COMPOSITIONS AND METHODS FOR PREPARING STAPHYLOCOCCUS AUREUS SEROTYPE 5 AND 8 CAPSULAR POLYSACCHARIDE CONJUGATE IMMUNOGENIC COMPOSITIONS

Cross Reference to Related Applications

[0001] This application claims the priority benefit of U.S. Provisional Patent Application Nos. 61/219,143 and 61/219,151, filed June 22, 2009, the entire disclosures of which are each hereby incorporated by reference herein in their entirety.

Field of the Invention

[0002] The invention relates generally to *Staphylococcus aureus* serotype 5 and 8 capsular polysaccharide conjugate immunogenic compositions and methods for their preparation and use.

Background of the Invention

[0003] Humans are a natural reservoir for Gram-positive *Staphylococcus aureus*. For example, *S. aureus* can colonize the skin, nares and throat, either permanently or transiently, without causing disease. *S. aureus* infections range from mild skin infections to endocarditis, osteomyelitis, bacteremia and sepsis. *S. aureus* also causes a majority of nosocomial infections, and its prevalence in community-onset infections is increasing. Moreover, in 2005, methicillin-resistant *S. aureus* (MRSA) infections were estimated at 31.8 per 100,000 individuals, including 16,650 deaths in the United States in 2005 (Klevens *et al.* (2007) *J. Am. Med.*

Assoc. 298:1763-1771). Disease subsequently occurs when individuals become immunocompromised due to breaches in immune barriers, such as during surgery, placement of indwelling catheters or other devices, trauma or wounds.

[0004] *S. aureus* produces a large number of extra- and intra-cellular antigens, including numerous toxins and enzymes. Of particular interest herein are capsular polysaccharide serotypes of *S. aureus* (*see*, Karakawa & Vann, "Capsular polysaccharides of *Staphylococcus aureus*," In: Weinstein & Fields, eds. Seminars in Infectious Disease. IV. Bacterial Vaccines. (New York, NY; Thieme Stratton; 1982. pp. 285-293), especially serotype 5 and 8 capsular polysaccharides. Epidemiological studies on a large number of strains of *S. aureus* isolated from individuals showed that 70% to 80% were either serotype 5 or 8 capsular polysaccharide (Arbeit *et al.* (1984) *Diagn. Microbiol. Infect. Dis.* 2:85-91). Unfortunately, the capsular polysaccharides are poor immunogens by themselves.

[0005] Staphylococcal infections and diseases dramatically increased in the last twenty years, as has use of intravascular devices and invasive procedures. The rise in disease incidence is more troubling because of a parallel rise of antibiotic resistance; therefore, there is an urgent need for immunogenic compositions to prevent Staphylococcal infections and diseases.

SUMMARY OF THE INVENTION

[0006] The present invention is directed towards immunogenic conjugates comprising a *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein, and methods for making such conjugates. *S. aureus* serotype 5 or 8 capsular polysaccharides may be obtained directly from the bacteria using isolation procedures known to those skilled in the art, may be produced using synthetic protocols, or may be recombinantly produced using genetic engineering procedures also known to those skilled in the art. In addition, the present invention provides methods for inducing an immune response against a *Staphylococcus* bacterium, methods for preventing a disease caused by a *Staphylococcus* bacterium, and methods for reducing the severity of at least one symptom of a disease caused by infection with a *Staphylococcus* bacterium.

[0007] In one embodiment, the invention comprises an immunogenic polysaccharide-protein conjugate comprising an isolated *Staphylococcus aureus*

serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein, wherein the polysaccharide has a molecular weight of between 20 kDa and 1000 kDa. In some embodiments, the immunogenic conjugate has a molecular weight of between 200 kDa and 5000 kDa. In one embodiment the polysaccharide portion of the immunogenic conjugate has a molecular weight range of between 70 kDa and 300 kDa. In one embodiment the immunogenic conjugate has a molecular weight range of between 500 kDa and 2500 kDa.

[0008] In one embodiment, the serotype 5 or 8 capsular polysaccharide has a degree of O-acetylation between 10-100%. In one embodiment, the degree of O-acetylation is between 50-100%. In one embodiment, the degree of O-acetylation is between 75-100%. In one embodiment, the immunogenic conjugate generates an antibody that is functional as measured by killing bacteria in either an animal efficacy model or via an opsonophagocytic killing assay.

[0009] In one embodiment, the immunogenic conjugate carrier protein comprises CRM_{197}. In one embodiment, the CRM_{197} is covalently linked to the polysaccharide through a carbamate linkage, an amide linkage, or both. In one embodiment, the molar ratio of conjugated lysines to CRM_{197} can be about 10:1 to about 25:1. In one embodiment, the conjugate comprises one covalent linkage between CRM_{197} and polysaccharide for at least every 5 to 10 saccharide repeat units of the polysaccharide. In one embodiment, the linkage between carrier protein and polysaccharide occurs for once in every 5 repeat units of the polysaccharide.

[0010] In one embodiment, the immunogenic conjugate comprising CRM_{197} comprises 5 to 22 lysines or 8 to 15 lysines covalently linked to the polysaccharide. In one embodiment, the immunogenic conjugate comprising CRM_{197} comprises 5 to 23 lysines or 8 to 12 lysines covalently linked to the polysaccharide.

[0011] In one embodiment, the immunogenic conjugate comprises a type 5 or 8 polysaccharide that is 10-100% O-Acetylated. In one embodiment, the immunogenic conjugate comprises a type 5 or 8 polysaccharide that is 50-100% O-Acetylated. In one embodiment, the immunogenic conjugate comprises a type 5 or 8 polysaccharide that is 75-100% O-Acetylated. In some embodiments,

the immunogenic composition can be used to generate antibodies that are functional in an animal efficacy model or an opsonophagocytic killing assay.

[0012] In one embodiment, the immunogenic conjugate comprises less than about 30% free type 5 or 8 polysaccharide compared to the total amount of type 5 or 8 polysaccharide.

[0013] In one embodiment, the immunogenic conjugate comprises less than about 20% free type 5 or 8 polysaccharide compared to the total amount of type 5 or 8 polysaccharide.

[0014] In one embodiment, the invention comprises an immunogenic composition comprising an immunogenic conjugate as described herein and at least one of an adjuvant, diluent, or carrier.

[0015] The adjuvant can be an aluminum-based adjuvant, such as one or more of aluminum phosphate, aluminum sulfate and aluminum hydroxide. In one embodiment the adjuvant comprises aluminum phosphate.

[0016] In one embodiment, the immunogenic composition comprises less than about 30% free type 5 or 8 polysaccharide compared to the total amount of type 5 or 8 polysaccharide.

[0017] In one embodiment, the immunogenic composition comprises less than about 20% free type 5 or 8 polysaccharide compared to the total amount of type 5 or 8 polysaccharide.

[0018] In one embodiment, the invention comprises a method of inducing an immune response to a *Staphylococcus aureus* serotype 5 or 8 capsular polysaccharide conjugate in a subject, comprising administering to the subject an immunologically effective amount of an immunogenic composition as described herein.

[0019] In one embodiment, the invention comprises a method of producing an immunogenic polysaccharide-protein conjugate comprising an isolated *Staphylococcus aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein, the method comprising the steps of: reacting an isolated *S. aureus* serotype 5 or 8 capsular polysaccharide with 1,1-carbonyl-di-(1,2,4-triazole) (CDT) in an organic solvent to produce an activated serotype 5 or 8 polysaccharide; and reacting the activated serotype 5 or 8 polysaccharide with a

carrier protein in an organic solvent to produce a serotype 5 or 8 polysaccharide:carrier protein conjugate.

[0020] In one embodiment, the method of activating *Staphylococcus aureus* serotype 5 or 8 capsular polysaccharide further comprises lyophilizing the isolated serotype 5 or 8 polysaccharide and re-suspending the lyophilized polysaccharide in an organic solvent. In one embodiment, the resuspended polysaccharide is activated and then directly reacted with the carrier protein. In one embodiment, the activated isolated serotype 5 or 8 polysaccharide is isolated prior to reacting with the carrier protein. In one embodiment, the isolated activated isolated serotype 5 or 8 polysaccharide is lyophilized to produce a lyophilized activated isolated serotype 5 or 8 polysaccharide prior to reacting the polysaccharide with carrier protein. In one embodiment, the method of producing an isolated polysaccharide-carrier protein conjugate comprises a step of lyophilizing the carrier protein to produce a lyophilized carrier protein prior to reacting the carrier protein with the polysaccharide. In one embodiment the method of producing an isolated polysaccharide-carrier protein conjugate comprises the step of re-suspending lyophilized activated isolated serotype 5 or 8 polysaccharide and lyophilized carrier protein in an organic solvent as part of the reaction of the activated isolated serotype 5 or 8 polysaccharide with a carrier protein.

[0021] In one embodiment the method of producing an isolated *S. aureus* type 5 or 8 capsular polysaccharide-carrier protein conjugate comprises the step of diluting the reaction mixture of activated polysaccharide and carrier protein and maintaining a pH of about 8.8 to about 9.2 for at least 4 hours at about 20°C to about 26°C.

[0022] In one embodiment, the reaction mixture of activated *S. aureus* type 5 or 8 capsular polysaccharide and carrier protein is maintained at a pH of about 9.0 for at least 4 hours at about 23°C.

[0023] In one embodiment the method of producing an isolated *S. aureus* type 5 or 8 capsular polysaccharide-carrier protein comprises the step of isolating the isolated serotype 5 or 8 polysaccharide-protein conjugate after it is produced.

[0024] In one embodiment, the organic solvent used in the method of producing an isolated *S. aureus* type 5 or 8 capsular polysaccharide-carrier protein conjugate

is a polar aprotic solvent. In one embodiment, the polar aprotic solvent is selected from the group consisting of dimethyl sulfoxide (DMSO), In one embodiment, the method of producing an isolated polysaccharide-carrier protein conjugate the organic solvent is DMSO.

[0025] In one embodiment, the method of producing isolated *S. aureus* type 5 capsular polysaccharide-carrier protein conjugate comprises the step of adjusting the water concentration of the reaction mixture comprising type 5 capsular polysaccharide and CDT in an organic solvent to between about 0.1 and 0.3%. In one embodiment, the water concentration of the reaction mixture comprising type 5 capsular polysaccharide and CDT in an organic solvent is adjusted to about 0.2%.

[0026] In one embodiment, the step of activating isolated *S. aureus* type 5 capsular polysaccharide comprises reacting the polysaccharide with about an amount of CDT that is 20 molar excess to the amount of polysaccharide present in the reaction mixture comprising type 5 capsular polysaccharide and CDT in an organic solvent.

[0027] In one embodiment, the method of producing isolated *S. aureus* type 8 capsular polysaccharide:carrier protein conjugate comprises the step of determining the water concentration of the reaction mixture comprising type 8 capsular polysaccharide. In one embodiment, the amount of CDT added to the reaction mixture to activate the polysaccharide is provided in about an amount of CDT that is equimolar to the amount of water present in the reaction mixture comprising type 8 capsular polysaccharide and CDT in an organic solvent.

[0028] In one embodiment, the amount of CDT added to the reaction mixture to activate the polysaccharide is provided in about an amount of CDT that is at a molar ratio of about 0.5:1 compared to the amount of water present in the reaction mixture comprising type 8 capsular polysaccharide and CDT in an organic solvent. In one embodiment, the amount of CDT added to the reaction mixture to activate the polysaccharide is provided in about an amount of CDT that is at a molar ratio of 0.75:1 compared to the amount of water present in the reaction mixture comprising type 8 capsular polysaccharide and CDT in an organic solvent.

[0029] In one embodiment, the method which comprises the step of isolating the activated polysaccharide comprises the step of diafiltration.

[0030] In one embodiment, the method which comprises lyophilization of the carrier protein, prior to lyophilization the carrier protein is diafiltered against NaCl and the w/w ratio of NaCl/protein carrier protein is adjusted to about 0.5 to about 1.5. In one embodiment, the ratio of NaCl to carrier protein is about 1.

[0031] In one embodiment, the carrier protein used in the method of producing an isolated *S. aureus* type 5 or 8 capsular polysaccharide-carrier protein conjugate comprises CRM_{197}.

[0032] In one embodiment, the CRM_{197} used in the method of producing an isolated *S. aureus* type 5 or 8 capsular polysaccharide-carrier protein conjugate is reacted with the activated serotype 5 or 8 polysaccharide at a ratio by weight of about 1:1.

[0033] In one embodiment, the method of producing an isolated *S. aureus* type 5 or 8 capsular polysaccharide-carrier protein conjugate comprises the step of mixing the type 5 or 8 capsular polysaccharide with imidazole or triazole prior to mixing with CDT in an organic solvent.

[0034] In one embodiment, the method of producing an isolated *S. aureus* type 5 or 8 capsular polysaccharide:carrier protein conjugate comprises the step of hydrolyzing the serotype 5 or 8 polysaccharide-carrier protein conjugate to remove unreacted activation groups.

[0035] In one embodiment, the invention provides a method of producing an immunogenic conjugate comprising an isolated *Staphylococcus aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein, the method comprising the steps of: reacting a *S. aureus* serotype 5 or 8 capsular polysaccharide with 3-(2-pyridyldithio)-propionyl hydrazide (PDPH) and a carbodiimide in an organic solvent to produce a PDPH-linked polysaccharide; reacting the PDPH-linked polysaccharide with a reducing agent to produce an activated polysaccharide; isolating the activated serotype 5 or 8 polysaccharide to produce an isolated activated serotype 5 or 8 polysaccharide; providing an activated carrier protein; reacting the isolated activated serotype 5 or 8 polysaccharide with the activated carrier protein to produce a serotype 5 or 8

polysaccharide-carrier protein conjugate; whereby an immunogenic conjugate comprising an isolated *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein is produced. In one embodiment, the activated carrier protein is isolated prior to reacting the activated carrier protein with the activated polysaccharide.

[0036] In one embodiment, the step of isolating the activated carrier protein further comprises lyophilizing the isolated activated serotype 5 or 8 polysaccharide to produce a lyophilized activated serotype 5 or 8 polysaccharide.

[0037] In one embodiment, the bromoacetic acid is a N-hydroxysuccinimide ester of bromoacetic acid (BAANS).

[0038] In one embodiment, the method of producing serotype 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH comprises the use of an organic solvent that is a polar aprotic solvent. In one embodiment, the polar aprotic solvent is selected from the group consisting of dimethyl sulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide, *N*-methyl-2-pyrrolidone, and hexamethylphosphoramide (HMPA), In one embodiment, the organic solvent is dimethyl sulfoxide (DMSO).

[0039] In one embodiment, the carbodiimide used in the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH is 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC).

[0040] In one embodiment, the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH and EDAC comprises the step of reacting the serotype 5 or 8 capsular polysaccharide with PDPH and EDAC in an organic at a polysaccharide:PDPH:EDAC ratio by weight of about 1:5:3.

[0041] In one embodiment, the reducing agent used in the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH and EDAC is dithiothreitol (DTT).

[0042] In one embodiment, activation of the carrier protein in the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH and EDAC comprises reacting the carrier protein with a bromoacetic acid.

[0043] In one embodiment, the step of isolating the activated serotype 5 or 8 polysaccharide in the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH and EDAC comprises diafiltration.

[0044] In one embodiment, the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH and EDAC comprises the step of hydrolyzing the serotype 5 or 8 polysaccharide-carrier protein conjugate to remove unreacted activation groups. In one embodiment, the step of hydrolyzing the serotype 5 or 8 polysaccharide-carrier protein conjugate comprises the addition of cysteamine hydrochloride.

[0045] In one embodiment, the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH and EDAC further comprises isolating the immunogenic conjugate comprising an isolated *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein.

[0046] In one embodiment, the isolation of the serotype 5 or 8 polysaccharide-carrier protein conjugate comprises diafiltration.

[0047] In one embodiment, the carrier protein used in the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH and EDAC comprises CRM_{197}.

[0048] In one embodiment, the CRM_{197} in the method of producing serotype 5 or 8 capsular polysaccharide-CRM_{197} conjugate which utilizes PDPH and EDAC is added in a ratio by weight of about 1:1 CRM_{197}:capsular polysaccharide molecule.

[0049] In one embodiment, activated type 5 or 8 capsular polysaccharide used in the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH and EDAC has a size between about 50 kd and about 500 kd.

[0050] In one embodiment, immunogenic conjugate produced in the method of producing serotype 5 or 8 capsular polysaccharide-carrier protein conjugate which utilizes PDPH and EDAC has a size between about between 400 kd and about 5000 kd.

[0051] In one embodiment, the invention provides an immunogenic composition comprising a type 5 or 8 capsular polysaccharide-carrier protein conjugate produced by any of the methods described herein.

[0052] In one embodiment, the invention provides an immunogenic composition comprising a type 5 or 8 capsular polysaccharide-carrier protein conjugate produced by any of the methods described herein and at least one of an adjuvant, diluent or carrier. In one embodiment, the immunogenic compositions comprise an aluminum based adjuvant that can be selected from the group consisting of aluminum phosphate, aluminum sulfate and aluminum hydroxide. In one embodiment, the immunogenic compositions described herein comprise the adjuvant aluminum phosphate.

[0053] The immunogenic compositions described herein can comprise less than 30% and less than 20% free type 5 or 8 polysaccharide compared to the total amount of type 5 or 8 polysaccharide. The immunogenic compositions described herein can be stored in water or a low ionic strength neutral pH buffer.

[0054] In one embodiment, the invention provides a method of reducing or preventing a Staphylococcal infection, disease or condition associated with a *Staphylococcus* bacteria in a subject, the method comprising the step of administering a therapeutically or prophylactically amount of an immunogenic composition as described herein to the subject. In one embodiment the infection, disease or condition is selected from the group consisting of invasive *Staphylococcus aureus*, sepsis and carriage.

[0055] In one embodiment, the invention provides a method of reducing or preventing a Staphylococcal infection in a subject undergoing a surgical procedure, the method comprising the step of administering a prophylactically effective amount of an immunogenic composition as described herein to the subject prior to the surgical procedure.

[0056] In one embodiment, the method of the invention comprises the substitution of CDI for CDT.

[0057] In one embodiment, the invention provides a *Staphylococcus aureus* Type 5 or 8 capsular polysaccharide having a molecular weight of between 50 kDa and 800 kDa covalently bound to a carrier protein; wherein the combined

molecular weight of the polysaccharide covalently bound to the carrier protein is between about 400 kDa and 5000 kDa.

[0058] In one embodiment, the polysaccharide covalently bound to carrier protein comprises a polysaccharide portion that has a molecular weight range of between 70 kDa and 300 kDa. In one embodiment, the polysaccharide covalently bound to carrier protein has a molecular weight range of between 500 kDa and 2500 kDa.

[0059] In one embodiment, the carrier protein portion of the polysaccharide covalently bound to carrier protein comprises CRM_{197}. In one embodiment the CRM_{197} is covalently linked to the polysaccharide through a carbamate linkage, an amide linkage, or both. In some embodiments, the molar ratio of conjugated lysines to CRM_{197} is about 10:1 to about 25:1. In some embodiments, the polysaccharide covalently bound to carrier protein comprises at least one covalent linkage between CRM_{197} at least at every 5 to 10 saccharide repeat units of the polysaccharide. In some embodiments, the polysaccharide covalently bound to carrier protein comprises at least one linkage between CRM_{197} and polysaccharide occurs at every 5 saccharide repeat units of the polysaccharide. In some embodiments, the CRM_{197} portion of the polysaccharide covalently bound to the CRM_{197} comprises 5 to 22 lysines covalently linked to the polysaccharide. In some embodiments, the CRM_{197} portion of the polysaccharide covalently bound to the CRM_{197} comprises 5 to 23 lysines covalently linked to the polysaccharide. In some embodiments, the CRM_{197} portion of the polysaccharide covalently bound to carrier protein of comprises 8 to 15 lysines covalently linked to the polysaccharide. In some embodiments, the CRM_{197} portion of the polysaccharide covalently bound to carrier protein of comprises 8 to 12 lysines covalently linked to the polysaccharide.

[0060] In one embodiment the invention provides an immunogenic composition comprising a *S. aureus* type 5 or 8 polysaccharide covalently bound to carrier protein as described herein and at least one of an adjuvant, diluent, or carrier.

[0061] In one embodiment, the invention provides a method of administering an immunogenic composition comprising a *S. aureus* type 5 or 8 polysaccharide

covalently bound to carrier protein as described herein to a subject to generate an immune response as described herein.

[0062] In one embodiment, the invention provides a method of isolating a polysaccharide with a molecular weight between 20 kDa and 1000 kDa.

[0063] In one embodiment, the invention provides an antibody generated by a capsular polysaccharide, an immunogenic conjugate, or an immunogenic composition of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0064] The present invention will be better understood and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

[0065] **Figure 1** shows a repeating polysaccharide structure of *S. aureus* serotype 8 capsular polysaccharide (N-acetyl mannosaminuronic acid is ManNAca, N-acetyl L-fucosamine is L-FucNAc, and N-acetyl D-fucosamine is D-FucNAc).

[0066] **Figure 2A** shows an analysis of fractions from ion exchange chromatography (Q-Sepharose) for *S. aureus* serotype 8 capsular polysaccharide (*O*-Acetyl Assay) and teichoic acid (phosphate assay); **Figure 2B** shows an analysis of fractions from ion exchange chromatography (Q-Sepharose) for *S. aureus* serotype 8 capsular polysaccharide by double immunodiffusion assay.

[0067] **Figure 3A** shows the effect of pH (3.5, 4 or 5) at 95°C on the reduction of *S. aureus* serotype 8 capsular polysaccharide molecular weight in heat treatment; **Figure 3B** shows the effect of temperature (55°C, 75°C or 95°C) at pH 3.5 on the reduction of *S. aureus* serotype 8 capsular polysaccharide molecular weight in heat treatment.

[0068] **Figure 4** shows the molecular weight of purified *S. aureus* serotype 8 capsular polysaccharide compared to serotype 5 capsular polysaccharide over time during heat treatment at pH 3.5 and 4.5, respectively, and 95°C.

[0069] **Figure 5** shows increased survival in mice that received a serotype 8 capsular polysaccharide-CRM_{197} conjugate (diamonds) compared to $AlPO_4$-treated controls (circles).

[0070] **Figure 6** shows a repeating polysaccharide structure of *S. aureus* serotype 5 polysaccharide (*N*-acetyl mannosaminuronic acid is ManNAcA, *N*-acetyl L-fucosamine is L-FucNAc, and *N*-acetyl D-fucosamine is D-FucNAcA).

[0071] **Figure 7A** shows an analysis of fractions from ion exchange chromatography (Q-Sepharose) for *S. aureus* serotype 5 polysaccharide (O-Acetyl Assay) and teichoic acid (phosphate assay); **Figure 7B** shows an analysis of fractions from ion exchange chromatography (Q-Sepharose) for *S. aureus* serotype 5 polysaccharide by double immunodiffusion assay.

[0072] **Figure 8A** shows the effect of pH (3.5, 4 or 5) at 95°C on the reduction of *S. aureus* serotype 5 capsular polysaccharide molecular weight in heat treatment; **Figure 8B** shows the effect of temperature (55°C, 75°C or 95°C) at pH 3.5 on the reduction of *S. aureus* serotype 5 capsular polysaccharide molecular weight in heat treatment.

[0073] **Figure 9** shows reduced pyelonephritis in mice that received a serotype 5 polysaccharide-CRM_{197} conjugate compared to PBS-treated controls (shaded area is the treated mice).

[0074] **Figure 10** shows colony forming units (CFU) recovered in kidneys after challenge with *S. aureus* PFESA0266 in mice vaccinated with high molecular weight (HMW) CP5-CRM, low molecular weight (LMW) CP5-CRM or PP5-CRM control

[0075] **Figure 11** shows a comparison of OPA titers (geomean) from serum obtained from mice vaccinated with different formulations of polysaccharide conjugate (high molecular weight (HMW) CP5-CRM, low molecular weight (LMW) CP5-CRM). Groups consisted of 5 to 9 mice.

DETAILED DESCRIPTION

Overview

[0076] The present invention relates to immunogenic conjugates comprising *S. aureus* serotype 5 or 8 capsular polysaccharides conjugated to carrier proteins and methods for their preparation and use. Novel features of the immunogenic conjugates of the invention include the molecular weight profiles of the polysaccharides and resulting conjugates, the ratio of conjugated lysines per

CRM_{197} carrier protein and number of lysines covalently linked to the polysaccharide, the number of covalent linkages between the carrier protein and the polysaccharide as a function of repeat units of the polysaccharide, and the relative amount of free polysaccharide compared to the total polysaccharide. The term "free polysaccharide" as used herein means a polysaccharide that is not conjugated to the carrier protein, but is nevertheless present in the conjugate composition.

