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(71) Applicant: **GLAXOSMITHKLINE BIOLOGICALS SA** [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE).

(72) Inventors: **CARBONI, Filippo**; Via Fiorentina 1, 53100 Siena (IT). **COZZI, Roberta**; Via Fiorentina 1, 53100 Siena (IT). **MARGARIT Y ROS, Immaculada**; Via Fiorentina 1, 53100 Siena (IT). **ROMAGNOLI, Giacomo**; Via Fiorentina 1, 53100 Siena (IT). **ROMANO, Maria Rosaria**; Via Fiorentina 1, 53100 Siena (IT).

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(54) Title: BACTERIAL IMMUNIZATION USING QBETA HAIRPIN NANOPARTICLE CONSTRUCTS

(57) Abstract: The present disclosure provides protein nanoparticles, constructs, and compositions for use in inducing an immunogenic response against a bacterial polysaccharide. Also provided are polynucleotides encoding the protein nanoparticles, host cells capable of expressing the protein nanoparticles, and methods of producing the protein nanoparticles.

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Bacterial Immunization Using Qbeta Hairpin Nanoparticle Constructs

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on July 20, 2022, is named VU67131P-US_SL.txt and is 184,011 bytes in size.

FIELD OF THE INVENTION

[01] The present disclosure relates to methods of producing protein nanoparticles. The present disclosure also relates to protein nanoparticles, polynucleotides, constructs, and compositions for use in inducing an immunogenic response against a bacterial polysaccharide. Also provided are methods of inducing an immunogenic response against a bacterial polysaccharide using the protein nanoparticles, constructs, and compositions of the present disclosure.

BACKGROUND

[02] *Streptococcus agalactiae* (also known as "Group B Streptococcus" or "GBS") is a β-hemolytic, encapsulated Gram-positive microorganism that is a major cause of neonatal sepsis and meningitis, particularly in infants born to women carrying the bacteria (Heath & Schuchat (2007)). The use of intrapartum antibiotic prophylaxis has reduced early-onset neonatal disease but has not significantly affected the incidence of late-onset (7-90 days after birth) neonatal GBS disease (*see, e.g.*, Baker (2013)). An effective vaccine designed for maternal administration during pregnancy is desirable to prevent GBS disease in infants; currently, no licensed GBS vaccine is available.

[03] The GBS capsule is a virulence factor that assists the bacterium in evading human innate immune defences. The GBS capsule consists of high molecular weight polymers made of multiple identical repeating units of four to seven monosaccharides and including sialic acid (N-acetylneuraminic acid) residues, referred to as Capsular

Polysaccharides (CPS). GBS can be classified into ten serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX) based on the chemical composition and the pattern of glycosidic linkages of the capsular polysaccharide repeating units. Non-typeable strains of GBS are also known to exist. Description of the structure of GBS CPS may be found in the published literature (*see e.g.*, WO2012/035519). The capsular polysaccharides of different GBS serotypes are chemically related but are antigenically different.

[04] GBS capsular polysaccharides (also referred to as capsular saccharides or CPS) have been investigated for use in vaccines. However, saccharides are T-independent antigens and are generally poorly immunogenic. Covalent conjugation of a saccharide to a carrier molecule (such as a monomeric protein carrier) can convert T-independent antigens into T-dependent antigens, thereby enhancing memory responses and allowing protective immunity to develop. Immune interference is a concern where a subject receives multiple different vaccines (either concurrently or sequentially) that contain the same carrier protein (*see*, *e.g.*, Findlow and Borrow (2016); Voysey et al., (2016); Dagan et al., Infect. Immun. 66:2093-2098 (1998)). Tetanus toxoid (TT), diphtheria toxoid (DT), and cross-reacting material 197 (CRM, or CRM197) are currently used as monomeric carrier proteins in marketed vaccines against *H. influenzae* and multiple strains of meningococcal bacteria. CRM197 is additionally found in marketed multivalent pneumococcal vaccines.

[05] GBS glycoconjugates of CPS serotypes Ia, Ib, II, III, IV and V conjugated to monomeric carrier proteins have separately been shown to be immunogenic in humans. Multivalent GBS vaccines have been described, *e.g.*, in WO2016-178123, WO2012-035519, and WO2014-053612. Clinical studies using monovalent or bivalent GBS glycoconjugate (saccharide + carrier protein) vaccines have previously been conducted with both non-pregnant adults and pregnant women. *See*, *e.g.*, Paoletti *et al.* (1996); Baker *et al.* (1999); Baker *et al.*, (2000); Baker *et al.* (2003); Baker *et al.* (2004).

[06] A pentavalent GBS glycoconjugate vaccine (serotypes Ia, Ib, II, III, and V, conjugated to monomeric carrier protein, and with or without aluminum phosphate adjuvant) has been evaluated in a phase I trial (NCT03170609). A GBS trivalent vaccine (serotypes Ia, Ib, and III) comprising conjugates of GBS CPS and the monomeric carrier protein CRM197 was evaluated for use in maternal vaccination in a phase 1b/2 clinical trial (NCT01193920); infants born to the vaccinated women were reported to have higher GBS serotype-specific

antibody levels (transplacentally transferred antibodies) until 90 days of age, compared with a placebo group (Madhi et al., Clin. Infect. Dis. 65(11):1897-1904 (2017).

[07] Typical monomeric carrier proteins used for the conjugation with bacterial saccharide antigens for the development of potential vaccines are the Tetanus Toxoid (TT), the genetically detoxified diphtheriae toxoid (CRM197) and GBS pili proteins (Nilo et al, (2015a) and Nilo et al, (2015b)).

[08] There is a continuing need for efficient, reproducible antigenic constructs and compositions comprising such constructs that are capable of inducing an immune response against GBS and other bacterial pathogens in human subjects.

SUMMARY OF THE INVENTION

[09] This summary is provided to introduce a selection of concepts that are further described below in the detailed description. This summary is not intended to identify key or essential features of the disclosed subject matter, nor should it be construed as limiting the scope of the disclosed subject matter.

[010] The present disclosure provides methods of producing a protein nanoparticle, such as a Virus-Like Particle (VLP), having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial or yeast saccharide, and wherein the VLP encapsulates at least one RNA polynucleotide. In the methods of the present disclosure, specifically designed RNA polynucleotides are efficiently encapsulated inside the VLP. The methods of the present disclosure produce reproducible RNA encapsulating VLPs, wherein the VLPs are conjugated to bacterial saccharides. In one embodiment, the produced protein nanoparticle is a Qβ VLP encapsulating at least one RNA polynucleotide. In one embodiment, the bacterial or yeast saccharide is selected from the group consisting of Acinetobacter species, Bacillus species, Bordetella species, Borrelia species, Burkholderia species, Campylobacter species, Candida species, Chlamydia species, Clostridium species, Corynebacterium species, Enterococcus species, Escherichia species, Francisella species, Haemophilus species, Helicobacter species, Klebsiella species, Legionella species, Listeria species, Neisseria species, Proteus species, Pseudomonas species, Salmonella species,

Shigella species, Staphylococcus species, Streptococcus species, Streptomyces species, Vibrio species, and Yersinia species.

[011] The present disclosure also provides protein nanoparticles, constructs, immunogenic compositions, pharmaceutical compositions, and vaccine compositions used for the manufacture of a medicament for inducing an immune response. Also provided are polynucleotides encoding the protein nanoparticles of the present disclosure and host cells capable of expressing the protein nanoparticles/and or polynucleotides.

[012] A further embodiment of the disclosure is a method of inducing an immune response in a human subject by administering to the subject an immunologically effective amount of one of the following: protein nanoparticles, immunogenic compositions, vaccine compositions, or pharmaceutical compositions of the present disclosure.

BRIEF DESCRIPTION OF THE FIGURES

[013] **FIG. 1** depicts the plasmid/vector (pET24_ Qβ CP_HP) used for the preparation of the Qβhp nanoparticles (Qβ VLP encapsulating the RNA hairpin). The pET24 plasmid contains *inter alia* a T7 promoter, a DNA polynucleotide sequence encoding the Qbeta nanoparticle coat protein (CP), which is genetically fused to a DNA polynucleotide encoding the RNA hairpin, and a DNA polynucleotide encoding an aptamer RNA.

[014] **FIG. 2** provides a graph of Labchip® analysis comparing the RNA profile in Qβhp prepared in the HTMC medium (lot 11) and Qβhp prepared in the M9 medium (lot 13 b) versus Qβwt (lot 9).

[015] **FIG. 3** illustrates oxidation of GBS serotype II capsular polysaccharide using $NaIO_4$.

[016] **FIG. 4** depicts the conjugation of modified GBS serotype II polysaccharides (as shown in **FIG. 3**) to Qβhp nanoparticles.

[017] **FIG. 5** provides graphs of SE-HPLC analysis of GBS PSII-Qβhp and Qβhp nanoparticles. **FIG. 5A** compares Qβhp nanoparticles (without conjugated saccharide)

prepared in HTMC medium (lot 11) with GBS PSII-Qβhp nanoparticles prepared in the same medium. **FIG. 5B** compares Qβhp nanoparticles (without conjugated saccharide) prepared in M9 medium (lot 13B) with GBS PSII-Qβhp nanoparticles prepared in the same medium.

[018] **FIG. 6** provides a negative stain TEM image of Qβhp nanoparticles conjugated to GBS PSII showing typical icosahedral symmetry with a diameter around 33nm (Scale bar = 200 nm).

[019] **FIG. 7.** provides an ELISA graph summarizing the results of the *in vivo* immunogenicity experiments. The ELISA assay compared GBS PSII-Qβhp (Qβ VLP encapsulating the RNA hairpin) with GBS PSII- Qβ wt (wild type) and GBS PSII-CRM after one dose (on days 21 and 42) and after two doses (on days 21 and 42) of injections.

DETAILED DESCRIPTION

[020] The present disclosure provides methods of producing a protein nanoparticle, such as a Virus-Like Particle (VLP), having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, and wherein the VLP encapsulates at least one RNA polynucleotide.

[021] The present disclosure also provides protein nanoparticles, constructs, immunogenic compositions, pharmaceutical compositions used for the manufacture of a medicament for inducing an immune response. Also provided are polynucleotides encoding the protein nanoparticles of the present disclosure and host cells capable of expressing the protein nanoparticles/and or polynucleotides.

Methods of Producing Protein Nanoparticles

[022] Provided herein are methods of producing protein nanoparticles, such as Virus-Like Particles (VLPs), wherein the protein nanoparticles encapsulate at least one specific RNA polynucleotide. RNA polynucleotides encapsulated within the VLPs can be single-stranded or double-stranded RNA (dsRNA). In some embodiments, the RNA is not a naturally occurring RNA. In some embodiments, the RNA contains thermal-stable tetraloops, G-quartets, and other motifs that confer desired folding propensities. VLPs containing RNA

polynucleotides are referred to as "RNA-VLPs." In some embodiment, the RNA-VLP is a Qβ RNA-VLP.

[023] In one embodiment, there is provided a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial or yeast saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; and (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination.

[024] In one embodiment, the vector is an expression vector. In general, expression of recombinantly encoded nanoparticles involves the preparation of an expression vector comprising a recombinant polynucleotide under the control of one or more promoters, such that the promoter stimulates transcription of the polynucleotide and promotes expression of the encoded polypeptide. "Recombinant Expression" as used herein refers to such a method.

[025] "Recombinant expression vectors" comprise a recombinant nucleic acid sequence operatively linked to control sequences capable of effecting expression of the gene product. "Control sequences" are nucleic acid sequences capable of effecting the expression of the nucleic acid molecules and need not be contiguous with the nucleic acid sequences, so long as they function to direct the expression thereof. Host cells comprise such recombinant expression vectors. In one embodiment, the vector of the present disclosure is designed to encode (i) at least one RNA polynucleotide; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer. In one embodiment, the vector is a single vector. In one

embodiment, the vector comprises more than one vector, such as two vectors. In one embodiment, a single vector encodes: (i) at least one RNA polynucleotide; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer. In one embodiment, one vector encodes the at least one bacteriophage VLP, and another vector encodes the at least one RNA polynucleotide and optionally the at least one aptamer. In one embodiment, the vector contains *inter alia* a T7 promotor, a DNA polynucleotide sequence encoding the Qbeta nanoparticle coat protein (CP), a DNA polynucleotide encoding the RNA hairpin, and optionally a DNA polynucleotide encoding an aptamer RNA, wherein the DNA polynucleotide sequence encoding the Qbeta nanoparticle coat protein is genetically fused to (i) the DNA polynucleotide encoding the RNA hairpin, and (ii) optionally the DNA polynucleotide encoding an aptamer RNA.

[026] Methods of expression of a vector in a host cell are known in the art. Various expression systems are known in the art, including those using human (e.g., HeLa) host cells, mammalian (e.g., Chinese Hamster Ovary (CHO)) host cells, prokaryotic host cells (e.g., *E. coli*), or insect host cells. The host cell is typically transformed with the recombinant nucleic acid sequence encoding the desired polypeptide product, cultured under conditions suitable for expression of the product, and the product purified from the cell or culture medium. Cell culture conditions are particular to the cell type and expression vector, as is known in the art.

[027] Host cells can be cultured in conventional nutrient media modified as appropriate and as will be apparent to those skilled in the art (e.g., for activating promoters). Culture conditions, such as temperature, pH and the like, may be determined using knowledge in the art, see e.g., Freshney (1994) and the references cited therein. In one embodiment of the present disclosure the host cell is cultured in HTMC growth medium (Cell Applications, Inc.). In one embodiment host cell is cultured in M9 medium (Cell Applications, Inc.). In bacterial host cell systems, a number of expression vectors are available including, but not limited to, multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene) or pET vectors (Novagen, Madison WI). In mammalian host cell systems, a number of expression systems, including both plasmids and viral-based systems, are available commercially.

[028] Eukaryotic or microbial host cells expressing the nanoparticles can be disrupted by any convenient method (including freeze-thaw cycling, sonication, mechanical disruption), and polypeptides and/or self-assembled nanoparticles can be recovered and purified from recombinant cell culture by any suitable method known in the art (including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography (e.g., using any of the tagging systems noted herein), hydroxyapatite chromatography, and lectin chromatography). High-performance liquid chromatography (HPLC) can be employed in the final purification steps.

