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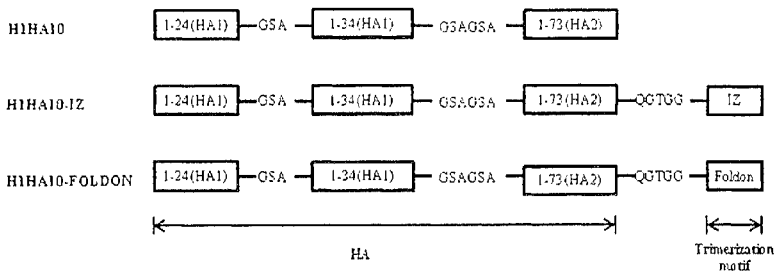

FIGURE 1

(57) Abstract: The present disclosure relates to a polypeptide comprising hemagglutinin stem domain fragments that can elicit broadly cross-reactive anti-influenza antibodies and confer protection against influenza virus. The disclosure also provides a method of preparing the polypeptide with biochemical and biophysical properties that enhance its immunogenic properties. Also provided are recombinant DNA constructs, vectors, and host cells comprising the nucleic acid encoding the polypeptide, as well as uses of the polypeptide, particularly in the prevention, and detection of influenza.

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TITLE OF THE INVENTION
POLYPEPTIDES FOR GENERATING ANTI-INFLUENZA ANTIBODIES AND USES THEREOF

FIELD OF INVENTION

[001] The present disclosure relates to the field of molecular biology, virology, and immunology. In particular, the present disclosure relates to polypeptides comprising two influenza HA1 stem fragments, and one HA2 fragment that elicit neutralizing antibodies against influenza virus. The present disclosure also provides a method for making the polypeptide, and uses thereof.

BACKGROUND OF THE INVENTION

[002] Seasonal influenza outbreaks across the globe cause an estimated 250,000 to 500,000 deaths annually. Current influenza vaccines need to be updated every few years because of antigenic drift (Pica *et al.*, *Annu Rev Med.*, 2013, *64*,189-202). Despite intensive monitoring, strain mismatch between vaccine formulation and influenza viruses circulating within the population has occurred in the past (Carrat *et al.*, *Vaccine*, 2007, *25(39-40)*, 6852-6862). Public health is further compromised when an unpredictable mixing event among influenza virus genomes leads to antigenic shift facilitating a potential pandemic outbreak. These concerns have expedited efforts towards developing a 'universal' flu vaccine.

[003] Neutralizing antibodies (nAbs) against hemagglutinin (HA) are the primary correlate for protection in humans and hence HA is an attractive target for vaccine development (Gerhard, *Curr Top Microbiol Immunol.*, 2001, *260*,171-190). The precursor polypeptide, HA0, is assembled into a trimer along the secretory pathway and transported to the cell surface. Cleavage of HA0 generates the disulfide linked HA1 and HA2 subunits. Mature HA has a globular head domain which mediates receptor binding and is primarily composed of the HA1 subunit while the stem domain predominantly comprises of the HA2 subunit. The HA stem is trapped in a metastable state and undergoes an extensive low-pH induced conformational rearrangement in the host-cell endosomes to adopt the virus-host membrane fusion competent state (Carr *et al.*, *Cell*, 1993, *73(4)*, 823-832; Skehel *et al.*, *Annu Rev Biochem.*, 2000, *69*, 531-569) .

[004] The antigenic sites on the globular head of HA are subjected to heightened immune pressure resulting in escape variants; thereby limiting the breadth of head-directed nAbs (Knossow *et al.*, *Immunology*, 2006, *119(1)*, 1-7). However, extensive efforts have resulted in the isolation of monoclonal antibodies which bind within the globular head and inhibit receptor attachment, which neutralize drifted variants of an HA subtype or heterosubtypic HA subtype

(Ekiert DC, *et al.*, *Nature*, 2012, *489(7417)*, 526-532; Hong M, *et al. J Virol.*, 2013, *87(22)*, 12471-12480; Krause JC, *et al.*, *J Virol.*, 2011, *85(20)*, 10905-10908; Lee PS, *et al.*, *Proc Natl Acad Sci U S A*, 2012, *109(42)*, 17040-17045; Ohshima N, *et al.*, *J Virol.*, 2011, *85(21)*, 11048-11057; Schmidt AG, *et al.*, *Proc Natl Acad Sci U S A*, 2013, *110(1)*, 264-269; Tsibane T, *et al.*, *PLoS Pathog* , 2012, *8(12)*, e1003067; Whittle JR, *et al.*, *Proc Natl Acad Sci U S A*, 2011, *108(34)*, 14216-14221; Wrammert J, *et al.*, *J Exp Med.*, 2011, *208(1)*, 181-193; Xu R, *et al.*, *Nat Struct Mol Biol.*, 2013, *20(3)*, 363-370).

[005] The HA stem is targeted by several neutralizing antibodies (bnAbs) with neutralizing activity against diverse influenza A virus subtypes (Julien JP *et al.*, *Immunol Rev.*, 2012, *250(1)*, 180-198). The epitopes of these bnAbs in the HA stem are more conserved across different influenza HA subtypes compared to the antigenic sites in the HA globular head (Ellebedy AH *et al.*, *Front Immunol.*, 2012, *3*, 53).