[0077] Methods for making the immunogenic conjugates of the invention involve covalent conjugation of the capsular polysaccharides with the carrier proteins using conjugation chemistry involving CDI (1,1-carbonyldiimidazole), CDT (1,1-carboyl-di-1,2,4-triazole) or PDPH (3-(2-pyridyldithio)-propionyl hydrazide). CDI is specific for CP8 conjugation only. Use of CDI/CDT results in a one-carbon or zero-carbon linker between capsular polysaccharide and carrier protein, while use of PDPH results in a covalent thioether bond between capsular polysaccharide and carrier protein.

[0078] Additional cross linkers for –SH (thiolated CP) to $-NH_2$ linkages include but are not limited to: sulfo-LC-SMPT; sulfo-LC-SMPT (4-sulfosuccinimidyl-6-methyl-a-(2-pyridyldithio)toluamido]hexanoate)); sulfo-KMUS (*N*-[k-maleimidoundecanoyloxy]sulfosuccinimide ester); sulfo-LC-SPDP (sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate) which cleaves by thiols; sulfo-SMPB (sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate); sulfo-SIAB (*N*-sulfosuccinimidyl[4-iodoacetyl]aminobenzoate); sulfo-EMCS ([N-e-maleimidocaproyloxy]sulfosuccinimide ester); EMCA (N-e-maleimidocaproic acid); sulfo-SMCC (sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate); sulfo-MBS (*m*-maleimidobenzoyl-N-hydroxysulfosuccinimide ester); sulfo-GMBS (N-[g-maleimidobutyryloxy]sulfosuccinimide ester); BMPA (N-β-maleimidopropionic acid); 2-immunothiolane hydrochloride; 3-(2-pyridyldithio) propionic acid N-succinimidyl ester; 3-malemidopropionic acid N-succinimidyl ester; 4-maleimidobutyric acid N-succinimidyl ester; SMPT (4-succinimidyloxycarbonyl-methyl-a-[2-pyridyldithio]toluene); LC-SMCC (succinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxy-[6-amidocaproate]); KMUA (N-k-maleimidoundecanoic acid); LC-SPDP (succinimidyl 6-(3-[2-

pyridyldithio]-propionamido)hexanoate); SMPH (succinimidyl-6-[β-maleimidopropionamido]hexanoate); SMPB (succinimidyl 4-[*p*-maleimidophenyl]butyrate); SIAB (*N*-succinimidyl[4-iodoacetyl]aminobenzoate); EMCS ([N-e-Maleimidocaproyloxy]succinimide ester); SMCC (succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate); MBS (*m*-Maleimidobenzoyl-N-hydroxysuccinimide ester); SBAP (succinimidyl 3-[bromoacetamido]propionate); BMPS (*N*-[β-maleimidopropyloxy]succinimide ester); AMAS N-(a-maleimidoacetoxy) succinimide ester); SIA (*N*-succinimidyl iodoacetate); and N-succinimidyl (4-iodoacetyl)-aminobenzoate.

[0079] The agents can also be crosslinked using crosslinkers for -SH to –OH groups. Such cross linkers include but are not limited to PMPI (N-[*p*-maleimidophenyl]isocyanate).

[0080] The compositions and methods described herein are useful in a variety of applications. For example, the conjugates can be used in the production of conjugate immunogenic compositions to protect recipients from *S. aureus* infections. Alternatively, the various conjugates can be used in the production of antibodies against bacterial capsular polysaccharides, which subsequently can be used in research and clinical laboratory assays, such as bacterial detection and serotyping. Such antibodies may also be used to confer passive immunity to a subject. In some embodiments, the antibodies produced against bacterial polysaccharides are functional in either an animal efficacy model or in an opsonophagocytic killing assay.

[0081] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. In describing the embodiments and claiming the invention, certain terminology will be used in accordance with the definitions set out below.

[0082] As used herein, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus, *e.g.*, references to "the method" includes one or more methods, and/or steps of the type described

herein and/or which will become apparent to one of ordinary skill in the art upon reading this disclosure and so forth.

[0083] As used herein, "about" means within a statistically meaningful range of a value such as a stated concentration range, time frame, molecular weight, temperature or pH. 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" will depend upon 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.

[0084] 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 closed ended. Accordingly, these terms refer to the inclusion of a particular ingredient or set of ingredients and the exclusion of all other ingredients.

Immunogenic conjugates

[0085] As described above, the present invention relates to immunogenic conjugates comprising *S. aureus* serotype 5 or 8 capsular polysaccharides

conjugated to carrier proteins. One embodiment of the invention provides immunogenic conjugates comprising a *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to carrier molecule or protein having one or more of the following features: the polysaccharide has a molecular weight of between 50 kDa and 700 kDa; the immunogenic conjugate has a molecular weight of between 500 kDa to 2500 KDa; and the conjugate comprises less than about 30% free polysaccharide relative to total polysaccharide. In some embodiments, the polysaccharide has a molecular weight of between 20 kDa and 1000 kDa. In some embodiments the immunogenic conjugate has a molecular weight of between 200 kDa and 5000 kDa. In other embodiments, the conjugate comprises less than about 25%, about 20%, about 15%, about 10%, or about 5% free polysaccharide relative to total polysaccharide.

[0086] The "conjugates" as used herein 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.

[0087] 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" refer 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 other processes known in the art.

[0088] The molecular weight of the *S. aureus* 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 of "high molecular weight capsular polysaccharides" is contemplated for use in the compositions and methods of the present invention. In one embodiment of the invention, high molecular weight serotype 5 or 8 capsular polysaccharide can be isolated and purified ranging from 20 kDa to 1000 kDa in molecular weight. In one embodiment of the invention, high molecular weight serotype 5 or 8 capsular polysaccharide can be isolated and purified ranging from 50 kDa to 700 kDa in molecular weight. In one embodiment of the invention, high molecular weight serotype 5 or 8 capsular polysaccharide can be isolated and purified ranging from 50 kDa to 300 kDa in molecular weight. In one embodiment, high molecular weight serotype 5 or 8 capsular polysaccharide can be isolated and purified ranging from 70 kDa to 300 kDa in molecular weight. In one embodiment, high molecular weight serotype 5 or 8 capsular polysaccharide can be isolated and purified ranging from 90 kDa to 250 kDa in molecular weight. In one embodiment, high molecular weight serotype 5 or 8 capsular polysaccharide can be isolated and purified ranging from 90 kDa to 150 kDa in molecular weight. In one embodiment, high molecular weight serotype 5 or 8 capsular polysaccharide can be isolated and purified ranging from 90 kDa to 120 kDa in molecular weight. In one embodiment, high molecular weight serotype 5 or 8 capsular polysaccharide can be isolated and purified ranging from 80 kDa to 120 kDa in molecular weight. Other ranges of high molecular weight serotype 5 or 8 capsular polysaccharide that can be isolated and purified by the methods of this invention include 70 kDa to 100 kDa in molecular weight; 70 kDa to 110 kDa in molecular weight; 70 kDa to 120 kDa in molecular weight; 70 kDa to 130 kDa in molecular weight; 70 kDa to 140 kDa in molecular weight; 70 kDa to 150 kDa in molecular weight; 70 kDa to 160 kDa in molecular weight; 80 kDa to 110 kDa in molecular weight; 80 kDa to 120 kDa in molecular weight; 80 kDa to 130 kDa in molecular weight; 80 kDa to 140 kDa in molecular weight; 80 kDa to 150 kDa in molecular weight; 80 kDa to 160 kDa in molecular weight; 90 kDa to 110 kDa in molecular weight; 90 kDa to 120 kDa in molecular weight; 90 kDa to 130 kDa in molecular weight; 90 kDa to 140 kDa in molecular weight; 90 kDa to 150 kDa in molecular weight; 90 kDa to 160 kDa in molecular weight; 100 kDa to 120 kDa in molecular weight; 100 kDa to 130 kDa in molecular weight;

100 kDa to 140 kDa in molecular weight; 100 kDa to 150 kDa in molecular weight; 100 kDa to 160 kDa in molecular weight; and similar desired molecular weight ranges. Any whole number integer within any of the above ranges is contemplated as an embodiment of the invention.

[0089] In one embodiment, the conjugate has a molecular weight of between about 50 kDa and about 5000 kDa in molecular weight. In one embodiment, the conjugate has a molecular weight of between about 200 kDa and about 5000 kDa in molecular weight. In one embodiment, the immunogenic conjugate has a molecular weight of between about 500 kDa and about 2500 kDa. In one embodiment, the immunogenic conjugate has a molecular weight of between about 500 kDa and about 2500 kDa. In one embodiment, the immunogenic conjugate has a molecular weight of between about 600 kDa and about 2800 kDa. In one embodiment, the immunogenic conjugate has a molecular weight of between about 700 kDa and about 2700 kDa. In one embodiment, the immunogenic conjugate has a molecular weight of between about 1000 kDa and about 2000 kDa; between about 1800 kDa and about 2500 kDa; between about 1100 kDa and about 2200 kDa; between about 1900 kDa and about 2700 kDa; between about 1200 kDa and about 2400 kDa; between about 1700 kDa and about 2600 kDa; between about 1300 kDa and about 2600 kDa; between about 1600 kDa and about 3000 kDa. Any whole number integer within any of the above ranges is contemplated as an embodiment of the invention

[0090] As used herein, "immunogenic" means an ability of an antigen (or an epitope of the antigen), such as a bacterial capsular polysaccharide or a conjugate immunogenic composition comprising the antigen, to elicit an immune response in a host such as a mammal, either humorally or cellularly mediated, or both. Accordingly, "immunogenic conjugate" or "conjugate" as used herein means any immunogenic conjugate containing an antigen or antigenic determinant (*i.e.*, epitope) of a bacterial capsular polysaccharide conjugated to a carrier molecule that can be used to elicit an immune response. The immunogenic conjugate may serve to sensitize the host by the presentation of the antigen in association with MHC molecules at a cell surface. In addition, antigen-specific T-cells or antibodies can be generated to allow for the future protection of an immunized

host. Immunogenic conjugates thus can protect the host from one or more symptoms associated with infection by the bacteria, or may protect the host from death due to the infection with the bacteria associated with the capsular polysaccharide. Immunogenic conjugates may also be used to generate polyclonal or monoclonal antibodies, which may be used to confer passive immunity to a subject. Immunogenic conjugates may also be used to generate antibodies that are functional as measured by the killing of bacteria in either an animal efficacy model or via an opsonophagocytic killing assay.

[0091] 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.

[0092] "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.

[0093] The term "antigen" generally refers to a biological molecule, usually a protein, peptide, polysaccharide or conjugate in an immunogenic composition, or 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 a 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. "Antigen" also 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, N.J. 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.*, US Patent No. 4,708,871; Geysen *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen *et al.* (1986) *Molec. Immunol.* 23:709-715; each of which is incorporated herein by reference as if set forth in its entirety. 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, "antigen" also can 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, so 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, *e.g.*, polyepitopes, flanking epitopes, and other recombinant or synthetically derived antigens (Bergmann *et al.* (1993) *Eur. J. Immunol.* 23:2777 2781; Bergmann *et al.* (1996) *J. Immunol.* 157:3242-3249; Suhrbier (1997) *Immunol. Cell Biol.* 75:402 408; Gardner *et al.* (1998) 12th World AIDS Conference, Geneva, Switzerland, Jun. 28 to Jul. 3, 1998).

[0094] A "protective" immune response refers to the ability of an immunogenic composition to elicit an immune response, either humoral or cell mediated, or both, which serves to protect a 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. In general, a "protective immune response" would include the induction of an increase in antibody levels specific for a particular antigen in at least 50% of subjects, including some level of measurable functional antibody responses to each antigen. 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, including some level of measurable functional antibody responses to each antigen. In certain embodiments, opsonising antibodies correlate with a protective immune response. Thus, protective immune response may be assayed by measuring the percent decrease in the bacterial count in an opsonophagocytosis assay, for instance those described below. Preferably, there is a decrease in bacterial count of at least 10%, 25%, 50%, 65%, 75%, 80%, 85%, 90%, 95% or more. The "immunogenic amount" of a particular conjugate in a composition are generally dosed based on total polysaccharide, conjugated and

non-conjugated for that conjugate. For example, a serotype 5 or 8 capsular polysaccharide conjugate with 20% free polysaccharide will have about 80 mcg of conjugated polysaccharide and about 20 mcg of non-conjugated polysaccharide in a 100 mcg dose. The protein contribution to the conjugate is usually not considered when calculating the dose of a conjugate. The amount of conjugate can vary depending upon the staphylococcal serotype. Generally, each dose will comprise 0.1 to 100 mcg of polysaccharide, particularly 0.1 to 10 mcg, and more particularly 1 to 10 mcg.

[0095] 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.

[0096] As shown in Figure 1, *S. aureus* serotype 5 and 8 capsular polysaccharides have the following structures: serotype 5 [→4)-β-D-ManNAcA-(1→4) -3-O-Ac -α-L-FucNAc-(1→3)-β-DFucNAc-(1→]$_n$. and serotype 8 [→3)-4-O-Ac-β-D-ManNAcA-(1→3)-α-L-FucNAc-(1→3)-β-DFucNAc-(1→]$_n$. *See*, Jones (2005) *Carbohydr. Res.* 340:1097-1106. Serotype 8 capsular polysaccharide has similar trisaccharide repeating units to serotype 5 capsular polysaccharide; however, they differ in the sugar linkages and in sites of *O*-acetylation, which produces serologically distinct patterns of immunoreactivity (Fournier *et al.* (1984) *Infect. Immun.* 45:87-93; and Moreau *et al.* (1990) *Carbohydr. Res.* 201:285-297). Serotype 8 and 5 capsular polysaccharides are therefore relatively complex carbohydrates that are water soluble, usually acidic, and were previously thought to have molecular weights of approximately 25 kDa (Fattom (1990) *Infect. Immun.* 58, 2367-2374).

[0097] In some embodiments, the serotype 5 and/or 8 capsular polysaccharides of the invention are O-acetylated. In some embodiments, the degree of

O-acetylation of type 5 capsular polysaccharide or oligosaccharide is 10-100%, 20-100%, 30-100%, 40-100%, 50-100%. 60-100%, 70-100%, 80-100%, 90-100%, 50- 90%, 60-90%, 70-90% or 80-90%. In some embodiments, the degree of O-acetylation of type 8 capsular polysaccharide or oligosaccharide is 10-100%, 20-100%, 30-100%, 40-100%, 50-100%. 60-100%, 70-100%, 80-100%, 90-100%, 50-90%, 60-90%, 70-90% or 80-90%. In some embodiments, the degree of O-acetylation of type 5 and type 8 capsular polysaccharides or oligosaccharides is 10-100%, 20-100%, 30-100%, 40-100%, 50-100%. 60-100%, 70-100%, 80-100%, 90-100%, 50-90%, 60-90%, 70-90% or 80-90%.

[0098] 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 and Jones 1996, Carbohydrate Research 296; 83-96, Jones and Lemercinier 2002, J Pharmaceutical and Biomedical analysis 30; 1233-1247, WO 05/033148 or WO 00/56357). Another commonly used method is described by Hestrin (1949) J. Biol. Chem. 180; 249-261.

[0099] In some embodiments, the serotype 5 and/or 8 capsular polysaccharides of the invention are used to generate antibodies that are 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. Functional killing may not be demonstrated using an assay that monitors the generation of antibodies alone, which is not indicative of the importance of O-acetylation in efficacy.

[0100] Capsular polysaccharides such as serotype 5 or 8 can be obtained directly from bacteria using isolation procedures known to one of ordinary skill in the art. *See, e.g.*, Fournier *et al.* (1984), *supra*; Fournier *et al.* (1987) *Ann. Inst. Pasteur/Microbiol.* 138:561-567; US Patent Application Publication No, 2007/0141077; and Int'l Patent Application Publication No. WO 00/56357; each of which is incorporated herein by reference as if set forth in its entirety). In addition, they can be produced using synthetic protocols. Moreover, serotype 5 or 8 capsular polysaccharide can be recombinantly produced using genetic engineering procedures also known to one of ordinary skill in the art (*see*, Sau *et al.* (1997) *Microbiology* 143:2395-2405; and US Patent No. 6,027,925; each of which is incorporated herein by reference as if set forth in its entirety).

[0101] One *S. aureus* strain that can be used to obtain isolated serotype 8 capsular polysaccharide is *S. aureus* R2 PFESA0286. This strain was selected by flow cytometry with rabbit anti-serotype 8 polysaccharide antibodies after cultivation of *S. aureus* PFESA0286 (American Type Culture Collection; Manassas, VA; ATCC Accession No. 49525;) in Modified Frantz Broth. Two populations, R1 and R2, were observed during flow cytometry. R1 and R2 were purified and re-cultured. R2 yielded a serotype 8 capsular polysaccharide. Flow cytometric analysis showed a homogenous fluorescence intensity. As such, R2 was selected for serotype 8 capsular polysaccharide production.

[0102] One *S. aureus* strain that can be used to obtain isolated serotype 5 capsular polysaccharide is *S. aureus* PFESA0266. This strain produces serotype 5 capsular polysaccharide during growth, and production peaks when cells are in a stationary phase. Other S. *aureus* type 5 or type 8 strains can be used to make the respective polysaccharides that are obtained either from established culture collections or clinical specimens.

[0103] Another component of the immunogenic conjugate of the invention is a carrier molecule or protein to which the bacterial capsular polysaccharide is conjugated. The term "protein carrier" or "carrier protein" 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 to an carrier can enhance the immunogenicity of the antigen. The conjugation can be performed by standard procedures. Preferred protein carriers for the antigens are toxins, toxoids or any mutant cross-reactive material (CRM) of the toxin from tetanus, diphtheria, pertussis, *Pseudomonas*, *E. coli*, *Staphylococcus* and *Streptococcus*. In one embodiment, a particularly preferred carrier is of diphtheria toxoid CRM_{197}, derived from *C. diphtheriae* strain C7 (β197), which produces CRM_{197} protein. This strain has ATCC accession No. 53281. A method for producing CRM_{197} is described in US Patent No. 5,614,382, which is incorporated herein by reference as if set forth in its entirety. Alternatively, a fragment or epitope of the protein carrier or other immunogenic protein can be used. For example, a haptenic antigen can be coupled to a T-cell epitope of a bacterial toxin, toxoid or CRM. *See*, US Patent Application No. 150,688, filed Feb. 1, 1988,

entitled "Synthetic Peptides Representing a T-Cell Epitope as a Carrier Molecule For Conjugate Vaccines"; incorporated herein by reference as if set forth in its entirety. Other suitable carrier proteins include inactivated bacterial toxins such as cholera toxoid (*e.g.*, as described in Int'l Patent Application No. WO 2004/083251), *E. coli* LT, *E. coli* ST, and exotoxin A from *Pseudomonas aeruginosa*. Bacterial outer membrane proteins such as outer membrane complex c (OMPC), porins, transferrin binding proteins, pneumolysin, pneumococcal surface protein A (PspA), pneumococcal adhesion protein (PsaA) or *Haemophilus influenzae* protein D also can be used. Other proteins, such as ovalbumin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or purified protein derivative of tuberculin (PPD) also can be used as carrier proteins.

[0104] Accordingly, in one embodiment, the carrier protein within the immunogenic conjugate of the invention is CRM_{197}, and the CRM_{197} is covalently linked to the capsular polysaccharide via a carbamate linkage, an amide linkage, or both. In some embodiments, the carrier protein within the immunogenic conjugate of the invention is CRM_{197}, and the CRM_{197} is covalently linked to the capsular polysaccharide via a thioether bond. The number of lysine residues in the carrier protein that become conjugated to a capsular polysaccharide can be characterized as a range of conjugated lysines. For example, in a given immunogenic composition, the CRM_{197} may comprise 5 to 15 lysines out of 39 covalently linked to the capsular polysaccharide. Another way to express this parameter is that 12% to 40% of CRM_{197} lysines are covalently linked to the capsular polysaccharide. For example, in a given immunogenic composition, the CRM_{197} may comprise 18 to 22 lysines out of 39 covalently linked to the capsular polysaccharide. Another way to express this parameter is that 40% to 60% of CRM_{197} lysines are covalently linked to the capsular polysaccharide. In some embodiments, the CRM_{197} comprises 5 to 15 lysines out of 39 covalently linked to CP8. Another way to express this parameter is that 12% to 40% of CRM_{197} lysines are covalently linked to CP8. In some embodiments, the CRM_{197} comprises 18 to 22 lysines out of 39 covalently linked to CP5. Another way to express this parameter is that 40% to 60% of CRM_{197} lysines are covalently linked to CP5.

[0105] As discussed above, the number of lysine residues in the carrier protein conjugated to the capsular polysaccharide can be characterized as a range of conjugated lysines, which may be expressed as a molar ratio. For example, the molar ratio of conjugated lysines to CRM_{197} in the CP8 immunogenic conjugate can be between about 18:1 to about 22:1. In one embodiment, the range of molar ratio of conjugated lysines to CRM_{197} in the CP8 immunogenic conjugate can be between about 15:1 to about 25:1. In one embodiment, the range of molar ratio of conjugated lysines to CRM_{197} in the CP8 immunogenic conjugate can be between about 14:1 to about 20:1; about 12:1 to about 18:1; about 10:1 to about 16:1; about 8:1 to about 14:1; about 6:1 to about 12:1; about 4:1 to about 10:1; about 20:1 to about 26:1; about 22:1 to about 28:1; about 24:1 to about 30:1; about 26:1 to about 32:1; about 28:1 to about 34:1; about 30:1 to about 36:1; about 5:1 to about 10:1; about 5:1 to about 20:1; about 10:1 to about 20:1; or about 10:1 to about 30:1. Also, the molar ratio of conjugated lysines to CRM197 in the CP5 immunogenic conjugate can be between about 3:1 and 25:1. In one embodiment, the range of molar ratio of conjugated lysines to CRM197 in the CP5 immunogenic conjugate can be between about 5:1 to about 20:1. In one embodiment, the range of molar ratio of conjugated lysines to CRM197 in the CP5 immunogenic conjugate can be between about 4:1 to about 20:1; about 6:1 to about 20:1; about 7:1 to about 20:1; about 8:1 to about 20:1; about 10:1 to about 20:1; about 11:1 to about 20:1; about 12:1 to about 20:1; about 13:1 to about 20:1; about 14:1 to about 20:1; about 15:1 to about 20:1; about 16:1 to about 20:1; about 17:1 to about 20:1; about 18:1 to about 20:1; about 5:1 to about 18:1; about 7:1 to about 16:1; or about 9:1 to about 14:1.

[0106] Another way to express the number of lysine residues in the carrier protein conjugated to the capsular polysaccharide can be as a range of conjugated lysines. For example, in a given CP8 immunogenic conjugate, the CRM_{197} may comprise 5 to 15 lysines out of 39 covalently linked to the capsular polysaccharide. Alternatively, this parameter can be expressed as a percentage. For example, in a given CP8 immunogenic conjugate, the percentage of conjugated lysines can be between 10% to 50%. In some embodiments, 20% to 50% of lysines can be covalently linked to CP8. Alternatively still, 30% to 50% of CRM_{197} lysines can

be covalently linked to CP8; 10% to 40% of CRM_{197} lysines; 10% to 30% of CRM_{197} lysines; 20% to 40% of CRM_{197} lysines; 25% to 40% of CRM_{197} lysines; 30% to 40% of CRM_{197} lysines; 10% to 30% of CRM_{197} lysines; 15% to 30% of CRM_{197} lysines; 20% to 30% of CRM_{197} lysines; 25% to 30% of CRM_{197} lysines; 10% to 15% of CRM_{197} lysines; or 10% to 12% of CRM_{197} lysines are covalently linked to CP8. Also, in a given CP5 immunogenic conjugate, the CRM_{197} may comprise 18 to 22 lysines out of 39 covalently linked to the capsular polysaccharide. Alternatively, this parameter can be expressed as a percentage. For example, in a given CP5 immunogenic conjugate, the percentage of conjugated lysines can be between 40% to 60%. In some embodiments, 40% to 60% of lysines can be covalently linked to CP5. Alternatively still, 30% to 50% of CRM_{197} lysines can be covalently linked to CP5; 20% to 40% of CRM_{197} lysines; 10% to 30% of CRM_{197} lysines; 50% to 70% of CRM_{197} lysines; 35% to 65% of CRM_{197} lysines; 30% to 60% of CRM_{197} lysines; 25% to 55% of CRM_{197} lysines; 20% to 50% of CRM_{197} lysines; 15% to 45% of CRM_{197} lysines; 10% to 40% of CRM_{197} lysines; 40% to 70% of CRM_{197} lysines; or 45% to 75% of CRM_{197} lysines are covalently linked to CP5.

