

MODIFIED INFLUENZA HEMAGGLUTININ PROTEINS AND USES THEREOF**CROSS REFERENCE TO RELATED APPLICATION**

[001] This application claims priority from U.S. Provisional Application Serial No. 61/426,390, filed December 22, 2010, which is hereby incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

[002] The present invention is generally directed to modified influenza hemagglutinin (HA) proteins and methods for making and using them, including their use in immunogenic compositions such as vaccines for the treatment and/or prevention of influenza infection.

DESCRIPTION OF TEXT FILE SUBMITTED ELECTRONICALLY

[003] The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: NOV_046_00US_SeqList_ST25.txt, date recorded: October 29, 2010, file size: 10 kilobytes).

BACKGROUND OF THE INVENTION

[004] Influenza virus is a member of Orthomyxoviridae family and contains a segmented negative-sense RNA genome. The influenza virion includes the following proteins: hemagglutinin (HA), neuraminidase (NA), matrix (M1), proton ion-channel protein (M2), nucleoprotein (NP), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), and nonstructural protein 2 (NS2). The HA, NA, M1, and M2 proteins are membrane associated, whereas NP, PB1, PB2, PA, and NS2 are associated with the nucleocapsid. The HA protein is an envelope glycoprotein responsible for virus attachment to the host cell and is a major source of immunodominant epitopes for virus neutralization and protective immunity. Accordingly, the HA protein is considered an important component for prophylactic influenza vaccines because of its relatively robust immunogenicity.

[005] Currently licensed vaccines that incorporate HA include the inactivated influenza A and B virus vaccines (*e.g.* trivalent vaccines for parenteral administration). The available commercial influenza vaccines are whole virus (WV) or subvirion (SV) virus vaccines. The WV vaccine contains intact, inactivated virions, whereas the SV vaccines are treated with

solvents and contain nearly all of the viral structural proteins and some of the viral envelopes. Furthermore, when a SV virus is treated with detergent and further purified, it contains mainly aggregates of HA, NA, and NP proteins.

[006] In addition to attenuated WV or SV influenza vaccines which comprise HA, several recombinant HA products have been developed as vaccine candidates. These approaches have focused on the expression, production, and purification of influenza HA proteins, including expression of these proteins using baculovirus infected insect cells.

[007] Recently, attempts to produce more effective vaccines have focused on the expression of HA in conjunction with one or more additional viral proteins (*e.g.* influenza M1 and/or NA) to form protein macromolecular particles, such as virus-like particles (VLPs) (Pushko *et al.*, 2005, *Vaccine* 23: 5751-9). VLPs mimic the overall structure of the virus particle without the requirement of containing potentially infectious material. VLPs lack a viral DNA or RNA genome, but retain the three-dimensional structure of an authentic virus.

[008] Although presently used HA-containing influenza VLPs have been well-tolerated following prophylactic administration, these VLP vaccines have exhibited questionable efficacy in certain populations, notably the young, the elderly, and individuals with chronic medical conditions. Accordingly, there is a need for improved influenza VLP vaccines for use in these highly susceptible patient populations. The present inventors have addressed this need by developing influenza HA proteins that induce enhanced immunogenicity, particularly when expressed in the context of an influenza VLP.

SUMMARY OF THE INVENTION

[009] The present inventors have observed that the removal of one or more glycosylation sites from an influenza hemagglutinin (HA) protein surprisingly improves the ability of the HA protein to induce an immune response. Moreover, the present inventors have observed that HA proteins which have been modified to remove one or more glycosylation sites still maintain their conformational structure and unexpectedly retain their ability to interact to with other influenza virus proteins such as M1 and NA when expressed in the context of an influenza VLP. Such glycosylation site modifications will therefore generally improve the immunogenicity of an HA-based influenza vaccine (*e.g.*, an influenza VLP vaccine) when produced in an appropriate expression system. Thus, this discovery also lessens the requirement for the amount of viral antigen to be used per dose of vaccine and concomitantly results in increased vaccine yields per batch of vaccine manufactured.

[010] Accordingly, in one aspect, the present invention provides hemagglutinin (HA) proteins which have been modified to remove one or more glycans from a location on the HA protein (*i.e.* at glycosylation sites). Such modified HA proteins can be expected to induce stronger immune responses against infection with influenza virus than their unmodified counterparts. The glycans selected for removal may be found in either the stem region or in the globular head of the HA protein. In a specific embodiment, one of more glycans targeted for removal is/are found in the within the receptor binding domain (RBD) of the globular head. In various embodiments described herein, the glycan to be removed is an N-linked glycan (*i.e.*, a glycan attached to a nitrogen of asparagine or arginine side chain).

[011] In one embodiment, the HA protein is modified by engineering the HA protein to harbor one or more amino acid mutations that removes one or more glycosylation sites from said HA protein. The one or more amino acid mutations that removes one or more glycosylation sites from the HA protein, in one embodiment, is one amino acid mutation, or two amino acid mutations, or three amino acid mutations, or four amino acid mutations, or five amino acid mutations. In one embodiment, the amino acid mutation(s) is/are made in the stem region. In another embodiment, the amino acid mutation(s) is/are made in the globular head region. In yet another embodiment, amino acid mutation(s) is/are made in both the stem region and the globular head region. In one embodiment, one or more of the amino acid mutations occurs within the receptor binding domain (RBD) within the globular head of the modified HA protein. In a further embodiment, all of the amino acid mutations occur in the RBD within the globular head of the modified HA protein. In a specific embodiment, one or more of the amino acid mutations is created by altering an NX(T/S) (SEQ ID NO: 1) glycosylation consensus sequence in the HA protein, wherein X is any amino acid and T/S is a threonine or serine residue. In an exemplary embodiment, the amino acid mutation is located at an amino acid position corresponding to amino acid residues selected from 170-172 and 181-183 of SEQ ID NO: 2.

[012] In an alternative embodiment, the HA protein is modified to remove one or more glycans via enzymatic treatment. In one embodiment, the HA proteins is modified to remove one or more glycans via treatment with endoglycosidases. In other embodiments, the HA protein is modified to remove one of more glycans via chemical treatment. In a specific embodiment, one or more glycans are removed chemically by a hydrazine-like chemical reaction.

[013] The modified influenza virus HA proteins of the present invention may be derived from interpandemic (*e.g.* annual or seasonal) influenza strains or alternatively, the HA

proteins may be derived from strains with the potential to cause a pandemic outbreak (*i.e.* influenza strains with new hemagglutinin compared to hemagglutinin in currently circulating strains, or influenza strains which are pathogenic in avian subjects and have the potential to be transmitted horizontally in the human population, or influenza strains which are pathogenic to humans). Depending on the particular season and on the nature of the HA antigen included in the vaccine, the influenza virus HA may be derived from one or more of the following subtypes: H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, or H16. In exemplary embodiments, the influenza virus HA is derived from H1, H2, H3, H5, H7, or H9 subtypes.

[014] In various embodiments described herein, the HA protein may be derived from an avian influenza virus strain. In one embodiment, the avian influenza virus strain is H5N1. In another embodiment, the avian influenza virus strain is H9N2. In yet another embodiment, the avian influenza virus strain is H7N3. In yet another embodiment, the avian influenza virus strain is H7N7.

[015] In alternative embodiments, the HA protein may be derived from a swine influenza virus strain. In one embodiment, the swine influenza virus strain is H1N1. In another embodiment, the swine influenza virus strain is H1N2. In yet another embodiment, the swine influenza virus strain is H3N2.

[016] In alternative embodiments, the HA protein may be derived from a human influenza virus strain. In one embodiment, the human influenza virus strain is H1N1. In another embodiment, the human influenza virus strain is a B strain.

[017] In additional embodiments, the immunogenic composition may comprise more than one modified HA protein. For instance, the immunogenic composition may comprise a modified HA protein derived an avian influenza virus strain and a modified HA protein derived from a swine influenza virus strain.

[018] In one embodiment, the modified HA protein exhibits hemagglutinin activity. Such modified HA proteins retain their ability to bind and agglutinate red blood cells (erythrocytes).

[019] In another aspect, the present invention provides virus-like particles (VLPs) comprising one or more modified influenza HA proteins. Such VLPs comprising modified influenza HA proteins can be expected to induce stronger immune responses against influenza virus than their VLP counterparts comprising unmodified influenza HA proteins. In one embodiment, the modified HA protein comprises one or more amino acid mutations that removes one or more glycosylation sites from said HA protein. In a further embodiment,

one or more of the amino acid mutations occurs within the receptor binding domain (RBD) of the modified HA protein. In a specific embodiment, one or more of the amino acid mutations is created by altering an NX(T/S) (SEQ ID NO: 1) glycosylation consensus sequence in the HA protein, wherein X is any amino acid and T/S is a threonine or serine residue. In an exemplary embodiment, the amino acid mutation is located at an amino acid position corresponding to amino acid residues selected from 170-172 and 181-183 of SEQ ID NO: 2.

[020] In various embodiments described herein, the VLPs of the invention may comprise additional influenza proteins, including, but not limited to, M1, M2, NA, NP, PB1, PB2, and NS2. In an exemplary embodiment, the VLPs of the invention comprise M1, NA, and a modified HA protein. In one embodiment, the M1 protein comprises the amino acid residues YKKL at the amino acid positions corresponding to positions 100-103 of SEQ ID NO: 3. In another embodiment, the M1 protein is derived from an avian influenza virus strain. In yet another embodiment, the M1 protein is derived from an avian influenza virus strain selected from H5N1, H9N2, H7N3, and H7N7. In a specific embodiment, the M1 protein is derived from the avian influenza virus strain A/Indonesia/5/05 (H5N1). In a further specific embodiment, the M1 protein derived from the avian influenza virus strain A/Indonesia/5/05 (H5N1) comprises SEQ ID NO: 3. In another embodiment, the M1 protein is derived from a swine influenza virus strain. In yet another embodiment, the M1 protein is derived a swine influenza virus strain selected from H1N1, H1N2, and H3N2. In a specific embodiment, the M1 protein is derived from the swine influenza virus strain A/California/04/09 (H1N1). In a further specific embodiment, the M1 protein derived from the swine influenza virus strain A/California/04/09 (H1N1) comprises SEQ ID NO: 4.

[021] The present invention also provides a method of inducing substantial immunity to influenza virus infection in a mammal susceptible to influenza comprising administering at least one effective dose of a vaccine comprising one or more HA proteins which have been modified to remove one or more glycosylation sites. In an exemplary embodiment, the present invention provides a method of inducing substantial immunity to influenza virus infection in a mammal susceptible to influenza comprising administering at least one effective dose of a vaccine comprising an influenza VLP, wherein the influenza VLP comprises one or more HA protein which have been modified to remove one or more glycosylation sites. In one embodiment, the mammal is a human. In one embodiment, said method comprises administering to an animal said vaccine orally, parenterally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

[022] The present invention also provides a method of formulating a vaccine that induces substantial immunity to influenza virus infection in a mammal susceptible to influenza, comprising adding to said formulation an effective dose of a vaccine comprising one or more HA proteins which have been modified to remove one or more glycosylation sites. In an exemplary embodiment, the present invention provides a method of formulating a vaccine that induces substantial immunity to influenza virus infection in a mammal susceptible to influenza, comprising adding to said formulation an effective dose of a vaccine comprising an influenza VLP, wherein the influenza VLP comprises one or more HA proteins which have been modified to remove one or more glycosylation sites.

[023] In another aspect, the present invention provides a method for producing a VLP derived from influenza by constructing one or more recombinant constructs that encode an HA protein which has been modified to remove one or more glycosylation sites, and at least one additional structural protein derived from influenza virus. In an exemplary embodiment, the VLP comprises M1, NA, and an HA protein which has been modified to remove one or more glycosylation sites. In one embodiment, the one or more recombinant constructs are used to transfect, infect, or transform a suitable host cell. In an exemplary embodiment, the recombinant construct(s) is/are baculovirus constructs. The host cell is cultured under conditions which permit the expression of the modified HA protein and at least one structural protein derived from influenza virus, and the VLP is formed in the host cell. The infected cell media containing the influenza VLP is harvested and the VLP is purified. In various embodiments described herein, the host cell may be a eukaryotic cell. In an exemplary embodiment, the host cell is an insect cell.