[029] In another embodiment, provided herein is a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the antigenic molecule, optionally a bacterial saccharide from a bacteria; (e) conjugating the antigenic molecule, for example the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by chemical conjugation (e.g., reductive amination), wherein the at least one recognition tag is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the polynucleotide of SEQ ID NO: 1. In one embodiment, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or 100% of the at least one RNA polynucleotide is encoded by the vector.

[030] Recognition tags for viral and bacteriophage coat proteins are known in the art and include, but are not limited to the Qbeta bacteriophage coat protein (*see*, *e.g.,* Witherell, et al., Biochemistry, 28:71-76 (1989)) and PP7 coat protein (*see, e.g.,* Lim and Peabody, Nucleic Acids Res., 30 (19) 4138-4144 (2002)). The recognition tag facilitates the efficient and spontaneous uptake and assembly of the RNA into VLPs. In one embodiment, the recognition tag is a 54-nucleotide RNA containing two hairpin structures specific for the bacteriophage Qbeta coat protein (SEQ ID NO: 2). Qβ Coat protein binds to the RNA hairpin structure during VLP assembly, which leads to the assembly of the RNA-VLP nanoparticle.

[031] In some embodiments, the at least one RNA polynucleotide contains a nucleotide sequence that facilitates the purification of the RNA by affinity chromatography. In one embodiment, the nucleotide sequence that facilitates purification forms an RNA aptamer. RNA aptamers can assist in the purification of the RNA through affinity chromatography with RNA binding proteins. In one embodiment, a sequence that assists in the purification of the RNA "RNA aptamer or aptamer" is located at the opposite end of the RNA polynucleotide to the bacteriophage or viral coat protein recognition tag (SEQ ID NO: 6) (Nucleic Acids Research, 2001, V01.29, No. 2 e4).

[032] In one embodiment, provided herein is a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from

a bacteria; (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination, wherein the aptamer is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the polynucleotide of SEQ ID NO: 5.

[033] In one embodiment, the VLP of the present disclosure is a Qβ or coat protein of Qβ VLP. Qβ VLP coat proteins are known in the art. SEQ ID NO: 4 is an exemplary nucleotide sequence for genes encoding the bacteriophage Qβ coat protein of the present disclosure.

[034] In one embodiment, provided herein is a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination, wherein the bacteriophage VLP comprises an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the amino acid sequence set out in SEQ ID NO: 3.

[035] In one embodiment, provided herein is a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a

bacteriophage Virus-Like Particle (VLP), wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination, wherein the vector comprises the polynucleotide of SEQ ID NO: 7 or SEQ ID NO: 8.

Purification

[036] Because of their distinctive size, properties and high stability, VLPs can be easily purified from the host. The purification of VLPs containing RNA "RNA-VLP" could be performed by known methods such as centrifugation, dialysis, chromatography, gel electrophoresis, affinity purification, filtration, precipitation, antibody capture, and combinations thereof. Nanoparticles may be expressed with a tag operable for affinity purification, such as a 6xHistidine tag as is known in the art. A His-tagged nanoparticle may be purified using, for example, Ni-NTA column chromatography or using anti-6xHis antibody fused to a solid support. The term "purified" as used herein refers to the separation or isolation of a defined product (e.g., a recombinantly expressed polypeptide) from a composition containing other components (e.g., a host cell or host cell medium). A polypeptide/protein nanoparticle composition that has been fractionated to remove undesired components, and which composition retains its biological activity is considered purified. A purified polypeptide/ protein nanoparticle retains its biological activity. Purified is a relative term and does not require that the desired product be separated from all traces of other components. Stated another way, "purification" or "purifying" refers to the process of removing undesired components from a composition or host cell or culture.

[037] A "substantially pure" preparation of polypeptides (or nanoparticles) or nucleic acid molecules is one in which the desired component represents at least 50% of the total polypeptide (or nucleic acid) content of the preparation. In certain embodiments, a substantially pure preparation will contain at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% or more of the total polypeptide (or nucleic acid) content of the preparation. Methods for quantifying the degree of purification of expressed polypeptides are known in the art and include, for example, determining the specific activity of an active fraction or assessing the number of polypeptides within a fraction by SDS/PAGE analysis.

Antigenic display

[038] Molecules, including antigenic molecules, attached to the exterior surface of the protein nanoparticles ("NP") of the present disclosure may be referred to herein as "display" or "displayed" molecules. Antigen-displaying nanoparticles preferably display multiple copies of antigenic molecules in an ordered array. It is theorized that an ordered multiplicity of antigens presented on a NP allows multiple binding events to occur simultaneously between the NP and a host's cells, which favors the induction of a potent host immune response. *See e.g.*, Lopez-Sagaseta *et al.*, (2016).

[039] Presentation of antigens on NP has been exploited to improve the immunogenicity of subunit protein antigens (Jardine et al, (2013); Correira et al, (2014)). In particular, Qβ nanoparticles have been used as a scaffold for a variety of haptens (including nicotinamide/alzheimer peptides/angiotensin) (Lopez-Sagaseta et al., (2015)). Qβ nanoparticles are also known to behave as scaffolds for short synthetic cancer (Wu et al., (2019)) or bacterial (Polonskaya et al, (2017)) carbohydrates antigens. However, the impact of conjugation of medium-long carbohydrates (medium length oligosaccharides and long length polysaccharides *per se* exposing multiple carbohydrate epitopes in sequence) on the onset of the elicited immune response is not predictable and has never been thoroughly explored.

Conjugation

[040] Conjugation of bacterial saccharides, such as GBS saccharides, to monomeric carrier proteins, has been widely reported (Paoletti *et al.* (1990)). Therefore, as used herein, the term "monomeric carrier protein" or "carrier protein" refers to an immunogenic protein which, when conjugated to a polysaccharide and administered to a subject, such as a human subject, will enhance an immune response in the subject, particularly the production of antibodies that bind specifically to the conjugated polysaccharide. The typical process for the production of bacterial glycoconjugates, such as GBS glycoconjugates, typically involves reductive amination of a purified saccharide to a monomeric carrier protein such as tetanus toxoid (TT) or CRM197 (Wessels et al. (1990)). The reductive amination involves an amine group on the side chain of an amino acid in the monomeric carrier and an aldehyde group in the saccharide. The conjugates can be prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508. In one embodiment of the present disclosure, the protein nanoparticle encapsulating at least one RNA polynucleotide is conjugated to the bacterial saccharide by reductive amination.

[041] As GBS capsular saccharides do not include an aldehyde group in their natural form then this is typically generated before conjugation by oxidation (*e.g.* periodate oxidation) of a portion (*e.g.* between 5 and 40%) of the saccharide's sialic acid residues [Wessels et al. (1990); US Patent No. 4,356,170]. GBS glycoconjugate vaccines prepared in this manner have been shown to be safe and immunogenic in humans for each of GBS serotypes Ia, Ib, II, III, and V (Paoletti & Kasper (2003)). An alternative conjugation process involves the use of $-NH_2$ groups in the saccharide (either from de-N-acetylation, or after introduction of amines) in conjunction with bifunctional linkers, as described (WO2006/082530). A further alternative process is described in WO96/40795 and Michon et al. (2006). In this process, the free aldehydes groups of terminal 2,5-anhydro-D-mannose residues from depolymerization of type II or type III capsular saccharides by mild cleavage through de-N-acetylation/nitrosation are used for conjugation by reductive amination.

[042] The conjugation method may rely on activation of the saccharide with cyanylate chemistry, such as with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate

(CDAP) to form a cyanate ester. The activated saccharide may thus be coupled directly or via a spacer (linker) group to an amino group on the protein nanoparticle. For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide or oligosaccharide which could be coupled to the protein nanoparticle via a thioether linkage obtained after reaction with a maleimide-activated protein nanoparticle (for example using GMBS) or a holoacetylated protein nanoparticle (for example using iodoacetimide or N-succinimidyl bromoacetatebromoacetate). Optionally, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or ADH and the amino-derivatised saccharide is conjugated to the protein nanoparticle using carbodiimide (e.g. EDAC or EDC) chemistry via a carboxyl group on the protein nanoparticle. Such conjugation methods are described in PCT published application WO 93/15760 Uniformed Services University and WO 95/08348 and WO 96/29094.

[043] Other suitable techniques use carbiinides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU. Many are described in WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by the reaction of a free hydroxyl group of the saccharide with CDI (Bethell et al J. Biol. Chem. 1979, 254; 2572-4, Hearn et al J. Chromatogr. 1981. 218; 509-18) followed by reaction of with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group' reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

[044] Following the conjugation (the reduction reaction and optionally the capping or quenching reaction), the glycoconjugates may be purified (enriched with respect to the amount of polysaccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration, ultrafiltration, precipitation/elution, column chromatography (ion-exchange chromatography, multimodal ion-exchange chromatography, DEAE, or hydrophobic interaction chromatography), and depth filtration. *See,* e.g., U.S. Pat. No. 6,146,902.

[045] In one embodiment, provided herein is a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the

antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination, wherein the bacterial or yeast saccharide is from a bacterial or yeast species selected from the group consisting of Acinetobacter species, Bacillus species, Bordetella species, Borrelia species, Burkholderia species, Campylobacter species, Candida species, Chlamydia species, Clostridium species, Corynebacterium species, Enterococcus species, Escherichia species, Francisella species, Haemophilus species, Helicobacter species, Klebsiella species, Legionella species, Listeria species, Neisseria species, Proteus species, Pseudomonas species, Salmonella species, Shigella species, Staphylococcus species, Streptococcus species, Streptomyces species, Vibrio species, and Yersinia species. In one embodiment, the bacterial saccharide is from a Streptococcus species selected from Streptococcus agalactiae (Group B Streptococcus, or GBS) and Streptococcus pneumoniae. In one embodiment, the bacterial saccharide is selected from the group consisting of GBS CPS serotype Ia, GBS CPS serotype Ib, GBS CPS serotype II, GBS CPS serotype III, GBS CPS serotype IV, and GBS CPS serotype V.

Protein Nanoparticles, Polynucleotides, and Host cells

[046] The protein nanoparticles of the present disclosure are produced using the methods discussed above. The protein nanoparticles may be analyzed using methods known in the art, *e.g.*, by crystallography, Dynamic Light Scattering (DLS), Nano-Differential Scanning Fluorimetry (Nano-DSF), and Electron Microscopy, to confirm production of suitable nanoparticles.

[047] The protein nanoparticles of the present disclosure are capable of self-assembly from subunit proteins, into nanoparticles, *i.e.,* particles of less than about 100nm in maximum diameter. Self-assembly of NPs refers to the oligomerization of polypeptide subunits into an ordered arrangement, driven by non-covalent interactions.

[048] The protein nanoparticles of the present disclosure have antigenic molecules conjugated to their exterior surface, wherein the antigen molecules are bacterial or yeast saccharides, and wherein the protein nanoparticles are bacteriophage Virus-Like Particles (VLPs) encapsulating at least one RNA polynucleotide. VLPs are supermolecular structures built from multiple viral protein molecules (polypeptide subunits) of one or more types. VLPs lack the viral genome and are therefore noninfectious. VLPs can often be produced in large quantities by recombinant expression methods.

[049] Examples of VLPs include those made of the viral capsid proteins of hepatitis B virus (Ulrich, et al., (1998)), measles virus (Wames, et al., Gene 160:173-178 (1995)), Sindbis virus, rotavirus (U.S. Pat. Nos. 5,071,651 and 5,374,426), foot-and-mouth-disease virus (Twomey, et al., (1995)), Norwalk virus (Jiang, et al., (1990); Matsui, et al., J. Clin. Invest. 87:1456-1461 (1991)), the retroviral GAG protein (WO 96/30523), the surface protein of Hepatitis B virus (WO 92/11291), and human papilloma virus (WO 98/15631).

[050] VLPs may also be made from recombinant proteins of an RNA-phage, such as from bacteriophage Qβ, bacteriophage R17, bacteriophage fr, bacteriophage GA, bacteriophage SP, bacteriophage MS2, bacteriophage M11, bacteriophage MX1, bacteriophage NL95, bacteriophage f2, and bacteriophage PP7.

[051] In one embodiment, provided herein is a protein nanoparticle which is a VLP made of coat proteins from the *E. coli* RNA bacteriophage Qbeta (Qβ). Qβ VLPs have an essentially icosahedral phage-like capsid structure with a diameter of about 30 nm. The capsid contains 180 copies of coat protein, linked in covalent pentamers and hexamers by disulfide bridges (Golmohammadi, et al., (1996)). Capsids or VLPs made from recombinant Qβ coat proteins may contain, however, subunits which are either not linked via disulfide bonds to other subunits within the capsid, or which are incompletely linked, meaning that such VLPs comprise fewer than the maximum number of possible disulfide bonds.

[052] The gene for the Qβ coat protein (CP) contains a "leaky" stop codon that occasionally results in a readthrough by the host ribosome producing a minor coat protein A_1. A_1 consists of the full-length coat domain connected by a flexible linker to the readthrough domain, a 196-amino acid C-terminal extension (Cui et al., (2017); Runnieks et al. (2011)).

[053] Qβ capsid proteins used to produce VLPs may include Qβ Coat Protein (CP) and Qβ A1 protein, and variants thereof, including variant proteins in which the N-terminal methionine is cleaved; C-terminal truncated forms of Qβ A1 missing as much as 100, 150 or 180 amino acids; variant proteins which have been modified by the removal of a lysine residue by deletion or substitution or by the addition of a lysine residue by substitution or insertion (see for example Qbeta-240, Qbeta-243, Qbeta-250, Qbeta-251 and Qbeta-259 disclosed in WO03/024481 (US 8,691,209; US9,950,055)). See also, *e.g.*, WO02/056905, WO03/024480. Typically, the percentage of Qβ A1 protein relative to CP in the VLP is limited, to ensure VLP formation. *See* Qbeta Coat Protein (CP) Protein Information Resource (PIR) Database, Accession No. VCBPQbeta; Qβ Al protein PIR Database Accession No. AAA16663. Qβ VLPs are hollow with pores 1.4 nm in diameter. The inventors of the present disclosure have efficiently packaged at least one RNA polynucleotide/RNA, such as RNA hairpin in the Qβ VLPs to increase efficiency, reproducibility, and immunogenicity of the Qβ VLP conjugates of the present disclosure. In one embodiment, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or 100% of the at least one RNA polynucleotide is encoded by the vector.

[054] In one embodiment, the nanoparticles of the present disclosure are produced by a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the

bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; and (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination.

