Title: OLIGOMERIC INFLUENZA IMMUNOGENIC COMPOSITIONS

Abstract: Described are proteins and immunogenic compositions for preventing infection with influenza viruses wherein the immunogenic compositions comprise immunogens such as the M2e and HA2 FP domains of influenza A virus and an immunopotentiator such as an Fc fragment of human IgG and optionally a stabilization sequence.

FIG. 1A  H5N1

FIG. 1B  H7N9
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OLIGOMERIC INFLUENZA IMMUNOGENIC COMPOSITIONS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. §119(e) to United States Provisional Patent Application 61/765,519 filed February 15, 2013, the entire contents of which is incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant No. R03 AI088449 awarded by United States National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present disclosure relates to the field of immunogenic compositions for the prevention of influenza infection.

BACKGROUND

[0004] The influenza A virus, which belongs to the Orthomyxoviridae family, can cause influenza in humans, birds or domesticated food animals. The virus can be further classified into different subtypes based on their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Of the 18 known HAs and 11 NAs, three HA subtypes (H1, H2, and H3) and two NA subtypes (N1 and N2) are most commonly found in humans. H1N1 and H3N2 are the major subtypes that cause human seasonal influenza and global pandemics of influenza. The influenza pandemic in 2009 was caused by influenza A virus H1N1 of swine origin. This has led to a growing concern regarding the pandemic potential of the highly pathogenic avian influenza (HPAI) H5N1 viruses. Thus the development of an effective and safe vaccine against divergent influenza A virus strains is urgently needed for the prevention of future outbreaks of influenza.

SUMMARY

[0005] Disclosed herein are proteins and immunogenic compositions for the prevention and treatment of infection with influenza viruses.

[0006] In one embodiment, a protein is provided comprising an immunogen sequence including an influenza A virus matrix protein M2e domain, or a fragment thereof, and an influenza A virus hemagglutinin fusion peptide (FP) domain, or a fragment thereof; and an immunopotentiator sequence.

[0007] In an embodiment, the FP domain is from influenza A virus hemagglutinin subtype 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18. In another embodiment,
the FP domain and the M2e domain are independently from an influenza A virus selected from an H1N1 virus, an H1N2 virus, an H2N2 virus, an H3N2 virus, an H5N1 virus, an H7N2 virus, an H7N3 virus, an H7N7 virus, an H7N9 virus, an H9N2 virus, or an H10N8 virus. In yet another embodiment, the amino acid sequence of the FP domain is at least 90% identical to one of SEQ ID NOs. 3, 7, 17, 18, 19, or 20. In another embodiment, the amino acid sequence of the M2e domain is at least 90% identical to one of SEQ ID NOs. 4, 8, 21, 22, 23, or 24. In certain embodiments, the immunogen comprises M2e-FP or FP-M2e.

[0008] In another embodiment, the protein further comprises a stabilization sequence. In another embodiment, the stabilization sequence is a foldon (Fd) or GCN4 sequence.

[0009] In another embodiment, the immunopotentiator sequence is an Fc fragment sequence of human IgG Fc, a C3d sequence, an *Onchocerca volvulus* ASP-1 sequence, a cholera toxin sequence, or a muramyl peptide sequence. In yet another embodiment, the immunopotentiator sequence is at least 90% identical to the human IgG Fc sequence of SEQ ID NO.10.

[0010] In another embodiment, the protein is a fusion protein. In one embodiment, the immunopotentiator sequence is linked to the C-terminus of the immunogen sequence. In yet another embodiment, the stabilization sequence is linked to the C-terminus of the immunogen sequence, and the immunopotentiator sequence is linked to the C-terminus of the stabilization sequence. In certain embodiments, the fusion protein comprises M2e-FP-Fc, FP-M2e-Fc, M2e-FP-FdFc, or FP-M2e-FdFc.

[0011] In another embodiment, the fusion protein further comprises a linker sequence disposed in at least one location from between the M2e and FP domains of the immunogen sequence, between the immunogen sequence and the stabilization sequence, and between the stabilization sequence and the immunopotentiator sequence. In another embodiment, the linker is (GGGGS)n, wherein n is an integer between 0 and 8. In one embodiment, n is 1.

[0012] Also provided is an immunogenic composition comprising a protein disclosed herein and at least one pharmaceutically acceptable excipient. In another embodiment, the immunogenic composition further comprises an adjuvant.

[0013] Further provided herein is a method of inducing an immune response against an influenza virus in a subject comprising administering an immunogenic composition or protein disclosed herein to the subject. In another embodiment, the immune response is a protective immune response. In another embodiment, the influenza virus is an influenza A virus, an influenza B virus, or an influenza C virus.
Further provided herein is a method of preventing infection of a subject with an influenza virus comprising administering an immunogenic composition or protein disclosed hereinto the subject. In another embodiment, the immune response is a protective immune response. In another embodiment, the influenza virus is an influenza A virus, an influenza B virus, or an influenza C virus.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1A-B depicts the schematic structures of constructed recombinant fusion proteins containing influenza A virus H5N1 M2e and FP domains, fused with foldon (Fd) and the fragment, crystallizable (Fc) portion of human IgG (M2e-FP-FdFc, also referred to as M2e-FP-FdFc), or with Fc (M2e-FP-Fc), or M2e with Fc alone (M2e-Fc), as well as H7N9 M2e and FP, fused with Fd and Fc of human IgG (H7N9 M2e-FP-FdFc). The highly conserved sequences of M2e and FP of H5N1 and H7N9 influenza A viruses as well as Fd sequence are listed on the top. Differences of amino acid residues in M2e and FP of H5N1 and H7N9 are underlined.

FIG. 2A-C depicts the characterization of recombinant M2e-FP fusion proteins by SDS-PAGE and Western blot. The purified H5N1 M2e-FP fusion proteins were analyzed by SDS-PAGE, followed by Coomassie Blue staining (FIG. 2A) and Western blot by using H5N1 M2e- (FIG. 2B) and HA2-specific MAbs (FIG. 2C), respectively. The protein molecular weight marker (kDa) is indicated on the left. The dilution of the MAbs is 1:3,000 for M2e (#16) and HA2 (1B12) of H5N1 virus.