[0107] The frequency of attachment of the capsular polysaccharide chain to a lysine on the carrier molecule is another parameter for characterizing conjugates of capsule polysaccharides. For example, in one embodiment, at least one covalent linkage between CRM_{197} and polysaccharide occurs for at least every 5 to 10 saccharide repeat units of the capsular polysaccharide. In another embodiment, there is at least one covalent linkage between CRM_{197} and capsular polysaccharide for every 5 to 10 saccharide repeat units; every 2 to 7 saccharide repeat units, every 3 to 8 saccharide repeat units; every 4 to 9 saccharide repeat units; every 6 to 11 saccharide repeat units; every 7 to 12 saccharide repeat units; every 8 to 13 saccharide repeat units; every 9 to 14 saccharide repeat units; every 10 to 15 saccharide repeat units; every 2 to 6 saccharide repeat units, every 3 to 7 saccharide repeat units; every 4 to 8 saccharide repeat units; every 6 to 10 saccharide repeat units; every 7 to 11 saccharide repeat units; every 8 to 12 saccharide repeat units; every 9 to 13 saccharide repeat units; every 10 to 14 saccharide repeat units; every 10 to 20 saccharide repeat units; or every 5 to 10

saccharide repeat units of the capsular polysaccharide. In another embodiment, at least one linkage between CRM_{197} and capsular polysaccharide occurs for every 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 saccharide repeat units of the capsular polysaccharide.

[0108] One embodiment of the invention provides an immunogenic composition comprising any of the immunogenic conjugates comprising a *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein described above.

[0109] 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 protect or treat a human susceptible to *S. aureus* infection, by means of administering the immunogenic compositions via a systemic, dermal or mucosal route or be used to generate a polyclonal or monoclonal antibody preparation that could be used to confer passive immunity on another subject. These administrations can include injection via the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or via mucosal administration to the oral/alimentary, respiratory or genitourinary tracts. In one embodiment, intranasal administration is used for the treatment or prevention of nasopharyngeal carriage of *S. aureus*, thus attenuating infection at its earliest stage. Immunogenic compositions may also be used to generate antibodies that are functional as measured by the killing of bacteria in either an animal efficacy model or via an opsonophagocytic killing assay.

[0110] 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.

[0111] The immunogenic compositions of the present invention may also include one or more of the following antigens: ClfA, ClfB, SdrC, SdrD, SdrE MntC/SitC/Saliva Binding Protein, IsdB, IsdA, Opp3a, DltA, HtsA, LtaS, SdrH, SrtA, SpA, SBI, alpha-hemolysin (hla), beta-hemolysin, fibronectin-binding protein A (fnbA), coagulase, map, Panton-Valentine leukocidin (pvl),

gamma-toxin (hlg), ica, immunodominant ABC transporter, RAP, autolysin, laminin receptors, IsaA/PisA, IsaB/PisB , SPOIIIE, SsaA, EbpS, SasF, SasH, EFB (FIB), FnbB, Npase, EBP, bone sialo binding protein II, aureolysin precursor (AUR)/Sepp1, Cna, TSST-1, mecA, dPNAG, GehD, EbhA, EbhB, SSP-1, SSP-2 HBP, vitronectin binding protein, HarA, Enterotoxin A, Enterotoxin B, Enterotoxin C1, and novel autolysin.

[0112] In one embodiment, the immunogenic compositions of the invention further comprise at least one of an adjuvant, a buffer, a cryoprotectant, a salt, a divalent cation, a non-ionic detergent, an inhibitor of free radical oxidation, a diluent or a carrier. In one embodiment, the adjuvant within the immunogenic composition of the invention is an aluminum-based adjuvant. In one embodiment, the adjuvant is an aluminum-based adjuvant selected from the group consisting of aluminum phosphate, aluminum sulfate and aluminum hydroxide. In one embodiment, the adjuvant is aluminum phosphate.

[0113] An adjuvant is a substance that enhances the immune response when administered together with an immunogen or antigen. A number of cytokines or lymphokines have been shown to have immune modulating activity, and thus may be useful in a manner the same or similar to adjuvants, including, but not limited to, the interleukins 1-α, 1-β, 2, 4, 5, 6, 7, 8, 10, 12 (*see, e.g.*, US 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.*, US Patent No. 5,078,996 and ATCC Accession Number 39900); macrophage colony stimulating factor (M-CSF); granulocyte colony stimulating factor (G-CSF); and 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 Caspases, including ICE.

[0114] Suitable adjuvants used to enhance an immune response may further include, without limitation, MPL™ (3-O-deacylated monophosphoryl lipid A, Corixa; Hamilton, MT), which is described in US 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, and those that are described in US Patent No. 6,113,918. One such AGP is 2-[(R)-3-Tetradecanoyloxytetradecanoylamino] ethyl 2-Deoxy-4-O-phosphono-3-O-[(R)-3-tetradecanoyoxytetradecanoyl]-2-[(R)-3-tetradecanoyloxytetradecanoyl-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).

[0115] Still other adjuvants include muramyl peptides, such as N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanine-2-(1'-2' dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE); oil-in-water emulsions, such as MF59 (US 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); incomplete Freund's adjuvant (IFA); aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate; Amphigen; Avridine; L121/squalene; D-lactide-polylactide/glycoside; pluronic polyols; killed *Bordetella*; saponins, such as Stimulon™ QS-21 (Antigenics, Framingham, MA.), described in US Patent No. 5,057,540, Iscomatrix® (CSL Limited, Parkville, Australia), described in US Patent No. 5,254,339, and immunostimulating complexes (ISCOMS); *Mycobacterium*

tuberculosis; bacterial lipopolysaccharides; synthetic polynucleotides such as oligonucleotides containing a CpG motif (*e.g.*, US Patent No. 6,207,646); IC-31 (Intercell AG, Vienna, Austria), described in EP Patent Nos. 1,296,713 and 1,326,634; a pertussis toxin (PT) or mutant thereof, a cholera toxin or mutant thereof (*e.g.*, US 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.*, US Patent Nos. 6,149,919, 7,115,730 and 7,291,588).

[0116] The immunogenic composition optionally can comprise a pharmaceutically acceptable carrier. 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. Water, saline solutions and aqueous dextrose and glycerol solutions can be employed as liquid carriers, particularly for injectable solutions. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. The formulation should suit the mode of administration.

[0117] 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 embodiment, up-regulation of the humoral and/or cell-mediated arms of the immune system is provided. Examples of certain immunomodulators include, *e.g.*, an adjuvant or cytokine, or Iscomatrix® (CSL Limited; Parkville, Australia), described in US Patent No. 5,254,339 among others. Non-limiting examples of adjuvants that can be used in the immunogenic composition of the present invention include the RIBI adjuvant system (Ribi Inc.; Hamilton, MT), alum, mineral gels such as aluminum hydroxide gel, oil-in-water emulsions, water-in-oil emulsions such as, *e.g.*, Freund's complete and incomplete adjuvants, Block copolymer (CytRx; Atlanta, GA), QS-21 (Cambridge Biotech Inc.; Cambridge,

MA), SAF-M (Chiron; Emeryville, CA), 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 in the immunogenic composition of the invention include modified SEAM62 and SEAM 1/2 formulations. Modified SEAM62 is an oil-in-water emulsion 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 mcg/ml Quil A, 100 mcg/ml cholesterol, and 0.5% (v/v) lecithin. Modified SEAM 1/2 is an oil-in-water emulsion comprising 5% (v/v) squalene, 1% (v/v) Span® 85 Detergent, 0.7% (v/v) polysorbate 80 detergent, 2.5% (v/v) ethanol, 100 mcg/ml Quil A, and 50 mcg/ml cholesterol. Other "immunomodulators" that can be included in the immunogenic composition include, *e.g.*, one or more interleukins, interferons, or other known cytokines or chemokines. In one embodiment, the adjuvant may be a cyclodextrin derivative or a polyanionic polymer, such as those described in US Patent Nos. 6,165,995 and 6,610,310, respectively. It is to be understood that the immunomodulator and/or adjuvant to be used will depend on the subject to which the immunogenic composition will be administered, the route of injection and the number of injections to be given.

[**0044**] The immunogenic compositions of the invention may further comprise one or more preservatives in addition to a plurality of staphylococcal capsular polysaccharide-protein conjugates. The FDA requires that biological products in multiple-dose (multi-dose) vials contain a preservative, with only a few exceptions. Vaccine products containing preservatives include vaccines containing benzethonium chloride (anthrax), 2-phenoxyethanol (DTaP, HepA, Lyme, Polio (parenteral)), phenol (Pneumo, Typhoid (parenteral), Vaccinia) and thimerosal (DTaP, DT, Td, HepB, Hib, Influenza, JE, Mening, Pneumo, Rabies). 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, thimerosal and phenylmercuric nitrate.

[**0118**] Formulations of the invention may further comprise one or more of a buffer, a salt, a divalent cation, a non-ionic detergent, a cryoprotectant such as a

sugar, and an anti-oxidant such as a free radical scavenger or chelating agent, or any multiple combination thereof. The choice of any one component, e.g., a chelator, may determine whether or not another component (e.g., a scavenger) is desirable. The final composition formulated for administration should be sterile and/or pyrogen free. The skilled artisan may empirically determine which combinations of these and other components will be optimal for inclusion in the preservative containing immunogenic compositions of the invention depending on a variety of factors such as the particular storage and administration conditions required.

[0119] In certain embodiments, a formulation of the invention which is compatible with parenteral administration comprises one or more physiologically acceptable buffers selected from, but not limited to, Tris (trimethamine), phosphate, acetate, borate, citrate, glycine, histidine and succinate. In certain embodiments, the formulation is buffered to within a pH range of about 6.0 to about 9.0, preferably from about 6.4 to about 7.4.

[0120] In certain embodiments, it may be desirable to adjust the pH of the immunogenic composition or formulation of the invention. The pH of a formulation of the invention may be adjusted using standard techniques in the art. The pH of the formulation may be adjusted to be between 3.0 and 8.0. In certain embodiments, the pH of the formulation may be, or may adjusted to be, between 3.0 and 6.0, 4.0 and 6.0, or 5.0 and 8.0. In other embodiments, the pH of the formulation may be, or may adjusted to be, about 3.0, about 3.5, about 4.0, about 4.5, about 5.0, about 5.5, about 5.8, about 6.0, about 6.5, about 7.0, about 7.5, or about 8.0. In certain embodiments, the pH may be, or may adjusted to be, in a range from 4.5 to 7.5, or from 4.5 to 6.5, from 5.0 to 5.4, from 5.4 to 5.5, from 5.5 to 5.6, from 5.6 to 5.7, from 5.7 to 5.8, from 5.8 to 5.9, from 5.9 to 6.0, from 6.0 to 6.1, from 6.1 to 6.2, from 6.2 to 6.3, from 6.3 to 6.5, from 6.5 to 7.0, from 7.0 to 7.5 or from 7.5 to 8.0. In a specific embodiment, the pH of the formulation is about 5.8.

[0121] In certain embodiments, a formulation of the invention which is compatible with parenteral administration comprises one or more divalent cations,

including but not limited to $MgCl_2$, $CaCl_2$ and $MnCl_2$, at a concentration ranging from about 0.1 mM to about 10 mM, with up to about 5 mM being preferred.

[0122] In certain embodiments, a formulation of the invention which is compatible with parenteral administration comprises one or more salts, including but not limited to sodium chloride, potassium chloride, sodium sulfate, and potassium sulfate, present at an ionic strength which is physiologically acceptable to the subject upon parenteral administration and included at a final concentration to produce a selected ionic strength or osmolarity in the final formulation. The final ionic strength or osmolality of the formulation will be determined by multiple components (e.g., ions from buffering compound(s) and other non-buffering salts. A preferred salt, NaCl, is present from a range of up to about 250 mM, with salt concentrations being selected to complement other components (e.g., sugars) so that the final total osmolarity of the formulation is compatible with parenteral administration (e.g., intramuscular or subcutaneous injection) and will promote long term stability of the immunogenic components of the immunogenic composition formulation over various temperature ranges. Salt-free formulations will tolerate increased ranges of the one or more selected cryoprotectants to maintain desired final osmolarity levels.

[0123] In certain embodiments, a formulation of the invention which is compatible with parenteral administration comprises one or more cryoprotectants selected from but not limited to disaccharides (e.g., lactose, maltose, sucrose or trehalose) and polyhydroxy hydrocarbons (e.g., dulcitol, glycerol, mannitol and sorbitol).

[0124] In certain embodiments, the osmolarity of the formulation is in a range of from about 200 mOs/L to about 800 mOs/L, with a preferred range of from about 250 mOs/L to about 500 mOs/L, or about 300 mOs/L - about 400 mOs/L. A salt-free formulation may contain, for example, from about 5% to about 25% sucrose, and preferably from about 7% to about 15%, or about 10% to about 12% sucrose. Alternatively, a salt-free formulation may contain, for example, from about 3% to about 12% sorbitol, and preferably from about 4% to 7%, or about 5% to about 6% sorbitol. If salt such as sodium chloride is added, then the effective range of

sucrose or sorbitol is relatively decreased. These and other such osmolality and osmolarity considerations are well within the skill of the art.

[0125] In certain embodiments, a formulation of the invention which is compatible with parenteral administration comprises one or more free radical oxidation inhibitors and/or chelating agents. A variety of free radical scavengers and chelators are known in the art and apply to the formulations and methods of use described herein. Examples include but are not limited to ethanol, EDTA, a EDTA/ethanol combination, triethanolamine, mannitol, histidine, glycerol, sodium citrate, inositol hexaphosphate, tripolyphosphate, ascorbic acid/ascorbate, succinic acid/succinate, malic acid/maleate, desferal, EDDHA and DTPA, and various combinations of two or more of the above. In certain embodiments, at least one non-reducing free radical scavenger may be added at a concentration that effectively enhances long term stability of the formulation. One or more free radical oxidation inhibitors/chelators may also be added in various combinations, such as a scavenger and a divalent cation. The choice of chelator will determine whether or not the addition of a scavenger is needed.

[0126] In certain embodiments, a formulation of the invention which is compatible with parenteral administration 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) and Polysorbate-20 (Tween 20), 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), with preferred components Polysorbate-80 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).

[0127] In certain embodiments, a formulation of the invention comprises one or more additional stabilizing agents suitable for parenteral administration, e.g., a reducing agent comprising at least one thiol (-SH) group (e.g., cysteine, N-acetyl cysteine, reduced glutathione, sodium thioglycolate, thiosulfate, monothioglycerol, or mixtures thereof). Alternatively or optionally, preservative-containing

immunogenic composition formulations of the invention may be further stabilized by removing oxygen from storage containers, protecting the formulation from light (e.g., by using amber glass containers).

[0128] Preservative-containing immunogenic composition formulations of the invention may comprise one or more pharmaceutically acceptable carriers or excipients, which includes any excipient that does not itself induce an immune response. Suitable excipients include but are not limited to macromolecules such as proteins, saccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, sucrose (Paoletti et al, 2001, *Vaccine*, 19:2118), trehalose, lactose and lipid aggregates (such as oil droplets or liposomes). Such carriers are well known to the skilled artisan. Pharmaceutically acceptable excipients are discussed, e.g., in Gennaro, 2000, Remington: The Science and Practice of Pharmacy, 20th edition, ISBN:0683306472.

[0129] Compositions of the invention may be lyophilized or in aqueous form, i.e. solutions or suspensions. Liquid formulations may advantageously be administered directly from their packaged form and are thus ideal for injection without the need for reconstitution in aqueous medium as otherwise required for lyophilized compositions of the invention.

[0130] Direct delivery of immunogenic compositions of the present invention to a subject may be accomplished by parenteral administration (intramuscularly, intraperitoneally, intradermally, subcutaneously, intravenously, or to the interstitial space of a tissue); or by rectal, oral, vaginal, topical, transdermal, intranasal, ocular, aural, pulmonary or other mucosal administration. In a preferred embodiment, parenteral administration is by intramuscular injection, e.g., to the thigh or upper arm of the subject. Injection may be via a needle (e.g. a hypodermic needle), but needle free injection may alternatively be used. A typical intramuscular dose is 0.5mL. Compositions of the invention may be prepared in various forms, e.g., for injection either as liquid solutions or suspensions. In certain embodiments, the composition may be prepared as a powder or spray for pulmonary administration, e.g. in an inhaler. In other embodiments, the composition may be prepared as a suppository or pessary, or for nasal, aural or ocular administration, e.g. as a spray, drops, gel or powder.

[0131] The amount of conjugate in each immunogenic composition dose is selected as an amount that induces an immunoprotective response without significant, adverse effects. Such amount can vary depending upon the staphylococcal serotype. Generally, each dose will comprise 0.1 to 100 µg of polysaccharide, particularly 0.1 to 10 µg, and more particularly 1 to 5 µg.

[0132] Optimal amounts of components for a particular immunogenic composition may 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.

Packaging and Dosage Forms

[0133] Immunogenic compositions of the invention may be packaged in unit dose or multi-dose form (e.g. 2 doses, 4 doses, or more). For multi-dose forms, vials are typically but not necessarily preferred over pre-filled syringes. Suitable multi-dose formats include but are not limited to: 2 to 10 doses per container at 0.1 to 2 mL per dose. In certain embodiments, the dose is a 0.5 mL dose. See, e.g., International Patent Application WO2007/127668, which is incorporated by reference herein.

Compositions may be presented in vials or other suitable storage containers, or may be presented in pre-filled delivery devices, e.g., single or multiple component syringes, which may be supplied with or without needles. A syringe typically but need not necessarily contains a single dose of the preservative-containing immunogenic composition of the invention, although multi-dose, pre-filled syringes are also envisioned. Likewise, a vial may include a single dose but may alternatively include multiple doses.

[0134] Effective dosage volumes can be routinely established, but a typical dose of the composition for injection has a volume of 0.5 mL. In certain embodiments, the dose is formulated for administration to a human subject. In certain embodiments, the dose is formulated for administration to an adult, teen, adolescent, toddler or infant (i.e., no more than one year old) human subject and may in preferred embodiments be administered by injection.

[0135] Liquid immunogenic compositions of the invention are also suitable for reconstituting other immunogenic compositions which are presented in lyophilized

form. Where an immunogenic composition is to be used for such extemporaneous reconstitution, the invention provides a kit with two or more vials, two or more ready-filled syringes, or one or more of each, with the contents of the syringe being used to reconstitute the contents of the vial prior to injection, or vice versa.

[0136] Alternatively, immunogenic compositions of the present invention may be lyophilized and reconstituted, e.g., using one of a multitude of methods for freeze drying well known in the art to form dry, regular shaped (e.g., spherical) particles, such as micropellets or microspheres, having particle characteristics such as mean diameter sizes that may be selected and controlled by varying the exact methods used to prepare them. The immunogenic compositions may further comprise an adjuvant which may optionally be prepared with or contained in separate dry, regular shaped (e.g., spherical) particles such as micropellets or microspheres. In such embodiments, the present invention further provides an immunogenic composition kit comprising a first component that includes a stabilized, dry immunogenic composition, optionally further comprising one or more preservatives of the invention, and a second component comprising a sterile, aqueous solution for reconstitution of the first component. In certain embodiments, the aqueous solution comprises one or more preservatives, and may optionally comprise at least one adjuvant (see, e.g., WO2009/109550 (incorporated herein by reference).

[0137] In yet another embodiment, a container of the multi-dose format is selected from one or more of the group consisting of, but not limited to, general laboratory glassware, flasks, beakers, graduated cylinders, fermentors, bioreactors, tubings, pipes, bags, jars, vials, vial closures (e.g., a rubber stopper, a screw on cap), ampoules, syringes, dual or multi-chamber syringes, syringe stoppers, syringe plungers, rubber closures, plastic closures, glass closures, cartridges and disposable pens and the like. The container of the present invention is not limited by material of manufacture, and includes materials such as glass, metals (e.g., steel, stainless steel, aluminum, etc.) and polymers (e.g., thermoplastics, elastomers, thermoplastic-elastomers). In a particular embodiment, the container of the format is a 5 mL Schott Type 1 glass vial with a butyl stopper. The skilled artisan will appreciate that the format set forth above is by no means an exhaustive list, but

merely serve as guidance to the artisan with respect to the variety of formats available for the present invention. Additional formats contemplated for use in the present invention may be found in published catalogues from laboratory equipment vendors and manufacturers such as United States Plastic Corp. (Lima, OH), VWR.

Methods for Making Immunogenic Conjugates

[0138] The present invention also includes methods of making the immunogenic conjugates described herein. Methods for making the immunogenic conjugates of the invention involve covalent conjugation of the capsular polysaccharides with the carrier proteins using conjugation chemistry involving CDI (1,1-carbonyldiimidazole), CDT (1,1-carboyl-di-1,2,4-triazole) or PDPH (3-(2-pyridyldithio)-propionyl hydrazide).

[0139] Accordingly, one embodiment of the invention provides a CDT-based method of making an immunogenic conjugate comprising a *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein, the method comprising the steps of: a) compounding a *S. aureus* serotype 5 or 8 capsular polysaccharide with imidazole or triazole to produce a compounded polysaccharide; b) reacting the compounded polysaccharide with CDT in an organic solvent and about 0.1% to about 0.3% w/v water to produce an activated serotype 5 or 8 capsular polysaccharide; c) purifying the activated serotype 5 or 8 capsular polysaccharide to produce a purified activated serotype 5 or 8 capsular polysaccharide; d) reacting the purified activated serotype 5 or 8 capsular polysaccharide with a carrier protein in the organic solvent to produce a serotype 5 or 8 capsular polysaccharide:carrier protein conjugate; and e) hydrolyzing the serotype 5 or 8 capsular polysaccharide:carrier protein conjugate to remove unreacted activation groups; whereby an immunogenic conjugate comprising a *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein is produced. In one embodiment, prior to step (d), the purified activated serotype 5 or 8 capsular polysaccharide is compounded with a carrier protein.

[0140] In one embodiment of the invention, another CDT-based method of making an immunogenic conjugate is provided comprising a *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein, the method comprising the steps of: a) compounding a *S. aureus* serotype 5 or 8 capsular

polysaccharide with imidazole or triazole to produce a compounded polysaccharide; b) reacting the compounded polysaccharide with CDT in an organic solvent and about 0.1% to about 0.3% w/v water to produce an activated serotype 5 or 8 capsular polysaccharide; c) reacting the activated serotype 5 or 8 capsular polysaccharide with a carrier protein in the organic solvent to produce a serotype 5 or 8 capsular polysaccharide:carrier protein conjugate; and d) hydrolyzing the serotype 5 or 8 capsular polysaccharide:carrier protein conjugate to remove unreacted activation groups; whereby an immunogenic conjugate comprising a *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein is produced.

[0141] In one embodiment, the organic solvent within the CDT-based methods of making an immunogenic conjugate is a polar aprotic solvent. In one embodiment, the organic solvent is a polar aprotic solvent selected from the group consisting of dimethyl sulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide, *N*-methyl-2-pyrrolidone, and hexamethylphosphoramide (HMPA). In one embodiment, the organic solvent is DMSO.

[0142] In one embodiment, the step of reacting the compounded polysaccharide with CDT within the CDT-based methods of making an immunogenic conjugate comprises providing about 20-fold molar excess of CDT compared to the polysaccharide.

In one embodiment, the step of purifying the activated serotype 5 or 8 capsular polysaccharide within the CDT-based methods of making an immunogenic conjugate comprises diafiltration.

[0143] In one embodiment, the carrier protein within the CDT-based methods of making an immunogenic conjugate is CRM_{197}. In one embodiment, the activated serotype 5 or 8 capsular polysaccharide within the methods of making an immunogenic conjugate is reacted with the CRM_{197} in a ratio by weight of about 1:1.

[0144] In one embodiment, the step of hydrolyzing the serotype 5 or 8 polysaccharide:carrier protein conjugate to remove unreacted activation groups within the CDT-based methods of making an immunogenic conjugate comprises dilution into a buffer and maintaining a pH of about 8.8 to about 9.2 for at least 4

hours at about 20°C to about 26°C. In one embodiment, the step of hydrolyzing the serotype 5 or 8 capsular polysaccharide:carrier protein conjugate comprises dilution into a buffer and maintaining a pH of about 9.0 for at least 4 hours at about 23°C.

[0145] In one embodiment, the serotype 5 or 8 capsular polysaccharide:carrier protein conjugate produced according to the CDT-based methods of making an immunogenic conjugate is purified. In one embodiment, purification of the serotype 5 or 8 capsular polysaccharide:carrier protein conjugate comprises diafiltration.

[0146] In one embodiment, prior to reacting the compounded polysaccharide with CDT within the CDT-based methods of making an immunogenic conjugate, the compounded serotype 5 or 8 polysaccharide is lyophilized and resuspended. In one embodiment, both the compounded polysaccharide and the carrier protein are separately lyophilized and re-suspended prior to reacting the compounded polysaccharide with CDT. In one embodiment, the lyophilized compounded polysaccharide and/or the lyophilized carrier protein are resuspended in an organic solvent. In one embodiment, the organic solvent is DMSO.

[0147] In one embodiment, prior to reacting the activated serotype 5 or 8 capsular polysaccharide compounded with a carrier protein within the CDT-based method of making an immunogenic conjugate, the purified activated serotype 5 or 8 capsular polysaccharide and the carrier protein are separately lyophilized and re-suspended. In one embodiment, the carrier protein is CRM_{197} and prior to lyophilization the CRM_{197} is diafiltered against NaCl. In one embodiment, prior to lyophilization the CRM_{197} is diafiltered against NaCl and the w/w ratio of NaCl/CRM is adjusted to about 0.5 to about 1.5.