[055] In one embodiment, the vector of the present disclosure is designed to encode (i) at least one RNA polynucleotide; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer. In one embodiment, the vector is a single vector. In one embodiment, the vector comprises more than one vector, such as two vectors. In one embodiment, a single vector encodes: (i) at least one RNA polynucleotide; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer. In one embodiment, one vector encodes the at least one bacteriophage VLP, and another vector encodes the at least one RNA polynucleotide and optionally the at least one aptamer.

[056] In one embodiment, the nanoparticles of the present disclosure are produced by a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; and (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination, wherein the at least one recognition tag is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%,

or 100% identical to the polynucleotide of SEQ ID NO: 1. In one embodiment, at least 50% of the at least one RNA polynucleotide is encoded by the vector.

[057] In one embodiment, the nanoparticles of the present disclosure are produced by a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; and (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination, wherein the aptamer is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the polynucleotide of SEQ ID NO: 5.

[058] In one embodiment, the nanoparticles of the present disclosure are produced by a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering

and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; and (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination, wherein the bacteriophage VLP comprises an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the amino acid sequence set out in SEQ ID NO: 3. In one embodiment, the RNA-VLP is a Qbeta RNA-VLP. In one embodiment, the vector contains inter alia a T7 promotor, a DNA polynucleotide sequence encoding the Qbeta nanoparticle coat protein (CP), a DNA polynucleotide encoding the RNA hairpin, and optionally a DNA polynucleotide encoding an aptamer RNA, wherein the DNA polynucleotide sequence encoding the Qbeta nanoparticle coat protein is genetically fused to (i) the DNA polynucleotide encoding the RNA hairpin, and (ii) optionally the DNA polynucleotide encoding an aptamer RNA.

[059] In one embodiment, the nanoparticles of the present disclosure are produced by a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; and (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination, wherein the bacterial saccharide is from a bacterial species selected from the group consisting of Acinetobacter species, Bacillus species, Bordetella species, Borrelia species, Burkholderia species, Campylobacter species, Candida species, Chlamydia species, Clostridium species,

Corynebacterium species, Enterococcus species, Escherichia species, Francisella species, Haemophilus species, Helicobacter species, Klebsiella species, Legionella species, Listeria species, Neisseria species, Proteus species, Pseudomonas species, Salmonella species, Shigella species, Staphylococcus species, Streptococcus species, Streptomyces species, Vibrio species, and Yersinia species. In one embodiment, the bacterial saccharide is from a Streptococcus species selected from Streptococcus agalactiae (Group B Streptococcus, or GBS) and Streptococcus pneumoniae. In one embodiment, the bacterial saccharide is selected from the group consisting of GBS CPS serotype Ia, GBS CPS serotype Ib, GBS CPS serotype II, GBS CPS serotype III, GBS CPS serotype IV, and GBS CPS serotype V.

[060] The NPs of the present disclosure may be used for any suitable purpose, such as for inducing an immune response in a subject. In one embodiment the subject is a human subject.

[061] The present inventors have surprisingly found that Qbeta RNA-VLP (Qbeta nanoparticles encapsulating a specific RNA polynucleotide) displaying bacterial capsular polysaccharide antigens efficiently induced specific immune responses, in particular antibody responses. Such responses could be induced in the absence of an adjuvant. Using, Qbeta RNA-VLP nanoparticles, strong immune responses to displayed bacterial capsular polysaccharides were achieved after a single administration in mice which were higher than the responses induced by a single administration of the bacterial saccharide-CRM197 conjugate. Qbeta RNA-VLP displaying bacterial capsular polysaccharide antigens displaying bacterial saccharide antigens induced in mice after one dose a comparable or higher immune response compared to two doses of the bacterial saccharide-CRM197 conjugates.

[062] Accordingly, the present disclosure provides a Qbeta RNA-VLP conjugated to a GBS saccharide antigen, such as a polysaccharide antigen, wherein the Qbeta RNA-VLP is capable of inducing an immune response to the saccharide antigen following a single dose, and wherein the immune response is higher than the immune response elicited by a single dose of a monomeric protein carrier, such as CRM197, displaying the same GBS saccharide. In another embodiment, the Qbeta RNA-VLP is capable of inducing an immune response to the GBS saccharide antigen following a single dose, wherein the immune response is higher or comparable to the immune response elicited by two doses of a monomeric protein carrier, such as CRM197, displaying the same GBS saccharide.

[063] In one embodiment, provided herein is a protein nanoparticle produced by the methods of the present disclosure.

[064] In one embodiment, provided herein are polynucleotides encoding the protein nanoparticles of the present disclosure.

[065] In one embodiment, provided herein are host cells comprising the polynucleotides encoding the protein nanoparticles of the present disclosure.

Compositions

[066] A further embodiment of the present disclosure is immunogenic compositions pharmaceutical compositions, or vaccine compositions, which comprise the nanoparticles of the present disclosure, wherein the nanoparticles display bacterial polysaccharide, such as GBS polysaccharide antigens, and a pharmaceutically acceptable diluent, or excipient. In certain instances, immunogenic compositions are administered to subjects to elicit an immune response that protects the subject against infection by a pathogen, or decreases symptoms or conditions induced by a pathogen. In the context of this disclosure, the term immunogenic composition will be understood to encompass compositions that are intended for administration to a subject or population of subjects for the purpose of eliciting a protective or palliative immune response against a bacterial pathogen, such as a *Acinetobacter* species, *Bacillus* species, *Bordetella* species, *Borrelia* species, *Burkholderia* species, *Campylobacter* species, *Candida* species, *Chlamydia* species, *Clostridium* species, *Corynebacterium* species, *Enterococcus* species, *Escherichia* species, *Francisella* species, *Haemophilus* species, *Helicobacter* species, *Klebsiella* species, *Legionella* species, *Listeria* species, *Neisseria* species, *Proteus* species, *Pseudomonas* species, *Salmonella* species, *Shigella* species, *Staphylococcus* species, *Streptococcus* species, *Streptomyces* species, *Vibrio* species, and *Yersinia* species.

[067] An "immunogenic composition" is a composition of matter suitable for administration to a human or non-human mammalian subject and which, upon administration of an immunologically effective amount, elicits a specific immune response, *e.g.*, against an antigen displayed on the protein nanoparticles. An immunogenic composition of the present disclosure can include one or more additional components, such as an adjuvant. An

"adjuvant" is an agent that enhances the production of an immune response in a non-specific manner. Common adjuvants include suspensions of minerals (alum, aluminum hydroxide, aluminum phosphate); saponins such as QS21; emulsions, including water-in-oil, and oil-in-water (and variants thereof, including double emulsions and reversible emulsions), liposaccharides, lipopolysaccharides, immunostimulatory nucleic acid molecules (such as CpG oligonucleotides), liposomes, Toll Receptor agonists, Toll-like Receptor agonists (particularly, TLR2, TLR4, TLR7/8 and TLR9 agonists), and various combinations of such components. While administration of an antigen displayed on protein nanoparticles may enhance a subject's immune response to the antigen (compared to administration of the antigen in the absence of the nanoparticles), as used herein, the nanoparticle scaffolds are not defined as an adjuvant.

[068] A pharmaceutical composition of the present disclosure can include an excipient. Numerous pharmaceutically acceptable diluents and/or pharmaceutically acceptable excipients are known in the art and are described, *e.g.*, in Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975). The adjective "pharmaceutically acceptable" indicates that the diluent or excipient is suitable for administration to a subject (*e.g.*, a human or non-human mammalian subject). In general, the nature of the diluent and/or excipient will depend on the particular mode of administration being employed. For instance, parenteral formulations usually include injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. In certain formulations (for example, solid compositions, such as powder forms), a liquid diluent is not employed. In such formulations, non-toxic solid components can be used, including for example, pharmaceutical grades of trehalose, mannitol, lactose, starch or magnesium stearate. Suitable solid components are typically large, slowly metabolized macromolecules such as proteins (*e.g.,* nanoparticles), polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles.

[069] Accordingly, suitable excipients can be selected by those of skill in the art to produce a formulation suitable for delivery to a subject by a selected route of administration.

[070] In one embodiment of the present disclosure, the immunogenic or pharmaceutical compositions comprising the protein nanoparticles of the present disclosure do not further comprise an adjuvant.

[071] Preparation of immunogenic compositions, such as vaccines, including those for administration to human subjects, is generally described in Pharmaceutical Biotechnology, Vol.61 Vaccine Design-the subunit and adjuvant approach, edited by Powell and Newman, Plenum Press, 1995. See also New Trends and Developments in Vaccines, edited by Voller *et al.*, University Park Press, Baltimore, Maryland, U.S.A. 1978.

[072] In one embodiment, provided herein are immunogenic compositions comprising a protein nanoparticles having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide as described herein. In one embodiment, the immunogenic composition further comprises an adjuvant. The protein nanoparticles are produced using the methods discussed earlier.

[073] In one embodiment, provided herein are immunogenic compositions comprising polynucleotides encoding the protein nanoparticle of the present disclosure. In one embodiment, the immunogenic composition further comprises an adjuvant.

[074] In one embodiment, provided herein are pharmaceutical compositions comprising a protein nanoparticles having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide as described herein. In one embodiment, the pharmaceutical composition further comprises an excipient.

[075] In one embodiment, provided herein are pharmaceutical compositions comprising polynucleotides encoding the protein nanoparticle of the present disclosure. In one embodiment, the pharmaceutical composition further comprises an excipient.

[076] In one embodiment, provided herein are vaccine compositions comprising a protein nanoparticles having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a

bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide as described herein.

[077] In one embodiment, provided herein is a vaccine composition comprising a polynucleotide encoding the protein nanoparticle of the present disclosure.

[078] In one embodiment, provided herein is a vaccine composition comprising an immunogenic composition or a pharmaceutical composition as described herein.

Prophylactic and Therapeutic Uses

[079] Bacterial infections have a large impact on public health. For example, GBS is a major cause of neonatal sepsis and meningitis in infants born to women carrying the bacteria. At birth, a neonate's immune system is still developing, and they are vulnerable to infection by vertically acquired and postnatally acquired GBS. Immunization of a female subject to produce antibodies that can, during pregnancy, be passively transferred across the placenta to a gestating infant is referred to herein as maternal immunization, maternal vaccination, or as a maternally administered vaccine. See, *e.g.*, Englund, 2007. Maternal immunization has been previously investigated using saccharide based vaccines, including meningococcal vaccines (see, *e.g.*,Shahid *et al.* 2002; Quimbao *et al.* 2007; O'Dempsey *et al.* 1996).

[080] In women who have not received a GBS vaccine, an inverse relationship has been reported between levels of naturally occurring GBS serotype-specific IgG antibodies at the time of delivery and the risk of neonatal infection. *See e.g.,* Lin *et al.* (2001), Lin *et al.* (2004), Baker et al. (2014), Dangor et al. (2015) and Fabbrini *et al.* (2016). Lin *et al.* (2001) report that neonates born to women who had levels of IgG GBS Ia antibody $\geqslant$5 μg/mL had an 88% lower risk (95% confidence interval, 7%–98%) of developing type-specific EOD, compared with neonates born to women who had levels <0.5 μg/mL. Baker *et al.* (2014) estimated that the absolute risk of a neonate contracting GBS EOD due to serotypes Ia, III and V would decrease by 70% if maternal CPS-specific antibody concentrations were equal or higher than 1 μg/ml. Fabbrini *et al.*, (2016) reported that maternal anti-capsular IgG concentrations above 1 μg/mL mediated GBS killing in vitro and were predicted to respectively reduce by 81% and 78% the risk of GBS Ia and III early-onset diseases in

Europe. Dangor *et al.* (2015) report that the risk of neonatal invasive GBS disease was less than 10% when maternal antibody concentrations were ≥6 μg/mL and ≥3 μg/mL for serotypes Ia and III, respectively. However, as noted in Kobayashi et al. (2016), it is unclear the extent to which correlates of protection may be inferred from the evaluation of natural immunity in observational studies.

[081] In some prior studies of maternal immunization against GBS, a boosting dose was administered one month (30 days) after the priming dose. *See* Madhi et al. (2016), Leroux-Roel et al. (2016). WO 2018/229708 reports that an extended period (more than 30 days) between prime and boost was beneficial in eliciting GBS serotype-specific maternal antibodies that could be transferred to a gestational infant, and that IgG titers in maternal sera from vaccinated women were predictive of the opsonophagocytic killing assay (OPKA) titers against GBS serotypes, indicating comparable functional activity of naturally-acquired and vaccine-induced GBS antibodies. In the study reported in Donders *et al.* (2016), more than 50% of women (Belgium and Canada) in both the vaccine and placebo groups had baseline GBS antibody concentrations below the lower limit of quantification (LLOQ) for Ia, Ib, and III serotypes. After vaccination, antibody GMCs were statistically higher for women who were at or above the LLOQ at baseline, compared with those below the LLOQ at baseline. Similarly, Heyderman (2016) reported undetectable antibody concentrations at baseline (<LLOQ) for about 69–80% of women against serotype Ia, 1–6% of women against serotype Ib, and 34–43% of women against serotype III. Antibody GMCs post-vaccination were higher in subjects who had baseline antibody concentrations >LLOQ.

[082] For effective vaccination of pregnant woman against GBS and other bacterial pathogens, a vaccine capable of eliciting a strong antibody response with a single dose in subjects who are seronegative at baseline is desirable.

[083] A further aspect of the present disclosure is a method of inducing an immune response in a mammalian subject, such as a human subject, wherein the immune response is specific for a bacterial antigenic molecule, such as a bacterial polysaccharide displayed on the surface of the protein nanoparticles of the present disclosure. The method comprises administering to a subject an immunologically effective amount of a protein nanoparticle displaying the bacterial antigenic molecule to which an immune response is desired. The subject may have a bacterial infection at the time of administration, or the administration may

be given prophylactically to a subject who does not have a bacterial infection at the time of administration.

[084] In one embodiment, the administered nanoparticles are produced by a method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; and (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination. In one embodiment, the at least one recognition tag is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the polynucleotide of SEQ ID NO: 1. In one embodiment, the bacteriophage VLP comprises an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the amino acid sequence set out in SEQ ID NO: 3. In one embodiment, the aptamer is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the polynucleotide of SEQ ID NO: 5. In one embodiment, the RNA-VLP is a Qbeta RNA-VLP. In one embodiment, the vector contains *inter alia* a T7 promotor, a DNA polynucleotide sequence encoding the Qbeta nanoparticle coat protein (CP), a DNA polynucleotide encoding the RNA hairpin, and optionally a DNA polynucleotide encoding an aptamer RNA, wherein the DNA polynucleotide sequence encoding the Qbeta nanoparticle

coat protein is genetically fused to (i) the DNA polynucleotide encoding the RNA hairpin, and (ii) optionally the DNA polynucleotide encoding an aptamer RNA.