[024] The invention also features a method of formulating a drug substance containing an influenza VLP which comprises one or more HA proteins which have been modified to remove one or more glycosylation sites, and at least one additional structural protein derived from influenza virus. In one embodiment, one or more recombinant constructs encoding a modified HA protein and at least one structural protein derived from influenza virus are used to transfect, infect, or transform a suitable host cell. In an exemplary embodiment, the recombinant construct(s) is/are baculovirus constructs. The host cell is cultured under conditions which permit the expression of the modified HA protein and at least one structural protein derived from influenza virus, and the VLP is formed in the host cell. The influenza VLP is isolated and purified and a drug substance is formulated containing the influenza VLP. The drug substance may further include an adjuvant. In addition, the invention provides a method for formulating a drug product, by mixing such a drug substance

containing an influenza VLP with a lipid vesicle, *i.e.*, a non-ionic lipid vesicle. Thus, the influenza VLPs may bud as enveloped particles from the infected cells. The budded influenza VLPs may be isolated and purified by ultracentrifugation or column chromatography as drug substances and formulated alone or with adjuvants such as Novasomes[®], a product of Novavax, Inc., as drug products such as vaccines. Novasomes[®], which provide an enhanced immunological effect, are further described in U.S. Pat. No. 4,911,928, which is incorporated herein by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[025] **Figure 1** depicts the sequence of the A/Indonesia/05/2005 (H5N1) HA1 sequence and illustrates the positions of glycans at amino acid residues 170-172 and 181-183.

[026] **Figure 2** is an SDS-PAGE of A/Indonesia/05/2005 (H5N1) influenza VLPs comprising HA proteins modified to remove a glycosylation site at amino acid positions 170-172 (lanes 4-6), at amino acid positions 181-183 (lane 2), or both amino acid positions 170-172 and 181-183 (lane 3).

DETAILED DESCRIPTION

Definitions:

[027] As used herein, the term “antigenic formulation” or “antigenic composition” refers to a preparation which, when administered to a vertebrate, especially a bird or a mammal, will induce an immune response.

[028] As used herein, the term “adjuvant” refers to a compound that, when used in combination with a specific immunogen (*e.g.* a VLP) in a formulation, augments or otherwise alters or modifies the resultant immune response. Modification of the immune response includes intensification or broadening the specificity of either or both antibody and cellular immune responses. Modification of the immune response can also mean decreasing or suppressing certain antigen-specific immune responses.

[029] As used herein, the term “avian influenza virus” refers to influenza viruses found chiefly in birds but that can also infect humans or other animals. In some instances, avian influenza viruses may be transmitted or spread from one human to another. An avian influenza virus that infects humans has the potential to cause an influenza pandemic, *i.e.*, morbidity and/or mortality in humans. A pandemic occurs when a new strain of influenza virus (a virus in which human have no natural immunity) emerges, spreading beyond individual localities, possibly around the globe, and infecting many humans at once.

[030] As used herein, the term “chimeric protein” refers to a construct that links at least two heterologous proteins into a single macromolecule (*e.g.*, a fusion protein).

[031] As used herein, an “effective dose” generally refers to that amount of the VLP of the invention sufficient to induce immunity, to prevent and/or ameliorate influenza virus infection or to reduce at least one symptom of influenza infection and/or to enhance the efficacy of another dose of a VLP. An effective dose may refer to the amount of the VLP sufficient to delay or minimize the onset of an influenza infection. An effective dose may also refer to the amount of the VLP that provides a therapeutic benefit in the treatment or management of influenza infection. Further, an effective dose is the amount with respect to the VLPs of the invention alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or management of an influenza viral infection. An effective dose may also be the amount sufficient to enhance a subject’s (*e.g.*, a human’s) own immune response against a subsequent exposure to influenza virus. Levels of immunity can be monitored, *e.g.*, by measuring amounts of neutralizing secretory and/or serum antibodies, *e.g.*, by plaque neutralization, complement fixation, enzyme-linked immunosorbent, or microneutralization assay. In the case of a vaccine, an “effective dose” is one that prevents disease or reduces the severity of symptoms.

[032] As used herein, the term “influenza VLP” refers to a VLP comprising at least one influenza protein. Said VLPs can comprise additional influenza and/or non-influenza proteins.

[033] As used herein, the term “hemagglutinin” (or “HA” or “HA protein” or “hemagglutinin protein”) refers to a polypeptide whose amino acid sequence includes at least one characteristic sequence of HA. A wide variety of HA sequences from influenza isolates are known in the art; indeed, the National Center for Biotechnology Information (NCBI) maintains a database (www.ncbi.nlm.nih.gov/genomes/FLU/flu.html) that, as of the filing of the present application included over 9000 HA sequences. Those of ordinary skill in the art, referring to this database, can readily identify sequences that are characteristic of HA polypeptides generally, and/or of particular HA polypeptides (*e.g.*, H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, or H16 polypeptides; or of HAs that mediate infection of particular hosts, *e.g.*, avian, camel, canine, cat, civet, environment, equine, human, leopard, mink, mouse, seal, stone martin, swine, tiger, whale, etc).

[034] As used herein, the term “hemagglutinin activity” refers to the ability of HA-containing proteins, VLPs, or portions thereof to bind and agglutinate red blood cells (erythrocytes).

[035] As used herein, the terms “N-linked glycan” and “N-linked oligosaccharide” refer to an oligosaccharide which is covalently bonded to a conjugate (e.g. HA) by an N-glycosidic linkage.

[036] As used herein, the term “neuraminidase activity” refers to the enzymatic activity of NA-containing proteins, VLPs, or portions thereof to cleave sialic acid residues from substrates including proteins such as fetuin.

[037] As use herein, the term “infectious agent” refers to microorganisms that cause an infection in a vertebrate. Usually, the organisms are viruses, bacteria, parasites and/or fungi. The term also refers to different antigenic variations of the same infectious agent.

[038] As used herein, the term “immune stimulator” refers to a compound that enhances an immune response via the body’s own chemical messengers (cytokines). These molecules comprise various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (e.g., granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immune stimulator molecules can be administered in the same formulation as the influenza VLPs, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect.

[039] As used herein, the term “immunity” refers to induction of the immune system of a vertebrate wherein said induction results in the prevention, amelioration, and/or reduction of at least one symptom of an infection in said vertebrate. Immunity may also refer to a hemagglutination inhibition (HI) titer of ≥ 40 when VLPs of the invention have been administered to a vertebrate and said VLPs have induced an immune response against a HA of an influenza virus.

[040] As used herein, the term “receptor binding domain” (or “RBD”) refers to an approximately 148 amino acid domain that includes the sialic acid-binding site of HA. A subregion of the HA receptor binding domain is known as the RBD-A (“top-of-head”) domain and is contained within amino acids 131 to 143, 170 to 182, 205 to 215, and 257 to 262 based upon a numbering scheme established for the HA from influenza strain A/South Carolina/1/1918. (Chih-Jen Wei *et al.*, 2010, *Sci. Transl Med* 2: 24ra21).

[041] As used herein, the term “seasonal influenza virus” refers to the influenza viral strains that have been determined to be passing within the human population for a given influenza

season based on epidemiological surveys conducted by National Influenza Centers worldwide. These epidemiological studies, and some isolated influenza viruses, are sent to one of four World Health Organization (WHO) reference laboratories, one of which is at the Centers for Disease Control and Prevention (CDC) in Atlanta for detailed testing. These laboratories test how well antibodies made to the current vaccine react to the circulating virus and new flu viruses. This information, along with information about flu activity, is summarized and presented to an advisory committee of the U.S. Food and Drug Administration (FDA) and at a WHO meeting. These meetings result in the selection of three viruses (two subtypes of influenza A viruses and one influenza B virus) to go into flu vaccines for the following fall and winter. The selection occurs in February for the northern hemisphere and in September for the southern hemisphere. Usually, one or two of the three virus strains in the vaccine changes each year.

[042] As used herein, the term “swine influenza virus” refers to influenza viruses found chiefly in pigs but that can also infect humans or other animals. In some instances, swine influenza viruses may be transmitted or spread from one human to another. A swine influenza virus that infects humans has the potential to cause an influenza pandemic, *i.e.*, morbidity and/or mortality in humans. A pandemic occurs when a new strain of influenza virus (a virus in which human have no natural immunity) emerges, spreading beyond individual localities, possibly around the globe, and infecting many humans at once.

[043] As used herein, the term “vaccine” refers to a preparation of dead or weakened pathogens, or of derived antigenic determinants that is used to induce formation of antibodies or immunity against the pathogen. A vaccine is given to provide immunity to the disease, for example, influenza, which is caused by influenza viruses. In addition, the term “vaccine” also refers to a suspension or solution of an immunogen (*e.g.* VLP) that is administered to a vertebrate to produce protective immunity, *i.e.*, immunity that prevents or reduces the severity of disease associated with infection. The present invention provides for vaccine compositions that are immunogenic and may provide protection against a disease associated with infection.

[044] As use herein, the term “vertebrate” or “subject” or “patient” refers to any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species. Farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks,

geese, and the like are also non-limiting examples. The terms “mammals” and “animals” are included in this definition. Both adult and newborn individuals are intended to be covered.

[045] As used herein, the term “virus-like particle” (VLP) refers to a structure that in at least one attribute resembles a virus but which has not been demonstrated to be infectious. Virus-like particle in accordance with the invention do not carry genetic information encoding for the proteins of virus-like particles. In general, virus-like particles lack a viral genome and, therefore, are noninfectious. In addition, virus-like particles can often be produced in large quantities by heterologous expression and can be easily purified.

Influenza Hemagglutinin (HA):

[046] Influenza viruses are RNA viruses which are characterized by a lipid membrane envelope containing two glycoproteins, hemagglutinin (HA) and neuraminidase (NA), embedded in the membrane of the virus particular. There are 16 known HA subtypes and 9 NA subtypes, and different influenza strains are named based on the number of the strain's HA and NA subtypes. Based on comparisons of amino acid sequence identity and of crystal structures, the HA subtypes have been divided into two main groups and four smaller clades. The different HA subtypes do not necessarily share strong amino acid sequence identity, but the overall 3-dimensional structures of the different HA subtypes are similar to one another, with several subtle differences that can be used for classification purposes. For example, the particular orientation of the membrane-distal subdomains in relation to a central α -helix is one structural characteristic commonly used to determine HA subtype (Russell *et al.*, *Virology*, 325:287, 2004).

[047] HA is a homotrimeric integral membrane glycoprotein. It is cylindrical in shape and contains an ectodomain comprised of a globular head and a stem region. Both the globular head and stem regions of HA carry N-linked oligosaccharides which can affect the functional properties of the protein. For instance, HA mutants lacking one or more stem oligosaccharides have shown reduced fusion activity, leading to the conclusion that oligosaccharides in the stem region preserve the metastable form of HA required for cell fusion (Ohuchi *et al.*, 1997, *J. Virology* 71: 3719-25). Furthermore, the presence of glycans near the proteolytic activation site of HA have been shown to modulate cleavage and influence the virulence of influenza virus (Deshpande *et al.*, 1987, *Proc Natl Acad Sci USA* 84: 36-40). Moreover, glycosylation of the influenza virus HA is important for protection against proteolytic degradation (Schwartz *et al.*, 1976, *J. Virology* 19: 782-91).

[048] Although little is known regarding how the structure and composition of glycans affect HA activity, the role of glycans in stabilizing the conformation of HA and their influence on the ability of HA to interact with other influenza proteins has been suggested (Wilson *et al.*, 1981, *Nature* 289: 373-8). Indeed, growth of viruses lacking one or more glycans from the globular head region was found to be impaired in MDCK cells as well as in embryonated chicken eggs (Wagner *et al.*, 2001, *Intl Cong Series* 1219: 375-82). This was due in large part to the limited release of progeny viruses from host cells as a result of enhanced receptor affinity of the mutated HA. *Id.* Furthermore, when glycans were eliminated from the HA stem, the resulting viruses showed temperate sensitive growth in cell culture and in chicken eggs. These viruses had decreased pH stability, indicating that glycans are important for maintaining the HA protein in a proper conformation.