FIG. 3A-B depicts an anti-influenza IgG antibody response induced by recombinant H5N1 M2e-FP fusion proteins. PBS was used as the negative control. FIG. 3A: H5N1 M2e-FP-specific IgG antibody responses were detected in mouse sera (1:6,400) collected at 10 days after each vaccination. The data are expressed as mean A450 ± standard deviation (SD) of 5 mice per group. FIG. 3B: Ability of IgG antibody binding to recombinant H5N1 M2e-FP fusion proteins, wherein the IgG antibody was detected in mouse sera collected at 10 days post-last vaccination. The data are presented as mean A450 ± SD of 5 mice per group at various dilution points.

FIG. 4A-B depicts the ability of IgG1 (FIG. 4A) and IgG2a (FIG. 4B) antibodies to bind to recombinant H5N1 M2e-FP fusion proteins, wherein the antibodies were detected in mouse sera collected at 10 days post-last vaccination. PBS was used as the negative control. The data are presented as mean A450 ± SD of 5 mice per group at various dilution points.

FIG. 5A-B depicts cross-clade protection induced by recombinant H5N1 M2e-FP fusion proteins against 10 lethal dose 50% (LD50) of H5N1 virus challenge. Vaccinated mice
were intranasally (i.n.) challenged with 10 LD<sub>50</sub> of H5N1 virus clade 1:A/Vietnam/1 194/2004 (VN/1 194) (FIG. 5A) or clade 2.3.4:A/Shenzhen/406H/06 (SZ/406H) (FIG. 5B), respectively, and survival rate (%) of the challenged mice was monitored for 2 weeks. The significance between survival curves from 8 mice per group was analyzed by Kaplan-Meier survival analysis with log-rank test. ‘*’ and ‘**’ indicate P < 0.05 and P < 0.01, respectively, as compared with the control group.

[0020] FIG. 6A-B depicts cross-clade protection induced by recombinant H5N1 M2e-FP fusion proteins against 3 LD<sub>50</sub> of H5N1 virus challenge. Vaccinated mice were i.n. challenged with 3 LD<sub>50</sub> of H5N1 virus clade 1:VN/1194 (FIG. 6A) or clade 2.3.4:SZ/406H (FIG. 6B), and survival rate (%) of the challenged mice were monitored for 2 weeks. The significance between survival curves from 8 mice per group was analyzed by Kaplan-Meier survival analysis with log-rank test. ‘**’ and ‘***’ indicate P <0.01 and P <0.001, respectively, as compared with the control group.

[0021] FIG. 7A-B depicts viral titers from lung tissues of mice vaccinated with recombinant H5N1 M2e-FP fusion proteins following lethal H5N1 virus challenge. Vaccinated mice were i.n. challenged with 10 LD<sub>50</sub> (FIG. 7A) or 3 LD<sub>50</sub> (FIG. 7B) of H5N1 virus clade 1:VN/1194 or clade 2.3.4:SZ/406H, respectively, and viral titers were detected 5 days post-challenge in the collected lung tissues. The data are expressed as Log<sub>10</sub>TCID<sub>50</sub>/g of lung tissues and presented as geometric mean titers (GMT) ± SD of 5 mice per group. The lower limit of detection is 1.5 Log<sub>10</sub>TCID<sub>50</sub>/g of tissues. The P values between different groups are indicated.

[0022] FIG. 8 depicts the detection of cross-reactivity of IgG antibody response induced by recombinant H5N1 M2e-FP fusion proteins with a recombinant H7N9 M2e-FP-FdFc protein. PBS was used as the negative control. The IgG antibody was detected in mouse sera collected at 10 days post-last vaccination. The data are presented as mean A450 ± SD of 5 mice per group at various dilution points.

**DEFINITION OF TERMS**

[0023] To facilitate an understanding of the following Detailed Description, Examples and appended claims it may be useful to refer to the following definitions. These definitions are non-limiting in nature and are supplied merely as a convenience to the reader.

[0024] Gene: A "gene" as used herein refers to at least a portion of a genetic construct having a promoter and/or other regulatory sequences required for, or that modify the expression of, the genetic construct.
Host: As used herein "host" refers to the recipient of the present immunogenic compositions. Exemplary hosts are mammals including, but not limited to, primates, rodents, cows, horses, dogs, cats, sheep, goats, pigs and elephants. In one embodiment of the present invention the host is a human. For the purposes of this disclosure host is synonymous with "vaccinee."

Immunogen: As used herein the term "immunogen" refers to any substrate that elicits an immune response in a host.

Immunogenic Composition: As used herein an "immunogenic composition" refers to an expressed protein or a recombinant vector, with or without an adjuvant, which expresses and/or secretes an immunogen in vivo and wherein the immunogen elicits an immune response in the host. The immunogenic compositions disclosed herein may or may not be immunoprotective or therapeutic. When the immunogenic compositions may prevent, ameliorate, palliate or eliminate disease from the host, then the immunogenic composition may optionally be referred to as a vaccine. However, the term immunogenic composition is not intended to be limited to vaccines.

Fusion Protein: As used herein, the term "fusion protein" refers to proteins created through the joining of two or more nucleic acid sequences which originally coded for separate proteins or peptides. Fusion proteins are typically created artificially by recombinant DNA technology. Expression of the combined nucleotide sequence results in a fusion protein including sequences from all the sources.

DETAILED DESCRIPTION

Development of an effective and safe immunogenic composition against divergent influenza A viruses (IAVs) is urgently needed for the prevention of future outbreaks of influenza, especially because of the pandemic potential of the divergent strains of highly pathogenic avian influenza (HPAI) H5N1 viruses. The present disclosure describes the development of an influenza immunogenic composition based on the extracellular domain of matrix protein 2 (M2e) and the "fusion peptide" domain (FP) of hemagglutinin (HA) 2 of IAV. This immunogenic composition induced strong immune responses, and extensive cross-protective immunity in immunized animals.

A universal influenza immunogenic composition that could provide heterosubtypic immunity would be a tremendous advance for public health. Disclosed herein is an influenza immunogenic composition fusion protein comprising two immunogens, an extracellular domain of M2e, and an FP domain of an IAV HA2 protein, which are then linked with an immunopotentiator. Optionally, a trimerization stabilization sequence is disposed
between the immunogen and the immunopoentiator. The immunogens can be in any order; however, the immunopotentiator is C-terminal to the immunogens.

[0031] As used herein with regard to influenza immunogenic compositions, the term "universal" refers to immunogenic compositions which can induce a protective immune response against influenza viruses of different clades or strains than the source of the immunogens. For example, an immunogenic composition comprised of sequences from H5N1 virus also protects against an H1N1 or an H7N9 virus. Additionally, immunogenic compositions comprised of sequences from one IAV also protect against all, or most, other IAVs. Additionally, immunogenic compositions comprised of sequences from IAVs also protect against influenza B or C viruses.

