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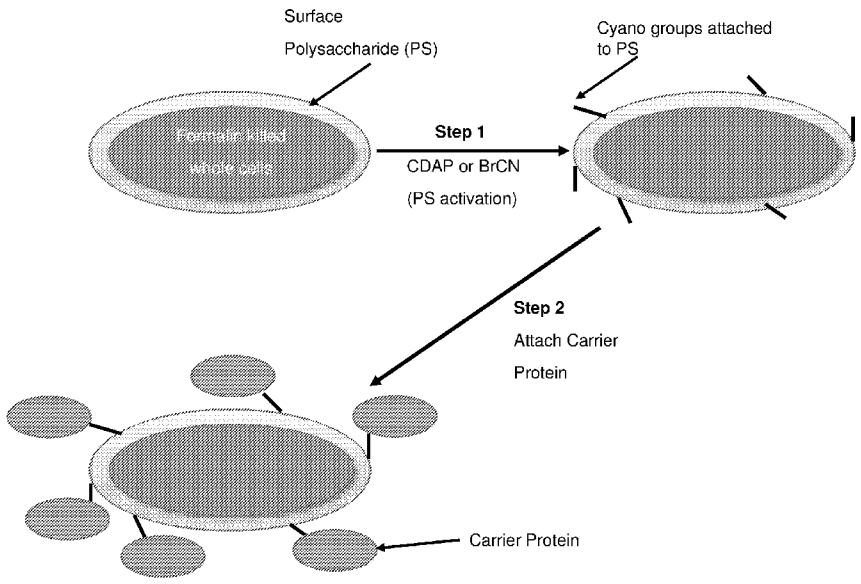
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(54) Title: WHOLE CELL-PROTEIN CONJUGATES AND METHODS OF MAKING THE SAME

FIG.1



(57) Abstract: Disclosed are immunogenic whole cell-protein conjugates containing a carrier protein and a whole cell having an exterior surface including a polyol antigen that is linked to the carrier protein through a linker. The linker can be covalent, or the linker can include an affinity pair. The covalent linker may be of formula: Z^1-C(Z^3)-N(H)-N(H)-(-C(O)-(L-C(O))$_n$-N(H)-N(H)-)$_m$-Z^2, where Z^1 is a bond to an oxygen atom in the polyol antigen, Z^2 is a bond to a carbonyl group in the carrier protein, Z^3 is O or NH, each of n and m is 0 or 1, and L, when present, is C_{2-6} alkanediyl or C_{6-10} arenediyl. Also disclosed are immunogenic whole-cell protein conjugates containing a carrier protein and a whole cell having an exterior surface including a polyol antigen that is covalently linked to the carrier protein through a linker formed by a reaction between a cyanate group bonded to

[Continued on next page]

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the polyol antigen and a hydrazine-activated carrier protein. Further disclosed are pharmaceutical compositions containing the immunogenic whole cell-protein conjugates, methods of preparing immunogenic whole cell-protein conjugates, methods of preparing polyol antigen-protein conjugates via whole cell-protein conjugates, and methods of generating an immune response in a subject using the disclosed pharmaceutical compositions.

WHOLE CELL-PROTEIN CONJUGATES AND METHODS OF MAKING THE SAME

STATEMENT AS TO FEDERALLY FUNDED RESEARCH

This invention was made with government support under grant number U19 AI109764 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

The invention relates to immunogenic whole cell-protein conjugates, pharmaceutical compositions containing immunogenic whole cell-protein conjugates, methods of making immunogenic whole cell-protein conjugates, methods of making immunogenic polysaccharide-protein conjugates via immunogenic whole cell-protein conjugates, and methods of generating an immune response in a subject using immunogenic whole cell-protein conjugates.

BACKGROUND OF THE INVENTION

Many antigens, particularly those associated with a microbial pathogen's surface such as capsule layers stimulate little or no immune response and complicate efforts to create effective immunogenic vaccines against those pathogens. Capsules are exterior surface components of microbes that are typically composed of polymers of organic compounds such as carbohydrates, amino acids, or alcohols. Capsules are quite diverse chemically. The monomeric units that make up capsules (e.g., carbohydrates) can be linked together in various molecular configurations and can be further substituted with phosphate, nitrogen, sulfate, and other chemical modifications. These chemical variations allow capsules to present numerous antigenic targets on the microbial exterior surface, thus allowing escape from the host immune response directed at these targets. Capsules can also be virulence factors which prevent microbes from being phagocytosed and killed by host leukocytes. Antibodies against capsules provide a potent defense against encapsulated organisms by fixing complement to the microbial exterior surface, which can result in their lysis or their opsonization, uptake, and killing by phagocytic host immune cells. The most potent antibodies against capsules are IgG antibodies. Capsules that fail to induce significant levels of IgG are called T-independent antigens. Covalent coupling of a protein to T-independent antigens such as capsules renders them "T-dependent" and such “conjugates” can elicit an IgG response.

There is a need for safe, synthetically accessible, cost-effective immunogenic conjugates directed to capsules and other T-independent antigens that do not evoke strong immune responses or IgG antibodies. Such immunogenic conjugates are needed to protect against various infectious diseases such as infection by anthrax, pneumococcus, influenzae Type B, meningococcus, streptococcus, mycobacteria, candida, malaria, and other bacterial, fungal and protozoan pathogens.

SUMMARY OF THE INVENTION

The invention relates to immunogenic whole cell-protein conjugates containing immunogenic whole cell-protein conjugate containing a carrier protein and a whole cell having an exterior surface including a polyol antigen that is linked to the carrier protein through a linker. The linker can be covalent, or the linker may include an affinity pair. The invention also provides methods of making such immunogenic conjugates using whole cells, methods of making polyol antigen-protein conjugates using whole cells, and methods of generating an immune response in a subject using the immunogenic conjugates.

In some embodiments, the linker in the whole cell-protein conjugates of the invention is formed by a reaction between a cyanate group bonded to the polyol antigen and a hydrazine-activated carrier protein. In certain embodiments, the linker can be represented by a formula:

$$Z^1–C(Z^3)–N(H)–N(H)–(–C(O)–(L–C(O))_n–N(H)–N(H)–)_m–Z^2,$$

where

Z^1 is a bond to an oxygen atom in the polyol antigen,

Z^2 is a bond to a carbonyl group in the carrier protein,

Z^3 is O or NH,

each of n and m is 0 or 1, and

L, when present, is C_{2-6} alkanediyl or C_{6-10} arenediyl.

In particular embodiments, the carrier protein is a diphtheria toxin, diphtheria toxoid, tetanus toxin, tetanus toxoid, *Pseudomonas aeruginosa* exotoxin A, cholera toxin B subunit, tetanus toxin fragment C, bacterial flagellin, pneumolysin, an outer membrane protein of *Neisseria menningitidis*, *Pseudomonas aeruginosa* Hcp1 protein, *Escherichia coli* heat labile enterotoxin, shiga-like toxin, human LTB protein, pneumolysin, listeriolysin O, a protein extract from whole bacterial cells, the dominant negative mutant (DNI) of the protective antigen of *Bacillus anthracis*, or *Escherichia coli* beta-galactosidase.

In certain embodiments, the whole cell is a *Pseudomonas aeruginosa* or *Streptococcal* cell (e.g., a *Streptococcus pneumonia* cell (e.g., *Streptococcus pneumonia* type 4). In further embodiments, the whole cell is a *Streptococcus pneumonia* type 1, 2, 3, 4, 5, 6A, 6B, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10F, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 21, 22F, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33D, 33F, 34, 35A, 35B, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, or 48. In other embodiments, the bacterial flagellin is the *Vibrio cholerae* flagellin protein. In yet other embodiments, the shiga-like toxin is the *Shigella* SltB2 protein. In still other embodiments, the carrier protein molecules are pneumolysin. In some embodiments, the carrier protein is listeriolysin O. In particular embodiments, the carrier protein is diphtheria toxin. In certain embodiments, the carrier protein is diphtheria toxoid. In further embodiments, the carrier protein is tetanus toxin. In yet further embodiments, the carrier protein is tetanus toxoid.

In other embodiments, the polyol antigen is a polysaccharide antigen. In yet other embodiments, the polyol antigen comprises at least 18 carbohydrate residues. In still other embodiments, the polyol antigen comprises a *Streptococcus pneumoniae* polysaccharide, *Francisella tularensis* polysaccharide, *Bacillus anthracis* polysaccharide, *Haemophilus influenzae* polysaccharide, *Salmonella typhi* polysaccharide, *Salmonella* species polysaccharide, *Shigella* polysaccharide, or *Neisseria meningitidis* polysaccharide. In some embodiments, the *Streptococcus pneumoniae* polysaccharide is capsular type 1, 2, 3, 4, 5, 6A, 6B, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10F, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 21, 22F, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33D, 33F, 34, 35A, 35B, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, or 48. In certain embodiments, the *Francisella tularensis* polysaccharide is O antigen. In particular embodiments, the polyol antigen is a microbial capsular polymer. In further embodiments, the whole cell is a heat inactivated whole cell pathogen or a chemically inactivated whole cell pathogen, or is chemically inactivated with formaldehyde or glutaraldehyde. In yet further embodiments, the immunogenic whole cell-protein conjugate, when administered to a mammal, elicits a T-cell dependent immune response in the mammal. In still further embodiments, the molar ratio of the polyol antigen to the carrier protein in the immunogenic whole cell-protein conjugate is 1 to 1.

In another aspect, the invention provides a pharmaceutical composition containing the immunogenic whole cell-protein conjugate of the invention and a pharmaceutically acceptable carrier or excipient. In some embodiments, the pharmaceutical composition does not comprise an adjuvant.

In yet another aspect, the invention provides a method of preparing an immunogenic conjugate (e.g., the immunogenic whole cell-protein conjugate of the invention) by contacting a hydrazine-activated carrier protein with a whole cell having an exterior surface including a polyol antigen bonded to at least one cyanate. In some embodiments, the method is for preparing a polyol antigen-carrier protein conjugate by contacting a hydrazine-activated carrier protein with a whole cell having an exterior surface including a polyol antigen bonded to at least one cyanate, and, after the contacting, lysing the whole cell to produce the polyol antigen-carrier protein conjugate. In particular embodiments, the method further includes purifying the polyol antigen-carrier protein conjugate away from unconjugated cellular components. In certain embodiments, the method further includes preparing the hydrazine-activated carrier protein by contacting a carrier protein with a hydrazine source agent. In particular embodiments, the method further includes preparing the whole cell having an exterior surface including a polyol antigen bonded to at least one cyanate by contacting an electrophilic source of cyanide with a whole cell including a polyol antigen. In further embodiments, the electrophilic source of cyanide is CDAP or cyanogen bromide.

In still another aspect, the invention provides a method of generating an immune response in a subject comprising administering the pharmaceutical composition of the invention to the subject, where the immunogenic whole cell-protein conjugate elicits a T-cell dependent immune response in the subject. In some embodiments, the subject is an infant, a child, or an adolescent. In certain embodiments, the

pharmaceutical composition is administered to the subject parenterally. In particular embodiments, the pharmaceutical composition is administered to the subject enterally (e.g., orally or through a rectal suppository), or nasally (e.g., through inhalation or nasal spray)).

In a further aspect, the invention provides an immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker Z^4–CH=N–[–N(H)–(–C(O)–(L–C(O))$_n$–N(H)–N(H)–)$_m$–]$_k$–Z^5,

wherein Z^4 is a bond to a polyol antigen,

Z^5 is a bond the carrier protein,

each of n, m, and k is independently 0 or 1, and

L, when present, is C_{2-6} alkanediyl or C_{6-10} arenediyl.

In a yet further aspect, the invention provides an immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker Z^1–C(O)–N(H)–(N(H))$_k$–L–N(H)–C(O)–CH_2–S–L^1–C(O)–Z^2,

wherein Z^1 is a bond to an oxygen atom in the polyol antigen,

Z^2 is a bond to an amino group in the carrier protein,

k is 0 or 1,

L is C_{2-6} alkanediyl, and

L^1 is C_{2-6} alkanediyl optionally substituted with a protected amino group.

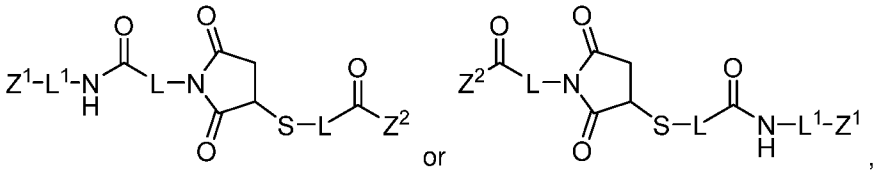
In a still further aspect, the invention provides an immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker Z^4–N(H)–C(O)–L–C(O)–N(H)–Z^5,

wherein Z^4 is a bond to a polyol antigen,

Z^5 is a bond the carrier protein,

L is C_{2-6} alkanediyl or C_{6-10} arenediyl.

In another aspect, the invention provides an immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker of formula:



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wherein Z^1 is a bond to an oxygen atom in the polyol antigen,

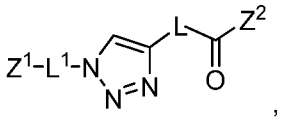
Z^2 is a bond to an amino group in the carrier protein,

each L is independently C_{2-6} alkanediyl, and

L^1 is C_{2-6} alkanediyl.

In yet another aspect, the invention provides an immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker Z^1–C(O)–N(H)–Z^5, wherein Z^1 is a bond to an oxygen atom in the polyol antigen, and Z^2 is a bond to a carrier protein.

In still another aspect, the invention provides an immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker



,

wherein Z^1 is a bond to an oxygen atom in the polyol antigen,
Z^2 is a bond to an amino group in the carrier protein, and
each of L and L^1 is independently C_{2-6} alkanediyl.

In a further aspect, the invention provides an immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is linked to said carrier protein through a linker comprising an affinity pair.

In some embodiments, the linker including an affinity pair is of formula:

$$Z^1–C(Z^3)–L^1–L–L^2–AP–Z^2,$$

where Z^1 is a bond to an oxygen atom in the polyol antigen,
Z^2 is a bond to a carrier protein,
Z^3 is O or NH,
L is C_{2-10} alkanediyl or –[–CH_2–CH_2–(–O–CH_2–CH_2–)$_n$]–, where n is an integer from 0 to 24,
L^1 is –N(H)– or –N(H)–N(H)–,
L^2 is –N(H)–, –C(O)–, –C(O)–N(H)–, or –N(H)–C(O)–,
AP is a complementary affinity pair.

In certain embodiments, the complementary affinity pair is a biotin/streptavidin, biotin/avidin, or biotin/neutravidin pair.

In particular embodiments, the carrier protein is a diphtheria toxin, diphtheria toxoid, tetanus toxin, tetanus toxoid, *Pseudomonas aeruginosa* exotoxin A, cholera toxin B subunit, tetanus toxin fragment C, bacterial flagellin, pneumolysin, an outer membrane protein of *Neisseria menningitidis*, *Pseudomonas aeruginosa* Hcp1 protein, *Escherichia coli* heat labile enterotoxin, shiga-like toxin, human LTB protein, pneumolysin, listeriolysin O, a protein extract from whole bacterial cells, a mutant form of these proteins that ablates toxicity, e.g., the dominant negative mutant (DNI) of the protective antigen of *Bacillus*

anthracis, or a microbial cytoplasmic protein, e.g., *Escherichia coli* beta-galactosidase.

In further embodiments, the whole cell is a *Pseudomonas aeruginosa* or *Streptococcal* cell. In yet further embodiments, the whole cell is a *Streptococcus pneumonia* cell. In still further embodiments, the whole cell is a *Streptococcus pneumonia* type 1, 2, 3, 4, 5, 6A, 6B, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10F, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 21, 22F, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33D, 33F, 34, 35A, 35B, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, or 48.

In other embodiments, the bacterial flagellin is the *Vibrio cholerae* flagellin protein. In yet other embodiments, the shiga-like toxin is the *Shigella* SltB2 protein. In some embodiments, the carrier protein molecules are pneumolysin. In certain embodiments, the carrier protein is listeriolysin O. In particular embodiments, the carrier protein is diphtheria toxin. In further embodiments, the carrier protein is diphtheria toxoid. In yet further embodiments, the carrier protein is tetanus toxin. In some embodiments, the carrier protein is tetanus toxoid. In certain embodiments, the polyol antigen is a polysaccharide antigen. In particular embodiments, the polyol antigen comprises at least 18 carbohydrate residues. In other embodiments, the polyol antigen comprises a *Streptococcus pneumoniae* polysaccharide, *Francisella tularensis* polysaccharide, *Bacillus anthracis* polysaccharide, *Haemophilus influenzae* polysaccharide, *Salmonella typhi* polysaccharide, *Salmonella* species polysaccharide, *Shigella* polysaccharide, or *Neisseria meningitidis* polysaccharide. In yet other embodiments, the *Streptococcus pneumoniae* polysaccharide is capsular type 1, 2, 3, 4, 5, 6A, 6B, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10F, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 21, 22F, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33D, 33F, 34, 35A, 35B, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, or 48. In still other embodiments, the *Francisella tularensis* polysaccharide is O antigen. In some embodiments, the polyol antigen is a microbial capsular polymer. In certain embodiments, the whole cell is a heat inactivated whole cell pathogen. In particular embodiments, the whole cell is a chemically inactivated whole cell pathogen. In further embodiments, the immunogenic whole cell-protein conjugate, when administered to a mammal, elicits a T-cell dependent immune response in said mammal. In yet further embodiments, the whole cell is a bacterial cell. In still further embodiments, the whole cell is a fungal cell. In particular embodiments, the fungal cell is *Candida*.

In a related aspect, the invention provides a pharmaceutical composition comprising the immunogenic whole cell-protein conjugate of the invention and a pharmaceutically acceptable carrier or excipient.

DEFINITIONS

By "administering," as used herein in conjunction with an immunogenic conjugate, is meant providing to a subject an immunogenic conjugate in a dose sufficient to induce an immune response in the subject, where the immune response results in the production of antibodies that specifically bind an

antigen contained in the immunogenic conjugate. Administering desirably includes parenteral administration (e.g., by subcutaneous, intramuscular, intravenous, or intradermal injection). While administering by a means that physically penetrates the dermal layer is desirable (e.g., a needle, airgun, or abrasion), the immunogenic conjugates of the invention can also be administered by transdermal absorption. Desirably, administration involves the inclusion of the appropriate immune adjuvants. Administering also includes enterally (for instance, by oral administration) by ingestion of an immunogenic conjugate in the form of e.g., a liquid, powder, capsule, or tablet. Administering may involve a single administration of an immunogenic conjugate or administering an immunogenic conjugate in multiple doses. Desirably, a second administration is designed to boost production of antibodies in a subject to reduce the likelihood of infection by an infectious agent. The frequency and quantity of the dosage of immunogenic conjugate depends on the specific activity of the immunogenic conjugate and can be readily determined by routine experimentation.

By "alkanediyl" is meant a divalent, saturated hydrocarbon group having a total of 2 to 6 carbon atoms. An alkanediyl may be linear or branched. Non-limiting examples of the alkanediyl groups are: ethane-1,2-diyl; propane-1,3-diyl; propane-1,2-diyl; 2-methyl-propane-1,3-diyl; butane-1,2-diyl; butane-1,3-diyl; and butane-1,4-diyl.

By "affinity pair" is meant a non-covalent complex of two complementary moieties. Preferably, the non-covalent complex has a dissociation constant of 10^{-13} M or less at *ca.* 25 °C in water. Affinity pairs are known in the art. For example, affinity pairs may include lectins/sugar structures, microbial toxins/receptors, antibodies/haptens, avidins/biotins or mimics, adhesins/receptors, maltose binding protein/maltose, and maltose binding protein/maltotriose. Non-limiting examples of lectin/sugar affinity pairs include concanavalin A/manose and concavalin A/glucose. Non-limiting examples of microbial toxin/receptor affinity pairs include toxins binding RGD peptide sequence/RGD peptides, heat labile enterotoxin of *E. coli*/L-galactose, and ricin toxin/galactose. Non-limiting examples of antibodies/haptens include antibodies/trinitrobenzene. Non-limiting examples of avidin/biotin affinity pairs include biotin/streptavidin, biotin/avidin, and biotin/neutravidin pairs. Non-limiting examples of avidin/biotin peptide mimics are known in the art (see, e.g., Dudak et al., *Molecules*, 16:774-789, 2011). Non-limiting examples of adhesin/receptor affinity pairs include Type I fimbriae (e.g., FimH) binding mannose residues.

By "arenediyl" is meant a divalent, cyclic, aromatic group having a total of 6 to 10 carbon atoms. Non-limiting examples of the arenediyl groups are phenylene and napthylene.

By "amino acid" is meant a residue in a polypeptide sequence that can be naturally occurring or synthetic. A naturally occurring amino acid is one encoded by the genetic code. A synthetic amino acid is one that is analogous in chemical structure to a naturally occurring amino acid; or one that has a different chemical structure from a naturally occurring amino acid yet functions similarly to a naturally occurring amino acid. Amino acids may be referred to herein by their single or three letter abbreviations. The single letter abbreviation for a particular amino acid, its corresponding amino acid, and three letter

abbreviation are as follows: A, alanine (Ala); C, cysteine (Cys); D, aspartic acid (Asp); E, glutamic acid (Glu); F, phenylalanine (Phe); G, glycine (Gly); H, histidine (His); I, isoleucine (Ile); K, lysine (Lys); L, leucine (Leu); M, methionine (Met); N, asparagine (Asn); P, proline (Pro); Q, glutamine (Gln); R, arginine (Arg); S, serine (Ser); T, threonine (Thr); V, valine (Val); W, tryptophan (Trp); and Y, tyrosine (Tyr).

By "antigen" as used herein is meant any molecule or combination of molecules that is /specifically bound by an antibody or an antibody fragment.

By "boost the production of antibodies" is meant the activation of memory B-cells that occurs during a second exposure to an antigen, called a "booster response," and is indicative of a long lived "secondary" memory immune response, resulting in the long lived production of antibodies.

By "carrier protein" is meant a protein used in an immunogenic conjugate that invokes an immune response to itself and/or to a polyol antigen covalently linked to a carrier protein. Desirably, the carrier protein contains an epitope recognized by a T-cell. In some embodiments, a carrier protein includes multi-antigenic peptides (MAPs), which are branched peptides and include lysine. Exemplary desirable carrier proteins include toxins and toxoids (chemical or genetic), which may be mutant. In certain embodiments, a carrier protein is diphtheria toxin or a mutant thereof, diphtheria toxoid, tetanus toxin or a mutant thereof, tetanus toxoid, *Pseudomonas aeruginosa* exotoxin A or a mutant thereof, cholera toxin B subunit, tetanus toxin fragment C, bacterial flagellin, pneumolysin, listeriolysin O (and related molecules), an outer membrane protein of *Neisseria menningitidis*, *Pseudomonas aeruginosa* Hcp1 protein, *Escherichia coli* heat labile enterotoxin, shiga-like toxin, human LTB protein, or a protein extract from whole bacterial cells or any other protein that can be conjugated by a peptide linker. Mutant forms of these proteins can also be carrier proteins, preferably when mutations ablate toxicity or remove epitopes that immunologically cross-react with human proteins. One example of such a protein is the dominant negative mutant (DNI) of the protective antigen of *Bacillus anthracis*. Pure nontoxic proteins such as *Escherichia coli* beta-galactosidase and other abundant microbial cytoplasmic proteins can also be carrier proteins. In particular embodiments, the carrier protein does not contain a sortase protein described in U.S. Serial No. 62/191,028. A carrier protein can also include one or more affinity purification tags. Affinity purification tags are known in the art; non-limiting examples of the purification tags are poly-His tags (e.g., oligohistidines having from 5 to 10 His repeating units or from 6 to 9 repeating units), poly-Arg tag, FLAG-tag, calmodulin-tag, S-tag, SBP-tag, TC tag, Strep-tag, VSV-tag (vesicular stomatitis virus tag), Xpress tag, Isopeptag, Halo-tag, GFP-tag, biotin carboxyl carrier protein, chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST), V5-tag, Myc-tag, and HA-tag. The affinity tags have been described in Terpe *Appl. Microbioll. Biotechnol.*, 60:523-533, 2003; Schmidt et al., *Nat. Protocol.*, 2:1528-1535, 2007; the disclosure of which is incorporated herein by reference.

By "conjugated" or "conjugation" is meant covalent linking of two or more molecules (e.g., a polyol antigen and a protein).

By "covalently linked" is meant the presence of a covalent linker bonded to two or more molecules (e.g., a polyol antigen and a carrier protein).

By "DNI" is meant the dominant negative mutant (DNI) protein, which is a mutated form of protective antigen (PA) of *B. anthracis*, as described by Benson et al. (*Biochemistry* 37:3941-3948, 1998).

By "electrophilic source of cyanide" is meant a compound that upon reaction with a hydroxyl of an alcohol produces a cyanate group. Non-limiting examples of the electrophilic sources of cyanide include 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) or cyanogen bromide.

By "hydrazine-activated carrier protein" is meant a carrier protein that is modified to include H_2N-NH- group.

By "hydrazine source agent" is meant a compound capable of reacting with a carrier protein to produce a hydrazine-activated carrier protein. In some embodiments, the formula of a hydrazine source agent is $H_2N–N(H)–(–C(O)–(L–C(O))_n–N(H)–N(H)–)_m–H$, in which each of n and m is 0 or 1, and L is C_{2-6} alkanediyl or C_{6-10} arenediyl. Non-limiting examples of hydrazine source agents are hydrazine and dicarboxylic acid dihydrazides (e.g., succinic acid dihydrazide or adipic acid dihydrazide).

By "infection" is meant the invasion of a subject by a microbe, e.g., a bacterium, fungus, parasite, or virus. The infection may include, for example, the excessive multiplication of microbes that are normally present in or on the body of a subject or multiplication of microbes that are not normally present in or on a subject. A subject is suffering from a microbial infection when an excessive amount of a microbial population is present in or on the subject's body or when the presence of a microbial population(s) is damaging the cells or causing pathological symptoms to a tissue of the subject.

By "infectious agent" is meant a microbe, e.g., a bacterium, fungus, parasite, or virus that is capable of causing an infection in a subject.

By "immunogenic" is meant the capability of a compound to induce an immune response in a subject. Desirably, the immune response is a T-cell dependent immune response that involves the production of IgG antibodies.

By "microbial capsular polymer" is meant a polymer present in or on the capsule coating of a microbe. Desirably, a microbial capsular polymer is a polyol antigen, e.g., a polysaccharide antigen (e.g., dextrans, alginates, phosphopolysaccharides, polysaccharides with an amino sugar with an N-acetyl substitution, or polysaccharides containing a sulfonylated sugar, or an O side chain of a lipopolysaccharide) or a polyalcohol antigen (e.g., teichoic acids).

By "polyalcohol antigen" is meant a polymer having a repeating unit that contains a sugar alcohol. Non-limiting examples of polyalcohols are teichoic acids.

By "polyglycine" is meant a $(Gly)_n$ sequence. Desirably n is between 2 and 20, or more desirably between 2 and 5, and even more desirably 5 glycine residues.

By "polyol antigen" is meant a polyalcohol antigen, a polysaccharide antigen, or a polypeptide antigen having at least one alcohol hydroxyl (e.g., in a side chain, such as in Ser or Thr).

By "polysaccharide antigen" is meant a polymer of saccharides (sugars) derived from capsules of encapsulated bacterial pathogens (e.g., *Streptococcus pneumoniae*, *Francisella tularensis*, *Bacillus anthracis*, *Haemophilus influenzae*, *Salmonella typhi*, *Salmonella* species, *Shigella*, or *Neisseria meningitides*) that is specifically bound by an antibody or an antibody fragment.