[085] In one embodiment, the administered protein nanoparticles display at least one bacterial saccharide. The bacterial saccharide may be a capsular saccharide or O-antigen saccharide. The bacterial saccharide, may be selected from a bacterial species selected from the group consisting of a *Acinetobacter* species, *Bacillus* species, *Bordetella* species, *Borrelia* species, *Burkholderia* species, *Campylobacter* species, *Candida* species, *Chlamydia* species, *Clostridium* species, *Corynebacterium* species, *Enterococcus* species, *Escherichia* species, *Francisella* species, *Haemophilus* species, *Helicobacter* species, *Klebsiella* species, *Legionella* species, *Listeria* species, *Neisseria* species, *Proteus* species, *Pseudomonas* species, *Salmonella* species, *Shigella* species, *Staphylococcus* species, *Streptococcus* species, *Streptomyces* species, *Vibrio* species, and *Yersinia* species.

[086] In one embodiment, the administered nanoparticles display bacterial saccharide antigens from at least two (i.e., two or more) pathogenic bacterial species or serotypes. This may be achieved by administering a mixture of nanoparticles, wherein each nanoparticle displays a bacterial saccharide antigen from a single bacterial species or serotype, or by administering a nanoparticle that displays bacterial saccharides from multiple (such as two, three, four, five or more) species or serotypes.

[087] In one embodiment, the administered nanoparticle displays GBS CPS antigens from at least two disease-causing GBS serotypes, such as from any of serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX. This may be achieved by administering a mixture of nanoparticles where each NP displays a single GBS serotype antigen, or by administering nanoparticle that display multiple GBS serotype antigens. The GBS antigens may be capsular polysaccharides or immunogenic fragments thereof, GBS glycoconjugates, or a mixture thereof.

[088] A further aspect of the present disclosure is a method of inducing an immune response for the purpose of preventing and/or treating a bacterial infection in a subject, comprising administering to the subject an immunologically effective amount of the nanoparticles of the present disclosure that display at least one bacterial antigenic molecule to which an immune response is desired, wherein the at least one antigen can induce a protective

or therapeutic immune response. Such nanoparticles may be within an immunogenic or pharmaceutical composition as described herein. In a specific embodiment, the administration is to a pregnant human subject, or one intending to become pregnant, and the method is to prevent bacterial infection in an infant born to the subject by transplacental transfer of maternal antibodies. In one embodiment of the disclosure, a single dose is administered to the subject. The dose may be adjuvant-free, or it may further comprise an adjuvant.

[089] A further aspect of the present disclosure is a method of inducing an immune response for the purpose of treating and/or preventing a GBS infection in a subject, comprising administering to the subject an immunologically effective amount of the nanoparticles of the present disclosure that display the GBS antigenic molecule to which an immune response is desired, wherein the antigens can induce a protective or therapeutic immune response. Such nanoparticles may be within an immunogenic or pharmaceutical composition as described herein. In a specific embodiment, the administration is to a pregnant human subject, or one intending to become pregnant, and the method is to prevent GBS infection in an infant born to the subject by transplacental transfer of maternal antibodies.

[090] In one embodiment, a single dose of the nanoparticle displaying the bacterial antigenic molecule is capable of inducing a protective or therapeutic immune response to bacterial infection. In another embodiment of the disclosure, a single dose is administered to the subject. In another embodiment, two doses are administered to the subject with an interval of at least 1 year, at least 2 years, at least 3 years, at least 4 years or at least 5 years between doses. The dose may be adjuvant-free, or it may further comprise an adjuvant.

[091] Another embodiment of the present disclosure is a method of immunizing a human female subject in order to decrease the risk of Group B *Streptococcus* (GBS) disease in an infant born to the subject, where the female receives both a priming dose and a boosting dose of a composition according to the present disclosure, and where the priming and the boosting dose each elicit in the subject IgG antibodies specific for the same disease-causing Group B *Streptococcus* serotype(s). In one embodiment, the boosting dose is administered more than thirty days after the priming dose. In one embodiment, GBS antigen component of the priming and/or the boosting dose comprises GBS CPS antigens from at least two disease-causing GBS serotypes, such as selected from serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII,

and IX. The priming and/or boosting dose may be adjuvant-free, or either or both may further comprise an adjuvant. In an embodiment of the present disclosure, the priming dose is administered to a non-pregnant female subject, and the boosting dose is administered to the subject when pregnant.

[092] Thus, in one embodiment, the nanoparticles and compositions of the present disclosure are utilized in methods of immunizing a subject to achieve a protective (prophylactic) immune response in both the subject and (via transplacental transfer of maternal antibodies) to an infant born to the subject.

[093] The immunogenic compositions of the disclosure are conventionally administered parenterally, *e.g.*, by injection, either subcutaneously, intraperitoneally, transdermally, or intramuscularly. Dosage treatment may be a single dose schedule or a multiple-dose schedule.

[094] Another embodiment of the present disclosure is a method of immunizing a human subject, where the subject receives both a priming dose and a boosting dose of a composition according to the present disclosure, and where the priming and the boosting dose each elicit in the subject IgG antibodies specific for the same disease-causing bacterial serotype(s). In one embodiment, the boosting dose is administered more than thirty days after the priming dose. In one embodiment, the bacterial antigen component of the priming and/or the boosting dose comprises bacterial CPS antigens from at least two disease-causing bacterial serotypes. The priming and/or boosting dose may be adjuvant-free, or either or both may further comprise an adjuvant.

[095] Another embodiment of the present disclosure is a method of immunizing a human female subject in order to decrease the risk of bacterial infection in an infant born to the subject, where the female receives both a priming dose and a boosting dose of a composition according to the present disclosure, and where the priming and the boosting dose each elicit in the subject IgG antibodies specific for the same disease-causing bacterial serotype(s). In one embodiment, the boosting dose is administered more than thirty days after the priming dose. In one embodiment, the bacterial antigen component of the priming and/or the boosting dose comprises bacterial CPS antigens from at least two disease-causing bacterial serotypes. The priming and/or boosting dose may be adjuvant-free, or either or both

may further comprise an adjuvant. In an embodiment of the present disclosure, the priming dose is administered to a non-pregnant female subject, and the boosting dose is administered to the subject when pregnant.

[096] The various features which are referred to in individual sections above apply, as appropriate, to other sections. Consequently, features specified in one section may be combined with features specified in other sections, as appropriate. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the disclosure (or aspects of the disclosure) described herein.

Exemplary Embodiments of the present disclosure include:

C1. A method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising: (a) engineering a vector comprising at least one polynucleotide to encode: (i) at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP; (ii) at least one bacteriophage VLP; and (iii) optionally at least one aptamer; (b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide (RNA-VLP) nanoparticle; (c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable; (d) extracting and purifying the bacterial saccharide from a bacteria; (e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination.

C2. The method of C1, wherein the at least one recognition tag is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the polynucleotide of SEQ ID NO: 1.

C3. The method of C1 or C2, wherein the bacteriophage VLP comprises an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the amino acid sequence set out in SEQ ID NO: 3.

C4. The method of any one of C1-C3, wherein the aptamer is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the polynucleotide of SEQ ID NO: 5.

C5. The method of any one of C1-C4, wherein the RNA-VLP is a Qβ RNA-VLP.

C6. The method of any one of C1-C5, wherein the bacterial saccharide is from a bacterial species selected from the group consisting of Acinetobacter species, Bacillus species, Bordetella species, Borrelia species, Burkholderia species, Campylobacter species, Candida species, Chlamydia species, Clostridium species, Corynebacterium species, Enterococcus species, Escherichia species, Francisella species, Haemophilus species, Helicobacter species, Klebsiella species, Legionella species, Listeria species, Neisseria species, Proteus species, Pseudomonas species, Salmonella species, Shigella species, Staphylococcus species, Streptococcus species, Streptomyces species, Vibrio species, and Yersinia species.

C7. The method of any one of C1-C6, wherein the bacterial saccharide is from a Streptococcus species selected from Streptococcus agalactiae (Group B Streptococcus, or GBS) and Streptococcus pneumoniae.

C8. The method of C7, wherein the bacterial saccharide is selected from the group consisting of GBS CPS serotype Ia, GBS CPS serotype Ib, GBS CPS serotype II, GBS CPS serotype III, GBS CPS serotype IV, and GBS CPS serotype V, VI, VII, VIII, and IX.

C9. The method of any one of C1-C8, wherein the at least one RNA polynucleotide is encoded by the vector.

C10. The method of any one of C1-C9, wherein at least 30% of at least one RNA polynucleotide is Qβ RNA.

C11. The method of any one of C1-C8, wherein less than 30% of the at least RNA polynucleotide is derived from the host cell.

C12. The method of any one of C1-C11, wherein the DNA polynucleotide encoding the bacteriophage VLP or virus coat protein of the VLP is genetically fused to (i) the DNA polynucleotide encoding the at least one RNA polynucleotide, and (ii) optionally the DNA polynucleotide encoding the at least one aptamer.

C13. The method of C12, wherein the bacteriophage VLP is a Qβ VLP, and wherein the at least one RNA polynucleotide is an RNA hairpin.

C14. A protein nanoparticle produced by the method of any one of C1-C13.

C15. The protein nanoparticle of C14, wherein the nanoparticle is capable of eliciting a protective immune response in a subject following a single dose.

C16. The protein nanoparticle of C14, wherein the nanoparticle is capable of eliciting a higher immune response to the bacterial saccharide after one dose compared to after one dose of a monomeric protein carrier, such as CRM197, conjugated to the same bacterial saccharide.

C17. The protein nanoparticle of C14, wherein the nanoparticle is capable of eliciting a higher or comparable immune response to the bacterial saccharide after one dose compared to after two doses of a monomeric protein carrier, such as CRM197, conjugated to the same bacterial saccharide.

C18. A polynucleotide encoding the protein nanoparticle of any one of C14-C17.

C19. A host cell comprising the polynucleotide of C18.

C20. An immunogenic composition comprising the protein nanoparticle of any one of C14-C17, or the polynucleotide of C18.

C21. The immunogenic composition of C20, further comprising an adjuvant.

C22. The immunogenic composition according to C21, wherein the adjuvant is selected from the group consisting of alum, aluminum hydroxide, aluminum phosphate, a saponin, a water-in-oil emulsion, an oil-in-water emulsion, a liposaccharide, a

lipopolysaccharide, an immunostimulatory nucleic acid molecules, a liposome, and a Toll Receptor or Toll-Like Receptor agonist.

C23. A pharmaceutical composition comprising the protein nanoparticle of any one of C14-C17, or the polynucleotide of C18 and an excipient.

C24. A vaccine composition comprising the protein nanoparticle of any one of C14-C17 or the polynucleotide of C18, or the immunogenic composition of C20-C22, or the pharmaceutical composition of C23.

C25. A method of inducing or increasing an immune response in a subject, comprising administering to the subject an immunologically effective amount of the protein nanoparticle of any one of C14-C17, or the polynucleotide of C18, or the immunogenic composition of C20-C22, or the pharmaceutical composition of C23, or the vaccine composition of C24.

C26. A method of preventing or treating a bacterial infection in a subject, comprising administering to the subject an immunologically effective amount of the protein nanoparticle of any one of C14-C17, or the polynucleotide of C18, or the immunogenic composition of C20-C22, or the pharmaceutical composition of C23, or the vaccine composition of C24.

C27. Use of the protein nanoparticle according to any one of C14-C17, or the polynucleotide of C18, or the immunogenic composition of C20-C22, or the pharmaceutical composition of C23, or the vaccine composition of C24 for the manufacture of a medicament for inducing an immune response in a subject.

C28. Use of the protein nanoparticle according to any one of C14-C17, or the polynucleotide of C18, or the immunogenic composition of C20-C22, or the pharmaceutical composition of C23, or the vaccine composition of C24 in the prevention or treatment of a disease in a subject.

C29. Use of the protein nanoparticle according to any one of C14-C17, or the polynucleotide of C18, or the immunogenic composition of C20-C22, or the pharmaceutical composition of C23, or the vaccine composition of C24 in the prevention or treatment of a bacterial infection in a subject.

C30. The use according to any one of C27-C29, or the method according to C25 or C26, wherein the subject receives a single administration of the protein nanoparticle of any one of C14-C17, or the polynucleotide of C18, or the immunogenic composition of C20-C22, or the pharmaceutical composition of C23, or the vaccine composition of C24.

C31. The use according to any one of C27-C29, or the method according to C25 or C26, wherein the subject receives an intramuscular administration.

C32. The use according to any one of C27-C29, or the method according to C25 or C26, wherein the subject is a human subject.

C33. The protein nanoparticle according to any one of C14-C17, or the polynucleotide of C18, or the immunogenic composition of C20-C22, or the pharmaceutical composition of C23, or the vaccine composition of C24 for use in the prevention or treatment of disease in a subject, optionally a human subject.

Definitions

[097] To facilitate review of the various embodiments of this disclosure, the following explanations of terms are provided. Additional terms and explanations are provided in the context of this disclosure. Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. The term "plurality" refers to two or more. The term “at least one” refers to one or more.

[098] Additionally, numerical limitations given with respect to concentrations or levels of a substance, such as solution component concentrations or ratios thereof, and reaction conditions such as temperatures, pressures, and cycle times are intended to be approximate. Unless specified otherwise, where a numerical range is provided, it is inclusive, i.e., the endpoints are included. Definitions of common terms in molecular biology can be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular*

Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

[099] "Nanoparticles (NP)" as used herein refers to particles of less than about 100nm in size (less than about 100nm in maximum diameter for spherical, or roughly spherical, particles).

[0100] "Virus-like particles (VLPs)" are multiprotein structures that mimic the organization and conformation of authentic native viruses but lack the viral genome. VLPs are considered NPs for purposes of this disclosure. A typical embodiment of a virus-like particle in accordance with the present disclosure is a viral capsid of a virus or bacteriophage. The terms "viral capsid" or "capsid", refer to a macromolecular assembly composed of viral protein subunits, such as 60, 120, 180, 240, 300, 360 or more than 360 viral protein subunits. VLPS are packaging systems that can be used to store RNA polynucleotides/target RNA. A VLP encapsulating the target RNA is referred to as RNA-VLP.

