherein the conjugates comprise capsular polysaccharides from group B streptococcus serotype VI and at least four additional serotypes selected from the group consisting of serotypes Ia, Ib, II, III, IV, V, VII, VIII and IX. In a particular embodiment, the immunogenic composition polysaccharide-protein conjugates, wherein the conjugates comprise capsular polysaccharides from group B streptococcus serotypes VI, VII, VIII and IX. In another embodiment, the immunogenic composition polysaccharide-protein conjugates, wherein the conjugates comprise capsular polysaccharides from group B streptococcus serotypes Ia, Ib, II, III, and VI. In yet another embodiment, the immunogenic composition polysaccharide-protein conjugates, wherein the conjugates comprise capsular polysaccharides from group B streptococcus serotype VI and at least five additional serotypes selected from the group consisting of serotypes Ia, Ib, II, III, IV, V, VII, VIII, and IX. In one such embodiment, the immunogenic composition comprises six polysaccharide-protein conjugates, wherein the conjugates comprise a capsular polysaccharide from group B streptococcus serotypes Ia, Ib, II, III, IV and VI.

In an embodiment, the immunogenic composition of the invention comprises from 1 to 10 different serotypes of *S. agalactiae*. Therefore, in an embodiment, the immunogenic composition of the invention is a 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10-valent GBS conjugate composition. In one such embodiment, the immunogenic composition is a monovalent GBS conjugate composition. In another embodiment, the immunogenic composition is a 2-, 3-, 4-, 5-, or 6-valent GBS conjugate composition. In yet another embodiment, the immunogenic composition is a 7-valent GBS conjugate composition. In a further embodiment, the immunogenic composition is an 8-valent GBS conjugate composition.

Accordingly, the present invention-relates to monovalent and/or multivalent immunogenic compositions comprising polysaccharide-protein conjugates comprising at least one, two, three, or four GBS capsular polysaccharide serotypes, such as at least five GBS capsular polysaccharide serotypes, at least six GBS capsular polysaccharide serotypes, at least seven GBS capsular polysaccharide serotypes, at least eight GBS capsular polysaccharide serotypes, or at least nine GBS capsular polysaccharide serotypes,. In a particular embodiment, the immunogenic composition comprises GBS capsular polysaccharide serotype VI.

The polysaccharide-protein conjugates may comprise the same or different protein carriers. In one embodiment, the conjugates comprise the same protein carrier and the saccharides are conjugated to the same molecule of the protein carrier (carrier molecules having 2 or more different polysaccharides conjugated to it). For example, see International Patent Appl. Publication WO2004/083251. In another embodiment, the one or more polysaccharides are each individually conjugated to different molecules of the protein carrier

(each molecule of protein carrier only having one type of polysaccharide conjugated to it). In such an embodiment, the capsular saccharide(s) are said to be individually conjugated to the carrier protein.

Optimal amounts of components for a particular immunogenic composition can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects can receive one or several booster immunizations adequately spaced.

The immunogenic compositions of the invention may further comprise one or more preservatives in addition to a plurality of capsular polysaccharide protein conjugates. The FDA requires that biological products in multiple-dose (multi-dose) vials contain a preservative, with only a few exceptions. The present invention contemplates the use of such multi-dose vials. Vaccine products containing preservatives include vaccines containing benzethonium chloride (anthrax), 2-phenoxyethanol (DTaP, HepA, Lyme, Polio (parenteral)), and phenol (Pneumo, Typhoid (parenteral). Preservatives approved for use in injectable drugs include, *e.g.*, chlorobutanol, m cresol, methylparaben, propylparaben, 2-phenoxyethanol, benzethonium chloride, benzalkonium chloride, benzoic acid, benzyl alcohol, phenol, and phenylmercuric nitrate.

In another aspect, the invention relates to a composition including at least one of any polysaccharide described herein and a pharmaceutically acceptable excipient, buffer, stabilizer, adjuvant, a cryoprotectant, a salt, a divalent cation, a non-ionic detergent, an inhibitor of free radical oxidation, a diluent or a carrier, or mixture thereof.

The immunogenic composition optionally comprises one or more physiologically acceptable buffers selected from, but not limited to HEPES, PIPES, MES, Tris (trimethamine), phosphate, acetate, borate, citrate, glycine, histidine and succinate. In a preferred embodiment, the buffer is histidine.

In one embodiment, the immunogenic composition comprises a buffer at a concentration of from about 5 mM to about 50 mM, about 5 mM to about 40 mM, about 5 mM to about 30 mM, about 5 mM to about 20 mM, about 5 mM to about 10 mM, about 10 mM to about 50 mM, about 10 mM to about 40 mM, about 10 mM to about 35 mM, about 10 mM to about 30 mM, about 10 mM to about 25 mM, about 10 mM to about 20 mM, about 10 mM to about 15 mM, about 15 mM to about 50 mM, about 15 mM to about 40 mM, about 15 mM to about 35 mM, about 15 mM to about 30 mM, about 15 mM to about 25 mM, or about 15 mM to about 20 mM. In a preferred embodiment, the immunogenic composition comprises a buffer at a concentration of about 10 mM to about 25 mM, and most preferably about 20 mM.

In another embodiment, the immunogenic composition comprises histidine at a concentration of about 20 mM.

In certain embodiments, the formulation is buffered to within a pH range of about 5.0 to about 7.1, such as about 5.3 to about 7.1, about 5.5 to about 7.0, about 6.0 to about 7.0, about

6.0 to about 6.5, about 6.3 to about 7.0, or about 6.5 to about 7.0. In another embodiment, the formulation is buffered to a pH of about 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, or 7.0. In a preferred embodiment, the formulation is buffered to a pH range of from about 6.0 to about 7.0, and most preferably about 6.5.

The immunogenic composition optionally comprises one or more non-ionic surfactants, including but not limited to polyoxyethylene sorbitan fatty acid esters, polysorbate-80 (TWEEN 80), polysorbate-60 (TWEEN 60), polysorbate-40 (TWEEN 40), polysorbate-20 (TWEEN 20), and polyoxyethylene alkyl ethers, including but not limited to BRIJ 58, BRIJ 35, as well as others such as TRITON X-100; TRITON X- 114, NP40, SPAN 85 and the PLURONIC series of non-ionic surfactants (e.g., PLURONIC 121). In one embodiment, the immunogenic composition comprises polysorbate-80 or polysorbate-40, preferably polysorbate-80 (PS80).

In one embodiment, the immunogenic composition comprises a surfactant at a concentration of from about 0.001% to about 2% (v/w), about 0.001% to about 1%, about 0.001% to about 0.5%, about 0.001% to about 0.1%, about 0.001% to about 0.05%, about 0.001% to about 0.01%, about 0.001% to 0.005%, about 0.005% to about 2%, about 0.005% to about 1%, about 0.005% to about 0.5%, about 0.005% to about 0.1%, about 0.005% to about 0.05%, about 0.005% to about 0.01%, about 0.01% to about 2%, about 0.01% to about 1%, about 0.01% to about 0.5%, about 0.01% to about 0.1%, about 0.01% to about 0.05%, about 0.01% to about 0.04%, about 0.01% to about 0.03%, about 0.015% to about 2%, about 0.015% to about 1%, about 0.015% to about 0.5%, about 0.015% to about 0.1%, about 0.015% to about 0.05%, about 0.015% to about 0.04%, about 0.015% to about 0.03%, about 0.02% to about 2%, about 0.02% to about 1%, about 0.02% to about 0.5%, about 0.02% to about 0.1%, about 0.02% to about 0.05%, about 0.02% to about 0.04%, about 0.02% to about 0.03%, about 0.05% to about 2%, about 0.05% to about 1%, about 0.05% to about 0.5%, about 0.05% to about 0.1%, about 0.1% to about 2%, about 0.1% to about 1%, about 0.1% to about 0.5% or about 0.1% to about 0.25%. In a preferred embodiment, the immunogenic composition comprises a surfactant at a concentration of about 0.01% to about 0.03%, and most preferably about 0.02%.

In another embodiment, the immunogenic composition comprises polysorbate-80 (PS80) at a concentration from about 0.001% to about 2% (with up to about 0.25% being preferred) or polysorbate-40 at a concentration from about 0.001% to 1% (with up to about 0.5% being preferred).

In an embodiment, the immunogenic composition comprises PS80 at a concentration of about 0.02%.

Pharmaceutically acceptable carriers are not to be confused with “carrier proteins”, which are used in attaching the carbohydrate of the invention to a protein and modify the immune response to that carbohydrate. To avoid confusion with the protein carriers herein described, the term pharmaceutically acceptable diluent will be preferred over pharmaceutically acceptable carriers, but these terms may occasionally be used interchangeably. The term

"pharmaceutically acceptable carrier" means a carrier approved by a regulatory agency of a Federal, a state government, or other regulatory agency, or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, including humans as well as non-human mammals. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Suitable pharmaceutically acceptable diluents include any and all conventional solvents, dispersion media, fillers, solid carriers, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Such pharmaceutically acceptable diluents can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin. Water, water for injection (WFI), sterile isotonic saline solutions, phosphate buffered saline, adjuvant suspensions, aqueous dextrose and glycerol solutions, and combination thereof, can be employed as liquid carriers, particularly for injectable solutions. Pharmaceutically acceptable diluents may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness in the body. The preparation and use of pharmaceutically acceptable diluents is well known in the art. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. In one embodiment, the diluent is water, water for injection (WFI), an adjuvant suspension, or saline. In a particular embodiment, the diluent is a suspension of any adjuvant described herein. In a preferred embodiment, the diluent is an aluminum-based adjuvant suspension, such as an aluminum phosphate suspension.

Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol, palatinit, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, glycine, arginine, lysine, sodium chloride (NaCl), dried skim milk, glycerol, propylene glycol, water, ethanol and the like. In a preferred embodiment, the excipient is NaCl.

In one embodiment, the immunogenic composition comprises an excipient at a concentration of from about 10 mM to about 500 mM, about 10 mM to about 450 mM, about 10 mM to about 400 mM, about 10 mM to about 350 mM, about 10 mM to about 300 mM, about 10 mM to about 250 mM, about 10 mM to about 200 mM, about 10 mM to about 150 mM, about 10 mM to about 100 mM, about 10 mM to about 50 mM, about 10 mM to about 30 mM, about 10 mM to about 20 mM, 20 mM to about 500 mM, about 20 mM to about 450 mM, about 20 mM to about 400 mM, about 20 mM to about 350 mM, about 20 mM to about 300 mM, about 20 mM to about 250 mM, about 20 mM to about 200 mM, about 20 mM to about 150 mM, about 20 mM to about 100 mM, about 20 mM to about 50 mM, about 20 mM to about 30 mM, 50 mM to about 500 mM, about 50 mM to about 450 mM, about 50 mM to about 400 mM, about 50 mM to about 350 mM, about 50 mM to about 300 mM, about 50 mM to about 250 mM, about 50 mM to about 200 mM, about 50 mM to about 150 mM, about 50 mM to about 100 mM, about 100 mM to about 500 mM, about 100 mM to about 450 mM, about 100 mM to about 400 mM, about 100

mM to about 350 mM, about 100 mM to about 300 mM, about 100 mM to about 250 mM, about 100 mM to about 200 mM, about 100 mM to about 150 mM, about 150 mM to about 500 mM, about 150 mM to about 450 mM, about 150 mM to about 400 mM, about 150 mM to about 350 mM, about 150 mM to about 300 mM, about 150 mM to about 250 mM, about 150 mM to about 200 mM, about 200 mM to about 500 mM, about 200 mM to about 450 mM, about 200 mM to about 400 mM, about 200 mM to about 350 mM, about 200 mM to about 300 mM, about 200 mM to about 250 mM, about 250 mM to about 500 mM, about 250 mM to about 450 mM, about 250 mM to about 400 mM, about 250 mM to about 350 mM, about 250 mM to about 300 mM, about 300 mM to about 500 mM, about 300 mM to about 450 mM, about 300 mM to about 400 mM, about 300 mM to about 350 mM, about 350 mM to about 500 mM, about 350 mM to about 450 mM, about 350 mM to about 400 mM, about 400 mM to about 500 mM, about 400 mM to about 450 mM, or about 450 mM to about 500 mM. In a preferred embodiment, the immunogenic composition comprises an excipient at a concentration of from about 10 mM to about 250 mM, and most preferably about 150 mM.

In another embodiment, the excipient is NaCl at a concentration of about 150 mM.

The composition, if desired, can also contain minor amounts of wetting, bulking, emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, lyophilized powder or cake, and the like. The formulation should suit the mode of administration. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the immunogenic compositions of the present invention is contemplated.

In an embodiment, the immunogenic composition is lyophilized, optionally in the presence of at least one excipient. In a preferred embodiment, the at least one excipient is selected from the group consisting of starch, glucose, lactose, sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol, palatinit, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, glycine, arginine, lysine, sodium chloride (NaCl), dried skim milk, glycerol, propylene glycol, water, and ethanol. In a preferred embodiment, the at least one excipient is selected from the group consisting of sucrose, mannitol, and glycine. In a particular embodiment, the at least one excipient is sucrose. In another embodiment, the lyophilized composition comprises an additional excipient. In one such embodiment, the additional excipient is mannitol or glycine.

In another embodiment, the lyophilized composition comprises about 1% (w/v) to about 10% (w/v) of at least one saccharide, such as about 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, 6.5%, 7.0%, 7.5%, 8.0%, 8.5%, 9.0%, 9.5% or 10.0%. In a preferred embodiment, the lyophilized composition comprises greater than about 5.5% (w/v) of at least one excipient, such as greater than about 7.0%(w/v). In a further embodiment, the lyophilized composition comprises about 1% (w/v) to about 10% (w/v) of an additional excipient, such as about 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, 6.5%, 7.0%, 7.5%, 8.0%,

8.5%, 9.0%, 9.5% or 10.0%. In a preferred embodiment, the lyophilized composition comprises about 1% (w/v) to about 10% (w/v) of the at least one excipient and about 1% (w/v) to about 10% (w/v) of the additional excipient.

In yet another embodiment, the lyophilized composition is reconstituted with water, water for injection (WFI), an adjuvant suspension, or saline. In a preferred embodiment, the diluent is an aluminum-based adjuvant suspension, such as an aluminum phosphate suspension.

In one embodiment, the composition includes an isolated polysaccharide described herein and a carrier molecule. Suitable carrier molecules may include proteins, polysaccharides, polylactic acids, polyglycollic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG.