By "protected amino group" is meant a group of formula $-N(R^{N1})_2$, where each R^{N1} is independently H or an *N*-protecting group, provided that at least one R^{N1} is an *N*-protecting group. *N*-protecting groups are known in the art. Commonly used *N*-protecting groups are disclosed in Greene, "Protective Groups in Organic Synthesis," 3rd Edition (John Wiley & Sons, New York, 1999), which is incorporated herein by reference. Other *N*-protecting groups include, but are not limited to, amino acids such as alanine, leucine, phenylalanine, and the like; sulfonyl-containing groups such as benzenesulfonyl, p-toluenesulfonyl, and the like; carbamate forming groups such as benzyloxycarbonyl, p-chlorobenzyloxycarbonyl, p-methoxybenzyloxycarbonyl, p-nitrobenzyloxycarbonyl, 2-nitrobenzyloxycarbonyl, p-bromobenzyloxycarbonyl, 3,4-dimethoxybenzyloxycarbonyl, 3,5-dimethoxybenzyl oxycarbonyl, 2,4-dimethoxybenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitro-4,5-dimethoxybenzyloxycarbonyl, 3,4,5-trimethoxybenzyloxycarbonyl, 1-(p-biphenylyl)-1-methylethoxycarbonyl, α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl, benzhydryloxy carbonyl, t-butyloxycarbonyl, diisopropylmethoxycarbonyl, isopropoxycarbonyl, ethoxycarbonyl, methoxycarbonyl, allyloxycarbonyl, 2,2,2-trichloroethoxycarbonyl, phenoxycarbonyl, 4-nitrophenoxy carbonyl, fluorenyl-9-methoxycarbonyl, cyclopentyloxycarbonyl, adamantyloxycarbonyl, cyclohexyloxycarbonyl, phenylthiocarbonyl, and the like, benzyl, triphenylmethyl, benzyloxymethyl, and the like.

By "CRM" we mean the nontoxic CRM197 mutant protein of diphtheria toxin.

By "Ply" we mean the *Streptococcus pneumoniae* toxin pneumolysin in either its wild type or mutant form including fusion proteins with other peptides and proteins required for stability or purification.

By "subject" is meant an animal that can be infected by a microbe. Desirably, a subject is a mammal such as a human, monkey, dog, cat, mouse, rat, cow, sheep, goat, or horse. In a desirable embodiment, the subject is a human, such as a human child. Desirably, the subject is a human infant, toddler, pre-pubescent child, pubescent child, young adult, or adult under the age of 55 years old.

By "T-cell independent antigen" is meant an antigen which results in the generation of antibodies without the cooperation of T lymphocytes. The T-cell independent antigen desirably directly stimulates B

lymphocytes without the cooperation of T lymphocytes. Exemplary desirable T-cell independent antigens include polysaccharides (e.g., alginic acid (alginate) or dextran) and polyalcohols (e.g., teichoic acids).

By "whole cell" is meant a chemically fixed bacterial or fungal cell. Fixation may be accomplished through exposure to an aldehyde containing compound such as formaldehyde, paraformaldehyde, or glutaraldehyde.

Advantages

Compared to existing immunogenic conjugate technologies, the immunogenic conjugates of the present invention are simple to make, less expensive, and more adaptive to different antigens of interest and carrier proteins than existing conjugate technologies. No capsular antigens need be purified to produce these conjugate immunogens. Because the immunogens are prepared on the surface of fixed cells, the microbial pathogen is irreversibly inactivated before synthesis of the conjugate is performed. Fixation can be accomplished through exposure to aldehyde containing compounds such as formaldehyde, paraformaldehyde, or glutaraldehyde. These chemicals stabilize proteins and other immunogens in conformations that are typically more immunogenic, less susceptible to degradation, and that do not require refrigeration (a cold chain) to maintain their immunological properties.

The immunogenic conjugates of the present invention do not require that each combination of carrier protein and the antigen intended to evoke an immune response be conjugated by a tailored ligation process unique to their respective chemical properties. Polysaccharide (PS)-protein immunogenic conjugates have been prohibitively expensive to produce and sell in the developing world, and conventional immunogenic conjugates are difficult to produce cheaply because of the highly specialized chemistry required for each antigen-protein conjugate from pure components. Thus, the present invention simplifies the method of making these conjugates from inactivated whole microbial cells, and carrier protein that does not need to be highly purified in order to react with whole cells activated by the described procedures. Hence, the present invention and reduces the cost of their preparation compared to current immunogenic conjugate technology.

The immunogenic conjugates of the present invention also address a need for immunogenic conjugates that can safely induce immunity against previously intractable antigens. Immunogenic conjugates containing TLR (Toll-like receptor) ligands have been shown to evoke immune responses for otherwise intractable antigens, but they tend to be unsafe because TLR ligands are often proinflammatory, toxic in even small doses, reactogenic, and likely to cause adverse symptoms compared to immunogenic conjugates of the invention.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a scheme showing CDAP- or BrCN-mediated activation of the surface of a fixed whole cell, followed by covalent linking a carrier protein to the activated surface of the fixed whole cell.

FIG. 2A is a FITC/SSC dot plot for pneumococcal PS4 cells treated with CDAP.

FIG. 2B is a FITC/SSC dot plot for pneumococcal PS4 cells treated with CDAP and with hydrazine-activated CRM197 protein.

FIG. 3A is an electron micrograph of untreated pneumococcal PS4 whole cells exposed to gold nanoparticles against CRM197.

FIG. 3B is an electron micrograph of pneumococcal PS4 whole cells treated with the hydrazine-activated CRM197 and exposed to gold nanoparticles against CRM197.

FIG. 3C is an electron micrograph of pneumococcal PS4 whole cells treated with CDAP and exposed to gold nanoparticles against CRM197.

FIG. 3D is an electron micrograph of pneumococcal PS4 whole cells treated with both CDAP and the hydrazine-activated CRM197 and then exposed to gold nanoparticles against CRM197.

FIG. 4 is a chart showing anti-PPS4 IgG titers at various dilutions for mice immunized with PBS control (PBS), formalin-fixed pneumococcal PS4 whole cells (FX), PPS4-CRM197 conjugates (CRM), and PREVNAR 13® vaccine (Pneumococcal 13-valent conjugate vaccine [diphtheria CRM197 protein]) (PREVNAR).

FIG. 5A is an image showing a Western blot analysis of CDAP mediated conjugation between a purified PPS4 capsule and hydrazine-labeled peptide. Different conditions were assayed, separated on 4-12% gradient SDS-PAGE, and immunoblotted against PPS4 (Green) and peptide (HA; Red).

FIG. 5B is an image showing Western blot analysis of CDAP mediated conjugation between a fixed whole PPS4 cell and hydrazine-labeled peptide. Different conditions were assayed, separated on 4-12% gradient SDS-PAGE, and immunoblotted against PPS4 (Green) and peptide (HA; Red).

FIG. 6A is an image showing immunogold electron microscopy visualization of unmodified, fixed PPS4 cells.

FIG. 6B is an image showing immunogold electron microscopy visualization of CDAP-treated, fixed PPS4 cells.

FIG. 6C is an image showing immunogold electron microscopy visualization of unmodified, fixed PPS4 cells incubated with hydrazine-SGSGSLPETGGYPYDVPDY (SEQ ID NO: 1).

FIG. 6D is an image showing immunogold electron microscopy visualization of CDAP-treated, fixed PPS4 cells incubated with hydrazine-SGSGSLPETGGYPYDVPDY (SEQ ID NO: 1).

FIG. 7 is a series of fluorescence microscopy images showing: in row A, untreated fixed cells; in row B, CDAP-treated cells; in row C, unmodified fixed cells incubated with hydrazine-SGSGSLPETGGYPYDVPDY (SEQ ID NO: 1); and, in row D, CDAP-treated fixed cells incubated with hydrazine-SGSGSLPETGGYPYDVPDY (SEQ ID NO: 1). The data in FIG. 7 demonstrate no significant loss of DNA in CDAP activated cells. Prior to imagine, the cells were stained for hydrazine-SGSGSLPETGGYPYDVPDY (SEQ ID NO: 1) (anti-HA) and spotted on 1% agar pads with 5 μM Syto 9 to stain intracellular DNA.

FIG. 8A is an image showing Western blot analysis of CDAP-mediated conjugation between fixed PPS4 cells and hydrazine activated CRM197. Separation is on 4-12% gradient SDS-PAGE, and immunoblotted against PPS4.

FIG. 8B is an image showing Western blot analysis of CDAP-mediated conjugation between fixed PPS4 cells and hydrazine activated CRM197. Separation is on 4-12% gradient SDS-PAGE, and immunoblotted against CRM197.

FIG. 9 is a chart providing Quantification of mean fluorescent intensity (MFI) for different conditions assayed (n=2) in fluorescent assisted cell sorting (FACS) analysis of CDAP mediated conjugation between fixed PPS4 cells and hydrazine activated CRM197.

FIG. 10A is an immunogold electron microscopy image of fixed, whole PPS4 cells.

FIG. 10B is an immunogold electron microscopy image of fixed, whole PPS4 cells incubated with hydrazine activated CRM197.

FIG. 10C is an immunogold electron microscopy image of CDAP-activated, fixed, whole PPS4 cells incubated with hydrazine activated CRM197.

FIG. 10D is an immunogold electron microscopy image of CDAP-activated, fixed, whole PPS4 cells incubated with hydrazine activated CRM197 without primary antibody incubation.

FIG. 11A is a chart showing ELISA response curves of serially diluted, enumerated samples towards CRM197. The enumerated samples are as follows: (1) fixed whole cells, (2) CDAP-treated cells, (3) activated CRM197 incubated with fixed whole cells, (4) CDAP-treated cells incubated with hydrazine activated CRM197, and (10x) is a 10x scale up of conjugations. The quantification is ELISA-based. The

tests were performed by assaying serially diluted samples for the presence of CRM197 (detection: AP-conjugated IgG secondary antibody; and visualization: by incubation with PNPP substrate detection at 405nm).

FIG. 11B is a chart quantifying ELISA responses of serially diluted, enumerated samples towards CRM197 compared to a CRM197 standard curve. The enumerated samples are as follows: (1) fixed whole cells, (2) CDAP-treated cells, (3) activated CRM197 incubated with fixed whole cells, (4) CDAP-treated cells incubated with hydrazine activated CRM197, and (10x) is a 10x scale up of conjugations. The quantification is ELISA-based. The tests were performed by assaying serially diluted samples for the presence of CRM197 (detection: AP-conjugated IgG secondary antibody; and visualization: by incubation with PNPP substrate detection at 405nm).

FIG. 12A is a chart showing ELISA response curves of serially diluted, enumerated samples towards Ply*. The enumerated samples are as follows: (1) fixed whole cells, (2) CDAP-treated cells, (3) activated Ply* incubated with fixed whole cells, (4) CDAP-treated cells incubated with hydrazine activated Ply*, and (10x) is a 10x scale up of conjugations. The quantification is ELISA-based. The tests were performed by assaying serially diluted samples for the presence of Ply* (detection: AP-conjugated IgG secondary antibody; and visualization: by incubation with PNPP substrate detection at 405nm).

FIG. 12B is a chart quantifying ELISA responses of serially diluted, enumerated samples towards Ply*. The enumerated samples are as follows: (1) fixed whole cells, (2) CDAP-treated cells, (3) activated Ply* incubated with fixed whole cells, (4) CDAP-treated cells incubated with hydrazine activated Ply*, and (10x) is a 10x scale up of conjugations. The quantification is ELISA-based. The tests were performed by assaying serially diluted samples for the presence of Ply* (detection: AP-conjugated IgG secondary antibody; and visualization: by incubation with PNPP substrate detection at 405nm).

FIG. 13 is a chart showing survival curves of mice in IP challenge against PPS4. Groups of 8 mice were infected with $1.7x10^7$ CFU PPS4 whole cells and monitored for the disease progression.

FIG. 14A is a FACS density plot showing consistent cell size by area for flow cytometrically analyzed CDAP-activated PPS4 cells before the CRM197 conjugation.

FIG. 14B is a FACS density plot showing consistent single cell size width for flow cytometrically analyzed CDAP-activated PPS4 cells before the CRM197 conjugation.

FIG. 14C is a FACS density plot showing anti-CRM197 antibody response to CDAP-activated PPS4 cells for flow cytometrically analyzed CDAP-activated PPS4 whole cells before the CRM197 conjugation.

FIG. 14D is a FACS density plot showing consistent cell size by area for flow cytometrically analyzed PPS4-CRM197 whole cell conjugates.

FIG. 14E is a FACS density plot showing consistent single cell size width for flow cytometrically analyzed PPS4-CRM197 conjugated cells.

FIG. 14F is a FACS density plot showing anti-CRM197 antibody response to PPS4-CRM197 whole cell conjugates for flow cytometrically analyzed PPS4-CRM197 conjugated cells.

FIG. 15A is a chart showing ELISA response curves of CDAP-activated PPS4 whole cells to the varied quantity of added CRM197.

FIG. 15B is a chart quantifying ELISA responses of CDAP-activated PPS4 whole cells to the varied quantity of added CRM197.

FIG. 16A is a chart showing ELISA response curves of PPS4 whole cells to the varied volume of the PPS4 resuspended sample.

FIG. 16B is a chart quantifying ELISA responses of PPS4 whole cells to the varied volume of the PPS4 resuspended sample.

FIG. 17A is a chart showing ELISA response curves of PPS4-CRM197 conjugated cells to the variation in the conjugation volume.

FIG. 17B is a chart quantifying ELISA responses of PPS4-CRM197 conjugated cells to the variation in the conjugation volume.

FIG. 18 is a chart showing anti-CRM197 titers at various dilutions for mice immunized with an adjuvant (control), formalin-fixed pneumococcal PS4 whole cells, PPS4-CRM197 whole cell conjugates, PPS4-Ply whole cell conjugates, and PREVNAR 13® vaccine (Pneumococcal 13-valent conjugate vaccine [diphtheria CRM197 protein]).

FIG. 19 is a chart showing anti-Ply* titers at various dilutions for mice immunized with an adjuvant (control), formalin-fixed pneumococcal PS4 whole cells, PPS4-CRM197 whole cell conjugates, PPS4-Ply whole cell conjugates, and PREVNAR 13® vaccine (Pneumococcal 13-valent conjugate vaccine [diphtheria CRM197 protein]).

FIG. 20 is a chart showing anti-PPS4 IgG titers at various dilutions for mice immunized with an adjuvant (control), formalin-fixed pneumococcal PS4 whole cells, PPS4-CRM197 whole cell conjugates, PPS4-Ply whole cell conjugates, and PREVNAR 13® vaccine (Pneumococcal 13-valent conjugate vaccine [diphtheria CRM197 protein]).

FIG. 21 is a chart showing anti-PPS4 IgG1* titers at various dilutions for mice immunized with an adjuvant (control), formalin-fixed pneumococcal PS4 whole cells, PPS4-CRM197 whole cell conjugates, PPS4-Ply whole cell conjugates, and PREVNAR 13® vaccine (Pneumococcal 13-valent conjugate vaccine [diphtheria CRM197 protein]).

FIG. 22 is a chart showing anti-PPS4 IgG2b titers at various dilutions for mice immunized with an adjuvant (control), formalin-fixed pneumococcal PS4 whole cells, PPS4-CRM197 whole cell conjugates, PPS4-Ply whole cell conjugates, and PREVNAR 13® vaccine (Pneumococcal 13-valent conjugate vaccine [diphtheria CRM197 protein]).

FIG. 23 is a chart showing anti-PPS4 IgG3 titers at various dilutions for mice immunized with an adjuvant (control), formalin-fixed pneumococcal PS4 whole cells, PPS4-CRM197 whole cell conjugates, PPS4-Ply whole cell conjugates, and PREVNAR 13® vaccine (Pneumococcal 13-valent conjugate vaccine [diphtheria CRM197 protein]).

FIG. 24 is a scheme showing conjugation of biotin to a whole cell.

FIG. 25A is an image for the Western blot analysis of CDAP-mediated conjugation between fixed PPS4 whole cells and NHS-PEG4-Biotin. Separation is on 4-12% gradient SDS-PAGE, and immunoblotted against PPS4.

FIG. 25B is an image for the Western blot analysis of CDAP-mediated conjugation between fixed PPS4 whole cells and NHS-PEG4-Biotin. Separation is on 4-12% gradient SDS-PAGE, and immunoblotted against biotin.

FIG. 26A is an image showing immunogold electron microscopy visualization of unmodified, fixed PPS4 whole cells.

FIG. 26B is an image showing immunogold electron microscopy visualization of CDAP-treated, fixed PPS4 whole cells conjugated to biotin using NHS-PEG4-Biotin.

FIG. 27 is a scheme illustrating a non-limiting example of the preparation and purification of a polyol antigen-carrier protein conjugate via a whole cell-carrier protein conjugate.

DETAILED DESCRIPTION

The invention provides whole cell-protein conjugates and methods of making and administering such compositions to provide immunity against T-cell independent antigens or antigens which normally invoke weak immune responses (e.g., polyol antigens, such as polysaccharides and polyalcohols). The whole cell-protein conjugates of the invention include a carrier protein and a whole cell having an exterior surface including a polyol antigen that is covalently linked to the carrier protein. Alternatively, the whole

cell-protein conjugates of the invention include a carrier protein and whole cell having an exterior surface including a polyol antigen that is linked to the carrier protein through a linker including a complementary affinity pair. The carrier protein can effectively display and facilitate a robust immune response to the polyol antigen. The immune response to the polyol antigen can also be enhanced in the immunogenic conjugate relative to the isolated polyol antigen or relative to the whole cell having an exterior surface including an unconjugated polyol antigen.

The invention also provides a method of preparing immunogenic polyol antigen-protein conjugates via whole cell-protein conjugates; this method permits preparation of immunogenic polyol antigen-protein conjugates with only a simple purification (e.g., affinity purification) of the polyol antigen-protein conjugates. Specifically, this method takes advantage of the conjugated protein to purify the polyol antigen-protein conjugate away from unconjugated cellular components in contrast to the complex purification techniques required for separation of an unconjugated polyol antigen from unconjugated cellular components produced by lysis of whole cells having an exterior surface including the unconjugated polyol antigen. Cell lysis methods are known in the art. Non-limiting examples of cell lysis methods include mechanical disruption methods, for example, freeze/thaw cycling, sonication, pressure, or filtration. Alternatively, enzymatic as well as detergent-mediated cell lysis methods are also known in the art.

Polysaccharides (PS) are polymers of saccharides (sugars). PS derived from capsules are the primary antigenic components involved in protective immunity against encapsulated bacterial pathogens, such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Salmonella typhi*, and *Haemophilus influenzae* Type B. Immunization of adolescents and adults with immunogenic conjugates based on microbial PS has been successful in reducing disease burden, but has proven less effective in providing protective immunity to infants and young children (i.e., children less than 24 months of age). Young children have not yet developed a mature adaptive immune repertoire and T cell-independent antigens, such as capsular PS, are poorly immunogenic and do not lead to long-term protective immune responses (i.e., an immunological memory response) in such young immunogenic conjugate recipients.

A T-cell independent antigen, such as PS, can be converted to a T-cell dependent antigen by chemical coupling of PS to protein; this process is called "conjugation" and involves the formation of covalent bonds between atoms in the PS structure and side chain atoms of amino acids present in the "carrier" protein. Such "immunogenic conjugates" more efficiently promote the induction of B-cell maturation and isotype switching leading to much higher levels of antibody with the correct anti-PS protective profile. Protective antibodies have high affinity for their PS antigens and typically are of the Immunoglobulin G (IgG) subclass—a long-lived antibody with complement fixing and opsonic effector activity.

Without wishing to be bound by a theory, a typical pathway for induction of an anti-PS IgG immune response can be exemplified by a conjugate containing a PS and tetanus toxoid as the carrier protein. In this model, only B-cells that display antibody receptors that recognize the PS bind the PS-

carrier protein conjugate. Thus, the carrier protein is bound to the surface of the B-cell that displays the correct PS binding specificity. The carrier protein-PS complex is taken up by these B-cells into the intracellular vacuolar compartment where the carrier is processed by proteolytic degradation. Peptides derived from the carrier protein are transported and loaded into the presentation groove of the MHC-Class II receptor (MHC-II). This MHC-II-carrier peptide complex is displayed on the surface of the B-cell. Upon recognition of the MHC-II-peptide complex by the T-cell receptor (TCR), T-cells become activated and secrete cytokines that provide "help" for the induction of B-cell differentiation. B-cells expand in numbers and differentiate into "plasma cells" which now secrete antibody. Initially Immunoglobulin M (IgM) is produced by plasma cells but eventually the T-cell help cause the plasma cells to class switch and produce other isotype classes of antibody such as IgG. This process continues with plasma cells undergoing mutational changes leading to production of antibody receptors that have even higher affinity for the PS-carrier protein conjugates. As antigen is cleared, only the higher affinity plasma cells are activated by residual PS-carrier protein conjugate remaining in circulation. The process of T-cell dependent maturation of plasma cells continues, leading to the expansion of plasma cell populations which produce high affinity antibodies of the IgG class. The expansion can be easily monitored by measuring the levels of anti-PS IgG antibodies in the serum of an immunized subject, e.g., a human.

Eventually the maturation and switching process leads to the production of Memory B-cells which are long lived and specific for the PS. Memory B-cells have a unique property in that they can be immediately activated if exposed to PS. Activation causes memory B-cells to multiply and quickly produce anti-PS IgG. The activation of memory B cells that occurs during a second exposure of to PS antigen is called a "booster response" and is indicative of a long lived "secondary" memory immune response. Primary immunization may stimulate the production of IgM antibodies and some IgG antibodies. Upon secondary immunization, i.e., the "booster" shot, memory cells already programmed by the first immunization are stimulated to produce large quantities of IgG, the memory immune response.

A T-cell independent antigen generally does not stimulate lasting immunity, i.e., the production of IgG antibodies, but may stimulate the production of less potent and more temporary IgM antibodies. As such, polyol antigens alone do not typically produce booster responses of IgG. However, polyol antigens do produce booster responses if primary immunization is performed with a conjugate containing a polyol antigen and a carrier protein because memory cells induced by the conjugate have already been programmed to produce IgG. Indeed, the booster response in immunized animals or humans is thought to mimic the protective response due to exposure to a microbe displaying the polyol; this long term memory is critical for an immunogenic conjugate to work in protecting immunized subjects years after their immunization with immunogenic conjugates. Thus, with conjugates containing a polyol antigen and a carrier protein are valued for (1) their ability to induce high levels of IgG against polyol antigens, and (2) their ability to induce memory immune responses against polyol antigens. Unconjugated polyol antigens either isolated or as part of a whole cell typically do not display these properties and thus are inferior antigens. The difficulty in synthesizing immunogenic conjugates and their cost of production has slowed the development of immunogenic conjugates for many bacterial diseases where an immune response to

polyol antigen may be protective.

Some biological polymers can activate a class of receptors termed Toll-like receptors (TLRs). Once activated, TLRs can induce production of cytokines by host cells and produce changes in the adaptive immune response. For example, lipopolysaccharides (LPS) are PS that are highly immunogenic and induce IgG and memory responses; the lipid A moiety of LPS is a TLR ligand and may be responsible for the immunological properties.

Carrier Proteins

Carrier proteins used in the immunogenic conjugates of the invention desirably are proteins that, either alone or in combination with an antigen, invoke an immune response in a subject. Desirably, the carrier protein contains at least one epitope recognized by a T-cell. Desirably, the epitope is capable of inducing a T-cell response in a subject, and induce B-cells to produce antibodies against the entire antigen of interest. Epitopes as used in describing this invention, include any determinant on an antigen that is responsible for its specific interaction with an antibody molecule or fragment thereof. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three-dimensional structural characteristics as well as specific charge characteristics. To have immunogenic properties, a protein or polypeptide generally is capable of stimulating T-cells. However, a carrier protein that lacks an epitope recognized by a T-cell may also be immunogenic.

By selecting a carrier protein that is known to elicit a strong immunogenic response, a diverse population of subjects can be treated by an immunogenic conjugate described herein. The carrier protein desirably is sufficiently foreign to elicit a strong immune response to the immunogenic conjugate. Typically, the carrier protein used is a molecule that is capable of imparting immunogenicity to the antigen of interest. In a desirable embodiment, a carrier protein is one that is inherently highly immunogenic. Thus, a carrier protein that has a high degree of immunogenicity and is able to maximize antibody production to the antigens complexed with it is desirable.

Various carrier proteins of the invention include, e.g., toxins and toxoids (chemical or genetic), which may or may not be mutant, such as anthrax toxin, PA and DNI (PharmAthene, Inc.), diphtheria toxoid (Massachusetts State Biological Labs; Serum Institute of India, Ltd.) or CRM 197, tetanus toxin, tetanus toxoid (Massachusetts State Biological Labs; Serum Institute of India, Ltd.), tetanus toxin fragment Z, exotoxin A or mutants of exotoxin A of *Pseudomonas aeruginosa*, bacterial flagellin, pneumolysin, an outer membrane protein of *Neisseria meningitidis* (strain available from the ATCC (American Type Culture Collection, Manassas, Va.)), *Pseudomonas aeruginosa* Hcp1 protein, *Escherichia coli* heat labile enterotoxin, shiga-like toxin, human LTB protein, a protein extract from whole bacterial cells, and any other protein that can be cross-linked by a peptide linker. Desirably, the carrier protein is the cholera toxin B subunit (available from SBL Vaccin AB), diphtheria toxin (Connaught, Inc.), tetanus toxin Fragment C (available from Sigma Aldrich), DNI, or beta-galactosidase from *Escherichia coli* (available from Sigma Aldrich). Other desirable carrier proteins include bovine serum albumin (BSA),

P40, and chicken riboflavin. (Unless otherwise indicated, the exemplary carrier proteins are commercially available from Sigma Aldrich.) Other exemplary carrier proteins are MAPs (multi-antigenic peptides), which are branched peptides. By using a MAP, cross-linking density is maximized because of multiple branched amino acid residues. An exemplary amino acid that can be used to form a MAP is, but is not limited to, lysine. Carrier proteins also include those from non-human source such as keyhole limpet hemocyanin (KLH). Mutant forms of all the above proteins are also included, in particular, if the mutation ablates toxicity or removes epitopes that immunologically cross-react with human proteins. These can be engineered for production through standard genetic techniques by investigators skilled in the art of protein engineering.

Both BSA and keyhole limpet hemocyanin (KLH) have commonly been used as carriers in the development of immunogenic conjugates when experimenting with animals. Carrier proteins which have been used in the preparation of therapeutic immunogenic conjugates include, but are not limited to, a number of toxins of pathogenic bacteria and their toxoids. Examples include diphtheria and tetanus toxins and their medically acceptable corresponding toxoids. Other candidates are proteins antigenically similar to bacterial toxins referred to as cross-reacting materials (CRMs). Carrier proteins of the invention may also include any protein not derived from humans and not present in any human food substance.

In another embodiment, DNI is used as the carrier protein because it is nontoxic leaving no need to detoxify the protein before use. Furthermore, the use of DNI is desirable because DNI may also induce a protective immune response to *B. anthracis*, in addition to the protective immune response to the antigen of interest.

Polyol Antigens

The immunogenic conjugate of the invention and the related compositions and methods of the invention can be used with any polyol antigen (e.g., a polysaccharide antigen or a polyalcohol antigen). Non-limiting examples of polyol antigens include polysaccharides (e.g., polysaccharides having at least 18 residues, or e.g., dextrans, alginates, phosphopolysaccharides, polysaccharides with an amino sugar with an N-acetyl substitution, or polysaccharides containing a sulfonylated sugar, or an O side chain of a lipopolysaccharide) and polyalcohols (e.g., teichoic acids).