[0148] One embodiment of the present invention provides a PDPH-based method of making an immunogenic conjugate comprising a *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein, the method comprising the steps of: a) reacting a *S. aureus* serotype 5 or 8 capsular polysaccharide with PDPH and a carbodiimide in an organic solvent to produce a PDPH-linked polysaccharide; b) reacting the PDPH-linked polysaccharide with a reducing agent to produce an activated polysaccharide; c) purifying the activated serotype 5 or 8

capsular polysaccharide to produce a purified activated serotype 5 or 8 capsular polysaccharide; d) reacting a carrier protein with a bromoacetic acid in an organic solvent to produce an activated carrier protein; e) purifying the activated carrier protein to produce a purified activated carrier protein; f) reacting the purified activated serotype 5 or 8 capsular polysaccharide with the purified activated carrier protein to produce a serotype 5 or 8 capsular polysaccharide:carrier protein conjugate; and g) hydrolyzing the serotype 5 or 8 capsular polysaccharide:carrier protein conjugate to remove unreacted activation groups; whereby an immunogenic conjugate comprising a *S. aureus* serotype 5 or 8 capsular polysaccharide conjugated to a carrier protein is produced.

[0149] In one embodiment, the bromoacetic acid used within the PDPH-based methods of making an immunogenic conjugate is a *N*-hydroxysuccinimide ester of bromoacetic acid (BAANS). In one embodiment, the carrier protein used within the PDPH-based methods of the invention is CRM_{197} and the BAANS is added in a CRM_{197}:BAANS ratio by weight of about 1:0.1 to about 1:0.5.

[0150] In one embodiment, the organic solvent within the PDPH-based methods of making an immunogenic conjugate is a polar aprotic solvent. In one embodiment, the organic solvent is a polar aprotic solvent selected from the group consisting of DMSO, DMF, dimethylacetamide, *N*-methyl-2-pyrrolidone, and HMPA. In one embodiment, the organic solvent is DMSO.

In one embodiment, the carbodiimide used within the PDPH-based methods of making an immunogenic conjugate is 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC). In one embodiment, the step of reacting the serotype 5 or 8 capsular polysaccharide with PDPH and EDAC in an organic solvent comprises maintaining a polysaccharide:PDPH:EDAC ratio by weight of about 1:5:3.

[0151] In one embodiment, the reducing agent used within the PDPH-based methods of making an immunogenic conjugate is dithiothreitol (DTT).

[0152] In one embodiment, the steps of purifying the activated serotype 5 or 8 capsular polysaccharide and purifying the carrier protein within the PDPH-based methods of making an immunogenic conjugate each comprise diafiltration.

[0153] In one embodiment, the carrier protein within the PDPH-based methods of making an immunogenic conjugate is CRM_{197}. In one embodiment, the

activated serotype 5 or 8 polysaccharide within the methods of making an immunogenic conjugate is reacted with the CRM_{197} in a ratio by weight of about 1:1.

[0154] In one embodiment, the step of hydrolyzing the serotype 5 or 8 polysaccharide:carrier protein conjugate to remove unreacted activation groups within the PDPH-based methods of making an immunogenic conjugate comprises the addition of cysteamine hydrochloride.

[0155] In one embodiment, the serotype 5 or 8 capsular polysaccharide:carrier protein conjugate produced according to the PDPH-based methods of making an immunogenic conjugate is purified. In one embodiment, purification of the serotype 5 or 8 capsular polysaccharide:carrier protein conjugate comprises diafiltration.

[0156] In one embodiment, prior to reacting the purified activated serotype 5 or 8 capsular polysaccharide with the purified activated carrier protein within the PDPH-based methods of making an immunogenic conjugate, both the purified activated polysaccharide and the purified activated carrier protein are separately lyophilized and re-suspended. In one embodiment, the lyophilized activated polysaccharide and/or the lyophilized activated carrier protein are resuspended in an organic solvent. In one embodiment, the organic solvent is DMSO.

[0157] As used herein, "lyophilization" means a dehydration process in which the bacterial capsular polysaccharide is frozen while the surrounding pressure is reduced in the presence of enough heat to allow frozen water to sublime directly from a solid phase to gas phase. Any method known in the art for lyophilizing polysaccharides can be used. *See, e.g.*, Harris & Angal (1989) "Protein Purification Methods," In: Kennedy & Cabral, eds. "Recovery Processes for Biological Materials" (John Wiley & Sons; 1993); US Patent No. 4,134,214; and Int'l Patent Application Publication No. WO 2003/086471; each of which is incorporated herein by reference as if set forth in its entirety. Optionally, a cryoprotectant can be included during lyophilization, such as for example sucrose, glucose, lactose, trehalose, arabinose, xylose, galactose, sorbitol or mannitol.

[0158] As used herein, "activate" and "activation" means that a bacterial capsular polysaccharide or carrier protein is modified in such a way that it is rendered

amenable to conjugation (*i.e.*, at least one moiety must be rendered capable of covalently bonding to the carrier molecule). For example, with respect to CDT-based conjugation methods of the present invention, the polysaccharide is activated in a low moisture environment (*e.g.*, in DMSO) to form triazole carbamate moieties with available hydroxyls and acyltriazole moieties with carboxylic acids. Activated polysaccharides may then be reacted with CRM_{197} protein, which leads to the nucleophilic displacement of the triazole by lysine residues within CRM_{197} and formation of a carbamate linkage (for activated hydroxyls) and the amide linkage (for activated carboxylic acids). By contrast, with respect to the PDPH-based conjugation methods of the present invention, both the carrier proteins and the polysaccharides are activated prior to conjugation: 1) activation of CRM_{197} involves introducing bromoacetyl groups into the CRM_{197} protein by reaction of amine groups with the N-hydroxysuccimide ester of bromoacetic acid; and 2) activation of polysaccharide involves coupling the carbodiimide-activated carboxylate groups of N-acetylmannosaminouronic acid in the polysaccharide to the hydrazide group of the sulfhydryl-reactive hydrazide heterobifunctional linker PDPH, followed by reduction with DTT. Activated polysaccharides may then be reacted with activated carrier protein such that thiols of PDPH-thiolated polysaccharides react with bromoacetyl groups of activated carrier protein resulting in a covalent thioether linkage formed by bromide displacement.

[0159] According to the methods of the invention, the capsular polysaccharides, carrier proteins, and/or polysaccharide-protein conjugates may be purified. Any method known in the art for purifying polysaccharides or proteins can be used, such as concentration/diafiltration, precipitation/elution, column chromatography and depth filtration. *See, e.g.*, Farrés *et al.* (1996) *Biotechnol. Tech.* 10:375-380; Gonçalves *et al.* In: Communicating Current Research and Educational Topics and Trends in Applied Microbiology (Antonio Mendez Vilas, ed. 1st ed. Badajoz, Espanha: Formatex; 2007. pp.450-457); Tanizaki *et al.* (1996) *J. Microbiol. Methods* 27:19-23; and US Patent No. 6,146,902; and US Patent Application Publication No. 2008/0286838; each of which is incorporated herein by reference as if set forth in its entirety.

[0160] As used herein, the term "isolated" or "purified" 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 polysaccharide, peptide or protein 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 protein or polysaccharide can 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 for example, the methods of purification of the capsular polysaccharides, as described herein. The language "substantially free of cellular material" includes preparations of a polysaccharide/polypeptide/protein in which the polysaccharide/polypeptide/protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, a polypeptide/protein, polysaccharide, or conjugate that is substantially free of cellular material or other compounds includes preparations of the polypeptide/protein, polysaccharide, or conjugate having less than about 30%, 20%, 10%, 5%, 2.5% or 1% (by dry weight) of contaminating protein, polysaccharide, or other compounds. 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%, 5%, 4%, 3%, 2%, or 1% 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%, 4%, 3%, 2%, or 1% (by dry weight) of chemical precursors or compounds other than polypeptide/protein or polysaccharide fragment of interest.

[0161] Immunogenic conjugates produced by any of the methods described herein may be stored in water or a low ionic strength neutral pH buffer or lyophilized into a dry powder.

Methods for Inducing an Immune Response and Protecting Against S. Aureus Infection

[0162] The present invention also includes methods of use for immunogenic compositions described herein. For example, one embodiment of the invention provides a method of inducing an immune response against *S. aureus* comprising administering to a subject an immunogenic amount of any of the immunogenic compositions described herein. One embodiment of the invention provides a method of protecting a subject against an infection with *S. aureus,* or a method of preventing infection with *S. aureus*, or a method of reducing the severity of or delaying the onset of at least one symptom associated with an infection caused by *S. aureus,* the methods comprising administering to a subject an immunogenic amount of any of the immunogenic compositions described herein. One embodiment of the invention provides a method of treating or preventing a Staphylococcal infection, disease or condition associated with a *Staphylococcus sp.* in a subject, the method comprising the step of administering a therapeutically or prophylactically effective amount of an immunogenic composition described herein to the subject. In some embodiments, the method of treating or preventing a Staphylococcal infection, disease or conditions comprises human, veterinary, animal, or agricultural treatment. Another embodiment provides a method of treating or preventing a Staphylococcal infection, disease or condition associated with a *Staphylococcus sp.* in a subject, the method comprising generating a polyclonal or monoclonal antibody preparation from the immunogenic composition described herein, and using said antibody preparation to confer passive immunity to the subject. One embodiment of the invention provides a method of preventing a Staphylococcal infection in a subject undergoing a surgical procedure, the method comprising the step of administering a prophylactically effective amount of an immunogenic composition described herein to the subject prior to the surgical procedure.

[0163] An "immune response" to an antigen or immunogenic composition is the development in a subject of a humoral and/or a cell-mediated immune response to molecules present in the antigen or vaccine composition of interest. For purposes of the present invention, a "humoral immune response" is an antibody-mediated immune response and involves the induction and generation of antibodies that recognize and bind with some affinity for the antigen in the immunogenic composition of the invention, while a "cell-mediated immune response" is one mediated by T-cells 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), CD1 or other non-classical MHC-like molecules. This activates antigen-specific CD4+ T helper cells or CD8+ cytotoxic T lymphocyte cells ("CTLs"). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by classical or non-classical MHCs 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 or other antigens in association with classical or non-classical 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 re-stimulation with antigen. Such assays are well known in the art. *See, e.g.*, Erickson *et al.* (1993) *J. Immunol.* 151:4189-4199; and Doe *et al.* (1994) *Eur. J. Immunol.* 24:2369-2376.

[0164] As used herein, "treatment" (including variations thereof, *e.g.*, "treat" or "treated") means any one or more of the following: (i) the prevention of infection

or re-infection, as in a traditional vaccine, (ii) the reduction in the severity of, or, in 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 treatment is the preferred mode. According to a particular embodiment of the present invention, compositions and methods are provided that treat, including prophylactically and/or therapeutically immunize, a host animal against a microbial infection (*e.g.*, a bacterium such as *Staphylococcus*). 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.

[0165] As used herein, "mammal" means a human or non-human animal. More particularly, mammal refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports and pet companion animals such as a household pet and other domesticated animal including, but not limited to, cattle, sheep, ferrets, swine, horses, rabbits, goats, dogs, cats, and the like. Preferred companion animals are dogs and cats. Preferably, the mammal is human.

[0166] An "immunogenic amount," and "immunologically effective amount," both of which are used interchangeably herein, 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.

[0167] The amounts of a particular conjugate in a composition is generally calculated based on total polysaccharide, conjugated and non-conjugated for that conjugate. For example, a CP5 conjugate with 20% free polysaccharide will have about 80 mcg of conjugated CP5 polysaccharide and about 20 mcg of non-conjugated CP5 polysaccharide in a 100 mcg CP5 polysaccharide dose. The protein contribution to the conjugate is usually not considered when calculating the dose of a conjugate. The amount of conjugate can vary depending upon the staphylococcal serotype. Generally, each dose will comprise 0.1 to 100 mcg of polysaccharide, particularly 0.1 to 10 mcg, and more particularly 1 to 10 mcg. The "immunogenic amount" of the different polysaccharide components in the

immunogenic composition, may diverge and each may comprise 1 mcg, 2 mcg, 3 mcg, 4 mcg, 5 mcg, 6 mcg, 7 mcg, 8 mcg, 9 mcg, 10 mcg, 15 mcg, 20 mcg, 30 mcg, 40 mcg, 50 mcg, 60 mcg, 70 mcg, 80 mcg, 90 mcg, or about 100 mcg of any particular polysaccharide antigen.

[0168] *S. aureus* "invasive disease" is the isolation of bacteria from a normally sterile site, where there is associated clinical signs/symptoms of disease. Normally sterile body sites include blood, CSF, pleural fluid, pericardial fluid, peritoneal fluid, joint/synovial fluid, bone, internal body site (lymph node, brain, heart, liver, spleen, vitreous fluid, kidney, pancreas, ovary) or other normally sterile sites. Clinical conditions characterizing invasive diseases include bacteremia, pneumonia, cellulitis, osteomyelitis, endocarditis, septic shock and more.

[0169] The effectiveness of an antigen as an immunogen, can be measured 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, or by measuring the levels of B-cell activity by measuring the levels of circulating antibodies specific for the antigen in serum. An immune response may also be detected by measuring the serum levels of antigen specific antibody induced following administration of the antigen, and more specifically, 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 administered. 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 is 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, *e.g.*, 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 procedures for measuring immunogenicity and/ or *in vivo* efficacy.

In certain embodiments, the term "about" means within 20%, preferably within 10%, and more preferably within 5%.

[0170] The invention further provides antibodies and antibody compositions which bind specifically and selectively to the serotype 5 or 8 capsular polysaccharides or immunogenic conjugates of the present invention. In some embodiments, antibodies are generated upon administration to a subject of the serotype 5 or 8 capsular polysaccharides or immunogenic conjugates of the present invention. In some embodiments, the invention provides purified or isolated antibodies directed against one or more of the serotype 5 or 8 capsular polysaccharides or immunogenic conjugates 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, cell line (such as hybridoma cells or other engineered cell lines for recombinant production of antibodies) or a transgenic animal that produces an antibody or antibody composition of the invention, using techniques well-known to those of skill in the art.

[0171] Antibodies or antibody compositions of the invention may be used in a method of treating or preventing a Staphylococcal infection, disease or condition associated with a *Staphylococcus sp.* 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 CP5, CP8 or a conjugate thereof.

[0172] Several animal models known in the art may be used to assess the efficacy of any one of the immunogenic compositions described herein. For example:

[0173] Passive Murine Sepsis Model: Mice are passively immunized intraperitoneally (i.p.) with immune IgG or monoclonal antibody. The mice are

challenged 24 hours later with a lethal dose of *S. aureus*. The bacterial challenge is administered intravenously (i.v. or i.p.) ensuring that any survival could be attributed to the specific *in vivo* interaction of the antibody with the bacteria. The bacterial challenge dose is determined to be the dose required to achieve lethal sepsis of approximately 20% of the un-immunized control mice. Statistical evaluation of survival studies can be carried out by Kaplan-Meier analysis.

[0174] Active Immunization and Challenge Model: In this model, mice are actively immunized subcutaneously (s.c.) with a target antigen at 0, 3 and 6 weeks (or a similar schedule known to those skilled in the art) and challenged with *S. aureus* at week 8 (or other similar schedule known to those skilled in the art) by the intravenous or intraperitoneal route. The bacterial challenge dose is calibrated to achieve approximately 20% survival in the control group over a 14 day period. Statistical evaluation of survival studies can be carried out by Kaplan-Meier analysis.

[0175] Passive Infectious Endocarditis Model: A passive immunization model for infectious endocarditis (IE) caused by *S. aureus* has previously been used to show that ClfA can induce protective immunity. *See*, Vernachio *et al.* (2006) *Antmicro. Agents & Chemo.* 50:511-518. In this model of IE, rabbits or rats are used to simulate clinical infections that include a central venous catheter, bacteremia, and hematogenous seeding to distal organs. Catheterized rabbits or rats with sterile aortic valve vegetations are administered a single intravenous injection of a monoclonal or polyclonal antibody specific for the target antigen. After 24 hours, the animals are challenged i.v. with heterologous staphylococcal strains or a MRSA strain. Then 48 hours after challenge, cardiac vegetations, kidneys and blood are harvested and cultured. The frequency of staphylococcal infection in cardiac valve vegetations, kidneys, and blood is then measured. In one study, when animals were challenged with either MRSE ATCC 35984 or MRSA PFESA0003, significant reductions in infection rate were shown using either the polyclonal antibody preparation or the monoclonal antibody to ClfA. *See*, Vernachio *et al., supra.*

[0176] Passive Infectious Endocarditis Model: The infectious endocarditis model has also been adapted for active immunization studies. Rabbits or rats are

immunized intramuscularly (i.m.) with target antigen and challenged with *S. aureus* two weeks later via the i.v. route.

[0177] Pyelonephritis Model: In the pyelonephritis model, mice are immunized on weeks 0, 3 and 6 (or a similar schedule known to those skilled in the art) with the target antigens. On week 8, the animals are challenged by, *e.g.*, i.p. injection of, e.g., 1.7 x 10^8 cfu *S. aureus* PFESA0266. After 48 hours, the kidneys and/or other tissues are harvested and cultured. Finally, colony forming units of challenge bacteria are enumerated in the kidneys and/or other tissues. This model evaluates systemic dissemination in the animal.

Monitoring functional antibodies using opsonophagocytic killing assays

[0178] Differentiated effector cells from a cell line (e.g. HL60s) or polymorphonuclear cells (PMNs) isolated from donor human blood using LYMPHOLYTE®-poly solution (Cedarlane laboratories limited, Ontario, Canada) as per manufacturer's protocol can be used for this assay. Effector cells were resuspended in assay buffer (Modified Eagle's media containing 1% bovine serum albumin) at approximately 2 X 10^7 cells/ml concentration and placed in 37° C incubator until ready to use. *S. aureus* strain PFESA0266 was grown overnight on tryptic soy agar plates. Bacterial cells were scraped, washed twice and resuspended in assay buffer containing 5% glycerol to an OD_{600} = 1, which equals to approximately 5 X 10^8 cfu/ml concentration. One ml aliquots of the bacterial suspension were frozen and stored at -40°C until ready to use. Frozen bacterial suspension were thawed and adjusted to a concentration of 10^6 cfu/ml in assay buffer and placed on ice. The assay was performed using a sterile 96 deep well 1 ml polypropylene plates. Two fold serial dilutions of antibody samples (50 µl) were prepared and followed by addition of 300 µl of assay buffer to the antibody mix. Bacteria were added (50 µl) to the plates and placed on a rotary shaker at 4 °C for 30 minutes. The opsonization step was followed by addition of 50 µl of human complement (1% final concentration). Finally, 50 µl of effector cells (10^7 cells/ml concentration) were added to the plate and the suspension mixed well by repeated pipetting. A 50µl aliquot of the suspension was 10 fold serially diluted in sterile 1% saponin solution, vortexed to minimize bacterial clumping and plated

on tryptic soy agar in duplicate. The assay plate was incubated at 37°C for 1 hour with continuous mixing using rotisserie style shaker. At the end of the incubation a 50µl aliquot of suspension was 10 fold serially diluted in sterile 1% saponin solution, mixed by vortexing to minimize bacterial clumping and plated on tryptic soy agar in duplicate. The percentage killing was calculated by determining the ratio of the number of cfu surviving at 60 minutes in wells with bacteria, antibodies, complement and effector cells to the number of cfu surviving in tubes lacking antibodies but containing bacteria, complement and effector cells. Controls containing bacteria, complement, and sera were included to adjust for any reduction in cfu due to clumping.

Complement adsorption

[0179] Serum from human donors adsorbed against *S. aureus* strains PFESA0266, PFESA0286 and PFESA0270 can be used as a source of complement in the assay. *S. aureus* strains were grown overnight on TSA plates at 37°C. Cells were scraped from the plate and resuspended in sterile PBS. Bacterial cells were centrifuged at 10,000 rpm for 10 minutes at 4°C and cell pellet was resuspended in human serum for adsorption. Serum was incubated with bacteria on a nutator at 4°C for 30 minutes. Cells were centrifuged, serum transferred to another tube containing bacteria and the adsorption step repeated again for 30 minutes. Finally, the cells were centrifuged and the serum passed through a 0.2 micron filter before 0.5 ml aliquots were frozen down in liquid nitrogen.

Method II - OPA using HL-60 cells

[0180] HL-60 cells were differentiated according to S. Romero-Steiner, et al., Clin Diagn Lab Immunol 4 (4) (1997), pp. 415–422. Harvested HL-60 cells were resuspended in assay buffer (Modified Eagle's media containing 1% bovine serum albumin) at approximately 10^8 cells/ml and placed in 37 °C incubator until ready to use. *S. aureus* was grown overnight on tryptic soy agar plates. Bacterial cells were scraped, washed twice and resuspended in assay buffer containing 5% glycerol to an OD_{600} = 1, which equals to approximately 5 X 10^8cfu/ml. One ml aliquots of the bacterial suspension were frozen and stored at -40°C until ready to

use. Frozen bacterial suspension were thawed and adjusted to a concentration of 10^6 cfu/ml in assay buffer and placed on ice. The assay was performed using a sterile 96 deep well 1 ml polypropylene plates. Two fold serial dilutions of monoclonal antibody samples (25µl) were prepared and followed by addition of 150 µl of assay buffer to the antibody suspension. Bacteria were added (25µl) to the plates and placed on a rotary shaker at 4 °C for 30 minutes followed by addition of 25 µl of human complement (1% final concentration). Finally, 25µl of HL-60 cells (10^7 cells/ml) were added to the plate and the suspension mixed well by repeated pipetting. A 25µl aliquot of the suspension was 10 fold serially diluted in sterile 1% saponin solution, mixed by vortexing to minimize bacterial clumping and plated on tryptic soy agar in duplicates. The assay plate was incubated at 37°C for 1 hour with continuous mixing using rotisserie style shaker. At the end of incubation a 25µl aliquot of suspension was 10 fold serially diluted in sterile 1% saponin solution, mixed by vortexing to and plated on tryptic soy agar in duplicate. The percentage killing was calculated by determining the ratio of the number of cfu surviving at 60 minutes in wells with bacteria, antibodies, complement and HL-60 cells to the number of cfu surviving in tubes lacking antibodies but containing bacteria, complement and HL-60 cells. Controls containing bacteria, complement and mAb was included to adjust for any reduction in cfu due to clumping.

[0181] The following examples are provided by way of illustration, not by way of limitation.

EXAMPLES

Example 1: Preparation of *S. aureus* Serotype 8 Capsular Polysaccharide.

[0182] In this example, production of various size ranges of *S. aureus* serotype 8 capsular polysaccharide is described. The structure *S. aureus* serotype 8 capsular polysaccharide repeat unit is shown in Figure 1. The methods described herein are effective in producing serotype 8 capsular polysaccharide with molecular weights ranging from about 20 kDa to 700 kDa. By proper selection of conditions, high molecular weight serotype 8 capsular polysaccharides can be isolated and purified ranging from 50 kDa to 700 kDa in molecular weight. For use in immunogenic

compositions, serotype 8 capsular polysaccharide can be isolated and purified ranging from 70 kDa to 300 kDa in molecular weight and many desired ranges. Based on growth characteristics and the quantity of capsule produced, strains PFESA0005 or PFESA0286 were used for the production of serotype 8 capsular polysaccharide. Capsules isolated from strains PFESA0005 or PFESA0286 were shown to be identical.

[0183] For production of serotype 8 capsular polysaccharides, the strains were grown in a complex medium consisting primarily of a carbon source (either lactose or sucrose), hydrolyzed soyflour as the nitrogen source, and trace metals. The strains were grown in bioreactors for 2 to 5 days.

[0184] Prior to autoclaving, a sample was removed to test the level of Staphylococcal enterotoxin B (SEB) in the culture. In the presence of 0.05% polysorbate 80, the concentration of SEB in the fermentation was 15-20 ng/ml. Previous experiments showed that autoclaving the culture for 1 hour reduced the level of SEB to less than 0.1 ng/ml, which is below the limit of detection for the TECRA kit.

[0185] Diafiltered, ethanol-fractionated polysaccharide was loaded onto a Q-Sepharose AEC column and eluted with a linear gradient of NaCl as described above. Fractions were analyzed by the *O*-acetyl assay and double immunodifusion test for presence of serotype 5 polysaccharide and phosphate assay for the presence of teichoic acid (TA). The presence of serotype 8 polysaccharide was detected in fractions 35 to 95 (Figures 2A-B).

[0186] To reduce contamination with teichoic acid, fractions 35 to 75 were pooled and any residual teichoic acid was oxidized with sodium-metaperiodate to allow its removal by 3K diafiltration against diH_2O.

[0187] Purification of serotype 8 capsular polysaccharide used for the preparation of conjugates was performed by two different methods that rely on elevated temperature and low pH to affect the release of capsule from the cell and reduce the molecular weight of the polysaccharide. The resulting molecular weight depended on the time, temperature and pH of the hydrolysis step.