The immunogenic compositions of the present invention can further comprise one or more additional "immunomodulators", which are agents that perturb or alter the immune system, such that either up-regulation or down-regulation of humoral and/or cell-mediated immunity is observed. In one particular embodiment, up-regulation of the humoral and/or cell-mediated arms of the immune system is preferred. Examples of certain immunomodulators include, for example, an adjuvant or cytokine, or ISCOMATRIX (CSL Limited, Parkville, Australia), described in U.S. Patent No. 5,254,339, among others. The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen as further described herein.

Non-limiting examples of adjuvants that can be used in the composition of the present invention include the RIBI adjuvant system (Ribi Inc., Hamilton, Mont.); mineral gels, such as aluminum hydroxide gel; water-in-oil emulsions, such as Freund's complete and incomplete adjuvants; Block copolymer (CytRx, Atlanta Ga.); SAF-M (Chiron, Emeryville, Calif.); AMPHIGEN® adjuvant; saponin; Quil A or other saponin fraction; monophosphoryl lipid A; and Avridine lipid-amine adjuvant. Non-limiting examples of oil-in-water emulsions useful as an adjuvant in the vaccine of the invention include MF59 (U.S. Patent No. 6,299,884) (containing 5% Squalene, 0.5% polysorbate 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA)), and SAF (containing 10% Squalene, 0.4% polysorbate 80, 5% pluronic-blocked polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion); modified SEAM62 (containing 5% (v/v) squalene (Sigma), 1% (v/v) SPAN® 85 detergent (ICI Surfactants), 0.7% (v/v) polysorbate 80 detergent (ICI Surfactants), 2.5% (v/v) ethanol, 200 µg/ml Quil A, 100 µg/ml cholesterol, and 0.5% (v/v) lecithin); and modified SEAM 1/2 (containing 5% (v/v) squalene, 1%

(v/v) SPAN® 85 detergent, 0.7% (v/v) polysorbate 80 detergent, 2.5% (v/v) ethanol, 100 µg/ml Quil A, and 50 µg/ml cholesterol).

Suitable adjuvants used to enhance an immune response further include, without limitation, MPL™ (3-O-deacylated monophosphoryl lipid A, Corixa, Hamilton, MT), which is described in U.S. Patent No. 4,912,094. Also suitable for use as adjuvants are synthetic lipid A analogs or aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa (Hamilton, MT), and which are described in U.S. Patent No. 6,113,918. One such AGP is 2-[(R)-3-Tetradecanoyloxy¬tetradecanoyl¬amino] ethyl 2-Deoxy-4-O-phosphono-3-O-[(R)-3-tetra¬decanoyoxy¬tetrade¬canoyl]-2-[(R)-3-tetradecanoyloxy¬tetradecanoyl-amino]-b-D-glucopyranoside, which is also known as 529 (formerly known as RC529). This 529 adjuvant is formulated as an aqueous form (AF) or as a stable emulsion (SE).

Still other adjuvants include a cyclodextrin derivative (U.S. Patent No. 6,165,995); a polyanionic polymer (U.S. Patent No. 6,610,310); muramyl peptides, such as N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), and N-acetyl-normuramyl-L-alanine-2-(1'-2' dipalmitoyl-sn-glycero-3-hydroxy¬phosphoryl¬oxy)-ethylamine (MTP-PE); Amphigen; Avridine; L121/squalene; D-lactide-polylactide/glycoside; pluronic polyols; killed Bordetella; saponins, such as Stimulon™ QS-21 (Antigenics, Framingham, MA.), described in U.S. Patent No. 5,057,540; Mycobacterium tuberculosis; bacterial lipopolysaccharides; synthetic polynucleotides such as oligonucleotides containing a CpG motif (*e.g.*, U.S. Patent No. 6,207,646); IC-31 (Intercell AG, Vienna, Austria), described in European Patent Nos. 1,296,713 and 1,326,634; a pertussis toxin (PT) or mutant thereof, a cholera toxin or mutant thereof (*e.g.*, U.S. Patent Nos. 7,285,281, 7,332,174, 7,361,355 and 7,384,640); or an *E. coli* heat-labile toxin (LT) or mutant thereof, particularly LT-K63, LT-R72 (*e.g.*, U.S. Patent Nos. 6,149,919, 7,115,730 and 7,291,588).

Other "immunomodulators" that can be included in the vaccine include, *e.g.*, one or more of the interleukins 1-α, 1-β, 2, 4, 5, 6, 7, 8, 10, 12 (see, e.g., U.S. Patent No. 5,723,127), 13, 14, 15, 16, 17 and 18 (and its mutant forms); the interferons-α, β and γ; granulocyte-macrophage colony stimulating factor (GM-CSF) (*see, e.g.*, U.S. Patent No. 5,078,996 and ATCC Accession Number 39900); macrophage colony stimulating factor (M-CSF); granulocyte colony stimulating factor (G-CSF); or the tumor necrosis factors α and β. Still other adjuvants that are useful with the immunogenic compositions described herein include chemokines, including without limitation, MCP-1, MIP-1α, MIP-1β, and RANTES; adhesion molecules, such as a selectin, *e.g.*, L-selectin, P-selectin and E-selectin; mucin-like molecules, *e.g.*, CD34, GlyCAM-1 and MadCAM-1; a member of the integrin family such as LFA-1, VLA-1, Mac-1 and p150.95; a member of the immunoglobulin superfamily such as PECAM, ICAMs, *e.g.*, ICAM-1, ICAM-2 and ICAM-3, CD2 and LFA-3; co-stimulatory molecules such as B7-1, B7-2,CD40 and CD40L; growth factors including vascular growth factor, nerve growth factor, fibroblast growth

factor, epidermal growth factor, PDGF, BL-1, and vascular endothelial growth factor; receptor molecules including Fas, TNF receptor, Flt, Apo-1, p55, WSL-1, DR3, TRAMP, Apo-3, AIR, LARD, NGRF, DR4, DR5, KILLER, TRAIL-R2, TRICK2, and DR6; and Caspase (ICE).

It is to be understood that the decision whether to use an immunomodulator and/or adjuvant or the choice of which immunomodulator and/or adjuvant to be used will depend on the subject to which the vaccine or immunogenic composition will be administered, the route of injection, and the number of injections to be given. For instance, if the subject has been exposed to the pathogen naturally, an adjuvant may not be required as the vaccine antigens can effectively induce a memory response. In certain embodiments, the immunogenic composition will include one or more adjuvants. In one embodiment, the immunogenic composition comprises Streptococcal C5a peptidase (SCP). In one embodiment, the immunogenic composition comprises an aluminum-based adjuvant. In one such embodiment, the aluminum adjuvant is aluminum hydroxide, aluminum phosphate, or aluminum hydroxyl phosphate. In a particular embodiment, the adjuvant is aluminum phosphate. In another embodiment of the invention, the immunogenic composition comprises QS-21 as the adjuvant.

In one embodiment, the immunogenic composition comprises an adjuvant at a concentration of from about 0.1 mg/ml to about 1.0 mg/ml, 0.1 mg/ml to about 0.9 mg/ml, 0.1 mg/ml to about 0.8 mg/ml, 0.1 mg/ml to about 0.7 mg/ml, 0.1 mg/ml to about 0.6 mg/ml, 0.1 mg/ml to about 0.5 mg/ml, 0.1 mg/ml to about 0.4 mg/ml, 0.1 mg/ml to about 0.3 mg/ml, 0.1 mg/ml to about 0.2 mg/ml, 0.25 mg/ml to about 0.95 mg/ml, 0.25 mg/ml to about 0.85 mg/ml, 0.25 mg/ml to about 0.75 mg/ml, 0.25 mg/ml to about 0.65 mg/ml, 0.25 mg/ml to about 0.55 mg/ml, 0.25 mg/ml to about 0.45 mg/ml, 0.25 mg/ml to about 0.35 mg/ml, 0.5 mg/ml to about 1.0 mg/ml, 0.5 mg/ml to about 0.9 mg/ml, 0.5 mg/ml to about 0.8 mg/ml, 0.5 mg/ml to about 0.75 mg/ml, 0.5 mg/ml to about 0.7 mg/ml, 0.5 mg/ml to about 0.65 mg/ml, 0.5 mg/ml to about 0.6 mg/ml, 0.75 mg/ml to about 1.0 mg/ml, 0.75 mg/ml to about 0.95 mg/ml, 0.75 mg/ml to about 0.9 mg/ml, and 0.75 mg/ml to about 0.85 mg/ml. In a preferred embodiment, the immunogenic composition comprises an adjuvant at a concentration of from about 0.25 mg/ml to about 0.75 mg/ml, and most preferably about 0.5 mg/ml.

In another embodiment, the adjuvant is an aluminum-based at a concentration of about 0.5 mg/ml. In one such embodiment, the aluminum-based adjuvant is aluminum phosphate or aluminum hydroxyl phosphate.

In one embodiment, the immunogenic composition comprises a polysaccharide-protein conjugate as described herein, a buffer, a surfactant, an excipient, and optionally an adjuvant, wherein the composition is buffered to a pH of about 6.0 to about 7.0.

In one such embodiment, the immunogenic composition comprises a GBS polysaccharide-protein conjugate, a buffer, a surfactant, an excipient, and optionally an adjuvant, wherein the composition is buffered to a pH of about 6.0 to about 7.0 and wherein the capsular polysaccharide has a sialic acid level of greater than about 60%.

In one embodiment, the immunogenic composition comprises a GBS polysaccharide-protein conjugate, histidine, polysorbate-80, sodium chloride, and optionally aluminum phosphate, wherein the composition is buffered to a pH of about 6.0 to about 7.0 and wherein the capsular polysaccharide has a sialic acid level of greater than about 60%.

In another embodiment, the immunogenic composition comprises about 5 mcg/ml to about 50 mcg/ml of a GBS polysaccharide-protein conjugate, about 10 mM to about 25 mM of histidine, about 0.01% to about 0.03% (v/w) of polysorbate-80, about 10 mM to about 250 mM of sodium chloride, and optionally about 0.25 mg/ml to about 0.75 mg/ml of aluminum as aluminum phosphate, wherein the capsular polysaccharide has a sialic acid level of greater than about 60%.

In one such embodiment, the immunogenic composition comprises at least one GBS polysaccharide-protein conjugate, a buffer, a surfactant, an excipient, and optionally an adjuvant, wherein the composition is buffered to a pH of about 6.0 to about 7.

In one such embodiment, the immunogenic composition comprises at least two GBS polysaccharide-protein conjugates, a buffer, a surfactant, an excipient, and optionally an adjuvant, wherein the composition is buffered to a pH of about 6.0 to about 7.0 and wherein the conjugates comprise capsular polysaccharides from group B streptococcus (GBS) serotype VI and at least one additional serotype selected from the group consisting of Ia, Ib, II, III, IV, V, VII, VIII, and IX.

In a preferred embodiment, the immunogenic composition comprises about 5 mcg/ml to about 50 mcg/ml each of at least two GBS polysaccharide-protein conjugates, about 10 mM to about 25 mM of histidine, about 0.01% to about 0.03% (v/w) of polysorbate-80, about 10 mM to about 250 mM of sodium chloride, and optionally about 0.25 mg/ml to about 0.75 mg/ml of aluminum as aluminum phosphate, wherein the conjugates comprise capsular polysaccharides from group B streptococcus (GBS) serotype VI and at least one additional serotype selected from the group consisting of Ia, Ib, II, III,IV, V, VII, VIII, and IX.

Evaluation of Immunogenic Compositions

Various *in vitro* tests are used to assess the immunogenicity of the immunogenic compositions of the invention. For example, an *in vitro* opsonic assay is conducted by incubating together a mixture of streptococcal cells, heat inactivated serum containing specific antibodies to the antigens in question, and an exogenous complement source. Opsonophagocytosis proceeds during incubation of freshly isolated polymorphonuclear cells (PMN's) or differentiated effector cells such as HL60s and the antibody/complement/streptococcal cell mixture. Bacterial cells that are coated with antibody and complement are killed upon opsonophagocytosis. Colony forming units (cfu) of surviving bacteria that are recovered from opsonophagocytosis are determined by plating the assay mixture. Titers are reported as the reciprocal of the highest dilution that gives 50% bacterial killing, as determined by comparison to assay controls.

A whole cell ELISA assay may also be used to assess *in vitro* immunogenicity and surface exposure of the antigen, wherein the bacterial strain of interest (*S. agalactiae*) is coated onto a plate, such as a 96 well plate, and test sera from an immunized animal is reacted with the bacterial cells. If an antibody, specific for the test antigen, is reactive with a surface exposed epitope of the antigen, it can be detected by standard methods known to one skilled in the art. Alternatively, flow cytometry may be used to measure surface exposure of capsular polysaccharide antigens and specificity of antibodies, including monoclonal antibodies.

An antigen demonstrating the desired *in vitro* activity may then be tested in an *in vivo* animal challenge model. In certain embodiments, immunogenic compositions are used in the immunization of an animal (*e.g.*, a mouse) by methods and routes of immunization known to those of skill in the art (*e.g.*, intranasal, parenteral, oral, rectal, vaginal, transdermal, intraperitoneal, intravenous, subcutaneous, etc.). Following immunization of the animal with a GBS immunogenic composition, the animal is challenged with a *Streptococcus agalactiae* strain and assayed for resistance to the streptococcal infection.

In one embodiment, pathogen-free mice are immunized and challenged with *S. agalactiae*. For example, mice are immunized with one or more doses of the desired antigen in an immunogenic composition. Subsequently, the mice are challenged with *S. agalactiae* and survival is monitored over time post challenge.

Methods of Use

"Immunocompromised", as used herein, refers to a subject suffering from a deficiency with respect to the cellular and/or humoral arm(s) of the immune system. Accordingly, the extent of deficiency in immune function varying from slight impairment in the immune process to complete immune suppression is contemplated.

The term "subject" refers to a mammal, bird, fish, reptile, or any other animal. The term "subject" also includes humans. The term "subject" also includes household pets. Non limiting examples of household pets include: dogs, cats, pigs, rabbits, rats, mice, gerbils, hamsters, guinea pigs, ferrets, birds, snakes, lizards, fish, turtles, and frogs. The term "subject" also includes livestock animals. Non limiting examples of livestock animals include: alpaca, bison, camel, cattle, deer, pigs, horses, llamas, mules, donkeys, sheep, goats, rabbits, reindeer, yak, chickens, geese, and turkeys.