[0032] Influenza type A viruses are divided into subtypes on the basis of two proteins on the surface of the virus: HA and neuraminidase (NA). For example, an H7N2 virus designates an IAV subtype that has an HA 7 protein and an NA 2 protein. Similarly an H5N1 virus has an HA 5 protein and an NA 1 protein. There are 18 known HA subtypes and 11 known NA subtypes. Many different combinations of HA and NA proteins are possible. No matter which strain the immunogens are from, the claimed immunogenic compositions protect against infection against other IAV strains. Additionally, the disclosed immunogenic compositions are protective against infection with influenza B and C viruses.

[0033] Influenza viruses are further classified by their serotype and are identified by the virus type, the geographical origins, the strain number, year of isolation, and virus subtype. For example, A/Anhui/1/2005(H5N1) refers to an IAV originating in Anhui, China in 2005, is strain number 1, and is an H5N1 subtype.

[0034] Hemagglutinin (HA) is a homotrimeric integral membrane glycoprotein of influenza virus. It is shaped like a cylinder, and is approximately 13.5 nanometers long. The three identical monomers that constitute HA are constructed into a central a helix coil; three spherical heads contain the sialic acid binding sites. HA monomers are synthesized as precursors that are then glycosylated and cleaved into two smaller polypeptides: the HA1 and HA2 subunits. Each HA monomer consists of a long, helical chain anchored in the membrane by HA2 and topped by a large HA1 globule. The most conserved region of HA is at the N-terminus of the HA2 subunit, a relatively hydrophobic sequence of amino acids (aa) referred to as the "fusion peptide" (FP). In one embodiment, the FP domain comprises the first 20-30 aa of the HA2 subunit of IAV. In other embodiments, the FP domain comprises the first 21-29 aa, the first 22-29 aa, the first 23-28 aa, the first 21 aa, the first 22 aa, the first 23 aa, the first 24 aa, the first 25 aa, the first 26 aa, the first 27 aa, the first 28 aa, or the first
29 aa of the HA2 subunit of IAV. Exemplary FP domains can be found in SEQ ID NOs, 3, 7, 17, 18, 19, and 20.

[0035] The influenza virus M2 protein is a transmembrane ion channel which allows protons to move through the viral envelope and acidify the core of the virus, which causes the core to disassemble and release the viral RNA and core proteins. The external domain of the M2 protein (M2e) is 23 aa long and has a highly conserved sequence. Exemplary M2e domains can be found in SEQ ID NOs, 4, 8, 21, 22, 23, and 24.

[0036] Previously designed influenza HA-based vaccines could not induce highly potent and broad neutralizing responses in the hosts, most likely because these vaccines could not properly maintain the stable and soluble trimeric conformation, or they lack efficient immunogenicity to induce high levels of neutralizing antibodies. The presently described immunogenic compositions have solved these problems by linking a Fc (fragment crystallizable) fragment of IgG to the IAV fragments, which results in enhanced immunogenicity of the immunogen to induce high levels of cross-protection against a broad spectrum of influenza viruses. In addition, the Fc fragment has tendency to form a non-covalent dimer through its disulfide bond, which may allow the fusion protein to form a dimer, hexamer or other form of oligomer, resulting in a more immunogenic molecule. In certain embodiments, where improvement of stability of conformation is needed, a stabilization or trimerization sequence allows the fusion protein to maintain a stable and soluble trimeric conformation.

[0037] The ability to induce antibodies against divergent strains of a particular virus solves the one strain-one vaccine problem that has been a significant hurdle for all manufacturers of influenza vaccines. Furthermore, the described immunogenic compositions do not require chicken eggs to grow the virus - a major advantage that not only significantly reduces manufacturing time and cost, but also allows pregnant women and persons allergic to chicken egg proteins to receive the vaccine. The disclosed proteins are instead produced by standard recombinant protein production means.

[0038] In one embodiment, the FP domain component of the instant immunogenic composition or protein comprises a sequence from an IAV HA of any subtype, i.e. subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, H17, or H18. In another embodiment, the FP domain component is from an IAV HA of subtype H1, subtype H2, subtype H3, subtype H5, subtype H7, or subtype H9. In another embodiment, the FP domain is from an H1N1 IAV, an H1N2 IAV, an H2N2 IAV, an H3N2 IAV, an H5N1 IAV, an H7N2 IAV, an H7N3 IAV, an H7N7 IAV, an H7N9 IAV, an H9N2 IAV, or an H10N8 IAV; or an FP sequence of any IAV. In another embodiment, the M2e component of the instant
immunogenic composition can comprise a sequence from an H1N1 IAV, an H1N2 IAV, an H2N2 IAV, an H5N1 IAV, an H7N2 IAV, an H7N3 IAV, an H7N7 IAV, an H7N9 IAV, an H9N2 IAV, or an H10N8 IAV; or an M2e sequence of any IAV. Amino acid and nucleic acid sequences for each of the above domains can be found in the Influenza Research Database (http://www.fludb.org/brc/home). The terms "protein" and "polypeptide" refer to similar structures and are used interchangeably herein.

Table 1. Amino acid sequences of immunopotentiator-linked oligomeric influenza immunogenic compositions.