[0101] "Qβ," "Qbeta," and "Qβ VLP" are used interchangeably herein. For the purpose of this disclosure, Qbeta is a VLP that is derived from a single-strand RNA bacteriophage. "Qβ VLPs" as used here, refers to VLPs which are hollow with pores 1.4 nm in diameter. Target RNA could be packaged/encapsulated in Qβ VLPs. Qβ VLPs encapsulating target RNA is referred to as "RNA- Qβ VLPs." "Qβhp" means Qβ VLP encapsulating the RNA hairpin.

[0102] "Virus-like particle of an RNA bacteriophage," as used herein, refers to a virus-like particle comprising, or preferably consisting essentially of, or consisting of, coat proteins, mutants or fragments thereof, of an RNA bacteriophage.

[0103] The term "recombinant VLP" as used herein refers to a VLP that is obtained by a process which comprises at least one step of recombinant DNA technology.

[0104] Viral "coat protein" and "capsid protein." The term viral "coat protein" is used interchangeably herein with viral "capsid protein," and refers to a protein, such as a subunit of a natural capsid of a virus, which is capable of being incorporated into a virus capsid or a VLP. For example, the specific gene product of the Coat Protein gene of RNA bacteriophage Qβ is referred to as " Qβ CP", whereas the "coat proteins" or "capsid proteins" of bacteriophage Qβ comprise the Qβ CP as well as the A1 protein.

[0105] As used herein the terms "protein" and "polypeptide" are used interchangeably. A protein or polypeptide sequence refers to a contiguous sequence of two or more amino acids linked by a peptide bond. The proteins and polypeptides of the present disclosure may comprise L-amino acids, D-amino acids, or a combination thereof.

[0106] The term "fragment," in reference to a polypeptide (or polysaccharide or oligosaccharide) antigen, refers to a contiguous portion (that is, a subsequence) of that polypeptide (or polysaccharide). An "immunogenic fragment" of a polypeptide, polysaccharide or oligosaccharide refers to a fragment that retains at least one immunogenic epitope (*e.g.*, a predominant immunogenic epitope or a neutralizing epitope).

[0107] As used herein, a "polypeptide subunit" of a nanoparticle, or "subunit", refers to a polypeptide that, in combination with other polypeptide subunits, self-assembles into a nanoparticle. The subunit may further comprise a polypeptide sequence which extends from the surface of the nanoparticle (*i.e.*, is 'displayed' by the nanoparticle), a purification tag, or other modifications as are known in the art and that do not interfere with the ability to self-assemble into a nanoparticle.

[0108] As used herein, a "variant" polypeptide refers to a polypeptide having an amino acid sequence which is similar, but not identical to, a reference sequence, wherein the biological activity of the variant protein is not significantly altered. Such variations in sequence can be naturally occurring variations or they can be engineered through the use of genetic engineering techniques as known to those skilled in the art. Examples of such techniques may be found, *e.g.*, in Sambrook *et al.,* Molecular Cloning--A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, pp. 9.31-9.57), or in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6.

[0109] Nucleic acid" herein means a polymeric form of nucleotides of any length, which contain deoxyribonucleotides, ribonucleotides, and/or their analogs. It includes DNA, RNA and DNA/RNA hybrids. It also includes DNA or RNA analogs, such as those containing modified backbones (e.g. peptide nucleic acids (PNAs) or phosphorothioates) or modified bases. Thus, the nucleic acid of the present disclosure includes mRNA, DNA, cDNA, recombinant nucleic acids, branched nucleic acids, plasmids, vectors, etc.

[0110] Nucleic acids can be in recombinant form, *i.e.*, a form that does not occur in nature. For example, the nucleic acid may comprise one or more heterologous nucleic acid sequences (*e.g.*, a sequence encoding another antigen and/or a control sequence such as a promoter or an internal ribosome entry site). The nucleic acid may be part of a vector *i.e.*, part of a nucleic acid designed for transduction/transfection of one or more cell types. Vectors may be, for example, "expression vectors," which are designed for expression of a nucleotide sequence in a host cell, or "viral vectors," which are designed to result in the production of a recombinant virus or virus-like particle.

[0111] "RNA" "(ribonucleic acid") and "RNA polynucleotide" are used interchangeably herein. RNA as used herein relates to a molecule which comprises ribonucleotide residues. The term "ribonucleotide" refers to a nucleotide containing ribose as its pentose component. The term "RNA" comprises double-stranded RNA, single stranded RNA, isolated RNA, synthetic RNA, recombinantly generated RNA, and modified RNA which differs from naturally occurring RNA by addition, deletion, substitution and/or alteration of one or more nucleotides. Nucleotides in RNA molecules can comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. "mRNA" (or messenger RNA") as used herein means "messenger-RNA" and relates to a transcript which is generated by using a DNA template and encodes a peptide or protein. Typically, mRNA comprises a protein coding region flanked by a 5'-UTR and a 3'-UTR. The term "antisense-RNA" relates to single-stranded RNA comprising ribonucleotide residues, which are complementary to the mRNA. The term "siRNA" means "small interfering RNA", which is a class of double-stranded RNA-molecules comprising about 20 to about 25 base pairs.

[0112] The term "vector" refers to a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. The vectors described herein can be expression vectors. The term "expression vector" refers to a vector that includes one or more expression control sequences.

[0113] The term "gene" refers to a DNA sequence that encodes through its template or messenger RNA a sequence of amino acids characteristic of a specific peptide, polypeptide, or protein. The term "gene" also refers to a DNA sequence that encodes an RNA

product. The term gene as used herein with reference to genomic DNA includes intervening, non-coding regions as well as regulatory regions and can include 5' and 3' ends.

[0114] As used herein, a "recognition tag" refers to a signal that directs a molecule to a specific cell, tissue, organelle, or intracellular region. The signal can be a polynucleotide, polypeptide, or carbohydrate moiety or can be an organic or inorganic compound sufficient to direct an attached molecule to a desired location.

[0115] As used herein, an "aptamer" or an "RNA aptamer" refers to a sequence that assist in the purification of the RNA. RNA aptamers can assist in the purification of the RNA through affinity chromatography with RNA binding proteins.

[0116] As used herein, an "antigen" is a molecule (such as a protein or saccharide), a compound, composition, or substance that stimulates an immune response by producing antibodies and/or a T cell response in a mammal, including compositions that are injected, absorbed or otherwise introduced into a mammal. The term "antigen" includes all related antigenic epitopes. The term "epitope" or "antigenic determinant" refers to a site on an antigen to which B and/or T cells respond. The "predominant antigenic epitopes" are those epitopes to which a functionally significant host immune response, *e.g.*, an antibody response or a T-cell response, is made. Thus, with respect to a protective immune response against a pathogen, the predominant antigenic epitopes are those antigenic moieties that when recognized by the host immune system result in protection from disease caused by the pathogen. The term "T-cell epitope" refers to an epitope that when bound to an appropriate MHC molecule is specifically bound by a T cell (via a T cell receptor). A "B-cell epitope" is an epitope that is specifically bound by an antibody (or B cell receptor molecule).

[0117] As used herein, the term "immunogenic" refers to the ability of a specific antigen, or a specific region thereof, to elicit an immune response to that antigen or region thereof when administered to a mammalian subject. The immune response may be humoral (mediated by antibodies) or cellular (mediated by cells of the immune system), or a combination thereof.

[0118] An "immune response" is a response of a cell of the immune system, such as a B cell, T cell, or monocyte, to a stimulus. An immune response can be a B cell response, which results in the production of specific antibodies, such as antigen specific neutralizing

antibodies. An immune response can also be a T cell response, such as a CD4+ response or a CD8+ response. In some cases, the response is specific for a particular antigen (that is, an "antigen-specific response"), such as a GBS antigen. A "protective immune response" is an immune response that inhibits a detrimental function or activity of a pathogen, prevents infection by a pathogen in an individual, or decreases symptoms that result from infection by the pathogen. A protective immune response can be measured, for example, by measuring resistance to pathogen challenge *in vivo*.

[0119] A "higher" immune response means an immune response that is higher than the immune response of a reference treatment. For example, IgG titers induced by a protein nanoparticle described herein (for example, as measured by Luminex/ELISA) are considered higher than the IgG titers of a reference treatment if the IgG titers are statistically higher at a p value of 0.05 or lower (such as, for example, $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.005$,or $p \leq 0.001$) when calculated by well-known methods, such as the Mann-Whitney Test. OPKA titers elicited by a nanoparticle described herein as measured in pooled sera are considered higher than a reference treatment where there is at least a 2-fold increase as compared to the reference treatment.

[0120] A "comparable" immune response means an immune response that does not meet the threshold of a higher (or lower) immune response. For example, comparable IgG titers between treatment groups would be those that are not statistically higher or lower than the immune response of a reference treatment at a p value of 0.05 or lower (such as, for example, $p < 0.05$, $p < 0.01$, $p < 0.005$,or $p < 0.001$). OPKA titers between treatment groups are considered comparable if there is less than a 2-fold difference between the groups.

[0121] An "effective amount" means an amount sufficient to cause the referenced effect or outcome. An "effective amount" can be determined empirically and in a routine manner using known techniques in relation to the stated purpose. An "immunologically effective amount" is a quantity of an immunogenic composition sufficient to elicit an immune response in a subject (either in a single dose or in a series). Commonly, the desired result is the production of an antigen (*e.g.*, pathogen)-specific immune response that is capable of or contributes to protecting the subject against the pathogen. Obtaining a protective immune response against a pathogen can require multiple administrations of the immunogenic composition; preferably a single administration is required.

[0122] As used herein, a "glycoconjugate" is a carbohydrate moiety (such as a polysaccharide or oligosaccharide) covalently linked to a moiety that is a different chemical species, such as a protein, peptide, lipid or lipid. A "GBS glycoconjugate", as used herein, refers to a conjugate of a GBS capsular saccharide molecule and a monomeric carrier protein molecule, including the carrier proteins TT, DT, and CRM197, but excluding a GBS capsular saccharide molecule conjugated to a polypeptide subunit of an NP, including a non-viral NP or VLP.

[0123] By "c-terminally" or "c-terminal" to, it is meant toward the c-terminus. Therefore, by "c-terminally adjacent" it is meant "next to" and on the c-terminal side (*i.e.,* on the right side if reading from left to right).

[0124] By "n-terminally" or "n-terminal" to, it is meant toward the n-terminus. Therefore, by "n-terminally adjacent" it is meant "next to" and on the n-terminal side (*i.e.,* on the left side if reading from left to right).

[0125] As used herein, a "recombinant" or "engineered" cell refers to a cell into which an exogenous DNA sequence, such as a cDNA sequence, has been introduced. A "host cell" is one that contains such an exogenous DNA sequence. "Recombinant" as used herein to describe a polynucleotide means a polynucleotide which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide.

[0126] A "subject" is a living multi-cellular vertebrate organism. In the context of this disclosure, the subject can be an experimental subject, such as a non-human mammal, *e.g.,* a mouse, a rat, or a non-human primate. Alternatively, the subject can be a human subject.

[0127] The term "comprises" means "includes." Thus, unless the context requires otherwise, the word "comprises," and variations such as "comprise" and "comprising" will be understood to imply the inclusion of a stated compound or composition (*e.g.*, nucleic acid, polypeptide, antigen) or step, or group of compounds or steps, but not to the exclusion of any

other compounds, composition, steps, or groups thereof. The abbreviation, "*e.g.*" is used herein to indicate a non-limiting example and is synonymous with the term "for example."

[0128] It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acid molecules or polypeptides are approximate and are provided for description. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Additionally, numerical limitations given with respect to concentrations or levels of a substance, such as an antigen, are intended to be approximate. Thus, where a concentration is indicated to be at least (for example) 200 pg, it is intended that the concentration be understood to be at least approximately (or "about" or "~") 200 pg.

[0129] The present disclosure is not limited to particular embodiments described herein. Certain features of the disclosure, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the disclosure, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments are specifically embraced by the present disclosure and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations are also specifically embraced by the present disclosure and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

[0130] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below.

[0131] The entire disclosure of published references, patents, and published patent applications cited herein are incorporated herein by reference in their entirety.

Examples

[0132] Many modifications and variations of the present disclosure are possible in light of the teachings of the present disclosure. It is, therefore, to be understood that, within

the scope of the appended claims, a skilled person in the art would recognize that the disclosure may be practiced otherwise than as specifically described. The illustrative embodiments and examples should not be construed as limiting the disclosure.

Example 1: Production of Qβ Hairpin (Qβhp) Nanoparticles

[0133] Qβ Virus-Like Particle (VLP) nanoparticles have an icosahedral phage-like capsid structure with a diameter of about 35 nm and are composed of 180 copies of coat protein linked in covalent pentamers and hexamers by disulfide bridges (Golmohammadi et al., (1996)). Qβ VLPs are hollow with pores 1.4 nm in diameter (Fang et al,. (2018)). Qβhp nanoparticles were produced by expression in *E. coli* cells using a "single- plasmid" expression system (pET24_QbetaCP_HP) which contains SEQ ID NO: 1, SEQ ID NO: 4, and optionally SEQ ID NO: 5 (**FIG. 1**). A skilled person would recognize that Qβhp (Qβ VLP encapsulating RNA hairpin) nanoparticles can also be produced by expression in *E. coli* cells using a "dual- plasmid" expression system.

[0134] Transformed *E. coli* (Stellar™, Takara Bio) host cells were grown, and the plasmid DNA was extracted and sequenced in order to confirm the sequence identity. The plasmid was transformed into two additional *E. coli* strains BL21DE3tlr and ClearColi™ (Lucigen), and the cells were cultured in two different mediums: HTMC (Cell Applications, Inc.) and M9 (Cell Applications, Inc.). Qβhp samples were purified from both HTMC and M9 medium using CAPTO Q column for ionic exchange chromatography with a NaCl salt gradient purification (from 0 to 1M NaCl). Fractions containing Qβhp were pooled and concentrated six times and further purified using size exclusion chromatography purification and negative cHT column. Fractions were run on SDS page gels, and those containing Qβhp were collected.