As used herein, "treatment" (including variations thereof, for example, "treat" or "treated") refers to any one or more of the following: (i) the prevention of infection or reinfection, as in a traditional vaccine, (ii) the reduction in the severity of or the elimination of symptoms, and (iii) the substantial or complete elimination of the pathogen or disorder in question. Hence, treatment may be effected prophylactically (prior to infection) or therapeutically (following infection). In the present invention, prophylactic or therapeutic treatments can be used. According to a particular embodiment of the present invention, compositions and methods are provided which treat, including prophylactically and/or therapeutically immunize, a host animal

against a microbial infection (*e.g.*, a bacterium such as *S. agalactiae*). The methods of the present invention are useful for conferring prophylactic and/or therapeutic immunity to a subject. The methods of the present invention can also be practiced on subjects for biomedical research applications.

In another aspect, the invention relates to a method of inducing an immune response against GBS in a subject by administering to the subject an effective amount of an immunogenic composition described herein. In one embodiment, the invention relates to a method of preventing or reducing a disease or condition associated with group B streptococcus in a subject by administering to the subject an effective amount of an immunogenic composition described herein. In an aspect, the invention relates to the immunogenic composition described herein for use as a medicament. In an aspect, the invention relates to the immunogenic composition described herein for use in a method of inducing an immune response against GBS in a subject. In a particular embodiment, the subject is a female planning to become pregnant or a pregnant female. In one such embodiment, the pregnant female is in her third trimester or second half of pregnancy, such as at least 20 weeks or at least 27 weeks gestation. In a preferred embodiment, the pregnant female is at 27 weeks to 36 weeks gestation. In another embodiment, the subject is an older adult, such as an adult 50 years of age or older, 65 years of age or older, and 85 years of age or older. In a further embodiment, the subject is immunocompromised. In one aspect, the subject may have a medical condition selected from the group consisting of obesity, diabetes, HIV infection, cancer, cardiovascular disease, or liver disease. In a preferred embodiment, the group B streptococcus is *S. agalactiae*.

An immunogenic or effective amount of an immunogenic composition can be determined by doing a dose response study in which subjects are immunized with gradually increasing amounts of the immunogenic composition and the immune response analyzed to determine the optimal dosage. Starting points for the study can be inferred from immunization data in animal models. The dosage amount can vary depending upon specific conditions of the individual. The amount can be determined in routine trials by means known to those skilled in the art.

An immunologically effective amount of the immunogenic composition in an appropriate number of doses is administered to the subject to elicit an immune response. The dosage amount can vary depending upon specific conditions of the individual, such as age and weight. This amount can be determined in routine trials by means known to those skilled in the art.

In one embodiment, patients being administered immunogenic compositions of the invention show a reduction in *S. agalactiae* carriage rates. Such reduction in carriage or a prolonged interval of time spent as a non-carrier following administration of an immunogenic composition is significant from a medical need perspective. For example, reduction in overall *S. agalactiae* carriage in carriers may be assessed following one dose of the immunogenic composition of the invention. For example, 1 day prior to administration of the immunogenic composition, a group of adults aged 18-50 years may be screened for carriage by nasal, throat,

axillary, rectal, perineal, and vaginal swabs followed by cultivation to determine their carriage state. Next, the group can be administered an immunogenic composition of the invention with a group receiving a control. Nasal, throat, axillary, rectal, perineal, and vaginal swabs performed weekly over a 12 week period, and monthly up to 6 months post administration of the immunogenic composition are performed and compared to placebo. One primary endpoint is to compare carriage rates in patients after administration of an immunogenic composition versus placebo at 3 month intervals post immunization.

Antibodies

An “antibody” is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule. As used herein, unless otherwise indicated by context, the term is intended to encompass not only intact polyclonal or monoclonal antibodies, but also engineered antibodies (*e.g.*, chimeric, humanized and/or derivatized to alter effector functions, stability and other biological activities) and fragments thereof (such as Fab, Fab’, F(ab’)2, Fv), single chain (ScFv) and domain antibodies, including shark and camelid antibodies), and fusion proteins comprising an antibody portion, multivalent antibodies, multispecific antibodies (*e.g.*, bispecific antibodies so long as they exhibit the desired biological activity) and antibody fragments as described herein, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site. An antibody includes an antibody of any class, such as IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant domain of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2 in humans. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Antibody fragments" comprise only a portion of an intact antibody, wherein the portion preferably retains at least one, preferably most or all, of the functions normally associated with that portion when present in an intact antibody.

"Functional activity" of an antibody or "functional antibody" as used herein refers to an antibody that, at a minimum, can bind specifically to an antigen. Additional functions are known in the art and may include additional components of the immune system that effect clearance or killing of the pathogen such as through opsonization, ADCC or complement-mediated cytotoxicity. After antigen binding, any subsequent antibody functions can be mediated through the Fc region of the antibody. The antibody opsonophagocytosis assay (OPA) is an *in vitro* assay designed to measure *in vitro* Ig complement-assisted killing of bacteria with effector cells

(white blood cells), thus mimicking a biological process. Antibody binding may also directly inhibit the biological function of the antigen it binds. In some embodiments, a “functional antibody” refers to an antibody that is functional as measured by the killing of bacteria in an animal efficacy model or an opsonophagocytic killing assay that demonstrates that the antibodies kill the bacteria.

In one aspect, the invention relates to an isolated antibody or fragment thereof that specifically binds to a polysaccharide described herein. An “isolated” antibody as used herein refers to an antibody that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In exemplary embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. An isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, an isolated antibody will be prepared by at least one purification step.

An antibody that “specifically binds to” or is “specific for” a particular polysaccharide or an epitope on a particular polysaccharide is one that binds to that particular polysaccharide or epitope on a particular polysaccharide without substantially binding to any other polysaccharide or polysaccharide epitope.

The word “label” when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody so as to generate a “labeled” antibody. The label may be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

The invention further provides antibodies and antibody compositions which bind specifically and selectively to one or more antigens of an immunogenic composition of the present invention. In some embodiments, antibodies are generated upon administration to a subject of an immunogenic composition of the present invention. In some embodiments, the invention provides purified or isolated antibodies directed against one or more of the antigens of an immunogenic composition of the present invention. In some embodiments, the antibodies of the present invention are functional as measured by killing bacteria in either an animal efficacy model or via an opsonophagocytic killing assay. In some embodiments, the antibodies of the invention confer passive immunity to a subject. The present invention further provides polynucleotide molecules encoding an antibody or antibody fragment of the invention, and a cell

or cell line (such as hybridoma cells or other engineered cell lines for recombinant production of antibodies) and a transgenic animal that produces an antibody or antibody composition of the invention, using techniques well-known to those of skill in the art.

Antibodies or antibody compositions of the invention may be used in a method of treating or preventing a streptococcal infection, disease or condition associated with *S. agalactiae* in a subject, the method comprising generating a polyclonal or monoclonal antibody preparation, and using said antibody or antibody composition to confer passive immunity to the subject. Antibodies of the invention may also be useful for diagnostic methods, *e.g.*, detecting the presence of or quantifying the levels of one or more antigens of the immunogenic compositions of the present invention.

Antibody responses to repeat structures such as a polysaccharide of the present invention may exhibit some unique features. For example, the regularity of the repeating units may mean that antigen molecules of vastly different molecular weights can bind to antibodies specific for the polysaccharide. Second, the repeat structures of the larger length polysaccharides are capable of inducing T-cell independent antibody responses. Therefore, when using polysaccharides conjugated to protein carriers having T-cell helper epitopes, both T-cell independent and T-cell dependent antibody responses can be stimulated. Therefore, immune response can be modified by appropriate selection of polysaccharide size and whether or not a carrier protein is used.

Polyclonal Antibodies

In certain embodiments, the anti-polysaccharide antibody is a polyclonal antibody. Polyclonal antibodies, as defined herein, refers to a mixture of antibodies having differing specificities derived from a preparation of serum and originating from different B-cell clones. The preparation and characterization of polyclonal antibodies are known in the art.

Polyclonal antibodies are raised in a subject, for example in a mammal, by administering one or more injections of an immunogen or immunogenic composition described herein and, if desired, an adjuvant, buffer, and/or diluent. A range of animal species may be used for the production of specific antisera. Typically an animal used for production of anti-saccharide polyclonal antisera is a nonhuman primate, a goat, a sheep, a rabbit, a mouse, a rat, a hamster or a guinea pig. Typically, the immunogen or immunogenic composition with or without the adjuvant is injected in the mammal by multiple injections. The immunogenic material may include a polysaccharide, oligosaccharide, polysaccharide, polysaccharide-protein conjugate described herein, or a larger assembly of immunogens. Typically, beginning 2-6 weeks after the first immunization, blood is collected from the immunized animal, allowed to clot and serum is harvested. The serum contains the anti-saccharide polyclonal antibodies from the immunized animal and is often referred to as antisera.

Monoclonal Antibodies

An anti-saccharide monoclonal antibody may be prepared through use of known hybridoma techniques. Typically, preparing monoclonal antibodies involves first immunizing a suitable target animal host with a selected immunogen comprising a polysaccharide, oligosaccharide, polysaccharide or polysaccharide-protein conjugate of the present invention. If desired, an adjuvant, buffer, and/or diluents may be included. The immunization is conducted in a manner sufficient to elicit B lymphocytes to produce or express antibodies that specifically bind to the polysaccharide or conjugate thereof. Alternatively, the lymphocytes are immunized *in vitro*.

The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. The source of the lymphocytes determines whether the monoclonal antibodies are of human or animal origin. In general, peripheral blood lymphocytes ("PBLs") are used if antibodies and cells of human origin are desired, and spleen cells or lymph node cells are used if non-human mammalian sources are desired.

Immortalized cell lines are typically transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells are cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Immortalized cell lines are chosen for practical considerations such as species of origin, fusion and growth characteristics. For example, suitable immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Examples of immortalized cell lines include: murine myeloma lines. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies.

The monoclonal antibody is secreted into the culture medium by the hybridoma cells. The culture medium is then assayed for the presence of monoclonal antibodies that recognize and bind the polysaccharide. The anti-polysaccharide binding specificity of particular monoclonal antibodies produced by the hybridoma cells is determined by one of numerous procedures that are well known in the art. For example, antibody binding specificity may be determined by immunoprecipitation, radioimmunoassay (RIA), western blot, enzyme-linked immunoabsorbent assay (ELISA) or surface plasmon resonance (*e.g.*, Biacore). The precise epitope recognized by the monoclonal antibody is determined by epitope mapping. Such techniques and assays are well known in the art.

After hybridoma cells producing antibodies with the desired specificity are identified, the clones are subcloned by limiting dilution and cultured using standard methods. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells are grown *in vivo* as ascites in a mammal. The monoclonal antibodies secreted by the subclones are isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

Alternatively, antibodies having the desired specificity and from the desired species of origin can be obtained through the use of phage display libraries. Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in the art.

Uses of Antibodies

In one aspect, the invention relates to use of an immunogenic composition described herein for producing a GBS antibody and/or antibody fragment. The polysaccharide-protein conjugates described herein and/or antibodies generated therefrom may be used in a variety of immunodiagnostic techniques known to those of skill in the art, including ELISA- and microarray-related technologies. In addition, these reagents may be used to evaluate antibody responses, including serum antibody levels, for example, to immunogenic polysaccharide conjugates. The assay methodologies of the invention may involve the use of labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, and/or secondary immunologic reagents for direct or indirect detection of a complex between an antigen or antibody in a biological sample and a corresponding antibody or antigen bound to a solid support.

The antibody or antibody fragment produced may also be useful in passive immunotherapy or for prophylaxis against a streptococcal infection.

Method of Producing a polysaccharide

In yet another aspect, the invention relates to a method for producing the polysaccharides described herein. The method includes culturing a GBS and collecting the polysaccharide produced by the bacterium. In one embodiment, the GBS includes *S. agalactiae*. The bacterium may be any strain of *S. agalactiae*. In a preferred embodiment, the bacterium is an encapsulated strain of *S. agalactiae*. *S. agalactiae* strains for use in the present invention include 090, A909 (ATCC Accession No. BAA-1138), 515 (ATCC Accession No. BAA-1177), B523, CJB524, MB 4052 (ATCC Accession No. 31574), H36B (ATCC Accession No. 12401), S40, S42, MB 4053 (ATCC Accession No. 31575), M709, 133, 7357, PFEGBST0267, MB 4055 (ATCC Accession No. 31576), 18RS21 (ATCC Accession No. BAA-1175), S16, S20, V8 (ATCC Accession No. 12973), DK21, DK23, UAB, 5401, PFEGBST0708, MB 4082 (ATCC Accession No. 31577), M132, 110, M781 (ATCC Accession No. BAA-22), D136C(3) (ATCC

Accession No. 12403), M782, S23, 120, MB 4316 (M-732; ATCC Accession No. 31475), M132, K79, COH1 (ATCC Accession No. BAA-1176), PFEGBST0563, 3139 (ATCC Accession No. 49446), CZ-NI-016, PFEGBST0961, 1169-NT1, CJB111 (ATCC Accession No. BAA-23), CJB112, 2603 V/R (ATCC Accession No. BAA-611), NCTC 10/81, CJ11, PFEGBST0837, 118754, 114852, 114862, 114866, 118775, B 4589, B 4645, SS1214, CZ-PW-119, 7271, CZ-PW-045, JM9130013, JM9130672, IT-NI-016, IT-PW-62, and IT-PW-64.

A polysaccharide described herein may be produced by culturing the GBS in an appropriate medium. An appropriate medium may include Columbia broth. The medium may include dextrose, hemin, and/or glucose. In an embodiment, the medium includes Columbia broth and dextrose. If *S. agalactiae* is cultured using Columbia broth and dextrose, preferably the temperature for culture is 20 to 40 °C, preferably 37 °C. In a preferred embodiment, the bacterium is cultured under aerobic conditions. In another preferred embodiment, the bacterium is cultured for 12 to 60 hours.