Exemplary polyol antigens of interest also include capsular organic polymers including those synthesized by microbes, e.g., bacteria, fungi, parasites, and viruses. Exemplary polyol antigens are those found on the exterior surface of bacterial organisms, such as *Bacillus* species (including *B. anthracis*) (Wang and Lucas, *Infect. Immun.* 72(9):5460-5463, 2004), *Streptococcus pneumoniae* (Bentley et al., *PLoS Genet.* 2(3):e31, Epub 2006; Kolkman et al., *J. Biochemistry* 123:937-945, 1998; and Kong et al., J. *Med. Microbiol.* 54:351-356, 2005), *Shigella* (Zhao et al., *Carbohydr. Res.* 342(9):1275-1279, Epub 2007), *Haemophilus influenzae*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Salmonella*, (including *Salmonella typhi*), *Streptococcus pyogenes*, *Escherichia coli* (Zhao et al., *Carbohydr. Res.* 342(9):1275-1279, Epub 2007), *Francisella tularensis*, and *Pseudomonas aeruginosa*, and fungal organisms such as *Cryptococcus* and *Candida*, as well as many other microorganisms (see,

e.g., Ovodov, *Biochemistry* (Mosc.) 71(9):937-954, 2006; Lee et al., *Adv. Exp. Med. Biol.* 491:453-471, 2001; and Lee, *Mol. Immunol.* 24(10):1005-1019, 1987).

The *Francisella tularensis* polysaccharide may be, e.g., the O antigen. The *Streptococcus pneumoniae* polysaccharide may be, e.g., one of capsular types described in Kong et al. (*J. Med. Microbiol.* 54:35-356, 2005). For example, *Streptococcus pneumoniae* polysaccharide capsular type may be, e.g., 1 (e.g., 1-g or 1-q), 2 (e.g., 2-g, 2-q, or 2-41 A), 3 (e.g., 3-g, 3-q, 3-c, or 3-nz), 4, 5 (e.g., 5-q, 5-c, 5-qap, or 5-g), 6A (e.g., 6A-g, 6A-cl, 6A-c2, 6A-n, 6A-qap, 6A-6B-g, 6A-6B-q, or 6A-6B-s), 6B (e.g., 6B-c, 6A-6B-g, 6A-6B- q, or 6A-6B-s), 7F (e.g., 7F-7A), 7A (e.g., 7A-cn or 7F-7A), 7B (e.g., 7B-40), 7C (e.g., 7C-19C-24B), 8 (e.g., 8-g or 8-s), 9A (e.g., 9A-9V), 9L, 9N, 9V (e.g., 9A-9V), 9V and 14, 1OF (e.g., 10F-q, lOF-ca, or lOF-lOC), 1OA (e.g., 10A-17A or 10A-23F), 1OB (e.g., lOB-lOC), HF, I IA (e.g., I IA-nz or 11A-11D-18F), HB (e.g., 1 IB-11C), HC (e.g., 1 IB-11C or HC-cn), HD (e.g., 11A-11D-18F), 12F (e.g., 12F-q or 12F-12A-12B), 12A (e.g., 12A-cn, 12A-46, or 12F-12A-12B), 12B (e.g., 12F-12A-12B), 13 (e.g., 13-20), 14 (e.g., 14-g, 14-q, 14-v, or 14-c), 15F (e.g., 15F-cnl or 15F-cn2), 15A (e.g., 15A-cal, 15A-ca2, or l5A-chw), 15B (e.g., 15B-C, 15B-15C, 15B-15C-22F-22A), 15C (e.g., 15C- ca, 15C-ql, 15C-q2, 15C-q3, 15C-S, 15B-15C, or 15B-15C-22F-22A), 16F (e.g., 16F-q or 16F-nz), 16A, 17F (e.g., 17F-n and 17F-35B-35C-42), 17A (e.g., 17A-ca or 10A-17A), 18F (e.g., 18F-ca, 18F-W, or 1 IA-I ID- 18F), 18A (e.g., 18A-nz or 18A-q), 18B (e.g., 18B-18C), 18C (e.g., 18B-18C), 19F (e.g., 19F-gl, 19F-g2, 19F-g3, 19F-q, 19F-n, or 19F-C), 19A (e.g., 19A-g, 19A-, or 19A-ca), 19B, 19C (e.g., 19C-cnl, 19C-cn2, or 7C- 19C-24B), 20 (e.g., 13-20), 21 (e.g., 21-ca or 21-cn), 22F (e.g., 15B-15C-22F-22A), 23F (e.g., 23F-C, 10A-23F, or 23F-23A), 23B (e.g., 23B-C or 23B-q), 24F (e.g., 24F-cnl, 24F- cn2, or 24F-cn3), 24A, 24B (e.g., 7C-19C-24B), 25F (e.g., 25F-38), 25A, 27, 28F (e.g., 28F-28A or 28F-cn), 28A (e.g., 28F-28A), 29 (e.g., 29-ca or 29-q), 31, 32F (e.g., 32F- 32A), 32A (e.g., 32A-cn or 32F-32A), 33F (e.g., 33F-g, 33F-q, 33F-chw, 33F-33B, or 33F-33A-35A), 33A (e.g., 33F-33A-35A), 33B (e.g., 33B-q, 33B-s, or 33F-33B), 33D, 34 (e.g., 34-ca or 34s), 35F (e.g., 35F-47F), 35A (e.g., 33F-33A-35A), 35B (e.g., 17F- 35B-35C-42), 36, 37 (e.g., 37-g or 37-ca), 38 (e.g., 25F-38), 39 (e.g., 39-cnl or 39-cn2), 40 (e.g., 7B-40), 41F (e.g., 41F-cn or 41F-s), 41A (e.g., 2-41A), 42 (e.g., 17B-35B-35C- 42), 43, 44, 45, 46 (e.g., 46-s or 12A-46), 47F (e.g., 35F-47F), 47 A, 48 (e.g., 48-cnl or 48-cn2), or GenBank Accession Number AF532714 or AF532715.

Alternatively, exemplary antigens of interest include killed whole cell encapsulated pathogens such as *Bacillus* species (including *B. anthracis*) (Wang and Lucas, *Infect. Immun.* 72(9):5460-5463, 2004), *Streptococcus pneumoniae* (Bentley et al., *PLoS Genet.* 2(3):e31, Epub 2006; Kolkman et al., *J. Biochemistry* 123:937-945, 1998; and Kong et al., J. *Med. Microbiol.* 54:351-356, 2005), *Shigella* (Zhao et al., *Carbohydr. Res.* 342(9):1275-1279, Epub 2007), *Haemophilus influenzae*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Salmonella*, (including *Salmonella typhi*), *Streptococcus pyogenes*, *Escherichia coli* (Zhao et al., *Carbohydr. Res.* 342(9):1275-1279, Epub 2007), *Francisella tularensis*, and *Pseudomonas aeruginosa*, and fungal organisms such as *Cryptococcus* and *Candida*, as well as many other microorganisms (see, e.g., Ovodov, *Biochemistry* (Mosc.) 71(9):937-954, 2006; Lee et al., *Adv. Exp. Med. Biol.* 491:453-471, 2001; and Lee, *Mol. Immunol.* 24(10):1005-1019, 1987). Whole cell pathogens may contain, but are not limited to, one or more of the aforementioned antigens of interest, e.g., microbial capsular organic polymers, O-antigen, and PGA.

Covalently Linked Immunogenic Conjugates

The immunogenic whole cell-protein conjugate of the invention can include a carrier protein and a whole cell having an exterior surface including a polyol antigen that is covalently linked to the carrier protein through a linker. The linker can be formed by a reaction between a cyanate group bonded to the polyol antigen and a hydrazine-activated carrier protein. The linker can be represented by a formula:

$$Z^1–C(Z^3)–N(H)–N(H)–(–C(O)–(L–C(O))_n–N(H)–N(H)–)_m–Z^2,$$

where Z^1 is a bond to an oxygen atom in the polyol antigen,

Z^2 is a bond to a carbonyl group in the carrier protein,

Z^3 is O or NH,

each of n and m is 0 or 1, and

L, when present, is C_{2-6} alkanediyl or C_{6-10} arenediyl.

The conjugation of a polyol antigen to a carrier protein may be performed using a cyanylation-mediated conjugation method. Specifically, contacting an antigen-cyanate with a hydrazine-activated carrier protein can produce a conjugate, where the antigen is linked to the carrier protein through the linker $Z^1–C(Z^3)–N(H)–N(H)–(–C(O)–(L–C(O))_n–N(H)–N(H)–)_m–Z^2$. The antigen-cyanate can be prepared through the cyanylation of the antigen with an electrophilic source of cyanide (e.g., cyanogen bromide or CDAP). The hydrazine-activated carrier protein can be prepared by reacting the carrier protein with $H_2N–N(H)–(–C(O)–(L–C(O))_n–N(H)–N(H)–)_m–H$ under the reaction conditions known in the art for hydrazide formation. For a general description of the cyanylation-mediated conjugation reactions, see *Bioconjugate Techniques*, 3rd ed.; eds.: Hermanson; Academic Press, London, UK, incorporated herein by reference. For example, one non-limiting cyanylation-mediated conjugation protocol includes dissolution of a hydrazine source agent (e.g., dicarboxylic acid dihydrazide, such as succinic acid dihydrazide or adipic acid dihydrazide) in about neutral pH phosphate buffer (e.g., pH of 7.2) containing sodium chloride (e.g., about 0.15M sodium chloride), dissolving a carrier protein in this solution, adding a peptide coupling agent (e.g., 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)), and allowing the resulting reaction mixture to react for a sufficient time period (e.g., about 2 to 4 hours). The resulting modified carrier protein can be purified, e.g., by dialysis or gel filtration using a desalting resin.

Advantageously, preparation of a whole cell-carrier protein conjugate can facilitate the preparation of polyol antigen-carrier protein conjugates, as, after the conjugation, the whole cell-carrier protein conjugate can be subjected to lysing conditions known in the art, and the resulting polyol antigen-carrier protein conjugate can be purified away from the unconjugated cellular components using, e.g., affinity purification using solid support having affinity for the carrier protein (e.g., if the carrier protein includes one or more affinity purification tags, or if the solid support includes antibodies specific to the carrier protein). A non-limiting example of the preparation of polyol antigen-carrier protein conjugates is illustrated in FIG. 27. Whole cells may be killed by a chemical, e.g., formaldehyde, treatment or by heat-inactivation and subsequently conjugated as described herein.

Furthermore, multiple polyol antigens can be coupled to a carrier protein, or a mixture of carrier proteins can be conjugated to the polyol antigen; both of these options may be achieved in a single

reaction or multiple sequential reactions. Thus, the method described herein permits multiplexing of the immunogenic conjugate, further reducing the cost of production.

The methods of preparing immunogenic compositions described herein may be used with any polyol antigen having an alcohol hydroxyl group capable of being cyanylated with an electrophilic source of cyanide (e.g., CDAP or cyanogen bromide). Tetanus toxoid is one possible carrier protein. This toxin is detoxified by treatment with formaldehyde, a reagent that reacts with amino groups of proteins. Other desirable carrier proteins include the cholera toxin B subunit (available from SBL Vaccin AB), diphtheria toxin, tetanus toxin Fragment C (available from Sigma Aldrich), DNI, or beta-galactosidase from *Escherichia coli* (available from Sigma Aldrich).

The immunogenic conjugates of the invention may be used to immunize against, for example, *Pneumococcus* infection, *Streptococcus* (groups A and B) infection, *Haemophilus influenzae* type B ("HiB") infection, meningococcal (e.g., *Neisseria meningitides*) infection, and may be used as O antigen immunogenic conjugates from Gram negative bacteria (e.g., *Pseudomonas aeruginosa*, *Francisella tularensis* (Thirumalapura et al., *J. Med. Microbiol.* 54:693-695, 2005; Vinogradov and Perry, *Carbohydr. Res.* 339:1643-1648, 2004; Vinogradov et al., *Carbohydr. Res.* 214:289-297, 1991), *Shigella* species, *Salmonella* species, *Acinetobacter* species, *Burkholderia* species, and *Escherichia coli*).

Other covalent linkers may be used in the whole cell-carrier protein conjugates of the invention. Provided below are further examples of covalent linkers that may be used in the whole cell-carrier protein conjugates of the invention described herein.

The linker can be formed by a reaction between an aldehyde group (-CHO) and an amino group ($-NH_2$) in a carrier protein or a hydrazine-activated carrier protein. The linker can be represented by a formula:

$$Z^4–CH=N–[–N(H)–(–C(O)–(L–C(O))_n–N(H)–N(H)–)_m–]_k–Z^5,$$

where Z^4 is a bond to a polyol antigen,

Z^5 is a bond the carrier protein,

each of n, m, and k is independently 0 or 1, and

L, when present, is C_{2-6} alkanediyl or C_{6-10} arenediyl.

The aldehyde group can be introduced into the polyol antigen having a *vicinal* diol moiety (e.g., as found in a polysaccharide antigen) using reactions and reaction conditions known in the art. A non-limiting example of a reaction whereby an aldehyde group is introduced into a polyol antigen includes a diol cleavage using sodium periodate.

The linker can be formed through a thioether formation. The linker can be represented by a formula:

$$Z^1–C(O)–N(H)–(N(H))_k–L–N(H)–C(O)–CH_2–S–L^1–C(O)–Z^2,$$

where Z^1 is a bond to an oxygen atom in the polyol antigen,

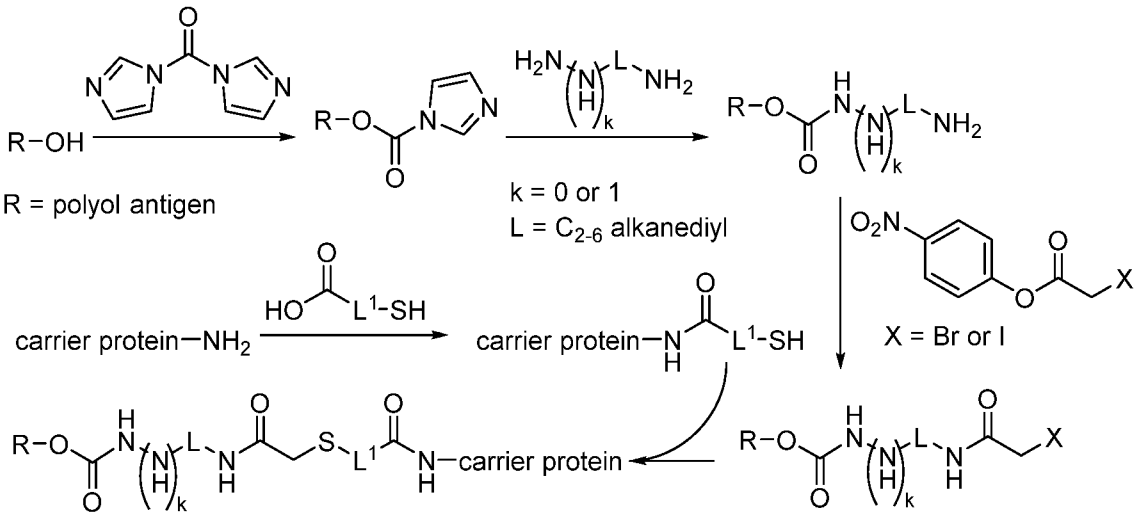
Z^2 is a bond to an amino group (e.g., N-terminus or a side chain amino) in the carrier protein,

k is 0 or 1,

L is C_{2-6} alkanediyl, and

L^1 is C_{2-6} alkanediyl optionally substituted with a protected amino group.

A non-limiting example of the thioether formation-based linking of a polyol antigen to a carrier protein is shown in the following scheme.



L^1 = C_{2-6} alkanediyl optionally substituted with a protected amino group

The linker can be formed through a reaction sequence including reductive amination and amidation. The linker can be represented by a formula:

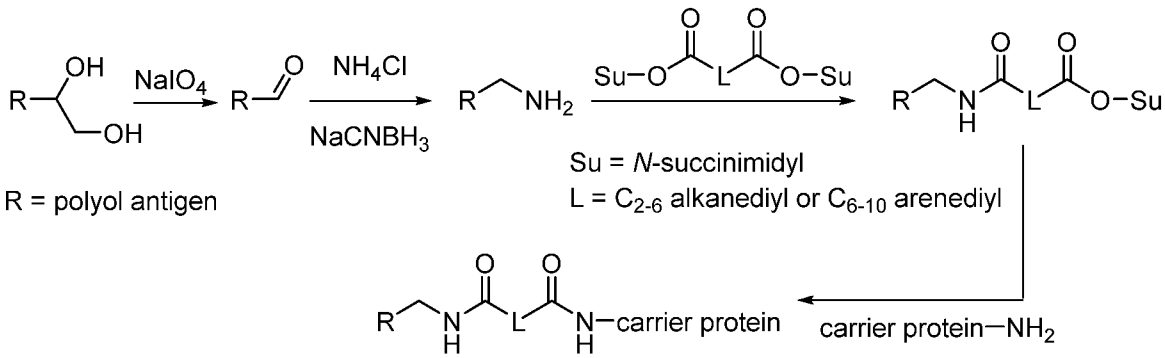
$$Z^4–N(H)–C(O)–L–C(O)–N(H)–Z^5,$$

where Z^4 is a bond to a polyol antigen,

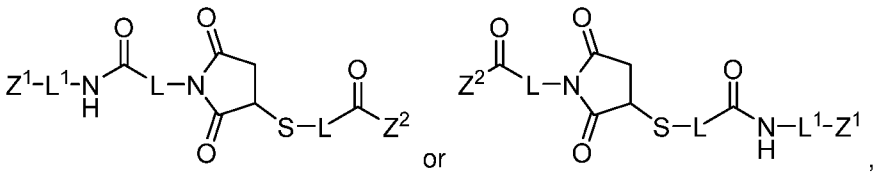
Z^5 is a bond the carrier protein,

L is C_{2-6} alkanediyl or C_{6-10} arenediyl.

A non-limiting example of the reaction sequence including reductive amination and amidation is shown in the following scheme.



The linker can be formed through a thiol-maleimide conjugation. The linker can be represented by a formula:



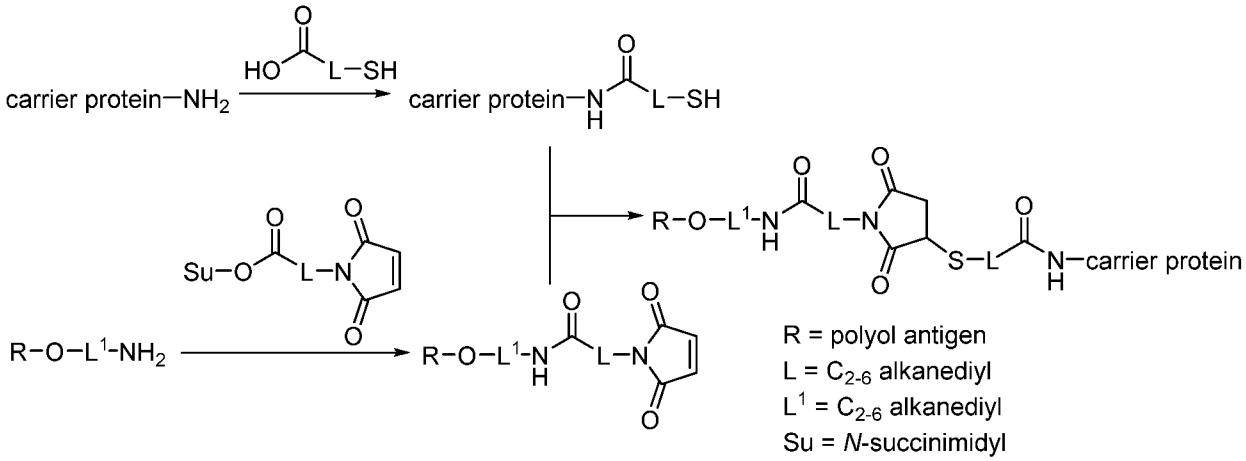
where Z^1 is a bond to an oxygen atom in the polyol antigen,

Z^2 is a bond to an amino group (e.g., N-terminus or a side chain amino) in the carrier protein,

each L is independently C_{2-6} alkanediyl, and

L^1 is C_{2-6} alkanediyl.

A non-limiting example of the thiol-maleimide conjugation is shown in the following scheme.

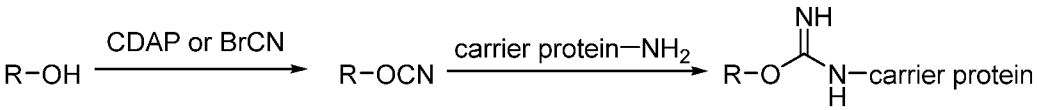


The linker can be formed through a reaction between a cyanylated polyol antigen and a carrier protein. The linker can be represented by a formula:

$$Z^1–C(O)–N(H)–Z^5,$$

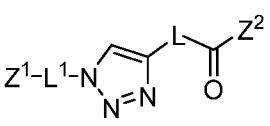
where Z^1 is a bond to an oxygen atom in the polyol antigen, and Z^2 is a bond to a carrier protein.

A non-limiting example of a reaction between a cyanylated polyol antigen and a carrier protein is shown in the following scheme.



R = polyol antigen

The linker can be formed using click chemistry (e.g., [3+2] dipolar cycloaddition). The linker can be represented by a formula:

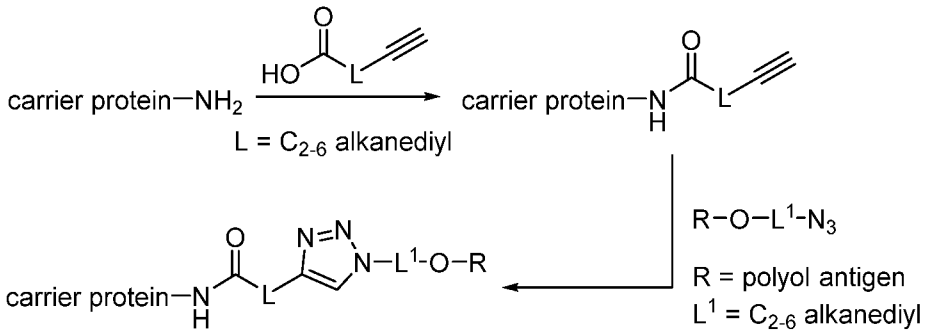


where Z^1 is a bond to an oxygen atom in the polyol antigen,

Z^2 is a bond to an amino group (e.g., N-terminus or a side chain amino) in the carrier protein, and

each of L and L^1 is independently C_{2-6} alkanediyl.

A non-limiting example of [3+2] dipolar cycloaddition-based conjugation is shown in the following scheme.



Methods for [3+2] dipolar cycloaddition are known in the art. Typically, the cycloaddition is performed in the presence of a catalytic amount of a copper salt (e.g., a Cu(I) salt).

Immunogenic Conjugates Linked Through an Affinity Pair

The immunogenic whole cell-protein conjugate of the invention can include a carrier protein and a whole cell having an exterior surface including a polyol antigen that is linked to the carrier protein through a linker containing a complementary affinity pair. The linker can be represented by a formula:

$$Z^1–C(Z^3)–L^1–L–L^2–AP–Z^2,$$

where Z^1 is a bond to an oxygen atom in the polyol antigen,

Z^2 is a bond to a carrier protein,

Z^3 is O or NH,

L is C_{2-10} alkanediyl or $–[–CH_2–CH_2–(–O–CH_2–CH_2–)_n]–$, where n is an integer from 0 to 24,

L^1 is –N(H)– or –N(H)–N(H)–,

L^2 is –N(H)–, –C(O)–, –C(O)–N(H)–, or –N(H)–C(O)–,

AP is a complementary affinity pair.

The complementary affinity pair may be a biotin/streptavidin, biotin/avidin, biotin/neutravidin, maltose binding protein/maltose, or maltose binding protein/maltotriose pair. One component of a complementary affinity pair may be covalently linked to the polyol antigen, and the complementary component from the said pair may be covalently linked to the carrier protein. The affinity pair-based linker can be formed by contacting such polyol antigen and carrier protein.

When biotin/streptavidin, biotin/avidin, or biotin/neutravidin pair is used, biotin may be covalently linked to the polyol antigen, and streptavidin, avidin, or neutravidin may be covalently linked to the carrier protein. A carrier protein linked to streptavidin, avidin, or neutravidin may be a fusion protein. Such fusion proteins may be prepared using techniques known in the art (e.g., recombinant fusion protein preparation).

When maltose binding protein/maltose or maltose binding protein/maltotriose pair is used, maltose or maltotriose may be covalently linked to the polyol antigen, and maltose binding protein may be covalently linked to the carrier protein. A carrier protein linked to maltose binding protein may be a fusion

protein. Such fusion proteins may be prepared using techniques known in the art (e.g., recombinant fusion protein preparation).

Methods of Preparing Polyol Antigen-Carrier Protein Conjugates

Polyol antigen-protein conjugates are difficult to produce cost-effectively because of the specialized chemistry required for their preparation and purification. Polyol antigen-protein conjugation by covalent linkage procedures have numerous drawbacks, including the need for either synthetic preparation of polyol antigens or for isolation of polyol antigens from cell lysates. Both approaches dramatically increase cost of manufacture of polyol antigen-protein conjugates. Thus, there is a need for a simplified, reproducible, cost-effective mechanism of producing polyol antigen-protein conjugates. This need is addressed by the methods of the invention. Specifically, the polyol antigen-protein conjugate can be prepared from a whole cell-protein conjugate through lysing the whole cells in the whole cell-protein conjugate according to methods known in the art and purifying the resulting polyol antigen-protein conjugate away from the unconjugated cellular components. The purification may include affinity purification, e.g., using metal ions immobilized on solid support, if the protein contains one or more poly-His tags, or using immunoprecipitation techniques (e.g., techniques utilizing a solid support having immobilized antibodies capable of binding to the carrier protein). Advantageously, this method of the invention takes advantage of the carrier protein structure to separate out the polyol antigen, which, typically otherwise, would require either a costly chemical preparation or a costly, complex purification using techniques known in the art.

Pharmaceutical Compositions and Methods of Use of the Immunogenic Conjugates

The immunogenic conjugates of the invention may be used in combination, for example, in pediatric immunizations. In addition, the immunogenic conjugates of the invention may be used to immunize against, for example, *Pneumococcus* infection, *Haemophilus influenzae* type B ("HiB") infection, *Streptococcus* (groups A and B) infection, meningococcal (e.g., *Neisseria meningitides*) infection, and may be used as O antigen immunogenic conjugates from Gram negative bacteria (e.g., *Pseudomonas aeruginosa*, *Francisella tularensis*, *Shigella* species, *Salmonella* species, *Acinetobacter* species, *Burkholderia* species, and *Escherichia coli*).

The immunogenic conjugate formulation desirably includes at least one immunogenic conjugate of the invention and a pharmaceutically acceptable carrier or excipient (e.g., aluminum phosphate, sodium chloride, or sterile water). An immunogenic conjugate composition may also include an adjuvant system for enhancing the immunogenicity of the formulation, such as oil in a water system and other systems known in the art or other pharmaceutically acceptable excipients. An immunogenic conjugate that is insoluble under physiological conditions is desirable to slowly release the antigen after administration to a subject. Such a complex desirably is delivered in a suspension containing pharmaceutically acceptable excipients. Alternatively, the immunogenic conjugate of the invention may also be soluble under physiological conditions.

Typically, the immunogenic conjugate may be provided in a volume of about 0.5 mL for subcutaneous injection, 0.1 mL for intradermal injection, or 0.002-0.02 mL for percutaneous administration. A 0.5 mL dose of the immunogenic conjugate may contain approximately 2-500 µg of the antigen covalently linked with approximately 2-500 µg of the carrier protein. In a desirable embodiment, in a 0.5 mL dose, approximately 10 µg of the antigen are conjugated with approximately 10 µg of the carrier protein. The molar ratio of a polyol antigen to carrier protein desirably is 1:1 (e.g., 1 part polyol antigen to 1 part carrier protein).