[006] A 'headless' stem domain immunogen offers an attractive solution. However, early attempts at expressing the HA2-subunit independently in a native, pre-fusion conformation have been unsuccessful. In the absence of the head domain, the HA2-subunit expressed in *E.coli* spontaneously adopts a low-pH conformation (Chen J, *et al.*, *Proc Natl Acad Sci U S A*, 1995, *92(26)*, 12205-12209) in which the functional epitopes of stem-directed bnAbs are disrupted. More recently, the entire HA stem region has been expressed in a pre-fusion, native-like conformation in both prokaryotic and eukaryotic systems adopting multiple strategies (Bommakanti G, *et al.*, *Proc Natl Acad Sci U S A*, 2010, *107(31)*, 13701-1370; Bommakanti G, *et al.*, *J Virol*, 2012, *86(24)*, 13434-13444; Lu Y *et al.*, *Proc Natl Acad Sci U S A*, 2014, *111(1)*, 125-130; Steel J, *et al.*, *MBio.*, 2010, *1(1)*).

[007] US6720409 provides an anti-human influenza virus antibody which recognizes the stem regions of haemagglutinin molecules of the H1N1 and H2N2 subtypes and has a neutralization activity but does not recognize the stem region of the H3N2 subtype and has no neutralization activity.

[008] WO2013011347 describes antibodies, and antigen binding fragments thereof, that specifically bind to an epitope in the stem region of an influenza A hemagglutinin trimer and neutralize a group 1 subtype and a group 2 subtype of influenza A virus.

[009] US5589174describes an anti-human influenza virus antibody is provided having the following characteristics: (a) specifically binds to the stem region of hemagglutinin of

human influenza A virus subtype H3N2; (b) does not specifically bind to the stem region of hemagglutinin of human influenza A virus subtypes H1N1 and H2N2; and (c) does not specifically bind to the stem region of hemagglutinin of human influenza B virus.

[0010] WO201377444 describes vaccine compositions and methods of producing and using the same, which compositions comprise a modified HA stem domain in trimeric configuration.

SUMMARY OF THE INVENTION

[0011] An aspect of the present disclosure relates to a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker.

[0012] An aspect of the present disclosure relates to a recombinant DNA construct comprising a polynucleotide fragment operably linked to a promoter, wherein said polynucleotide fragment encodes a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker.

[0013] An aspect of the present disclosure relates to a recombinant vector comprising a recombinant DNA construct comprising a polynucleotide fragment operably linked to a promoter, wherein said polynucleotide fragment encodes a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an

amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker.

[0014] An aspect of the present disclosure relates to a recombinant host cell comprising a a recombinant vector comprising a recombinant DNA construct comprising a polynucleotide fragment operably linked to a promoter, wherein said polynucleotide fragment encodes a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker, wherein the recombinant host cell is selected from the group consisting of a bacterial cell, fungal cell, and mammalian cell, preferably *E.coli*.

[0015] An aspect of the present disclosure relates to an influenza vaccine comprising a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker.

[0016] An aspect of the present disclosure relates to a method to produce a vaccine against influenza, said method comprising: (a) expressing a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker, in a host cell, and (b) purifying the expressed polypeptide from step (a).

[0017] An aspect of the present disclosure relates to a method to vaccinate an individual against influenza, said method comprising administering a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker, to an individual such that said polypeptide elicits an immune response against influenza virus.

[0018] An aspect of the present disclosure relates to a method of detection of influenza virus in a host, said method comprising: (a) obtaining a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker, (b) contacting serum from a host with the polypeptide from step (a), and (c) carrying out an ELISA test, wherein formation and detection of antibody and polypeptide complexes is indicative of presence of influenza virus in said host.

[0019] An aspect of the present disclosure relates to an influenza vaccine comprising a polynucleotide fragment encoding a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker.

[0020] An aspect of the present disclosure relates to an influenza vaccine comprising a polypeptide, said polypeptide comprising a first subunit, a second subunit, and a third subunit,

wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker.

[0021] In an aspect of the present disclosure, there is provided a use of a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, and wherein each subunit is connected by a linker as a vaccine against influenza.

[0022] In an aspect of the present disclosure, there is provided a method of administering a vaccine to a subject in need of creating an immune response against influenza in said subject cell tissue, said method comprising of: a) obtaining a therapeutically or prophylactically effective amount of an influenza vaccine comprising a polynucleotide fragment encoding a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker; or an influenza vaccine comprising said polypeptide; or said polypeptide; and b) administering said effective amount of influenza vaccine or polypeptide to said subject, wherein said method creates an immune response against influenza.

[0023] These and other features, aspects, and advantages of the present subject matter will be better understood with reference to the following description and appended claims. This summary is provided to introduce a selection of concepts in a simplified form. This summary is

not intended to identify key features or essential features of the claimed subject matter, nor is it intended to be used to limit the scope of the claimed subject matter.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

[0024] The following drawings form part of the present specification and are included to further illustrate aspects of the present disclosure. The disclosure may be better understood by reference to the drawings in combination with the detailed description of the specific embodiments presented herein.

[0025] Figure 1 depicts the fragments of the HA stem used in construct of the polypeptide connected by flexible, soluble linkers, and derivatives with C-terminal trimerization motif, in accordance with an embodiment of the present disclosure.

[0026] Figure 2 depicts the spectra of the polypeptides as determined by CD-spectroscopy, in accordance with an embodiment of the present disclosure.

[0027] Figure 3 depicts the NMR spectra of H1HA10-Foldon, in accordance with an embodiment of the present disclosure.

[0028] Figure 4 depicts the oligomeric state of polypeptides, in accordance with an embodiment of the present disclosure.

[0029] Figure 5 depicts the limited proteolysis resistance of polypeptides, in accordance with an embodiment of the present disclosure.

[0030] Figure 6A-F depicts the breadth of the antibody response elicited by the polypeptides as determined by ELISA, in accordance with an embodiment of the present disclosure.