[0188] Characterization of serotype 8 capsular polysaccharide was performed using the techniques specified in Table 1.

Table 1: Characterization Assays for Purified *S. aureus* Serotype 8 Capsular Polysaccharides.

Specificity	Assay
Residual Protein	Lowry colorimetric assay
Residual Nucleic acids	260nm scan
Residual Teichoic Acid	Phosphate colorimetric assay
Residual Peptidoglycan	HPAEC-PAD
Size	SEC-MALLS
Composition	HPAEC-PAD
Identity	1H-NMR or reaction with specific mAb
O-acetylation	1H-NMR
Concentration	MALLS-RI or HPAEC-PAD

[0189] Capsule polysaccharides produced by the methods described below result in pure well characterized polysaccharides with low levels of protein, nucleic acid, peptidoglycan and teichoic acid contaminants.

[0190] In the first method, following release of the capsule polysaccharide from the cell and reduction of molecular weight, the preparation was treated with a cocktail of enzymes (*e.g.*, ribonuclease, deoxyribonuclease, lysozyme and protease) to digest impurities. After incubation, residual impurities are precipitated by the addition of ethanol (final concentration about 25%). After removal of the residual ethanol, a solution containing capsule polysaccharide was loaded onto an anion exchange column (Q-Sepharose) and eluted with a linear salt gradient. Fractions containing capsule polysaccharide were pooled and treated with sodium meta-periodate. This treatment resulted in the oxidative hydrolysis of residual teichoic acid contaminant, but did not affect serotype 8 capsular polysaccharide. The reaction was quenched by the addition of ethylene glycol. The material was concentrated and diafiltered against dH_2O to remove any residual reagents and by-products.

[0191] The second method was used to produce capsule polysaccharide without the use of enzymes to digest the various cell-derived impurities. In this method, following release of the capsule polysaccharide from the cell and reduction of molecular weight, the hydrolyzed fermentation broth was clarified by microfiltration followed by ultrafiltration and diafiltration. The solution was treated with activated carbon to remove impurities. After carbon treatment, the

material was treated with sodium meta-periodate to oxidize residual teichoic acid followed by quenching with propylene glycol. The material was concentrated and diafiltered against dH_2O to remove any residual reagents and by-products.

[0192] Preparations produced using either method resulted in pure capsular polysaccharides with low levels of protein, nucleic acid and teichoic acid contaminants. The methods described can be used to produce specific ranges of the desired high molecular weight polysaccharides by varying the conditions of hydrolysis. Examples of capsular polysaccharide obtainable by the methods described herein are shown in Table 2 below. Batches of purified serotype 8 capsular polysaccharide had high purity as indicated by no teichoic acid (TA), peptidoglycan and low residual protein. *See*, Table 2. The range of lower molecular weights spanned 20.4 kDa to 65.1 kDa, and the purified polysaccharides were highly O-acetylated (~100%). The levels of nucleic acid contamination were low (0.12-2.45%).

Table 2: Characterization of Serotype 8 Capsular Polysaccharide Preparations.

Sample	Total CP8 Purified	MW	Protein	Nuc. Acid	O-Acetyl
	mg	(kDa) (g/mol)	(Lowry) % (w/w)	(260 nm scan) % (w/w)	NMR %
1	310	27.0	1.2	0.94	100
2	438	29.0	2.4	2	100
3	179	20.4	0.37	0.12	108

[0193] *Molecular Weight Selection of Capsular Polysaccharides:* A kinetic analysis demonstrated that a broad range of molecular weights of capsule polysaccharides can be generated by the methods described herein. Initially, larger polysaccharides were produced by the bacterial cells, and subsequently, a desired molecular weight range selected and then purified by manipulation of the pH and heat conditions of the heat and hydrolysis steps.

[0194] Heat treatment of *S. aureus* fermentation broth is a process step between fermentation and capsular polysaccharide recovery. This process step uses heat to treat pH-adjusted broth for a specified period. The goals of the heat treatment at

low pH were to kill cells, inactivate enterotoxins, release cell bound polysaccharide and reduce molecular weight to the desired size. Among these goals, the reduction of molecular weight was the slowest in terms of processing time required in this step. Therefore, the other goals were inevitably achieved within the treatment time considered.

[0195] *Heat Treatment:* pH and temperature conditions for selecting various molecular weight ranges of capsule polysaccharides were determined. A 15L Biolafitte Fermenter was used for these studies. The fermentation broth was transferred to the fermenter by a peristaltic pump. Using an agitation speed of about 200 rpm, the broth pH was adjusted with concentrated sulfuric acid. Then, the broth temperature was raised to the set value. The heat treatment time started as soon as the temperature reached the set point. When the desired treatment time was reached, the broth was cooled to room temperature. In-process samples were taken to determine polysaccharide concentration and molecular weight by HPLC and SEC-MALLS systems, respectively. The molecular weight (MW) data was used in the kinetic analysis. The MW profiles were determined over time at pH 3.5, 4.0 and 5.0. *See*, Figure 3A.

[0196] The kinetics of mild acid hydrolysis of polysaccharides was conducted using purified serotype 8 capsular polysaccharide obtained from the process. The purified polysaccharide solution was adjusted to the desired pH for the experiment with sulfuric acid. About 1.5 mL of the solution was transferred to each of the 15mL centrifuge tubes. The tubes were placed in an oil bath equipped with a precision temperature control system. The tube was taken out at a predetermined time interval and was quenched in an ice bucket. At the end of the experiment, an aliquot of 1M Tris buffer (pH 7.5) was added to the sample to adjust the pH back to about 7. The samples were analyzed by a SEC-MALLS system. The MW data was used in the kinetic analysis. The effect of temperature on the MW profile of CP8 at pH 3.5 was determined over time. *See*, Figure 3B.

Results

[0197] As shown in Figure 3A, a lower pH was more effective in reducing the molecular weight of the polysaccharide. Molecular weights between 300 kDa and 600 kDa can be generated using a pH of 5 at 95°C for between 15 minutes and 120

minutes. Likewise, molecular weights between 250 kDa and 450 kDa can be generated using a pH of 4 at 95°C for between 15 minutes and 120 minutes. Moreover, molecular weights between 120 kDa and 450 kDa can be generated using a pH of 3.5 at 95 °C for between 15 minutes and 120 minutes.

[0198] As shown in Figure 3B, the higher the temperature, the faster the rate of hydrolysis and broader the range of the molecular weights of polysaccharide produced with time. Use of a lower temperature, 55°C versus 95°C at the same pH, produces a narrower range of polysaccharide molecular weights.

[0199] Furthermore, Figure 4 demonstrates a correlation between the molecular weight of purified CP8 with the treatment time for mild acid (pH 3.5 at 95°C) hydrolysis. The purified polysaccharide is the final product obtained from the recovery process detailed previously. As also shown in Figure 4, an increase in time of heat treatment of the *S. aureus* PFESA0005 strain at pH 3.5 resulted in smaller molecular weight CP8, whereas shorter heat treatment times at pH 3.5 resulted in higher molecular weight CP8. The size of the serotype 8 capsular polysaccharides ranged from about 80 kDa to about 220 kDa depending on the length of time of heat treatment at pH 3.5. The correlation between the time of heat treatment at low pH and size of the purified CP8, as shown in Figure 4, allows for an estimation of the treatment time required to produce purified polysaccharide with a specified range of molecular weight.

[0200] It is important to note that as demonstrated above the full range of molecular weights of serotype 8 capsular polysaccharides from 20 kDa to more than 500 kDa can be produced, released and purified. The methods therefore may be used to produce specific ranges of desired high molecular weight capsule polysaccharides such as is shown in Table 3. The relatively narrow range of molecular weight polysaccharide produced where the peak molecular weights range from 87 kDa to 108 kDa represents a well characterized range of molecular weights that may be obtained by the methods described herein. A particularly advantageous range of high molecular weight polysaccharides, ranging from 70 kDa to 300 kDa or from 70 kDa to 150 kDa, is useful in making immunogenic compositions by conjugating the capsular polysaccharide to a carrier molecule or protein (*see*, Table 3). The conditions used to generate the CP8 capsule

polysaccharide having a molecular weight range of from about 80 to 120 kDa are as follows: 95°C, pH 3.5 for 300 minutes.

Table 3: Production of Specific Range of High Molecular Weight Serotype 8 Capsular Polysaccharide.

Run	Serotype 8 Capsular Polysaccharide MW (kDa)
1	98
2	89
3	108
4	108
5	89
6	100
1	99
2	113
3	105
4	100
5	87

Example 2: Conjugation of Serotype 8 Capsular Polysaccharides to CRM_{197}.

[0201] This example describes processes and characterization assays used in the production of *S. aureus* serotype 8 capsular polysaccharide-CRM_{197} conjugates. Different conjugation chemistries were developed for conjugating *S. aureus* serotype 8 capsular polysaccharide to this carrier protein. For example, conjugation using PDPH (3-(2-pyridyldithio)-propionyl hydrazide) results in a covalent thioether bond between the CP and the carrier protein. Alternatively, conjugation using CDI/CDT (1,1-carboyldiimidazole/1,1-carboyl-di-1,2,4-triazole) results in a one-carbon or zero-carbon linker between CP and carrier protein.

Conjugation of Serotype 8 Capsular Polysaccharide to CRM_{197} by PDPH Conjugation Chemistry.

[0202] The PDPH conjugation chemistry is a multi-step process that involves activation of the polysaccharide, removal of the thiol protecting group, purification of the activated polysaccharide intermediate, activation and purification of the CRM_{197} protein, and conjugation of the activated components followed by purification. After introduction of a thiol group containing linker to the polysaccharide and a haloacetyl group to the CRM_{197} protein carrier, *S. aureus*

serotype 8 capsular polysaccharide was linked to the protein carrier through a thioether bond. Bromoacetyl groups were introduced into the CRM_{197} protein by reaction of amine groups with the N-hydroxysuccimide ester of bromoacetic acid. To generate thiolated polysaccharide, the carbodiimide activated carboxylate groups of N-acetylmannosaminouronic acid in the polysaccharide were coupled to the hydrazide group of the sulfhydryl-reactive hydrazide heterobifunctional linker 3-(2-pyridyldithio)-propionyl hydrazide (PDPH). Thiols of PDPH-thiolated polysaccharide, generated by reduction with DTT and purified by SEC on a Sephadex G25 column, reacted with bromoacetyl groups of activated protein resulting in covalent thioether linkage formed by bromine displacement between polysaccharide and the carrier protein. Non-reacted bromoacetyl groups were "capped" with cysteamine hydrochloride (2-aminoethanethiol hydrochloride). The reaction mixture was then concentrated and diafiltered. The remaining unconjugated bromoacetyl groups were capped with cysteamine hydrochloride to ensure no reactive bromoacetyl groups were left after conjugation. This formed a covalent bond between the thiol end of cysteamine and the acetyl group on the lysine residue after displacement of bromine.

[0203] *1. Thiolation of S. aureus Serotype 8 Capsular Polysaccharide with PDPH*: The polysaccharide was first activated by thiolation with PDPH. The polysaccharide was mixed with a freshly prepared PDPH stock solution (250 mg/mL in DMSO), an EDAC stock solution (90 mg/mL in diH_2O), and MES buffer stock solution (0.5M, pH 4.85) to make the final solution 0.1 M MES, and 2 and 4 mg polysaccharide/mL while maintaining a polysaccharide:PDPH:EDAC ratio by weight of 1:0.6:1.25 for serotype 8 capsular polysaccharide. This mixture was incubated for 1 hour at room temperature and then dialyzed against a 1000X volume of distilled H_2O four times using a 3500 MWCO dialysis device at between 4°C and 8°C to remove unreacted PDPH. The PDPH-linked polysaccharide was made 0.2 M DTT and incubated at room temperature for 3 hours or overnight at between 4°C and 8°C. Excess DTT as well as the by-products of the reaction were separated from the activated saccharide by SEC using Sephadex G25 resin and distilled water as the mobile phase. Fractions were assayed by the DTDP assay for thiol groups and thiol-positive fractions that eluted

near the void volume of the column were pooled. The pool of fractions was assayed by the PAHBAH and the O-acetyl assays to determine the degree of activation which is expressed as a molar percent of the repeat units containing a thiol group (molar concentration of thiols/molar concentration of repeat units). The activated polysaccharide was lyophilized and stored at -25°C until needed for conjugation.

[0204] The results from the reproducibility of serotype 8 polysaccharide thiolation with PDPH are shown in Table 4. Degree of activation of serotype 8 polysaccharide was in the range 12% to 16%, which corresponds to approximately one linker molecule attached per ten capsular polysaccharide repeat units to one linker molecule per five repeat units.

Table 4: Activation of Serotype 8 Capsular Polysaccharide with PDPH – Reproducibility Study.

Serotype 8 polysaccharide-PDPH	Activation (% M_{SH}/M_{RU})	Scale mg	Yield mg (%, w/w)
1	14	36	30 (83)
2	16	30	27 (91)
4	16	38	42 (110)
5	12	40	44 (110)

[0205] *2. Carrier protein activation:* Separately, the carrier protein was activated by bromoacetylation. CRM_{197} was diluted to 5 mg/mL with 10 mM phosphate buffered 0.9% NaCl pH 7 (PBS) and then made 0.1 M $NaHCO_3$ pH 7.0 using 1 M stock solution. The N-hydroxysuccinimide ester of bromoacetic acid (BAANS) was added at a CRM_{197}:BAANS ratio 1:0.25 (w:w) using a BAANS stock solution of 20 mg/mL DMSO. This reaction mixture was incubated at between 4 and 8°C for 1 hour then purified using SEC on Sephadex G-25. The purified activated CRM_{197} was assayed by the Lowry assay to determine the protein concentration and then diluted with PBS to 5 mg/mL. Sucrose was added to 5% wt/vol as a cryoprotectant and the activated protein was frozen and stored at -25°C until needed for conjugation.

[0206] Bromoacetylation of lysine residues of CRM_{197} was very consistent, resulting in the activation of 19 to 25 lysines from 39 lysines available (*see*, Table 5). The reaction produced high yields of activated protein.

Table 5: Yields and Degree of Bromoacetylation of CRM_{197}.

Preparation	Lysines Activated (n=)	Scale (mg)	Yield (% w/w)
1	24	23	85
2	20	38	87
3	19	35	77
4	22	35	94
5	23	35	87
6	25	48	104

[0207] *3. Coupling Reaction:* Once the activated capsule polysaccharide and activated carrier protein were prepared, the two were combined in a conjugation reaction. The lyophilized and thiolated polysaccharide was dissolved in 0.16 M borate pH 8.95, mixed with thawed bromoacetylated CRM_{197} and distilled water to make the final solution 0.1 M borate, 1:1 wt/wt ratio of CRM_{197}:polysaccharide, and 1 mg/mL polysaccharide. This mixture was incubated at room temperature for between 16 and 24 hours. Unreacted bromoacetyl groups on the protein were capped by adding cysteamine hydrochloride at a ratio of CRM_{197}:cysteamine of 1:2 (wt/wt) using a 135 mg/mL stock solution of cysteamine dissolved in 0.1 M borate pH 8.95 and incubated for 4 hours at room temperature. The capsule polysaccharide-CRM_{197} conjugate (conjugate) was purified by diafiltering 50-fold against 0.9% NaCl using a 100K polyethersulfone ultrafilter.

[0208] The results from the reproducibility of serotype 8 capsular polysaccharide thiolation studies with PDPH demonstrated that the degree of activation of the polysaccharide was in the range 12 to 16 % which corresponds to approximately one linker molecule attached per ten polysaccharide repeat units to one linker molecule per five repeat units.

Conjugation of Serotype 8 Capsular Polysaccharide to CRM_{197} by CDI/CDT Conjugation Chemistry.

[0209] CDI and CDT afford a one-step conjugation process where the polysaccharide is activated in an anhydrous environment (DMSO) to form imidazole or triazole carbamate moieties with available hydroxyls and acylimidazole or acyltriazole moieties with carboxylic acids. Addition of a protein carrier (in DMSO) leads to the nucleophilic displacement of the imidazole or triazole by lysine and formation of a carbamate linkage (for activated hydroxyls) and the amide linkage (for activated carboxylic acids).

[0210] Both CDI and CDT conjugation chemistries produced serotype 8 capsular polysaccharide covalently linked to the carrier protein, which was indicated by the presence of the saccharide and protein in the fractions from size exclusion chromatography, and by amino acid analysis of glycolaldehyde capped or cysteamine hydrochloride capped conjugate.

[0211] Summary of the results from the preparation of several lots of conjugates prepared by both PDPH and CDI/CDT chemistries for capsular serotype 8 with polysaccharide size in the range of 20 kDa to 40 kDa are shown in Table 6 below. There were no significant differences in the free capsule polysaccharide, ratio of polysaccharide-protein and yields of conjugates generated by these two conjugation methods. The antigenicity of conjugated serotype 8 capsular polysaccharide was not altered by conjugation as portrayed by identity precipitin line between conjugates and native polysaccharide.

Table 6: Characterization of Serotype 8 Capsular Polysaccharide-CRM197 Prepared by Two Conjugation Chemistries.

Chemistry	CP Yield (%)	Protein Yield (%)	Output Ratio	Free sugar (%)	Free Protein (%)	Lysines Modified	Size (MW or Kd (% < 0.3), sacc/prot))
CDI/CDT	46-62	54-55	0.8-0.9	22-25	< 1	7-8	34/57 to 60/57
PDPH	34-70	61-83	0.6-0.9	15-41	ND	11-16	74-92%

[0212] As shown above, the methods described herein may be used to produce specific ranges of desired high molecular weight capsule polysaccharides. We sought to prepare conjugates from a pre-selected range of high molecular weight

that could be filtered and purified serotype 8 capsular polysaccharide for use in immunogenic compositions. Table 7 summarizes the analysis of serotype 8 capsular polysaccharide conjugates where the serotype 8 capsule polysaccharide ranged in molecular weight from about 80 kDa to 120 kDa and the imidazole conjugation chemistry was utilized. The molecular weights of the resulting conjugates ranged from 595 kDa to 1708 kDa. The number of conjugated lysines per CRM_{197} ranged from a high of 9 to a low of 3. The free capsule polysaccharide ranged from a high of 6% to a low of 2%.

Table 7: Conjugates With Preselected Molecular Weight Range of Serotype 8 Capsular Polysaccharide.

Run	Poly MW (kDa)	Yield (%)	Free Sacc. (%)	MW by SEC-MALLS (kDa)	Lysines Modified
1	99	88	6	943	4
2	113	73	5	841	3
3	105	79	3	719	7
4	100	86	2	630	9
5	87	90	3	595	6

[0213] Both conjugation chemistries produce serotype 8 capsular polysaccharide covalently linked to carrier protein. There were no significant differences in free capsule polysaccharide, ratio of serotype 8 capsular polysaccharide:protein and yields of conjugates generated by these two methods.

Example 3: One Pot Versus Complex CDI/CDT Process.

[0214] As described above, methods for making the immunogenic conjugates of the invention involve covalent conjugation of the capsular polysaccharides with the carrier proteins using conjugation chemistry involving CDI (1,1-carbonyldiimidazole), CDT (1,1-carboyl-di-1,2,4-triazole) or PDPH (3-(2-pyridyldithio)-propionyl hydrazide). Use of CDI/CDT results in a one-carbon or zero-carbon linker between capsular polysaccharide and carrier protein, while use of PDPH results in a covalent thioether bond between capsular polysaccharide and carrier protein.

[0215] The PDPH-based method was a multi-step process that involved activation of the polysaccharide, removal of a thiol protecting group on the polysaccharide, purification of the activated polysaccharide intermediate, activation and purification of the protein carrier, and conjugation of the activated components followed by purification. In this method, *S. aureus* serotype 8 capsular polysaccharides were reacted with PDPH and a carbodiimide in an aqueous solution such as 0.1M MES to produce PDPH-linked polysaccharides. The PDPH-linked polysaccharides were reacted with a reducing agent to produce activated polysaccharides that were then purified. Carrier proteins were reacted with bromoacetic acid N-hydroxysuccinimide ester in an aqueous solution to produce activated carrier proteins that were then purified. The purified activated serotype 8 polysaccharides were then reacted with the purified activated carrier proteins to produce serotype 8 polysaccharide:carrier protein conjugates.

[0216] In contrast, the CDI- and CDT-based methods were one or two step conjugation processes, in which the capsular polysaccharide was activated in an anhydrous environment (*i.e.*, DMSO) to form imidazole or triazole carbamate moieties with available hydroxyls and acylimidazole or acyltriazole moieties with carboxylic acids. Addition of the protein carrier (in DMSO) lead to a nucleophilic displacement of the imidazole or triazole by lysine and formation of a carbamate linkage (for activated hydroxyls) and the amide linkage (for activated carboxylic acids). Accordingly, two CDI- or CDT-based methods were developed: a more complex process and a simpler one-pot process. In the more complex process, *S. aureus* serotype 8 capsular polysaccharides were compounded with imidazole or triazole, lyophilized, and then reacted with CDI or CDT in an organic solvent (such as DMSO) to produce activated serotype 8 polysaccharides. The activated serotype 8 polysaccharides were purified and then reacted with carrier proteins in the organic solvent to produce serotype 8 polysaccharide-carrier protein conjugates. The one-pot process was similar to the complex process except that the activated serotype 8 polysaccharides were not purified prior to the reaction with carrier proteins.

CDI/CDT Complex Process.

[0217] *Activation of Polysaccharide:* Serotype 8 polysaccharide was mixed with 10 g triazole/g serotype 8 and lyophilized. The resulting cake was dissolved in DMSO at 2.0 mg serotype 8 polysaccharide/mL. The water content was determined. A freshly prepared stock solution of CDT at 100 mg/mL in DMSO was added to achieve a molar amount of CDT equivalent to the water. Alternatively, the amount of CDT added may be adjusted to achieve a higher or lower degree of activation. This was held 30 minutes at 23°C.

[0218] *Purification of Activated Serotype 8 Polysaccharide:* The solution of activated serotype 8 (ACP8) was poured into 25 volumes of water to destroy excess CDT. This was concentrated to its original volume on a 10 kDa PES membrane at approximately 1 mg/cm^2 and diafiltered against water for at least 10 volumes. This step was completed in less than 4 hours. The diafiltered material was mixed with 10 g triazole/g of original serotype 8 polysaccharide and lyophilized.

[0219] *Preparation of Lyophilized CRM:* CRM was diafiltered against 0.4% NaCl/5% sucrose at constant volume on a 10 kDa PES membrane for at least 10 volumes. The protein concentration was determined and sufficient diafiltration buffer was added to bring the protein concentration to 5.0 g/L, thus affording a w/w ratio of NaCl/CRM = 0.8. The CRM was lyophilized.

[0220] *Conjugation:* Activated, diafiltered serotype 8 polysaccharide was dissolved in DMSO at 1 mg/mL. Borate solution at 100 mM was added to achieve 2% v/v.

[0221] CRM was resuspended at 2 mg/mL and, when dissolution was complete, combined with the ACP8 solution. This was allowed to react at 23°C for 20 hours.

[0222] The conjugate reaction was poured into 24 volumes of 5 mM borate pH 9.0 and allowed to stir at room temperature for 1 hour. It was then adjusted to pH 7.5 with 0.5 M phosphate buffer, pH 6.5. This was filtered through a 5 micron filter and concentrated to the original volume on a 300 kDa PES membrane at a load of ~ 1 mg/cm^2 and diafiltered against at least 10 volumes of water. The resulting concentrate was filtered through a 0.22 micron filter and stored at 2°C-8°C.

CDI/CDT One Pot Process.

[0223] *CRM_{197} Matrix Exchange:* CRM_{197} was diafiltered to exchange from the bulk matrix of approximately 10 mM phosphate/80 mM NaCl/15% sucrose, pH 7 to 5 mM imidazole/0.72 % NaCl/15 mM octyl-β-D-glucoside, pH 7. The exchange allowed the removal of phosphate and sucrose which are detrimental to the conjugation and defines the sodium chloride content carried into the conjugation. Octyl-β-D-glucopyranoside is added prevent particle formation after sterile filtration.

[0224] The matrix of the CRM_{197} was exchanged by tangential flow filtration against 5 mM imidazole/0.72%/15 mM octyl-B-D-glucopyranoside pH 7 through 10 diavolumes using 10K MWCO PES membranes at a retentate concentration of approximately 4 mg/mL. Typical membrane challenge was 2 grams/ft^2 and the target final CRM_{197} concentration in the matrix was 6 mg/mL. The CRM_{197} was stored at 2°C-8°C.

[0225] *Activation/Conjugation:* The activation/conjugation process for *S. aureus* serotype 8 capsular polysaccharide consisted of the following steps: 1) Matrix exchange of the CRM197; 2) Compounding of polysaccharide; 3) Shell freezing and lyophilization of CRM_{197} and compounded polysaccharide; 4) Dissolution of the lyophilized polysaccharide and CRM_{197}; 5) Activation of the polysaccharide; 6) Conjugation of the activated polysaccharide to CRM_{197}; and 7) Purification of the conjugate (dilution, diafiltration, sterile filtration).