A polysaccharide may be collected from the obtained culture by using a method known in the art to collect a target substance from a culture, such as, for example, heating, enzyme treatment, centrifugation, precipitation, treatment with activated carbon, and/or filtration. (See, for example, U.S. Patent Appl. Pub. Nos. 2006/0228380, 2006/0228381, 2007/0184071, 2007/0184072, 2007/0231340, and 2008/0102498; Int'l Patent Appl. Pub. No. WO 2008/118752). In one embodiment, the culture containing the bacterium and polysaccharide is centrifuged and treated with an enzyme, such as, for example, lysozyme, RNase, DNase, Pronase, mutanolysin, and combinations thereof. For example, in one embodiment, an appropriate organic solvent is added to the obtained supernatant to precipitate proteins, and the precipitate is removed by centrifugation. Then a polysaccharide may be precipitated by further adding an appropriate organic solvent to the supernatant, and the polysaccharide may be collected by centrifugation. More specifically, a polysaccharide described herein may be obtained by adding ethanol at a final concentration of about 25 volume % to the supernatant from which the bacterium has been removed, removing a precipitation that contains protein by centrifugation, further adding ethanol to a final concentration of about 75 volume % thereto, and then collecting a precipitate by centrifugation. The resulting precipitate may be dried with nitrogen. The resulting precipitate may be resuspended in Tris and 0.05% Na azide.

A further aspect of the invention provides a novel method, using organic reagents such as derivatized hydroxyl amine compounds, for the isolation of largely intact high molecular weight CPs while preserving N- and O-acetyl groups. Since this method does not lyse the cells, the CPs isolated by centrifugation is minimally contaminated with intracellular components and may lead to higher overall yield. Moreover, these reagents cleave the group B antigen impurity to very small fragments due to its multiple phospodiester linkages, which can be easily removed by diafiltration.

In one embodiment, the CP is isolated by reacting a hydroxyl amine with a cell paste comprising a capsular polysaccharide producing bacterium. In an embodiment, the method further comprises the step of centrifuging. In another embodiment, the method further comprises the step of filtering.

In an aspect of the invention, the hydroxyl amine may be those listed in Table 2 in Example 2. In a preferred embodiment, the hydroxyl amine is selected from the group consisting of dibenzyl hydroxylamine; diethyl hydroxylamine; hydroxylamine; ethylenediamine; triethylenetetramine; 1,1,4,7,10,10 hexamethyl triethylene tetramine; and 2,6,10,Trimethyl 2,6,10 triazaundecane.

In one aspect of the invention, the concentration of hydroxyl amine is about 5 mM to about 200 mM, such as about 5 mM to about 150 mM, about 5 mM to about 100 mM, about 5 mM to about 75 mM, about 5 mM to about 50 mM, about 5 mM to about 25 mM, about 5 mM to about 10 mM, 10 mM to about 200 mM, such as about 10 mM to about 150 mM, about 10 mM to about 100 mM, about 10 mM to about 75 mM, about 10 mM to about 50 mM, about 10 mM to about 25 mM, about 25 mM to about 200 mM, about 25 mM to about 150 mM, about 25 mM to about 100 mM, about 25 mM to about 75 mM, about 25 mM to about 50 mM, about 50 mM to about 200 mM, about 50 mM to about 150 mM, 50 mM to about 100 mM, and about 50 mM to about 75 mM.

In another aspect, the pH of the reaction is maintained at about 5.5 to about 9.5, such as about 5.5 to about 9.0, about 5.5 to about 8.5, about 5.5 to about 8.0, about 5.5 to about 7.5, about 5.5 to about 7.0, about 5.5 to about 6.5, about 6.0 to about 9.5, about 6.0 to about 9.0, about 6.0 to about 8.5, about 6.0 to about 8.0, about 6.0 to about 7.5, about 6.0 to about 7.0, about 6.5 to about 9.5, about 6.5 to about 8.5, about 6.5 to about 8.0, about 6.5 to about 7.5, about 7.0 to about 9.5, about 7.0 to about 9.0, 7.0 to about 8.5, and about 7.0 to about 8.0.

In a further aspect of the invention, the reaction takes place at a temperature of about 20°C to about 85°C, such as about 20°C to about 80°C, about 20°C to about 75°C, about 20°C to about 70°C, about 20°C to about 65°C, about 20°C to about 60°C, about 20°C to about 55°C, about 20°C to about 50°C, about 25°C to about 85°C, about 25°C to about 80°C, about 25°C to about 75°C, about 25°C to about 70°C, about 25°C to about 65°C, about 25°C to about 60°C, about 25°C to about 55°C, about 25°C to about 50°C, about 30°C to about 85°C, about 30°C to about 80°C, about 30°C to about 75°C, about 30°C to about 70°C, about 30°C to about 65°C, about 30°C to about 60°C, about 30°C to about 55°C, about 30°C to about 50°C, about 35°C to about 85°C, about 35°C to about 80°C, about 35°C to about 75°C, about 35°C to about 70°C, about 35°C to about 65°C, about 35°C to about 60°C, about 35°C to about 55°C, about 40°C to about 85°C, about 40°C to about 80°C, about 40°C to about 75°C, about 40°C to about 70°C, about 40°C to about 65°C, about 40°C to about 60°C, about 45°C to about 85°C, about 45°C to about 80°C, about 45°C to about 75°C, about 45°C to about 70°C, about 45°C to about 65°C, about 50°C to about 85°C, about 50°C to about 80°C, about 50°C to about 75°C, about 50°C to

about 70°C, about 55°C to about 85°C, about 55°C to about 80°C, about 55°C to about 75°C, about 60°C to about 85°C, and about 65°C to about 85°C.

In yet another aspect, the reaction time is about 10 hours to about 90 hours, such as about 10 hours to about 85 hours, about 10 hours to about 80 hours, about 10 hours to about 75 hours, about 10 hours to about 70 hours, about 10 hours to about 60 hours, about 10 hours to about 50 hours, about 10 hours to about 40 hours, about 10 hours to about 30 hours, about 10 hours to about 25 hours, about 10 hours to about 20 hours, about 10 hours to about 15 hours, about 15 hours to about 90 hours, about 15 hours to about 85 hours, about 15 hours to about 80 hours, about 15 hours to about 75 hours, about 15 hours to about 70 hours, about 15 hours to about 60 hours, about 15 hours to about 50 hours, about 15 hours to about 40 hours, about 15 hours to about 30 hours, 15 hours to about 20 hours, such as about 20 hours to about 90 hours, about 20 hours to about 85 hours, about 20 hours to about 80 hours, about 20 hours to about 75 hours, about 20 hours to about 70 hours, about 20 hours to about 60 hours, about 20 hours to about 50 hours, about 20 hours to about 40 hours, about 20 hours to about 30 hours, and about 20 hours to about 25 hours.

Alternatively, in another embodiment of the invention, the polysaccharide is chemically synthesized. The polysaccharide may be chemically synthesized according to conventional methods.

In yet another embodiment of the invention the polysaccharide is prepared by expression in a surrogate host after cloning and expressing a biosynthetic pathway to produce the polysaccharide. For example, a host cell may be modified to produce a polysaccharide having structural similarity to a polysaccharide described herein, wherein a repeating unit of the polysaccharide produced in the host cell is partially identical to a repeating unit of a polysaccharide described herein. A polysaccharide is structurally similar to a polysaccharide described herein if, for example, a repeating unit of the polysaccharide has a missing branch, is heterogeneous in size and/or is heterogeneous in branching arrangement, as compared to a repeating unit of a polysaccharide described herein. Preferably, the host cell is a bacterial host cell.

EXAMPLES

The following examples demonstrate some embodiments of the present invention. However, it is to be understood that these examples are for illustration only and do not purport to be wholly definitive as to conditions and scope of this invention. It should be appreciated that when typical reaction conditions (*e.g.*, temperature, reaction times, etc.) have been given, the conditions both above and below the specified ranges can also be used, though generally less conveniently. All parts and percents referred to herein are on a weight basis and all temperatures are expressed in degrees centigrade unless otherwise specified.

Furthermore, the following examples were carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. As noted above, the following examples are presented for illustrative purpose, and should not be construed in any way limiting the scope of this invention.

Example 1. Preparation of Polysaccharide-Protein Conjugates with De-O-Acetylated Polysaccharides

S. agalactiae strains for respective serotypes were fermented in submerged culture with pH-control in a defined medium. The procedures and media used were optimized through experimentation and were extensions of basic techniques previously described in von Hunolstein, C. et al., Appl. Micro. Biotech. 38(4):458-462 (1993). The capsular polysaccharide was removed from the cells by NaOH treatment. After clarification, a series of UF/DF, precipitation, and carbon filtration steps afforded the purified polysaccharide. *See, e.g.*, U.S. Patent No. 8,652,480 Reductive amination chemistry was used to conjugate the activated polysaccharide to CRM_{197}. *See, e.g.*, U.S. Patent No. 5,360,897.

Example 2. Conjugation of GBS Capsular Polysaccharides by Reductive Amination Activating Polysaccharide

Polysaccharide oxidation was carried out in 100 mM potassium phosphate buffer (pH 6.0 ± 0.5) by sequential addition of calculated amount of 500 mM potassium phosphate buffer (pH 6.0) and water for injection (WFI) to give final polysaccharide concentration of 2.0 g/L. If required, the reaction pH was adjusted to pH 6.0, approximately. After pH adjustment, the reaction temperature was adjusted to 23 °C. Oxidation was initiated by the addition of approximately 0.25 molar equivalents of sodium periodate. The oxidation reaction was performed at 5 ± 3 °C during 16 hrs, approximately.

Concentration and diafiltration of the activated polysaccharide was carried out using 5K MWCO ultrafiltration cassettes. Diafiltration was performed against 20-fold diavolumes of WFI. The purified activated polysaccharide was then stored at 5 ± 3°C. The purified activated saccharide is characterized, *inter alia*, by (i) saccharide concentration by colorimetric assay; (ii) aldehyde concentration by colorimetric assay; (iii) degree of oxidation; and (iv) molecular weight by SEC-MALLS.

The degree of oxidation (DO = moles of sugar repeat unit / moles of aldehyde) of the activated polysaccharide was determined as follows:

The moles of sugar repeat unit are determined by various colorimetric methods, for example, by using the Anthrone method. By the Anthrone method, the polysaccharide is first broken down to monosaccharides by the action of sulfuric acid and heat. The Anthrone reagent reacts with the hexoses to form a yellow-green colored complex whose absorbance is read

spectrophotometrically at 625nm. Within the range of the assay, the absorbance is directly proportional to the amount of hexose present.

The moles of aldehyde are also determined simultaneously, using the MBTH colorimetric method. The MBTH assay involves the formation of an azine compound by reacting aldehyde groups (from a given sample) with a 3-methyl-2-benzothiazolone hydrazone (MBTH assay reagent). The excess 3-methyl-2-benzothiazolone hydrazone oxidizes to form a reactive cation. The reactive cation and the azine react to form a blue chromophore. The formed chromophore is then read spectroscopically at 650 nm.

Compounding Activated Polysaccharide with Sucrose Excipient, and Lyophilizing

The activated polysaccharide was compounded with sucrose to a ratio of 25 grams of sucrose per gram of activated polysaccharide. The bottle of compounded mixture was then lyophilized. Following lyophilization, bottles containing lyophilized activated polysaccharide were stored at -20 ± 5°C. Calculated amount of CRM_{197} protein was shell-frozen and lyophilized separately. Lyophilized CRM_{197} was stored at -20 ± 5°C.

Reconstituting Lyophilized Activated Polysaccharide and Carrier Protein

Lyophilized activated polysaccharide was reconstituted in anhydrous dimethyl sulfoxide (DMSO). Upon complete dissolution of polysaccharide, an equal amount of anhydrous DMSO was added to lyophilized CRM_{197} for reconstitution.

Conjugating and Capping

Reconstituted activated polysaccharide was combined with reconstituted CRM_{197} in the reaction vessel, followed by mixing thoroughly to obtain a clear solution before initiating the conjugation with sodium cyanoborohydride. The final polysaccharide concentration in reaction solution was approximately 1 g/L. Conjugation was initiated by adding 1.0 – 1.5 MEq of sodium cyanoborohydride to the reaction mixture and incubating at 23 ± 2 °C for 20-48 hrs. The conjugation reaction was terminated by adding 2 MEq of sodium borohydride ($NaBH_4$) to cap unreacted aldehydes. This capping reaction continued at 23 ± 2°C for 3 ± 1 hrs.

Purifying the Conjugate

The conjugate solution was diluted 1:10 with chilled 5 mM succinate-0.9% saline (pH 6.0) in preparation for purification by tangential flow filtration using 100-300K MWCO membranes.

The diluted conjugate solution was passed through a 5 µm filter, and diafiltration was performed using 5 mM succinate / 0.9% saline (pH 6.0) as the medium. After the diafiltration was completed, the conjugate retentate was transferred through a 0.22µm filter. The conjugate was diluted further with 5 mM succinate / 0.9% saline (pH 6), to a target saccharide concentration of approximately 0.5 mg/mL. Alternatively, the conjugate is purified using 20 mM Histidine-0.9% saline (pH 6.5) by tangential flow filtration using 100-300K MWCO membranes. Final 0.22µm filtration step was completed to obtain the immunogenic conjugate.

Example 3. Conjugation of GBS Capsular Polysaccharides by CDAP (1-Cyano-4-Dimethylaminopyridinium tetrafluoroborate)

The GBS polysaccharide was dialyzed against WFI to remove buffer salt and lyophilized. The lyophilized polysaccharide (100 mg) was dissolved in WFI (4-5 mg/mL) and activated with CDAP (100 mg; 100 mg/mL in acetonitrile: water, 9:1) for about 30 seconds. After activation, 0.2M Triethylamine (4 mL) was added and stirred for about 2.5 minutes followed by addition of tetanus toxoid (TT) (150 mg, 3 mg/mL in saline). The pH of the reaction was adjusted to 8.8 and stirred at room temperature for 24 hours. The conjugation reaction was quenched with 2M glycine, conjugate was passed through a 5 µm filter and purified using saline by tangential flow filtration using 100K MWCO membrane. Final 0.22µm filtration step was completed to obtain the immunogenic conjugate.

Example 4: Effects of Varying Conjugation Conditions on GBS Polysaccharide-CRM_{197} Conjugates

GBS serotypes VI, VII, VIII and IX conjugates may be generated by deliberately varying periodate oxidation/reductive amination chemistry (PO/RAC) conditions, including the solvent for the reagent (aqueous medium versus DMSO), varying levels of sialic acid in the initial polysaccharide, and degree of oxidation/saccharide epitope modification. In general, conjugates produced using DMSO as the solvent are found to have lower levels of unreacted (free) polysaccharide, higher conjugate molecular weight, and higher saccharide/protein ratios than conjugates produced using aqueous medium.