For whole cell immunogenic conjugates formulated for enteral administration, the formulation includes at least one immunogenic conjugate and a pharmaceutically acceptable buffer (e.g., bicarbonate buffer) as a pharmaceutically acceptable carrier for neutralization of gastric acid. In this formulation, the immunogenic conjugate preparation is mixed with a buffer, e.g., 5.6 g of sodium hydrogen carbonate granules dissolved in 150 mL of sterile water.

Typically the immunogenic conjugate contains about 1 mg of whole cell pathogen immunogenic conjugate in a single dose for oral administration. A 1 mg dose of the immunogenic conjugate may contain at least 1 x 10^9 whole cell pathogens or a range of 1 x 10^9 to 1 x 10^{11} whole cell pathogens covalently linked with approximately 2-500 µg of the carrier protein.

In particular, the immunogenic conjugates of the invention may be administered to a subject enterally (for instance, by oral administration) by ingestion of an immunogenic conjugate in the form of a e.g., liquid, powder, capsule, or tablet. Immunogenic conjugates of the invention may be administered, one or more times, often including a second administration designed to boost production of antibodies in a subject to prevent infection by an infectious agent. The frequency and quantity of immunogenic conjugate dosage depends on the specific activity of the immunogenic conjugate and can be readily determined by routine experimentation. The age at which the first dosage is administered generally is two-years. The immunogenic conjugates of the invention may be administered according to the following exemplary schedule.

Age of first dosage	**Dosage schedule**
2 – 6 years of age	Three oral doses at least one week apart.
6 years of age and older	Two oral doses at least one week apart.

A booster dose is desirably given as early as six months following the last dose in subjects who are at a high risk for infection; or five years after the last dose to previously immunized adults and children above two years of age.

If there is a concern that the peptides or conjugates may be degraded in the stomach, the immunogenic conjugate may be desirably administered parenterally (e.g., by subcutaneous, intramuscular, intravenous, or intradermal injection). While delivery by a means that physically penetrates the dermal layer is desirable (e.g., a needle, airgun, or abrasion), the immunogenic conjugates

of the invention can also be administered by transdermal or transmucosal absorption.

In particular, the immunogenic conjugates of the invention may be administered to a subject, e.g., by intramuscular injection, intradermal injection, or transcutaneous immunization with appropriate immune adjuvants. Immunogenic conjugates of the invention may be administered, one or more times, often including a second administration designed to boost production of antibodies in a subject to prevent infection by an infectious agent. The frequency and quantity of immunogenic conjugate dosage depends on the specific activity of the immunogenic conjugate and can be readily determined by routine experimentation. While the age at which the first dosage is administered generally is two-months, an immunogenic conjugate may be administered to infants as young as six weeks of age. For children who are beyond the age of a routine infant vaccination schedule, the immunogenic conjugates of the invention may be administered according to the following exemplary schedule.

Age of first dosage	**Dosage schedule**
6 weeks-5 years of age	Total of four 0.5 ml doses; the first three at least eight weeks apart and the fourth at least six months after the third dose.
6-17 years of age	One 0.5 ml dose.
50 years of age and older	One 0.5 ml dose.

A booster dose is desirably given as early as four years following the last dose in subjects who are at a high risk for infection; or 10 years after the last dose to previously immunized adults and children above fifteen years of age.

The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier immediately prior to use. Immunogenic conjugates of the invention can be formulated in pharmacologically acceptable vehicles, e.g., alum hydroxide gel, adjuvant preparation, or saline, and then administered, e.g., by intramuscular injection, intradermal injection, or transcutaneous immunization with appropriate immune adjuvants.

The invention also provides kits that include an immunogenic conjugate described herein. The kits of the invention can also include instructions for using the kits in the immunization methods described herein.

The efficacy of the immunization schedule may be determined by using standard methods for measuring the antibody titer in the subject. In general, mean antibody titers (desirably IgG titers) of approximately 1 µg/ml are considered indicative of long-term protection.

The whole cell-carrier protein conjugates are desirably between 100 nm and 100 µm in diameter. Viruses can be 100 nm in diameter and are immunogenic. Whole bacteria are 1-10 µm in diameter and are also immunogenic. A small clump of bacteria can be about 100 µm in diameter.

The invention is described herein below by reference to specific examples, embodiments and figures, the purpose of which is to illustrate the invention rather than to limit its scope. The following examples are not to be construed as limiting.

EXAMPLES

Example 1: Preparation of Whole Cell-Carrier Protein (CRM197) Conjugates through Cyanylation-mediated Conjugation

Commercially available CRM197 was hydrazine-activated using adipic acid dihydrazide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling agent. The resulting hydrazine-activated CRM197 was purified by size exclusion chromatography. Then, formalin-killed pneumococcal PS4 cells (PPS4) were treated with CDAP to form pheumococcal PS4 cells having a surface with polysaccharide antigens modified to contain cyanate groups. The CDAP-treated pneumococcal PS4 cells were then treated with the hydrazine-activated CRM197 to produce a whole cell PPS4-CRM197 conjugate. The formation of the whole cell PPS4-CRM197 conjugate was confirmed by flow cytometry (see FIGS. 2A and 2B) and electron microscopy using gold nanoparticles against CRM197 (see FIGS. 3A, 3B, 3C, and 3D).

The whole cell-carrier protein conjugate preparation through cyanylation-mediated conjugation was also assessed for scalability. As is illustrated in the below table, scaling up the reaction 10-fold did not reduce the recovery of whole cells, as measured by the optical density.

	Added	**Recovered**	**Percent recovered**	**Protein added**	**Protein/ OD**
0.1 OD cells	194.0	54.8	28.3%	139.2	1392.0
1.0 OD cells	1940.4	621.6	32.0%	1318.8	1318.8

Example 2: Immunogenicity of PPS4-CRM197 Whole Cell Conjugates

BALB/c mice were immunized by intraperitoneal (I.P.) injections twice with a 3-week rest period with 200µL of PPS4-CRM197 prepared in Example 1, PREVNAR 13® vaccine, or phosphate buffered saline (control). Anti-PPS4 IgG titers were then obtained for these mice (see FIG. 4). As illustrated in FIG. 4, despite lacking adjuvant, PPS4-CRM197 produced anti-PPS4 IgG titers that were comparable to those observed for PREVNAR 13®, which includes an adjuvant. FIG. 4 further shows that anti-PPS4 IgG titers for PPS4-CRM197 were significantly more robust than those observed for unconjugated fixed whole cells.

Example 3: Combination of Immunogenic Conjugates with Adjuvants

The PS-carrier protein immunogenic conjugate can be modified to further stimulate the immune response, and ultimately improve the efficacy of the immunization, by addition of an adjuvant. The immunogenic conjugate can be absorbed by an alum adjuvant such as aluminum hydroxide gel. Additionally, the immunogenic can be combined with an emulsion adjuvant such as squalene based oil in

water nano emulsion. Adjuvants such as these can be used to create a delivery system for the immunogenic conjugate and function to create depots that trap the conjugated antigen-carrier protein at the site of injection to allow for its slow release. This allows for extended stimulation of the immune system by enabling the immunogenic conjugate to persist at the site of injection, increasing the recruitment and activation of immune cells.

Example 4: Preparation of Whole Cell-Carrier Protein (CRM197 or Pneumolysin) Conjugates through Cyanylation-mediated Conjugation

Growth and Fixation of PPS4 cells

PPS4 bacteria were grown on Todd Hewitt with yeast media (THY: 37g/L Todd Hewitt Broth; Fluka Analytical 101565165, 5g/L Bacto™ Yeast Extract; BD Biosciences 212750). Bacteria were first grown on agar plates for 48h @37°C in a 5% CO_2 chamber, then colonies were transferred to liquid media for 18h, to mid log phase (OD_{600}~0.2). Bacteria were subsequently pelleted, washed with PBS, and resuspended in 1% Formaldehyde (PBS). Fixation was performed overnight with rocking at 4°C. Fixed bacteria are then washed 2x with PBS, and resuspended in sterile PBS and stored at 4°C.

Activation of Carrier Proteins with Hydrazine Moiety

In order to improve the efficiency of conjugation between carrier proteins and cyano-activated carbohydrates, the proteins carboxylic acids are modified with adipic acid dihydrazide to produce a terminal hydrazide group. Briefly, the carrier protein was resuspended (5mg/mL) in 0.1M sodium phosphate, 0.15M NaCl, pH 7.2. Fresh adipic acid dihydrazide (40mg/mL) was added, and vortexed thoroughly. 20 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added and vortexed till dissolved. The protein was incubated for 3h at room temperate. The protein was then purified by buffer exchange chromatography, utilizing a PD-10 desalting column (GE healthcare), and resuspended in reaction buffer; 0.1M $Na_2B_4O_7 \cdot 10H_2O$ (Sodium Borate), 4mM $C_{24}H_{39}NaO_4$ (Sodium deoxycholate) pH 9.0.

Purification of Pneumolysin (Ply*)

A catalytically dead mutant form of pneumolysin was cloned with a 6-His C terminal epitope into pET100_D-TOPO, and expressed in *E. coli*. *E. coli* cells were grown to midlog phase, induced with 1mM IPTG, and grown for 3 hrs. Cell pellet was resuspended in 50 mL of binding buffer (300mM NaCl, 20mM Tris, 20mM Imidazole, pH 7.8), and cells lysed by cell disruptor (Pressure Biosciences) at 25 kPa. Cell debris was removed by centrifugation, and the supernatant was loaded onto a Ni^{2+}-NTA 5mL column. The column was washed with wash buffer (300mM NaCl, 20mM Tris, 50mM Imidazole) and eluted with elution buffer (300mM NaCl, 20mM Tris, 500mM Imidazole, pH 7.8). Buffer exchange was utilized to store Ply in 0.1M sodium phosphate, 0.15M NaCl, pH 7.2

Synthesis of CDAP Conjugated Purified PPS4 Capsule

The protocol was based on Battaglinia and Pallarola (2011). Briefly, 0.5 mL of 2 mg/mL purified PPS4 capsule was resuspended in 0.1M sodium borate pH 9.0, 20 µL of CDAP (100mg/mL in acetonitrile) was added and vortexed for 30s, followed by 20 µL 0.2M triethylamine, and incubated for 150

s. 500 µL of hydrazine-activated peptide was incubated with the CDAP activated capsule, and incubated for 2.5 hrs at room temperature with rocking. The reaction is quenched by addition of 50 µL 1M ethanolamine. The conjugate was separated from the non-conjugated peptide by size exclusion chromatography, and stored in PBS. The CDAP-activated, purified PPS4 capsule was analyzed using Western blot (FIG. 5A).

Synthesis of Whole cell conjugates (Wcc)

Formalin fixed cells were washed with reaction buffer and resuspended at a concentration of 0.1 OD cells/ 0.3mL buffer. CDAP (100mg/mL in acetonitrile) was added at a ratio of 100 mL/ OD cells for 2.5 mins with shaking at room temperature, followed by addition of an equal volume of 0.2M triethylamine. The reaction was incubated with shaking for 8 mins, and the cells were pelleted by centrifugation, washed with reaction buffer, and resuspended at a ratio of 0.2 OD/mL reaction buffer. Hydrazine activated peptides or proteins were added to the CDAP activated cells (2mg/mL) at a ratio of 1 mL/OD bacteria, and incubated for 3h at room temperature with rocking. The excess remaining activated polysaccharides are quenched by adding 200 µL 1M ethanolamine, 1h at room temperature. Cells were then pelleted and washed for 30 mins with 0.1% Saponin to remove any unconjugated protein remaining. Cells were pelleted, resuspended in 1% Kolliphor (PBS), and stored at 4°C. The CDAP-activated whole cells were tested to confirm that conjugation chemistry is feasible on the cell surface; the cells were treated with hydrazine-SGSGSLPETGGYPYDVPDY (SEQ ID NO: 1) and analyzed by Western blot (FIG. 5B). FIG. 5B (lane 4) shows that elevated levels of the peptide are present in the CDAP-activated cells, thereby suggesting an efficient conjugation between the cell and peptide. For reference, FIG. 5A shows Western blot analysis for conjugation of hydrazine-SGSGSLPETGGYPYDVPDY (SEQ ID NO: 1) to a CDAP-activated, purified PPS4 capsule.

In order to visualize the conjugation between cells and hydrazine-SGSGSLPETGGYPYDVPDY (SEQ ID NO: 1), we employed immunogold electron microscopy directed towards the hydrazine-activated peptide (FIGs. 6A-6D). Similar to the results observed in FIG. 5B, little to no reactivity was observed for unmodified (FIG. 6A) or CDAP activated cells (FIG. 6B), while some reactivity was observed when the peptide was incubated in the absence of CDAP (FIG. 6C). However, CDAP activated cells were found to be coated with immunogold on their surface (FIG. 6D), revealing efficient conjugation. In combination with observations by Western blot, it can be concluded that CDAP can be utilized on formalin fixed cells, and hydrazine activated peptides can be conjugated in an efficient manner.

Further, cellular stability of CDAP treated cells was assessed by the analysis for the presence of DNA using fluorescent microscopy (FIG. 7). No significant differences were observed for number of cells with DNA in any of the conditions tested, thereby indicating that CDAP treatment does not cause formalin fixed cells to lyse.

Next, conjugation of full size carrier protein to a CDAP-activated whole cell was evaluated using Western blot. CDAP treated PPS4 cells lost specific bands when assayed with a whole cell antibody (FIG. 8A), some unspecific binding of the hydrazine activated protein was observed attached to the cell

surface (FIG. 8B, Lane 3), and attachment of more protein onto CDAP activated cells was observed (FIG, 8B, Lane 4). This observation confirms that CDAP can mediate conjugation of activated proteins onto fixed bacterial cells.

To determine the frequency of CDAP mediated conjugation of CRM197 to PPS4 cells occurs, fluorescence-assisted cells sorting (FACS) was employed (FIGs. 14A-14F). Bacterial cells were gated initially to eliminate debris, followed by a second gating selection for single cell bacteria only (FIGs. 14A-14F). As shown in FIGs. 14C, incubation of CDAP activated cells with hydrazine activated CRM resulted in a significant shift in the fluorescence of the population. To quantify the shift of fluorescence in the population of fixed cells, the mean fluorescent intensity (MFI) was calculated for different cellular treatments (FIG. 14F, n=2). Consistent with the previous experiments with a hydrazine modified peptide, very little signal was observed from unmodified and CDAP activated cells towards the protein, and some unspecific attachment of the activated protein to cells was observed. In contrast, when activated cells and CRM were incubated, a significantly stronger MFI is observed, suggesting hydrazine-activated proteins can be specifically conjugated to formalin fixed cells.

To confirm CRM197 is conjugated in a similar manner as hydrazine-SGSGSLPETGGYPYDVPDY (SEQ ID NO: 1), we employed immunogold electron microscopy directed towards the protein (FIGs. 10A-10D). Consistent with the electron microscopy previously shown, immunogold deposition was only observed in significant quantities when both CDAP activated cells and hydrazine activated CRM197 are incubated together (FIG. 10C). Together, these results confirm that CDAP activated cells can be conjugated with hydrazine activated proteins in a specific manner.

To quantify the amount of CRM197 conjugated to PPS4 cells, an ELISA based detection method was developed, and the reaction was optimized for pH, detergent, CDAP added, CRM197 added, buffer volumes, and conjugation volume (FIGs. 15A and 15B). The reaction was then scaled up with the optimized parameters to produce sufficient quantities required for animal vaccinations (FIGs. 11A and 11B). Consistent with previous results, very little CRM197 was detected on unmodified or CDAP activated PPS4 cells, and a small amount of protein was detected when added alone. For both the CDAP and CRM modified cells, a virtually identical quantity of protein is observed for the same unit of bacterial cells in small scale and the 10x scaled up samples.

Using the same parameters described herein for CRM197, PPS4 cells were conjugated in a CDAP dependent manner with Ply* (FIGs. 12A and 12B).

Western blot Analysis of Wcc Conjugation

Treated PPS4 cells were denatured at 95°C with Bolt sample buffer, separated on an 4-12% SDS-PAGE gel, and transferred to a nitrocellulose membrane. The membrane was blocked for 1 hr in Odyssey blocking buffer (Licor), followed by 1 hr incubation in primary solution; 50% blocking buffer, 50% PBST, anti-PPS4 (SSI; 16747) and anti-HA (Cell Signaling Technology; 2367S), both at a dilution of

1/1000. Membranes were washed 3 times with PBST for 5 mins, and incubated in the dark with secondary solution (50% blocking buffer, 50% PBST, IRDye 680 LT Goat anti-mouse and IRDye 800CW goat anti-rabbit both at a dilution of 1/15,000) for 1 hr at room temperature with rocking. The membrane was washed 2x with PBST, one with PBS, and imaged with an Odyssey CLx imaging system.

Visualization of Wcc's by Fluorescent Microscopy

Treated cells were blocked for 1 hr at room temperature with shaking in PBS+ 2mg/mL BSA. The cells were subsequently incubated with anti-HA antibody (1/1,000 in 100 µL PBST) for 1 hr, washed 3x with PBST, and incubated for 1 hr with Alexa Flor 647 goat anti-mouse IgG (1/1,000; A21235). Stained cells were spotted on a 1% agar pad containing 3 µM Syto 9, and visualized with a Nikon Eclipse Ti fluorescent microscope.

Characterization of Wcc by Fluorescence Assisted Cell Sorting (FACS) and Immunogold Electron Microscopy

Quantification of the Wcc mean fluorescence intensity (MFI) was done by FACS analysis. Whole cell conjugates were blocked in solution with PBS +2mg/mL BSA for 1h at room temperature with shaking. Blocked cells were pelleted, and resuspended in 100µL of primary antibody (anti-CRM antibody, 1/1,000 dilution; Abcam 53828) in PBST +2mg/mL BSA for 1h with shaking. Cells were then washed 3x 5 mins with PBST, and incubated in 100µL secondary antibody (AlexaFlor 488 goat-anti rabbit, 1/1000 dilution, Life Technologies) in PBST +2mg/mL BSA for 1h with shaking in the dark. Cells were washed 3x 5 mins with PBST, resuspended in 1 mL PBST, and analyzed on a BD FACSCanto flow cytometry system.

For Electron microscopy imagining, the same workup was followed, with the exception that the secondary antibody employed was 15nm pAg (1/100 dilution), and visualized with a Tecnai G^2 Spirit BioTWIN electron microscope.

ELISA Based Quantification of CFU Quantification and Carrier Protein Attached to Cell Surface

Treated cells were diluted to a concentration of 0.1 OD/mL in 0.1M Sodium bicarbonate pH 9.0, as well as a protein standard were serially diluted by half in 96 well plates, 100 µL of each well was transferred to Nunc-Immuno™ Microwell™ 96 well plates, and incubated overnight at 4°C to facilitate binding to the surface. The wells were washed with MQH_20, incubated with 100 µL of 10µM Syto 9 for 30 mins in the dark with rocking, washed 3x with MQH_20, with a final addition of 100 µL of MQH_20. Biotek Synergy plate reader was utilized to detect Syto 9 signal (excitation 480nm, emission 500nm). Fixed cells in samples was determined via comparison of calculated line of best fit against standardized dilutions of fixed cells.

The plate was blocked with 125 µL PBS+2mg/mL BSA for 2 hrs, incubated with primary antibody (anti-CRM; ab53828 or anti-Ply; ab71810) 1/5,000 and 1/4,000 respectively for 2 hrs. Next, washed 3x 5 min with PBST, then secondary antibody incubation for 2 hrs (1/4,000, goat anti-rabbit IgG-AP Goat anti-mouse IgG-AP; Southern Biotechnologies), washed 3x 5 mins PBST, and visualized by development of

colour of PnPP substrate. Protein conjugated to cells was determined via comparison of calculated lines of best fit between protein standard and test group.

Example 5: Immunogenicity of Whole Cell-Carrier Protein Conjugates in Vivo

Eight week old female BALB/c mice were purchased from Jackson Labs (Location). All mice were housed in sterile cages and were provided one week for acclimatization prior to the beginning of experiments.

Groups of eight mice were immunized by intraperitoneal (I.P.) injections twice with a 3-week rest period with 200µL of (1) the vaccine prepared in Example 4 (Wcc-CRM197 or Wcc-Ply) with 0.05% Aluminum phosphate gel adjuvant (Adju-Phos®), or (2) Prevnar (which contains aluminum phosphate adjuvant). The components of each dose are summarized in Table 1.

Table 1

	PPS4 Cells	PPS4 Capsule (µg)	CRM (µg)	Ply (µg)
Alum Only	(-)	(-)	(-)	(-)
Fixed Cells	$2x10^7$	10.2 ±0.02	(-)	(-)
Wcc-CRM	$2x10^7$	10.2 ±0.02	3.7 ±0.8	(-)
Wcc-Ply	$2x10^7$	10.2 ±0.02	(-)	17.4 ±2.4
Prevnar	(-)	0.33	5.1*	(-)

Doses of bacterial CFU were calculated by plate count prior to formalin fixation, and prior to conjugation to carrier protein. For Prevnar vaccinated group, 75 µL was diluted to volume for each mouse. Sera were collected from the mice 2 weeks after the second vaccination dose. The IgG responses of pooled murine sera against different components post-vaccination are summarized in Table 2.

Table 2

	CRM	Ply*	Capsule (-) Whole Cell	PPS4 Capsule				
	IgG	IgG	IgG	IgG	IgG1	IgG2a	IgG2b	IgG3
Group								
Alum only	<100	<100	<100	<100	<100	<100	<100	<100
Fixed Cells	<100	<100	51,200	<100	<100	<100	<100	<100
Wcc-CRM	204,800	<100	<100	25,600	25,600	<100	<100	400
Wcc-Ply	<100	1,638,400	800	12,800	12,800	<100	100	800

Prevnar	204,800	<100	100	12,800	12,800	<100	<100	100

Both Prevnar and Wcc-CRM197 vaccinated mice had robust IgG immune responses against CRM197, whereas only Wcc-Ply mice responded towards Ply. Further, a robust IgG immune response towards an unencapsulated strain of TIGR4 Streptococcus pneumoniae was only observed in mice vaccinated with unmodified fixed whole cells. A low level response was observed for the Wcc-Ply mice, which could be attributed to the naturally occurring Ply in PPS4 cells. The results in Table 2 indicate that Wccs produce a robust glycoconjugate-style vaccination response towards the PPS4 capsule, whereas fixed cells can only produce antibodies against cellular proteins.

The mice were given one week rest after sera collection prior to bacterial challenge.

ELISA Quantification of Immune Response of Vaccinated Mice

To immobilize the antigen of interest, Nunc-Immuno™ Microwell™ 96 well plates were incubated with 100 µL of antigen per well in 0.1M Sodium Bicarbonate pH 9.0 (For PPS4 capsule, CRM, and Ply 10 µg/mL) overnight at 4°C . The plates were then blocked with 125 µL PBS with 2% BSA for 3h at room temperature with gentle rocking. Concurrently, murine sera were absorbed with 10 µL/mL purified cell wall polysaccharide (CWPS Multi, SSI68866) in PBST+2% BSA for 3h at room temperature. The absorbed sera were serially diluted at a ratio of ½ in a 96 well plate with PBST +2% BSA. The blocked antigen plate was washed 3x 5 mins with PBST, followed by the transfer of 100 µL of diluted murine sera, and incubated for 2h at room temperature with gentle rocking. After incubation, the plate was washed 3x with PSBT for 5 mins. Secondary antibodies were diluted 1:3,000 (Goat anti-mouse IgG, Human ads-AP; 1030-04, Goat anti-mouse IgG1, Human ads-AP; 1070-04, Goat anti-mouse IgG2b, Human ads-AP; 1090-04, Goat anti-mouse IgG3, Human ads-AP; 1100-04, all from Southern Biotechnologies) in PBST +2% BSA, and incubated for 2h at room temperature. The ELISA plates were washed 3x 5mins with PBST, and immunological response was detected using 1-Step™ PnPP (Thermo Scientific; 37621). Results are presented as reverse titers, and cut off was determined as above the signal of 2x the blanked control with 3x the standard deviation.

Bacterial Challenge of Immunized Mice

The mice were challenged with $1.7x10^7 \pm 8.6x10^6$ CFU (0.5 mL) of *S. pneumonia* (serotype 4, TIGR4) injected IP under isoflurane anesthesia. This dose is *ca.* 30-fold greater than the calculated LD50. FIG. 23 shows the survival curves for the mice. Concentration of bacteria prior to administration as well as post-administration was quantified based on CFU observed on THY plates. Mice were monitored daily or more as needed. Moribund mice were humanely euthanized.

Example 6: Whole Cell Conjugates Based on Affinity Pairs

10^7 formalin fixed streptococcus pneumoniae serotype 4 (PPS4) cells were activated with 10 mg of CDAP, pelleted, and resuspended in 600 µL of 0.1 M sodium borate, 4 mM sodium deoxycholate, and pH 9 buffer. 200 µg of the EZ Link (NH_2-PEG2-Biotin) was incubated with the cells with rocking for 3

hours, and the reaction was quenched with 100 µL of 1 M ethanolamine. Pelleted cells were resuspended in 0.1% tween 80.

From this point, $5^{*}10^{6}$ cells were solubilized in loading buffer, and separated on a 4-12% gradient SDS-PAGE gel. The gel was transferred to a nitrocellulose membrane, and assayed with a anti-biotin antibody. The lanes of the western blot (FIGs. 25A and 25B) are as follows:

1. Unmodified formalin fixed PPS4 cells
2. CDAP activated PPS4 cells
3. Unactivated PPS4 cells incubated with NH_2-PEG2-Biotin
4. CDAP activated PPS4 cells incubated with NH_2-PEG2-Biotin

As observed by Western blot, only CDAP activated cells incubated with NH_2-PEG2-Biotin has anti-biotin reactivity, demonstrating that the whole cell conjugates can be prepared by attaching a handle—a portion of an affinity pair.

For the immunogold protocol, the same cells utilized for western blot were instead incubated for 1 h with 2mg/mL BSA in PBS for blocking. The cells were subsequently incubated with ant-biotin antibody at a dilution of 1/1000 for 1 h in PBST. Cells were washed with PBST 3 times for 5 mins each, and incubated with anti-rabbit immunogold labelled antibody. Cells were washed again 3x5mins with PBST, and analyzed by electron microscopy. Only the presence of immunogold in the CDAP activated PPS4 cells incubated with NH_2-PEG2-Biotin could be observed, thus further supporting the availability of biotin on the PPS4 cell surface for conjugation to any streptavidin-fusion protein.

OTHER EMBODIMENTS

All publications and patents cited in this specification are incorporated herein by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference in its entirety.

Various modifications and variations of the described invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the art are intended to be within the scope of the invention.

Other embodiments are in the claims.

CLAIMS

What is claimed is:

1. An immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker Z^1–C(Z^3)–N(H)–N(H)–(–C(O)–(L–C(O))$_n$–N(H)–N(H)–)$_m$–Z^2,

wherein Z^1 is a bond to an oxygen atom in said polyol antigen,

Z^2 is a bond to a carbonyl group in said carrier protein,

Z^3 is O or NH,

each of n and m is 0 or 1, and

L, when present, is C_{2-6} alkanediyl or C_{6-10} arenediyl.

2. An immunogenic whole-cell protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen thereon, wherein said polyol antigen is covalently linked to said carrier protein through a linker formed by a reaction between a cyanate group bonded to said polyol antigen and a hydrazine-activated carrier protein.