[0031] Figure 7 depicts the binding competition of polypeptides with the bnAb CR6261, in accordance with an embodiment of the present disclosure.

[0032] Figure 8A-B depicts the protection effected by polypeptide in mice against lethal homologous challenge, in accordance with an embodiment of the present disclosure.

[0033] Figure 9 depicts the subtype specific and limited cross group protection afforded *in-vivo* by polypeptides, in accordance with an embodiment of the present disclosure.

[0034] Figure 10A-B depicts the thermal stability of polypeptides, in accordance with an embodiment of the present disclosure.

[0035] Figure 11A-C depicts the fluorescence emission spectra of polypeptides, in accordance with an embodiment of the present disclosure.

[0036] Figure 12A-D depicts the binding kinetics of polypeptides at different concentrations, in accordance with an embodiment of the present disclosure.

DETAILED DESCRIPTION OF THE INVENTION

[0037] Those skilled in the art will be aware that the present disclosure is subject to variations and modifications other than those specifically described. It is to be understood that the present disclosure includes all such variations and modifications. The disclosure also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any or more of such steps or features.

Definitions

[0038] For convenience, before further description of the present disclosure, certain terms employed in the specification, and examples are collected here. These definitions should be read in the light of the remainder of the disclosure and understood as by a person of skill in the art. The terms used herein have the meanings recognized and known to those of skill in the art, however, for convenience and completeness, particular terms and their meanings are set forth below.

[0039] The articles "a", "an" and "the" are used to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

[0040] The terms "comprise" and "comprising" are used in the inclusive, open sense, meaning that additional elements may be included. It is not intended to be construed as "consists of only".

[0041] Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated element or step or group of element or steps but not the exclusion of any other element or step or group of element or steps.

[0042] The term "including" is used to mean "including but not limited to". "Including" and "including but not limited to" are used interchangeably.

[0043] 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 disclosure belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the disclosure, the preferred methods, and materials are now described. All publications mentioned herein are incorporated herein by reference.

[0044] The term "bnAbs/bnAb" refers to broad neutralizing antibodies.

[0045] The term "nAbs/nAb" refers to neutralizing antibodies.

[0046] The term "IZ" refers to isoleucine zipper.

[0047] H1HA10 refers to a polypeptide having amino acid sequence as set forth in SEQ ID NO: 35, encoded by a nucleotide sequence as set forth in SEQ ID NO: 41.

[0048] H1HA10-IZ refers to a polypeptide having amino acid sequence as set forth in SEQ ID NO: 36, encoded by a nucleotide sequence as set forth in SEQ ID NO: 42.

[0049] H1HA1-Foldon refers to a polypeptide having amino acid sequence as set forth in SEQ ID NO: 37, encoded by a nucleotide sequence as set forth in SEQ ID NO: 43.

[0050] NCH1HA10-Foldon refers to a polypeptide having amino acid sequence as set forth in SEQ ID NO: 38, encoded by a nucleotide sequence as set forth in SEQ ID NO: 44.

[0051] pH1HA10-Foldon refers to a polypeptide having amino acid sequence as set forth in SEQ ID NO: 39, encoded by a nucleotide sequence as set forth in SEQ ID NO: 45.

[0052] H5HA10-Foldon refers to a polypeptide having amino acid sequence as set forth in SEQ ID NO: 40, encoded by a nucleotide sequence as set forth in SEQ ID NO: 46.

[0053] The term "immunogen/test immunogen" refers to the polypeptide as described herein and the terms immunogen or polypeptide may be used interchangeably.

Sequence description

[0054] SEQ ID NO: 1 depict the amino acid sequence of H1N1 A/Puerto Rico/8/34 HA1 stem fragment.

[0055] SEQ ID NO: 2 depict the amino acid sequence of H1N1 A/Puerto Rico/8/34 HA1 stem fragment.

[0056] SEQ ID NO: 3 depict the amino acid sequence of H1N1 A/Puerto Rico/8/34 HA2 stem fragment.

[0057] SEQ ID NO: 4 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 1.

[0058] SEQ ID NO: 5 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 2.

[0059] SEQ ID NO: 6 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 3.

[0060] SEQ ID NO: 7 depict the amino acid sequence of H1N1 A/Puerto Rico/8/34 or H1NI A/New Caledonia/20/99HA1 stem fragment.

[0061] SEQ ID NO: 8 depict the amino acid sequence of H1NI A/California/04/2009 HA1 stem fragment.

[0062] SEQ ID NO: 9 depict the amino acid sequence of H5N1 A/Viet Nam/1203/2004 HA1 stem fragment.

[0063] SEQ ID NO: 10 depict the modified amino acid sequence of H1N1 A/Puerto Rico/8/34 HA1 stem fragment.

[0064] SEQ ID NO: 11 depict the modified amino acid sequence of H1NI A/New Caledonia/20/99HA1 stem fragment.

[0065] SEQ ID NO: 12 depict the modified amino acid sequence of H1NI A/California/04/2009 HA1 stem fragment.

[0066] SEQ ID NO: 13 depict the modified amino acid sequence of H5N1 A/Viet Nam/1203/2004 HA1 stem fragment.

[0067] SEQ ID NO: 14 depict the modified amino acid sequence of H1N1 A/Puerto Rico/8/34 HA2 stem fragment.

[0068] SEQ ID NO: 15 depict the modified amino acid sequence of H1NI A/New Caledonia/20/99 HA2 stem fragment.

[0069] SEQ ID NO: 16 depict the modified amino acid sequence of H1NI A/California/04/2009 HA2 stem fragment.