A conjugation process that produces conjugates with lower levels of unreacted (free) polysaccharide is advantageous and preferable. It is well known that high levels of unreacted (free) polysaccharide may cause an excessive T-cell independent immune response, which has the potential to dilute the T-cell dependent response generated by the polysaccharide-protein conjugate, thereby lowering the immunogenic response generated by the conjugate.

GBS polysaccharides may be chemically desialylated by methods known in the art (see Chaffin, D.O, et al., J Bacteriol 187(13):4615-4626 (2005)) to generate conjugate variants to determine the impact of % desialylation on immunogenicity. In general, desialylation of more than about 40% (i.e. sialic acid levels less than about 60%) has a negative impact on immunogenicity.

Similarly in most cases, a degree of oxidation of less than about 5, or saccharide epitope modification greater than about 20%, has a negative impact on immunogenicity. Since oxidation occurs through the sialic acid on the capsular polysaccharide, it is found that saccharide epitope modification greater than about 20% reduces the sialic acid content, which results in reduced immunogenicity.

Conversely, conjugates having a variety of saccharide/protein ratio or polysaccharide molecular weight are found to produce an immunogenic response in mice, indicating a relatively broad range of acceptance criteria with regard to these attributes.

Additional conjugate variants may be generated using alternative chemistry routes. One alternative chemistry includes generating conjugates by reacting the polysaccharide with carbonylditriazole (CDT), and carrying out the conjugation reaction in DMSO. In another alternative chemistry, conjugates may be generated by oxidation of the polysaccharide using TEMPO [(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl] reagent (instead of sodium periodate) followed by conjugation using reductive amination chemistry (TEMPO/RAC) in DMSO, as detailed in Examples above. Conjugates generated by these alternative chemistries are found to be immunogenic in mice, indicating the suitability of alternative chemistry routes besides PO/RAC. However, some conjugation chemistries may perform better with some serotypes than others.

OPAs were performed as per Buurman, E.T., et al., J. Infect. Dis., Jun 5;220(1):105-115 (2019).

Example 5: Monovalent Conjugate Vaccines Produced Functional and Binding Antibody Responses in Rabbits

Rabbits (4-5 per group) were vaccinated at 0, 3, and 6 weeks with 20 mcg based on polysaccharide weight of monovalent conjugates of GBS CPS serotypes VI, VII, VIII or IX conjugated to different carrier proteins (CRM197, tetanus toxoid (TT), SCP) formulated with 20 mcg of QS21 per conjugate dose. Sera were assessed at baseline and at week 10 for anti-CPS IgG titers by direct binding Luminex immunoassay (dLIA) with CPS coated microspheres.

FIG. 1 shows the average binding activity profile of serially diluted sera from two rabbits administered the GBS CPS serotype VI-CRM197 conjugate. Interpolated EC50 serum dilution titer for GBS CPS VI shown in FIG. 1 was determined using a sigmoidal dose response curve fit (variable slope) (Graphpad Prism). Table 1 shows antibody titers generated for GBS CPS serotype VI conjugated to CRM197, SCP and TT. Immunization with GBS CPS serotype VI conjugated to CRM197 generated measurable antibody titers that were specific for serotype VI; negligible binding was seen for serotype VI induced sera to other serotype when assessed by direct binding Luminex immunoassay.

Table 1. IgG Direct Binding Luminex Titers Post-dose Two (Week 10)

Conjugate	rabbits	GMT (EC_{50})[1]	Responders[2]
GBS-VI CRM	5	1517	5/5
GBS-VI SCP	5	711	4/5
GBS-VI TT	4	148	3/4

[1]Geometric mean EC50 serum dilution titer (non-responders not included)
[2]Rabbits with serum titers $\leq$50 counted as non-responders

As shown in FIG. 2A-2C and Table 2, conjugates of GBS CPS serotypes VII, VIII or IX conjugated to TT similarly induced robust IgG antibody titers against the cognate polysaccharide following immunization.

Table 2. Serotype VII, VIII and IX Conjugate Immunogenicity

Conjugate	rabbits	GMT (EC_{50})[1]	Responders[2]
GBS-VII TT	4	831	4/4
GBS-VIII TT	4	63	2/4
GBS-IX TT	4	941	4/4

[1]Geometric mean EC50 serum dilution titer (non-responders not included)
[2]Rabbits with serum titers $\leq$50 counted as non-responders

FIG. 3A and 3B show the average binding activity profile of serially diluted sera from two rabbits administered the GBS CPS VII-TT conjugate and GBS CPS IX-TT conjugate, respectively. Cross-reactivity was seen for GBS CPS VII-TT conjugate induced antibodies against GBS CPS serotypes V and IX antigen, and for GBS CPS IX-TT conjugate induced antibodies against GBS CPS VI and VII antigens. Interpolated EC50 serum dilution titers indicated in FIG 3A (GBS CPS V, VII and IX) and 3B (GBS CPS VI, VII and IX) were determined using a sigmoidal dose response curve fit (variable slope) (Graphpad Prism).

GBS CPS serotype VI conjugates with CRM197 or C5a peptidase (SCP) as the carrier protein were further assessed for induction of functional bactericidal antibodies that could kill by opsonophagocytic uptake (OPA). Rabbits were immunized with GBS CPS VI conjugated to CRM197 at 0, 3 and 6 weeks, or with CPS VI conjugated to SCP at 0, 3, 6, 13 weeks. Sera from rabbits immunized with GBS VI-CRM197 or GBS VI-SCP, taken at weeks 13 and 16 respectively, were assessed for OPA titers with HL-60 phagocytic cells (Table 3).

Table 3. GBS VI-CRM197 and GBS VI-SCP Cross-Protective OPA Killing of Heterologous Serotypes

	GBS VI-CRM197 antisera			GBS VI-SCP antisera		
Serotype	**Wk 0**	**Wk 13**	**Wk 13+SCP**	**Wk 0**	**Wk 16**	**Wk 16+SCP**
III	NA	131	249	5	522	5
VIII	26	38	25	8	213	5
IX	NA	33	123	12	494	5
VI	5	10075	8510	5	1771	1533

Sera from rabbits immunized with GBS VI-CRM197 or VI:SCP demonstrated killing activity by OPA with target strains expressing serotype VI capsule. Preadsorption of GBS VI-SCP-induced sera with soluble SCP protein prior to OPA analysis marginally reduced the observed titer, which demonstrates specificity. Negligible cross-reactive killing by GBS VI-

CRM197-induced sera was seen for target strains expressing heterologous serotypes III, VIII or IX. By comparison, sera from serotype GBS VI-SCP immunized rabbits demonstrated measurable titers against strains expressing heterologous serotypes III, VIII or IX. Adsorption of SCP-specific antibodies with soluble SCP effectively lowered observed titers supporting the capacity of anti-SCP antibodies to effect functional activity across isolates in the context of diverse capsule types.

These results indicate that functional activity by anti-CPS antibodies is largely confined to the homologous serotype, and that anti-SCP carrier antibodies can impart a moderate but broad level of CPS-independent cross-functional activity among diverse isolates expressing different CPS types.

Example 6: Multivalent Conjugate Vaccines Produced Binding Antibody Responses in Rabbits

A single rabbit was vaccinated with CRM197 conjugates of serotypes Ia, Ib, II, III, IV, V and VI using doses of 20 mcg of each antigen and 20 mcg of QS21 adjuvant administered at weeks 0, 3, 6 and 9. IgG titers in sera were assessed at week 11 using the Luminex assay described in Example 5. Three-fold serial dilutions of the sera were tested starting at 1:15.

FIG. 4 shows the serum responses for the seven serotype Ia, Ib, II, III, IV, V and VI-CRM197 conjugates administered (open symbols) and three serotypes absent (VII, VIII, IX) from the formulation (closed symbols). The results demonstrate that a seven-valent GBS glycoconjugate vaccine that includes emerging serotype VI can induce antigen-specific IgG antibodies to all seven capsular polysaccharide components. Responses to the serotype VII and IX polysaccharides can be attributed to the cross-reactivity of antibodies to the serotype V antigen, which shares a common epitope within the branched sialylated side chain of the repeat unit (Berti, F., et al. (2014) JBC 289:34 23437-23448).

Aspects of the Invention

The following clauses describe additional embodiments of the invention.

C1. An immunogenic polysaccharide-protein conjugate comprising a group B streptococcus (GBS) capsular polysaccharide and a carrier protein, wherein the capsular polysaccharide has a sialic acid level of greater than about 60%.

C2. The immunogenic conjugate of C1, wherein the capsular polysaccharide is selected from the group consisting of serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX.

C3. The immunogenic conjugate of C2, wherein the capsular polysaccharide is serotype VI.

C4. The immunogenic conjugate of C2, wherein the capsular polysaccharide is serotype VII.

C5. The immunogenic conjugate of C2, wherein the capsular polysaccharide is serotype VIII.

C6. The immunogenic conjugate of C2, wherein the capsular polysaccharide is serotype IX.

C7. The immunogenic conjugate of any one of C1-C6, wherein the capsular polysaccharide has a sialic acid level of greater than about 95%.

C8. The immunogenic conjugate of any one of C1-C7, wherein the capsular polysaccharide has a sialic acid level of about 100%.

C9. The immunogenic conjugate of any one of C1-C8, wherein the capsular polysaccharides has at least about 0.6 mM sialic acid per mM of polysaccharide.

C10. The immunogenic conjugate of any one of C1-C9, wherein the capsular polysaccharides has at least about 0.65 mM sialic acid per mM of polysaccharide.

C11. The immunogenic conjugate of any one of C1-C10, wherein the capsular polysaccharides has at least about 0.7 mM sialic acid per mM of polysaccharide.

C12. The immunogenic conjugate of any one of C1-C11, wherein the capsular polysaccharides has at least about 0.75 mM sialic acid per mM of polysaccharide.

C13. The immunogenic conjugate of any one of C1-C12, wherein the capsular polysaccharides has at least about 0.8 mM sialic acid per mM of polysaccharide

C14. The immunogenic conjugate of any one of C1-C13, wherein the capsular polysaccharides has at least about 0.85 mM sialic acid per mM of polysaccharide.

C15. The immunogenic conjugate of any one of C1-C14, wherein the capsular polysaccharides has at least about 0.9 mM sialic acid per mM of polysaccharide .

C16. The immunogenic conjugate of any one of C1-C15, wherein the capsular polysaccharides has at least about 0.95 mM sialic acid per mM of polysaccharide.

C17. The immunogenic conjugate of any one of C1-C16, wherein the capsular polysaccharide has a molecular weight of between about 5 kDa and about 1,000 kDa.

C18. The immunogenic conjugate of any one of C1-C17, wherein the capsular polysaccharide has a molecular weight of between about 25 kDa and about 750 kDa.

C19. The immunogenic conjugate of any one of C1-C18, wherein the capsular polysaccharide has a molecular weight of between about 25 kDa and about 400 kDa.

C20. The immunogenic conjugate of any one of C1-C19, wherein the capsular polysaccharide has a molecular weight of between about 25 kDa and about 200 kDa.

C21. The immunogenic conjugate of any one of C1-C20, wherein the capsular polysaccharide has a molecular weight of between about 100 kDa and about 400 kDa.

C22. The immunogenic conjugate of any one of C1-C21, wherein the molecular weight of the conjugate is between about 300 kDa and about 20,000 kDa.

C23. The immunogenic conjugate of any one of C1-C22, wherein the molecular weight of the conjugate is between about 1,000 kDa and about 15,000 kDa.

C24. The immunogenic conjugate of any one of C1-C28, wherein the molecular weight of the conjugate is between about 1,000 kDa and about 10,000 kDa.

C25. The immunogenic conjugate of any one of C1-C24, wherein the polysaccharides are each individually conjugated to the carrier protein.

C26. The immunogenic conjugate of any one of C1-C25, wherein the carrier protein is CRM_{197} or tetanus toxoid.

C27. The immunogenic conjugate of any one of C1-C26, wherein the carrier protein is CRM_{197}.

C28. A method of isolating a capsular polysaccharide comprising reacting an organic reagent with a cell broth comprising a capsular polysaccharide producing bacterium.

C29. The method of C28, wherein the bacterium is not lysed.

C30. The method of C28 or C29, wherein the bacterium is heat killed.

C31. The method of any one of C28-C30, wherein the method further comprises the step of centrifuging to provide a cell paste.

C32. The method of any one of C28-C31, wherein the method further comprises the step of filtering.

C33. The method of C32, wherein said filtering step is a diafiltration.

C34. The method of C28, wherein the bacterium is *Streptococcus agalactiae.*

C35. An immunogenic composition comprising the immunogenic polysaccharide-protein conjugate of any one of C1-C27.

C36. An immunogenic composition comprising a polysaccharide-protein conjugate, wherein the conjugate comprises capsular polysaccharide from group B streptococcus (GBS) serotype VI conjugated to a carrier protein.

C37. An immunogenic composition comprising one or more polysaccharide-protein conjugates, wherein the conjugates comprise capsular polysaccharides from group B streptococcus (GBS) serotype VI and at least one additional serotype selected from the group consisting of Ia, Ib, II, III, IV, V, VII, VIII, and IX.

C38. The immunogenic composition of C37, wherein the at least one additional serotype is Ia.

C39. The immunogenic composition of C38, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype Ib.

C40. The immunogenic composition of C38 or C39, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype II.

C41. The immunogenic composition of any one of C38 to C40, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype III.

C42. The immunogenic composition of any one of C38-C41, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype IV.

C43. The immunogenic composition of any one of C38-C42, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype V.

C44. The immunogenic composition of any one of C38-C43, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype VII.

C45. The immunogenic composition of any one of C38-C44, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype VIII.

C46. The immunogenic composition of any one of C38-C45, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype IX.

C47. The immunogenic composition of C36, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype VII.

C48. The immunogenic composition of C36 or C47, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype VIII.

C49. The immunogenic composition of any one of C46-C48, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype IX.

C50. The immunogenic composition of C37, wherein the at least one additional serotype is VII.

C51. The immunogenic composition of C50, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype VIII.

C52. The immunogenic composition of any one of C50 or C51, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype IX.

C53. The immunogenic composition of C38, wherein the at least one additional serotype is VIII.

C54. The immunogenic composition of C53, wherein the composition further comprises a conjugate comprising a capsular polysaccharide from GBS serotype IX.