3. The immunogenic whole cell-protein conjugate of claim 1 or 2, wherein said carrier protein is a diphtheria toxin, diphtheria toxoid, tetanus toxin, tetanus toxoid, *Pseudomonas aeruginosa* exotoxin A, cholera toxin B subunit, tetanus toxin fragment C, bacterial flagellin, pneumolysin, an outer membrane protein of *Neisseria menningitidis*, *Pseudomonas aeruginosa* Hcp1 protein, *Escherichia coli* heat labile enterotoxin, shiga-like toxin, human LTB protein, pneumolysin, listeriolysin O, a protein extract from whole bacterial cells, the dominant negative mutant (DNI) of the protective antigen of *Bacillus anthracis*, or *Escherichia coli* beta-galactosidase.

4. The immunogenic whole cell-protein conjugate of any one of claims 1 to 3, wherein said whole cell is a *Pseudomonas aeruginosa* or *Streptococcal* cell.

5. The immunogenic whole cell-protein conjugate of claim 4, wherein said whole cell is a *Streptococcus pneumonia* cell.

6. The immunogenic whole cell-protein conjugate of claim 5, wherein said whole cell is a *Streptococcus pneumonia* type 1, 2, 3, 4, 5, 6A, 6B, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10F, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 21, 22F, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33D, 33F, 34, 35A, 35B, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, or 48.

7. The immunogenic whole cell-protein conjugate of claim 3, wherein said bacterial flagellin is the *Vibrio cholerae* flagellin protein.

8. The immunogenic whole cell-protein conjugate of claim 3, wherein said shiga-like toxin is the *Shigella* SltB2 protein.

9. The immunogenic whole cell-protein conjugate of claim 3, wherein said carrier protein molecules are pneumolysin.

10. The immunogenic whole cell-protein conjugate of claim 3, wherein said carrier protein is listeriolysin O.

11. The immunogenic whole cell-protein conjugate of claim 3, wherein said carrier protein is diphtheria toxin.

12. The immunogenic whole cell-protein conjugate of claim 3, wherein said carrier protein is diphtheria toxoid.

13. The immunogenic whole cell-protein conjugate of claim 3, wherein said carrier protein is tetanus toxin.

14. The immunogenic whole cell-protein conjugate of claim 3, wherein said carrier protein is tetanus toxoid.

15. The immunogenic whole cell-protein conjugate of any one of claims 1 to 14, wherein said polyol antigen is a polysaccharide antigen.

16. The immunogenic whole cell-protein conjugate of any one of claims 1 to 15, wherein said polyol antigen comprises at least 18 carbohydrate residues.

17. The immunogenic whole cell-protein conjugate of claim 15, wherein said polyol antigen comprises a *Streptococcus pneumoniae* polysaccharide, *Francisella tularensis* polysaccharide, *Bacillus anthracis* polysaccharide, *Haemophilus influenzae* polysaccharide, *Salmonella typhi* polysaccharide, *Salmonella* species polysaccharide, *Shigella* polysaccharide, or *Neisseria meningitidis* polysaccharide.

18. The immunogenic whole cell-protein conjugate of claim 17, wherein said *Streptococcus pneumoniae* polysaccharide is capsular type 1, 2, 3, 4, 5, 6A, 6B, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10F, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 21, 22F, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33D, 33F, 34, 35A, 35B, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, or 48.

19. The immunogenic whole cell-protein conjugate of claim 17, wherein said *Francisella tularensis* polysaccharide is O antigen.

20. The immunogenic whole cell-protein conjugate of any one of claims 15 to 19, wherein said polyol antigen is a microbial capsular polymer.

21. The immunogenic whole cell-protein conjugate of any one of claims 1 to 20, wherein said whole cell is a heat inactivated whole cell pathogen.

22. The immunogenic whole cell-protein conjugate of any one of claims 1 to 20, wherein said whole cell is a chemically inactivated whole cell pathogen.

23. The immunogenic whole cell-protein conjugate of any one of claims 1 to 22, wherein said immunogenic whole cell-protein conjugate, when administered to a mammal, elicits a T-cell dependent immune response in said mammal.

24. The immunogenic whole cell-protein conjugate of any one of claims 1 to 23, wherein the molar ratio of said polyol antigen to said carrier protein in said immunogenic whole cell-protein conjugate is 1 to 1.

25. A pharmaceutical composition comprising the immunogenic whole cell-protein conjugate of any one of claims 1 to 24 and a pharmaceutically acceptable carrier or excipient.

26. The pharmaceutical composition of claim 25, wherein said pharmaceutical composition does not comprise an adjuvant.

27. A method of preparing the immunogenic whole cell-protein conjugate of any one of claims 1 to 24, said method comprising contacting a hydrazine-activated carrier protein with a whole cell having an exterior surface comprising a polyol antigen bonded to at least one cyanate.

28. A method of preparing a polyol antigen-carrier protein conjugate, said method comprising contacting a hydrazine-activated carrier protein with a whole cell having an exterior surface comprising a polyol antigen bonded to at least one cyanate, and, after said contacting, lysing said whole cell to produce said polyol antigen-carrier protein conjugate.

29. The method of claim 28, said method further comprising purifying said polyol antigen-carrier protein conjugate away from unconjugated cellular components.

30. The method of any one of claims 27 to 29, said method further comprising preparing said hydrazine-activated carrier protein by contacting a carrier protein with a hydrazine source agent.

31. The method of any one of claims 27 to 30, said method further comprising preparing said whole cell having an exterior surface comprising a polyol antigen bonded to at least one cyanate by contacting an electrophilic source of cyanide with a whole cell comprising a polyol antigen.

32. The method of claim 31, wherein said electrophilic source of cyanide is CDAP.

33. The method of claim 31, wherein said electrophilic source of cyanide is cyanogen bromide.

34. A method of generating an immune response in a subject comprising administering the pharmaceutical composition of claim 25 or 26 to said subject, wherein said immunogenic whole cell-protein conjugate elicits a T-cell dependent immune response in said subject.

35. The method of claim 34, wherein said subject is an infant, a child, or an adolescent.

36. The method of claim 34 or 35, wherein said pharmaceutical composition is administered to said subject parenterally.

37. The method of claim 34 or 35, wherein said pharmaceutical composition is administered to said subject orally.

38. An immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker Z^4–CH=N–[–N(H)–(–C(O)–(L–C(O))$_n$–N(H)–N(H)–)$_m$–]$_k$–Z^5,

wherein Z^4 is a bond to a polyol antigen,

Z^5 is a bond the carrier protein,

each of n, m, and k is independently 0 or 1, and

L, when present, is C_{2-6} alkanediyl or C_{6-10} arenediyl.

39. An immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker Z^1–C(O)–N(H)–(N(H))$_k$–L–N(H)–C(O)–CH_2–S–L^1–C(O)–Z^2,

wherein Z^1 is a bond to an oxygen atom in the polyol antigen,

Z^2 is a bond to an amino group in the carrier protein,

k is 0 or 1,

L is C_{2-6} alkanediyl, and

L^1 is C_{2-6} alkanediyl optionally substituted with a protected amino group.

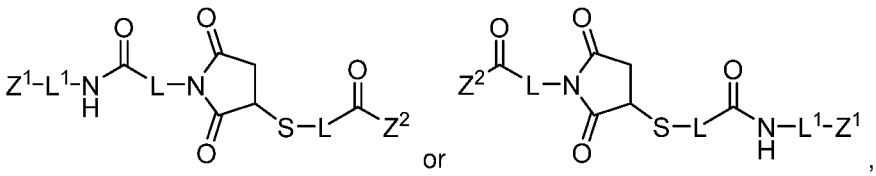
40. An immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker Z^4–N(H)–C(O)–L–C(O)–N(H)–Z^5,

wherein Z^4 is a bond to a polyol antigen,

Z^5 is a bond the carrier protein,

L is C_{2-6} alkanediyl or C_{6-10} arenediyl.

41. An immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker of formula:



wherein Z^1 is a bond to an oxygen atom in the polyol antigen,

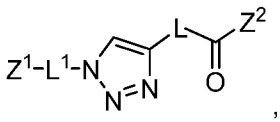
Z^2 is a bond to an amino group in the carrier protein,

each L is independently C_{2-6} alkanediyl, and

L^1 is C_{2-6} alkanediyl.

42. An immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker Z^1–C(O)–N(H)–Z^5, wherein Z^1 is a bond to an oxygen atom in the polyol antigen, and Z^2 is a bond to a carrier protein.

43. An immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is covalently linked to said carrier protein through a linker



wherein Z^1 is a bond to an oxygen atom in the polyol antigen,

Z^2 is a bond to an amino group in the carrier protein, and

each of L and L^1 is independently C_{2-6} alkanediyl.

44. An immunogenic whole cell-protein conjugate comprising a carrier protein and a whole cell having an exterior surface comprising a polyol antigen, wherein said polyol antigen is linked to said carrier protein through a linker comprising an affinity pair.

45. The immunogenic whole cell-protein conjugate of claim 44, wherein the linker is of formula:

$$Z^1–C(Z^3)–L^1–L–L^2–AP–Z^2,$$

where Z^1 is a bond to an oxygen atom in the polyol antigen,

Z^2 is a bond to a carrier protein,

Z^3 is O or NH,

L is C_{2-10} alkanediyl or –[–CH_2–CH_2–(–O–CH_2–CH_2–)$_n$]–, where n is an integer from 0 to 24,

L^1 is –N(H)– or –N(H)–N(H)–,

L^2 is –N(H)–, –C(O)–, –C(O)–N(H)–, or –N(H)–C(O)–,
AP is a complementary affinity pair.

46. The immunogenic whole cell-protein conjugate of claim 44, wherein the complementary affinity pair is a biotin/streptavidin, biotin/avidin, or biotin/neutravidin pair.

47. The immunogenic whole cell-protein conjugate of any one of claims 38-45, wherein said carrier protein is a diphtheria toxin, diphtheria toxoid, tetanus toxin, tetanus toxoid, *Pseudomonas aeruginosa* exotoxin A, cholera toxin B subunit, tetanus toxin fragment C, bacterial flagellin, pneumolysin, an outer membrane protein of *Neisseria menningitidis*, *Pseudomonas aeruginosa* Hcp1 protein, *Escherichia coli* heat labile enterotoxin, shiga-like toxin, human LTB protein, pneumolysin, listeriolysin O, a protein extract from whole bacterial cells, the dominant negative mutant (DNI) of the protective antigen of *Bacillus anthracis*, or *Escherichia coli* beta-galactosidase.

48. The immunogenic whole cell-protein conjugate of any one of claims 38 to 47, wherein said whole cell is a *Pseudomonas aeruginosa* or *Streptococcal* cell.

49. The immunogenic whole cell-protein conjugate of claim 48, wherein said whole cell is a *Streptococcus pneumonia* cell.

50. The immunogenic whole cell-protein conjugate of claim 49, wherein said whole cell is a *Streptococcus pneumonia* type 1, 2, 3, 4, 5, 6A, 6B, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10F, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 21, 22F, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33D, 33F, 34, 35A, 35B, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, or 48.

51. The immunogenic whole cell-protein conjugate of claim 46, wherein said bacterial flagellin is the *Vibrio cholerae* flagellin protein.

52. The immunogenic whole cell-protein conjugate of claim 46, wherein said shiga-like toxin is the *Shigella* SltB2 protein.

53. The immunogenic whole cell-protein conjugate of claim 46, wherein said carrier protein molecules are pneumolysin.

54. The immunogenic whole cell-protein conjugate of claim 46, wherein said carrier protein is listeriolysin O.

55. The immunogenic whole cell-protein conjugate of claim 46, wherein said carrier protein is diphtheria toxin.

56. The immunogenic whole cell-protein conjugate of claim 46, wherein said carrier protein is diphtheria toxoid.

57. The immunogenic whole cell-protein conjugate of claim 46, wherein said carrier protein is tetanus toxin.

58. The immunogenic whole cell-protein conjugate of claim 46, wherein said carrier protein is tetanus toxoid.

59. The immunogenic whole cell-protein conjugate of any one of claims 38 to 58, wherein said polyol antigen is a polysaccharide antigen.

60. The immunogenic whole cell-protein conjugate of any one of claims 38 to 59, wherein said polyol antigen comprises at least 18 carbohydrate residues.

61. The immunogenic whole cell-protein conjugate of claim 60, wherein said polyol antigen comprises a *Streptococcus pneumoniae* polysaccharide, *Francisella tularensis* polysaccharide, *Bacillus anthracis* polysaccharide, *Haemophilus influenzae* polysaccharide, *Salmonella typhi* polysaccharide, *Salmonella* species polysaccharide, *Shigella* polysaccharide, or *Neisseria meningitidis* polysaccharide.

62. The immunogenic whole cell-protein conjugate of claim 61, wherein said *Streptococcus pneumoniae* polysaccharide is capsular type 1, 2, 3, 4, 5, 6A, 6B, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10F, 11A, 11B, 11C, 11D, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 21, 22F, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33D, 33F, 34, 35A, 35B, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, or 48.

63. The immunogenic whole cell-protein conjugate of claim 61, wherein said *Francisella tularensis* polysaccharide is O antigen.

64. The immunogenic whole cell-protein conjugate of any one of claims 38 to 63, wherein said polyol antigen is a microbial capsular polymer.

65. The immunogenic whole cell-protein conjugate of any one of claims 38 to 64, wherein said whole cell is a heat inactivated whole cell pathogen.

66. The immunogenic whole cell-protein conjugate of any one of claims 38 to 65, wherein said whole cell is a chemically inactivated whole cell pathogen.

67. The immunogenic whole cell-protein conjugate of any one of claims 38 to 66, wherein said immunogenic whole cell-protein conjugate, when administered to a mammal, elicits a T-cell dependent immune response in said mammal.

68. The immunogenic whole cell-protein conjugate of any one of claims 1-24 and 38-67, wherein said whole cell is a bacterial cell.

69. The immunogenic whole cell-protein conjugate of any one of claims 1-24 and 38-67, wherein said whole cell is a fungal cell.

70. The immunogenic whole cell-protein conjugate of claim 69, wherein fungal cell is *Candida*.

71. A pharmaceutical composition comprising the immunogenic whole cell-protein conjugate of any one of claims 38 to 70 and a pharmaceutically acceptable carrier or excipient.

FIG.1

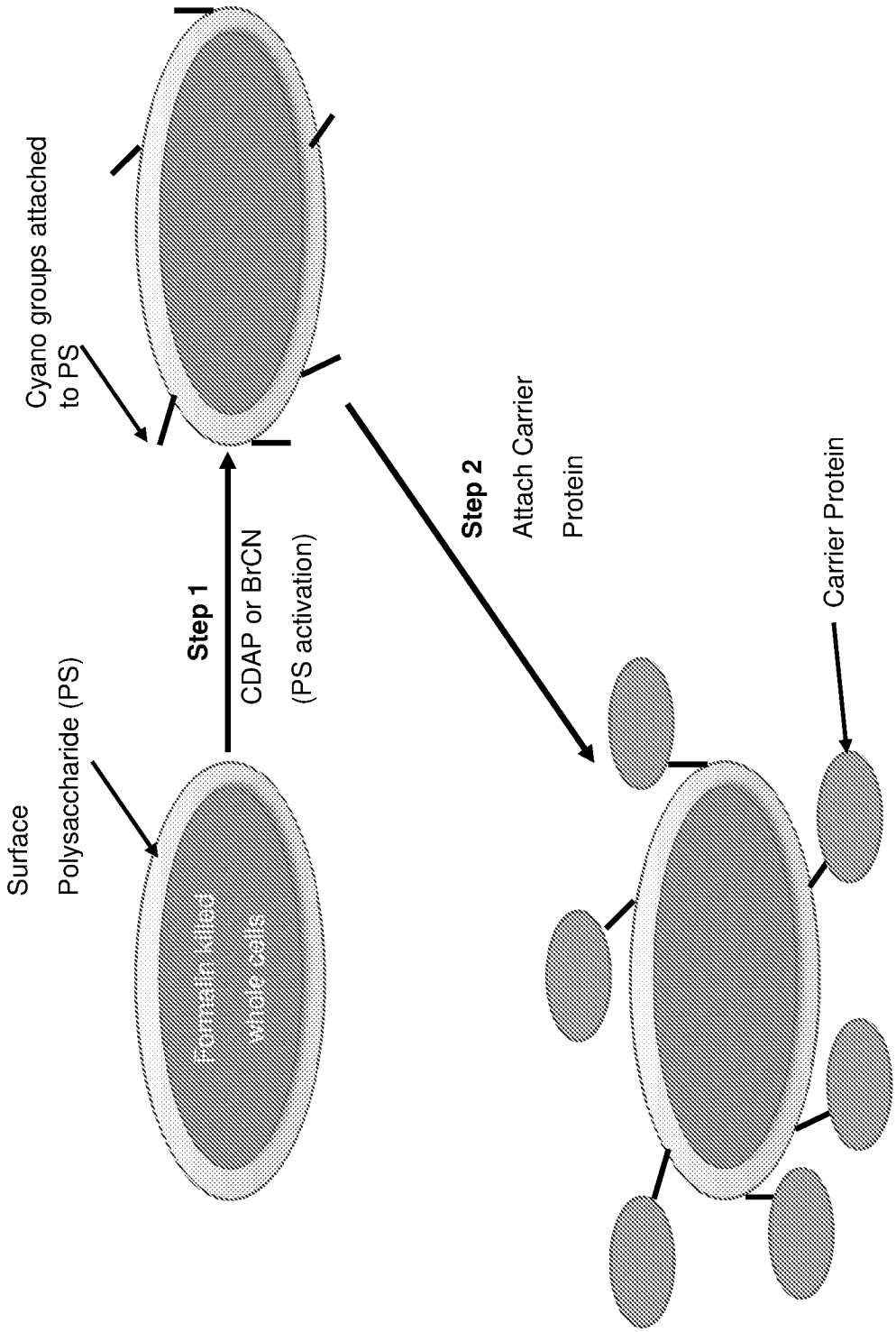

Surface
Polysaccharide (PS)
Step 1
CDAP or BrCN
(PS activation)
Cyano groups attached
to PS
Step 2
Attach Carrier
Protein
Carrier Protein

FIG. 2B

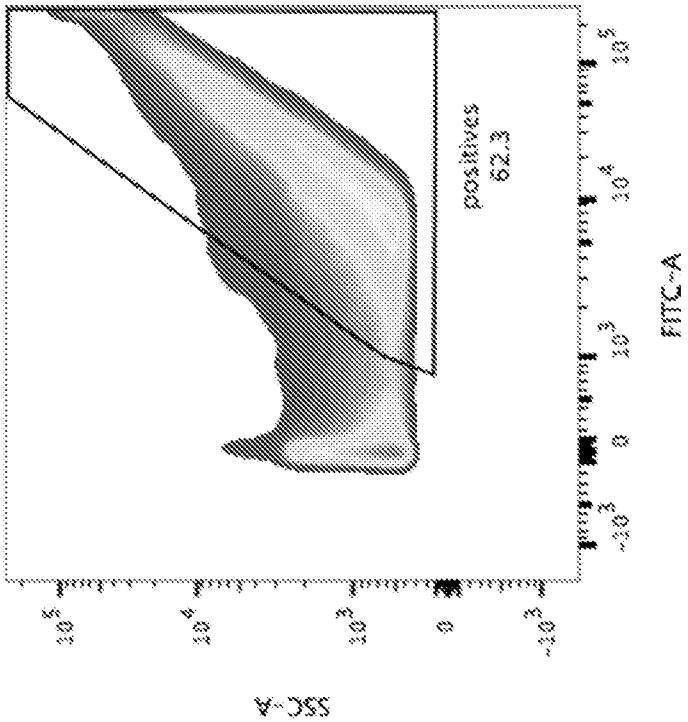


FIG. 2A

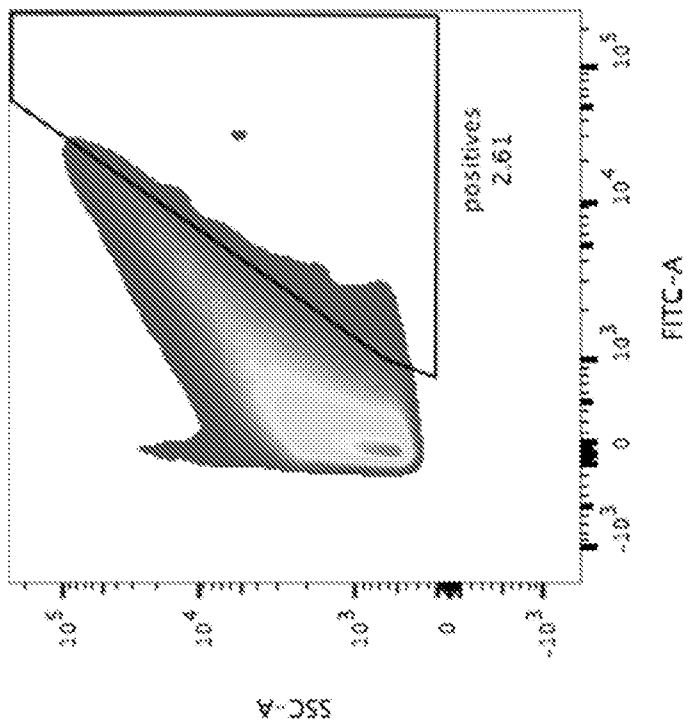


FIG. 3B

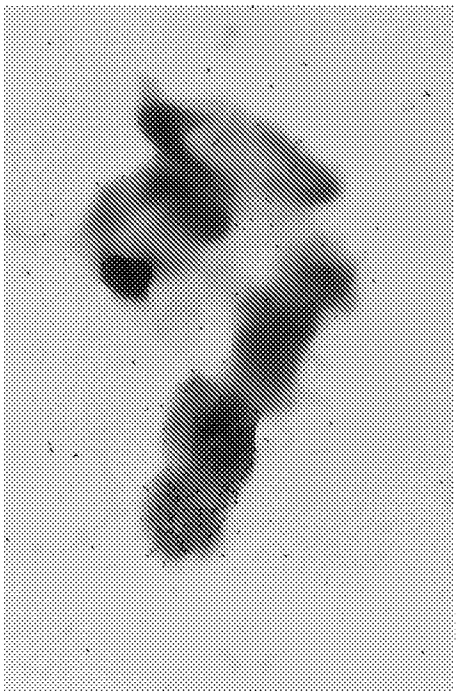


FIG. 3D

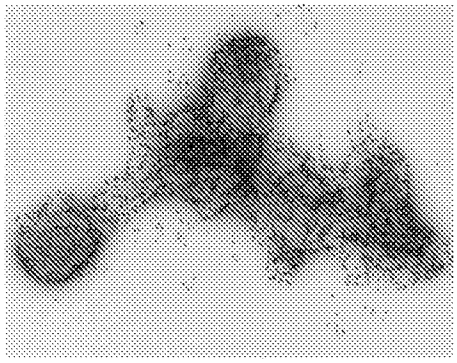


FIG. 3A

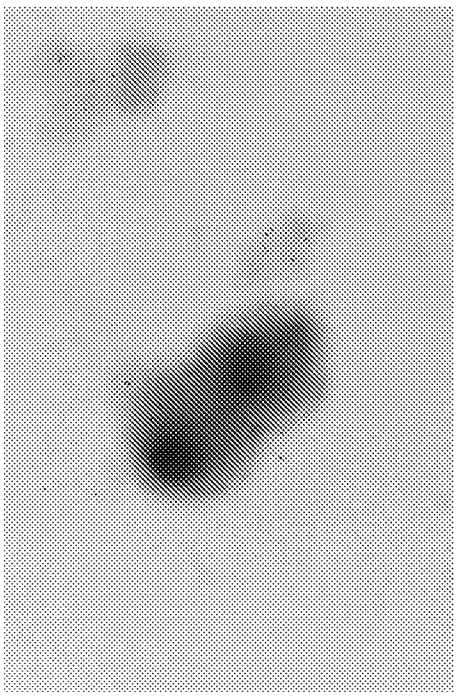


FIG. 3C

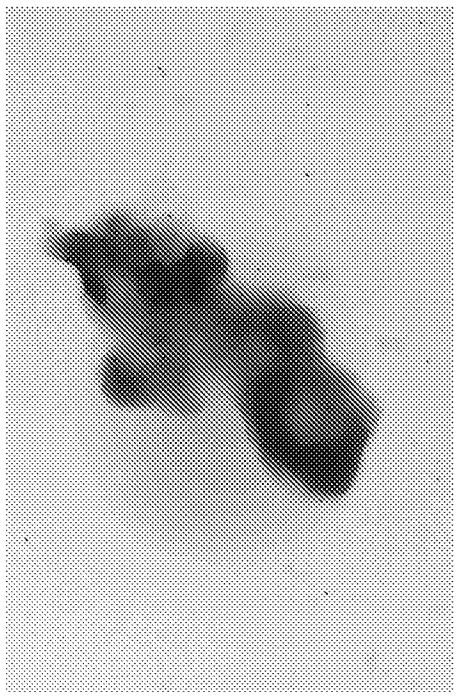


FIG. 4

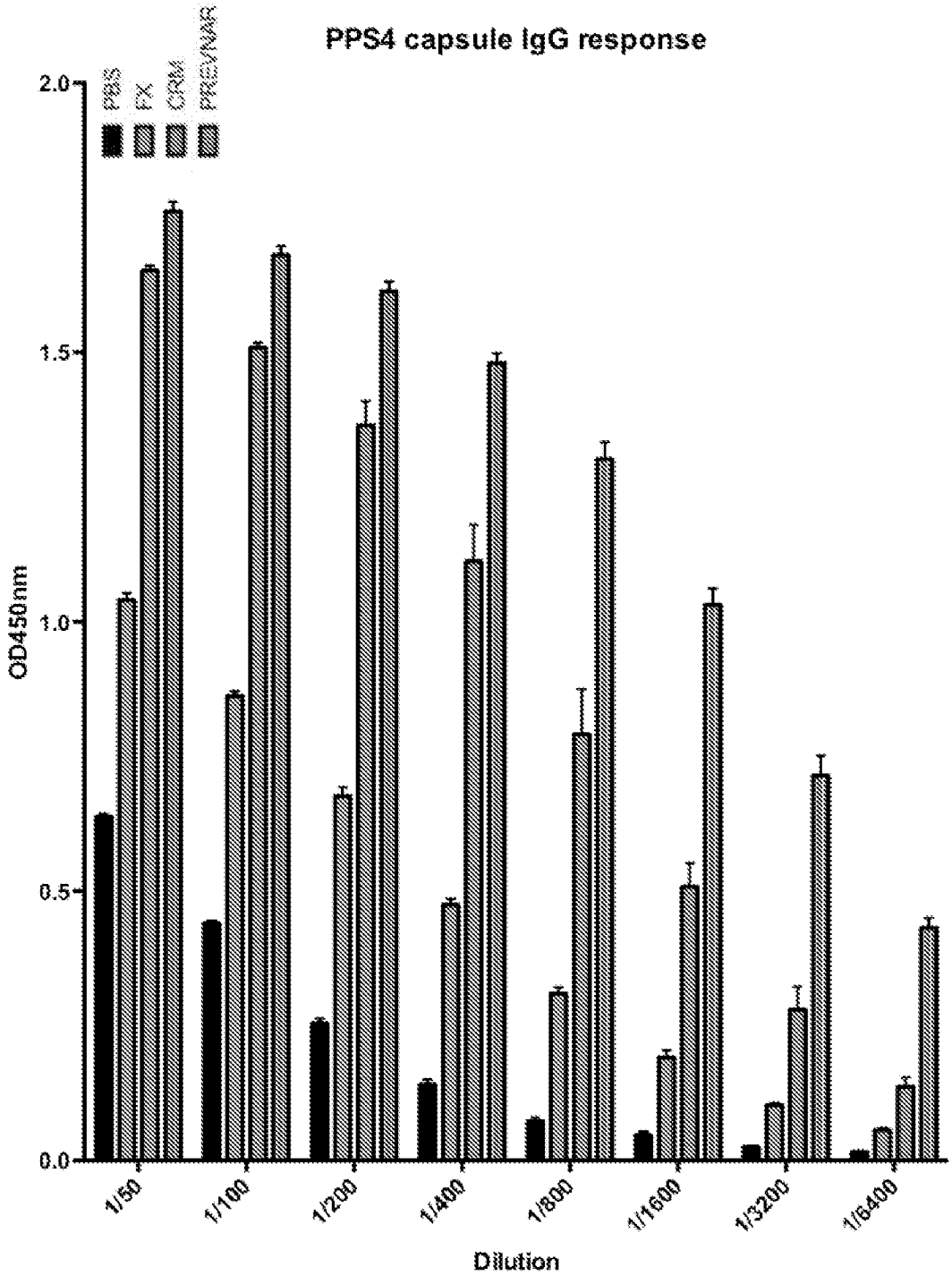

PPS4 capsule IgG response
PBS
FX
CRM
PREVNAR
OD450nm
2.0
1.5
1.0
0.5
0.0
1/50
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
Dilution