Example 2: Characterization of Qβhp Nanoparticles

[0135] SE-HPLC was used to estimate the purity of the protein. SE-HPLC was carried out using SRT-C 2000 column with fluorometric detection (excitation at 227nm and emission at 335nm). Running conditions were flow rate 0.5 mg/mL, run time 40 minutes, 100 mM NaPi, 100 mM Na2SO4, pH 7.2 as running buffer and injection volume20 µL. Both samples (nanoparticles purified from HTMC and M9 medium) were injected in a protein concentration of 0.3 mg/mL protein based. LabChip®, an Automated Electrophoresis

System, was used to evaluate the profile of the RNA. LabChip® analysis was carried out by applying the procedure reported in the LabChip®GXII manual protocol for RNA detection (RNA assay kit). LabChip® analysis of Qβhp purified from M9 (lot 13b), and HTMC medium (lot 11) compared to the Q Beta wild type (Qβwt; lot 9) is depicted in **FIG. 2**, where the main RNA peak at about 800 nt is present in the Qβhp samples, while the Qβwt sample presents a more complex pattern.

[0136] BCA and RiboGreen assays (Turner BioSystems) were used to measure the protein content and the RNA content, respectively. The endotoxin level of purified Qβhp was measured using the LAL test (Pierce LAL chromogenic endotoxin quantification kit). **Table 1** summarizes the characterization of Qβhp nanoparticles purified from M9 and HTMC medium. As demonstrated in Table 1, the Qβhp sample that was grown in M9 medium provided a higher protein yield and higher purity, and lower endotoxin level as compared to the Qβhp sample that was grown in the HTMC medium.

Table 1

Sample	Protein Yield	RNA Yield	Purity SE-HPLC			LAL-test
	mg/g	%RNA	260 nm	280 nm	FLR	(EU/μg)
Qβhp from HTMC	1.2	23%	86%	87%	87%	6.5
Qβhp from M9	3.3	16%	99%	99%	97%	0.3

[0137] The RNA contents of the Qβhp nanoparticles were further tested using next-generation sequencing (NGS). **Table 2** compares NGS analysis of Qβhp with Q Beta wild type (Qβ wt).

Table 2

Sample	Plasmid RNA (from vector)		RNA Genomic (from Ecoli)		
	Total RNA	Qβ RNA	Total RNA	tRNA	rRNA
Qβ wt	24%	8%	72%	0.36%	19.3%
Qβ wt-PSII	24%	7%	72%	0.31%	20.5%
Qβ Hairpin Lot.11-HTMC	52%	34%	48%	0.14%	11.7%
Qβ Hairpin-PSII Lot.01	51%	35%	48%	0.12%	12.1%
Qβ Hairpin Lot.13B-M9	75%	52%	25%	0.12%	3.3%

As shown in Table 2, at least 50% of the Total RNA in Qβ Hairpin samples is plasmid RNA, and at least 30% of the plasmid RNA is Qβ RNA. Conversely, the Qbeta wild type (Qβ wt) samples contained up to 24% plasmid RNA and up to 8% Qβ RNA.

Example 3: Conjugation of GBS Capsular polysaccharide to Qβhp

[0138] Polysaccharides of GBS CPS serotype II (molecular weight ~100 kDa) were produced based on the previously described procedure (Wessels et al. (1990)). Oxidation of GBS serotype II capsular polysaccharide was carried out using 5% of $NaIO_4$, as shown in **FIG. 3**. The oxidized polysaccharides were purified using a desalting column. The identity and structural conformity of the resulting polysaccharides were assessed by ^1H NMR. Total saccharide was quantified using HPAEC-PAD or Colorimetric assay (NeuNAc-based).

[0139] The oxidized polysaccharides were then conjugated to Qβhp (5-10mg/mL) at room temperature for 72 hours by reductive amination in the presence of NaBH3CN, using a w/w ratio between saccharide and Qβhp of 1.5:1, as illustrated in **FIG. 4**. The Qβhp conjugated to saccharides were purified by ammonium sulfate precipitation followed by serial centrifugal filtration (100kDa).

Example 4: Characterization of GBS saccharide Qβhp conjugates

[0140] HPAEC-PAD and BCA were used to estimate saccharide (total and free) and protein content of purified Qβhp nanoparticles conjugated to GBS saccharide, as reported in **Table 3**.

Table 3

Conjugated Sample	Medium	Saccharide (µg/mL)	Protein (µg/mL)	Saccharide/ protein (w/w)	%Free saccharide
PSII- Qβhp	Grown in HTMC medium	62.6	611.0	0.10	<12.3
PSII- Qβhp	Grown in M9 medium	73.1	535.7	0.14	13.2

[0141] The conjugates were further characterized in terms of size/structure by SE-HPLC. SE-HPLC was carried out using SRT-C 2000 column by Sepax with fluorometric detection (excitation at 227nm and emission at 335nm). Running conditions were flow rate 0.5 mg/mL, run time 40 minutes, 100 mM NaPi, 100 mM Na2SO4, pH 7.2 as running buffer and injection volume20 µL. All samples were injected in a protein concentration of 0.3 mg/mL protein based. **FIG. 5A** shows the SE-HPLC analysis of GBS PSII-Qβhp nanoparticle conjugate (Lot 11-HTMC medium) and the Qβhp nanoparticle (no conjugated saccharide). **FIG. 5B** shows the SE-HPLC analysis of GBS PSII-Qβhp nanoparticle conjugate (Lot 13B-M9 medium) and the Qβhp nanoparticle (no conjugated saccharide).

[0142] Qβhp conjugates were also characterized by transmission electron microscopy (TEM) analysis, using negative stain (NS). For analysis by negative staining, Qβhp conjugates were loaded onto copper 300-square mesh grids of carbon/formvar (Agar Scientific) rendered hydrophilic by glow discharge (Quorum Q150). The excess solution was blotted off using Whatman filter Paper No.1 and then the grids were negatively stained with NanoW. Micrographs were acquired using a Tecnai G2 Spirit Transmission Electron Microscope at 87000x magnification equipped with a CCD 2kx2k camera.

[0143] **FIG. 6** demonstrates the analysis by negative staining of Qβhp nanoparticles conjugated to GBS PSII. As shown in **FIG. 6,** Qβhp nanoparticles conjugated to GBS PSII

appear as highly symmetrical structures with a diameter around 33nm, with thin, elongated appendages corresponding to PSII attached to the Qβhp surface.

Example 5: *In vivo* immunization

[0144] An *in vivo* mouse immunization study (Study 1) was conducted using GBS serotype II antigen, either conjugated to CRM197 carrier protein, Qβ wild type (Qβwt), or conjugated to Qβhp. Immunizations and blood draws were carried out according to the schedule set forth in **Table 4**.

Table 4

Day	Action
0	Blood Draw 1 (pre-immunization)
1	Immunization 1
21	Blood Draw 2 (post first immunization)
22	Immunization 2
42	Blood Draw Final (post first or second immunization)

[0145] In Study 1, six groups of ten female mice each (CD1 strain, Charles River) were studied. In Groups 2, 4, 6 each mouse was immunized twice intramuscularly, while in Groups 1, 3, 5 each mouse was immunized once intramuscularly with the formulations as shown in **Table 5**. Immunizations were carried out on Day 1 and Day 22. Blood was drawn from each mouse on Day 0 (pre-immunization), Day 21, and Day 42, as described in **Table 4**.

Table 5

Group	Antigen 1 Dose	Antigen 2 Dose	Antigen dose	Adjuvant
1	PSII-CRM	-	0.5 µg GBSII	Alum 2 mg/mL
2	PSII-CRM	PSII-CRM	0.5 µg GBSII	Alum 2 mg/mL
3	PSII-Qβ (wt)	-	0.5 µg GBSII	Alum 2 mg/mL
4	PSII-Qβ (wt)	PSII-Qβ (wt)	0.5 µg GBSII	Alum 2 mg/mL
5	PSII-Qβhp grown in HTMC	-	0.5 µg GBSII	Alum 2 mg/mL

6	PSII-Qβhp grown in HTMC	PSII-Qβhp grown in HTMC	0.5 µg GBSII	Alum 2 mg/mL

[0146] Serum antibody titers in serums were measured by Luminex and Opsonophagocytic Killing (OPKA) assays. **Table 6** shows the Geometric Mean IgG Titers in sera as measured by Luminex for Study 1, along with Opsonophagocytic Killing Titers obtained with pooled sera from each group.

Table 6 - Study 1 IgG and OPKA Titers

					Luminex IgG GMT Titer in Sera [RLU/ml]			**OPKA titers**	
Group	**Mice CD1**	**Antigen 1 Dose**	**Antigen 2 Dose**	**Adjuvant (Alum 2 mg/ mL)**	**PI _Day0**	**Post1 _day21**	**Post _day42**	**Post1 _day21**	**Post _day42**
1	1-10	PSII-CRM	-	Alum	<LLOQ	23	35	128	207
2	11-20	PSII-CRM	PSII-CRM	Alum	<LLOQ	54	776	159	2032
3	21-30	PSII-Qβ (wt)	-	Alum	<LLOQ	748	6487	1595	7025
4	31-40	PSII-Qβ (wt)	PSII-Qβ (wt)	Alum	<LLOQ	618	29963	1379	11253
5	41-50	PSII-Qβhp-HTMC	-	Alum	<LLOQ	357	4313	321	2045
6	51-60	PSII-Qβhp-HTMC	PSII-Qβhp-HTMC	Alum	<LLOQ	231	7205	453	6382

Luminex Lower Limit of Quantification (LLOQ) = 20.4 Relative Luminex Units/ml; <LLOQ = 10.2

[0147] Luminex assay was performed using streptavidin-derivatized magnetic microspheres (Radix Biosolutions, USA) coupled with biotinylated type II native polysaccharide (Buffi et al., (2019)). Following equilibration at RT, 1.25 million microspheres were transferred to LoBind tubes (Eppendorf) and placed into a magnetic separator for 2 min in the dark. Microspheres were washed with PBS containing 0.05% TWEEN™ 20 (Calbiochem) and biotin-PSII was added to the microspheres at a final

concentration of 1 μg/ml in PBS, 0.05% TWEEN™ 20, 0.5% BSA (Sigma-Aldrich). The biotin-PSII–microspheres were incubated for 60 minutes at Room Temperature (RT) in the dark and washed twice with PBS, 0.05% TWEEN™ 20. Coupled microspheres were suspended in 500 μl of PBS, 0.05% TWEEN™ 20, 0.5% BSA, and stored at 4°C.

[0148] Eight 3-fold serial dilutions of a standard hyperimmune serum or test samples were prepared in PBS, pH 7.2, 0.05% TWEEN™ 20, 0.5% BSA. Each serum dilution (50 μl) was mixed with an equal volume of conjugated microspheres (3,000 microspheres/region/well) in a 96-well Greiner plate (Millipore Corporation) and incubated for 60 min at RT in the dark. After incubation, the microspheres were washed three times with 200 μl PBS. Each well was loaded with 50 μl of 2.5 μg/ml anti-mouse IgG secondary antibody (Jackson Immunoresearch), in PBS, pH 7.2, 0.05% TWEEN™ 20, 0.5% BSA and incubated for 60 min with continuous shaking. After washing, microspheres were suspended in 100 μl PBS and shaken before the analysis with a Luminex 200 instrument. Data were acquired in real time by Bioplex Manager TM Software (BioRad).

[0149] The functional activity of the sera was determined by Opsonophagocytic Killing Assay (OPKA) as previously described (Chatzikleanthous (2020)). HL60 cells were grown in RPMI 1640 with 10% fetal calf serum, incubated at 37 °C, 5% CO2. HL-60 cells were differentiated to neutrophils with 0.78% dimethylformamide (DMF) and after 4−5 days were used as source of phagocytes. The assay was conducted in 96-well microtiter plate, in a total volume of 125 μL/well. Each reaction contained heat inactivated test serum (12.5 μL), GBS II strain 5401 (6 × 104 colony forming units [CFU]), differentiated HL-60 cells (2 × 106 cells) and 10% baby rabbit complement (Cederlane) in Hank's balanced salt solution red (Gibco). For each serum sample, six serial dilutions were tested. Negative controls lacked effector cells or contained either negative sera or heat inactivated complement. After reaction assembly, plates were incubated at 37 °C for 1 hour under shaking. Before (T0) and after (T60) incubation, the mixtures were diluted in sterile water and plated in Trypticase Soy Agar plates with 5% sheep blood (Becton Dickinson). Each plate was then incubated overnight at 37°C with 5% of CO2; CFUs were counted the next day. OPKA titer was expressed as the reciprocal serum dilution leading to 50% killing of bacteria and the % of killing is calculated as follows

$$\%killing = \frac{T_{0-}\ T_{60}}{T_0}$$

where T_0 is the mean of the CFU counted at T_0 and T_{60} is the average of the CFU counted at T_{60} for the two replicates of each serum dilution.

[0150] As shown in Table 6 and FIG.7, Post-1 IgG Luminex titers after 21 days in groups 3, 4, 5, and 6 receiving PSII-Qβwt or PSII-Qβhp conjugates were higher than post-1 titers from group 1 and 2 receiving PSII-CRM. After 42 days of one-dose PSII-Qβwt or PSII-Qβhp conjugates (groups 3 and 5), IgG Luminex titers were higher or comparable to the group 2 receiving two PSII-CRM vaccine doses. In addition, after one dose of vaccine after 42 days, OPK titers in pooled sera from animals receiving one dose of PSII-Qβwt or PSII-Qβhp conjugates (groups 3 and 5) were above 3-5 fold higher than those receiving one dose of PSII-CRM (group 1). In addition, after one dose of vaccine after 42 days, OPK titers in pooled sera from animals receiving one dose of PSII-Qβwt or PSII-Qβhp conjugates (groups 3 and 5) were higher or comparable to the group 2 receiving two doses of PSII-CRM vaccine.