C55. The immunogenic composition of C54, wherein the at least one additional serotype is IX.

C56. An immunogenic composition comprising polysaccharide-protein conjugates comprising at least four GBS capsular polysaccharide serotypes selected from the group consisting of Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX.

C57. The immunogenic composition of C56, wherein the composition comprises at least two GBS capsular polysaccharide serotypes.

C58. The immunogenic composition of C56, wherein the composition comprises at least three GBS capsular polysaccharide serotypes.

C59. The immunogenic composition of C56, wherein the composition comprises at least four GBS capsular polysaccharide serotypes.

C60. The immunogenic composition of C56, wherein the composition comprises at least five GBS capsular polysaccharide serotypes.

C61. The immunogenic composition of C56, wherein the composition comprises at least six GBS capsular polysaccharide serotypes.

C62. The immunogenic composition of C56, wherein the composition comprises at least seven GBS capsular polysaccharide serotypes.

C63. The immunogenic composition of C56, wherein the composition comprises at least eight GBS capsular polysaccharide serotypes.

C64. The immunogenic composition of C56, wherein the composition comprises at least nine GBS capsular polysaccharide serotypes.

C65. The immunogenic composition of any one of C35-C64, wherein the composition further comprises a pharmaceutically acceptable excipient, buffer, stabilizer, adjuvant, a

cryoprotectant, a salt, a divalent cation, a non-ionic detergent, an inhibitor of free radical oxidation, a carrier, or a mixture thereof.

C66. The immunogenic composition of any one of C35-C65, wherein the composition further comprises a buffer.

C67. The immunogenic composition of C66, wherein the buffer is selected from the group consisting of HEPES, PIPES, MES, Tris (trimethamine), phosphate, acetate, borate, citrate, glycine, histidine and succinate.

C68. The immunogenic composition of C67, wherein the buffer is histidine.

C69. The immunogenic composition of any one of C35-C95, wherein the composition further comprises a surfactant.

C70. The immunogenic composition of C96, wherein the surfactant is selected from the group consisting of polyoxyethylene sorbitan fatty acid esters, polysorbate-80, polysorbate-60, polysorbate-40, polysorbate-20, and polyoxyethylene alkyl ethers.

C71. The immunogenic composition of C70, wherein the surfactant is polysorbate-80.

C72. The immunogenic composition of any one of C35-C71, wherein the composition further comprises an excipient.

C73. The immunogenic composition of C72, wherein the excipient is selected from the group consisting of starch, glucose, lactose, sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol, palatinit, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, glycine, arginine, lysine, sodium chloride (NaCl), dried skim milk, glycerol, propylene glycol, water, ethanol.

C74. The immunogenic composition of C73, wherein the excipient is sodium chloride.

C75. The immunogenic composition of any one of C35-C74, wherein the composition further comprises an adjuvant.

C76. The immunogenic composition of C75, wherein the adjuvant is an aluminum-based adjuvant or QS-21.

C77. The immunogenic composition of C76, wherein the aluminum-based adjuvant is selected from the group consisting of aluminum phosphate, aluminum hydroxyl phosphate, and aluminum hydroxide.

C78. The immunogenic composition of C77, wherein the adjuvant is aluminum phosphate.

C79. The immunogenic composition of C78, wherein the adjuvant is aluminum hydroxyl phosphate.

C80. The immunogenic composition of any one of C35-C79, wherein the composition comprises a buffer, a surfactant, an excipient, and optionally an adjuvant, wherein the composition is buffered to a pH of about 6.0 to about 7.0.

C81. The immunogenic composition of any one of C35-C80, wherein the composition comprises histidine, polysorbate-80, sodium chloride, and optionally aluminum phosphate, wherein the composition is buffered to a pH of about 6.0 to about 7.0.

C82. The immunogenic composition of any one of C35-C81, wherein the composition comprises about 10 mM to about 25 mM of histidine, about 0.01% to about 0.03% (v/w) of polysorbate-80, about 10 mM to about 250 mM of sodium chloride, and optionally about 0.25 mg/ml to about 0.75 mg/ml of aluminum as aluminum phosphate.

C83. The immunogenic composition of any one of C35-C82, wherein the composition comprises a dose of about 5 mcg/ml to about 50 mcg/ml.

C84. The immunogenic composition of any one of C35-C83, wherein the composition is lyophilized, optionally in the presence of at least one excipient.

C85. The immunogenic composition of C84, wherein the at least one excipient is selected from the group consisting of starch, glucose, lactose, sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol, palatinit, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, glycine, arginine, lysine, sodium chloride (NaCl), dried skim milk, glycerol, propylene glycol, water, and ethanol.

C86. The immunogenic composition of C85, wherein the at least one excipient is sucrose.

C87. The immunogenic composition of any one of C84-C86, wherein the composition comprises about 1%(w/v) to about 10% (w/v) of the at least one excipient.

C88. The immunogenic composition of any one of C84-C87, wherein the composition comprises an additional excipient.

C89. The immunogenic composition of C88, wherein the additional excipient is mannitol or glycine.

C90. The immunogenic composition of C88 or C89, wherein the composition comprises about 1% (w/v) to about 10% (w/v) of the additional excipient.

C91. The immunogenic composition of any one of C84-C90, wherein the composition is reconstituted with water, water for injection (WFI), an adjuvant suspension, or saline.

C92. An immunogenic composition of any one of C44-C91 for use as a medicament.

C93. An immunogenic composition of any one of C35-C92 for use in a method of inducing an immune response against GBS in a subject.

C94. The immunogenic composition of C93, wherein the subject is a female planning to become pregnant or a pregnant female.

C95. The immunogenic composition of C94, wherein the female is in her second half of pregnancy.

C96. The immunogenic composition of C95, wherein the pregnant female is at least at 20 weeks gestation.

C97. The immunogenic composition of C96, wherein the pregnant female is at 27 weeks to 36 weeks gestation.

C98. The immunogenic composition of C97, wherein the subject is an adult 50 years of age or older.

C99. The immunogenic composition of C98, wherein the subject is an adult 65 years of age or older.

C100. The immunogenic composition of C99, wherein the subject is an adult 85 years of age or older.

C101. The immunogenic composition of any one of C90-C100, wherein the subject is immunocompromised.

C102. The immunogenic composition of C101, wherein the subject has a medical condition selected from the group consisting of obesity, diabetes, HIV infection, cancer, cardiovascular disease, or liver disease.

C103. The immunogenic composition of any one of C93-102, wherein the group B streptococcus is *Streptococcus agalactiae*.

C104. A method of inducing an immune response against group B streptococcus comprising administering to a subject an effective amount of the immunogenic composition of any one of C35-C103.

C105. A method of preventing or reducing a disease or condition associated with group B streptococcus in a subject comprising administering to a subject an effective amount of the immunogenic composition of any one of C35-C104.

C106. The method of C104 or C105, wherein the subject is a female planning to become pregnant or a pregnant female.

C107. The method of C106, wherein the female is in her second half of pregnancy.

C108. The method of C106 or C107, wherein the pregnant female is at least at 20 weeks gestation.

C109. The method of any one of C106-C108, wherein the pregnant female is at 27 weeks to 36 weeks gestation.

C110. The method of C104 or C105, wherein the subject is an adult 50 years of age or older.

C111. The method of C110, wherein the subject is an adult 65 years of age or older.

C112. The method of C110 or C111, wherein the subject is an adult 85 years of age or older.

C113. The method of any one of C104-C112, wherein the subject is immunocompromised.

C114. The method of C113, wherein the subject has a medical condition selected from the group consisting of obesity, diabetes, HIV infection, cancer, cardiovascular disease, or liver disease.

C115. The method of any one of the preceding paragraphs, wherein the group B streptococcus is *Streptococcus agalactiae*.

C116 . An antibody that binds to a capsular polysaccharide in the immunogenic conjugate of any one of C1-C27.

C117. A composition comprising the antibody of C116.

C118. A method of conferring passive immunity to a subject comprising the steps of:

(a) generating an antibody preparation using the immunogenic composition of any preceding paragraph; and

(b) administering the antibody preparation to the subject to confer passive immunity.

C119. A method of making an immunogenic polysaccharide-protein conjugate of any one of C1-C27 comprising the steps of:

(a) reacting the GBS capsular polysaccharide with an oxidizing agent resulting in an activated polysaccharide; and

(b) reacting the activated polysaccharide with the carrier protein resulting in a polysaccharide-protein conjugate.

C120. The method of C119, wherein step (b) is carried out in a polar aprotic solvent.

C121. The method of C120, wherein the solvent is selected from the group consisting of dimethylsulfoxide (DMSO), sulfolane, dimethylformamide (DMF), and hexamethylphosporamide (HMPA).

C122. The method of C121, wherein the solvent is dimethylsulfoxide (DMSO).

C123. The method of any one of C119-C122, wherein the polysaccharide is reacted with 0.01 to 10.0 molar equivalents of the oxidizing agent.

C124. The method of any one of C119-C123, wherein the oxidizing agent is a periodate.

C125. The method of C124, wherein the periodate is sodium periodate.

C126. The method of any one of C119-C125, wherein the oxidation reaction of step (a) is between 1 hour and 50 hours.

C127. The method of any one of C119-C126, wherein the temperature of the oxidation reaction is maintained between about 2°C and about 25°C.

C128. The method of any one of C119-C127, wherein the oxidation reaction is carried out in a buffer selected from the group consisting of sodium phosphate, potassium phosphate, 2-(N-morpholino)ethanesulfonic acid (MES), and Bis-Tris.

C129. The method of C128, wherein the buffer has a concentration of between about 1 mM and about 500 mM.

C130. The method of any one of C119-C129, wherein the oxidation reaction is carried out at a pH between about 4.0 and about 8.0.

C131. The method of C119, wherein the oxidizing agent is 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO).

C132. The method of C131, wherein N-chlorosuccinimide (NCS) is a cooxidant.

C133. The method of any one of C119-C132, wherein step (a) further comprises quenching the oxidation reaction by addition of a quenching agent.

C134. The method of any one of C119-C133, wherein the concentration of polysaccharide is between about 0.1 mg/mL and about 10.0 mg/mL.

C135 . The method of any one of C119-C161, wherein the degree of oxidation of the activated polysaccharide is between 5 and 25.

C136 . The method of any one of C119-C135, wherein the method further comprises the step of lyophilizing the activated polysaccharide.

C137. The method of C136, wherein the activated polysaccharide is lyophilized in the presence of a saccharide selected from the group consisting of sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol and palatinit.

C138. The method of any one of C119-C137, wherein step (b) comprises:

(1) compounding the activated polysaccharide with a carrier protein, and

(2) reacting the compounded activated polysaccharide and carrier protein with a reducing agent to form a GBS capsular polysaccharide-carrier protein conjugate.

C139. The method of C138, wherein the concentration of activated polysaccharide in step (2) is between about 0.1 mg/mL and about 10.0 mg/mL.

C140. The method of C137 or C138, wherein the initial ratio (weight by weight) of activated polysaccharide to carrier protein is between 5:1 and 0.1:1.

C141. The method of any one of C138-C139, wherein the reducing agent is selected from the group consisting of sodium cyanoborohydride, sodium triacetoxyborohydride, sodium or zinc borohydride in the presence of Bronsted or Lewis acids, pyridine borane, 2-picoline borane, 2,6-diborane-methanol, dimethylamine-borane, t-BuMe^{i}PrN-BH_3, benzylamine-BH_3 or 5-ethyl-2-methylpyridine borane (PEMB).

C142. The method of C141, wherein the reducing agent is sodium cyanoborohydride.

C143. The method of any one of C138-C142, wherein the quantity of reducing agent is between about 0.1 and about 10.0 molar equivalents.

C144. The method of any one of C138-C143, wherein the duration of reduction reaction of step (2) is between 1 hour and 60 hours.

C145. The method of any one of C138-C144, wherein the temperature of the reduction reaction is maintained between 10°C and 40°C.

C146. The method of any one of C119-C145, wherein the method further comprises a step (step (c)) of capping unreacted aldehyde by addition of a borohydride.

C147. The method of C146, wherein the quantity of borohydride is between about 0.1 and about 10.0 molar equivalents.

C148. The method of C146, wherein the borohydride is selected from the group consisting of sodium borohydride ($NaBH_4$), sodium cyanoborohydride, lithium borohydride, potassium borohydride, tetrabutylammonium borohydride, calcium borohydride, and magnesium borohydride.

C149. The method of C146, wherein the borohydride is sodium borohydride ($NaBH_4$).

C150. The method of C146, wherein the duration of capping step is between 0.1 hours and 10 hours.

C151. The method of any one of C146-C149, wherein the temperature of the capping step is maintained between about 15°C and about 45°C.

C152. The method of any one of C119-C151, wherein the method further comprises the step of purifying the polysaccharide-protein conjugate.

C153. The method of any one of C119-C152, wherein the polysaccharide-protein conjugate comprises less than about 40% of free polysaccharide compared to the total amount of polysaccharide.

C154. The method of any one of C119-C153, wherein the ratio (weight by weight) of polysaccharide to carrier protein in the conjugate is between about 0.5 and about 3.0.

C155. The method of any one of C119-C154, wherein the degree of conjugation of the conjugate is between 2 and 15.

C156. A method of making a polysaccharide-protein conjugate comprising the steps of:
(a) reacting an isolated GBS capsular polysaccharide with an oxidizing agent;
(b) quenching the oxidation reaction of step (a) by addition of a quenching agent resulting in an activated GBS capsular polysaccharide;
(c) compounding the activated GBS capsular polysaccharide with a carrier protein,
(d) reacting the compounded activated GBS capsular polysaccharide and carrier protein with a reducing agent to form a GBS capsular polysaccharide-carrier protein conjugate, and
(e) capping unreacted aldehyde by addition of sodium borohydride ($NaBH_4$),
wherein steps (c) and (d) are carried out in DMSO.

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CLAIMS

1. An immunogenic composition comprising a polysaccharide-protein conjugate comprising a group B streptococcus (GBS) capsular polysaccharide selected from serotypes VI, VII, VIII, and IX and a carrier protein.

2. The immunogenic composition of claim 1, wherein the GBS capsular polysaccharide comprises serotype VI.

3. The immunogenic composition of claim 1, wherein the GBS capsular polysaccharide comprises serotype VII.

4. The immunogenic composition of claim 1, wherein the GBS capsular polysaccharide comprises serotype VIII.

5. The immunogenic composition of claim 1, wherein the GBS capsular polysaccharide comprises serotype IX.

6. The immunogenic composition of any one of claims 1-5, further comprising at least one additional serotype selected from serotypes Ia, Ib, II, III, IV, and V.