[0070] SEQ ID NO: 17 depict the modified amino acid sequence of H5N1 A/Viet Nam/1203/2004 HA2 stem fragment.

[0071] SEQ ID NO: 18 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 7.

[0072] SEQ ID NO: 19 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 8.

[0073] SEQ ID NO: 20 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 9.

[0074] SEQ ID NO: 21 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 10.

[0075] SEQ ID NO: 22 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 11.

[0076] SEQ ID NO: 23 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 12.

[0077] SEQ ID NO: 24 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 13.

[0078] SEQ ID NO: 25 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 14.

[0079] SEQ ID NO: 26 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 15.

[0080] SEQ ID NO: 27 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 16.

[0081] SEQ ID NO: 28 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 17.

[0082] SEQ ID NO: 29 depict the nucleotide sequence encoding a linker peptide having amino acid sequence as set forth in SEQ ID NO: 30.

[0083] SEQ ID NO: 30 depict the amino acid sequence of linker.

[0084] SEQ ID NO: 31 depict the isoleucine-zipper (IZ) amino acid sequence.

[0085] SEQ ID NO: 32 depict the Foldon amino acid acid sequence.

[0086] SEQ ID NO: 33 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 31.

[0087] SEQ ID NO: 34 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 32.

[0088] SEQ ID NO: 35 depict the amino acid sequence of H1HA10 (H1N1 A/Puerto Rico/8/34).

[0089] SEQ ID NO: 36 depict the amino acid sequence of H1HA1-IZ.

[0090] SEQ ID NO: 37 depict the amino acid sequence of H1HA1-Foldon.

[0091] SEQ ID NO: 38 depict the amino acid sequence of NCH1HA1-Foldon (H1N1 A/New Caledonia/20/99).

[0092] SEQ ID NO: 39 depict the amino acid sequence of pH1HA1-Foldon (H1N1 A/California/04/2009).

[0093] SEQ ID NO: 40 depict the amino acid sequence of H5HA10-Foldon (H5N1 A/Viet Nam/1203/2004).

[0094] SEQ ID NO: 41 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 35.

[0095] SEQ ID NO: 42 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 36.

[0096] SEQ ID NO: 43 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 37.

[0097] SEQ ID NO: 44 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 38.

[0098] SEQ ID NO: 45 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 39.

[0099] SEQ ID NO: 46 depict the nucleotide sequence encoding a polypeptide having amino acid sequence as set forth in SEQ ID NO: 40.

[00100] SEQ ID NO: 47 depict the amino acid sequence of linker.

[00101] SEQ ID NO: 48 depict the nucleotide sequence encoding a linker peptide having amino acid sequence as set forth in SEQ ID NO: 47.

[00102] SEQ ID NO: 49 depict amino acid sequence of linker.

[00103] SEQ ID NO: 50 depict amino acid sequence of linker.

[00104] SEQ ID NO: 51 depict amino acid sequence of linker.

[00105] SEQ ID NO: 52 depict the nucleotide sequence encoding a linker peptide having amino acid sequence as set forth in SEQ ID NO: 49.

[00106] SEQ ID NO: 53 depict the nucleotide sequence encoding a linker peptide having amino acid sequence as set forth in SEQ ID NO: 50.

[00107] SEQ ID NO: 54 depict the nucleotide sequence encoding a linker peptide having amino acid sequence as set forth in SEQ ID NO: 51.

[00108] In an embodiment of the present disclosure, there is provided a polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the

amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker.

[00109] In an aspect of the present disclosure, there is provided a polypeptide comprising a first subunit, a second subunit, and a third subunit connected by linkers, wherein the amino acid sequence of the first subunit shares 70%, 75%, 80%, 85%, 90%, 95%, or 100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1.

[00110] In a preferred embodiment of the present disclosure, the amino acid sequence of the first subunit is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9.

[00111] In a preferred embodiment of the present disclosure, the nucleotide sequence encoding the first subunit is selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, and SEQ ID NO: 20.

[00112] In an aspect of the present disclosure, there is provided a polypeptide comprising a first subunit, a second subunit, and a third subunit connected by linkers, wherein the amino acid sequence of the second subunit shares 70%, 75%, 80%, 85%, 90%, 95%, or 100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2.

[00113] In a preferred embodiment of the present disclosure, the amino acid sequence of the second subunit is selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, and SEQ ID NO: 13.

[00114] In a preferred embodiment of the present disclosure, the nucleotide sequence encoding the second subunit is selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.

[00115] In an aspect of the present disclosure, there is provided a polypeptide comprising a first subunit, a second subunit, and a third subunit connected by linkers, wherein the amino acid sequence of the third subunit shares 70%, 75%, 80%, 85%, 90%, 95%, or 100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3.

[00116] In a preferred embodiment of the present disclosure, the amino acid sequence of the third subunit is selected from the group consisting of SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17.

[00117] In a preferred embodiment of the present disclosure, the nucleotide sequence encoding the third subunit is selected from the group consisting of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, and SEQ ID NO: 28.

[00118] In as aspect of the present disclosure, there is provided a polypeptide as described herein, wherein the first, second, and third subunit is optionally modified.

[00119] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the first subunit is unmodified.

[00120] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the second subunit is modified.

[00121] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the third subunit is modified.

[00122] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the second subunit having amino acid sequence atleast 70% similar to a sequence as set forth in SEQ ID NO: 2 is modified at amino acid residues selected from the group consisting of I9, V12, I14, and C17.

[00123] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid modifications in the second subunit having amino acid sequence as set forth in SEQ ID NO: 2 are I9T, V12T, I14N, and C17S.