<table>
<thead>
<tr>
<th>SEQ ID</th>
<th>N.0.1</th>
<th>[A/Anhui/1/2005(H5N1) HA1:</th>
</tr>
</thead>
</table>
|        |       | MEKIVLAVSLVKSDQICGYHANNSTEVQDTIMEKNVTVAQDILEKTHNGKLCDDLGVMKLRLDCSVGAGWLLGNPMCDEFINVPEWSYIVEKAAPANDLCYPGNYNDYEELKLLSRINHFEEKIQIPKSSWDHASEGSSVGGStcYGTGSFFRNVWLKINNTYPIKSYNSYNNTQEDLLILWGHHNSDAAGTQLKQNTTYSISVGTSTLNNQRLVFKJATRSKVNGQNRMDDFWTILKPDNIAFSNESEAYPKIKGDSAVKSEVGYNCNTKCGTPIGAINSSMPFHNHIPLTTGECPKYKSNLVLATGLRNSPLERRRRKQRLFGAIAGFIEGGWQGMVGWDGYHYHSHNEQGSGYAADKESTQKIAIDGVTVKNSIDKMNTOFEAVGREFNNLERRIONDCQKMEFDGLDVTVYNAELVLMPERLTRLDHDSNKNKYDVKRLQRDNAELGNGCFEFYHKCDNCEMESVRNGTYDYPQYSEEARLKEEISGVKLESIGTYQILSITYSVASSSLAIVMAGLSLWMCNNGSLQCRICI
| SEQ ID | N.0.2 | [A/Anhui/1/2005(H5N1) HA2: |
|        |       | GLFGAIAGFIEGGWQGMVGWDGYHYHSHNEQGSGYAADKESTQKIAIDGVTVKNSIDKMNTOFEAVGREFNNLERRIONDCQKMEFDGLDVTVYNAELVLMPERLTRLDHDSNKNKYDVKRLQRDNAELGNGCFEFYHKCDNCEMESVRNGTYDYPQYSEEARLKEEISGVKLESIGTYQILSITYSVASSSLAIVMAGLSLWMCNNGSLQCRICI
| SEQ ID | N.0.3 | H5N1 FP: |
|        |       | GLFGAIAGFIEGGWQGMVGWDGYHYHSHN
| SEQ ID | N.0.4 | H5N1 M2e: |
|        |       | MSLLTEVETPTREPWHNCDSASS
| SEQ ID | N.0.5 | [A/Anhui/1/2013(H7N9) HA1: |
|        |       | MNTQILVFAlAIIIPTNADKCIKCLGHAVNSGTKVNTLTERGVEVNAETETERTNPICRSGKRTVDLGCCGELLGTIPDPOCDPLAFQSAELNRRREGSCDVPCGKFVNEFLAILQIRESGGIDKEAMGFTYSGIRTNGATACSRRSGSFSFYAEKMWLSSNTDANAPDOMTSYKNTRKSPALIWHVGHHSVSTAEQKYLGSNKLKTVGSSNYQSFVPSGAQVGNLSCRDIFHWMLNPDNTVTNSFANGAFIAPDRASFLRGKSMGSSQGSVQVBDANCEGDCYHSVGTLISNLPGQINDRSAVGCGRPYVKQRSLLALTGMKTVPEIPKGRLFGAIAGFIEGWEGIDGWYFHRHQNAQGEGAADYKSTQSAIDQTGKLNRLIEKTNQFQELIDNEFNEVEKIQIGNVINWTRDSITEVSWYNAELVAMENQHOTTADSEMDKLYERVKVQDRENAEDEGTCGEFIFHCKCDDCMASIRNTYDHSKRYEAMQNRIQDVPKLVSSGYKYDVILWFSFAGSCELLAIVMGVLFVICVKNGNMRCTICI
| SEQ ID | N.0.6 | [A/Anhui/1/2013(H7N9) HA2: |
|        |       | GLFGAIAGFIEGGWQGMVGWDGYHYHSHN

8
SEQ ID NO.7  H7N9 FP:
GLFGAIAGFIENGWEGLIDGWGYFRHQN

SEQ ID NO.8  H7N9 M2e:
MSLLTEVETPTRGWCNCGSSE

SEQ ID NO.9  [Foldon (Fd)]:
GYipeAPRQQAyVRKDEGVWLLSTFL

SEQ ID NO.10 [human IgG Fc (hFc)]:
RSDKTHTCPPCPAPELLGGPSVFLFPPKPKDLMISRTPEVTVCLVMDVPVSHEDPEVKFNWYVE
DGVEVHNAKTTPREEQYNSTYRVSVTLYQLDVHDFQWLNGKEYKCKVSNKALPPIEKTISAK
QGPREPQVYTLPPSEREMTKNQVSLCLVKQYFSPDIAVEWEHNAQPENNYKTTTPVLSDDGFS
FSLYKSLTVDKSRQVQGNVFCVSMHEALHNYTQKSLSPGK

SEQ ID NO.11 [mouse IgG Fc (mFc)]:
RSPPRGPTIKPCAPCPAPNLGGSVFLFPPKPKDLMISRTPEVTVCLVMDVPVSHEDPEVKFNWYVE
DGVEVHNAKTTPREEQYNSTYRVSVTLYQLDVHDFQWLNGKEYKCKVSNKALPPIEKTISAK
QGPREPQVYTLPPSEREMTKNQVSLCLVKQYFSPDIAVEWEHNAQPENNYKTTTPVLSDDGFS
FSLYKSLTVDKSRQVQGNVFCVSMHEALHNYTQKSLSPGK

SEQ ID NO.12 [rabbit IgG Fc (rFc)]:
RSPRPQTVKPCAPCPAPNLGGSVFLFPPKPKDLMISRTPEVTVCLVMDVPVSHEDPEVKFNWYVE
DGVEVHNAKTTPREEQYNSTYRVSVTLYQLDVHDFQWLNGKEYKCKVSNKALPPIEKTISAK
QGPREPQVYTLPPSEREMTKNQVSLCLVKQYFSPDIAVEWEHNAQPENNYKTTTPVLSDDGFS
FSLYKSLTVDKSRQVQGNVFCVSMHEALHNYTQKSLSPGK

SEQ ID NO.17  H1 FP [A/California/06/2009(H1N1)1]:
GLFAJAGFIEGGWTVGMDGWYHYHQN

SEQ ID NO.18  H2 FP [A/Japan/305/1957(H2N2)1]:
GLFAJAGFIEGGWGVQGMDGWYHYHNSN

SEQ ID NO.19  H3 FP [A/Arizona/08/2009(H3N2)1]:
GIFAAIAGFIENGWGMMDGWYGFRHQN

SEQ ID NO.20  H9 FP [A/Hong Kong/1 074/99(H9N2)1]:
GLFAJAGFIEGGWGPGLAVGWYGFQHNSN

SEQ ID NO.21  H1 M2e [A/California/06/2009(H1N1)1]:
MSLLTEVETPTRSEWCRCSSD

SEQ ID NO.22  H2 M2e [A/Japan/305/1957(H2N2)1]:
MSLLTEVETPIRSEWCRCSSD

SEQ ID NO.23  H3 M2e [A/Arizona/08/2009(H3N2)1]:
MSLLTEVETPIRSEWCRCSSD

SEQ ID NO.24  H9 M2e [A/Hong Kong/1 074/99(H9N2)1]:
MSLLTEVETLTRNGWECCKCSSD
SEQ ID NO.25 [Human C3d (aa residues 1002-1303 in C3)]:
HLIVTPSGCQRGMGMPVTIVAVHYLDLTEQWEKFGLEKROIAGALEIKKGYTQQALAFROP
SSAFAFVKRAPSTWLAYKSFVSLAVNIAIDSQVLCGAVWLIKWLEKQPDGVIQVDAPV
XHQEMIGFLRNNKEDMALTAFVLISLQEOAKDICEEQVNLPSLGSITKAGDFLEANYMNLQ
SYTVIAIGYALAMQRKPLNNKFLTTKDKKNRWEQGQLYNEVTASYALLALLQKDFF
VPPWRWLNQERYGGGSTQATFMVFQALAYQKDBAPDQHELNLDDVSLQLPSR