Modified Hemagglutinin (HA) Proteins:

[049] The present inventors have found that influenza virus-like particles (VLPs) comprising HA proteins deficient in one or more glycans can be made and surprisingly retain HA and NA activity and potency. Such a result is unexpected, particularly given the purported role of glycans in maintaining HA conformation and stability. Moreover, the modified HA proteins, when expressed alone, or in the context of an influenza VLP, have enhanced immunogenicity as compared to their unmodified counterparts. Accordingly, in one aspect, the present application describes the generation of modified HA proteins with increased immunogenicity that can facilitate the production of improved vaccines.

[050] In accordance with the invention, any number of modifications can be made to the HA proteins to remove a glycan from a location on the HA protein (*i.e.* a glycosylation site), and in one embodiment, multiple glycans (*e.g.*, two, three, four, five or six glycans) can be removed to result in an increased ability to stimulate an immune response to influenza virus infection. In an exemplary embodiment, the one or more glycans selected for removal is an N-linked glycan. In a further embodiment, only the N-linked glycan is removed. Such modifications can occur as a result of engineered point mutations, frame shift mutations, deletions, or insertions, with one or more (*e.g.*, one, two, three, four, five, six, seven or more) point mutations preferred. In alternative embodiments, one or more glycans can be removed via enzymatic or chemical treatments. For instance, HA proteins may be modified to remove one or more N-linked glycans via treatment with endoglycosidases. In other embodiments, an N-linked glycan can be removed chemically by a hydrazine-like chemical reaction.

[051] In one aspect, one or more glycosylation sites may be removed from an HA protein by mutating the nucleic acid sequence encoding said HA protein. Mutations to remove one or more glycans may be introduced into the HA proteins of the present invention using any methodology known to those skilled in the art. For example, oligonucleotide directed mutagenesis may be used to create the modified HA proteins which allows for all possible classes of base pair changes at any determined site along the encoding DNA molecule. In general, this technique involves annealing an oligonucleotide complementary (except for one or more mismatches) to a single stranded nucleotide sequence coding for the HA protein of interest. The mismatched oligonucleotide is then extended by DNA polymerase, generating a double-stranded DNA molecule which contains the desired change in sequence in one strand. The changes in sequence can, for example, result in the deletion, substitution, or insertion of an amino acid. The double-stranded polynucleotide can then be inserted into an appropriate expression vector, and a mutant or modified polypeptide can thus be produced. The above-described oligonucleotide directed mutagenesis can, for example, be carried out via PCR

[052] HA proteins for use in the compositions and methods of the invention include any HA protein having the ability to stimulate an immunogenic response to influenza virus infection. Such HA proteins include H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. As will be understood by one of ordinary skill in the art, HA proteins modified to remove a glycosylation site may be obtained by recombinant or genetic engineering techniques that are routine and well-known in the art. Modified HA proteins can, for example, be obtained by mutating the gene or genes encoding the HA protein of interest by site-directed or random mutagenesis. Such mutations may include point mutations, deletion mutations and insertional mutations. For example, one or more point mutations (*e.g.*, substitution of one or more amino acids with one or more different amino acids) may be used to construct modified HA proteins of the invention.

[053] The invention further includes homologous HA proteins which are 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical at the amino acid level to a wild-type HA protein which has been modified to remove one or more glycosylation sites and exhibits an increased ability to stimulate an immune response to influenza. The invention also includes nucleic acid molecules which encode the above described HA proteins.

[054] The invention also includes fragments of modified HA proteins which comprise at least 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, or 600 amino acid residues and retain one or more activities associated with the modified HA proteins. Such fragments may

be obtained by deletion mutations using recombinant techniques that are routine and well-known in the art, or by enzymatic digestion of the HA protein(s) of interest using any of a number of well-known proteolytic enzymes. The invention further includes nucleic acid molecules which encode the above described modified HA proteins and HA protein fragments.

[055] In one aspect, targeted amino acid substitutions are made at one or more glycosylation sites in an HA protein of interest. In one embodiment according to this aspect, one or more amino acid mutations in the NX(T/S) N-linked glycosylation consensus sequence is made to abolish the glycosylation signal sequence. Such mutations will generally prevent N-linked glycosylation and improve the immunogenicity of said HA protein.

[056] Methods of identifying N-linked glycosylation sites in the HA protein of interest are known in the art. For instance, the NetNGlyc 1.0 server (Technical University of Denmark) can be used to predict NA N-linked glycosylation sites. The software compares the matching sequence to the database of known N-linked glycosylation sites and provides a glycosylation potential score. A score of greater than 0.5 indicates a potential site and less than 0.5 indicates an unlikely site for glycosylation. Putative glycosylation sites identified using the NetNGlyc 1.0 software can then be searched against the NCBI protein database for matching sequences in naturally occurring influenza viruses using BLAST.

[057] In one embodiment, one or more glycosylation site(s) is/are removed from the globular head region. In another embodiment, one or more glycosylation site(s) is/are found removed from the stem region. In yet another embodiment, one or more glycosylation site(s) is/are removed from both the globular head region and the stem region. In an exemplary embodiment, one or more glycosylation site(s) is/are removed from the top of the globular head in a region known as the receptor-binding domain (RBD), an approximately 148 amino acid domain which harbors the sialic acid-binding site. The RBD, also known as the receptor binding site (RBS), is at the membrane distal end of each HA monomer and its specificity for sialic acid and the nature of its linkage to a vicinal galactose residue determines host-range restriction. In a specific embodiment, one or more glycosylation site(s) is removed from a sub-region of the RBD known as RBD-A. The RBD-A ("top-of-head" domain) is contained within amino acids 131 to 143, 170 to 182, 205 to 215, and 257 to 262 based upon a numbering scheme established for the HA from influenza strain A/South Carolina/1/1918 (GenBank Accession No. AF117241) (Chih-Jen Wei *et al.*, 2010, *Sci. Transl Med* 2: 24ra21).

[058] In one embodiment, amino acid substitutions are made at one or more NX(T/S) N-linked glycosylation consensus sequences in the RBD, wherein X is any amino acid and (T/S)

is either a threonine or serine residue. In an exemplary embodiment, one or more amino acid substitutions is/are made at position corresponding to an amino acid residue selected from 170-172 and 181-183 of the A/Indonesia/5/05 HA protein (SEQ ID NO: 2). Thus, one or more of the naturally occurring amino acids at these positions may be substituted with any other amino acid including Ala, Asn, Arg, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. A specific example of an HA protein which exhibits an increased ability to stimulate an immune response to influenza virus infection is an HA protein in which (1) the threonine at position 172 has been replaced with an alanine, and/or (2) the threonine at position 183 has been replaced with alanine. As will be understood by those art equipped with the disclosure of the present application, amino acid mutations at positions corresponding to asparagine residues 170 and/or 181 of the A/Indonesia/5/05 HA protein (SEQ ID NO: 2) can be made to achieve a similar result.

[059] In accordance with the invention, one or more mutations may be made in any HA protein of interest in order to increase the ability of the HA protein to stimulate an immune response to influenza virus infection. Such mutations include point mutations, frame shift mutations, deletions and insertions. In one embodiment, one or more point mutations, resulting in one or more amino acid substitutions, are used to produce HA proteins having an enhanced ability to stimulate an immune response to influenza virus infection. In an exemplary embodiment of the invention, one or more mutations at positions equivalent or corresponding to position N170 (*e.g.* N170D), T172 (*e.g.* T172A), N181 (*e.g.*, N181D) and/or T183 (*e.g.* T183A) of the A/Indonesia/5/05 (SEQ ID NO: 2) HA protein may be made to produce the desired result in other HA proteins of interest.

[060] The corresponding positions of the HA proteins identified herein (*e.g.* the A/Indonesia/5/05 HA of SEQ ID NO: 2) may be readily identified for other HA proteins by one of skill in the art. Thus, given the defined region and the assays described in the present application, one with skill in the art can make one or a number of modifications which would result in an increased ability to stimulate an immune response to influenza virus infection in any HA protein of interest.

[061] In a preferred embodiment, the modified HA proteins have one or more amino acid substitutions selected from positions corresponding to N170, T172, N181, and/or T183 as compared to the wild-type HA proteins. In other embodiments, the modified HA proteins have additional amino acid substitutions at other positions as compared to the respective wild-type HA proteins. Thus, modified HA proteins may have at least about 2, 3, 4, 5, 6, 7 or more different residues in other positions as compared to the respective wild-type HA

proteins. As will be appreciated by those of skill in the art, the number of additional positions that may have amino acid substitutions will depend on the wild-type HA protein used to generate the variants. For example, HA from the X31 influenza strain shows seven N-linked glycosylation sites (Ermonval *et al.*, 2000, *Glycobiology* 10: 77-87). Thus, in some instances, as many as 7 or more different N-glycans may be removed via targeted amino acid substitutions.

[062] As described above, in alternative embodiments, one or more glycans can be removed via enzymatic treatments. For instance, HA proteins may be modified to remove one or more N-linked glycans via treatment with endoglycosidases. As used herein, an “endoglycosidase” is used to refer to an enzyme, or a functional part, derivative and/or analogue thereof, capable of significantly cleaving sugar moieties from a substrate comprising an oligosaccharide. The use of such enzymes to release oligosaccharides from glycoproteins (*e.g.*, HA) or glycolipids is described in U.S. Patent No. 7,632,654, which is herein incorporated by reference in its entirety. Examples of endoglycosidases suitable for removal of N-glycans include endoglycosidases D, F, F1, F2, and H.

[063] In other embodiments, one or more N-linked glycans can be removed chemically. In one embodiment, the chemical reaction is mediated by one or more hydrazine-like reagents. Methods for the release of N-linked glycans using hydrazine and hydrazine-like reagents are found in U.S. Patent No. 5,539,090, which is herein incorporated by reference in its entirety. Briefly, before subjecting HA to the influence of the hydrazine reagent (*i.e.* hydrazinolysis), the HA protein and the hydrazine reagent are preferably prepared as follows. The glycoconjugate is rendered essentially salt-free, for example by dialysis, gel filtration, or chromatography in a suitable system. Once salt-free, HA is rendered essentially anhydrous, for example by lyophilization, and the water content reduced to at least that achieved at equilibrium under lyophilization conditions of 25 millibar at 25 °C. The water content of the hydrazine reagent is likewise relevant, and should not exceed 4% v/v. Thus, most commercially available samples of anhydrous hydrazine reagent are suitable, though drying of hydrazine reagent can be achieved through one of numerous reported processes. Suitable hydrazine reagent is then added to the appropriately prepared sample in an air-tight vessel. The reaction between HA and the hydrazine reagent can be initiated by the input of energy, either microscopically or macroscopically, for example by raising the temperature. For any method of input of energy the optimal conditions for reaction can be deduced from various experimental approaches. In this disclosure the preferred method of initiating the reaction is through increase in temperature to a steady state.

[064] In yet other embodiments, alkaline solutions can be used to remove N-linked glycans. The N-glycosidic linkages attaching N-glycans to a conjugate are alkali labile, and alkaline solutions can therefore be employed for release of N-glycans. For example, incubation with 1M NaOH at 100 °C for 4 hours has been successfully employed, as described in U.S. Patent No. 5,539,090, which is herein incorporated by reference in its entirety.