7. The immunogenic composition of claim 1, wherein the polysaccharide-protein conjugate comprises a GBS capsular polysaccharide serotype VI, and at least one additional serotype selected from serotypes Ia, Ib, II, III, IV, V, VII, VIII, and IX.

8. The immunogenic composition if claim 1, wherein the polysaccharide-protein conjugate comprises a GBS capsular polysaccharide serotype VII, and at least one additional serotype selected from serotypes Ia, Ib, II, III, IV, V, VI, VIII, and IX.

9. The immunogenic composition of claim 1, wherein the polysaccharide-protein conjugate comprises a GBS capsular polysaccharide serotype VIII, and at least one additional serotype selected from serotypes Ia, Ib, II, III, IV, V, VI, VII, and IX.

10. The immunogenic composition of claim 1, wherein the polysaccharide-protein conjugate comprises a GBS capsular polysaccharide serotype IX, and at least one additional serotype selected from serotypes Ia, Ib, II, III, IV, V, VI, VII, and VIII.

11. The immunogenic composition of any one of claims 1-10, wherein the capsular polysaccharide has a sialic acid level of greater than about 60%, greater than about 95%, or about 100%.

12. The immunogenic composition of any one of claims 1-11, wherein the capsular polysaccharide has at least about 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, or 0.95 mM sialic acid per mM of polysaccharide.

13. The immunogenic composition of any one of claims 1-12, wherein the capsular polysaccharide has a molecular weight of between about 5 kDa and about 1,000 kDa, between about 25 kDa and about 750 kDa, between about 25 kDa and about 400 kDa, between about 25 kDa and about 200 kDa, or between about 100 kDa and about 400 kDa.

14. The immunogenic composition of any one of claims 1-13, wherein the molecular weight of the conjugate is between about 300 kDa and about 20,000 kDa, between about 1,000 kDa and about 15,000 kDa, or between about 1,000 kDa and about 10,000 kDa.

15.The immunogenic composition of any one of claims 1-14, wherein the capsular polysaccharide is between about 0% and about 40% O-acetylated.

16. The immunogenic composition of any one of claims 1-15, wherein the capsular polysaccharide is less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% O-acetylated.

17. The immunogenic composition of any one of claims 1-16, wherein the capsular polysaccharide has at least about 0.1, 0.2, 0.3, 0.35 or about 0.4 mM O-acetate per mM saccharide repeating unit.

18. The immunogenic composition of any one of claims 1-16, wherein the capsular polysaccharide has less than about 0.01, 0.02, 0.03, 0.04, or 0.05 mM O-acetate per mM saccharide repeating unit.

19. The immunogenic composition of any one of claims 1-18, wherein the carrier protein is selected from CRM_{197}, Diphtheria toxoid (DT), tetanus toxoid (TT), and Streptococcal C5a peptidease (SCP).

20. The immunogenic composition of any one of claims 1-19, wherein the composition further comprises a pharmaceutically acceptable excipient, buffer, stabilizer, adjuvant, a cryoprotectant, a salt, a divalent cation, a non-ionic detergent, an inhibitor of free radical oxidation, a carrier, or a mixture thereof.

21. The immunogenic composition of claim 20, wherein the buffer is selected from HEPES, PIPES, MES, Tris (trimethamine), phosphate, acetate, borate, citrate, glycine, histidine and succinate.

22. The immunogenic composition of claim 21, wherein the buffer is histidine.

23. The immunogenic composition of any one of claims 1-22, wherein the composition further comprises a surfactant.

24. The immunogenic composition of claim 23, wherein the surfactant is selected from polyoxyethylene sorbitan fatty acid esters, polysorbate-80, polysorbate-60, polysorbate-40, polysorbate-20, and polyoxyethylene alkyl ethers.

25. The immunogenic composition of any one of claims 1-24, wherein the composition further comprises an excipient.

26. The immunogenic composition of claim 25, wherein the excipient is selected from starch, glucose, lactose, sucrose, trehalose, raffinose, stachyose, melezitose, dextran, mannitol, lactitol, palatinit, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, glycine, arginine, lysine, sodium chloride (NaCl), dried skim milk, glycerol, propylene glycol, water, and ethanol.

27. The immunogenic composition of any one of claims 1-26, wherein the composition further comprises an adjuvant.

28. An immunogenic composition of any one of claims 1-27, for use as a medicament.

29. An immunogenic composition of any one of claims 1-28, for use in a method of inducing an immune response against group B streptococcus in a subject.

30. The immunogenic composition of claim 29, wherein the group B streptococcus is *Streptococcus agalactiae*.

31. A method of inducing an immune response against group B streptococcus comprising administering to a subject an effective amount of the immunogenic composition of any one of claims 1-27.

32. A method of preventing, treating or reducing a disease or condition associated with group B streptococcus in a subject comprising administering to a subject an effective amount of the immunogenic composition of any one of claims 1-27.

33. The method of claim 31 or 32, wherein the group B streptococcus is *Streptococcus agalactiae*.

34. A method of producing an antibody comprising administering the immunogenic composition of any one of claims 1-27 to a subject.

35. An antibody produced by the method of claim 34.

36. A method of conferring passive immunity to a subject comprising the steps of:

generating an antibody preparation using the immunogenic composition of any one of claims 1-27; and

administering the antibody preparation to the subject to confer passive immunity.

FIG. 1

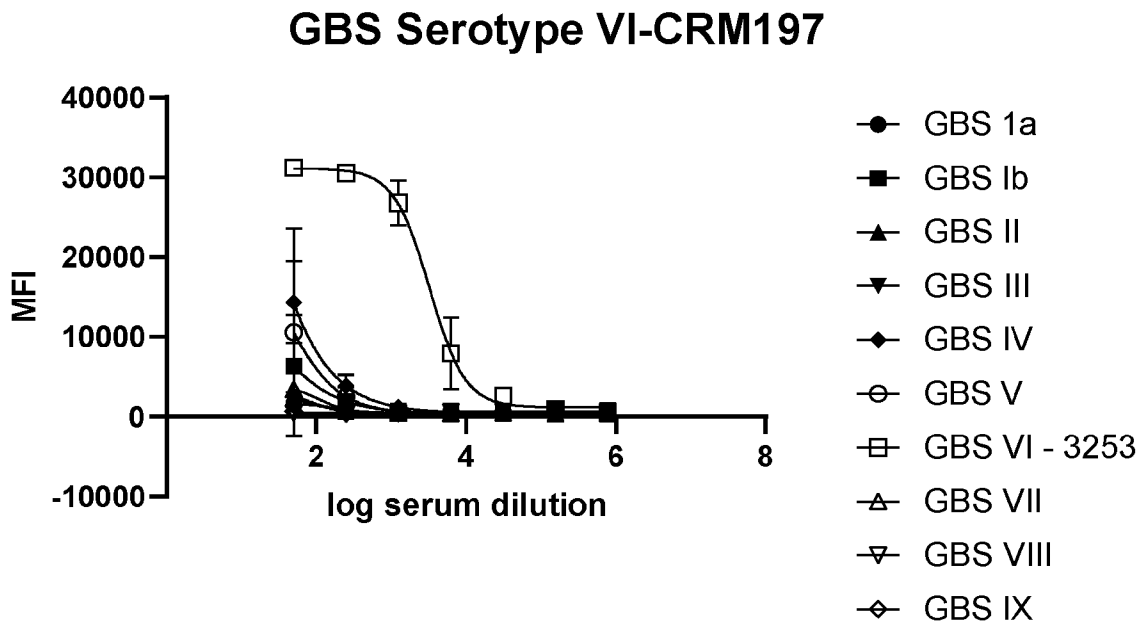

GBS Serotype VI-CRM197
MFI
40000
30000
20000
10000
0
-10000
2
4
6
8
log serum dilution
GBS 1a
GBS Ib
GBS II
GBS III
GBS IV
GBS V
GBS VI - 3253
GBS VII
GBS VIII
GBS IX

FIG. 2A

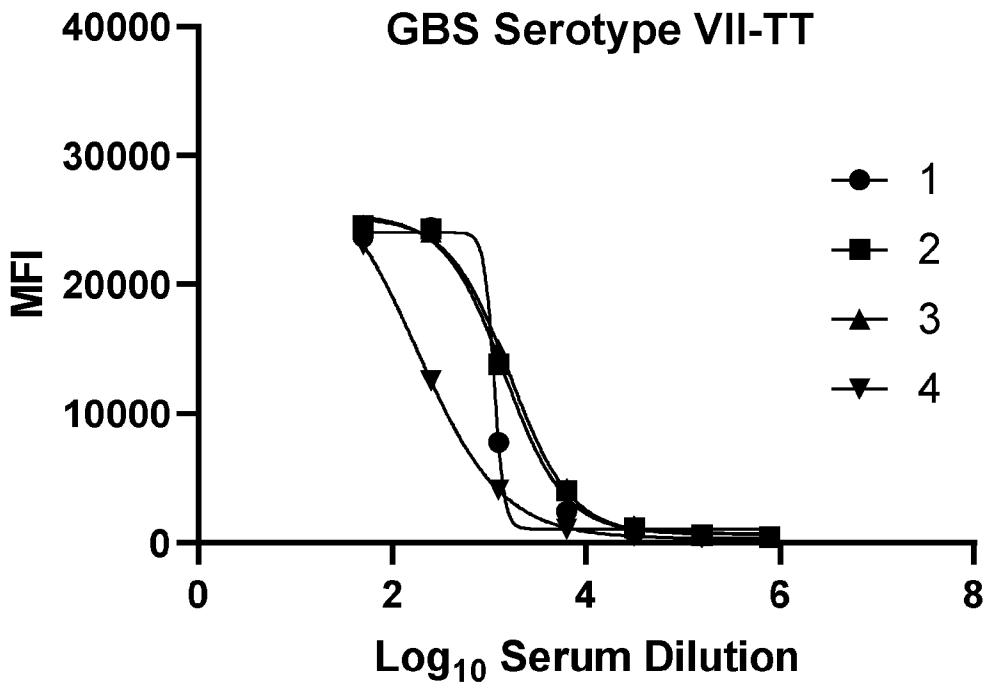

GBS Serotype VII-TT
MFI
40000
30000
20000
10000
0
0
2
4
6
8
Log10 Serum Dilution
1
2
3
4

FIG. 2B

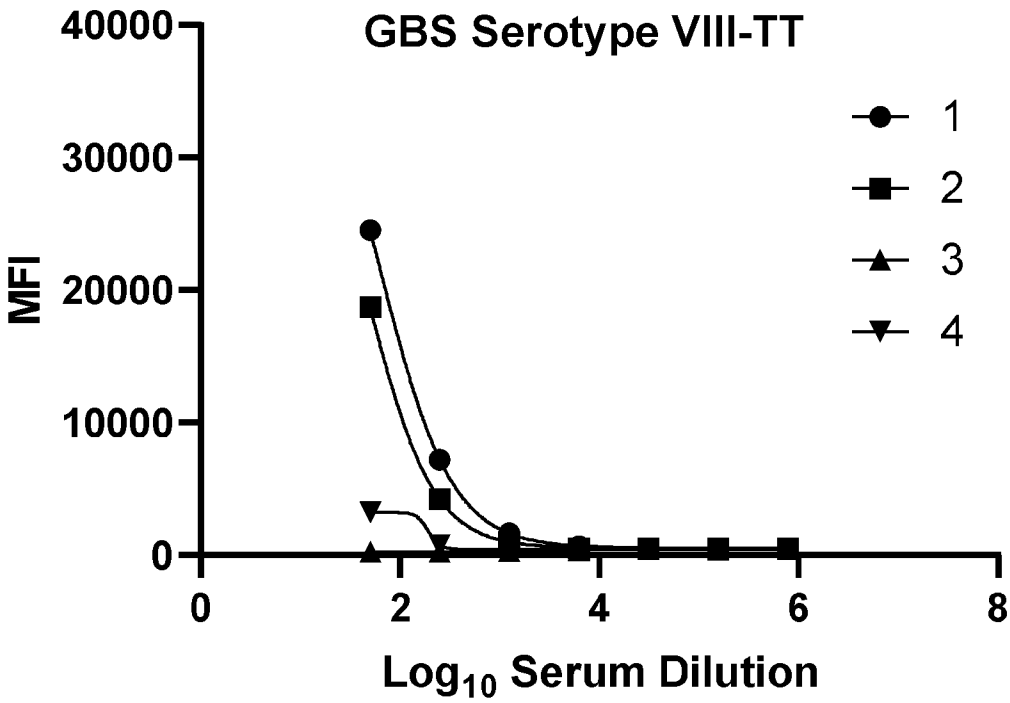

GBS Serotype VIII-TT
MFI
40000
30000
20000
10000
0
0
2
4
6
8
Log10 Serum Dilution
1
2
3
4

FIG. 2C

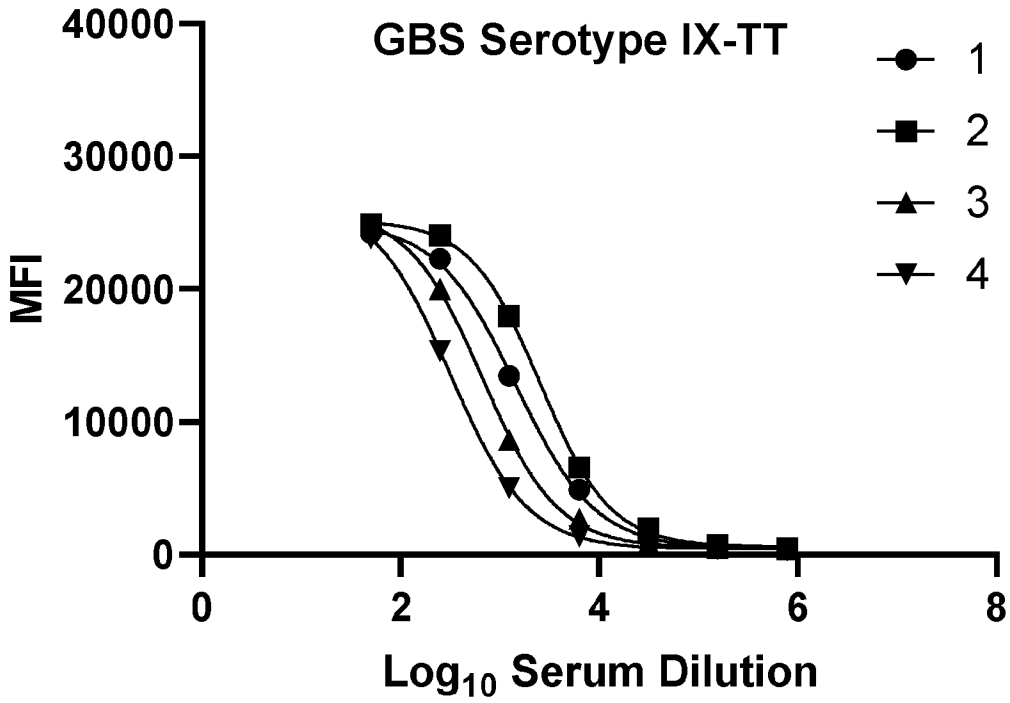

GBS Serotype IX-TT
MFI
40000
30000
20000
10000
0
0
2
4
6
8
Log10 Serum Dilution
1
2
3
4