SEQ ID NO.26 [Cholera toxin b subunit (amino acid 1-124)]:
MTPQNITDLCAEYHNTQIHTLNDKIFSYTESLAGKREMAITFKNGATFQVEVPGSQQHDSQK
KAIERMKDLTLRIAYLTEAKVEKCWNKNKTPRAIAASAN

[0039]  In one embodiment, the stabilization sequence comprises a sequence that stabilizes the HA sequence in the trimer or oligomer configuration. As used herein, the terms stabilization sequence, trimeric motif and trimerization sequence are interchangeable and equivalent. Suitable stabilization sequences include, but are not limited to, a 27 aa region of the C-terminal domain of T4 fibritin sequence (a foldon-like sequence); (GYIPEAPRDQAY VRKDGEXVLLSTFL, SEQ ID NO. 9 or GSGYIPEAPRDQAYVRKDGEXVLLSTFL, SEQ ID NO. 13), a GCN4 sequence (MKQIEKEDKIEILSKYHIEIARI KKLIGEV; SEQ ID NO. 14) sequence, an IQ sequence (RMKQIEKIEIESKQKKIEIARIKK; SEQ ID NO. 15) or an Iz sequence (IKKEIIEAIKKIEEAIKKKIEAIK; SEQ ID NO. 16). Other suitable stabilization methods include, but are not limited to, 2,2-bipyridine-5-carboxylic acid (BPy), disulfide bonds, and facile ligation.

[0040]  In another embodiment, the immunopotentiator comprises a sequence to enhance the immunogenicity of the immunogenic composition. Suitable immunopotentiators include, but are not limited to, an Fc fragment of human IgG (SEQ ID NO.10), a C3d (SEQ ID NO.25), a complement fragment that promotes antibody formation binding to antigens enhancing their uptake by dendritic cells and B cells, an Ov-ASP-1 (Onchocerca volvulus homologue of the activation associated secreted gene family) (see US 20060039921 , which is incorporated by reference herein for all it discloses regarding Ov-ASP-1 adjuvants), a cholera toxin (SEQ ID NO.26), a muramyl dipeptide, and fragments thereof.

[0041]  In one embodiment, the immunopotentiator is an immunoglobulin Fc fragment. The immunoglobulin molecule consists of two light chains (LCs) and two heavy chains (HCs) held together by disulfide bonds such that the chains form a Y shape. The base of the Y (carboxyl terminus of the HC) plays a role in modulating immune cell activity. This region is called the Fc region, and is composed of two HCs that contribute two or three constant domains depending on the class of the antibody. By binding to specific proteins, the Fc region generates an appropriate immune response for a given
antigen. The Fc region also binds to various cell receptors, such as Fc receptors, and other immune molecules, such as complement proteins. By doing this, it mediates different physiological effects including opsonization, cell lysis, and degranulation of mast cells, basophils and eosinophils.

[0042] Exemplary immunogenic compositions are found in FIG. 1. In certain embodiments, the M2e domain and FP domain sequences of the fusion protein are linked through a flexible linker comprising \((\text{GGGGS})_n\) (SEQ ID NO.27), wherein \(n\) is an integer between 0 and 8. In certain embodiments, \(n\) is 0, \(n\) is 1, \(n\) is 2, \(n\) is 3, \(n\) is 4, \(n\) is 5, \(n\) is 6, \(n\) is 7, or \(n\) is 8. Additionally, the FP and Fd and/or Fc portions of the fusion protein are linked through a flexible linker comprising \((\text{GGGGS})_n\), wherein \(n\) is an integer between 0 and 8. In certain embodiments, \(n\) is 0, \(n\) is 1, \(n\) is 2, \(n\) is 3, \(n\) is 4, \(n\) is 5, \(n\) is 6, \(n\) is 7, or \(n\) is 8.

[0043] In additional embodiments the use of conservatively modified variants of the immunogenic compositions is provided. The variants described herein maintain the biological activity of the parent or source molecule.

[0044] As used herein, a “conservative substitution” involves a substitution of one amino acid for another found in one of the following conservative substitutions groups: Group 1: alanine (Ala), glycine (Gly), serine (Ser), threonine (Thr); Group 2: aspartic acid (Asp), glutamic acid (Glu); Group 3: asparagine (Asn), glutamine (Gin); Group 4: arginine (Arg), lysine (Lys), histidine (His); Group 5: isoleucine (Ile), leucine (Leu), methionine (Met), valine (Val); and Group 6: phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp).

[0045] Additionally, amino acids can be grouped into conservative substitution groups by similar function or chemical structure or composition (e.g., acidic, basic, aliphatic, aromatic, sulfur-containing). For example, an aliphatic grouping may include, for purposes of substitution, Gly, Ala, Val, Leu, and Ile. Other groups containing amino acids that are considered conservative substitutions for one another include: sulfur-containing: Met and cysteine (Cys); acidic: Asp, Glu, Asn, and Gin; small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro, and Gly; polar, negatively charged residues and their amides: Asp, Asn, Glu, and Gin; polar, positively charged residues: His, Arg, and Lys; large aliphatic, nonpolar residues: Met, Leu, Ile, Val, and Cys; and large aromatic residues: Phe, Tyr, and Trp.

[0046] Modifications (which do not normally alter primary sequence) include in vivo, or in vitro chemical derivatization of polypeptides or proteins, e.g., acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide or protein during its synthesis and processing or in further processing steps; e.g. by exposing the polypeptide or protein to enzymes which affect
glycosylation, e.g., mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated aa residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

[0047] Also included are proteins which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties. Analogs of such proteins include those containing residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring synthetic amino acids. The proteins disclosed herein are not limited to products of any of the specific exemplary processes listed herein.

[0048] Thus, disclosed herein are an M2e domain sequence, an FP domain sequence, an immunopotentiator sequence, a human Fc sequence, a Fd sequence, a GCN4 sequence, an IQ sequence, an IZ sequence, a fusion protein sequence, or a protein sequence having one or several aa deletions, substitutions and/or additions and which maintain the biological activity of the unsubstituted peptide or protein.