[065] It is understood that various strains of influenza virus can act as “sources” for genetic material encoding HA proteins suitable for use in methods and compositions described herein. In various embodiments described herein, the modified HA proteins of the invention may be derived from any of the known HA sequences from influenza isolates known in the art. For instance, the National Center for Biotechnology Information (NCBI) maintains a database (www.ncbi.nlm.nih.gov/genomes/FLU/flu.html) of known HA sequences. In addition, a database of influenza HA wild-type or MDCK cell and egg-passaged sequences is available from the Global Initiative on Sharing All Influenza Database (GISAID) EpiFlu database. Those of ordinary skill in the art, referring to these databases, can readily identify sequences that are characteristic of HA polypeptides generally, and/or of particular HA polypeptides (*e.g.*, H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, or H16 polypeptides); or of HAs that mediate infection of particular hosts, (*e.g.*, avian, camel, canine, equine, feline, human, leopard, mink, mouse, seal, swine, tiger, whale, etc). Examples of subtypes comprising such HA proteins modifiable by the methods described herein include, but are not limited, to A/New Caledonia/20/99 (H1N1) A/Indonesia/5/05 (H5N1), A/chicken/New York/1995, A/herring gull/DE/677/88 (H2N8), A/Texas/32/2003, A/mallard/MN/33/00, A/duck/Shanghai/1/2000, A/northern pintail/TX/828189/02, A/Turkey/Ontario/6118/68 (H8N4), A/shoveler/Iran/G54/03, A/chicken/Germany/N/1949 (H10N7), A/duck/England/56 (H11N6), A/duck/Alberta/60/76 (H12N5), A/Gull/Maryland/704/77 (H13N6), A/Mallard/Gurjev/263/82, A/duck/Australia/341/83 (H15N8), A/black-headed gull/Sweden/5/99 (H16N3), B/Lee/40, C/Johannesburg/66, A/PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), A/Solomon Islands 3/2006 (H1N1), A/Brisbane 10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004, B/Florida/4/2006, A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), A/Teal/HongKong/W312/97 (H6N1), A/Equine/Prague/56 (H7N7), A/HongKong/1073/99 (H9N2). A representative list of HA proteins modifiable by the methods of the present invention may be found in US 2010/0239610, which is herein incorporated by reference in its entirety. In one embodiment, the HA sequence selected for targeted modification is an egg-adapted HA sequence. Methods of obtaining and identifying

egg-adapted HA sequences are known in the art (Robertson *et al.*, 1991, *J. Gen. Virology* 72: 2671-7).

[066] Also encompassed within the scope of the present invention are chimeric HA proteins comprising the transmembrane domain and/or cytoplasmic tail of a modified influenza HA protein fused to a protein from an infective agent. In another embodiment, the transmembrane domain and/or cytoplasmic tail of the modified influenza HA protein extends from the N or C-terminus to approximately 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 to about 50 amino acids past the transmembrane domain and is fused to said protein from another infectious agent. Infectious agents can be viruses, bacteria, fungi and/or parasites. Non-limiting examples of viruses from which said infectious agent proteins can be derived from are the following: coronavirus (*e.g.* the agent that causes SARS), hepatitis viruses A, B, C, D & E3, human immunodeficiency virus (HIV), herpes viruses 1, 2, 6 & 7, cytomegalovirus, varicella zoster, papilloma virus, Epstein Barr virus, parainfluenza viruses, respiratory syncytial virus (RSV), human metapneumovirus, adenoviruses, bunya viruses (*e.g.* hanta virus), coxsackie viruses, picorna viruses, rotaviruses, rhinoviruses, rubella virus, mumps virus, measles virus, Rubella virus, polio virus (multiple types), adeno virus (multiple types), parainfluenza virus (multiple types), avian influenza (various types), shipping fever virus, Western and Eastern equine encephalomyelitis, Japanese encephalomyelitis, fowl pox, rabies virus, slow brain viruses, rous sarcoma virus, Papovaviridae, Parvoviridae, Picomaviridae, Poxviridae (such as Smallpox or Vaccinia), Reoviridae (*e.g.*, Rotavirus), Retroviridae (HTLV-I, HTLV-II, Lentivirus), Togaviridae (*e.g.*, Rubivirus), Newcastle disease virus, West Nile fever virus, Tick borne encephalitis, yellow fever, chikungunya virus, and dengue virus (all serotypes).

Vaccines Comprising Modified HA Proteins:

[067] The present application provides influenza HA proteins modified to remove one or more glycosylation sites. In various aspects described herein, the modified HA proteins of the present invention have utility in immunogenic compositions such as vaccines for the treatment and/or prevention of influenza infection.

[068] Since influenza infection can be prevented by providing neutralizing antibodies to a vertebrate, a vaccine comprising a modified influenza HA protein may induce, when administered to a vertebrate, neutralizing antibodies *in vivo*. The modified influenza HA proteins are favorably used for the prevention and/or treatment of influenza infection. Thus, another aspect of this disclosure concerns a method for eliciting an immune response against

influenza. The method involves administering an immunologically effective amount of a composition containing a modified influenza HA protein to a subject (such as a human or animal subject). Administration of an immunologically effective amount of the composition elicits an immune response specific for epitopes present on the modified influenza HA protein. Such an immune response can include B cell responses (e.g., the production of neutralizing antibodies) and/or T cell responses (e.g., the production of cytokines). Preferably, the immune response elicited by the modified influenza HA protein includes elements that are specific for at least one conformational epitope present on the modified influenza HA protein.

[069] Vaccines comprising modified HA proteins within the scope of this invention include inactivated influenza vaccines, attenuated live virus vaccines, and virus-like particles (VLPs).

[070] Three types of inactivated influenza vaccine are currently used in the world: whole virus, split product, and surface antigen or "subunit" vaccines. These vaccines all contain the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) of the influenza virus strains that are expected to circulate in the human population in the upcoming season. These strains, which are incorporated into the vaccine, are grown in embryonated hens' eggs and the viral particles are subsequently purified before further processing. In one embodiment, one or more glycosylation sites are removed from existing egg-adapted HA sequences to improve the immunogenicity of existing influenza vaccines. Methods for preparing egg-adapted HA sequences are described in U.S. Patent No. 5,162,112, which is herein incorporated by reference in its entirety.

[071] Additionally, the modified HA protein may be derived from one or more of the multivalent vaccines for influenza (e.g., monovalent, divalent, or trivalent). In one aspect, the modified HA protein may be derived from one of the trivalent inactivated vaccines (TIV) for influenza. The standard components of TIV include hemagglutinin (HA) and neuraminidase from three different strains of influenza virus. Examples of TIV which may be used include, but are not limited to, Fluzone, Fluvirin, Fluarix, FluLaval, FluBlok, FluAd, Influvac, and Fluvax.

[072] In one aspect, the invention provides for vaccines comprising a composition of a modified HA protein which is presented to the immune system and is capable of inducing an immune response in an individual. In some embodiments, the composition further comprises an adjuvant or a carrier. In another embodiment, a modified HA protein described herein comprises one or more components of at least one trivalent inactivated influenza vaccine (TIV). In some embodiments, the TIV is selected from the group consisting of Fluzone,

Fluvirin, Fluarix, FluLaval, FluBlok, FluAd, Influvac, and Fluvax. In additional embodiments, a modified HA protein described herein may be administered in conjunction with a TIV is selected from the group consisting of Fluzone, Fluvirin, Fluarix, FluLaval, FluBlok, FluAd, Influvac, and Fluvax.

Virus-Like Particles Comprising Modified HA Proteins:

[073] The invention also encompasses influenza virus-like particles (VLPs) comprising a modified HA protein that can be formulated into vaccines or antigenic formulations for protecting vertebrates (e.g. humans) against influenza infection or at least one disease symptom thereof. The VLP may comprise a modified HA protein derived from one, or more than one subtype, including H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16 or fragment or portion thereof. Examples of subtypes comprising such HA proteins include A/New Caledonia/20/99 (H1N1) A/Indonesia/5/05 (H5N1), A/chicken/New York/1995, A/herring gull/DE/677/88 (H2N8), A/Texas/32/2003, A/mallard/MN/33/00, A/duck/Shanghai/1/2000, A/northern pintail/TX/828189/02, A/Turkey/Ontario/6118/68 (H8N4), A/shoveler/Iran/G54/03, A/chicken/Germany/N/1949 (H10N7), A/duck/England/56 (H11N6), A/duck/Alberta/60/76 (H12N5), A/Gull/Maryland/704/77 (H13N6), A/Mallard/Gurjev/263/82, A/duck/Australia/341/83 (H15N8), A/black-headed gull/Sweden/5/99 (H16N3), B/Lee/40, C/Johannesburg/66, A/PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), A/Solomon Islands 3/2006 (H1N1), A/Brisbane 10/2007 (H3N2), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004, B/Florida/4/2006, A/Singapore/1/57 (H2N2), A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), A/Teal/HongKong/W312/97 (H6N1), A/Equine/Prague/56 (H7N7), A/HongKong/1073/99 (H9N2).

[074] In an aspect of the invention, the HA protein may be an H1, H2, H3, H5, H6, H7 or H9 subtype. In an another aspect, the H1 protein may be from the A/New Caledonia/20/99 (H1N1), A/PuertoRico/8/34 (H1N1), A/Brisbane/59/2007 (H1N1), or A/Solomon Islands 3/2006 (H1N1) strain. The H3 protein may also be from the A/Brisbane 10/2007 (H3N2) or A/Wisconsin/67/2005 (H3N2) strain. In a further aspect of the invention, the H2 protein may be from the A/Singapore/1/57 (H2N2) strain. The H5 protein may be from the A/Anhui/1/2005 (H5N1), A/Vietnam/1194/2004 (H5N1), or A/Indonesia/5/2005 strain. In an aspect of the invention, the H6 protein may be from the A/Teal/HongKong/W312/97 (H6N1) strain. The H7 protein may be from the A/Equine/Prague/56 (H7N7) strain. In an aspect of the invention, the H9 protein is from the A/HongKong/1073/99 (H9N2) strain. In a further

aspect of the invention, the HA protein may be from an influenza virus may be a type B virus, including B/Malaysia/2506/2004 or B/Florida/4/2006.

[075] In some embodiments, the VLPs comprising one or more modified influenza HA proteins further comprise additional influenza proteins, such as M1, NA, M2, and NP. The HA, NA, M1, M2, and NP proteins may be derived from any strain of influenza virus, including pandemic and seasonal strains.

[076] The VLPs of the present invention may comprise an avian influenza M1 protein. In one embodiment, the avian influenza M1 protein is derived from an H9N2 influenza strain. In various embodiments described herein, the influenza M1 can be isolated from any one of the H9N2 influenza viruses selected from the group consisting of A/quail/Hong Kong/G1/97, A/Hong Kong/1073/99, A/Hong Kong/2108/03, Duck/HK/Y280/97, CK/HK/G9/97, Gf/HK/SSP607/03, Ph/HK/CSW1323/03, Wdk/ST/4808/01, CK/HK/NT142/03, CK/HK/WF126/03, Sck/HK/WF285/03, CK/HK/YU463/03, CK/HK/YU577/03, Sck/HK/YU663/03, Ck/HK/CSW161/03, and GF/HK/NT101/03. In an exemplary embodiment, the H9N2 influenza strain is A/Hong Kong/1073/99. In another embodiment, the avian influenza M1 protein is derived from an H5N1 influenza strain. In various embodiments described herein, the influenza M1 can be isolated from any one of the H5N1 influenza viruses selected from the group consisting of A/Vietnam/1194/04, A/Vietnam/1203/04, A/Hongkong/213/03, A/Indonesia/2/2005, A/Bar headed goose/Quinghai/1A/2005, A/Anhui/1/2005, and A/Indonesia/5/05. In an exemplary embodiment, the H5N1 strain is A/Indonesia/5/05.

[077] In alternative embodiments, the VLPs of the present invention may comprise an influenza M1 protein from a non-avian source, such as from an H1N1 strain of swine-origin. In one embodiment, the M1 protein is derived from influenza A/California/04/09. In other embodiments, the M1 proteins may be derived from influenza A/California/08/09 or influenza A/Mexico/4108/09.