FIG. 3A

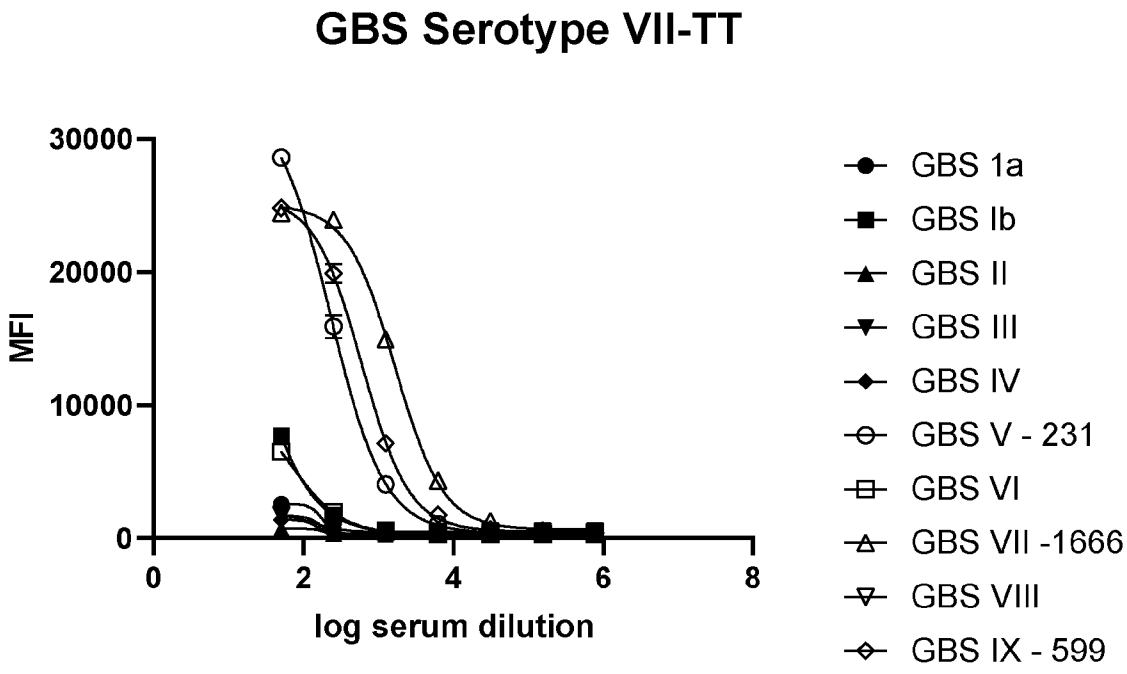
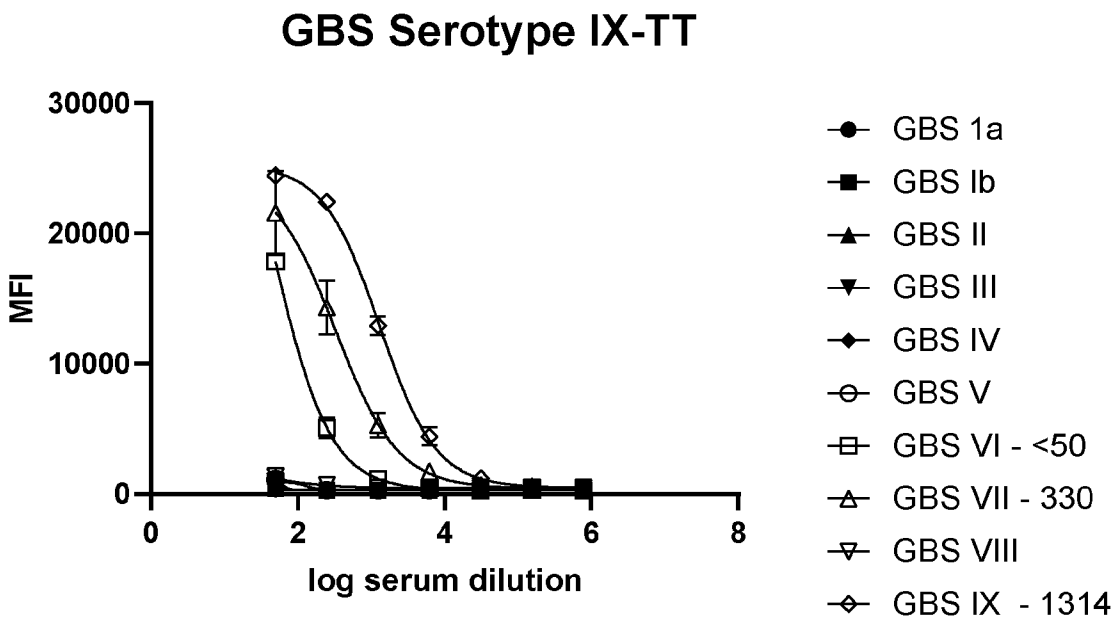

GBS Serotype VII-TT
30000
20000
10000
0
MFI
0
2
4
6
8
log serum dilution
GBS 1a
GBS Ib
GBS II
GBS III
GBS IV
GBS V - 231
GBS VI
GBS VII -1666
GBS VIII
GBS IX - 599

FIG. 3B


GBS Serotype IX-TT
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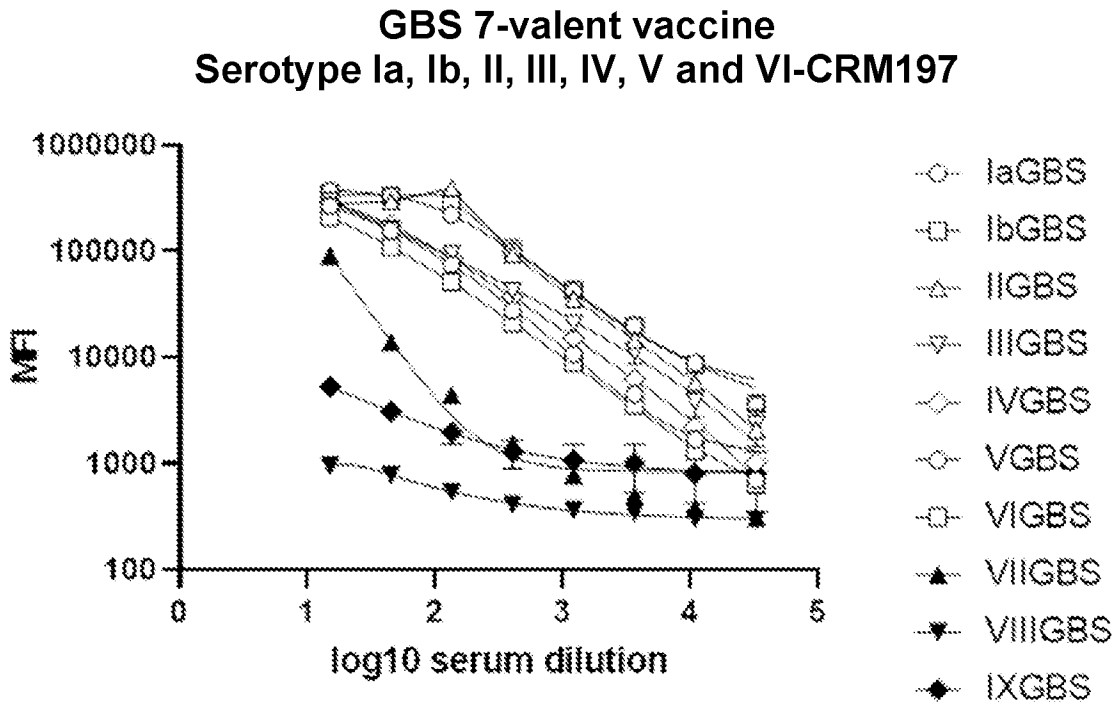
20000
10000
0
MFI
0
2
4
6
8
log serum dilution
GBS 1a
GBS Ib
GBS II
GBS III
GBS IV
GBS V
GBS VI - <50
GBS VII - 330
GBS VIII
GBS IX - 1314

FIG. 4


GBS 7-valent vaccine
Serotype Ia, Ib, II, III, IV, V and VI-CRM197
MFI
1000000
100000
10000
1000
100
0
1
2
3
4
5
log10 serum dilution
IaGBS
IbGBS
IIGBS
IIIGBS
IVGBS
VGBS
VIGBS
VIIGBS
VIIIGBS
IXGBS

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/057714

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/09 A61P31/04
ADD. A61K39/00

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)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PAOLETTI LAWRENCE C. ET AL: "Surface Structures of Group B Streptococcus Important in Human Immunity", MICROBIOLOGY SPECTRUM, vol. 7, no. 2, 12 April 2019 (2019-04-12), XP055856398, DOI: 10.1128/microbiolspec.GPP3-0001-2017 Retrieved from the Internet: URL:https://journals.asm.org/doi/pdf/10.1128/microbiolspec.GPP3-0001-2017> [retrieved on 2021-11-09] page 19; table 2 -----	1,2,19
X	WO 2018/087635 A1 (PFIZER [US]) 17 May 2018 (2018-05-17) page 4, line 23 - page 5, line 3 examples 1, 3, 5-6, 9-10 -----	1,2,6, 11-27, 34-36

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

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"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
9 November 2021	19/01/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Malamoussi, A

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2021/057714

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2017/001586 A1 (GLAXOSMITHKLINE BIOLOGICALS SA [BE]) 5 January 2017 (2017-01-05) tables; page 42 page 43, line 2 - line 4	1,2,6,7, 11-36
A	Paoletti Lawrence C ET AL: "Conjugate Vaccines against Group B Streptococcus Types IV and VII", The Journal of infectious diseases, 1 July 2002 (2002-07-01), pages 123-126, XP055856222, Chicago, IL DOI: 10.1086/341073 Retrieved from the Internet: URL:https://watermark.silverchair.com/186-1-123.pdf?token=AQECAHi208BE49Ooan9kkhW_Ercy7Dm3ZL_9Cf3qfKAc485ysgAAAtQwggLQBgkqhkiG9w0BBwagggLBMIICvQIBADCCArYGCSqGSIb3DQEHATAeBglghkgBZQMEAS4wEQQMu736NPILil5P-cNnAgEQgIICh_VhuwPaqg7xHtoxzzArS6Hv6PwORRwWwQQt1tFBolSlPnvVKadrXtmXsulCYH9HdWc3wmcwdYAQLpDpw_xDgLKRV1 [retrieved on 2021-10-29] abstract page 123, right-hand column, paragraph 1 - page 124, left-hand column, paragraph 1 page 125, left-hand column, paragraph 1	1,2,6,7, 11-36
X	Paoletti Lawrence C ET AL: "Synthesis and Preclinical Evaluation of Glycoconjugate Vaccines against Group B Streptococcus Types VI and VIII", The Journal of infectious diseases, 1 September 1999 (1999-09-01), pages 892-895, XP055856442, Chicago, IL DOI: 10.1086/314955 Retrieved from the Internet: URL:https://watermark.silverchair.com/180-3-892.pdf?token=AQECAHi208BE49Ooan9kkhW_Ercy7Dm3ZL_9Cf3qfKAc485ysgAAAtQwggLQBgkqhkiG9w0BBwagggLBMIICvQIBADCCArYGCSqGSIb3DQEHATAeBglghkgBZQMEAS4wEQQMtdodWblRwYGBM_-NAgEQgIICh71WDR-GAZOIR6lXTmQkLKpxqyceG9WY6nfPjvoL8C0O7GDKufXzm61uhWk0990FuYRNVULEBFqJrkguIvL6fWly2L [retrieved on 2021-10-29] abstract tables 1-2 "Discussion"	1,2,6,7, 19,20, 28-33

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2004/011027 A1 (BAXTER INT [US]; BAXTER HEALTHCARE SA [CH]) 5 February 2004 (2004-02-05) claims 1, 5, 6 -----	1,2,6,7, 11-36
A	GILCHUK PAVLO ET AL: "Discovering protective CD8 T cell epitopes-no single immunologic property predicts it!", CURRENT OPINION IN IMMUNOLOGY, ELSEVIER, OXFORD, GB, vol. 34, 6 February 2015 (2015-02-06), pages 43-51, XP029605146, ISSN: 0952-7915, DOI: 10.1016/J.COI.2015.01.013 abstract -----	1,2,6,7, 11-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2021/057714

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.:
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:

see additional sheet

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 an 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.:

2, 7(completely); 1, 6, 11-36(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 2, 7(completely); 1, 6, 11-36(partially)

relating to an immunogenic composition comprising a polysaccharide-protein conjugate comprising a group B streptococcus (GBS) capsular polysaccharide from serotype VI and a carrier protein.

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2. claims: 3, 8(completely); 1, 6, 11-36(partially)

relating to an immunogenic composition comprising a polysaccharide-protein conjugate comprising a group B streptococcus (GBS) capsular polysaccharide from serotype VII and a carrier protein.

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3. claims: 4, 9(completely); 1, 6, 11-36(partially)

relating to an immunogenic composition comprising a polysaccharide-protein conjugate comprising a group B streptococcus (GBS) capsular polysaccharide from serotype VIII and a carrier protein.

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4. claims: 5, 10(completely); 1, 6, 11-36(partially)

relating to an immunogenic composition comprising a polysaccharide-protein conjugate comprising a group B streptococcus (GBS) capsular polysaccharide from serotype IX and a carrier protein.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2021/057714

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