[00124] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the third subunit having amino acid sequence atleast 70% similar to a sequence as set forth in SEQ ID NO: 3 is modified at amino acid residues selected from the group consisting of V26, F70, F23, L33, S14, and N42.

[00125] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid modifications in the third subunit having amino acid sequence as set forth in SEQ ID NO: 3 are V26, F70A, F23D, L33D, S14T, and N42K.

[00126] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the first subunit shares atleast 70%-100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 4, wherein the nucleotide sequence encoding the second subunit shares atleast 70%-100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 5, and wherein the nucleotide

sequence encoding the third subunit shares atleast 70%-100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 6.

[00127] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the first subunit shares 70%, 75%, 80%, 85%, 90%, 95%, or 100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 4.

[00128] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the second subunit shares 70%, 75%, 80%, 85%, 90%, 95%, or 100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 5.

[00129] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the third subunit shares 70%, 75%, 80%, 85%, 90%, 95%, or 100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 6.

[00130] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the first subunit is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9.

[00131] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the second subunit is selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, and SEQ ID NO: 13.

[00132] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the third subunit is selected from the group consisting of SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17.

[00133] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the first subunit is selected from the group consisting of SEQ ID NO: SEQ ID NO: 18, SEQ ID NO: 19, and SEQ ID NO: 20.

[00134] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the second subunit is selected from the group consisting of SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.

[00135] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the third subunit is selected from the group consisting of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, and SEQ ID NO: 28.

[00136] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the linker of variable length has amino acid sequence as set forth in SEQ ID NO: 30.

[00137] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the linker having amino acid sequence as set forth in SEQ ID NO: 30 is as set forth in SEQ ID NO: 29.

[00138] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the linker of variable length has amino acid sequence as set forth in SEQ ID NO: 49.

[00139] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the linker having amino acid sequence as set forth in SEQ ID NO: 49 is as set forth in SEQ ID NO: 52.

[00140] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the linker of variable length has amino acid sequence as set forth in SEQ ID NO: 50.

[00141] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the linker having amino acid sequence as set forth in SEQ ID NO: 50 is as set forth in SEQ ID NO: 53.

[00142] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the linker of variable length has amino acid sequence as set forth in SEQ ID NO: 51.

[00143] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the linker having amino acid sequence as set forth in SEQ ID NO: 51 is as set forth in SEQ ID NO: 54.

[00144] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the linker of variable length has amino acid sequence as set forth in SEQ ID NO: 47.

[00145] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the linker having amino acid sequence as set forth in SEQ ID NO: 47 is as set forth in SEQ ID NO: 48.

[00146] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the linker of variable length is GGG.

[00147] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the linker having amino acid sequence GGG is GGTGGCGGT.

[00148] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the linker of variable length is GSG.

[00149] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the linker having amino acid sequence GGG is GGCTCTGGT.

[00150] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the linker of variable length is GSS.

[00151] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the linker having amino acid sequence GGG is GGTTCTTCC.

[00152] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the linker of variable length is GSA.

[00153] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the linker having amino acid sequence GSA is GGCAGCGCG.

[00154] In an embodiment of the present disclosure, the number of amino acid residues as set forth in SEQ ID NO: 30 or SEQ ID NO: 47 or SEQ ID NO 49 or SEQ ID NO: 50 or SEQ ID NO: 51 is modified to contain 1 less amino acid residue.

[00155] In an embodiment of the present disclosure, the number of amino acid residues as set forth in SEQ ID NO: 30 or SEQ ID NO: 47 or SEQ ID NO 49 or SEQ ID NO: 50 or SEQ ID NO: 51 is modified to contain 1-3 extra amino acid residues.

[00156] In an embodiment of the present disclosure, the linker having amino acid sequence selected from the group consisting of GSA, GGG, GSG, and GSS is modified to contain 1 less amino acid residue.

[00157] In an embodiment of the present disclosure, the linker having amino acid sequence selected from the group consisting of GSA, GGG, GSG, and GSS is modified to contain 1-3 extra amino acid residues.

[00158] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the linkers of variable length are GSA or SEQ ID NO: 47 or as set forth in SEQ ID NO: 30.

[00159] In an embodiment of the present disclosure, the nucleotide sequence encoding the amino acid sequence of the linkers as described herein is codon optimized for host specific expression.

[00160] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, further comprising a C-terminal trimerization domain.

[00161] In an embodiment of the present disclosure, the amino acid sequence of the C-terminal trimerization motif is selected from the group consisting of SEQ ID NO: 31, and SEQ ID NO: 32.

[00162] In an embodiment of the present disclosure, the nucleotide sequence encoding the C-terminal trimerization motif is selected from the group consisting of SEQ ID NO: 33, and SEQ ID NO: 34.

[00163] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the polypeptide is selected from the group consisting of SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39 and SEQ ID NO: 40.

[00164] In a preferred embodiment of the present disclosure, there is provided a polypeptide comprising a C-terminal trimerization motif as described herein, wherein the amino acid sequence of the C-terminal trimerization motif is as set forth in SEQ ID NO: 32.

[00165] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the nucleotide sequence encoding the polypeptide is selected from the group consisting of SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45 and SEQ ID NO: 46.

[00166] In a preferred embodiment of the present disclosure, the polypeptide as described herein is soluble.

[00167] In a preferred embodiment of the present disclosure, there polypeptide as described herein lacks disulfide bridges.

[00168] In a preferred embodiment of the present disclosure, there polypeptide as described herein lacks glycosylated amino acid residues.