[078] The present inventors have found that the use of influenza M1 protein comprising the YKKL L-domain sequence at amino acid positions corresponding to residues 100-103 of SEQ ID NO: 3 increases the efficiency of influenza VLP production. Thus, in exemplary embodiments described herein, the VLPs of the present invention comprise an influenza M1 protein that contains the YKKL L-domain sequence. In one embodiment, the influenza M1 protein comprising the YKKL L-domain sequence at amino acid positions corresponding to residues 100-103 of SEQ ID NO: 3 is an avian influenza M1 protein. In another embodiment, the influenza M1 protein comprising the YKKL L-domain sequence at amino

acid positions corresponding to residues 100-103 of SEQ ID NO: 3 is a non-avian influenza M1 protein. In a further embodiment, the non-avian influenza M1 protein is a swine influenza M1 protein. In a further embodiment, the swine influenza M1 protein is derived from influenza strain A/California/04/09. In an exemplary embodiment, the M1 protein derived from A/California/04/09 is comprised of SEQ ID NO: 4. Methods of making VLPs utilizing M1 proteins harboring the putative YKKL L-domain are described in commonly owned and co-pending U.S. Application Serial No. 12/832,657.

[079] The corresponding positions of the influenza M1 proteins identified herein (*e.g.* the YKKL amino acids at positions corresponding to residues 100-103 of SEQ ID NOs: 3 and 4) may be readily identified in other influenza M1 proteins by one of skill in the art. Thus, given the defined region and the assays described in the present application, one with skill in the art can identify an influenza M1 protein in nature exhibiting an increased ability to mediate VLP formation. Likewise, given the disclosures of the present application, one with skill in the art would be able to make one or more modifications which would result in an increased ability to mediate VLP formation, in any influenza M1 protein of interest. Thus, influenza M1 proteins that occur in nature with the YKKL amino acids at positions corresponding to residues 100-103 of SEQ ID NOs: 3 and 4, as well as those that have been mutated either naturally or through genetic engineering to contain the YKKL L-domain at positions corresponding to residues 100-103 of SEQ ID NOs: 3 and 4 fall within the scope of this invention

[080] In one embodiment, the VLPs may comprise proteins from at least two different influenza viruses. For example, the VLPs may comprise a modified HA derived from an avian influenza virus (*i.e.* H5N1) and an NA from a swine influenza virus (*i.e.* H1N1). In an alternative embodiment, the VLPs may comprise modified HA proteins derived from more than one type of influenza virus (*i.e.* H5N1 and H1N1). In these embodiments, said VLPs are multivalent VLPs capable of inducing an immune response to several proteins and therefore, several strains of influenza virus. In another embodiment, said multivalent VLPs comprise an influenza M1 protein. In one embodiment, the M1 protein comprises an L-domain sequence comprising YKKL at amino acid positions corresponding to residues 100-103 of SEQ ID NO: 3. In another embodiment, the M1 protein is a swine influenza M1 protein. In a further embodiment, the swine influenza M1 protein is derived from influenza strain A/California/04/09. In an exemplary embodiment, the M1 protein derived from A/California/04/09 is comprised of SEQ ID NO: 4. In yet another embodiment, the M1 protein is an avian influenza M1 protein. In a further embodiment, the avian influenza M1

protein is derived from influenza strain A/Indonesia/5/05. In an exemplary embodiment, the M1 protein derived from influenza strain A/Indonesia/5/05 is comprised of SEQ ID NO: 3.

[081] The invention also encompasses variants of the said proteins expressed on or in the VLPs of the invention. The variants may contain alterations in the amino acid sequences of the constituent proteins. The term “variant” with respect to a protein refers to an amino acid sequence that is altered by one or more amino acids with respect to a reference sequence. The variant can have “conservative” changes, wherein a substituted amino acid has similar structural or chemical properties, *e.g.*, replacement of leucine with isoleucine. Alternatively, a variant can have “nonconservative” changes, *e.g.*, replacement of a glycine with a tryptophan. Analogous minor variations can also include amino acid deletion or insertion, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without eliminating biological or immunological activity can be found using computer programs well known in the art, for example, DNASTAR software.

[082] Natural variants can occur due to mutations in the proteins. These mutations may lead to antigenic variability within individual groups of infectious agents, for example influenza. Thus, a person infected with an influenza strain develops antibody against that virus, as newer virus strains appear, the antibodies against the older strains no longer recognize the newer virus and reinfection can occur. The invention encompasses all antigenic and genetic variability of proteins from infectious agents for making the VLPs.

[083] General texts which describe molecular biological techniques, which are applicable to the present invention, such as cloning, mutation, cell culture and the like, include Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, Vol. 152 Academic Press, Inc., San Diego, Calif. (“Berger”); Sambrook *et al.*, *Molecular Cloning--A Laboratory Manual* (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2000 (“Sambrook”) and *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (“Ausubel”). These texts describe mutagenesis, the use of vectors, promoters and many other relevant topics related to, *e.g.*, the cloning and mutating HA, NA and/or M1. Thus, the invention also encompasses using known methods of protein engineering and recombinant DNA technology to improve or alter the characteristics of the proteins expressed on or in the VLPs of the invention. Various types of mutagenesis can be used to produce and/or isolate variant nucleic acids that encode for protein molecules and/or to further modify/mutate the proteins in or on the VLPs of the invention. They include but are not limited to site-directed, random point mutagenesis, homologous recombination (DNA

shuffling), mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA or the like. Additional suitable methods include point mismatch repair, mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis, mutagenesis by total gene synthesis, double-strand break repair, and the like. Mutagenesis, *e.g.*, involving chimeric constructs, is also included in the present invention. In one embodiment, mutagenesis can be guided by known information of the naturally occurring molecule or altered or mutated naturally occurring molecule, *e.g.*, sequence, sequence comparisons, physical properties, crystal structure or the like.

[084] The invention further comprises protein variants which show substantial biological activity, *e.g.*, able to elicit an effective antibody response when expressed on or in VLPs of the invention. Such variants include deletions, insertions, inversions, repeats, and substitutions selected according to general rules known in the art so as have little effect on activity.

[085] Methods of cloning said proteins are known in the art. For example, the gene encoding a specific virus protein can be isolated by RT-PCR from polyadenylated mRNA extracted from cells which had been infected with a virus (DNA or RNA virus) or PCR from cells which had been infected with a DNA virus. The resulting product gene can be cloned as a DNA insert into a vector. The term “vector” refers to the means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating. In many, but not all, common embodiments, the vectors of the present invention are plasmids or bacmids.

[086] Thus, the invention comprises nucleotides that encode proteins cloned into an expression vector that can be expressed in a cell that induces the formation of VLPs of the invention. An “expression vector” is a vector, such as a plasmid that is capable of promoting expression, as well as replication of a nucleic acid incorporated therein. Typically, the nucleic acid to be expressed is “operably linked” to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter and/or enhancer. In one embodiment, the vector comprises nucleotides that encode for M1, NA, and a modified HA.

[087] In some embodiments, said proteins may comprise, mutations containing alterations that produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded protein or how the proteins are made. Nucleotide variants can be produced for a variety of reasons, *e.g.*, to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by insect cells such as Sf9 cells). See U.S. patent publication 2005/0118191, herein incorporated by reference in its entirety for all purposes.

[088] In addition, the nucleotides can be sequenced to ensure that the correct coding regions were cloned and do not contain any unwanted mutations. The nucleotides can be subcloned into an expression vector (*e.g.* baculovirus) for expression in any cell. The above is only one example of how the influenza proteins can be cloned. A person with skill in the art understands that additional methods are available and are possible.

[089] The invention also provides for constructs and/or vectors that comprise nucleotides that encode for the influenza proteins described above. The constructs and/or vectors that comprise influenza proteins should be operatively linked to an appropriate promoter, such as the AcMNPV polyhedrin promoter (or other baculovirus), phage lambda PL promoter, the *E. coli* lac, phoA and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs are non-limiting examples. Other suitable promoters will be known to the skilled artisan depending on the host cell and/or the rate of expression desired. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome-binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

[090] Expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418, or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin, or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Among vectors preferred are virus vectors, such as baculovirus, poxvirus (*e.g.*, vaccinia virus, avipox virus, canarypox virus, fowlpox virus, raccoonpox virus, swinepox virus, etc.), adenovirus (*e.g.*, canine adenovirus), herpesvirus, and retrovirus. Other vectors that can be used with the invention comprise vectors for use in bacteria, which comprise pQE70, pQE60 and pQE-9, pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5. Among preferred eukaryotic vectors are pFastBac1 pWINEO, pSV2CAT, pOG44, pXT1, and pSG,

pSVK3, pBPV, pMSG, and pSVL. Other suitable vectors will be readily apparent to the skilled artisan.

[091] Next, the recombinant constructs mentioned above could be used to transfect, infect, or transform and can express influenza proteins, into eukaryotic cells and/or prokaryotic cells. Thus, the invention provides for host cells that comprise a vector (or vectors) that contain nucleic acids which code for influenza proteins, and permit the expression of said constructs in said host cell under conditions which allow the formation of VLPs.

[092] Among eukaryotic host cells are yeast, insect, avian, plant, *C. elegans* (or nematode), and mammalian host cells. Non limiting examples of insect cells are, *Spodoptera frugiperda* (Sf) cells, e.g. Sf9, Sf21, *Trichoplusia ni* cells, e.g. High Five cells, and *Drosophila* S2 cells. Examples of fungi (including yeast) host cells are *S. cerevisiae*, *Kluyveromyces lactis* (*K. lactis*), species of *Candida* including *C. albicans* and *C. glabrata*, *Aspergillus nidulans*, *Schizosaccharomyces pombe* (*S. pombe*), *Pichia pastoris*, and *Yarrowia lipolytica*. Examples of mammalian cells are COS cells, baby hamster kidney cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, and African green monkey cells, CV1 cells, HeLa cells, MDCK cells, Vero, and Hep-2 cells. *Xenopus laevis* oocytes, or other cells of amphibian origin, may also be used. Prokaryotic host cells include bacterial cells, for example, *E. coli*, *B. subtilis*, and mycobacteria.

[093] Vectors, e.g., vectors comprising polynucleotides of influenza proteins, can be transfected into host cells according to methods well known in the art. For example, introducing nucleic acids into eukaryotic cells can be by calcium phosphate co-precipitation, electroporation, microinjection, lipofection, and transfection employing polyamine transfection reagents. In one embodiment, said vector is a recombinant baculovirus. In another embodiment, said recombinant baculovirus is transfected into a eukaryotic cell. In a preferred embodiment, said cell is an insect cell. In another embodiment, said insect cell is a Sf9 cell.

[094] In another embodiment, said vector and/or host cell comprise nucleotides that encode one or more modified HA proteins, and one or more additional influenza proteins selected from M1, NA, M2, and NP. In an exemplary embodiment, the vector and/or host cell comprises nucleotides that encode one or more modified HA proteins, and M1 and/or NA proteins. In another embodiment, said vector and/or host cell consists essentially of one or more modified HA proteins, and M1 and/or NA proteins. In a further embodiment, said vector and/or host cell consists of one or more modified HA proteins, and M1 and/or NA

proteins. These vectors and/or host cells may contain additional markers, such as an origin of replication, selection markers, etc.

[095] The invention also provides for constructs and methods that will further increase the efficiency of VLPs production. For example, the addition of leader sequences to the M1, HA, and/or NA proteins, can improve the efficiency of protein transporting within the cell. For example, a heterologous signal sequence can be fused to M1, HA, and/or NA. In one embodiment, the signal sequence can be derived from the gene of an insect preprotein and fused to M1, HA, and/or NA. In another embodiment, the signal peptide is the chitinase signal sequence, which works efficiently in baculovirus expression systems.

[096] The invention also provides for methods of producing VLPs of the invention, said methods comprising expressing nucleic acids encoding one or more modified HA proteins, and one or more additional influenza proteins selected from M1, NA, M2, and NP in a cell culture expression system and purifying said VLPs from the cell culture supernatant.