[0049] As used herein, polypeptide sequences which are substantially the same typically share more than 95% aa identity. It is recognized, however, that proteins (and DNA or mRNA encoding such proteins) containing less than the above-described level of homology arising as splice variants or that are modified by conservative aa substitutions (or substitution of degenerate codons) are contemplated to be within the scope of the present disclosure. As readily recognized by those of skill in the art, various ways have been devised to align sequences for comparison, e.g., Blosum 62 scoring matrix, as described by Henikoff and Henikoff in Proc. Natl. Acad. Sci. USA 89:10915 (1992). Algorithms conveniently employed for this purpose are widely available (see, for example, Needleman and Wunsch in J. Mol. Bio. 48:443 (1970).

[0050] Therefore, within the scope of the present disclosure are aa sequences 85%-100% identical to a M2e domain sequence, an FP domain sequence, an immunopotentiator sequence, a human Fc sequence, a Fd sequence, a GCN4 sequence, an IQ sequence, an IZ sequence, a fusion protein sequence, or a protein sequence disclosed herein. In aspects of this embodiment, an aa sequence is at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to an M2e domain sequence, an FP domain sequence, an immunopotentiator sequence, a human Fc sequence, a Fd sequence, a GCN4 sequence, an IQ sequence, an IZ sequence, a fusion protein sequence, or a protein sequence disclosed herein.

[0051] The following expression systems are suitable for use in expressing the disclosed proteins, immunogens, immunopotentiators, and stabilization sequences:
mammalian cell expression systems such as, but not limited to, the pcDNA and GS Gene expression systems; insect cell expression systems such as, but not limited to, Bac-to-Bac, baculovirus, and DES expression systems; and Escherichia coli expression systems including, but not limited to, pET, pSUMO, and GST expression systems.

[0052] Various advantages are associated with expression of proteins in mammalian cell expression systems. The mammalian cell expression system is a relatively mature eukaryotic system for expression of recombinant proteins. It is more likely to achieve correctly folded soluble proteins with proper glycosylation, making the expressed protein maintain its native conformation and keep sufficient bioactivity. This system can either transiently or stably express recombinant antigens, and promote signal synthesis. Recombinant proteins expressed in this way may keep good antigenicity and immunogenicity. However, both insect and bacterial expression systems provide inexpensive and efficient expression of proteins which may be appropriate under certain conditions.

[0053] The optimal purification systems are dependent on whether a tag is linked or fused with the fusion proteins. If the fusion proteins are fused with IgG Fc, Protein A, or Protein G, affinity chromatography is used for the purification. If the fusion proteins are fused with GST proteins, GST columns will be used for the purification. If the fusion proteins link with 6xHis tag at the N- or C- terminal, the expressed proteins can be purified using His tag columns. If no tag is linked with the fusion protein, the expressed protein could be purified using fast protein liquid chromatography (FPLC), high performance liquid chromatography (HPLC), or other chromatography.

[0054] In certain embodiments, the immunogenic compositions further comprise or are administered with an adjuvant. Adjuvants suitable for use in animals include, but are not limited to, Freund's complete or incomplete adjuvants, Sigma Adjuvant System (SAS), and Ribi adjuvants. Adjuvants suitable for use in humans include, but are not limited to, MF59 (an oil-in-water emulsion adjuvant), Montanide ISA 51 or 720 (a mineral oil-based or metabolizable oil-based adjuvant), aluminum hydroxide, -phosphate or -oxide, HAVLOGEN® (an acrylic acid polymer-based adjuvant, Intervet Inc., Millsboro, DE), polycrylic acids, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as BAYOL™ or MARCOL™ (Esso Imperial Oil Limited, Canada), or a vegetable oil such as vitamin E acetate, saponins, and Onchocerca volvulus activation-associated protein-1 (Ov-ASP-1) (see US 20060039921, which is incorporated by reference herein for all it discloses regarding Ov-ASP-1 adjuvants). However, components with adjuvant activity are widely known and, generally, any adjuvant may be utilized that does not adversely interfere with the efficacy or safety of the vaccine and/or immunogenic composition.
Immunogenic compositions according to the various embodiments disclosed herein can be prepared and/or marketed in the form of a liquid, frozen suspension, or in a lyophilized form. Typically, vaccines and/or immunogenic compositions prepared according to the present disclosure contain a pharmaceutically acceptable carrier or diluent customarily used for such compositions. Carriers include, but are not limited to, stabilizers, preservatives and buffers. Suitable stabilizers are, for example SPGA, Tween compositions (such as are available from A.G. Scientific, Inc., San Diego, CA), carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Non-limiting examples of suitable buffers include alkali metal phosphates. Suitable preservatives are thimerosal, merthiolate and gentamicin. Diluents include water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

Also disclosed herein are methods for inducing an immune response to an influenza virus using the disclosed proteins or immunogenic compositions. Generally, the protein or immunogenic composition may be administered subcutaneously, intradermally, submucosally, or intramuscularly in an effective amount to prevent infection from the influenza virus of interest and/or treat an infection from the influenza virus. An effective amount is defined as an amount of immunizing protein or immunogenic composition that will induce immunity in the vaccinated animals against challenge by a virulent virus. Immunity is defined herein as the induction of a significant higher level of protection in a population of the animals after vaccination compared to an unvaccinated group.

Protective immune responses can include humoral immune responses and cellular immune responses. Protection against influenza is believed to be primarily conferred through serum antibodies (humoral immune response) directed to the surface proteins, with mucosal IgA antibodies and cell-mediated immune responses also playing a role. Cellular immune responses are useful in protection against influenza virus infection with CD4+ and CD8+ T cell responses well documented. CD8+ immunity is of particular importance in killing virally infected cells.

Further, in various formulations of the proteins and/or immunogenic compositions, suitable excipients, stabilizers and the like may be added as are known by persons of ordinary skill in the art.