[00169] In a preferred embodiment of the present disclosure, there polypeptide as described herein is thermotolerant.

[00170] In a preferred embodiment of the present disclosure, there polypeptide as described herein elicits/boosts broadly cross-reactive, anti-influenza antibodies.

[00171] In a preferred embodiment of the present disclosure, there polypeptide as described herein is folded and predominantly alpha-helical.

[00172] In a preferred embodiment of the present disclosure, there polypeptide as described herein has native, neutral pH like confirmation.

[00173] In an embodiment of the present disclosure, there is provided a recombinant DNA construct comprising a polynucleotide fragment operably linked to a promoter, wherein said polynucleotide fragment encodes a polypeptide as described herein.

[00174] In an embodiment of the present disclosure, there is provided a recombinant vector comprising a recombinant DNA construct comprising a polynucleotide fragment operably linked to a promoter, wherein said polynucleotide fragment encodes a polypeptide as described herein.

[00175] In an embodiment of the present disclosure, there is provided a recombinant host cell comprising a recombinant DNA construct comprising a polynucleotide fragment operably linked to a promoter, wherein said polynucleotide fragment encodes a polypeptide as described herein.

[00176] In a preferred embodiment of the present disclosure, there is provided a recombinant host cell comprising a recombinant vector, wherein the recombinant vector comprises a recombinant DNA construct comprising a polynucleotide fragment operably linked to a promoter, wherein said polynucleotide fragment encodes a polypeptide as described herein.

[00177] In an embodiment of the present disclosure, the recombinant host cell is a bacterial cell.

[00178] In a preferred embodiment of the present disclosure, the recombinant host cell is *E.coli*.

[00179] In an embodiment of the present disclosure, the recombinant host cell is a fungal cell.

[00180] In an embodiment of the present disclosure, the recombinant host cell is a plant cell.

[00181] In an embodiment of the present disclosure, the recombinant host cell is a mammalian cell.

[00182] In an embodiment of the present disclosure, there is provided an influenza vaccine comprising a polypeptide as described herein.

[00183] In an embodiment of the present disclosure, there is provided an influenza vaccine comprising a polynucleotide fragment encoding a polypeptide as described herein.

[00184] In an embodiment of the present disclosure, there is provided a method to produce a vaccine against influenza, said method comprising (a) expressing a polypeptide as described herein in a host cell as described herein, and (b) purifying the expressed polypeptide from step (a).

[00185] In an embodiment of the present disclosure, the polypeptide expressed by a method as described herein is present in the soluble fraction of the cell lysate.

[00186] In an embodiment of the present disclosure, the polypeptide expressed by a method as described herein is thermotolerant.

[00187] In an embodiment of the present disclosure, the polypeptide expressed by a method as described herein has native, neutral pH like confirmation.

[00188] In an embodiment of the present disclosure, the polypeptide expressed by a method as described herein elicits/boosts broadly cross-reactive, anti-influenza antibodies.

[00189] In an embodiment of the present disclosure, there is provided a method to vaccinate an individual against influenza, said method comprising administering a polypeptide as described herein to an individual such that said polypeptide elicits an immune response against influenza virus.

[00190] In an embodiment of the present disclosure, there is provided a use of a polypeptide as described herein to isolate antibodies in animals that are specific to influenza virus stem region.

[00191] In an embodiment of the present disclosure, the polypeptide used to isolate antibodies as described herein is selected from the group consisting of polypeptides having amino acid sequence as set forth in SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 39, and SEQ ID NO: 40.

[00192] In an embodiment of the present disclosure, the polypeptide isolate antibodies as described herein is encoded by a nucleotide fragment having nucleotide sequence selected from

the group consisting of SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, and SEQ ID NO: 46.

[00193] In an embodiment of the present disclosure, there is provided a method of detection of influenza virus in a host, said method comprising (a) obtaining a polypeptide as described herein, (b) contacting serum from a host with the polypeptide from step (a), and (c) carrying out an ELISA test, wherein formation and detection of antibody and polypeptide complexes is indicative of presence of influenza in said host.

[00194] In an embodiment of the present disclosure, the polypeptide used in a method of detection of influenza in a host as described herein is selected from the group consisting of polypeptides having amino acid sequence as set forth in SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO" 37, SEQ ID NO: 39, and SEQ ID NO: 40.

[00195] In an embodiment of the present disclosure, the polypeptide used in a method of detection of influenza in a host cell as described herein is encoded by a nucleotide fragment having nucleotide sequence selected from the group consisting of SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, and SEQ ID NO: 46.

[00196] In a preferred embodiment of the present disclosure, the host is a mammal.

[00197] In an embodiment of the present disclosure, the host is a bird.

[00198] In an embodiment of the present disclosure, the host is a poultry bird.

[00199] In an embodiment of the present disclosure, none of the introduced amino acid modifications in SEQ ID NO: 2 or SEQ ID NO: 3 are in the epitopes of stem directed broad neutralizing antibodies, CR6261, F10, or FI6v3 as described previously in the prior art.

[00200] In an embodiment of the present disclosure, a polypeptide lacking a C-terminal trimerization motif as described herein is predominantly present as monomeric conformer in solution.

[00201] In an embodiment of the present disclosure, a polypeptide having a C-terminal trimerization motif as described herein, wherein the amino acid sequence of the C-terminal trimerization motif is as set forth in SEQ ID NO: 31 is a mixture of stable trimeric and monomeric conformers in solution, wherein the percentage of stable trimeric conformers in said solution is in the range of 60%-70%, and the percentage of stable monomeric conformers in said solution is in the range of 30%-40%.