[097] In some embodiments, the nucleic acids encoding the one or more modified HA proteins, and M1 and/or NA proteins are expressed in a eukaryotic cell under conditions that permit the formation of VLPs. In one embodiment, the eukaryotic cell utilized for expression is selected from the group consisting of yeast, insect, amphibian, avian, and mammalian cells. In an exemplary embodiment, the eukaryotic cell is an insect cell. In one embodiment, the cell culture expression system is the baculovirus expression system.

[098] Depending on the expression system and host cell selected, VLPs are produced by growing host cells transformed by an expression vector under conditions whereby the recombinant proteins are expressed and VLPs are formed. The selection of the appropriate growth conditions is within the skill of a person with skill of one of ordinary skill in the art.

[099] Methods to grow cells engineered to produce VLPs of the invention include, but are not limited to, batch, batch-fed, continuous and perfusion cell culture techniques. Cell culture means the growth and propagation of cells in a bioreactor (a fermentation chamber) where cells propagate and express protein (*e.g.* recombinant proteins) for purification and isolation. Typically, cell culture is performed under sterile, controlled temperature and atmospheric conditions in a bioreactor. A bioreactor is a chamber used to culture cells in which environmental conditions such as temperature, atmosphere, agitation and/or pH can be monitored. In one embodiment, said bioreactor is a stainless steel chamber. In another embodiment, said bioreactor is a pre-sterilized plastic bag (*e.g.* Cellbag®, Wave Biotech, Bridgewater, NJ). In other embodiment, said pre-sterilized plastic bags are about 50 L to 1000 L.

[0100] VLPs are then isolated using methods that preserve the integrity thereof, such as by gradient centrifugation, *e.g.*, cesium chloride, sucrose and iodixanol, as well as standard purification techniques including, *e.g.*, ion exchange and gel filtration chromatography.

[0101] The following is an example of how VLPs of the invention can be made, isolated and purified. Usually VLPs are produced from recombinant cell lines engineered to create a VLP when said cells are grown in cell culture (see above). A person of skill in the art would understand that there are additional methods that can be utilized to make and purify VLPs of the invention, thus the invention is not limited to the method described.

[0102] Production of VLPs of the invention can start by seeding Sf9 cells (non-infected) into shaker flasks, allowing the cells to expand and scaling up as the cells grow and multiply (for example from a 125 ml flask to a 50 L Wave bag). The medium used to grow the cell is formulated for the appropriate cell line (preferably serum free media, *e.g.* insect medium ExCell-420, JRH). Next, said cells are infected with recombinant baculovirus at the most efficient multiplicity of infection (*e.g.* from about 1 to about 3 plaque forming units per cell). Once infection has occurred, the M1, such as avian influenza M1 and at least one influenza heterologous protein (*i.e.* a modified HA protein of the present invention and/or NA protein) are expressed from the virus genome, self assemble into VLPs and are secreted from the cells approximately 24 to 72 hours post infection. Usually, infection is most efficient when the cells are in mid-log phase of growth ($4-8 \times 10^6$ cells/ml) and are at least about 90% viable.

[0103] VLPs of the invention can be harvested approximately 48 to 96 hours post infection, when the levels of VLPs in the cell culture medium are near the maximum but before extensive cell lysis. The Sf9 cell density and viability at the time of harvest can be about 0.5×10^6 cells/ml to about 1.5×10^6 cells/ml with at least 20% viability, as shown by dye exclusion assay. Next, the medium is removed and clarified. NaCl can be added to the medium to a concentration of about 0.4 to about 1.0 M, preferably to about 0.5 M, to avoid VLP aggregation. The removal of cell and cellular debris from the cell culture medium containing VLPs of the invention can be accomplished by tangential flow filtration (TFF) with a single use, pre-sterilized hollow fiber 0.5 or 1.00 μ m filter cartridge or a similar device.

[0104] Next, VLPs in the clarified culture medium can be concentrated by ultrafiltration using a disposable, pre-sterilized 500,000 molecular weight cut off hollow fiber cartridge. The concentrated VLPs can be diafiltrated against 10 volumes pH 7.0 to 8.0 phosphate-buffered saline (PBS) containing 0.5 M NaCl to remove residual medium components.

[0105] The concentrated, diafiltered VLPs can be further purified on a 20% to 60% discontinuous sucrose gradient in pH 7.2 PBS buffer with 0.5 M NaCl by centrifugation at $6,500 \times g$ for 18 hours at about 4°C to about 10°C. Usually VLPs will form a distinctive visible band between about 30% to about 40% sucrose or at the interface (in a 20% and 60% step gradient) that can be collected from the gradient and stored. This product can be diluted to comprise 200 mM of NaCl in preparation for the next step in the purification process. This product contains VLPs and may contain intact baculovirus particles.

[0106] Further purification of VLPs can be achieved by anion exchange chromatography, or 44% isopycnic sucrose cushion centrifugation. In anion exchange chromatography, the sample from the sucrose gradient (see above) is loaded into column containing a medium with an anion (*e.g.* Matrix Fractogel EMD TMAE) and eluted via a salt gradient (from about 0.2 M to about 1.0 M of NaCl) that can separate the VLP from other contaminants (*e.g.* baculovirus and DNA/RNA). In the sucrose cushion method, the sample comprising the VLPs is added to a 44% sucrose cushion and centrifuged for about 18 hours at 30,000 g. VLPs form a band at the top of 44% sucrose, while baculovirus precipitates at the bottom and other contaminating proteins stay in the 0% sucrose layer at the top. The VLP peak or band is collected.

[0107] The intact baculovirus can be inactivated, if desired. Inactivation can be accomplished by chemical methods, for example, formalin or β -propyl lactone (BPL). Removal and/or inactivation of intact baculovirus can also be largely accomplished by using selective precipitation and chromatographic methods known in the art, as exemplified above. Methods of inactivation comprise incubating the sample containing the VLPs in 0.2% of BPL for 3 hours at about 25 °C to about 27 °C. The baculovirus can also be inactivated by incubating the sample containing the VLPs at 0.05% BPL at 4 °C for 3 days, then at 37 °C for one hour.

[0108] After the inactivation/removal step, the product comprising VLPs can be run through another diafiltration step to remove any reagent from the inactivation step and/or any residual sucrose, and to place the VLPs into the desired buffer (*e.g.*, PBS). The solution comprising VLPs can be sterilized by methods known in the art (*e.g.*, sterile filtration) and stored in the refrigerator or freezer.

[0109] The above techniques can be practiced across a variety of scales. For example, T-flasks, shake-flasks, spinner bottles, up to industrial sized bioreactors. The bioreactors can comprise either a stainless steel tank or a pre-sterilized plastic bag (for example, the system

sold by Wave Biotech, Bridgewater, NJ). A person with skill in the art will know what is most desirable for their purposes.

[0110] Expansion and production of baculovirus expression vectors and infection of cells with recombinant baculovirus to produce recombinant influenza VLPs can be accomplished in insect cells, for example Sf9 insect cells as previously described. In a preferred embodiment, the cells are Sf9 infected with recombinant baculovirus engineered to produce VLPs of the invention.

Pharmaceutical or Vaccine Formulations and Administration:

[0111] In another aspect, the present invention provides antigenic formulation comprising VLPs comprising one or more modified HA proteins. The VLP may comprise a modified HA protein derived from one, or more than one subtype, including H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16 or fragment or portion thereof. In one embodiment, the VLP may comprise additional influenza virus proteins including, but not limited to, M1, M2, NA, and NP. In an exemplary embodiment, the invention comprises an antigenic formulation comprising a VLP comprising a modified HA protein, a M1 protein, and an NA protein. In one embodiment, the formulations of the invention may comprise one or more VLPs as described herein in combination with, or formulated with, one or more purified or partially purified modified HA antigens. For instance, in one embodiment, a formulation comprises a VLP comprising a modified HA protein from influenza A/California/04/09 in combination with purified modified HA protein from that same virus. In another formulation, the VLP is combined with a homologous or heterologous HA or NA. In another embodiment, separate formulations of the at least one VLP and at least one HA or NA are made and administered to a patient or subject concurrently or separately. In some embodiments, antibody production as measured by HA antibody titers or other measure is superior when the VLP and HA are administered in a single or separate formulation to the same patient or subject (or animal)

[0112] Said formulations of the invention comprise a formulation comprising VLPs of the invention and a pharmaceutically acceptable carrier or excipient. Pharmaceutically acceptable carriers include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, and combinations thereof. A thorough discussion of pharmaceutically acceptable carriers, diluents, and other excipients is presented in Remington's Pharmaceutical Sciences (Mack Pub. Co. N.J. current edition). The formulation

should suit the mode of administration. In another embodiment, the formulation is suitable for administration to humans, preferably is sterile, non-particulate, and/or non-pyrogenic.

[0113] The pharmaceutical compositions useful herein contain a pharmaceutically acceptable carrier, including any suitable diluent or excipient, which includes any pharmaceutical agent that does not itself induce the production of an immune response harmful to the vertebrate receiving the composition, and which may be administered without undue toxicity and a VLP of the invention. As used herein, the term “pharmaceutically acceptable” means being approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia, European Pharmacopoeia, or other generally recognized pharmacopoeia for use in mammals, and more particularly in humans. These compositions can be useful as a vaccine and/or antigenic compositions for inducing a protective immune response in a vertebrate.

[0114] The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a solid form, such as a lyophilized powder suitable for reconstitution, a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

[0115] The invention also comprises a vaccine comprising a VLP comprising one or more modified HA proteins. The VLP may comprise a modified HA protein derived from one, or more than one subtype, including H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16 or fragment or portion thereof. In one embodiment, the VLP may comprise additional influenza virus proteins including, but not limited to, M1, M2, NA, and NP. In an exemplary embodiment, the invention comprises a vaccine comprising a VLP comprising a modified HA protein, a M1 protein, and an NA protein.

[0116] The invention also provides for a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the vaccine formulations of the invention. In one embodiment, the kit comprises two containers, one containing VLPs and the other containing an adjuvant. In another embodiment, the kit comprises two containers, one containing freeze dried VLPs and the other containing a solution to resuspend said VLPs. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[0117] The invention also provides that the VLP formulation be packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of composition. In one embodiment, the VLP composition is supplied as a liquid, in another embodiment, as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water or saline, to the appropriate concentration for administration to a subject. In one embodiment, said container comprises at least about 50 µg/ml, more preferably at least about 100 µg/ml, at least about 200 µg/ml, at least 500 µg/ml, or at least 1 mg/ml of an antigen associated with VLPs of the invention.

[0118] In an alternative embodiment, the VLP composition is supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the VLP composition. The liquid form of the VLP composition is supplied in a hermetically sealed container at least about 50 µg/ml, more preferably at least about 100 µg/ml, at least about 200 µg/ml, at least 500 µg/ml, or at least 1 mg/ml of an antigen associated with VLPs of the invention.

[0119] Generally, VLPs of the invention are administered in an effective amount or quantity (as defined above) sufficient to stimulate an immune response against one or more infectious agents. Preferably, administration of the VLP of the invention elicits immunity against an infectious agent. Typically, the dose can be adjusted within this range based on, *e.g.*, age, physical condition, body weight, sex, diet, time of administration, and other clinical factors. The prophylactic vaccine formulation is systemically administered, *e.g.*, by subcutaneous or intramuscular injection using a needle and syringe, or a needle-less injection device. Alternatively, the vaccine formulation is administered intranasally, either by drops, large particle aerosol (greater than about 10 microns), or spray into the upper respiratory tract. While any of the above routes of delivery results in an immune response, intranasal administration confers the added benefit of eliciting mucosal immunity at the site of entry of many viruses, including influenza.

[0120] Thus, the invention also comprises a method of formulating a vaccine or antigenic composition that induces immunity to an infection or at least one symptom thereof to a mammal, comprising adding to said formulation an effective dose of VLPs of the invention.