SEQUENCE LISTINGS

SEQ ID NO 1: Deoxyribonucleic acid sequence of recognition tag for the bacteriophage VLP:

TTCCTCGTGCTTAGTAACTAAGGATGAAATGCATGTCTAAGACAGCATCTTCGC

SEQ ID NO 2: Ribonucleic acid sequence of recognition tag for the bacteriophage VLP:

UUCCUCGUGCUUAGUAACUAAGGAUGAAAUGCAUGUCUAAGACAGCAUCUUC GC

SEQ ID NO 3: Amino acid sequence of bacteriophage Qbeta VLP:

MAKLETVTLGNIGKDGKQTLVLNPRGVNPTNGVASLSQAGAVPALEKRVT VSVSQPSRNR KNYKVQVKIQNPTACTANGSCDPSVTRQAYADVTFSFTQY STDEERAFVRTELAALLASPLLIDAIDQLNPAY

SEQ ID NO 4: Nucleic acid sequence of bacteriophage Qbeta VLP:

ATGGCAAAATTAGAGACTGTTACTTTAGGTAACATCGGGAAAGATGGAAAACAA ACTCTGGTCCTCAATCCGCGTGGGGTAAATCCCACTAACGGCGTTGCCTCGCTTT CACAAGCGGGTGCAGTTCCTGCGCTGGAGAAGCGTGTTACCGTTTCGGTATCTCA GCCTTCTCGCAATCGTAAGAACTACAAGGTCCAGGTTAAGATCCAGAACCCGAC CGCTTGCACTGCAAACGGTTCTTGTGACCCATCCGTTACTCGCCAGGCATATGCT GACGTGACCTTTTCGTTCACGCAGTATAGTACCGATGAGGAACGAGCTTTTGTTC GTACAGAGCTTGCTGCTCTGCTCGCTAGTCCTCTGCTGATCGATGCTATTGATCA GCTGAACCCAGCGTATTGA

SEQ ID NO 5: Deoxyribonucleic acid sequence of aptamer

CAGCAAGTTCCGCAACCGTATCAAAACGTAAATTACTCGGAC

SEQ ID NO 6: Ribonucleic acid sequence of aptamer

CAGCAAGUUCCGCAACCGUAUCAAAACGUAAAUUACUCGGAC

SEQ ID NO 7: Deoxyribonucleic acid sequence of a plasmid containing aptamer, Qbeta VLP, and recognition tag for the bacteriophage VLP:

CAGCAAGTTCCGCAACCGTATCAAAACGTAAATTACTCGGACTAATGAATGGCC
AAACTGGAAACCGTTACGCTGGGTAATATTGGTAAAGATGGTAAACAGACCCTG
GTTCTGAATCCGCGTGGTGTTAATCCGACCAATGGTGTTGCAAGCCTGAGCCAGG
CAGGCGCAGTTCCGGCACTGGAAAAACGTGTTACCGTTAGCGTTAGCCAGCCGA
GCCGTAATCGTAAAAACTATAAAGTTCAGGTGAAAATCCAGAATCCGACCGCAT
GTACCGCAAATGGTAGCTGTGATCCGAGCGTTACCCGTCAGGCATATGCAGATGT
TACCTTTAGTTTTACCCAGTATAGCACCGATGAAGAACGTGCATTTGTTCGTACC
GAACTGGCAGCACTGCTGGCAAGTCCGCTGCTGATTGATGCAATTGATCAGCTGA
ATCCGGCATATTAATGATTCCTCGTGCTTAGTAACTAAGGATGAAATGCATGTCT
AAGACAGCATCTTCGC

SEQ ID NO 8: Deoxyribonucleic acid sequence of a plasmid containing Qbeta VLP and recognition tag for the bacteriophage VLP:

ATGGCCAAACTGGAAACCGTTACGCTGGGTAATATTGGTAAAGATGGTAAACAG
ACCCTGGTTCTGAATCCGCGTGGTGTTAATCCGACCAATGGTGTTGCAAGCCTGA
GCCAGGCAGGCGCAGTTCCGGCACTGGAAAAACGTGTTACCGTTAGCGTTAGCC
AGCCGAGCCGTAATCGTAAAAACTATAAAGTTCAGGTGAAAATCCAGAATCCGA
CCGCATGTACCGCAAATGGTAGCTGTGATCCGAGCGTTACCCGTCAGGCATATGC
AGATGTTACCTTTAGTTTTACCCAGTATAGCACCGATGAAGAACGTGCATTTGTT
CGTACCGAACTGGCAGCACTGCTGGCAAGTCCGCTGCTGATTGATGCAATTGATC
AGCTGAATCCGGCATATTAATGATTCCTCGTGCTTAGTAACTAAGGATGAAATGC
ATGTCTAAGACAGCATCTTCGC

We claim:

1. A method of producing a protein nanoparticle having an antigenic molecule conjugated to its exterior surface, wherein the antigenic molecule is a bacterial saccharide, wherein the protein nanoparticle is a bacteriophage Virus-Like Particle (VLP), and wherein the VLP encapsulates at least one RNA polynucleotide, the method comprising:

a) engineering a vector comprising at least one DNA polynucleotide to encode:

i. at least one RNA polynucleotide, wherein the at least one RNA polynucleotide comprises at least one recognition tag for the bacteriophage VLP or virus coat protein of the VLP;

ii. at least one bacteriophage VLP; and

iii. optionally at least one aptamer;

b) expressing the vector in a host cell, wherein the host cell is cultured under conditions conductive to the expression and self-assembly of the bacteriophage VLP encapsulating the at least one RNA polynucleotide to form a (RNA-VLP) nanoparticle;

c) recovering and purifying the RNA-VLP nanoparticle from the host cell or the culture medium in which the host cell is grown, as is suitable;

d) extracting and purifying the bacterial saccharide from a bacteria;

e) conjugating the bacterial saccharide to the exterior of the RNA-VLP nanoparticle by reductive amination.

2. The method of claim 1, wherein the at least one recognition tag is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the polynucleotide of SEQ ID NO: 1.

3. The method of claim 1 or claim 2, wherein the bacteriophage VLP comprises an amino acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the amino acid sequence set out in SEQ ID NO: 3.

4. The method of any one of claims 1-3, wherein the aptamer is encoded by a polynucleotide that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to the polynucleotide of SEQ ID NO: 5.

5. The method of any one of claims 1-4, wherein the RNA-VLP is a Qβ RNA-VLP.

6. The method of any one of claims 1-5, wherein the bacterial saccharide is from a bacterial species selected from the group consisting of Acinetobacter species, Bacillus species, Bordetella species, Borrelia species, Burkholderia species, Campylobacter species, Chlamydia species, Clostridium species, Corynebacterium species, Enterococcus species, Escherichia species, Francisella species, Haemophilus species, Helicobacter species, Klebsiella species, Legionella species, Listeria species, Neisseria species, Proteus species, Pseudomonas species, Salmonella species, Shigella species, Staphylococcus species, Streptococcus species, Streptomyces species, Vibrio species, and Yersinia species.

7. The method of any one of claims 1-6, wherein the bacterial saccharide is from a Streptococcus species selected from *Streptococcus agalactiae* (Group B Streptococcus, or GBS) and *Streptococcus pneumoniae*.

8. The method of claim 7, wherein the bacterial saccharide is selected from the group consisting of GBS CPS serotype Ia, GBS CPS serotype Ib, GBS CPS serotype II, GBS CPS serotype III, GBS CPS serotype IV, and GBS CPS serotype V.

9. The method of any one of claims 1-8, wherein at least 50% of the at least one RNA polynucleotide is encoded by the vector.

10. A protein nanoparticle produced by the method of any one of claims 1-9.

11. The protein nanoparticle of claim 10, wherein the nanoparticle is capable of eliciting a higher immune response to the bacterial saccharide after one dose compared to after one dose of a monomeric protein carrier, such as CRM197, conjugated to the same bacterial saccharide.

12. The protein nanoparticle of claim 10, wherein the nanoparticle is capable of eliciting a higher or comparable immune response to the bacterial saccharide after one dose compared to after two doses of a monomeric protein carrier, such as CRM197, conjugated to the same bacterial saccharide.

13. A polynucleotide encoding the protein nanoparticle of any one of claims 10-12.

14. A host cell comprising the polynucleotide of claim 13.

15. An immunogenic composition comprising the protein nanoparticle of any one of claims 10-12, or the polynucleotide of claim 13.

16. The immunogenic composition of claim 15, further comprising an adjuvant.

17. A pharmaceutical composition comprising the protein nanoparticle of any one of claims 10-12, or the polynucleotide of claim 13 and an excipient.

18. A vaccine composition comprising the protein nanoparticle of any one of claims 10-12, or the polynucleotide of claim 13, or the immunogenic composition of claims 15-16, or the pharmaceutical composition of claim 17.

19. A method of inducing or increasing an immune response in a human subject, comprising administering to the human subject an immunologically effective amount of the protein nanoparticle of any one of claims 10-12, or the polynucleotide of claim 13, or the immunogenic composition of claims 15-16, or the pharmaceutical composition of claim 17, or the vaccine composition of claim 18.

20. A method of preventing or treating a bacterial infection in a human subject, comprising administering to the subject an immunologically effective amount of the protein nanoparticle of any one of claims 10-12, or the polynucleotide of claim 13, or the immunogenic composition of claims 15-16, or the pharmaceutical composition of claim 17, or the vaccine composition of claim 18.

21. Use of the protein nanoparticle of any one of claims 10-12, or the polynucleotide of claim 13, or the immunogenic composition of claims 15-16, or the pharmaceutical

composition of claim 17, or the vaccine composition of claim 18 for inducing an immune response in a human subject.

22. The protein nanoparticle of any one of claims 10-12, or the polynucleotide of claim 13, or the immunogenic composition of claims 15-16, or the pharmaceutical composition of claim 17, or the vaccine composition of claim 18 for use in the prevention or treatment of disease in a subject, optionally a human subject.

FIG. 1

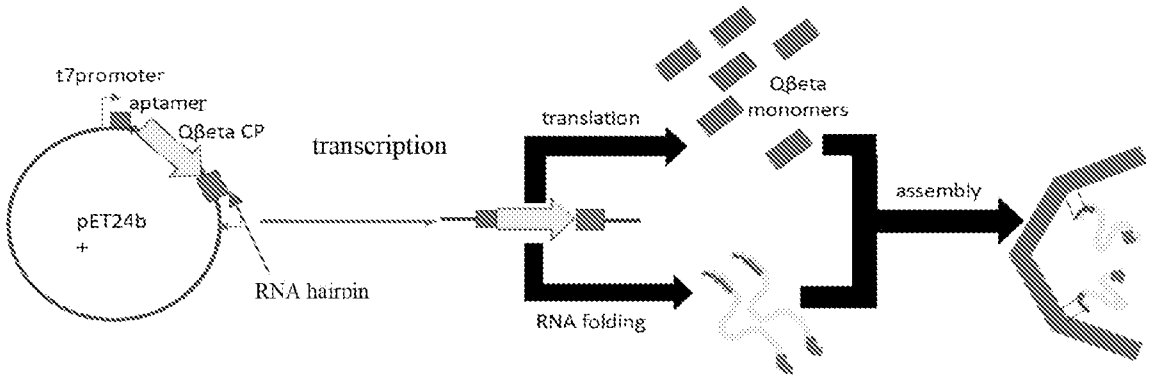

t7promoter
aptamer
Qβeta CP
pET24b
+
RNA hairpin
transcription
translation
Qβeta
monomers
assembly
RNA folding

FIG. 2

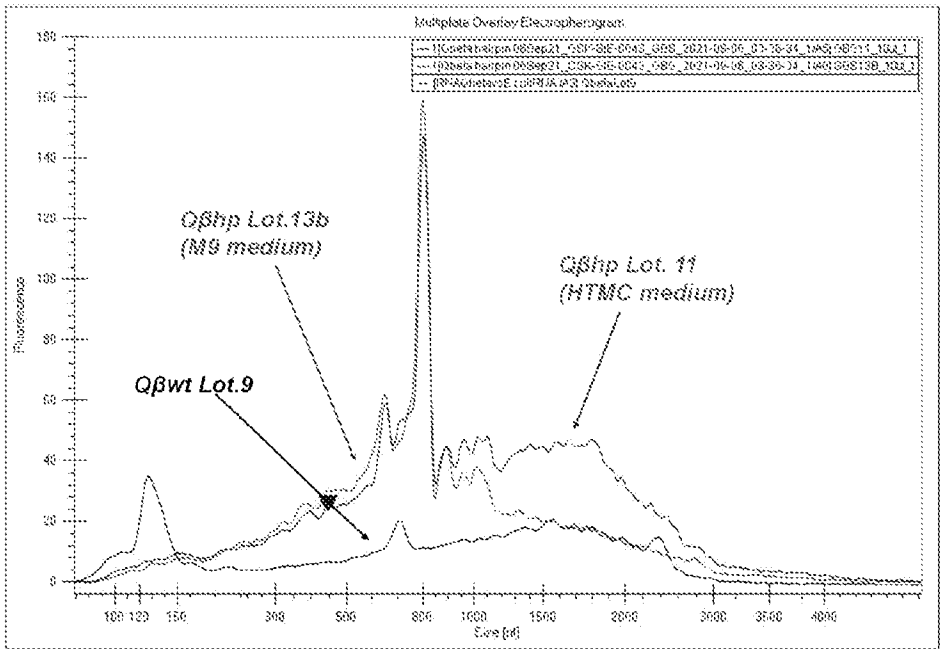

Multiplate Overlay Electropherogram
Qβhp Lot.13b
(M9 medium)
Qβhp Lot. 11
(HTMC medium)
Qβwt Lot.9
Fluorescence
Size [nt]

FIG. 3

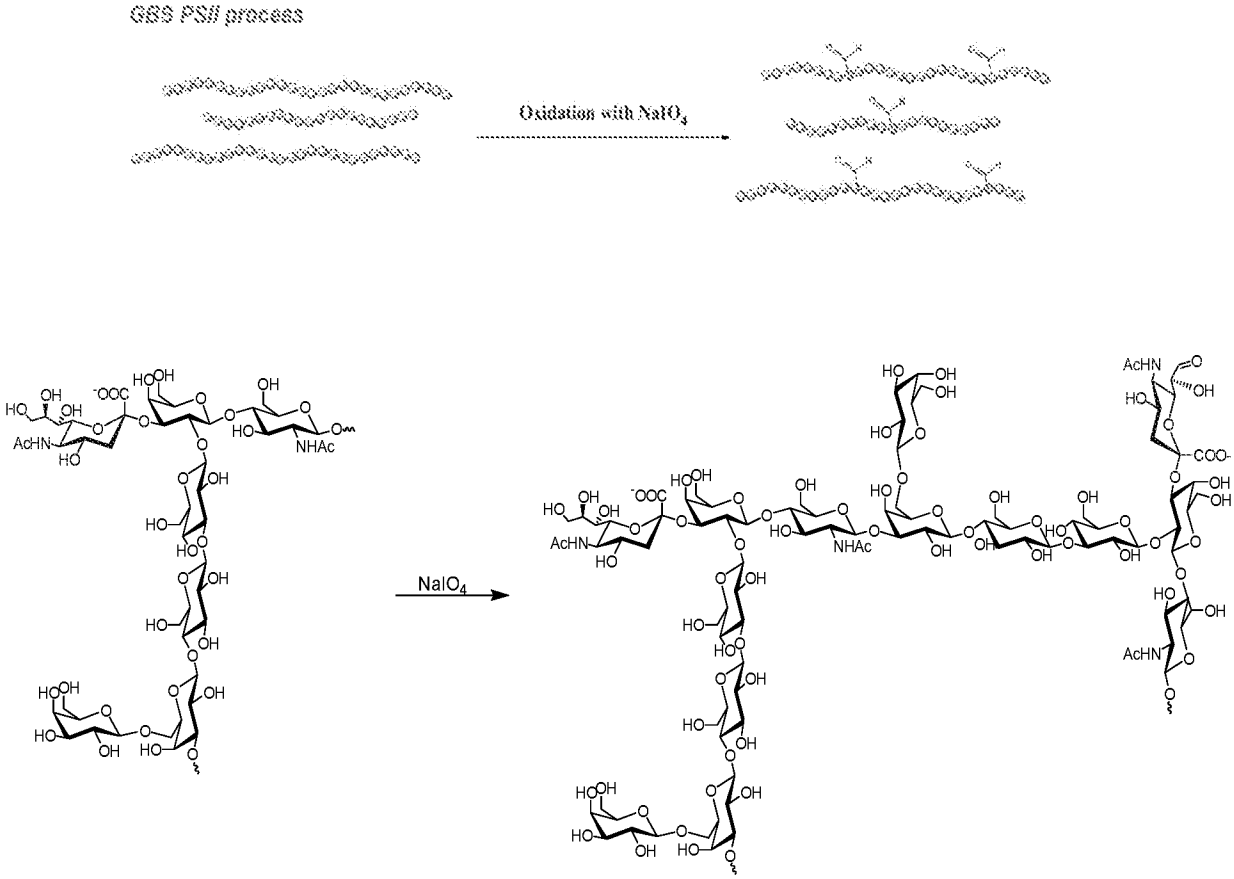


FIG 4.