[0226] The polysaccharide was compounded with 10 grams of 1,2,4- triazole excipient per gram of polysaccharide. The excipient was added as a powder to the polysaccharide, with a solution obtained after less than 15 minutes of mixing at ambient temperature.

[0227] The compounded polysaccharide and CRM_{197} were shell frozen separately using a -75°C ethanol bath. The volume per 1L bottle was approximately 500 mL.

[0228] For the polysaccharide dissolution, DMSO was added to the individual lyophilization bottles of the polysaccharide to obtain a suspension and then transferred to the activation/conjugation reaction vessel for heating. DMSO was added to obtain 2 g/L concentration. A clear solution was obtained as the

suspension reached approximately 45°C with mixing. The solution was then cooled to 23°C ± 2°C.

[0229] For the CRM_{197} dissolution, DMSO was added to the individual lyophilization bottles containing the CRM_{197} to obtain a suspension and then transferred to a second vessel for mixing. DMSO was added to obtain 2 g/L concentration. A clear solution was typically obtained in les than 15 minutes.

[0230] The polysaccharide/DMSO solution was sampled for Karl Fischer analysis to determine moisture content. CDT was prepared as a 100 mg/mL solution in DMSO and was added based on determined moisture content. Continuous addition of the CDT solution was performed over about 5 minutes at 23°C ± 2°C with mixing. The reaction was allowed to proceed for a minimum of 30 minutes at 23°C ± 2°C. The reaction was sampled to determine activation level (UV 220/205 nm) and then 100 mM sodium borate, pH 9 was added to obtain a 1.5% aqueous solution. The reaction solution was then stirred for a minimum of 30 minutes at 23°C ± 2°C.

[0231] For conjugation of the activated polysaccharide to CRM_{197}, DMSO was added to target a 0.8 mg/mL reaction concentration. The dissolved CRM_{197} in DMSO was then added to the activated polysaccharide solution with mixing. The reaction was stirred for a minimum of 4 hours at 23°C ± 2°C.

[0232] The reaction solution was 10X diluted by its addition into 5mM sodium tetraborate, pH 9 with mixing to hydrolyze residual activation groups. The diluted solution was passed through a 5 µm filter and concentrated to a target retentate concentration of 2 g/L. Tangential flow filtration was performed using 300K regenerated cellulose membranes through 20 diavolumes with 5 mM succinate, pH 7. Typical membrane challenge was 1 gram/ft^2. The purified conjugate was passed through a 0.22 micron filter and stored at 2°C-8°C.

Example 4: Conjugation of Serotype 8 Capsular Polysaccharide Using One-Pot and Complex Conjugation Process.

[0233] This example demonstrates that pre-selected range of molecular weights of capsule polysaccharides can be used for conjugation in either the one-pot or complex process. The larger polysaccharides are initially produced by the

bacterial cells, and the resulting molecular weight range purified can be controlled by pH and heat of the hydrolysis process in Example 1 (as shown in Table 3).

[0234] In this example, eight batches where the serotype 8 capsule polysaccharide ranged in molecular weight from about 80 kDa to about 120 kDa were selected and conjugation was performed using activation with 1,1-carbonyl-di-(1,2,4-triazole) for serotype 8 capsular polysaccharide. *See*, Table 8. The molecular weights of the resulting conjugates ranged from 595 kDa to 1708 kDa. The number of conjugated lysines per CRM ranged from a high of 13 to a low of 3. The free sugar ranged from a high of 11% to a low of 1%.

Table 8: Serotype 8 Capsular Polysaccharide Conjugates Prepared with 80 kDa to 120 kDa Capsular Polysaccharides.

Process	Run	Poly MW (kDa)	Sacc Yield (%)	Free Sugar (%)	MW by SEC-MALLS (kDa)	Lysine
One Pot	1	98	86	1	751	11
	2	89	80	1	675	13
	3	108	76	4	1073	5.0
	4	108	69	4	819	5.2
	5	89	85.1	8	1708	10
	6	100	94.0	11	1577	5
Complex	1	99	88	6	943	4
	2	113	73	5	841	3
	3	105	79	3	719	7
	4	100	86	2	630	9
	5	87	90	3	595	6

Example 5: Evaluation of the Conjugated Native- And Base-Treated Serotype 8 Capsular Polysaccharide In A Murine Bacteremia Model.

[0235] The importance of O-acetyl groups present on native serotype 8 capsular polysaccharide before conjugation for induction of functional antibody responses was evaluated for capsular polysaccharide conjugates. Serotype 8 capsular polysaccharide was de-O-Acetylated under mild basic conditions, and both NMR and Ion Chromatography (IC) confirmed absence of O-acetylation in serotype 8 capsular polysaccharide de-O-Ac-CRM. The CP8 de-O-Ac-CRM conjugate was prepared by conjugation of de-O-Ac CP8 polysaccharide to CRM by PDPH chemistry as described in Example 2.

[0236] The serotype 8 capsular polysaccharide conjugate unexpectedly showed no measurable acetyl groups by IC method. This could be attributed to differences in the structure, sites of O-acetylation compared to other *S. aureus* capsular polysaccharides, which in turn could cause the removal or modification of acetyl groups in serotype 8 capsular polysaccharides during conjugation.

[0237] The murine bacteremia model was used to evaluate efficacy of the native versus base-treated serotype 8 capsular polysaccharide conjugated to CRM_{197}. Groups of female BALB/c mice (15/group) were vaccinated at weeks 0, 3 and 6 with 1 mcg serotype 8 capsular polysaccharide de-O-Ac-CRM or 1 μg serotype 8 capsular polysaccharide O-Ac-CRM. Vaccines were formulated with 22 mcg $AlPO_4$. Animals were challenged with *S. aureus* PFESA0003, and bacteria were enumerated from the blood three hours later. The data showed that there was a statistically significant ($p=0.0362$) reduction in bacterial cfu recovered from the blood of animals immunized with untreated native serotype 8 capsular polysaccharide conjugate as determined by the Student t-Test (Table 9). In animals that were immunized with base-treated serotype 8 capsular polysaccharide conjugate, the bacterial cfu recovered from blood were similar to the saline control group.

Table 9: Serotype 8 Capsular Polysaccharide-CRM_{197} Conjugate Reduces Bacteremia Caused By *S. aureus* PFESA0003 In Mice.

Antigen	Strain/Dose	LogCFU/Blood	Significance (p value)
Saline	PFESA0003 1.14×10^8	4.35	
CP8 de-O-Ac-CRM		4.45	
CP8 O-Ac-CRM		3.93	0.03

Example 6: Evaluation of the Conjugated Native And Base Treated Serotype 8 Capsular Polysaccharide In A Murine Bacteremia Model.

[0238] Serotype 8 capsular polysaccharide conjugates were evaluated for their ability to protect mice in a pyelonephritis model. Bacterial counts in the blood of mice receiving i.p. *S. aureus* challenge were significantly reduced as compared to controls immunized with PBS.

[0239] Two studies were conducted to evaluate efficacy of CP8-CRM_{197} conjugate in the murine bacteremia model, described previously, after challenge with *S. aureus* PFESA0268 (Type 8). The first study (Figure 5) showed a significant reduction of bacteremia (p=0.0308). For the study, groups of 6-8 week old Swiss Webster mice (n=30) were actively immunized by subcutaneous injection with 1 μg serotype 8 capsular polysaccharide-CRM_{197} and saline both formulated with 100 μg $AlPO_4$ at 0, 2 and 4 weeks and challenged at week 6 by the intravenous route with *S. aureus* PFESA0268 (Type 8). Prior challenge experiments were conducted to optimize the dose of challenge strain at the age mice reach after three vaccinations. Statistical evaluation of survival studies was carried out by Kaplan-Meier analysis.

Example 7: Opsonic Activity Of Sera From Mice Immunized With Native And Chemically Modified Serotype 8 Capsular Polysaccharide Conjugates.

[0240] Select mouse sera (n=5) with high serotype 8 capsular polysaccharide titers from a vaccination study were compared for opsonic activity using PFESA0005 strain. The OPA results (Table 10) show that only conjugates prepared by the conjugation of native serotype 8 capsular polysaccharide elicited opsonic antibodies in mice. It is noteworthy that the de-OAc serotype 8 capsular polysaccharide conjugate was immunogenic in mice but the antibodies elicited were not opsonic in this assay. OPA titers are reported as reciprocal of dilution at which 40% killing was observed.

Table 10: Opsonic Activity of Native Serotype 8 Capsular Polysaccharide vs. de-O-Ac Serotype 8 Capsular Polysaccharide-CRM Immunized Mouse Sera.

De-O-Ac Serotype 8 Capsular Polysaccharide-CRM		Serotype 8 Capsular Polysaccharide-CRM	
OP titer Wk0 sera	OP titer Wk8 sera	OP titer Wk0 sera	OP titer Wk8 sera
<50	<50	50	150
<50	<50	<50	1350
<50	<50	<50	450
<50	<50	<50	1350
<50	<50	<50	4050

Example 8: Killing of *S. aureus* Strains by Serotype 8 Conjugate Antisera can be Inhibited by Addition of Native Serotype 8 Capsular Polysaccharide.

[0241] To confirm the specificity of the killing, the opsonophagocytic assay was performed in the presence of native serotype 8 capsular polysaccharide or unrelated pneumococcal polysaccharide (Pn 14 poly), essentially as described above.

[0242] The results (Table 11) showed that the presence of native serotype 8 capsular polysaccharide in reaction mixture inhibited opsonophagocytic killing of *S. aureus* PFESA0286 (Type 8). These results confirm that opsonophagocytic killing by immune sera is mediated by capsule specific antibodies.

Table 11: Addition Serotype 8 Capsular Polysaccharide Inhibits Opsonophagocytic Killing of *S. aureus* by Immune Sera.

Monkey	Sera Sample	OPA titer
	Wk0	<50
	Wk8	4050
02D133	Wk0 + 20 μg CP8 poly	<50
	Wk8 + 20 μg CP8 poly	<50
	Wk0 + 20 μg Pn14 poly	<50
	Wk8 + 20 μg Pn 14 poly	4050
	Wk0	<50
	Wk8	4050
A4N122	Wk0 + 20 μg CP8 poly	<50
	Wk8 + 20 μg CP8 poly	<50
	Wk0 + 20 μg Pn14 poly	<50
	Wk8 + 20 μg Pn 14 poly	1350

SUMMARY

[0243] All conjugation chemistries produced serotype 8 capsular polysaccharide covalently linked to the carrier protein CRM_{197}. There were no significant differences in free saccharide, ratio of serotype 8 capsular polysaccharide:protein and yields of conjugates generated by these methods.

Example 9: Preparation of *S. aureus* Serotype 5 Capsular Polysaccharide.

[0244] In this example, production of various size ranges of *S. aureus* serotype 5 capsular polysaccharide is described. The structure of *S. aureus* serotype 5 capsular polysaccharide repeat unit is shown in Figure 6. The methods described

herein are effective in producing serotype 5 capsular polysaccharide with molecular weights ranging from about 20 kDa to 800 kDa. By proper selection of conditions, high molecular weight serotype 5 capsular polysaccharides can be isolated and purified ranging from 50 kDa to 800 kDa in molecular weight. For use in immunogenic compositions, serotype 5 capsular polysaccharide can be isolated and purified ranging from 70 kDa to 300 kDa in molecular weight, from 70 kDa to 150 kDa and many other desired ranges. Strain PFESA0266 was chosen for serotype 5 capsular polysaccharide production based on growth characteristics and the quantity of capsule produced.

[0245] For production of serotype 5 capsular polysaccharide, strain PFESA0266 was grown in a complex medium consisting primarily of a carbon source (either lactose or sucrose), hydrolyzed soyflour as the nitrogen source, and trace metals. The strain was grown in bioreactors for 2 to 5 days.

[0246] Fermentation of the PFESA0266 strain was carried out as detailed above. At the time of harvest, OD_{600} of the culture was 7.38. The culture was autoclaved for 1 hour and after cooling, the culture was processed as described above to separate cells from supernatant material. Approximately 1 L each of filtered and concentrated supernatant and cells were recovered.

[0247] Prior to autoclaving, a sample was removed to test the level of Staphylococcal enterotoxin B (SEB) in the culture. In the presence of 0.05% polysorbate 80, the concentration of SEB in the fermentation was 15-20 ng/ml. Previous experiments showed that autoclaving the culture for 1 hour reduced the level of SEB to less than 0.1 ng/ml, which is below the limit of detection for the TECRA kit.

[0248] Diafiltered, ethanol-fractionated polysaccharide was loaded onto a Q-Sepharose AEC column and eluted with a linear gradient of NaCl as described above. Fractions were analyzed by the *O*-acetyl assay and double immunodifusion test for presence of serotype 5 polysaccharide and phosphate assay for the presence of teichoic acid. The presence of serotype 5 polysaccharide was detected in fractions 60 to 105 (Figures 7A-B). To reduce contamination with teichoic acid, fractions 60 to 85 were pooled and any residual teichoic acid was oxidized with sodium-metaperiodate to allow its removal by 3K diafiltration against diH_2O.

[0249] Purification of serotype 5 capsular polysaccharide used for the preparation of conjugates was performed by two different methods that rely on elevated temperature and low pH to affect the release of capsule from the cell and reduce the molecular weight of the polysaccharide. The resulting molecular weight depended on the time, temperature and pH of the hydrolysis step.

[0250] Characterization of serotype 5 capsular polysaccharide was performed using the techniques specified in Table 1, *supra*.

[0251] Capsule polysaccharides produced by the methods described below result in pure polysaccharides with low levels of protein, nucleic acid, peptidoglycan and teichoic acid contaminants.

[0252] In the first method, following release of the capsule polysaccharide from the cell and reduction of molecular weight, the preparation was treated with a cocktail of enzymes (*e.g.*, ribonuclease, deoxyribonuclease, lysozyme and protease) to digest impurities. After incubation, residual impurities were precipitated by the addition of ethanol (final concentration about 25%). After removal of the residual ethanol, a solution containing capsule polysaccharide was loaded onto an anion exchange column (Q-Sepharose) and eluted with a linear salt gradient. Fractions containing capsule polysaccharide were pooled and treated with sodium meta-periodate. This treatment resulted in the oxidative hydrolysis of residual teichoic acid contaminant, but did not affect serotype 5 capsular polysaccharide. The reaction was quenched by the addition of ethylene glycol. The material was concentrated and diafiltered against distilled water (dH_20) to remove any residual reagents and by-products.

[0253] The second method was used to produce capsule polysaccharide without the use of enzymes to digest the various cell-derived impurities. In this method, following release of the capsule polysaccharide from the cell and reduction of molecular weight, the hydrolyzed fermentation broth was clarified by microfiltration followed by ultrafiltration and diafiltration. The solution was treated with activated carbon to remove impurities. After carbon treatment, the material was treated with sodium meta-periodate to oxidize residual teichoic acid followed by quenching with propylene glycol. The material was concentrated and diafiltered against dH_2O to remove any residual reagents and by-products.

[0254] Preparations produced using either method resulted in pure capsular polysaccharides with low levels of protein, nucleic acid and teichoic acid contaminants. The methods described can be used to produce specific ranges of the desired high molecular weight polysaccharides by manipulating the conditions of hydrolysis.

[0255] Examples of capsular polysaccharide obtainable by the methods described herein are shown in Table 12 below. Batches of purified serotype 5 capsular polysaccharide had high purity as indicated by no teichoic acid (TA), peptidoglycan and low residual protein. *See*, Tables 12 and 13. The range of molecular weights spanned 132.7 kDa to 800 kDa and the purified polysaccharides were highly O-acetylated, ranging from 90%-100%, and were 100% N-acetylated. The yields of serotype 5 capsular polysaccharide purification were 39% to 63%, and the size of purified serotype 5 polysaccharide varied from 35 kDa to 65 kDa (*see*, Table 12). The level of teichoic acid (TA) contamination was acceptable and levels of residual proteins and nucleic acid were also in acceptable ranges. NMR spectra of serotype 5 polysaccharide were identical to ones reported in the literature.

Table 12: Serotype 5 Capsular Polysaccharide Characterization.

Sample	Total CP5 Purified	Yield	MW	Protein	Nuc. Acid	O-acetyl
	mg	%	(kDa) (g/mol)	% (w/w)	(260 nm scan) % (w/w)	NMR (%)
1	101	39	47	0	0.5	94
2	91	48	65	1.2	2.5	96
3	578	63	35	2.5	0.7	75

Table 13: Additional Serotype 5 Capsular Polysaccharide Characterization.

Sample	MW (kDa)	CP5 (mg/ml)	O-acetyl NMR (%)	Identity NMR	N-acetyl NMR (%)
1	800.1	3.164	100	Pass	100
2	132.7	1.172	90	Pass	100
3	335.4	0.975	90	Pass	100
4	366.8	0.865	90	Pass	ND

ND = not detected

[0256] *Molecular Weight Selection of Capsular Polysaccharides*: A kinetic analysis demonstrated that a broad range of molecular weights of capsule polysaccharides can be generated by the methods described herein. Initially, larger polysaccharides were produced by the bacterial cells, and subsequently, a desired molecular weight range selected and then purified by manipulation of the pH and heat conditions of the heat and hydrolysis steps.

[0257] Heat treatment of *S. aureus* fermentation broth is a process step between fermentation and capsular polysaccharide recovery. This process step uses heat to treat pH-adjusted broth for a specified period. The goals of the heat treatment at low pH were to kill cells, inactivate enterotoxins, release cell bound polysaccharide and reduce molecular weight to the desired size. Among these goals, the reduction of molecular weight was the slowest in terms of processing time required in this step. Therefore, the other goals were inevitably achieved within the treatment time considered.

[0258] *Heat Treatment*: pH and temperature conditions for selecting various molecular weight ranges of capsule polysaccharides were determined. A 15L Biolafitte Fermenter was used for these studies. The fermentation broth was transferred to the fermenter by a peristaltic pump. Using an agitation speed of about 200 rpm, the broth pH was adjusted with concentrated sulfuric acid. Then, the broth temperature was raised to the set value. The heat treatment time started as soon as the temperature reached the set point. When the desired treatment time was reached, the broth was cooled to room temperature. In-process samples were taken to determine polysaccharide concentration and molecular weight by HPLC and SEC-MALLS systems, respectively. The molecular weight (MW) data was

used in the kinetic analysis. The MW profiles were determined over time at pH 3.5, 4.0 and 5.0. *See*, Figure 8A.

[0259] The kinetics of mild acid hydrolysis of polysaccharides was conducted using purified serotype 8 capsular polysaccharide obtained from the process. The purified polysaccharide solution was adjusted to the desired pH for the experiment with sulfuric acid. About 1.5 mL of the solution was transferred to each of the 15mL centrifuge tubes. The tubes were placed in an oil bath equipped with a precision temperature control system. The tubes were taken out at a predetermined time intervals and quenched in an ice bucket. At the end of the experiment, an aliquot of 1M Tris buffer (pH 7.5) was added to the sample to adjust the pH back to about 7. The samples were analyzed by a SEC-MALLS system. The MW data was used in the kinetic analysis. The effect of temperature on MW profile of CP5 at pH 4.5 was determined over time. See, Figure 8B.

Results

[0260] As shown in Figure 8A, a lower pH was more effective in reducing the molecular weight of the polysaccharide. In this example, ranges of molecular weights between about 300 kDa and about 600 kDa can be generated using a pH of 5 at 95 °C for between 15 minutes and 120 minutes. *See* Figure 8A. Likewise, choosing a pH of 4.5 at 95 °C for between 15 minutes and 120 minutes can yield polysaccharide molecular weight ranges between 200 kDa and 400 kDa. In addition, choosing a pH of 4.0 at 95 °C for between 15 minutes and 120 minutes can yield polysaccharide molecular weight ranges between 120 kDa and 300 kDa.

[0261] As shown in Figure 8B, the higher the temperature, the faster the rate of hydrolysis and broader the molecular weights of polysaccharide produced with time. Expressed another way, use of a lower temperature, 55 °C versus 95 °C at the same pH, produces a narrower range of polysaccharide molecular weights.

[0262] Furthermore, Figure 4 demonstrates a correlation between the molecular weight of purified serotype 5 capsular polysaccharide with the treatment time for mild acid (pH 4.5 at 95°C) hydrolysis. The purified polysaccharide is the final product obtained from the recovery process detailed previously. As also shown in Figure 4, an increase in time of heat treatment of the *S. aureus* PFESA0266 strain at pH 4.5 resulted in smaller molecular weight serotype 8 capsular polysaccharide,

whereas shorter heat treatment times at pH 4.5 resulted in higher molecular weight serotype 5 capsular polysaccharide. The size of serotype 5 capsular polysaccharides ranged from about 90 kDa to about 220 kDa depending on the length of time of heat treatment at pH 4.5. A correlation between the time of heat treatment at low pH and size of the purified serotype 5 capsular polysaccharides, as shown in Figure 4, allows for an estimation of the treatment time required to produce purified polysaccharide with a specified range of molecular weight.

[0263] As demonstrated above, the full range of molecular weights of serotype 5 capsular polysaccharides from 20 kDa to more than 500 kDa can be produced, released and purified. The methods described may be used to produce specific ranges of desired high molecular weight capsule polysaccharides such as is shown in Table 14. The relatively narrow range of molecular weight polysaccharide produced where the peak molecular weights range from 63 kDa to 142 kDa represents a well characterized range of molecular weights that may be obtained by the methods described herein. A particularly advantageous range of high molecular weight polysaccharides, ranging from 70 kDa to 300 kDa or from 70 kDa to 150 kDa, is useful in making immunogenic compositions by conjugating the capsular polysaccharide to a carrier molecule or protein. The conditions used to generate the CP5 capsule polysaccharide having a molecular weight range of from about 100 to 140 kDa are as follows: 95°C, pH 4.5 for 135 minutes. Different combinations of pH, temperature, and time, however, will also generate CP5 molecules with a molecular weight range of about 100 to 140 kDa.

Table 14: Production of Specific Range of High Molecular Weight Serotype 5 Capsular Polysaccharide.

Run	Serotype 5 Capsular Polysaccharide MW (kDa)
1	142
2	108
3	142
4	108
5	ND
6	ND
7	63
8	72
9	74

Run	Serotype 5 Capsular Polysaccharide MW (kDa)
10	63
11	ND

ND = not done

Example 10: Conjugation of Serotype 5 Capsular Polysaccharides to CRM_{197}.

[0264] This example describes processes and characterization assays used in the production of *S. aureus* serotype 5 capsular polysaccharide-CRM_{197} conjugates. Different conjugation chemistries were developed for conjugating *S. aureus* serotype 5 capsular polysaccharide to this carrier protein. For example, conjugation using PDPH (3-(2-pyridyldithio)-propionyl hydrazide) results in covalent thioether bond between the CP and the carrier protein; whereas conjugation using CDT (1,1-carboyl-di-1,2,4-triazole) results in a one-carbon or zero-carbon linker between the capsular polysaccharide and carrier protein.

Conjugation of Serotype 5 Capsular Polysaccharide to CRM_{197} by PDPH Conjugation Chemistry.

[0265] The PDPH conjugation chemistry is a multi-step process that involves activation of the polysaccharide, removal of the thiol protecting group, purification of the activated polysaccharide intermediate, activation and purification of the CRM_{197} protein, and conjugation of the activated components followed by purification. After introduction of a thiol group containing linker to the polysaccharide and a haloacetyl group to the CRM_{197} protein carrier, *S. aureus* serotype 5 capsular polysaccharide was linked to the protein carrier through a thioether bond. Bromoacetyl groups were introduced into the CRM_{197} protein by reaction of amine groups with the N-hydroxysuccimide ester of bromoacetic acid. To generate thiolated polysaccharide, the carbodiimide activated carboxylate groups of *N*-acetylmannosaminouronic acid in the polysaccharide were coupled to the hydrazide group of the sulfhydryl-reactive hydrazide heterobifunctional linker 3-(2-pyridyldithio)-propionyl hydrazide (PDPH). Thiols of PDPH-thiolated polysaccharide, generated by reduction with DTT and purified by SEC on a Sephadex G25 column, reacted with bromoacetyl groups of activated protein resulting in covalent thioether linkage formed by bromine displacement between

polysaccharide and the carrier protein. Non-reacted bromoacetyl groups were "capped" with cysteamine hydrochloride (2-aminoethanethiol hydrochloride). The reaction mixture was then concentrated and diafiltered. The remaining unconjugated bromoacetyl groups were capped with cysteamine hydrochloride to ensure no reactive bromoacetyl groups were left after conjugation. This formed a covalent bond between the thiol end of cysteamine and the acetyl group on the lysine residue after displacement of bromine.