[0121] Methods of administering a composition comprising VLPs (vaccine and/or antigenic formulations) include, but are not limited to, parenteral administration (*e.g.*, intradermal, intramuscular, intravenous and subcutaneous), epidural, and mucosal (*e.g.*, intranasal and oral or pulmonary routes or by suppositories). In a specific embodiment, compositions of the present invention are administered intramuscularly, intravenously, subcutaneously,

transdermally or intradermally. The compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucous, colon, conjunctiva, nasopharynx, oropharynx, vagina, urethra, urinary bladder and intestinal mucosa, etc.) and may be administered together with other biologically active agents. In some embodiments, intranasal or other mucosal routes of administration of a composition comprising VLPs of the invention may induce an antibody or other immune response that is substantially higher than other routes of administration. In another embodiment, intranasal or other mucosal routes of administration of a composition comprising VLPs of the invention may induce an antibody or other immune response that will induce cross protection against other strains or organisms that cause infection. For example, a VLP comprising influenza protein, when administered to a vertebrate, can induce cross protection against several influenza strains. Administration can be systemic or local.

[0122] In yet another embodiment, the vaccine and/or antigenic formulation is administered in such a manner as to target mucosal tissues in order to elicit an immune response at the site of immunization. For example, mucosal tissues such as gut associated lymphoid tissue (GALT) can be targeted for immunization by using oral administration of compositions which contain adjuvants with particular mucosal targeting properties. Additional mucosal tissues can also be targeted, such as nasopharyngeal lymphoid tissue (NALT) and bronchial-associated lymphoid tissue (BALT).

[0123] Vaccines and/or antigenic formulations of the invention may also be administered on a dosage schedule, for example, an initial administration of the vaccine composition with subsequent booster administrations. In particular embodiments, a second dose of the composition is administered anywhere from two weeks to one year, preferably from about 1, about 2, about 3, about 4, about 5 to about 6 months, after the initial administration. Additionally, a third dose may be administered after the second dose and from about three months to about two years, or even longer, preferably about 4, about 5, or about 6 months, or about 7 months to about one year after the initial administration. The third dose may be optionally administered when no or low levels of specific immunoglobulins are detected in the serum and/or urine or mucosal secretions of the subject after the second dose. In a preferred embodiment, a second dose is administered about one month after the first administration and a third dose is administered about six months after the first administration. In another embodiment, the second dose is administered about six months after the first administration. In another embodiment, said VLPs of the invention can be administered as

part of a combination therapy. For example, VLPs of the invention can be formulated with other immunogenic compositions, antivirals and/or antibiotics.

[0124] The dosage of the pharmaceutical formulation can be determined readily by the skilled artisan, for example, by first identifying doses effective to elicit a prophylactic or therapeutic immune response, *e.g.*, by measuring the serum titer of virus specific immunoglobulins or by measuring the inhibitory ratio of antibodies in serum samples, or urine samples, or mucosal secretions. Said dosages can be determined from animal studies. A non-limiting list of animals used to study the efficacy of vaccines include the guinea pig, hamster, ferrets, chinchilla, mouse and cotton rat. Most animals are not natural hosts to infectious agents but can still serve in studies of various aspects of the disease. For example, any of the above animals can be dosed with a vaccine candidate, *e.g.* VLPs of the invention, to partially characterize the immune response induced, and/or to determine if any neutralizing antibodies have been produced. For example, many studies have been conducted in the mouse model because mice are small size and their low cost allows researchers to conduct studies on a larger scale.

[0125] In addition, human clinical studies can be performed to determine the preferred effective dose for humans by a skilled artisan. Such clinical studies are routine and well known in the art. The precise dose to be employed will also depend on the route of administration. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal test systems.

[0126] As also well known in the art, the immunogenicity of a particular composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Adjuvants have been used experimentally to promote a generalized increase in immunity against unknown antigens (*e.g.*, U.S. Pat. No. 4,877,611). Immunization protocols have used adjuvants to stimulate responses for many years, and as such, adjuvants are well known to one of ordinary skill in the art. Some adjuvants affect the way in which antigens are presented. For example, the immune response is increased when protein antigens are precipitated by alum. Emulsification of antigens also prolongs the duration of antigen presentation. The inclusion of any adjuvant described in Vogel *et al.*, "A Compendium of Vaccine Adjuvants and Excipients (2nd Edition)," herein incorporated by reference in its entirety for all purposes, is envisioned within the scope of this invention.

[0127] Exemplary, adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant. Other adjuvants comprise GMCSF, BCG,

aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion also is contemplated. MF-59, Novasomes[®], MHC antigens may also be used.

[0128] In one embodiment of the invention, the adjuvant is a paucilamellar lipid vesicle having about two to ten bilayers arranged in the form of substantially spherical shells separated by aqueous layers surrounding a large amorphous central cavity free of lipid bilayers. Paucilamellar lipid vesicles may act to stimulate the immune response several ways, as non-specific stimulators, as carriers for the antigen, as carriers of additional adjuvants, and combinations thereof. Paucilamellar lipid vesicles act as non-specific immune stimulators when, for example, a vaccine is prepared by intermixing the antigen with the preformed vesicles such that the antigen remains extracellular to the vesicles. By encapsulating an antigen within the central cavity of the vesicle, the vesicle acts both as an immune stimulator and as a carrier for the antigen. In another embodiment, the vesicles are primarily made of nonphospholipid vesicles. In another embodiment, the vesicles are Novasomes[®]. Novasomes[®] are paucilamellar nonphospholipid vesicles ranging from about 100 nm to about 500 nm. They comprise Brij 72, cholesterol, oleic acid and squalene. Novasomes[®] have been shown to be an effective adjuvant for influenza antigens (*see*, U.S. Patents 5,629,021, 6,387,373, and 4,911,928, herein incorporated by reference in their entireties for all purposes).

[0129] Another method of inducing an immune response can be accomplished by formulating the VLPs of the invention with "immune stimulators." These are the body's own chemical messengers (cytokines) to increase the immune system's response. Immune stimulators include, but not limited to, various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (*e.g.*, granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immunostimulatory molecules can be administered in the same formulation as the RSV VLPs, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect. Thus in one embodiment, the invention comprises antigenic and vaccine formulations comprising an adjuvant and/or an immune stimulator.

[0130] Thus, one embodiment of the invention comprises a formulation comprising a VLP and adjuvant and/or an immune stimulator. In another embodiment, said adjuvant are Novasomes[®]. In another embodiment, said formulation is suitable for human administration. In another embodiment, the formulation is administered to a vertebrate orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously or subcutaneously. In another embodiment, different VLPs are blended together to create a multivalent formulation. These VLPs may comprise modified HA proteins derived from different strains of influenza virus.

[0131] While stimulation of immunity with a single dose is preferred, additional dosages can be administered by the same or different route to achieve the desired effect. In neonates and infants, for example, multiple administrations may be required to elicit sufficient levels of immunity. Administration can continue at intervals throughout childhood, as necessary to maintain sufficient levels of protection against infections. Similarly, adults who are particularly susceptible to repeated or serious infections, such as, for example, health care workers, day care workers, family members of young children, the elderly, and individuals with compromised cardiopulmonary function may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored, for example, by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to elicit and maintain desired levels of protection.

Methods of Stimulating an Immune Response:

[0132] As mentioned above, the VLPs of the invention are useful for preparing compositions that stimulate an immune response that confers immunity to infectious agents. Both mucosal and cellular immunity may contribute to immunity to infectious agents and disease. Antibodies secreted locally in the upper respiratory tract are a major factor in resistance to natural infection. Secretory immunoglobulin A (sIgA) is involved in protection of the upper respiratory tract and serum IgG in protection of the lower respiratory tract. The immune response induced by an infection protects against reinfection with the same virus or an antigenically similar viral strain. For example, influenza undergoes frequent and unpredictable changes; therefore, after natural infection, the effective period of protection provided by the host's immunity may only be a few years against the new strains of virus circulating in the community.

[0133] VLPs of the invention can induce on immunity in a vertebrate (*e.g.* a human) when administered to said vertebrate. The immunity results from an immune response against

VLPs of the invention that protects or ameliorates infection or at least reduces a symptom of infection in said vertebrate. In some instances, if the said vertebrate is infected, said infection will be asymptomatic. The response may be not a fully protective response. In this case, if said vertebrate is infected with an infectious agent, the vertebrate will experience reduced symptoms or a shorter duration of symptoms compared to a non-immunized vertebrate.

[0134] The invention comprises a method of inducing immunity in a vertebrate comprising administering to said vertebrate a VLP comprising one or more modified HA proteins. The VLP may comprise a modified HA protein derived from one, or more than one subtype, including H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16 or fragment or portion thereof. In one embodiment, the VLP may comprise additional influenza virus proteins including, but not limited to, M1, M2, NA, and NP. In an exemplary embodiment, the invention comprises a vaccine comprising a VLP comprising a modified HA protein, a M1 protein, and an NA protein. In one embodiment, the immune response induced is a humoral immune response. In another embodiment, the immune response induced is a cellular immune response.

[0135] As used herein, an “antibody” is a protein comprising one or more polypeptides substantially or partially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as γ , μ , α , δ , or ϵ , which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD, and IgE, respectively. A typical immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light” (about 25 kD) and one “heavy” chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. Antibodies exist as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases.

[0136] In another embodiment, the invention comprises a method of inducing a protective cellular response to an infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of VLPs of the invention, wherein said VLPs comprise one or more modified HA proteins. The VLP may comprise a modified HA protein derived from one, or more than one subtype, including H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16 or fragment or portion thereof. In one embodiment, the VLP may comprise additional influenza virus proteins including, but not limited to, M1,

M2, NA, and NP. In an exemplary embodiment, the invention comprises a vaccine comprising a VLP comprising a modified HA protein, a M1 protein, and an NA protein.

[0137] As mentioned above, the VLPs of the invention can prevent or reduce at least one symptom of influenza in a subject when administered to said subject. Most symptoms of influenza are well known in the art. Thus, the method of the invention comprises the prevention or reduction of at least one symptom associated with an influenza. A reduction in a symptom may be determined subjectively or objectively, *e.g.*, self assessment by a subject, by a clinician's assessment or by conducting an appropriate assay or measurement (*e.g.* body temperature), including, *e.g.*, a quality of life assessment, a slowed progression of an infection or additional symptoms, reduced severity of symptoms, or suitable assays (*e.g.* antibody titer and/or T-cell activation assay). The objective assessment comprises both animal and human assessments.

[0138] The invention comprises a method of preventing and/or reducing an infection with influenza or symptom thereof, comprising administering to said vertebrate a VLP of the invention.

[0139] A strategy for the control of infectious diseases during an outbreak, *e.g.* influenza, is the universal vaccination of healthy individuals, including children. For example, vaccination with current influenza vaccines of approximately 80% of schoolchildren in a community has decreased respiratory illnesses in adults and excess deaths in the elderly (Reichert *et al.*, 2001). This concept is known as community immunity or "herd immunity" and is thought to play an important part of protecting the community against diseases. Because vaccinated people have antibodies that neutralize an infectious agent, *e.g.* influenza virus, they are much less likely to transmit said agent to other people. Thus, even people who have not been vaccinated (and those whose vaccinations have become weakened or whose vaccines are not fully effective) often can be shielded by the herd immunity because vaccinated people around them are not getting sick. Herd immunity is more effective as the percentage of people vaccinated increases. It is thought that approximately 95% of the people in the community must be protected by a vaccine to achieve herd immunity. People who are not immunized increase the chance that they and others will get the disease.

[0140] Thus, the invention also comprises a method of reducing the severity of influenza in a population, comprising administering a VLP of the invention to enough individuals in said population in order to prevent or decrease the chance of transmission to another individual in said population. The invention also encompasses a method of inducing immunity to an influenza virus to a population or a community in order to reduce the incidence of infections

among immunocompromised individuals or non-vaccinated individual by administering VLPs of the invention to a population in a community. In one embodiment, most school-aged children are immunized by administering the VLPs of the invention. In another embodiment, most healthy individuals in a community to are immunized by administering the VLPs of the invention. In another embodiment, VLPs of the invention are part of a “dynamic vaccination” strategy. Dynamic vaccination is the steady production of a low-efficacy vaccine that is related to an emerging pandemic strain, but due to an antigenic drift may not provide complete protection in a mammal (see Germann *et al.*, 2006).