[00202] In an embodiment of the present disclosure, there is provided a polypeptide having a C-terminal trimerization motif having amino acid sequence as set forth in SEQ ID NO: 32, wherein atleast 90% of said polypeptide is predominantly present as trimeric conformer in solution.

[00203] In an embodiment of the present disclosure, there is provided a polypeptide having a C-terminal trimerization motif having amino acid sequence as set forth in SEQ ID NO: 32, wherein atleast 95% of said polypeptide is predominantly present as trimeric conformer in solution.

[00204] In an embodiment of the present disclosure, the polypeptide as described herein shows limited resistance to proteolysis.

[00205] In an embodiment of the present disclosure, the polypeptide as described herein binds with stem directed broad neutralizing antibody CR6261 as described previously in the prior art.

[00206] In a preferred embodiment of the present disclosure, the polypeptide as described herein binds with stem directed broad neutralizing antibody CR6261 with sub-micromolar affinity.

[00207] In an embodiment of the present disclosure, C-terminal trimerization motifs assist in folding of H1HA10.

[00208] In an embodiment of the present disclosure, the degree of proteolytic resistance is as follows: H1HA10<H1HA10-IZ<H1HA10-Foldon.

[00209] In an embodiment of the present disclosure, all polypeptides as described herein elicit higher cross reactive antibody titers compared to mice immunized with PR8 virus.

[00210] In a preferred embodiment of the present disclosure, H1HA10-Foldon elicits highest cross reactive antibody titers.

[00211] In an embodiment of the present disclosure, the polypeptides as described herein protect mice against lethal homologous challenge.

[00212] In a preferred embodiment of the present disclosure, H1HA10 provides atleast approximately 2 fold higher protection to mice against lethal homologous challenge compared to naïve group.

[00213] In a more preferred embodiment of the present disclosure, H1HA10-IZ provides atleast approximately 2 fold higher protection to mice against lethal homologous challenge compared to naïve group.

[00214] In a most preferred embodiment of the present disclosure, H1HA10-Foldon provides approximately 2 fold higher protection to mice against lethal homologous challenge compared to naïve group.

[00215] In an embodiment of the present disclosure, the polypeptides as described herein confer robust subtype specific protection *in-vivo*.

[00216] In an embodiment of the present disclosure, the polypeptides as described herein confer limited cross group protection *in-vivo*.

[00217] In an embodiment of the present disclosure, H1HA10-Foldon shows thermal stability from approximately 300K-350K.

[00218] In an embodiment of the present disclosure, the polypeptides as described herein bind the confirmation-specific influenza hemagglutinin stem directed bnAb CR6261 as described previously in the prior art.

[00219] In an embodiment of the present disclosure, the first subunit of the polypeptide as described herein comprises of amino acids 18-41 of HA1 stem of influenza virus.

[00220] In an embodiment of the present disclosure, the second subunit of the polypeptide as described herein comprises of amino acids 290-323 of HA1 stem of influenza virus.

[00221] In an embodiment of the present disclosure, the third subunit of the polypeptide as described herein comprises of amino acids 41-113 of HA2 stem of influenza virus.

[00222] In an embodiment of the present disclosure, amino acid residues of other strains of influenza virus that are homologous to the modified amino acids in the subunits of the polypeptide as described herein may be modified to yield an immunogen with biochemical or biophysical properties similar to the polypeptide as described herein.

[00223] In an embodiment of the present disclosure, the NCBI-Flu database accession number for H1N1 A/Puerto Rico 8/1934 is ABD77675.1.

[00224] In an embodiment of the present disclosure, the NCBI-Flu database accession number for H1N1 A/New Caledonia/20/1999 is ACF41878.1.

[00225] In an embodiment of the present disclosure, the NCBI-Flu database accession number for H1N1 A/California/04/2009 is ACS45305.1.

[00226] In an embodiment of the present disclosure, the NCBI-Flu database accession number for H1N1 A/Viet Nam/1203/2004 is ABW90125.1.

[00227] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the first subunit has atleast 24 contiguous amino acids selected from amino acid residues 12-55 of HA1 stem of H1NI A/Puerto Rico 8/1934.

[00228] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the first subunit as set forth in SEQ ID NO: 1 shares 100% sequence homology with amino acid residues 18-41 of HA1 stem of H1NI A/Puerto Rico 8/1934.

[00229] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the second subunit has atleast 34 contiguous amino acids selected from amino acid residues 280-340 of HA1 stem of H1NI A/Puerto Rico 8/1934.

[00230] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the second subunit as set forth in SEQ ID NO: 2 shares 100% sequence homology with amino acid residues 290-323 of HA1 stem of H1NI A/Puerto Rico 8/1934.

[00231] In an embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the third subunit has atleast 73 contiguous amino acids selected from amino acid residues 30-130 of HA2 stem of H1NI A/Puerto Rico 8/1934.

[00232] In a preferred embodiment of the present disclosure, there is provided a polypeptide as described herein, wherein the amino acid sequence of the third subunit as set forth in SEQ ID NO: 3 shares 100% sequence homology with amino acid residues 41-113 of HA2 stem of H1NI A/Puerto Rico 8/1934.

[00233] In an embodiment of the present disclosure, there is provided an influenza vaccine comprising polypeptide, said polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker.

[00234] In an embodiment of the present disclosure, there is provided an influenza vaccine comprising a polynucleotide fragment encoding a polypeptide comprising a first subunit, a

second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, and wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, wherein each subunit is connected by a linker.

[00235] In a preferred embodiment of the present disclosure, there is provided an influenza vaccine comprising a polynucleotide fragment as described herein, wherein the nucleotide sequence of the polynucleotide fragment is as set forth in SEQ ID NO: 35.