[0266] 1. *Thiolation of S. aureus Serotype 5 Capsular Polysaccharide with PDPH*: The polysaccharide was first activated by thiolation with PDPH. The polysaccharide was mixed with a freshly prepared PDPH stock solution (250 mg/mL in DMSO), an EDAC stock solution (90 mg/mL in diH_2O), and MES buffer stock solution (0.5M, pH 4.85) to make the final solution 0.1 M MES, and 2 mg and 4 mg capsular polysaccharide/mL while maintaining a capsular polysaccharde:PDPH:EDAC ratio by weight of 1:5:3 for serotype 5 capsular polysaccharide. This mixture was incubated for 1 hour at room temperature and then dialyzed against a 1000X volume of distilled H_2O four times using a 3500 MWCO dialysis device at between 4°C and 8°C to remove unreacted PDPH. The PDPH-linked polysaccharide was made 0.2 M DTT and incubated at room temperature for 3 hours or overnight at between 4°C and 8°C. Excess DTT as well as the by-products of the reaction were separated from the activated saccharide by SEC using Sephadex G25 resin and distilled water as the mobile phase. Fractions were assayed by the DTDP assay for thiol groups and thiol-positive fractions that eluted near the void volume of the column were pooled. The pool of fractions was assayed by the PAHBAH and the O-acetyl assays to determine the degree of activation, which is expressed as a molar percent of the repeat units containing a thiol group (molar concentration of thiols/molar concentration of repeat units). The activated polysaccharide was lyophilized and stored at -25°C until needed for conjugation.

[0267] The results from the reproducibility of serotype 5 polysaccharide thiolation with PDPH are shown in Table 15. Degree of activation of serotype 5 polysaccharide was in the range 11% to 19%, which corresponds to approximately

one linker molecule attached per ten capsular polysaccharide repeat units to one linker molecule per five repeat units.

Table 15: Activation of Serotype 5 Capsular Polysaccharide with PDPH – Reproducibility Study.

Serotype 5 polysaccharide-PDPH	Activation (% M_{SH}/M_{RU})	Scale mg	Yield mg (%, w/w)
1	11	23	19.6 (85)
2	13	30	28 (93)
3	19	30	23 (77)
4	15	32	29 (90)

[0268] 2. *Carrier protein activation*: Separately, the carrier protein was activated by bromoacetylation. CRM_{197} was diluted to 5 mg/mL with 10 mM phosphate buffered 0.9% NaCl pH 7 (PBS) and then made 0.1 M $NaHCO_3$ pH 7.0 using 1 M stock solution. The *N*-hydroxysuccinimide ester of bromoacetic acid (BAANS) was added at a CRM_{197} :BAANS ratio 1:0.25 (w:w) using a BAANS stock solution of 20 mg/mL DMSO. This reaction mixture was incubated at between 4 and 8°C for 1 hour then purified using SEC on Sephadex G-25. The purified activated CRM_{197} was assayed by the Lowry assay to determine the protein concentration and then diluted with PBS to 5 mg/mL. Sucrose was added to 5% wt/vol as a cryoprotectant and the activated protein was frozen and stored at -25°C until needed for conjugation.

[0269] Bromoacetylation of lysine residues of CRM_{197} was very consistent, resulting in the activation of 19 to 25 lysines from 39 lysines available (*see*, Table 16). The reaction produced high yields of activated protein.

Table 16: Yields and Degree of Bromoacetylation of CRM_{197}.

Preparation	Lysines Activated (n=)	Scale (mg)	Yield (% w/w)
1	24	23	85
2	20	38	87
3	19	35	77
4	22	35	94
5	23	35	87

Preparation	Lysines Activated (n=)	Scale (mg)	Yield (% w/w)
6	25	48	104

[0270] 3. *Coupling Reaction*: Once the activated capsule polysaccharide and activated carrier protein were prepared, the two were combined in a conjugation reaction. The lyophilized and thiolated polysaccharide was dissolved in 0.16 M borate pH 8.95, mixed with thawed bromoacetylated CRM_{197} and distilled water to make the final solution 0.1 M borate, 1:1 wt/wt ratio of CRM_{197}:polysaccharide, and 2 mg/mL serotype 5 capsular polysaccharide. This mixture was incubated at room temperature for between 16 and 24 hours. Unreacted bromoacetyl groups on the protein were capped by adding cysteamine hydrochloride at a ratio of CRM_{197}:cysteamine of 1:2 (wt/wt) using a 135 mg/mL stock solution of cysteamine dissolved in 0.1 M borate pH 8.95 and incubated for 4 hours at room temperature. The capsule polysaccharide-CRM_{197} conjugate (conjugate) was purified by diafiltering 50-fold against 0.9% NaCl using a 100K polyethersulfone ultrafilter.

[0271] The results from the reproducibility of serotype 5 capsular polysaccharide thiolation studies with PDPH demonstrated that the degree of activation was in the range of 11% to 19 %, which corresponds to approximately one linker molecule attached per ten CP repeat units to one linker molecule per five repeat units.

Conjugation of Serotype 5 Capsular Polysaccharide to CRM_{197} by CDT Conjugation Chemistry.

[0272] CDT affords a one-step conjugation process where the polysaccharide is activated in an anhydrous environment (DMSO) to form triazole carbamate moieties with available hydroxyls and acylimidazole or acyltriazole moieties with carboxylic acids. Addition of a protein carrier (in DMSO) leads to the nucleophilic displacement of the triazole by lysine and formation of a carbamate linkage (for activated hydroxyls) and the amide linkage (for activated carboxylic acids). The reaction solution is diluted 10-fold into an aqueous solution in preparation for purification by tangential flow filtration.

[0273] CDT conjugation chemistry produced serotype 5 capsular polysaccharide covalently linked to the carrier protein, which was indicated by the presence of the saccharide and protein in the fractions from size exclusion chromatography, and by amino acid analysis of glycolaldehyde capped or cysteamine hydrochloride capped conjugate.

[0274] A summary of the results from the preparation of several lots of conjugates prepared by both PDPH and CDT chemistries for serotype 5 capsular polysaccharide sizes in the range of 20 kDa to 40 kDa is shown in Table 17 below. There were no significant differences in the free capsule polysaccharide, ratio of polysaccharide:protein and yields of conjugates generated by these conjugation chemistries. The antigenicity of conjugated serotype 5 capsular polysaccharide was not altered by conjugation as portrayed by identity precipitin line between conjugates and native polysaccharide.

Table 17: Characterization of Serotype 5 Capsular Polysaccharide-CRM_{197} Prepared by Two Conjugation Chemistries.

Chemistry	CP Yield (%)	Protein Yield (%)	Output Ratio	Free sugar (%)	Free Protein (%)	Lysines Modified	Size (MW or Kd (% < 0.3), sacc/prot))
CDT	19-27	35	0.5-0.8	10-40	<1	18-22	38/61 to 76/74
PDPH	26-52	40-99	0.4-1.0	23-50	ND	ND	7.5×10^5 to 2.3×10^6

ND = not detected

[0275] As shown above, the methods described herein may be used to produce specific ranges of desired high molecular weight capsule polysaccharides. We sought to prepare conjugates from a pre-selected range of high molecular weight that could be filtered and purified serotype 5 capsular polysaccharide for use in immunogenic compositions. Table 18 summarizes the analysis of serotype 5 capsular polysaccharide conjugates where the serotype 5 capsule polysaccharide ranged in molecular weight from about 92 kDa to about 119 kDa and activated with triazole (CDT). The molecular weights of the resulting conjugates ranged from 1533 kDa to 2656. The number of conjugated lysines per CRM_{197} ranged from a high of 22 to a low of 15. The free capsule polysaccharide ranged from a high of 18% to a low of 11%.

Table 18: Conjugates With Preselected Molecular Weight Range of Serotype 5 Capsular Polysaccharide.

Run	Poly MW (kDa)	Yield (%)	Free Sacc. (%)	MW by SEC-MALLS (kDa)	Lysines Modified
1	121	63	11	2130	19
2	92	72	16	1533	22
3	119	74	14	2656	15
4	115	63	18	1911	15

[0276] Both conjugation chemistries produce serotype 5 capsular polysaccharide covalently linked to carrier protein. There were no significant differences in free capsule polysaccharide, ratio of serotype 5 capsular polysaccharide:protein and yields of conjugates generated by these two methods.

Example 11: Complex versus One-Pot CDT Processes.

[0277] As described above, methods for making the immunogenic conjugates of the invention involve covalent conjugation of the capsular polysaccharides with the carrier proteins using conjugation chemistry involving CDT (1,1-carboyl-di-1,2,4-triazole) or PDPH (3-(2-pyridyldithio)-propionyl hydrazide). Use of CDT resulted in a one-carbon or zero-carbon linker between capsular polysaccharide and carrier protein, while use of PDPH results in a five carbon linker containing covalent thioether bond between capsular polysaccharide and carrier protein.

[0278] The PDPH-based method was a multi-step process that involved activation of the polysaccharide, removal of a thiol protecting group on the polysaccharide, purification of the activated polysaccharide intermediate, activation and purification of the protein carrier, and conjugation of the activated components followed by purification. In this method, *S. aureus* serotype 5 capsular polysaccharides were reacted with PDPH and a carbodiimide in an organic solvent such as DMSO to produce PDPH-linked polysaccharides. The PDPH-linked polysaccharides were reacted with a reducing agent to produce activated polysaccharides that were then purified. Carrier proteins were reacted with bromoacetic acid in an organic solvent to produce activated carrier proteins that were then purified. The purified activated serotype 5 polysaccharides were

then reacted with the purified activated carrier proteins to produce serotype 5 polysaccharide:carrier protein conjugates.

[0279] In contrast, the CDT-based methods were one-step conjugation processes, in which the capsular polysaccharide was activated in an anhydrous environment (*i.e.*, DMSO) to form triazole carbamate moieties with available hydroxyls and acylimidazole or acyltriazole moieties with carboxylic acids. Addition of the protein carrier (in DMSO) lead to a nucleophilic displacement of the imidazole or triazole by lysine and formation of a carbamate linkage (for activated hydroxyls) and the amide linkage (for activated carboxylic acids), thereby permitting the conjugation to proceed in "one pot." Accordingly, two CDT-based methods were developed: a more complex process and a simpler one-pot process. In the more complex process, *S. aureus* serotype 5 capsular polysaccharides were compounded with imidazole or triazole and then reacted with CDT in an organic solvent (such as DMSO) and about 0.2% w/v water to produce activated serotype 5 polysaccharides. The activated serotype 5 polysaccharides were purified and then reacted with carrier proteins in the organic solvent to produce serotype 5 polysaccharide:carrier protein conjugates. The one-pot process was similar to the complex process except that the activated serotype 5 polysaccharides were not purified prior to the reaction with carrier proteins.

CDT Complex process

[0280] *Activation of Serotype 5 Capsular Polysaccharide*: Serotype 5 capsular polysaccharide was mixed with 10g triazole/g serotype 5 capsular polysaccharide and lyophilized. The resulting cake was dissolved in DMSO at 2.0 mg serotype 5 capsular polysaccharide/mL. The water content was determined and adjusted to 0.2%. A freshly prepared stock solution of CDT at 100 mg/mL in DMSO was added to achieve a 20 fold molar excess amount of CDT compared to the amount of CP5. Alternatively, the amount of CDT added may be adjusted to achieve a higher or lower degree of activation. This was held 30 minutes at 23°C.

[0281] *Purification of Activated Serotype 5 Capsular Polysaccharide*: The solution of activated serotype 5 capsular polysaccharide (ACP5) was poured into 25 volumes of water to destroy excess CDT. This was concentrated to its original volume on a 10 kDa PES membrane at approximately 1 mg/cm2 and diafiltered

against water for at least 10 volumes. This step was completed in less than 4 hours. The diafiltered material was mixed with 10 g triazole/g of original serotype 5 polysaccharide and lyophilized.

[0282] *Preparation of Lyophilized CRM*: CRM was diafiltered against 0.4% NaCl/5% sucrose at constant volume on a 10 kDa PES membrane for at least 10 volumes. The protein concentration was determined and sufficient diafiltration buffer was added to bring the protein concentration to 5.0 g/L, thus affording a w/w ratio of NaCl/CRM = 0.8. The CRM was lyophilized.

[0283] *Conjugation*: Activated, diafiltered serotype 5 capsular polysaccharide was dissolved in DMSO at 1 mg/mL. Borate solution at 100 mM was added to achieve 2% v/v.

[0284] CRM was resuspended at 2 mg/mL and, when dissolution was complete, combined with the ACP5 solution. This was allowed to react at 4°C for 20 hours.

[0285] The conjugate reaction was poured into 24 volumes of 5 mM borate pH 9.0 and allowed to stir at room temperature for 1 hour. It was then adjusted to pH 7.5 with 0.5 M phosphate buffer, pH 6.5. This was filtered through a 5 micron filter and concentrated to the original volume on a 300 kDa PES membrane at a load of ~ 1 mg/cm^2 and diafiltered against at least 10 volumes of water. The resulting concentrate was filtered through a 0.22 micron filter and stored at 2°C-8°C.

CDT One Pot Process

[0286] CRM_{197} *Matrix Exchange*: CRM_{197} was diafiltered to exchange from the bulk matrix of approximately 10 mM phosphate/80 mM NaCl/15% sucrose, pH 7 to 5 mM imidazole/0.72 % NaCl,/15 mM octyl-β-D-glucopyranoside, pH 7. The exchange allowed the removal of phosphate and sucrose which are detrimental to the conjugation and defines the sodium chloride content carried into the conjugation. Octyl-β-D-glucopyranoside is added prevent particle formation after sterile filtration.

The matrix of the CRM_{197} was exchanged by tangential flow filtration against 5 mM imidazole/0.72% Nacl/15 mM octyl-β-D-glucopyranoside, pH 7 through 10 diavolumes using 10K MWCO PES membranes at a retentate concentration of approximately 4 mg/mL. Typical membrane challenge was 2 grams/ft2 and the

target final CRM_{197} concentration in the matrix was 6 mg/mL. The CRM_{197} was stored at 2°C-8°C.

[0287] *Activation/Conjugation*: The activation/conjugation process for *S. aureus* serotype 5 capsular polysaccharide consisted of the following steps: 1) Compounding of polysaccharide; 2) Shell freezing and lyophilization of CRM_{197} and compounded polysaccharide; 3) Dissolution of the lyophilized polysaccharide and CRM_{197}; 4) Activation of the polysaccharide; 5) Conjugation of the activated polysaccharide to CRM_{197}; and 6) Purification of the conjugate (dilution, diafiltration, sterile filtration).

[0288] The polysaccharide was compounded with 10 grams of 1,2,4- triazole excipient per gram of polysaccharide. The excipient was added as a power the polysaccharide, with a solution obtained after less than 15 minutes of mixing at ambient temperature.

[0289] The compounded polysaccharide and CRM_{197} were shell frozen separately using a -75°C ethanol bath. The volume per 1L bottle was approximately 500 mL.

[0290] For the polysaccharide dissolution, DMSO was added to the individual lyophilization bottles of the polysaccharide to obtain a suspension and then transferred to the activation/conjugation reaction vessel for heating. DMSO was added to obtain 2 g/L concentration. A clear solution was obtained after 5-10 minutes of mixing.

[0291] For the CRM_{197} dissolution, DMSO was added to the individual lyophylization bottles containing the CRM_{197} to obtain a suspension and then transferred to a second vessel for mixing. DMSO was added to obtain 2 g/L concentration. A clear solution was typically obtained in less than 15 minutes.

[0292] The polysaccharide/DMSO solution was sampled for Karl Fischer analysis to determine moisture content. CDT was prepared as a 100 mg/mL solution in DMSO and was added at a 5 molar excess to type 5 polysaccharide (the complex process used 20 molar equivalents CDT while the one-pot process used 5 molar equivalents CDT:CP5). Continuous addition of the CDT solution was performed over about 5 minutes at 23°C + 2°C with mixing. The reaction was allowed to proceed for a minimum of 30 minutes at 23°C ± 2°C. The reaction was

sampled to determine activation level (UV 220/205 nm) and then 100 mM sodium borate, pH 9 was added to obtain a 1.5% aqueous solution. The reaction solution was then stirred for a minimum of 30 minutes at 23°C + 2°C.

[0293] For conjugation of the activated polysaccharide to CRM_{197}, DMSO was added to target a 0.55 mg/mL reaction concentration. The dissolved CRM_{197} in DMSO was then added to the activated polysaccharide solution with mixing. The reaction was stirred for a minimum of 16 hours at 23°C + 2°C.

[0294] The reaction solution was 10X diluted with 5 mM sodium tetraborate, pH 8.6 to obtain a final diluted pH of 9 ± 0.2. The solution was stirred a 23 ± 3°C for a minimum of 4 hours. The diluted solution was passed through a 5 µm filter and concentrated to a target retentate concentration of 2 g/L. Tangential flow filtration was performed using 300K regenerated cellulose membranes through 20 diavolumes with 5 mM succinate, pH 7. Typical membrane challenge was 1 gram/ft2. The purified conjugate was passed through a 0.22 micron filter and stored at 2°C-8°C.

Example 12: Conjugation of Serotype 5 Capsular Polysaccharide Using One-Pot and Complex Conjugation Process.

[0295] This example demonstrates that pre-selected range of molecular weights of capsule polysaccharides can be used for conjugation in either the one -pot or complex process. The larger polysaccharides are initially produced by the bacterial cells and the resulting molecular weight range purified can be controlled by pH and heat of the hydrolysis process in Example 9. In this example, eight batches where the serotype 5 capsular polysaccharide ranged in molecular weight from about 90 kDa to about 140 kDa were selected and conjugation was performed using activation with triazole (CDT) in either the one-pot or complex processes described above. *See*, Table 19. The molecular weights of the resulting conjugates ranged from 1125 kDa to 2656 kDa. The number of conjugated lysines per CRM ranged from a high of 22 to a low of 15. The free sugar ranged from a high of 23% to a low of 11%.

Table 19: Serotype 5 Capsular Polysaccharide Conjugates Prepared with 90 kDa to 140 kDa Capsular Polysaccharides.

Process	Run	Poly MW (kDa)	Sacc Yield (%)	Free Sugar (%)	MW by SEC-MALLS (kDa)	Lysine
One Pot	1	142	93	11	1932	19
	2	108	93	14	1117	20
	3	142	85	17	1609	15
	4	108	86	23	1125	15
Complex	1	121	63	11	2130	19
	2	92	72	16	1533	22
	3	119	74	14	2656	15
	4	115	63	18	1911	15

Example 13: Serotype 5 Capsular Polysaccharide Conjugates Consistently Exhibit Protection in Murine Pyelonephritis Model.

[0296] Serotype 5 capsular polysaccharide conjugates were evaluated for their ability to protect mice in a pyelonephritis model. Bacterial counts in the blood of mice receiving i.p. *S. aureus* challenge were significantly reduced as compared to controls immunized with PBS.

[0297] All six individual studies showed a significant reduction in cfu/ml kidneys in immunized animals (Figure 9). When these studies were pooled for meta analysis, the overall significance for the studies as a whole increased to below 0.0001. The data showed consistent reduction of kidney colonization after active vaccinations with the capsular polysaccharide conjugate.

Example 14: Serotype 5 Capsular Polysaccharide Conjugates Prepared by Different Conjugation Chemistries Protect Mice Against Experimental Infections.

[0298] Active immunization studies in the murine pyelonephritis model were conducted with serotype 5 capsular polysaccharide conjugates prepared either by PDPH or CDT chemistry. The methods for conjugating capsular polysaccharides to CRM_{197} are described above. Results showed that both conjugates reduce colonization in mice compared to the sham immunized animals (Table 20).

Table 20: Effect of PDPH vs. CDT Conjugation on Protection Against *S. aureus* Challenge in Pyelonephritis Model.

Study #	Antigens	Strain/Dose	logCFU/Kidney	Significance
Study 1	Saline+$AlPO_4$	PFESA0266	5.53 ± 1.90	--
	1 mcg CP5-CRM (PDPH) +$AlPO_4$	2×10^8	3.01 ± 1.83	$p < 0.001$
	1 mcg CP5-CRM (CDT) +$AlPO_4$		1.67 ± 0.23	$p < 0.0001$
Study 2	Saline+$AlPO_4$	PFESA0266s	6.17 ± 1.76	--
	1 mcg CP5-CRM (PDPH) +$AlPO_4$	2.7×10^8	3.06 ± 1.69	$p < 0.0001$
	1 mcg CP5-CRM (CDT) +$AlPO_4$		1.87 ± 0.69	$p < 0.0001$

Example 15: Active Immunization of Serotype 5 Capsular Polysaccharide Conjugate Protects Rats in a Rat Endocarditis Model.

[0299] Four studies were conducted with CP5-CRM_{197} PDPH conjugate. The serotype 5 capsular polysaccharide conjugates significantly reduced recovered CFU after challenge with *S. aureus* PFESA0266 in both the heart and kidneys in 2 of 3 experiments (Table 21). In the third study, the Geometric Mean Titer (GMT) anti-CP5 titer was the lowest of the three experiments, but it was only slightly lower than in the previous experiment.

Table 21: Serotype 5 Capsular Polysaccharide-CRM_{197} Immunization Reduces CFU in a Rat Endocarditis Model.

		Log CFU Recovered		Significance		GMT
Immunogenic Composition	**Challenge Strain/Dose**	**Heart**	**Kidney**	**Heart**	**Kidney**	**CP Titer**
1 mcg CP5-CRM	PFESA0266	4.34± 1.78	3.92± 1.73			103,000
1 mcg PP5-CRM	2.21×10^8 cfu	7.94± 0.78	6.77± 0.79	$p<0.001$	$p<0.05$	
1 mcg CP5-CRM	PFESA0266	4.43± 2.30	3.11± 2.33			51,000
Saline	6.5×10^7 cfu	5.63± 2.48	4.19± 2.05	No	No	

		Log CFU Recovered		Significance		GMT
1 mcg CP5-CRM	PFESA0266	4.01± 2.49	3.90± 1.92			67,000
Saline	4.0 x 10^8 cfu	7.52± 1.38	6.52± 1.17	p<0.0002	p<0.0002	

Example 16: Enhanced Immunogenicity of HMW CP5 Conjugate Vaccines in Mice Compared to LMW CP5 Conjugate Vaccines

[0300] A murine pyelonephritis study was conducted to evaluate the immunogenicity and effectiveness of different CP5 conjugate formulations. Two formulations were tested: The first formulation was composed of a high molecular weight (HMW) CP5 (approximately 300 kDa) conjugated to CRM197. The second formulation contained a low molecular weight (LMW) CP5 (approximately 25 kDa) conjugated to CRM197. Three dose levels were tested for the HMW vaccine (1, 0.1 and 0.01 mcg). The LMW vaccine was tested at 1 mcg. A negative control group was also included composed of a polysaccharide conjugate vaccine derived from *Streptococcus pneumoniae* conjugated to CRM197 (PP5). Polysaccharides were formulated with 22mcg AlPO4 on weeks 0, 3 and 6 and challenged with *S. aureus* PFESA0266 on week 8. Kidneys were harvested and bacterial colonies enumerated 48 hours after challenge. Both vaccines were effective at generating an immune response and a reduction in CFUs of *S. aureus* PFESA0266 was observed from the kidneys of mice vaccinated with the 1 μg groups of both HMW and LMW vaccine. This was dosage dependent as demonstrated by reduced effectiveness from the lower vaccine dosages (Figure 10). The CFU readout was not sensitive enough to detect a difference in efficacy for the HMW and LMW vaccines. Sera from the mice were therefore tested by OPA. OPA titers were defined as the dilution of serum required to kill 40% of *S. aureus* Strain PFESA0266 in an OPA assay. Enhanced OPA titers were seen for the HMW vaccine compared to the LMW formulation (Figure 11).

Example 17: Capsule Polysaccharide Conjugates Comprising High Molecular Weight Polysaccharides Show Enhanced Immunogenicity Compared To Conjugates Comprising Low Molecular Weight Polysaccharides.

[0301] Non human primate (NHP) studies were conducted to evaluate the immunogenicity of different capsule conjugate formulations. Two formulations were tested at two different dosage levels (2 and 20 μg). The first formulation contained a high molecular weight (HMW) polysaccharide (approximately 130 kDa) conjugated to CRM_{197}. The second formulation contained a low molecular weight (LMW) polysaccharide (approximately 25 kDa) conjugated to CRM_{197}. Groups of five primates were vaccinated with a single dose of either vaccine and immune titers were monitored prior to vaccination and two weeks post vaccination. OPA titers were defined as the dilution of serum required to kill 40% of *S. aureus* Strain PFESA0266 in an OPA assay. Antibody titers were also monitored by ELISA. Enhanced activity was seen for the HMW vaccine compared to the LMW formulation (Table 22), evidenced by a ten fold rise in antibody titers for the HMW vaccine compared to the LMW vaccine. The OPA responder rate for the NHPs that received the HMW vaccine were also higher (80% compared to 40%).

Table 22. Enhanced Immunogenicity is observed for HMW polysaccharide conjugate vaccines compared to LMW polysaccharide conjugate vaccine.