FIG. 5A

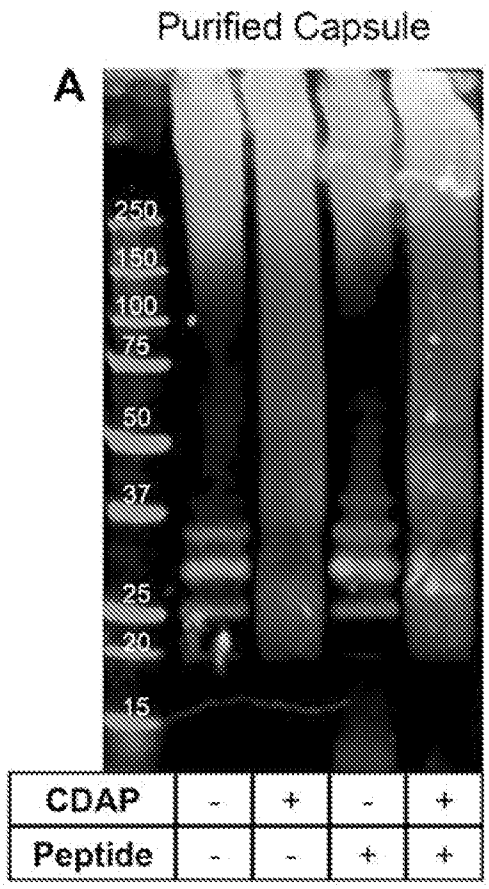
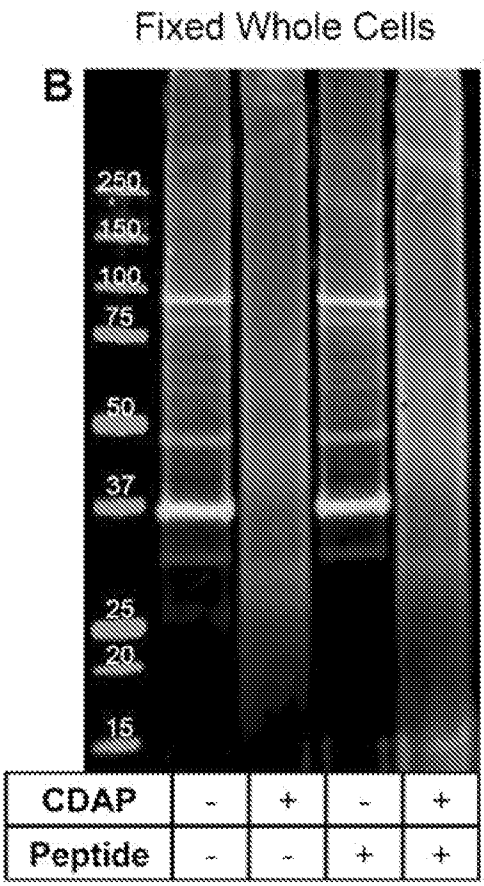


FIG. 5B



Green: α-PPS4 Capsule
Red: α-Peptide

FIG. 6B

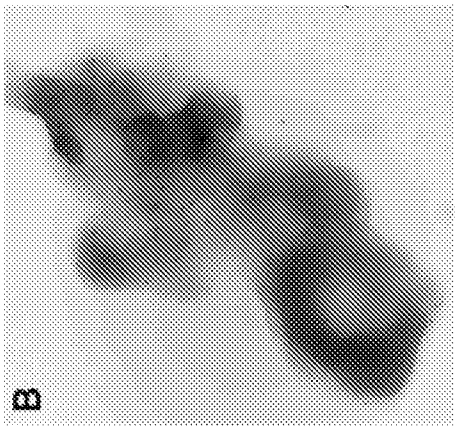


FIG. 6D

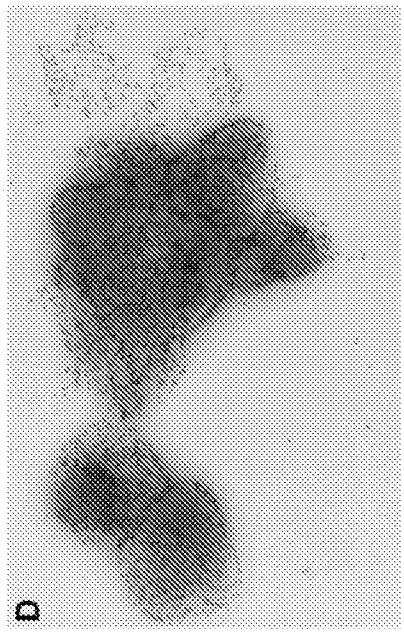


FIG. 6A

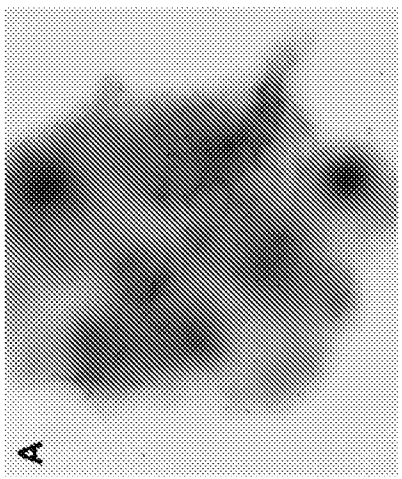


FIG. 6C

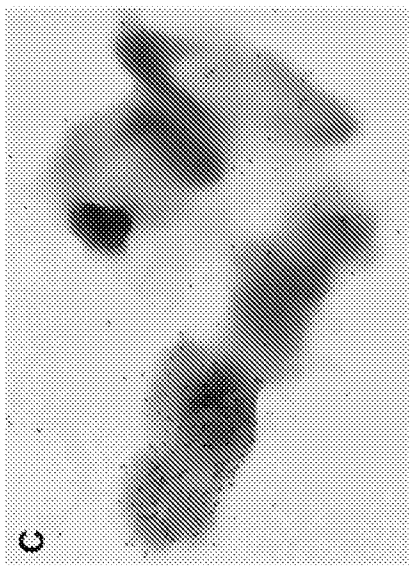


FIG. 7

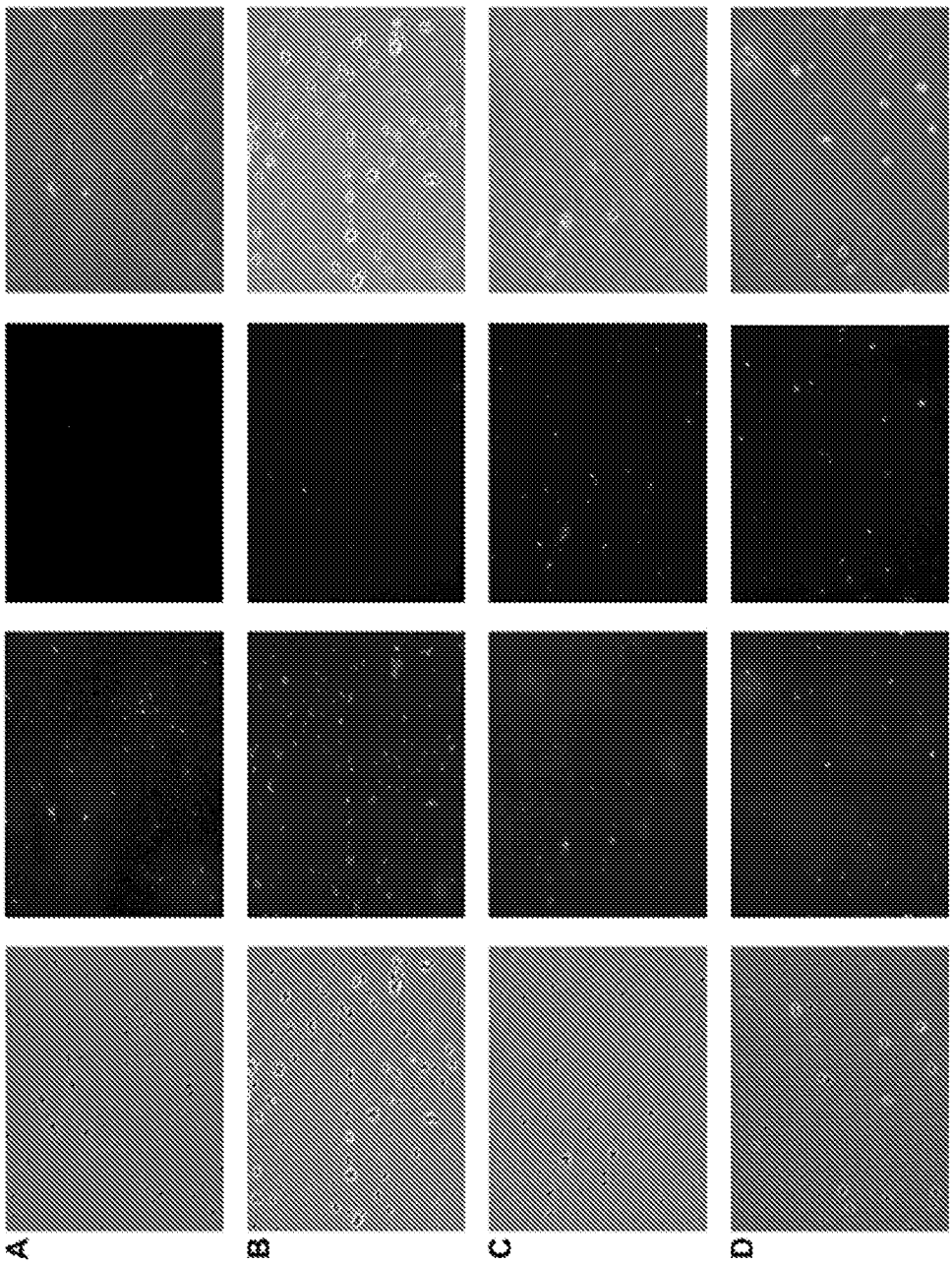


FIG. 8A

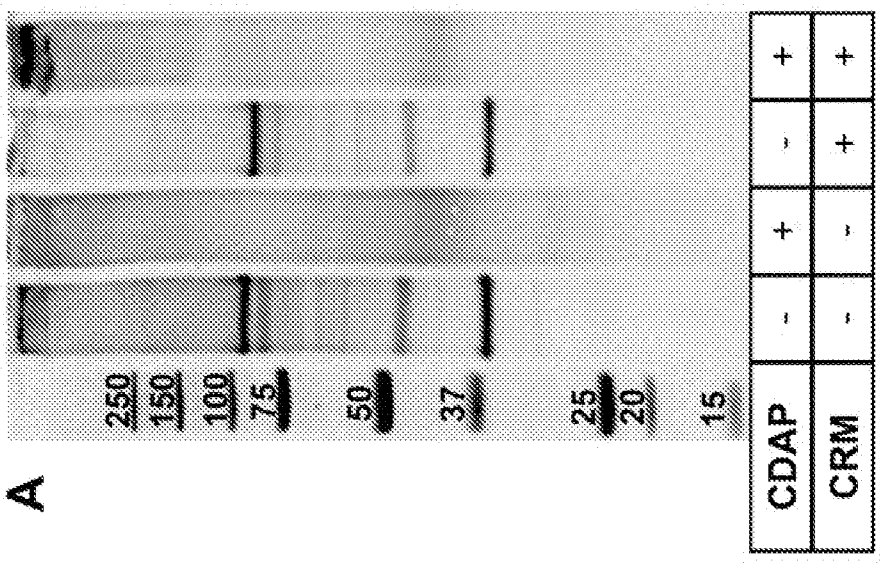

A
250
150
100
75
50
37
25
20
15
CDAP - + - +
CRM - - + +
α-PPS4

FIG. 8B

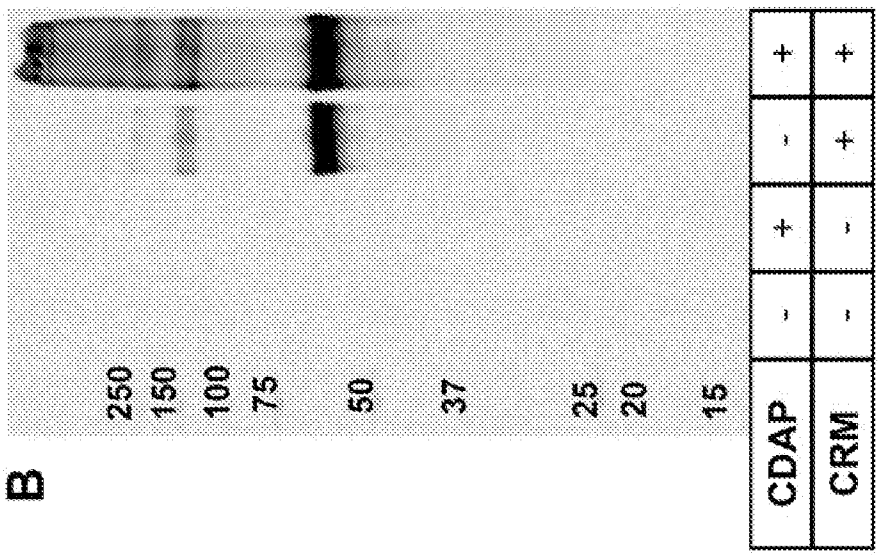

B
250
150
100
75
50
37
25
20
15
CDAP - + - +
CRM - - + +
α-CRM

FIG. 9

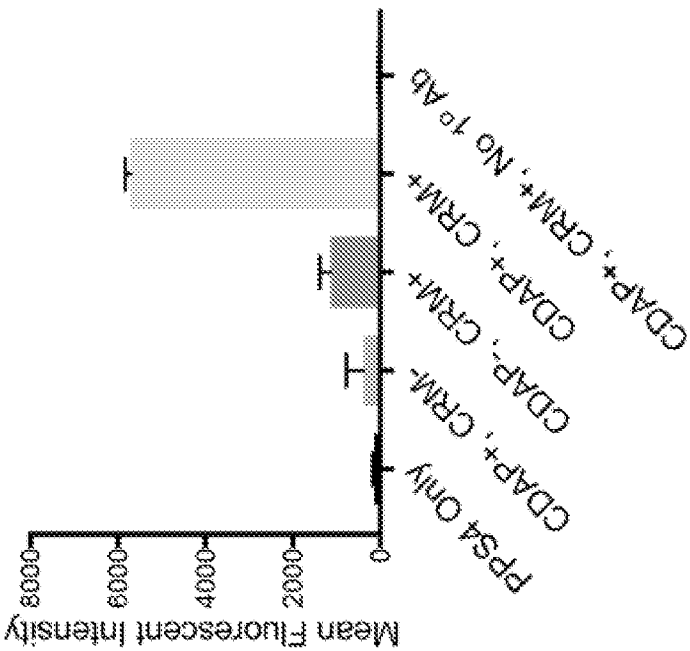

Mean Fluorescent Intensity
8000
6000
4000
2000
0
PPS4 Only
CDAP+, CRM-
CDAP-, CRM+
CDAP+, CRM+
CDAP+, CRM+, No TF Ab

FIG. 10B

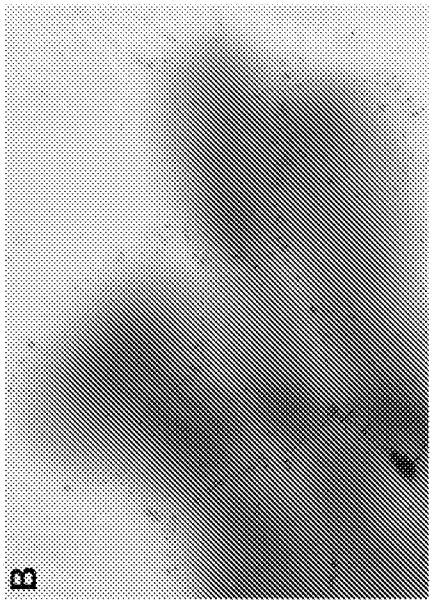


FIG. 10D

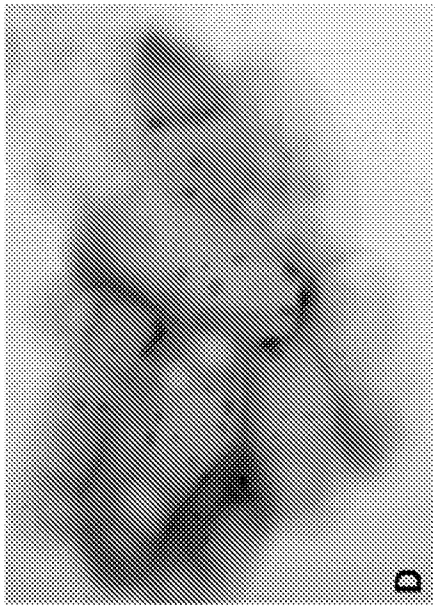


FIG. 10A

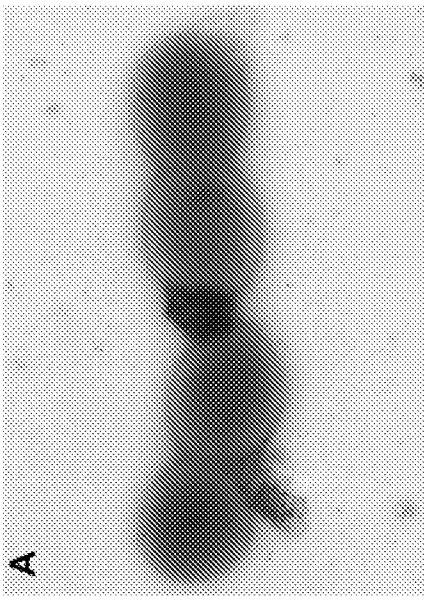
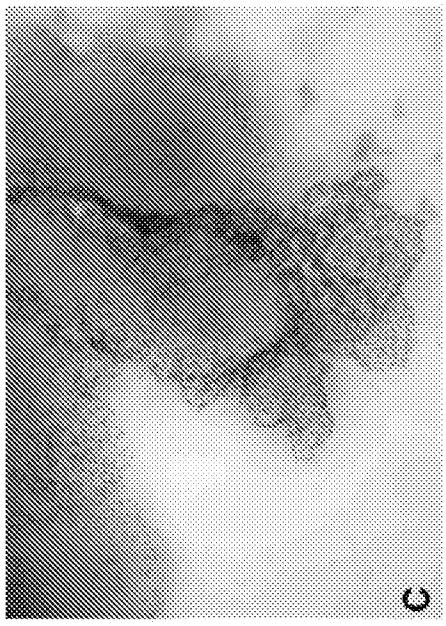


FIG. 10C



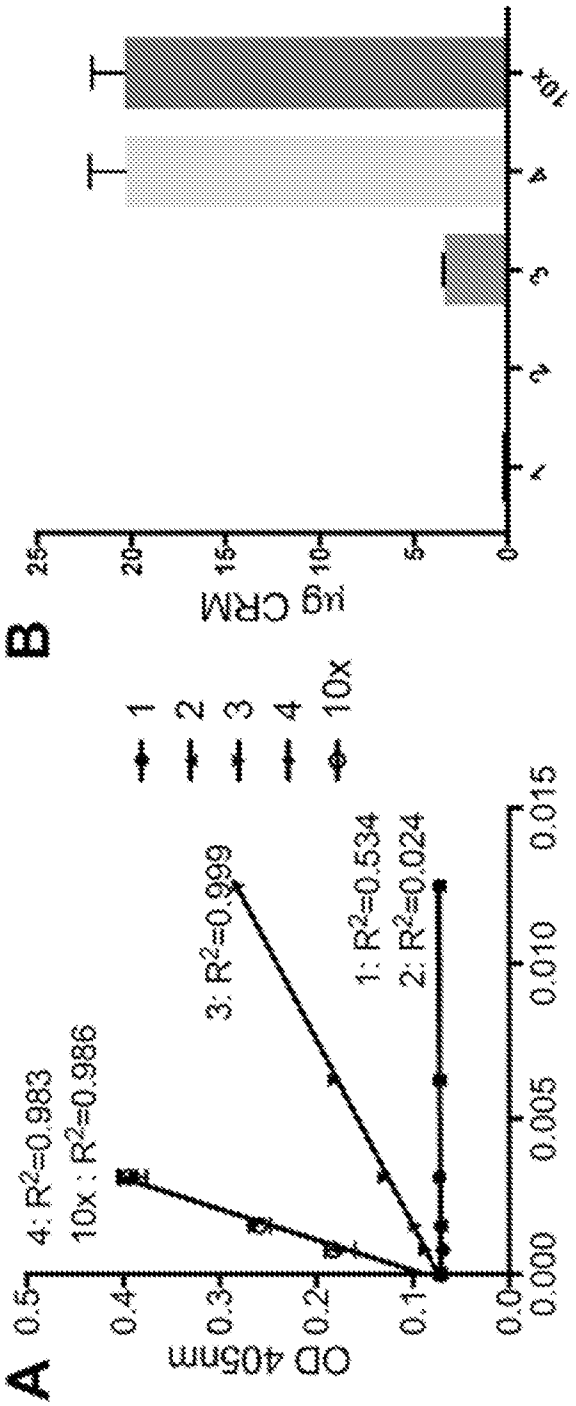


FIG. 11A

FIG. 11B

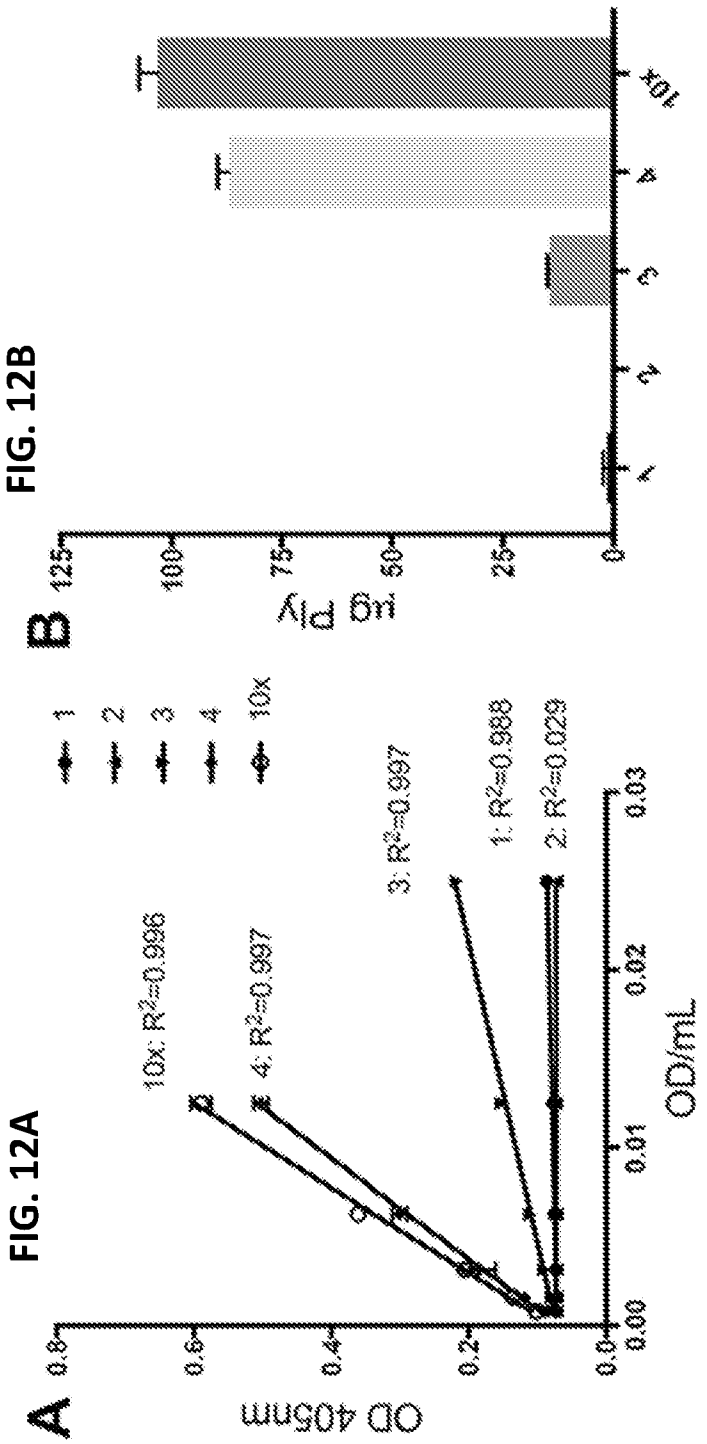


FIG. 12A

FIG. 12B

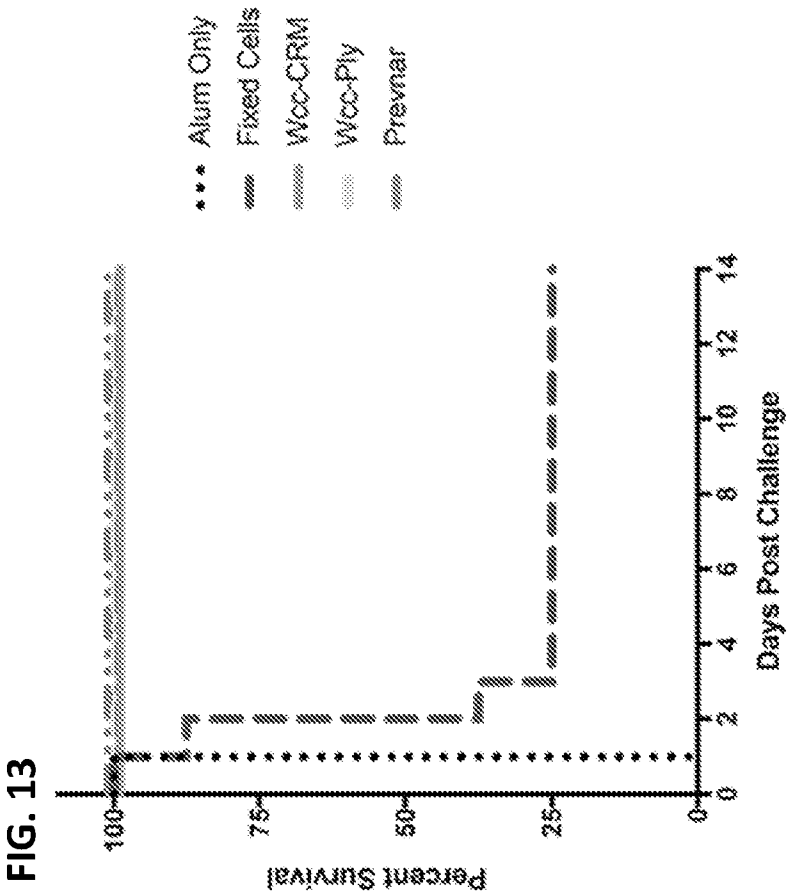

Alum Only
Fixed Cells
Wcc-CRM
Wcc-Ply
Prevnar
Percent Survival
100
75
50
25
0
0
2
4
6
8
10
12
14
Days Post Challenge