[0141] This invention is further illustrated by the following examples that should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference for all purposes.

EXAMPLES

Example 1

Generating Influenza Virus-Like Particles (VLPs) with Modified Hemagglutinin (HA) Proteins

[0142] This example describes the production of influenza H5N1 VLPs made with HA proteins modified to remove one or more glycosylation sites. To construct the modified HA proteins, one or both of the NX(T/S) sequences at amino acid positions 170-172 and 181-183 of the A/Indonesia/05/2005 (H5N1) HA1 (SEQ ID NO: 2) protein were mutated (Figure 1). Specifically, threonine to alanine mutations were made at positions 172 and/or 183 of the HA1 protein.

[0143] After the HA gene was modified, the modified HA gene was co-expressed with the NA and M1 genes derived from A/Indonesia/5/2005 (H5N1). The HA, NA, and M1 genes were expressed in *Spodoptera frugiperda* Sf9 insect cells using the baculovirus expression system. The expression products of infected Sf9 cells were characterized by SDS-PAGE analysis. As Figure 2 shows, the HA, NA, and M1 proteins of expected molecular weights (64 kd, 60 kd, and 31 kd, respectively) were detected. The presence of M1 and HA bands in the same lane is indicative of HA associating with M1 association, a hallmark of VLP formation. VLPs comprising HA proteins modified to remove a glycosylation site at amino acid positions 170-172 (lanes 4-6), at amino acid positions 181-183 (lane 2), or both amino

acid positions 170-172 and 181-183 (lane 3) formed correctly. These data provide evidence that it is possible to make influenza VLPs comprising HAs modified to remove one or more glycosylation sites, a surprising result given the reported alterations to protein's conformation that occur following modifications to the glycosylation pattern.

Example 2

Influenza VLPs with Modified Hemagglutinin (HA) Proteins Retain Hemagglutinin and Neuraminidase Activity

[0144] In this example, the hemagglutinin and neuraminidase activities of the HA and NA proteins, respectively, in wild-type A/Indonesia/05/2005 (H5N1) VLPs were compared to those same activities from H5N1 VLPs comprising HA proteins modified to remove one or more glycosylation sites at positions 170-172 and 181-183 of the A/Indonesia/05/2005 HA protein (SEQ ID NO: 2).

[0145] To determine the hemagglutination activity of the influenza VLPs, a series of 2-fold dilutions of sucrose gradient fractions containing wild-type influenza VLPs or influenza VLPs comprising modified HA proteins were prepared. Next, these VLPs were mixed with guinea pig red blood cells in PBS and incubated at 4°C for 1 to 16 hr. The extent of hemagglutination was determined visually, and the highest dilution capable of agglutinating guinea pig red blood cells was determined. The highest hemagglutination titer observed for the wild-type influenza VLPs was 1:4096. In comparison, influenza VLPs comprising an HA protein with a single T172A glycosylation site modification exhibited a hemagglutination titer of 1:2048, while influenza VLPs comprising an HA protein with T172A and T183A glycosylation site modifications exhibited a hemagglutination titer of 1:1024. These results demonstrate that H5N1 VLPs comprising HA proteins modified to remove one or more glycosylation sites retain hemagglutination activity.

[0146] In addition, the amount of neuraminidase activity in influenza VLP-containing sucrose gradient fractions was determined using a neuraminidase assay in which the NA protein acts on the substrate fetuin to release sialic acid. The amount of sialic acid liberated is determined chemically with thiobarbituric acid to produce a pink color in proportion to free sialic acid and the amount of the chromophore is measured using a spectrophotometer at a wavelength of 594 nm. Neuraminidase activity was detected in wild-type influenza VLPs, in VLPs comprising an HA protein with a single T172A glycosylation site modification, and in influenza VLPs comprising an HA protein with T172A and T183A glycosylation site

modifications, indicating that H5N1 VLPs comprising HA proteins modified to remove one or more glycosylation sites retain hemagglutination activity.

Example 3

Identification of N-Linked Glycosylation Sites Using the NetNGlyc 1.0 Software

[0147] In this example, the NetNGlyc prediction analysis software was used to identify N-Linked glycosylation sites in the top of the globular head in three seasonal influenza strains.

[0148] The first strain for analysis in this example was A/California/07/2009 (H1N1). The NetNGlyc prediction software identified glycosylation sites at positions 28 (with the accompanying amino acid sequence “NSTD”), 40 (NVTV), 304 (NTSL), and 557 (NGSL). Of these sites, none are located in the “top-of-head” region. Thus, HA from the pandemic A/California/07/2009 (H1N1) does not harbor any glycosylation sites in the region located near the top of the HA globular head.

[0149] The second strain for analysis in this example was A/Perth/16/2009 (H3N2). The NetNGlyc prediction software identified glycosylation sites at positions 24 (NSTA), 38 (NGTI), 79 (NCTL), 142 (NWTG), 149 (NGTS), 181 (NVTM), 262 (NSTG), and 301 (NGSI). Of these sites, four are located in the “top-of-head” region: 142, 149, 181, and 262. Based upon these results, four proposed mutation sites were suggested: N142D, T151A, T183A, and N262D. Of these four proposed mutations, N142D, T151A, and N262D are naturally occurring. In contrast, the T183A mutation is not naturally occurring, suggesting that this glycosylation site is very conserved.

[0150] The third strain for analysis in this example was B/Brisbane/60/2008. The NetNGlyc prediction software identified glycosylation sites at positions 40 (NVTG), 74 (NCTD), 160 (NITN), 181 (NKTA), 212 (NETQ), 248 (NQTE), 319 (NKSK), 348 (NGTK), and 578 (NVSC). Of these sites, four are located in the “top-of-head” region: 160, 181, 212, and 248. Based upon these results, four proposed mutation sites were suggested: T162A, T183A, T214A (egg-based mutation), and N248D. Of these four proposed mutations, T214A and N248D are naturally occurring. In contrast, the T162A and T183A are not naturally occurring, suggesting that this glycosylation site is very conserved.

[0151] The foregoing detailed description has been given for clearness of understanding only and no unnecessary limitations should be understood therefrom as modifications will be obvious to those skilled in the art. It is not an admission that any of the information provided

herein is prior art or relevant to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

[0152] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0153] Although the application has been broken into sections to direct the reader's attention to specific embodiments, such sections should not be construed as a division amongst embodiments. The teachings of each section and the embodiments described therein are applicable to other sections.

[0154] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

CLAIMS:

1. A virus-like particle (VLP) comprising a recombinant influenza virus hemagglutinin (HA) protein, wherein said HA protein comprises one or more amino acid mutations that removes one or more glycosylation sites from said HA protein.
2. The VLP of claim 1, wherein said HA protein is derived from an avian influenza virus strain.
3. The VLP of claim 2, wherein said avian influenza virus strain is H5N1.
4. The VLP of claim 2, wherein said avian influenza virus strain is H9N2.
5. The VLP of claim 1, wherein said HA protein is derived from a swine influenza virus strain.
6. The VLP of claim 5, wherein said swine influenza virus strain is H1N1.
7. The VLP of claim 1, wherein said HA protein is derived from a seasonal influenza virus strain.
8. The VLP of claim 7, wherein said seasonal influenza virus strain is a type A influenza virus strain.
9. The VLP of claim 7, wherein said seasonal influenza virus strain is a type B influenza virus strain.
10. The VLP of claim 1, wherein said HA protein is derived from a pandemic influenza virus strain.
11. The VLP of claim 1, wherein said HA protein is derived from a pre-pandemic influenza virus strain.

12. The VLP of any of claims 1-11, wherein said HA protein exhibits hemagglutinin activity.
13. The VLP of any of claims 1-12, wherein said HA protein is a chimeric protein.
14. The VLP of any of claims 1-13, wherein said one or more amino acid mutations occurs within the receptor binding domain (RBD) of said HA protein.
15. The VLP of any of claim 1-14, wherein said one or more amino acid mutations alters an NX(T/S) (SEQ ID NO: 1) sequence in said HA protein, wherein X is any amino acid and T/S is a threonine or serine residue.
16. The VLP of claim 15, wherein said amino acid mutation is located at an amino acid position corresponding to amino acid residues selected from 170-172 and 181-183 of SEQ ID NO: 2.
17. The VLP of any of claims 1-15, wherein said VLP comprises an influenza matrix (M1) protein.
18. The VLP of claim 17, wherein said M1 protein is derived from an avian influenza virus strain.
19. The VLP of claim 18, wherein said avian influenza virus strain is H9N2.
20. The VLP of claim 18, wherein said avian influenza virus strain is H5N1.
21. The VLP of claim 20, wherein said avian influenza virus strain is A/Indonesia/5/05.
22. The VLP of claim 21, wherein said M1 protein from A/Indonesia/5/05 comprises SEQ ID NO: 3.
23. The VLP of claim 17, wherein said M1 protein is derived from a swine influenza virus strain.

24. The VLP of claim 23, wherein said swine influenza virus strain is H1N1.
25. The VLP of claim 17, wherein said M1 protein comprises the amino acid residues YKKL at the amino acid positions corresponding to positions 100-103 of SEQ ID NO: 3.
26. The VLP of any of claims 1-25, wherein said VLP comprises an influenza neuraminidase (NA) protein.
27. The VLP of claim 26, wherein said NA protein exhibits neuraminidase activity.
28. The VLP of any of claims 1-27, wherein said VLP induces a stronger immune response in a mammal than a parental VLP comprising an HA protein which has not been mutated to remove one or more glycosylation sites.
29. A vaccine comprising a VLP according to any of claims 1-28, wherein said vaccine induces substantial immunity to influenza virus infection in a mammal susceptible to influenza.
30. The vaccine of claim 29, wherein said mammal is human.
31. A method of inducing substantial immunity to an influenza virus infection in a mammal susceptible to influenza, said method comprising administering to said mammal at least one effective dose of a vaccine, said vaccine comprising a VLP according to any of claims 1-28.
32. The method of claim 31, wherein said mammal is human.
33. A method of formulating a vaccine that induces substantial immunity to influenza virus infection in a mammal susceptible to influenza, comprising adding to said formulation an effective dose of a VLP according to any of claims 1-28, wherein said vaccine induces substantial immunity to influenza virus infection in said mammal.
34. A method of producing a VLP, the method comprising:

a) constructing a recombinant baculovirus construct encoding a recombinant influenza virus hemagglutinin (HA) protein, wherein said HA protein comprises one or more amino acid mutations that removes one or more glycosylation sites from said HA protein;

b) transfecting, infecting or transforming a suitable host cell with said recombinant baculovirus, and culturing the host cell under conditions which permit the expression of said HA protein;

c) allowing the formation of a VLP in said host cell;

d) harvesting infected cell media containing said VLP; and

e) purifying the VLP.

35. The method of claim 34, wherein said HA protein is derived from an avian influenza virus.

36. The method of claim 35, wherein said avian influenza virus is H5N1.

37. The method of claim 35, wherein said avian influenza virus is H9N2.

38. The method of claim 34, wherein said HA protein is derived from a swine influenza virus.

39. The method of claim 38, wherein said swine influenza virus is H1N1.

40. The method of claim 34, wherein said HA protein is derived from a seasonal influenza virus.

41. The method of claim 40, wherein said seasonal influenza virus is a type A influenza virus.

42. The method of claim 40, wherein said seasonal influenza virus is a type B influenza virus.

43. The method of any of claims 34-42, wherein said HA protein is a chimeric protein.

44. The method of any of claims 34-43, wherein said one or more amino acid mutations occurs within the receptor binding domain (RBD) of said HA protein.
45. The method of any of claims 34-44, wherein the amino acid mutation alters an NX(T/S) (SEQ ID NO: 1) sequence in said HA protein, wherein X is any amino acid and T/S is a threonine or serine residue.
46. The method of claim 45, wherein said amino acid mutation is located at an amino acid position corresponding to amino acid residues selected from 170-172 and 181-183 of SEQ ID NO: 2.