	CP5-CRM197 dose level (mcg) per animal	**Geometric Mean of PD1***	**OPA Responder Rate (%)±**
HMW (125 kDa)	20	32	80
	2	21	80
LMW (25 kDa)	20	3	40
	2	8	40
* Fold rise calculated from CP5 ELISA titer 2 weeks post vaccination compared to pre vaccine titers. ± Responder rate calculated from monkeys generating a rise in OPA titer following a single dose of vaccine 2 weeks post vaccination. Each group contained 5 Rhesus maccaques and vaccines were formulated with AlPO4 (250 mcg/dose)			

Example 18: Polysaccharide O-Acetylation Is Important For Induction Of Protective Antibody Responses To Serotype 5 Capsular Polysaccharide Conjugate.

[0302] To evaluate the importance of O-acetylation of serotype 5 capsular polysaccharide, the native capsular polysaccharide was de-O-acetylated (dOAc) and conjugated to CRM_{197} (dOAc-CRM_{197}) using PDPH conjugation chemistry, as discussed above. The efficacy of dOAcCP-CRM_{197} conjugate was compared side-by-side with CP5-CRM_{197} in a murine pyelonephritis model.

[0303] Immunization with conjugates lacking O-acetyl groups (dOAc CP5-CRM) failed to reduce recovered bacterial CFU in the kidneys. These data (Table 23) indicate that O-acetylation is important for elicitation of functional antibodies against CP5.

Table 23: Immunization with De-O-Acetylated Serotype 5 Capsular Polysaccharide Conjugates Did Not Protect Mice from Kidney Colonization.

Study #	Antigens	Strain/Dose	LogCFU/Kidney	Significance
Study 1	1 mcg PP5-CRM	PFESA0266	3.89 ± 2.24	
	1 mcg dOAc CP5-CRM	7×10^8	4.20 ± 1.75	
	1 mcg CP5-CRM		1.75 ± 0.39	p-value < 0.008
Study 2	Saline	PFESA0266	5.08 ± 1.96	
	1 mcg dOAc CP5-CRM	2.4×10^8	5.89 ± 1.29	
	1 mcg CP5-CRM		2.93 ± 2.11	p-value < 0.02

Example 19: Confirmation of the Importance of O-Acetylation as Functional Epitope of Serotype 5 Capsular Polysaccharide By OPA Using Monoclonal Antibodies With Known Specificities.

[0304] Serotype 5 capsular polysaccharide monoclonal antibodies with specificities to OAc+ (CP5-7-1), OAc+/- (CP5-5-1) and OAc- (CP5-6-1) were evaluated for OP killing activity against the type 5 strain PFESA0266 (Table 24). Monoclonal antibodies to serotype 8 capsular polysaccharide (CP8-3-1 specific to CP8 OAc+) were used as negative control.

[0305] The OAc-specific anti-CP5 mAb CP5-7-1 mediated killing of *S. aureus* PFESA0266 (Table 24). Also monoclonal antibody CP5-5-1, which recognizes epitopes shared by both CP5 OAc+ and CP5 OAc-, mediated killing of the PFESA0266 strain. The monoclonal antibody specific for epitopes present on serotype 5 OAc- capsular polysaccharide did not mediate killing of the PFESA0266 strain. These results indicate that O-Acetyl epitopes on serotype 5 capsular polysaccharide are necessary to elicit functional activity of serotype 5-specific antibodies.

[0306] Antibodies need to be functional as measured by the killing of bacteria in an animal efficacy model or an opsonophagocytic killing assay that demonstrates the antibodies kill the bacteria. Functional killing may not be demonstrated using an assay that monitors the generation of antibodies alone, which is not indicative of the importance of O-acetylation in efficacy.

Table 24: Monoclonal Antibodies Specific to O-Acetylated (+) Serotype 5 Capsular Polysaccharide and O- and de-O-Acetylated (+/-) Serotype 5 Capsular Polysaccharide Are Opsonic Against *S. aureus* PFESA0266 (Type 5).

CP5-5-1 (O-Ac +/-) (mcg)				**CP5-6-1 (O-Ac -) (mcg)**				**CP5-7-1 (O-Ac +) (mcg)**				**CP8-3-1 (-control) (mcg)**			
20	**10**	**5**	**2.5**	**20**	**10**	**5**	**2.5**	**20**	**10**	**5**	**2.5**	**20**	**10**	**5**	**2.5**
28	33	30	21	-12	-5	-12	-5	31	46	49	55	-18	-3	-13	-5

Data reported as percent killing and was calculated by determining the ratio of the number of CFU surviving at 60 minutes in wells with bacteria, antibodies, complement and HL-60 cells to the number of CFU surviving in wells lacking antibodies but containing bacteria, complement and HL-60 cells.

Example 20: Enhanced Immunogenicity is Observed for CP5 Conjugates Composed of High Molecular Weight Polysaccharides Compared to Low Molecular Weight Polysaccharides in Non Human Primates (NHP).

[0307] Non human primate (NHP) studies were conducted to evaluate the immunogenicity of different capsule conjugate formulations. Two formulations were tested at two different dosage levels (2 and 20 μg). The first formulation was composed of a high molecular weight (HMW) polysaccharide (approximately 130

kDa) conjugated to CRM197. The second formulation contained a low molecular weight (LMW) polysaccharide (approximately 25 kDa) conjugated to CRM197. Groups of five primates were vaccinated with a single dose of either vaccine and immune titers were monitored prior to vaccination and two weeks post vaccination. OPA titers were defined as the dilution of serum required to kill 40% of S. aureus Strain PFESA0266 in an OPA assay. Antibody titers were also monitored by ELISA. Enhanced activity was seen for the HMW vaccine compared to the LMW formulation (Table 25). There was a three to ten fold rise in antibody titers for the HMW vaccine compared to the LMW vaccine. The OPA responder rate for the NHPs that received the HMW vaccine were also higher (80% compared to 40%).

Table 25: Enhanced Immunogenicity is observed for HMW polysaccharide conjugate vaccines compared to LMW polysaccharide conjugate vaccine.

	CP5-CRM197 dose level (mcg) per animal	**Geometric Mean of PD1***	**OPA Responder Rate (%)±**
HMW (125 kDa)	20	32	80
	2	21	80
LMW (25 kDa)	20	3	40
	2	8	40

* Fold rise calculated from CP5 ELISA titer 2 weeks post vaccination compared to pre vaccine titers. ± Responder rate calculated from monkeys generating a rise in OPA titer following a single dose of vaccine 2 weeks post vaccination. Each group contained 5 Rhesus maccaques and vaccines were formulated with AlPO4 (250 mcg/dose)

SUMMARY

[0308] Both conjugation chemistries, described herein, produced serotype 5 capsular polysaccharide covalently linked to the carrier protein CRM197. There were no significant differences in free saccharide, ratio of serotype 5 polysaccharide:protein and yields of conjugates generated by these two methods.

[0309] All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the

same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

[0310] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, certain changes and modifications may be practiced within the scope of the appended claims.

What is Claimed is:

1. An immunogenic polysaccharide-protein conjugate comprising an isolated *Staphylococcus aureus* serotype 8 capsular polysaccharide conjugated to a carrier protein, wherein:

a) the polysaccharide has a molecular weight of between 20 kDa and 1000kDa.

2. The immunogenic conjugate of claim 1, wherein the immunogenic conjugate has a molecular weight of between 200 kDa and 5000 kDa.

3. An immunogenic polysaccharide-protein conjugate comprising an isolated *Staphylococcus aureus* serotype 5 capsular polysaccharide conjugated to a carrier protein, wherein:

a) the polysaccharide has a molecular weight of between 20 kDa and 1000kDa.

4. The immunogenic conjugate of claim 3, wherein the immunogenic conjugate has a molecular weight of between 200 kDa and 5000 kDa.

5. The immunogenic conjugate of any one of claims 1-4, wherein the polysaccharide has a molecular weight range of between 70 kDa and 300 kDa.

6. The immunogenic conjugate of any one of claims 1-5, wherein the conjugate has a molecular weight range of between 500 kDa and 2500 kDa.

7. The immunogenic conjugate of any one of claims 1-4, wherein the polysaccharide has a degree of O-acetylation between 10-100%

8. The immunogenic conjugate of claim 7, wherein the polysaccharide has a degree of O-acetylation between 50-100%.

9. The immunogenic conjugate of claim 7, wherein the polysaccharide has a degree of O-acetylation between 75-100%.

10. The immunogenic conjugate of any of claims 7-9, wherein the immunogenic conjugate can produce antibodies that are functional as measured by the killing of bacteria in either an animal efficacy model or an opsonophagocytic killing assay.

11. The immunogenic conjugate of any of claims 1-10, wherein the carrier protein is CRM_{197}.

12. The immunogenic conjugate of claim 11, wherein the CRM_{197} is covalently linked to the polysaccharide through a carbamate linkage, an amide linkage, or both.

13. The immunogenic conjugate of claim 11 or 12, wherein the molar ratio of conjugated lysines to CRM_{197} is about 10:1 to about 25:1.

14. The immunogenic conjugate of claim 13, wherein at least one covalent linkage between CRM_{197} and polysaccharide occurs at least at every 5 to 10 saccharide repeat units of the polysaccharide.

15. The immunogenic conjugate of claim 13 or 14, wherein at least one linkage between CRM_{197} and polysaccharide occurs at every 5 saccharide repeat units of the polysaccharide.

16. The immunogenic conjugate of any of claims 11-15, wherein the CRM_{197} comprises 5 to 22 lysines covalently linked to the polysaccharide.

17. The immunogenic conjugate of claim 16, wherein the CRM_{197} comprises 8 to 15 lysines covalently linked to the polysaccharide.

18. The immunogenic conjugate of any of claims 1-17 which comprises less than 30% free type 5 or 8 polysaccharide compared to the total amount of type 5 or 8 polysaccharide.

19. The immunogenic conjugate of claim 18 which comprises less than 20% free type 5 or 8 polysaccharide compared to the total amount of type 5 or 8 polysaccharide.

20. An immunogenic composition comprising the immunogenic conjugate of any of claims 1-19 and at least one of an adjuvant, diluent, or carrier.

21. The immunogenic composition of claim 20, wherein the adjuvant is an aluminum-based adjuvant.

22. The immunogenic composition of claim 21, wherein the adjuvant is selected from the group consisting of aluminum phosphate, aluminum sulfate and aluminum hydroxide.

23. The immunogenic composition of claim 21, wherein the adjuvant is aluminum phosphate.

24. The immunogenic composition of any of claims 20-23 which comprises less than 30% free type 5 or 8 polysaccharide compared to the total amount of type 5 or 8 polysaccharide.

25. The immunogenic composition of claim 24 which comprises less than 20% free type 5 or 8 polysaccharide compared to the total amount of type 5 or 8 polysaccharide.

26. A method of inducing an immune response to a *Staphylococcus aureus* serotype 5 or serotype 8 capsular polysaccharide conjugate in a subject, comprising administering to the subject an immunologically effective amount of the immunogenic composition of any of claims 20-25.

27. A method of producing an immunogenic polysaccharide-protein conjugate comprising an isolated *Staphylococcus aureus* serotype 8 capsular polysaccharide conjugated to a carrier protein, the method comprising the steps of:

a) reacting an isolated *S. aureus* serotype 8 capsular polysaccharide with carbonyl ditriazole (CDT) in an organic solvent to produce an activated serotype 8 polysaccharide; and

b) reacting the activated serotype 8 polysaccharide with a carrier protein in an organic solvent to produce a serotype 8 polysaccharide:carrier protein conjugate;

wherein an immunogenic conjugate comprising a *S. aureus* serotype 8 capsular polysaccharide-carrier protein conjugate is produced.

28. A method of producing an immunogenic polysaccharide-protein conjugate comprising an isolated *Staphylococcus aureus* serotype 5 capsular polysaccharide conjugated to a carrier protein, the method comprising the steps of:

a) reacting an isolated *S. aureus* serotype 5 capsular polysaccharide with carbonyl ditriazole (CDT) in an organic solvent to produce an activated serotype 8 polysaccharide; and

b) reacting the activated serotype 5 polysaccharide with a carrier protein in an organic solvent to produce a serotype 5 polysaccharide:carrier protein conjugate;

wherein an immunogenic conjugate comprising a *S. aureus* serotype 5 capsular polysaccharide-carrier protein conjugate is produced.

29. The method of claim 27 or claim 28, which further comprises:

lyophilizing the isolated polysaccharide; and re-suspending the lyophilized polysaccharide in an organic solvent.

30. The method of any one of claims 27-29 wherein the activated isolated polysaccharide is isolated from the activation reaction prior to reacting with the carrier protein.

31. The method of claim 30, wherein step b) comprises:

i) lyophilizing the isolated activated isolated polysaccharide to produce a lyophilized activated isolated polysaccharide;

ii) lyophilizing the carrier protein to produce a lyophilized carrier protein; and

iii) re-suspending the lyophilized activated isolated polysaccharide and the lyophilized carrier protein in an organic solvent to produce an activated isolated polysaccharide mixed with a carrier protein.

32. The method of any of claims 27-31, which further comprises dilution of the reaction mixture of step b) into a buffer and maintaining a pH of about 8.8 to about 9.2 for at least 4 hours at about 20°C to about 26°C.

33. The method of any of claims 27-31, which further comprises dilution of the reaction mixture of step b) into a buffer and maintaining a pH of about 9.0 for at least 4 hours at about 23°C.

34. The method of any of claims 27-33, further comprising isolating the *S. aureus* serotype capsular polysaccharide-carrier protein conjugate.

35. The method of any of claims 27-34, wherein the organic solvent is a polar aprotic solvent.

36. The method of claim 35, wherein the polar aprotic solvent is selected from the group consisting of dimethyl sulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide, *N*-methyl-2-pyrrolidone, and hexamethylphosphoramide (HMPA).

37. The method of claim 36, wherein the organic solvent is dimethyl sulfoxide (DMSO).

38. The method of any of claims 27 and 29-37 wherein the step of reacting the polysaccharide with CDT comprises determining the water present in the *S. aureus* serotype 8 capsular polysaccharide and adjusting the CDT concentration to about 1:1 molar ratio of CDT:water in the organic solvent.

39. The method of any of claims 27 and 29-38 wherein the step of reacting the polysaccharide with CDT comprises determining the water present

in the *S. aureus* serotype 8 capsular polysaccharide and adjusting the CDT concentration to about 0.5:1 molar ratio of CDT:water in the organic solvent.

40. The method of any of claims 27 and 29-38 wherein the step of reacting the polysaccharide with CDT comprises determining the water present in the *S. aureus* serotype capsular 8 polysaccharide and adjusting the CDT concentration to about 0.75:1 molar ratio of CDT:water in the organic solvent.

41. The method of any of claims 28-37, wherein the step of reacting the serotype 5 polysaccharide with CDT comprises providing about 20-fold molar excess of CDT compared to the polysaccharide.

42. The method of any of claims 28-37 and 41 wherein the serotype 5 polysaccharide CDT in an organic solvent mixture is adjusted to a water concentration between 0.1 and 0.3%.

43. The method of claim 42 wherein the water concentration is adjusted to 0.2%.

44. The method of any of claims 29-43, wherein the step of isolating the activated serotype polysaccharide comprises diafiltration.

45. The method of any of claims 27-44, wherein prior to lyophilization the carrier protein is diafiltered against NaCl and the w/w ratio of NaCl/protein carrier protein is adjusted to about 0.5 to about 1.5.

46. The method of any of claims 27-45, wherein the carrier protein is CRM_{197}.

47. The method of claim 46, wherein the activated serotype polysaccharide is reacted with the CRM_{197} in a ratio by weight of about 1:1.

48. The method of any of claims 27-47, wherein the isolated *S. aureus* serotype capsular polysaccharide is mixed with imidazole or triazole prior to mixing with CDT in an organic solvent.

49. The method of any of claims 27-48 which further comprises hydrolyzing the serotype polysaccharide-carrier protein conjugate to remove unreacted activation groups.

50. A method of making an immunogenic conjugate comprising an isolated *Staphylococcus aureus* serotype 8 capsular polysaccharide conjugated to a carrier protein, the method comprising the steps of:

a) reacting a *S. aureus* serotype 8 capsular polysaccharide with 3-(2-pyridyldithio)-propionyl hydrazide (PDPH) and a carbodiimide in an organic solvent to produce a PDPH-linked polysaccharide;

b) reacting the PDPH-linked polysaccharide with a reducing agent to produce an activated polysaccharide;

c) isolating the activated serotype 8 polysaccharide to produce an isolated activated serotype 8 polysaccharide;

d) providing an activated carrier protein;

e) reacting the isolated activated serotype 8 polysaccharide with the activated carrier protein to produce a serotype 8 polysaccharide-carrier protein conjugate; whereby an immunogenic conjugate comprising an isolated *S. aureus* serotype 8 capsular polysaccharide conjugated to a carrier protein is produced.

51. A method of making an immunogenic conjugate comprising an isolated *Staphylococcus aureus* serotype 5 capsular polysaccharide conjugated to a carrier protein, the method comprising the steps of:

a) reacting a *S. aureus* serotype 5 capsular polysaccharide with 3-(2-pyridyldithio)-propionyl hydrazide (PDPH) and a carbodiimide in an organic solvent to produce a PDPH-linked polysaccharide;

b) reacting the PDPH-linked polysaccharide with a reducing agent to produce an activated polysaccharide;

c) isolating the activated serotype 5 polysaccharide to produce an isolated activated serotype 5 polysaccharide;

d) providing an activated carrier protein;

e) reacting the isolated activated serotype 5 polysaccharide with the activated carrier protein to produce a serotype 5 polysaccharide-carrier protein conjugate; whereby an immunogenic conjugate comprising an isolated *S. aureus* serotype 5 capsular polysaccharide conjugated to a carrier protein is produced.

52. The method of claim 50 or claim 51 wherein the activated carrier protein is isolated prior to reacting the activated carrier protein with the activated polysaccharide.

53. The method of any one of claims 50-52,wherein:

step c) further comprises lyophilizing the isolated activated serotype 8 polysaccharide to produce a lyophilized activated serotype polysaccharide.

54. The method of any of claims 50-53, wherein the organic solvent is a polar aprotic solvent.

55. The method of claim 54, wherein the polar aprotic solvent is selected from the group consisting of dimethyl sulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide, *N*-methyl-2-pyrrolidone, and hexamethylphosphoramide (HMPA).

56. The method of claim 55, wherein the polar aprotic solvent is dimethyl sulfoxide (DMSO).

57. The method of any of claims 50-56, wherein the carbodiimide is 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC).

58. The method of any of claims 50-57, wherein the step of reacting the serotype capsular polysaccharide with PDPH and EDAC in an organic solvent comprises maintaining a polysaccharide:PDPH:EDAC ratio by weight of about 1:5:3.

59. The method of any of claims 50-58, wherein the reducing agent is dithiothreitol (DTT).

60. The method of any of claims 50-59 wherein activation of the carrier protein comprises reacting the carrier protein with a bromoacetic acid.

61. The method of claim 60 wherein the bromoacetic acid comprises the N-hydroxysuccinimide ester of bromoacetic acid (BAANS).

62. The method of any of claims 50-61, wherein the step of isolating the activated serotype polysaccharide comprises diafiltration.

63. The method of any of claims 50-62 wherein the step of isolating the activated carrier protein comprises diafiltration.

64. The method of any of claims 50-63 which further includes the step of hydrolyzing the serotype polysaccharide-carrier protein conjugate to remove unreacted activation groups.

65. The method of claim 64 wherein the step of hydrolyzing the serotype polysaccharide-carrier protein conjugate comprises the addition of cysteamine hydrochloride.

66. The method of any of claims 50-65 which further comprises isolating the immunogenic conjugate comprising an isolated *S. aureus* serotype capsular polysaccharide conjugated to a carrier protein.

67. The method of claim 66 wherein the isolation of the serotype polysaccharide:carrier protein conjugate comprises diafiltration.

68. The method of any of claims 50-67, wherein said carrier protein is CRM_{197}.

69. The method of claim 68, wherein the activated serotype polysaccharide is reacted with the CRM_{197} in a ratio by weight of about 1:1.

70. The method of any of claims 27-69 wherein the activated polysaccharide has a size between about 20 kDa and about 1000 kDa.

71. The method of claim 70, wherein the activated polysaccharide has a size between about 50 kDa and 500 kDa.

72. The method of any of claims 27-71 wherein the immunogenic polysaccharide-protein conjugate has a size between 400 kDa and about 5000 kDa.

73. An immunogenic conjugate produced by the method of any of claims 27-72.

74. The immunogenic conjugate of claim 73 which comprises more than about 1% free polysaccharide and less than about 30% free polysaccharide.

75. The immunogenic conjugate of claim 73 which comprises less than about 20% free polysaccharide.

76. An immunogenic composition comprising the immunogenic conjugate of any of claims 73-75 and at least one of an adjuvant, diluent, or carrier.

77. The immunogenic composition of claim 76, wherein the adjuvant is an aluminum-based adjuvant.

78. The immunogenic composition of claim 77, wherein the adjuvant is selected from the group consisting of aluminum phosphate, aluminum sulfate and aluminum hydroxide.

79. The immunogenic composition of claim 78 wherein the adjuvant is aluminum phosphate.

80. The immunogenic composition of any of claims 76-79 which comprises less than 30% free type polysaccharide compared to the total amount of type polysaccharide.

81. The immunogenic composition of claim 80 which comprises less than 20% free type polysaccharide compared to the total amount of type polysaccharide.

82. A *S. aureus* Type 8 capsular polysaccharide having a molecular weight of between 20 kDa and 1000 kDa covalently bound to a carrier protein; wherein the combined molecular weight of the polysaccharide covalently bound to the carrier protein is between about 200 kDa and 5000 kDa.

83. A *S. aureus* Type 5 capsular polysaccharide having a molecular weight of between 20 kDa and 1000 kDa covalently bound to a carrier protein; wherein the combined molecular weight of the polysaccharide covalently bound to the carrier protein is between about 200 kDa and 5000 kDa.

84. The polysaccharide covalently bound to carrier protein of claim 82 or claim 83 wherein the polysaccharide has a molecular weight range of between 70 kDa and 300 kDa.

85. The polysaccharide covalently bound to carrier protein of claim 82 or claim 83 that has a molecular weight range of between 500 kDa and 2500 kDa.

86. The polysaccharide covalently bound to carrier protein of claim 82 or claim 83, wherein the carrier protein is CRM_{197}.

87. The polysaccharide covalently bound to carrier protein of claim 86, wherein the CRM_{197} is covalently linked to the polysaccharide through a carbamate linkage, an amide linkage, or both.

88. The polysaccharide covalently bound to carrier protein of claim 86 or claim 87, wherein the molar ratio of conjugated lysines to CRM_{197} is about 10:1 to about 25:1.

89. The polysaccharide covalently bound to carrier protein of any one of claim 88, wherein at least one covalent linkage between CRM_{197} and

polysaccharide occurs at least at every 5 to 10 saccharide repeat units of the polysaccharide.

90. The polysaccharide covalently bound to carrier protein of claim 88 or claim 89, wherein at least one linkage between CRM_{197} and polysaccharide occurs at every 5 saccharide repeat units of the polysaccharide.

91. The polysaccharide covalently bound to carrier protein of any of claims 88-89 wherein the CRM_{197} comprises 5 to 22 lysines covalently linked to the polysaccharide.

92. The polysaccharide covalently bound to carrier protein of claim 91, wherein the CRM_{197} comprises 8 to 15 lysines covalently linked to the polysaccharide.

93. An immunogenic composition comprising the polysaccharide covalently bound to carrier protein of any of claims 82-92 and at least one of an adjuvant, diluent, or carrier.

94. The immunogenic composition of claim 93, wherein the adjuvant is an aluminum-based adjuvant.

95. The immunogenic composition of claim 94, wherein the adjuvant is selected from the group consisting of aluminum phosphate, aluminum sulfate and aluminum hydroxide.

96. The immunogenic composition of claim 95, wherein the adjuvant is aluminum phosphate.

97. A method of reducing or preventing a Staphylococcal infection, disease or condition associated with a *Staphylococcus* bacteria in a subject, the method comprising the step of administering an immunologically effective amount of an immunogenic composition of any of claims 20-25 and 76-81 and 93-96 to the subject.

98. The method of claim 97, wherein the infection, disease or condition is selected from the group consisting of invasive *Staphylococcus aureus*, sepsis and carriage.

99. A method of reducing or preventing a Staphylococcal infection in a subject undergoing a surgical procedure, the method comprising the step of administering an immunologically effective amount of an immunogenic composition of any of any of claims 20-25 and 76-81 and 93-96 to the subject prior to the surgical procedure.

100. The method of any one of claims 97-99, wherein the subject is a human, a household pet, or a livestock animal.

101. A method of isolating *S. aureus* capsular polysaccharide with molecular weight from 20-1000 kDa comprising the step of subjecting a bacterial cell suspension to an acidic heat treatment.

102. An antibody generated by a capsular polysaccharide, an immunogenic conjugate, or an immunogenic composition of the present invention.

103. The antibody of claim 102, wherein the antibody is functional as measured by killing bacteria in an animal efficacy model or an opsonophagocytic killing assay.