FIG. 13

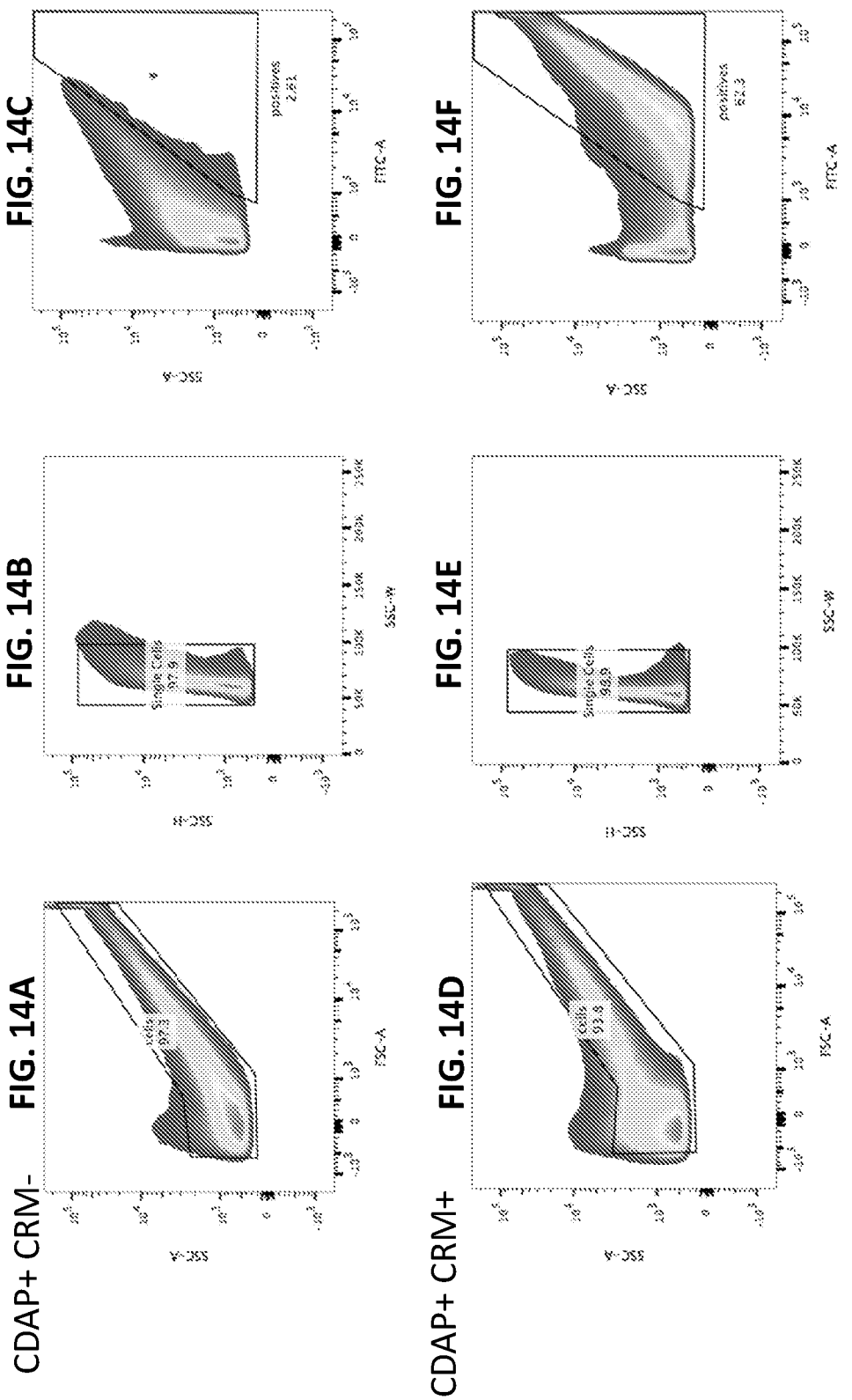

CDAP+ CRM-
FIG. 14A
FIG. 14B
FIG. 14C
CDAP+ CRM+
FIG. 14D
FIG. 14E
FIG. 14F
FSC-A
SSC-A
SSC-W
SSC-H
FITC-A
Single Cells
97.5
positives
2.61
cells
93.8
Single Cells
98.9
positives
82.3

FIG. 15B

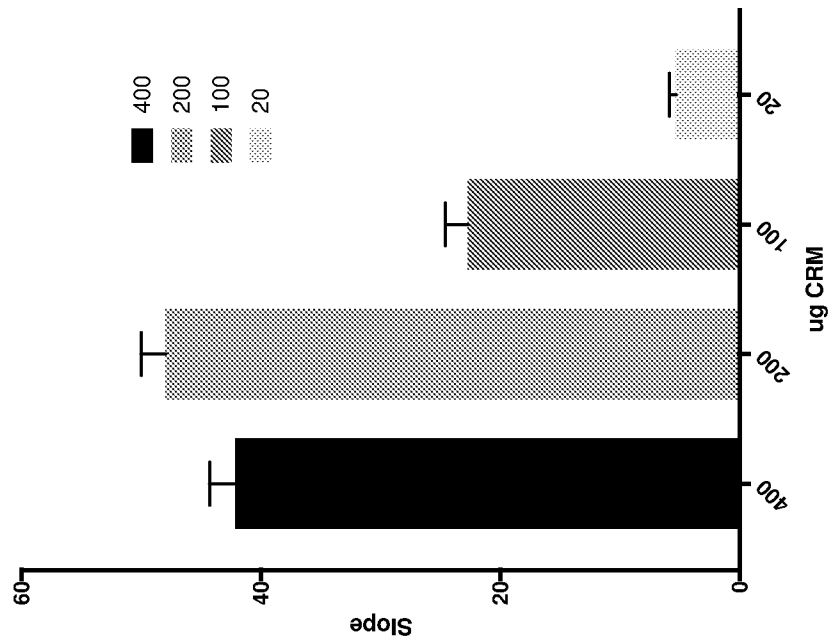


FIG. 15A

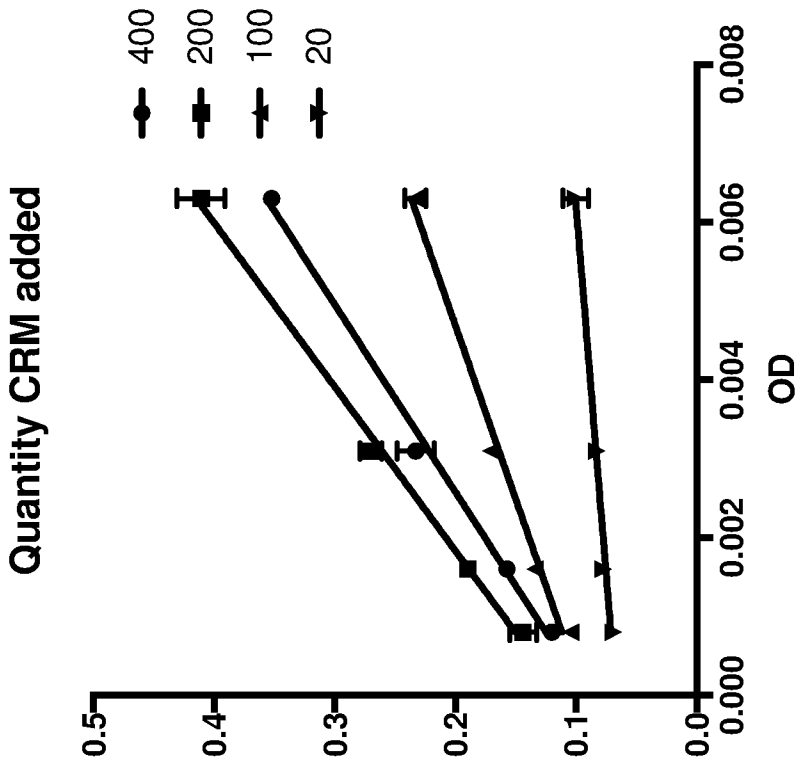


FIG. 16B

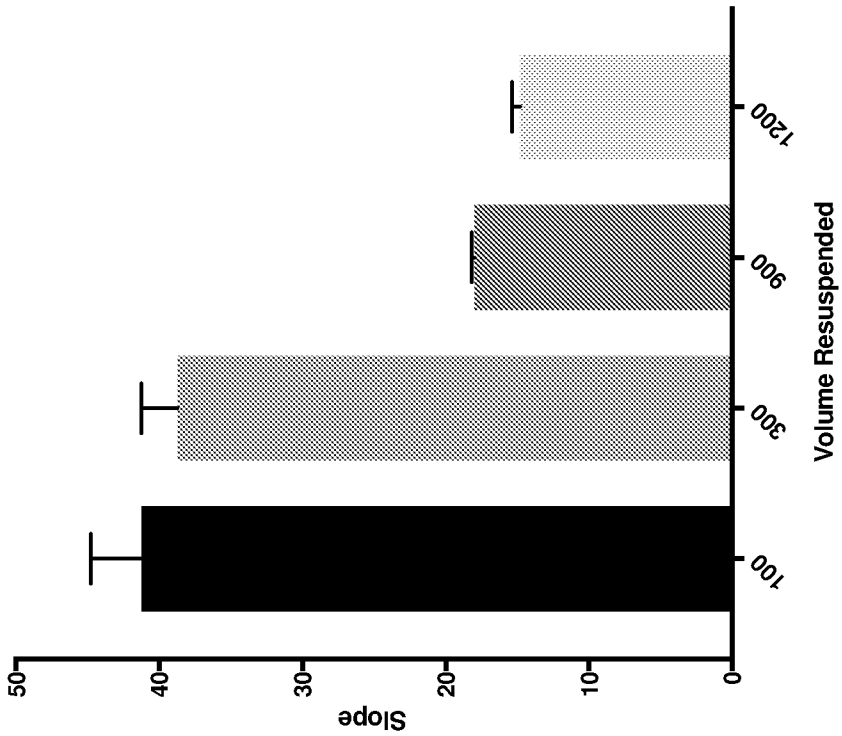


FIG. 16A

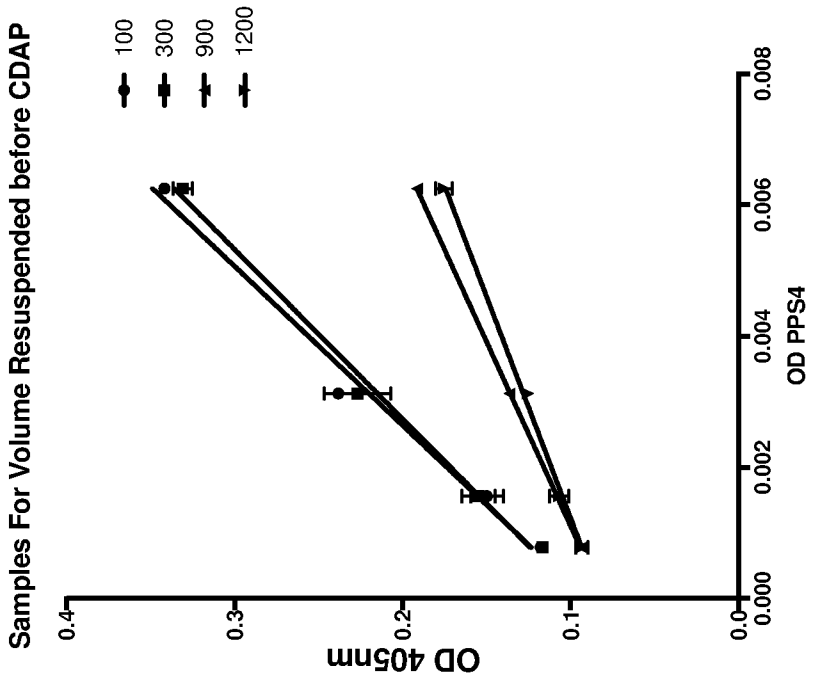


FIG. 17B

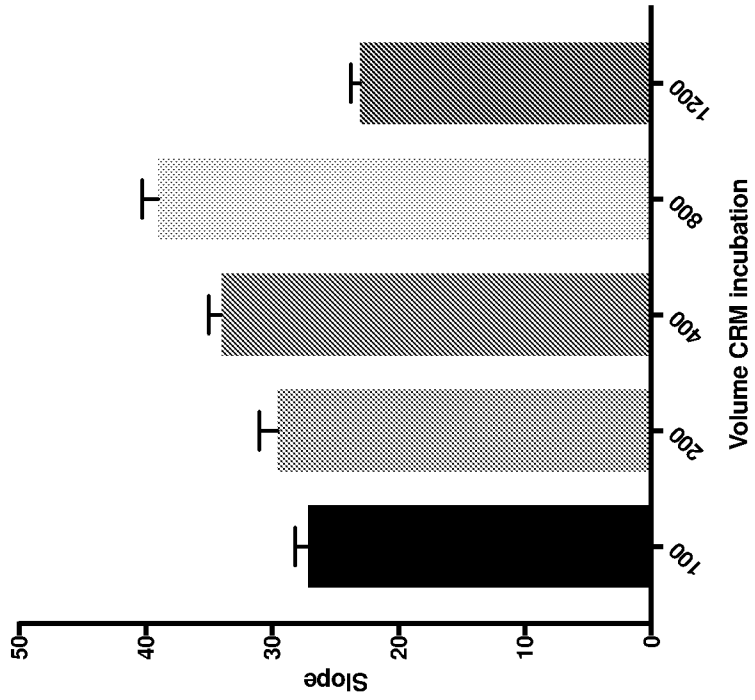
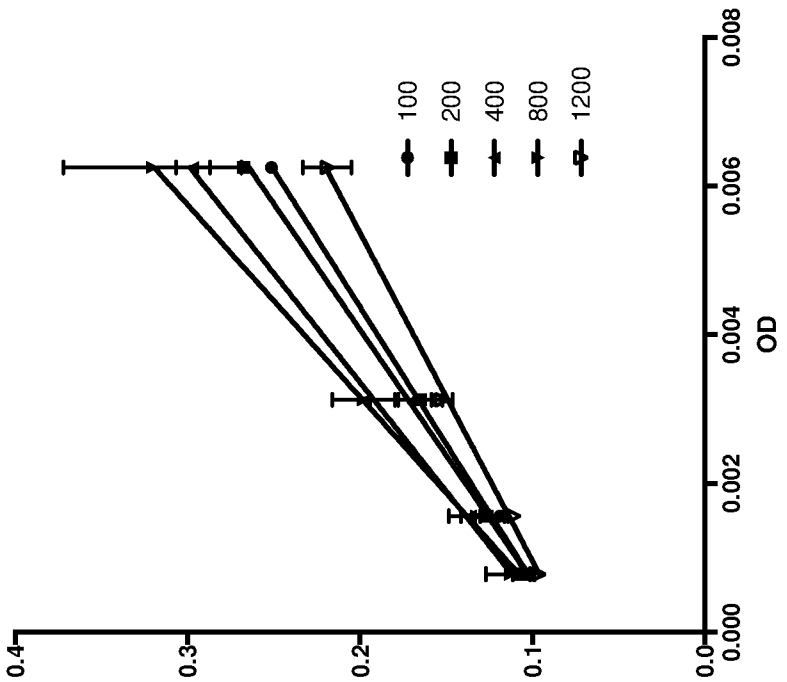


FIG. 17A



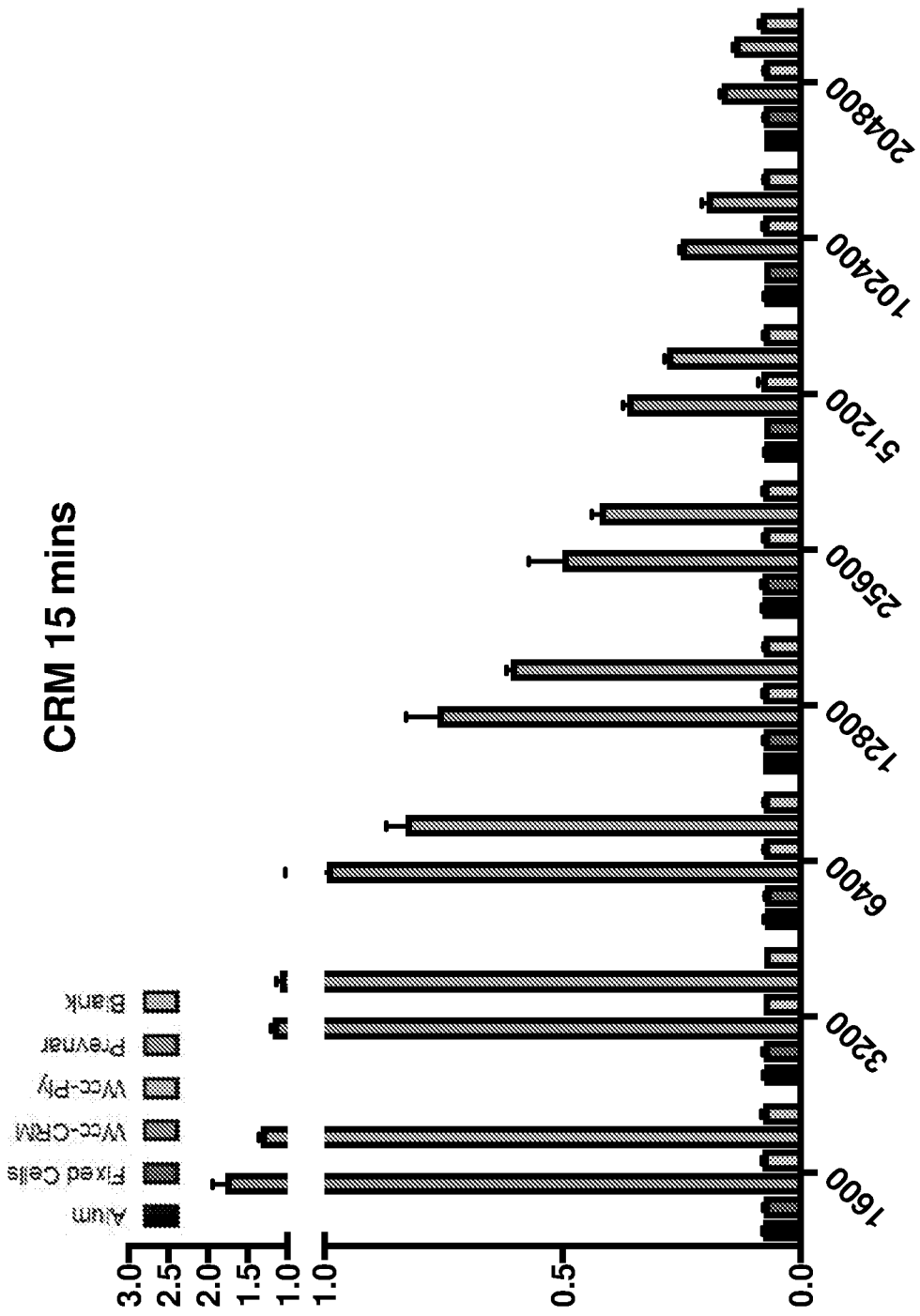

CRM 15 mins
Alum
Fixed Cells
Vcc-CRM
Vcc-Ply
Prevnar
Blank
3.0
2.5
2.0
1.5
1.0
1.0
0.5
0.0
1600
3200
6400
12800
25600
51200
102400
204800

FIG. 18

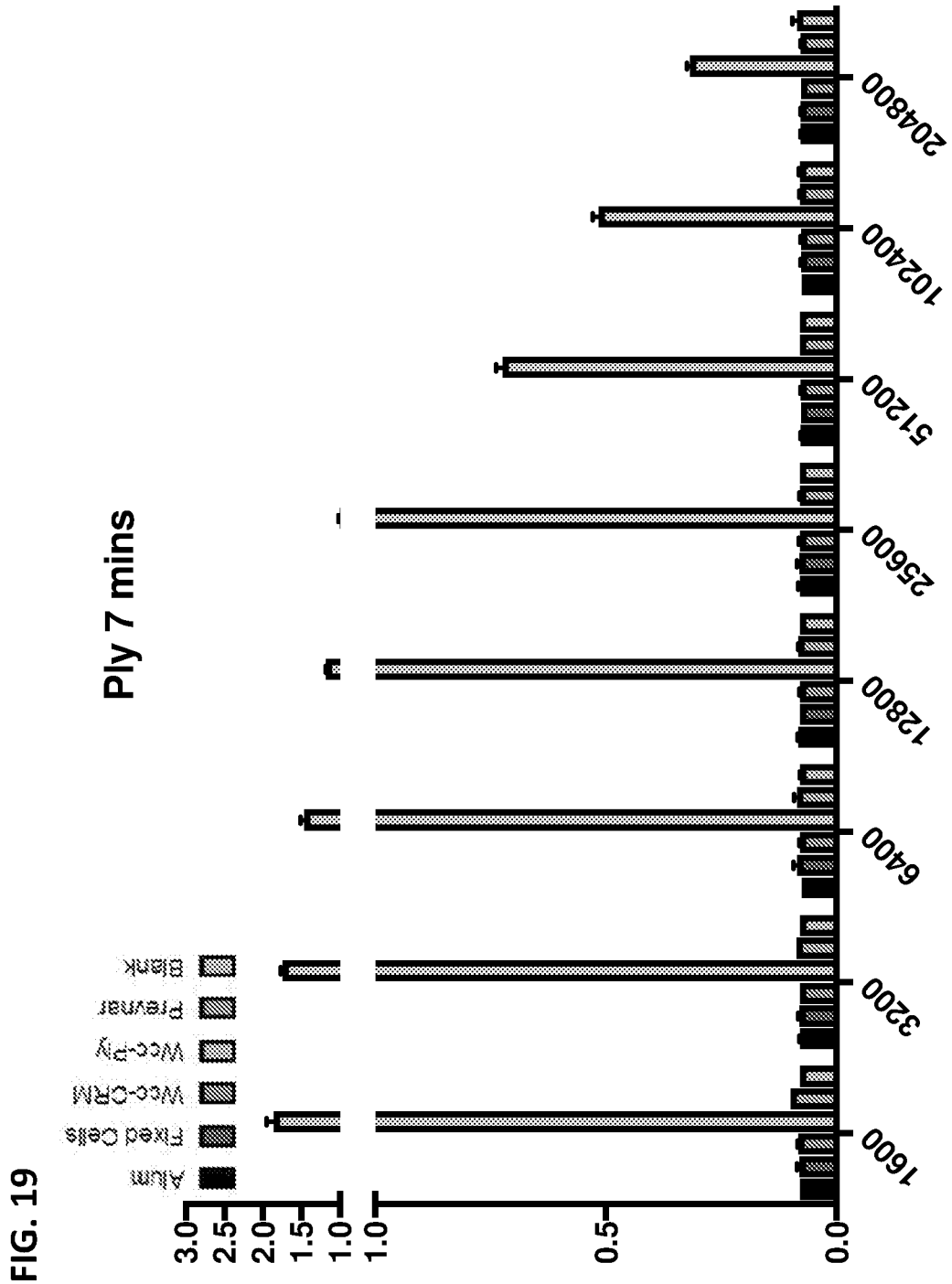
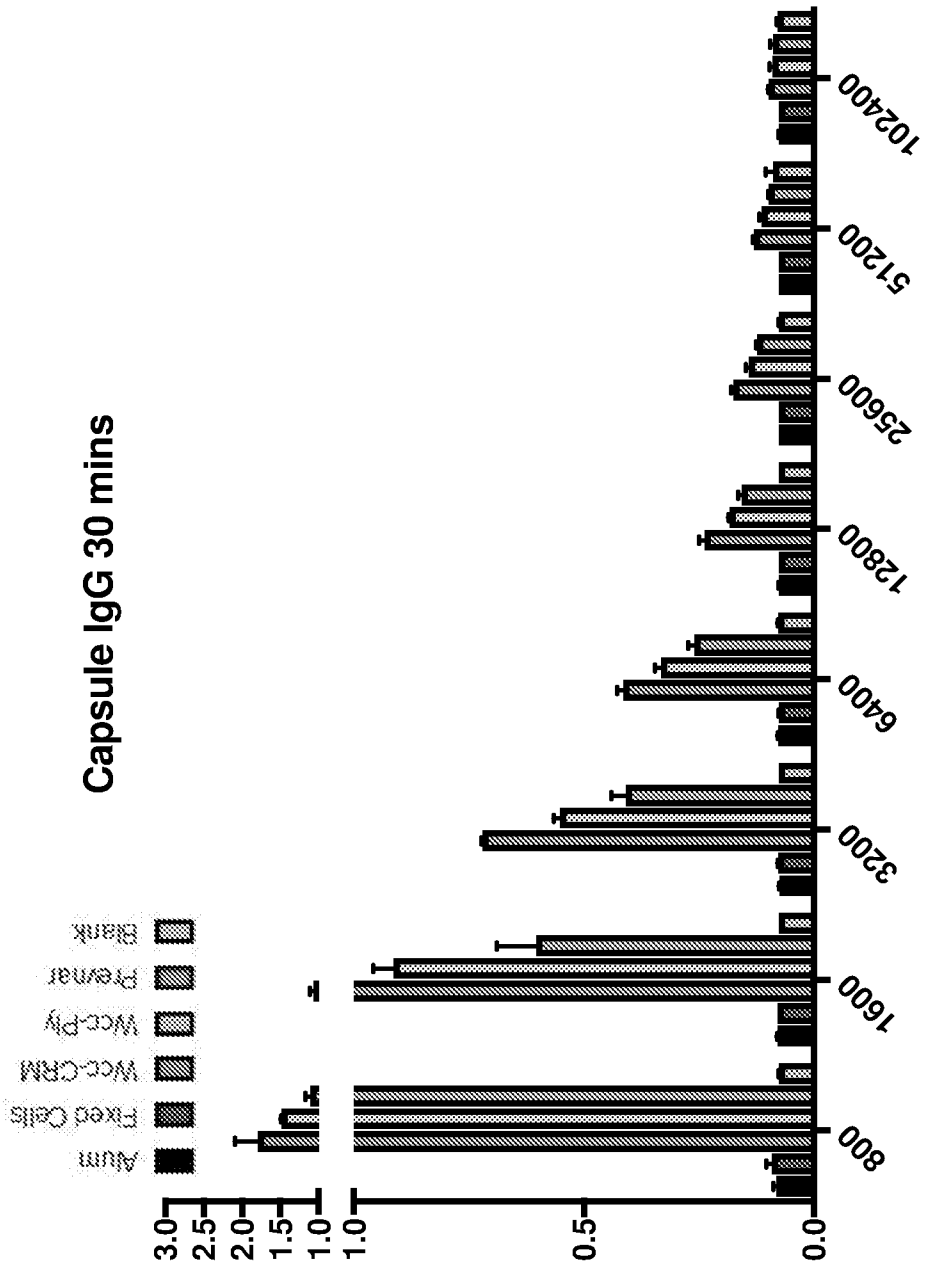

Ply 7 mins
Alum
Fixed Cells
Wcc-CRM
Wcc-Ply
Prevnar
Blank
3.0
2.5
2.0
1.5
1.0
1.0
0.5
0.0
1600
3200
6400
12800
25600
51200
102400
204800

FIG. 19

FIG. 20


Capsule IgG 30 mins
Alum
Fixed Cells
Wcc-CRM
Wcc-Ply
Prenar
Blank
3.0
2.5
2.0
1.5
1.0
0.5
0.0
800
1600
3200
6400
12800
25600
51200
102400