The disclosed proteins, immunogenic compositions, and methods may be used to prevent influenza virus infection in a subject susceptible to influenza virus such as, but not limited to, a human, a primate, a domesticated animal, an animal in the wild, or a bird.
EXAMPLES

Example 1

Construction and testing of recombinant immunogenic proteins of H5N1 virus

[0060] The genes encoding the 24 aa M2e domain (residues 1-24 of M2e) and the 28-aa FP domain (residues 1-28 of HA2) of an H5N1 IAV fused with Fd were amplified by PCR from a plasmid containing M2e and a full-length HA plasmid of IAV H5N1 (A/Anhui/1/2005 (H5N1)) as the templates and fused together with the Fd fragment using overlapping primers, followed by insertion into the pFUSE-hlgG1-Fc2 expression vector, generating H5N1 M2e-FP-FdFc (hereinafter M2e-FP-FdFc). For comparison, the amplified H5N1 M2e-FP genes or M2e genes were also directly inserted into the above expression vector, forming M2e-FP-Fc, and M2e-Fc, respectively (FIG. 1). H7N9 M2e-FP-FdFc (e.g., M2e-FP-FdFc) was constructed by PCR amplifying M2e and FP sequences of an H7N9 (A/Anhui/1/2013(H7N9)) influenza virus, and fusing with Fd using overlapping primers, and then inserting into the pFUSE-hlgG1-Fc2 expression vector. The sequence-confirmed recombinant plasmids were transfected into mammalian 293T cells by calcium phosphate method, and related recombinant proteins were purified by Protein A affinity chromatography using collected culture supernatant. The expressed proteins were confirmed for expression and specificity by SDS-PAGE and Western blot using M2e- and HA2-specific mAbs (FIG. 2).

[0061] Three purified H5N1 M2e-FP fusion proteins were then used to immunize mice three times at three week intervals, and sera were collected before immunization and 10 days post-each vaccination to detect the production of M2e- and FP-specific antibodies and subtypes (FIGS. 3-4). The vaccinated mice were detected for cross-clade protection against challenge of H5N1 virus covering clade 1:AA/ietNam/1194/2004 (VN/1194) or clade 2.3.4:A/Shenzhen/406H/06 (SZ/406H) using 10 lethal dose 50% (10 LD_{50}) and 3 LD_{50}, respectively (FIGs. 5-6). The challenged mice were monitored for 2 weeks, after which the survival rate (%) was calculated. Virus titers were also detected in the collected mouse lung tissues at 5 days post-virus challenge (FIG. 7).

[0062] The experiment results showed that the recombinant H5N1 M2e-FP fusion proteins could be expressed in the culture supernatant of transfected cells with high purity, and recognized by mAbs specifically targeting M2e and FP domains of IAVs. All three fusion proteins, particularly M2e-FP fused with Fd and Fc (M2e-FP-FdFc), were able to form high-molecular-weight molecules with conformational structures (FIG. 2). These fusion proteins, especially M2e-FP-FdFc, induced highly potent M2e and/or FP-specific IgG antibodies and IgG1 and IgG2a subtypes in the vaccinated mouse sera, reaching the highest antibody titers after the last vaccination. In general, M2e-FP-FdFc protein induced a relatively stronger
antibody response than M2e-FP-Fc or M2e-Fc, potentially due to the addition of Fd and Fc in promoting the formation of high molecular weight molecules, accordingly promoting immunogenicity (FIGs. 3-4). Importantly, recombinant fusion proteins of Me-FP were capable of protecting vaccinated mice against tested strains of VN/1 194 and SZ/406H H5N1 viruses at 10 LD_{50} and 3 LD_{50}, respectively. Particularly, the M2e-FP-FdFc protein completely cross-protected all vaccinated mice against challenge with clade 1:VN/1 194 and clade 2.3.4:SZ/406H virus at the lethal dose of 3 LD_{50}, while it still cross-protected 70% of vaccinated mice against these two virus challenges at the lethal dose of 10 LD_{50}. In comparison, M2e-FP-Fc, especially M2e-Fc, provided significantly lower protection rate. No mice in the PBS control group survived the challenge of two virus strains after day 9-1 1 (FIGs. 5-6). Accordingly, lower level of virus titers were detected in the mice vaccinated with M2e-FP-FdFc, followed by M2e-FP-Fc, and M2-Fc proteins, after lethal challenge with 3 LD_{50} and 10 LD_{50} of VN/1 194 and SZ/406H H5N1 virus, respectively. However, virus titer in the PBS control group was still significantly higher than the vaccination groups in all tested mice (FIG. 7). Detection of the cross-reactivity of these vaccinated mouse sera with H7N9 M2e-FP-FdFc protein showed that all three H5N1 fusion proteins were able to induce in vaccinated mice IgG antibody responses that cross-reacted with a recombinant protein containing conserved sequences of M2e and FP domains of a newly emerged avian IAV H7N9 (FIG. 8), suggesting the potential protection of these H5N1 fusion proteins against H7N9 IAV infection.

[0063] The above results indicate that the expressed recombinant influenza immunogenic compositions, which contain highly conserved epitope sequences of M2e and FP, and fused with Fd and Fc (M2e-FP-FdFc), has a great potential to be developed into a "universal" influenza vaccine against divergent influenza viruses, including IAVs, suggesting its ability in the prevention of future influenza pandemic.

[0064] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as
precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0065] The terms "a," "an," "the" and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0066] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0067] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0068] Specific embodiments disclosed herein may be further limited in the claims using consisting of or consisting essentially of language. When used in the claims, whether as filed
or added per amendment, the transition term "consisting of" excludes any element, step, or ingredient not specified in the claims. The transition term "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s). Embodiments of the invention so claimed are inherently or expressly described and enabled herein.

[0069] Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above-cited references and printed publications are individually incorporated herein by reference in their entirety.

[0070] In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described.
We claim:

1. A protein comprising:
   an immunogen sequence including an influenza A virus matrix protein M2e
domain, or a fragment thereof, and an influenza A virus hemagglutinin fusion peptide (FP)
domain, or a fragment thereof; and
   an immunopotentiator sequence.

2. The protein of claim 1, wherein the FP domain is from influenza A virus
   hemagglutinin subtype 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18.

3. The protein of claim 1, wherein the FP domain and the M2e domain are
   independently from an influenza A virus selected from an H1N1 virus, an H1N2 virus, an
   H2N2 virus, an H3N2 virus, an H5N1 virus, an H7N2 virus, an H7N3 virus, an H7N7 virus,
   an H7N9 virus, an H9N2 virus, or an H10N8 virus.

4. The protein of claim 1, wherein the amino acid sequence of the FP domain is
   at least 90% identical to one of SEQ ID NOs. 3, 7, 17, 18, 19, or 20.

5. The protein of claim 1, wherein the amino acid sequence of the M2e domain
   is at least 90% identical to one of SEQ ID NOs. 4, 8, 21, 22, 23, or 24.