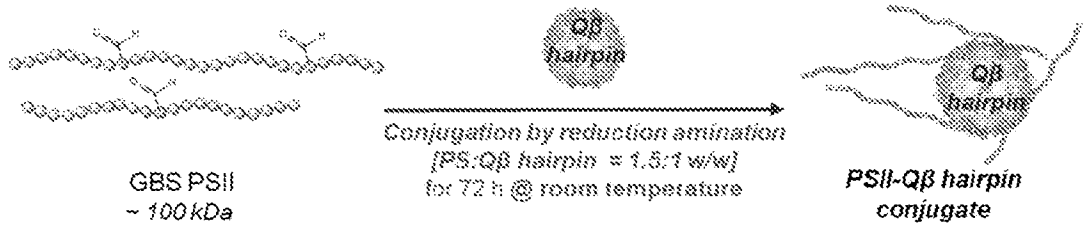

Qβ hairpin
Conjugation by reduction amination
[PS:Qβ hairpin = 1.5:1 w/w]
for 72 h @ room temperature
Qβ hairpin
GBS PSII
~ 100 kDa
PSII-Qβ hairpin conjugate

FIG. 5A

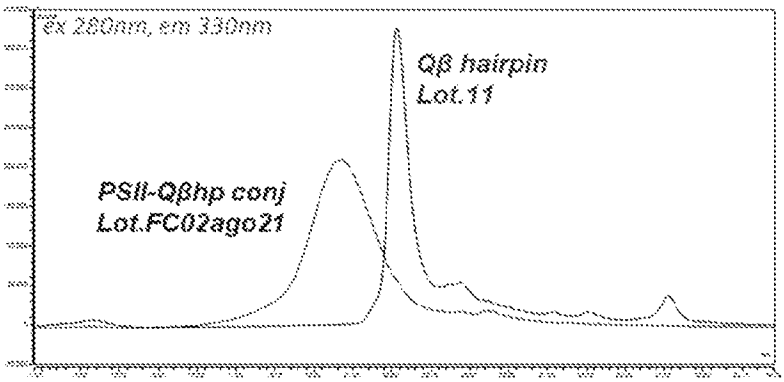

Ex 280nm, em 330nm
Qß hairpin
Lot.11
PSII-Qßhp conj
Lot.FC02ago21

FIG. 5B

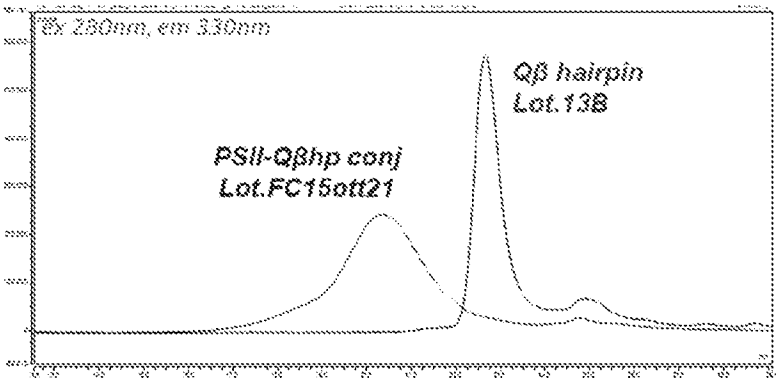

Ex 280nm, em 330nm
Qß hairpin
Lot.13B
PSII-Qßhp conj
Lot.FC15ott21

FIG. 6

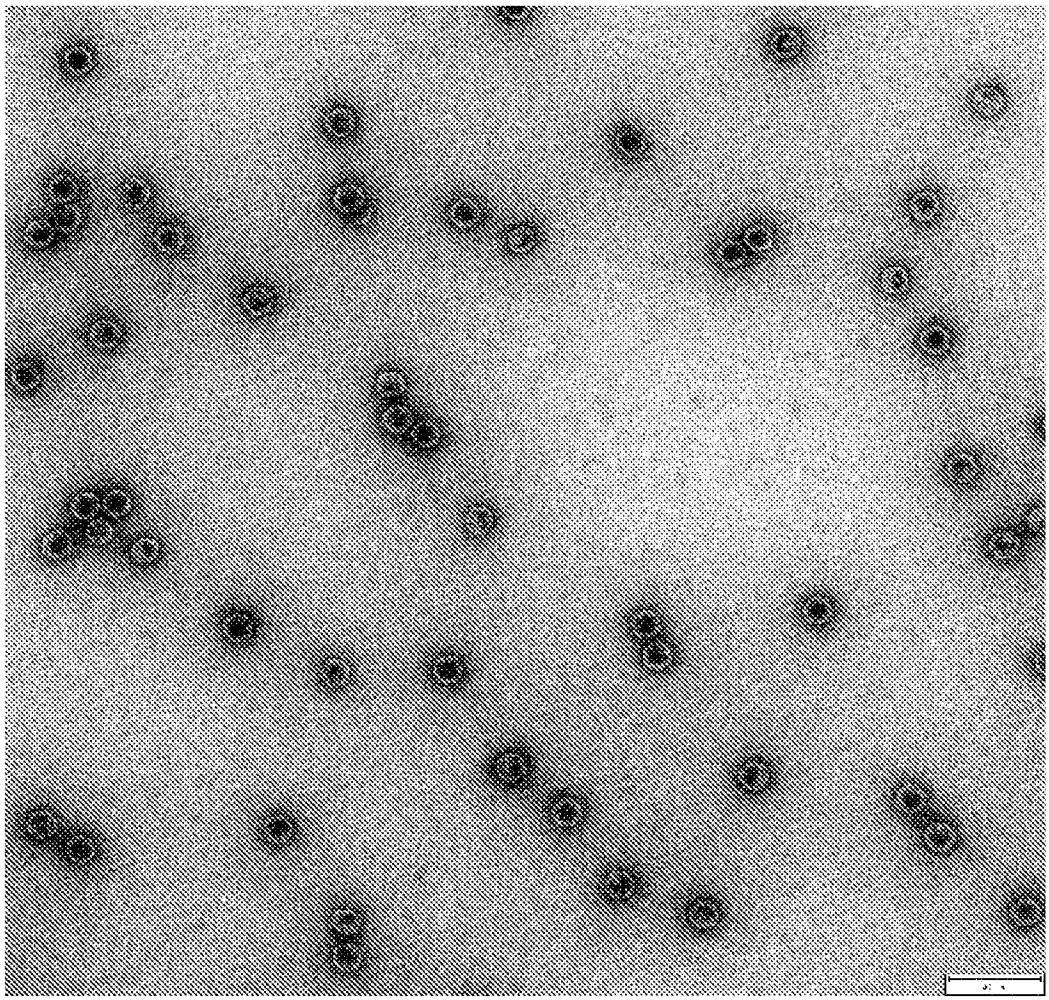
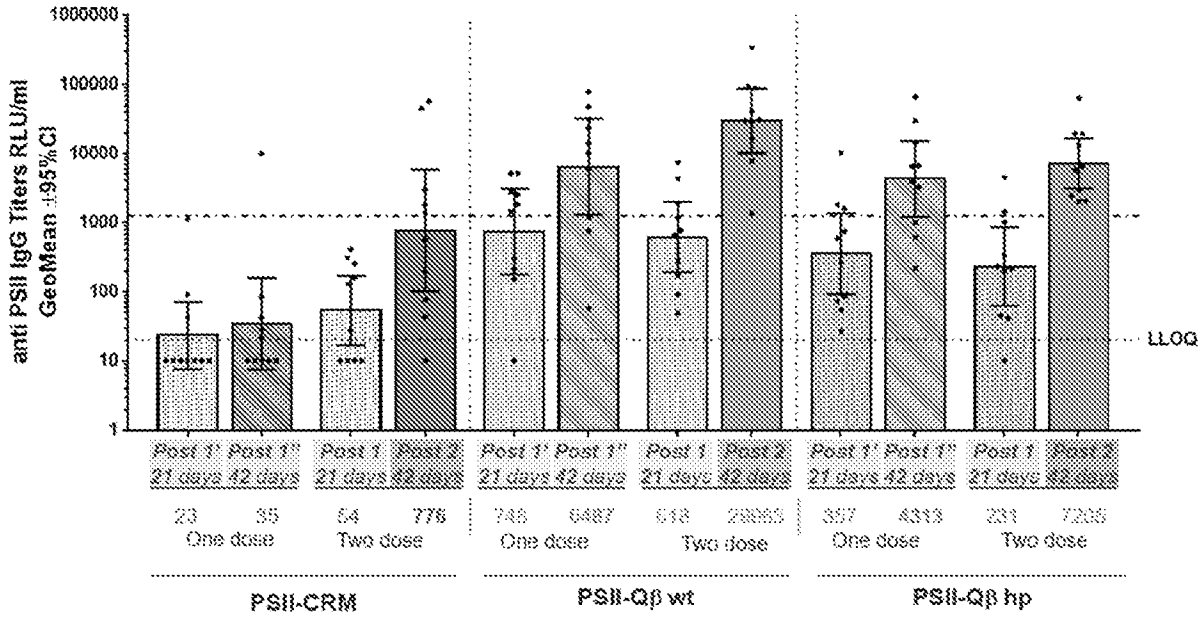


FIG. 7



LLOQ: lower limit of quantification (20.4 RLU/ml)
Preimmune sera:IgG titer <LLOQ

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2022/062086

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

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 3 650 044 A1 (ETH ZUERICH [CH]; UNIV OF BERN [CH]) 13 May 2020 (2020-05-13) paragraphs [0001], [0002], [0005], [0006], [0007], [0011], [0012], [0017], [0025], [0030], [0033], [0034], [0039], [0042], [0053] - [0057]; figures 1,2; example 1 ----- -/--	10-22 1-9

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

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2023	09/03/2023

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

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2022/062086

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROHOVIE MARCUS J. ET AL: "Virus-like particles: Next-generation nanoparticles for targeted therapeutic delivery : ROHOVIE et al.", BIOENGINEERING & TRANSLATIONAL MEDICINE, vol. 2, no. 1, 1 March 2017 (2017-03-01), pages 43-57, XP055952266, ISSN: 2380-6761, DOI: 10.1002/btm2.10049 Retrieved from the Internet: URL:https://api.wiley.com/onlinelibrary/tdm/v1/articles/10.1002%2Fbtm2.10049> page 2, paragraphs 1,3; table 1 page 3, last paragraph - page 4, paragraph 1; figure 1; table 2 page 4, paragraphs 3,4 table 3 page 7, paragraph 9 -----	1-22
A	FANG PO-YU ET AL: "RNA: packaged and protected by VLPs", RSC ADVANCES, vol. 8, no. 38, 1 January 2018 (2018-01-01), pages 21399-21406, XP093027765, DOI: 10.1039/C8RA02084A Retrieved from the Internet: URL:https://pubs.rsc.org/en/content/articlepdf/2018/ra/c8ra02084a> page 1, paragraph 5; figure 1 page 2, paragraph 7; figure 2 page 5, paragraphs 8,9 page 6, paragraphs 8,10 page 7, paragraph 1-4 -----	1-22
Y	WO 2015/038746 A1 (GEORGIA TECH RES INST [US]) 19 March 2015 (2015-03-19) page 10, lines 10-15 page 11, line 20 - page 12, line 35 page 14, line 20 - line 25 example 1 sequences 1,2,4 claims 1,16 ----- -/--	1-6,9

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2022/062086

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Bayliss Marc ET AL: "Assessments of hepatitis B virus-like particles and Crm197 as carrier proteins in melioidosis glycoconjugate vaccines", bioRxiv, 9 April 2020 (2020-04-09), pages 1-20, XP093027769, DOI: 10.1101/2020.04.08.031658 Retrieved from the Internet: URL:https://www.biorxiv.org/content/10.1101/2020.04.08.031658v1.full.pdf [retrieved on 2023-02-28] abstract lines 62-69,78-80,87,152-153,289-291,385-392 -----	1-22
Y	US 2017/072043 A1 (KAPRE SUBHASH V [US]) 16 March 2017 (2017-03-16) paragraphs [0006] - [0008], [0011] - [0012], [0020] - [0023]; claims 1-8,16; example 1 -----	7,8

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2022/062086

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2022/062086

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 3650044 A1	13-05-2020	EP 3650044 A1	13-05-2020
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		US 2021113680 A1	22-04-2021
		WO 2017044932 A1	16-03-2017
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