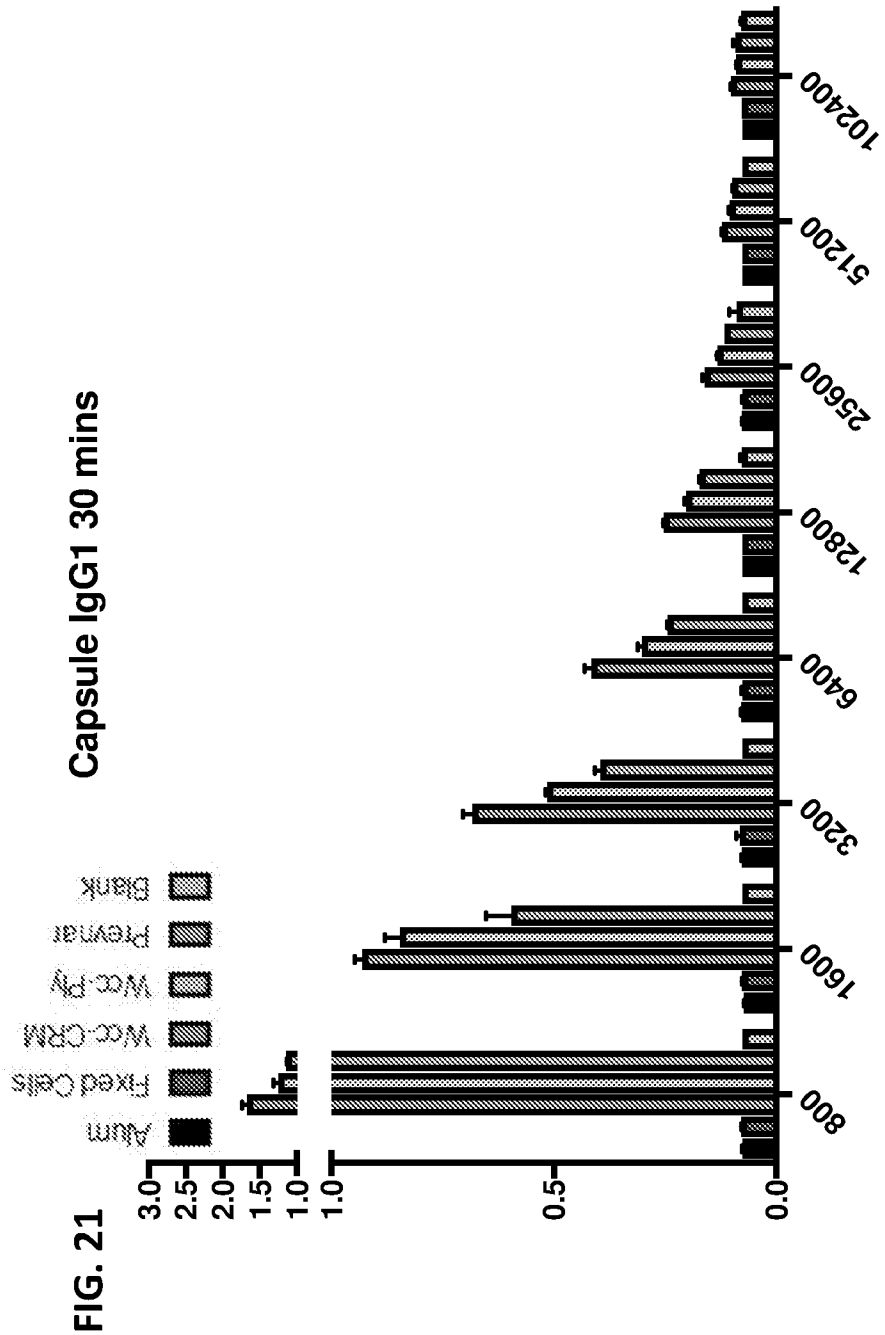

Capsule IgG1 30 mins
Alum
Fixed Cells
Wcc-CRM
Wcc-PV
Prevnar
Blank
3.0
2.5
2.0
1.5
1.0
1.0
0.5
0.0
800
1600
3200
6400
12800
25600
51200
102400

FIG. 21

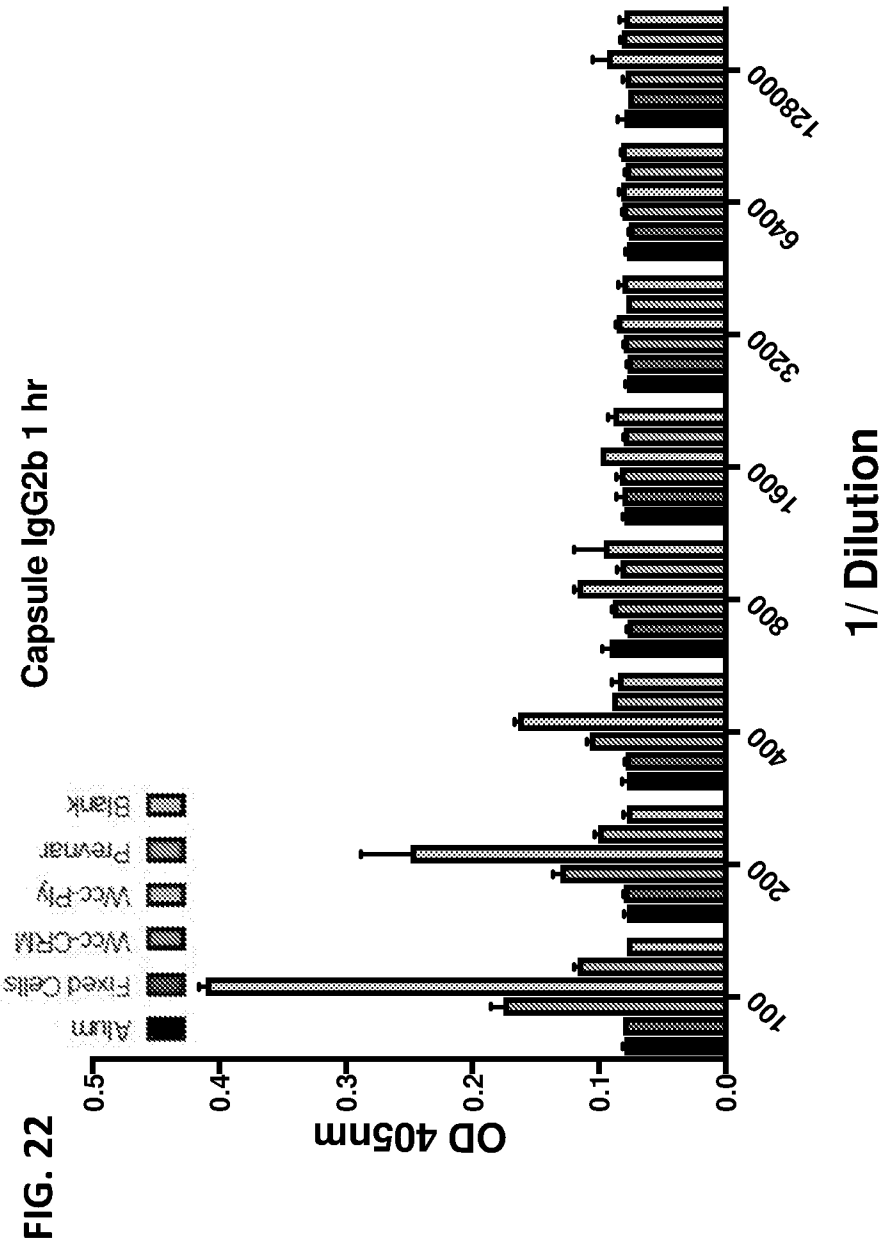

Capsule IgG2b 1 hr
Alum
Fixed Cells
Wcc-CRIII
Wcc-Ply
Pneumar
Blank
OD 405nm
0.0
0.1
0.2
0.3
0.4
0.5
100
200
400
800
1600
3200
6400
128000
1/ Dilution

FIG. 22

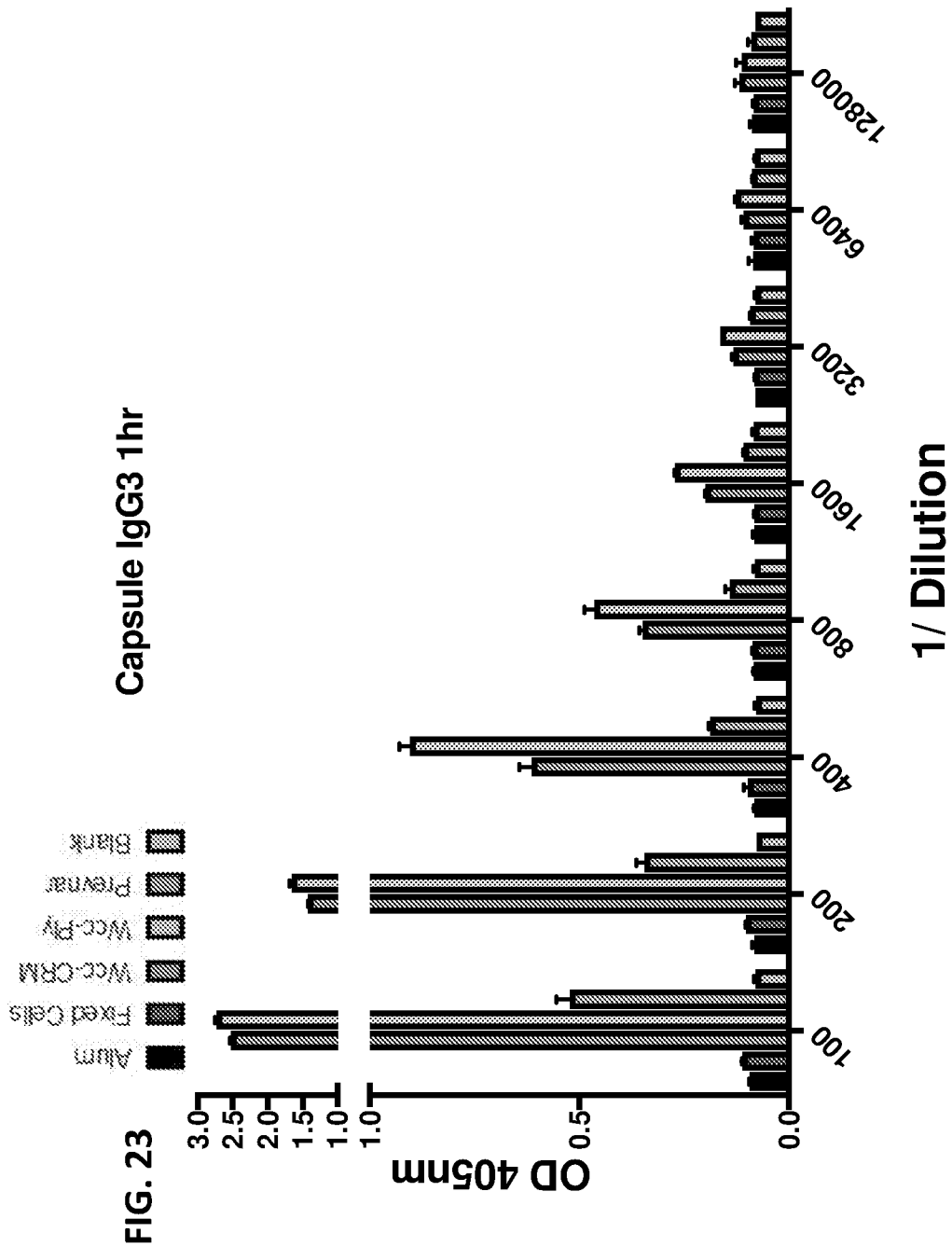

Capsule IgG3 1hr
Alum
Fixed Cells
Wcc-CRM
Wcc-PN
Prevnar
Blank
OD 405nm
3.0
2.5
2.0
1.5
1.0
1.0
0.5
0.0
100
200
400
800
1600
3200
6400
12800
1/ Dilution

FIG. 23

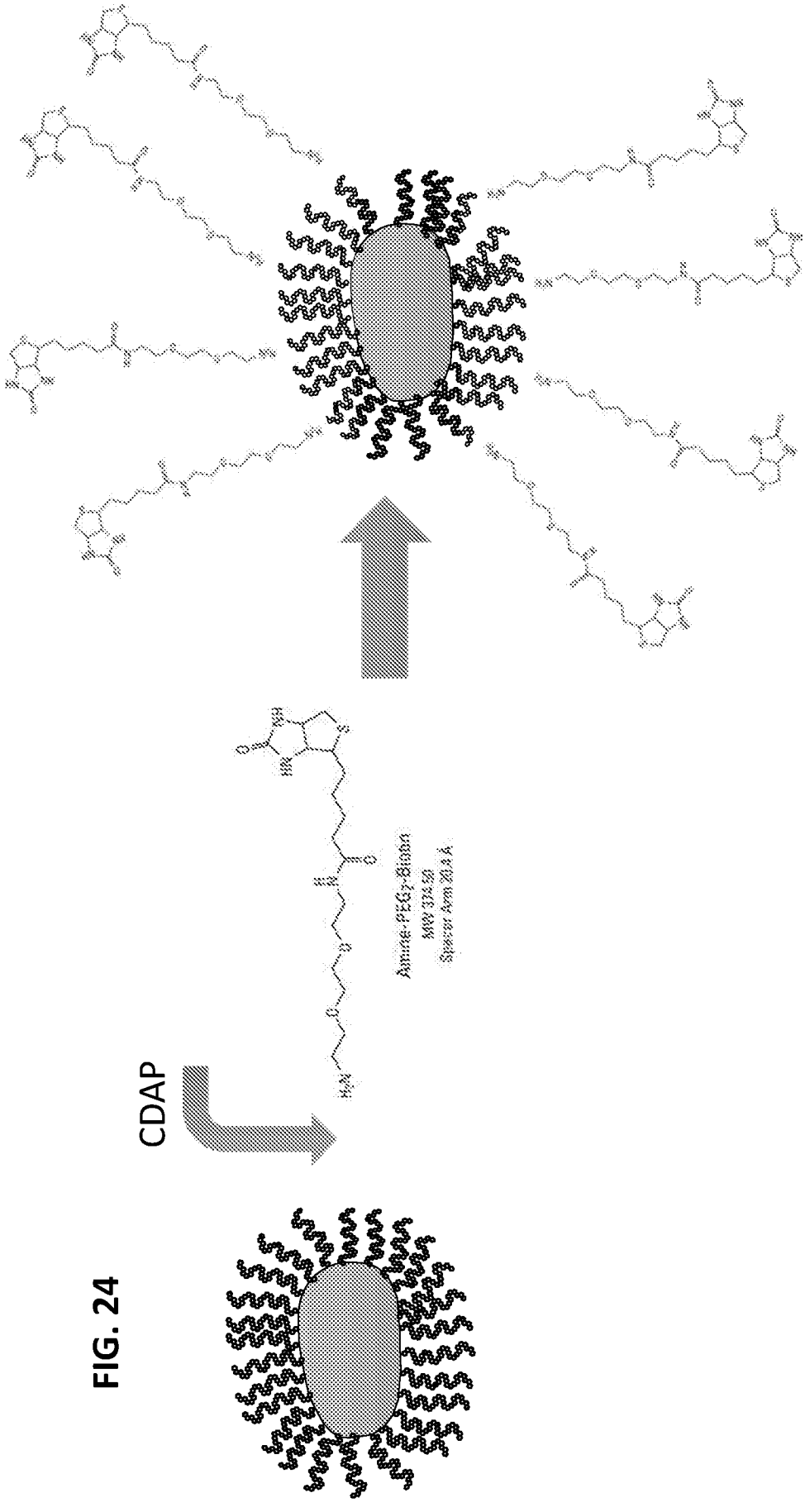
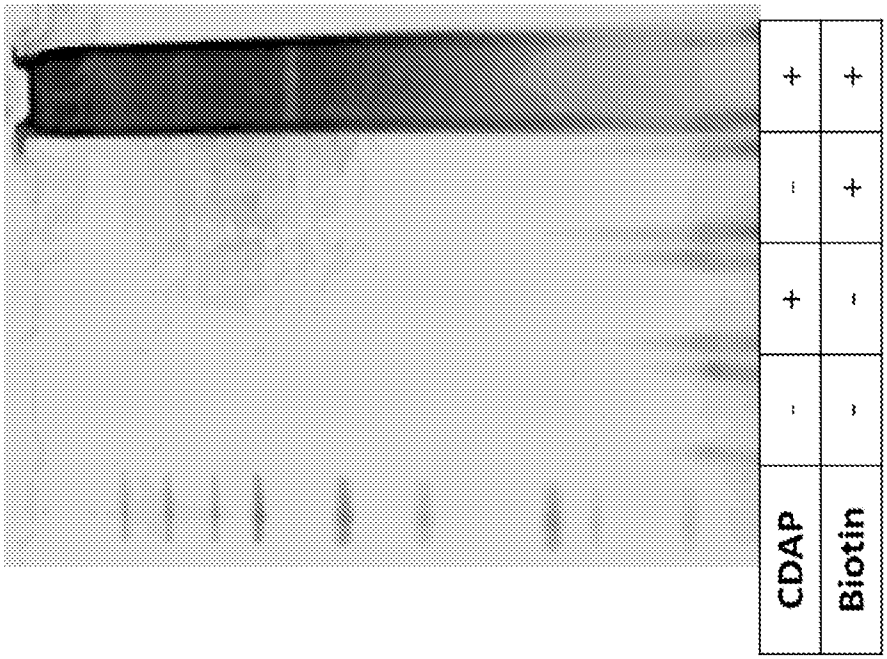


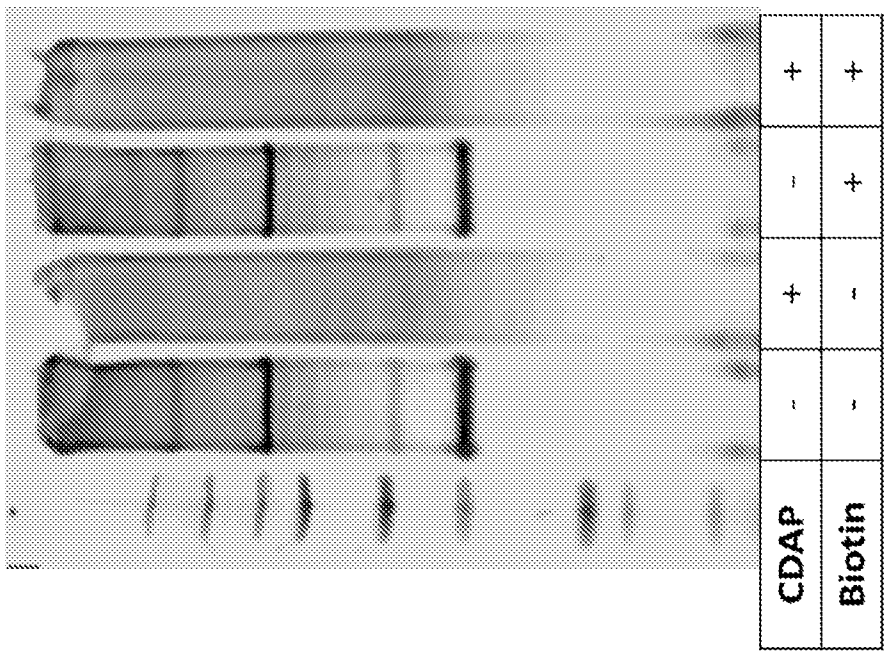
FIG. 24

FIG. 25B



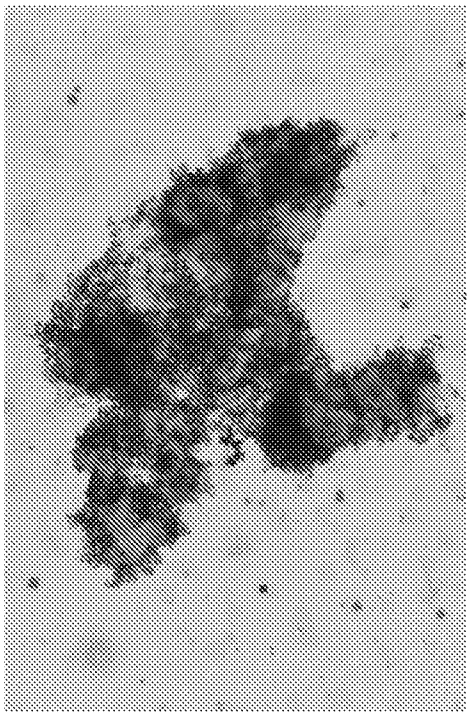
Anti-Biotin

FIG. 25A



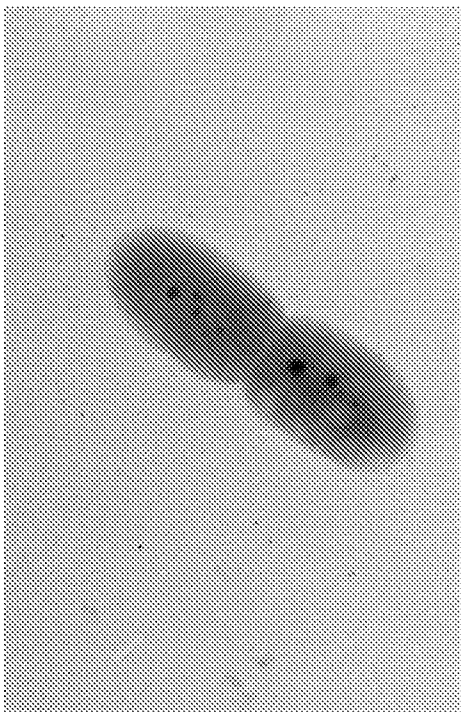
Anti-PPS4

FIG. 26B



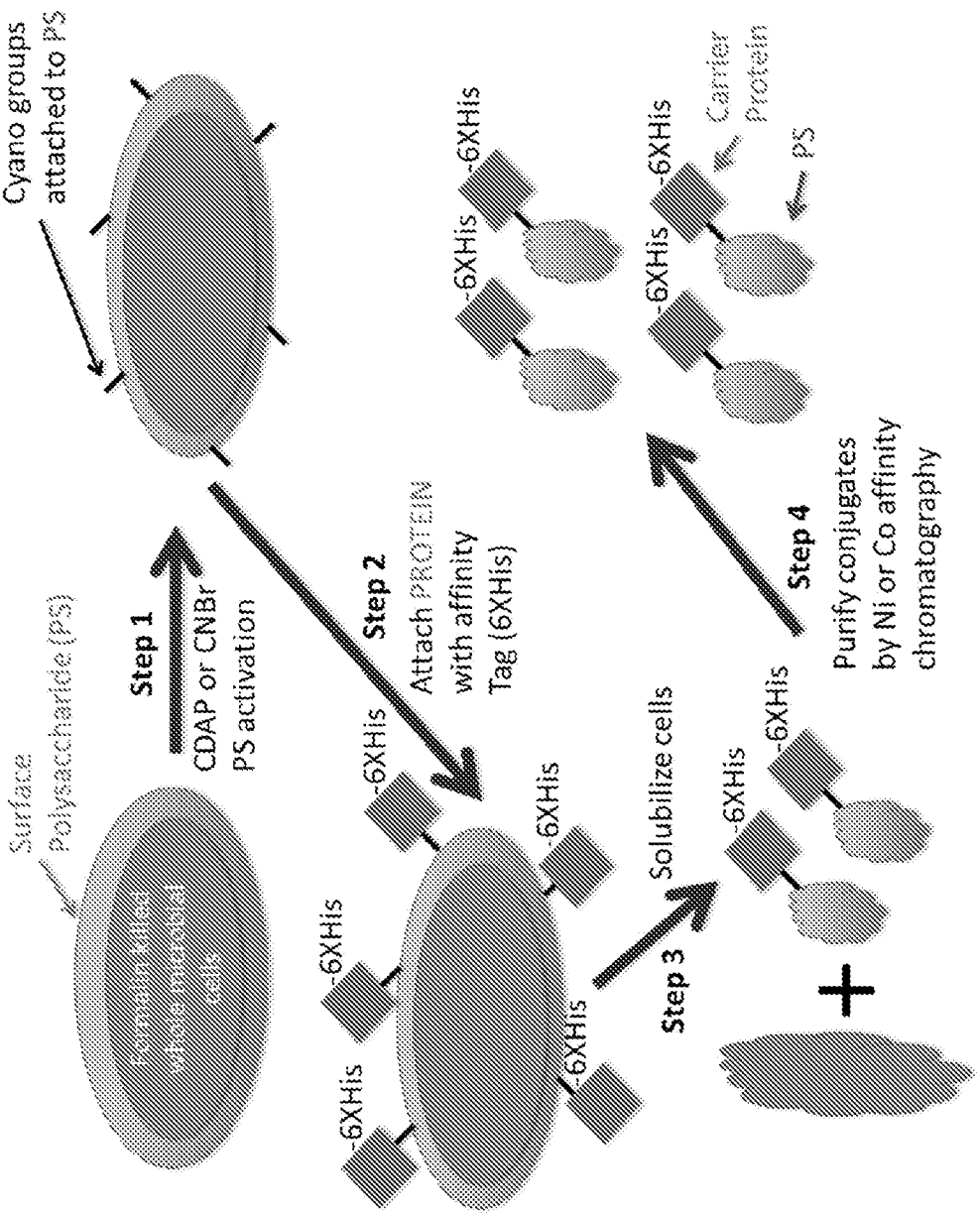
CDAP and Biotin

FIG. 26A



Fixed Cells

FIG. 27


Surface Polysaccharide (PS)
Step 1
CDAP or CNBr
PS activation
Cyano groups attached to PS
Step 2
Attach PROTEIN with affinity Tag (6XHis)
-6XHis
Solubilize cells
Step 3
Step 4
Purify conjugates by Ni or Co affinity chromatography
Carrier Protein
PS

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/041258

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/00; A61K 39/02; A61K 39/116; A61K 39/385; A61K 39/395; A61P 31/04 (2017.01)

CPC - A61K 39/116; A61K 39/385; A61K 47/6415; A61K 47/646; A61K 2039/6037; A61K 2039/62; C07K 1/084 (2017.08)

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)

See Search History document

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

USPC - 424/184.1; 424/193.1; 424/194.1; 424/197.11; 424/203.1; 424/234.1; 530/395; 530/409 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0195922 A1 (LEE) 02 August 2012 (02.08.2012) entire document	1-3, 11-14, 28, 29, 38-40, 42, 47
--		
Y		7-10, 41, 43-46, 51-58
Y	US 2016/0136257 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 19 May 2016 (19.05.2016) entire document	7-10, 51-54
Y	US 2011/0212466 A1 (DESHAYES et al) 01 September 2011 (01.09.2011) entire document	41
Y	US 2009/0240030 A1 (JU et al) 24 September 2009 (24.09.2009) entire document	43
Y	US 2014/0275482 A1 (POLUKHTIN) 18 September 2014 (18.09.2014) entire document	44-46, 51-58
A	ARSLAN et al. "Cyclodextrin Mediated Polymer Coupling via Thiol–Maleimide Conjugation: Facile Access to Functionalizable Hydrogels," RSC Advances, 28 October 2014 (28.10.2014), Vol. 4, Pgs. 1-9. entire document	1-3, 7-14, 28, 29, 38-47, 51-58
A	PASSWELL et al. "Safety and Immunogenicity of Improved Shigella O-Specific Polysaccharide-Protein Conjugate Vaccines in Adults in Israel," Infect. Immun. 30 March 2001 (31.03.2001), Vol. 69, No. 3, Pgs. 1351-1357. entire document	1-3, 7-14, 28, 29, 38-47, 51-58
A	WANG et al. "Synthesis of Neisseria meningitidis Serogroup W135 Capsular Oligosaccharides for Immunogenicity Comparison and Vaccine Development," Angewandte Chemie International Edition, 26 August 2013 (26.08.2013), Vol. 52, Pgs. 9157-9161. entire document	1-3, 7-14, 28, 29, 38-47, 51-58

☐ Further documents are listed in the continuation of Box C. ☐ 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
27 September 2017	17 OCT 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/041258

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

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

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

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

3. ☒ Claims Nos.: 4-6, 15-27, 30-37, 48-50, 59-71
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.