6. The protein of claim 1, wherein the protein further comprises a stabilization
   sequence.

7. The protein of claim 4, wherein the stabilization sequence is a foldon (Fd) or
   GCN4 sequence.

8. The protein of claim 1, wherein the immunopotentiator sequence is an Fc
   fragment sequence of human IgG Fc, a C3d sequence, an *Onchocerca volvulus* ASP-1
   sequence, a cholera toxin sequence, or a muramyl peptide sequence.

9. The protein of claim 1, wherein the immunopotentiator sequence is at least
   90% identical to the human IgG Fc sequence of SEQ ID NO.10.

10. The protein of claim 1, wherein the protein is a fusion protein.

11. The protein of claim 10, wherein the immunogen comprises M2e-FP

12. The protein of claim 10, wherein the immunogen comprises FP-M2e.

13. The protein of claim 10, wherein the immunopotentiator sequence is linked to
   the C-terminus of the immunogen sequence.
14. The protein of claim 10, wherein the stabilization sequence is linked to the C-terminus of the immunogen sequence, and the immunopotentiator sequence is linked to the C-terminus of the stabilization sequence.

15. The protein of claim 11, wherein the fusion protein comprises M2e-FP-Fc.

16. The protein of claim 12, wherein the fusion protein comprises FP-M2e-Fc.

17. The protein of claim 11, wherein the fusion protein comprises M2e-FP-FdFc.

18. The protein of claim 12, wherein the fusion protein comprises FP-M2e-FdFc.

19. The protein of claim 10, wherein the fusion protein further comprises a linker sequence disposed in at least one location from between the M2e and FP domains of the immunogen sequence, between the immunogen sequence and the stabilization sequence, and between the stabilization sequence and the immunopotentiator sequence.

20. The protein of claim 19, wherein the linker is (GGGGS)ₙ, wherein n is an integer between 0 and 8.

21. The protein of claim 20, wherein n is 1.

22. An immunogenic composition comprising the protein of any one of claims 1-15 and at least one pharmaceutically acceptable excipient.

23. The immunogenic composition of claim 16 wherein the immunogenic composition further comprises an adjuvant.

24. A method of inducing an immune response against an influenza virus in a subject comprising:
   - administering the immunogenic composition of claim 16 to the subject.

25. The method of claim 19, wherein the immune response is a protective immune response.

26. The method of claim 19, wherein the influenza virus is an influenza A virus, an influenza B virus, or an influenza C virus.

27. A method of preventing infection of a subject with an influenza virus comprising:
   - administering the immunogenic composition of claim 16 to the subject.

28. The method of claim 27, wherein the immune response is a protective immune response.

29. The method of claim 27, wherein the influenza virus is an influenza A virus, an influenza B virus, or an influenza C virus.
**FIG. 1A  H5N1**

MSLLTEVEPTPRNEWECRCSDSSE  SEQ ID NO:4

GLFGAIAGFIEGGWQGMVDGWGYGHSSN  SEQ ID NO:3

GYIPEAPRDQAYVRKDGWEWLLSTFL  SEQ ID NO:9

M2e-FP-FdFc

M2e  FP  Fd  Fc

GGGGS linker  SEQ ID NO:25

M2e-FP-Fc

M2e  FP  Fc

M2e-Fc

M2e  Fc

**FIG. 1B  H7N9**

MSLLTEVEPTPRTWECNCSGSSE  SEQ ID NO:8

GLFGAIAGFIEGNGWEDIWYGERHQN  SEQ ID NO:7

M2e-FP-FdFc

M2e  FP  Fd  Fc
FIG. 7A

Viral titers (log10TCID50/g)

- M2e-FP-FdFc
- M2e-FP-Fc
- M2e-Fc
- Control

H5N1 virus challenge

FIG. 7B

Viral titers (log10TCID50/g)

- M2e-FP-FdFc
- M2e-FP-Fc
- M2e-Fc
- Control

H5N1 virus challenge
**INTERNATIONAL SEARCH REPORT**

**CLASSIFICATION OF SUBJECT MATTER**

C07K 14/11(2006.01) i, A61K 39/145(2006.01) i

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

**FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

C07K 14/11; A61K 39/395; A61P 31/16; A61K 39/385; A61K 39/145; A61K 47/48

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & keywords: influenza, immunogenic composition, matrix protein (M2e), hemagglutinin fusion peptide (HA2 FP)

**DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>DU et al., &quot;Research and development of universal influenza vaccines&quot; Microbes and Infection, Vol. 12, No. 4, pp. 280-286 (April 2010) See page 281, right column - page 283, right column; and tables 1-2.</td>
<td>1-23</td>
</tr>
<tr>
<td>A</td>
<td>WO 2011-082087 A2 (KJ BIOSCIENCES, LLC) 07 July 2011 See paragraphs [0031], [0033]; claims 1, 7-19, 24-25, 27-29, 30; and figures 1 and 3.</td>
<td>1-23</td>
</tr>
<tr>
<td>A</td>
<td>DU et al., &quot;Critical H1 neutralizing domain of H5N1 influenza in an optimal conformation induces strong cross-protection &quot; Plos One, Vol. 8, No. 1, Article No. e53568, pp.1-13 (January 2013) See page 2, right column.</td>
<td>1-23</td>
</tr>
<tr>
<td>A</td>
<td>US 2010-0150954 A1 (MILLER et al.) 17 June 2010 See claim 1.</td>
<td>1-23</td>
</tr>
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</table>

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search: 27 May 2014 (27.05.2014)

Date of mailing of the international search report: 02 June 2014 (02.06.2014)

Name and mailing address of the ISA/KR

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Form PCT/ISA/210 (second sheet) (July 2009)
1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:

   a. a sequence listing filed or furnished
   -□ on paper
   -☒ in electronic form

   b. time of filing or furnishing
   -□ contained in the international application as filed
   -☒ filed together with the international application in electronic form
   □ furnished subsequently to this Authority for the purposes of search

2. In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
### Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. **X** Claims Nos.: 24-29  
   because they relate to subject matter not required to be searched by this Authority, namely:  
   Claims 24-29 pertain to methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

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

3.  
   **□** Claims Nos.:  
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

**Remark on Protest**

- **□** The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- **□** The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- **□** No protest accompanied the payment of additional search fees.
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
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<tr>
<td>WO 2011-082087 A2</td>
<td>07/07/2011</td>
<td>CN 102711794 A</td>
<td>03/10/2012</td>
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