[00236] In an embodiment of the present disclosure, there is provided an influenza vaccine as described herein, said influenza vaccine further comprising of pharmaceutically acceptable carriers, diluents, and excipients.

[00237] In an embodiment of the present disclosure, there is provided a use of a polypeptide as described herein as a vaccine against influenza.

[00238] In an embodiment of the present disclosure, there is provided a method of administering a vaccine to a subject in need of creating an immune response against influenza in said subject cell tissue, said method comprising of: a) obtaining a therapeutically or prophylactically effective amount of an influenza vaccine as described herein; and b) administering said effective amount of influenza vaccine to said subject, wherein said method creates an immune response against influenza.

[00239] In an embodiment of the present disclosure, there is provided a method of administering a vaccine to a subject in need of creating an immune response against influenza in said subject cell tissue, said method comprising of: a) obtaining a therapeutically or prophylactically effective amount of a polypeptide as described herein; and b) administering said effective amount of polypeptide to said subject, wherein said method creates an immune response against influenza.

[00240] In an embodiment of the present disclosure, there is provided a method of administering a vaccine to a subject in need of creating an immune response against influenza in said subject cell tissue as described herein, wherein said administration is oral.

[00241] In an embodiment of the present disclosure, there is provided a method of administering a vaccine to a subject in need of creating an immune response against influenza in said subject cell tissue as described herein, wherein said administration is intramuscular.

[00242] In an embodiment of the present disclosure, there is provided a method of administering a vaccine to a subject in need of creating an immune response against influenza in said subject cell tissue as described herein, wherein said administration is intraperitoneal.

[00243] In an embodiment of the present disclosure, it was surprisingly found that the polypeptides as described herein confer superior protection against influenza infection, capable of eliciting/boosting neutralizing antibodies against influenza virus, and the polypeptides are soluble, all of which are significant improvements over prior attempts as disclosed in the prior art.

[00244] Although the subject matter has been described in considerable detail with reference to certain preferred embodiments thereof, other embodiments are possible.

EXAMPLES

[00245] The disclosure will now be illustrated with working examples, which is intended to illustrate the working of disclosure and not intended to take restrictively to imply any limitations on the scope of the present disclosure. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods, devices and materials are described herein. It is to be understood that this disclosure is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary.

Example 1

Materials and Methods

[00246] Sequence analysis: All non-identical, full-length flu sequences (H1N1:4241 sequences and H5N1:182 sequences, derived from human hosts) were obtained from the NCBI-Flu Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). H1N1 sequences were clustered at 99% homology using CD-HIT (Cluster Database at High Identity with Tolerance) to filter-out 465 unique, representative H1N1 sequences which were used for further analysis

(Huang Y *et al.*, *Bioinformatics*, 2010, *26(5)*, 680-682). These H1N1 sequences were multiply aligned using ClustalX (Higgins DG *et al.*, *Gene*, 1988, *73(1)*, 237-244). H1N1 (n=465) and H5N1 (n=182) sequences were also simultaneously aligned using ClustalX. The quality score for each column in the alignment file is a measure of residue conservation at that position. The quality scores were binned and mapped onto the crystal structure of H1N1 A/PR/8/34 HA [Protein Data Bank (PDB) ID: 1RU7].

[00247] Cloning, expression, and protein purification: The gene sequence of the polypeptide "H1HA10" was synthesized and cloned in the expression vector pET-28a (+)between NdeI and BamHI restriction sites. Gene sequences corresponding to the trimerization motifs isoleucine-zipper (IZ) and foldon were synthesized with flanking KpnI and HindIII restriction sites. H1HA10-IZ and H1HA10-Foldon, derivatives of the preliminary construct were generated by cloning the trimerization motifs at the C-terminus of H1HA10. The stop-codon in H1HA10 was mutated to generate a unique KpnI restriction site using site-directed mutagenesis to facilitate cloning of the trimerization motifs. Cloning was confirmed by sequencing. Gene sequences corresponding to NCH1HA10-Foldon, pH1HA10-Foldon and H5HA10-Foldon were synthesized and cloned in the expression vector pET-28a (+) between NdeI and HindIII restriction sites. All constructs were codon-optimized for expression in *E.coli*.

[00248] The proteins were over-expressed in *E.coli* BL21(DE3) cells and purified from the soluble fraction of the cell culture lysate. All proteins in the present disclosure were purified using a similar protocol. Briefly, a single colony of *E.coli* BL21(DE3) transformed with the plasmid of interest was inoculated into 50ml of Tartoff-Hobbs HiVegTM media (HiMedia). The primary culture was grown over-night at 37°C. 2L of Tartoff-Hobbs HiVegTM media (500ml×4) (HiMedia) was inoculated with 1% of the primary inoculum and grown at 37°C until an OD_{600} of ~0.6-0.8 was reached. Cells were then induced with 1mM isopropyl-β-thiogalactopyranoside (IPTG) and grown for another 12-16hours at 20°C. Cells were harvested at 5000g and resuspended in 100ml of phosphate-buffered saline (PBS, pH 7.4). The cell suspension was lysed by sonication on ice and subsequently centrifuged at 14,000g. The supernatant was incubated with buffer-equilibrated Ni-NTA resin (GE HealthCare) for 2hours at 4°C under mild-mixing conditions to facilitate binding. The protein was eluted using an imidazole gradient (in PBS, pH 7.4) under gravity flow. Fractions containing the protein of interest were pooled and dialysed against PBS (pH 7.4) containing 1mM EDTA. The dialysed protein was concentrated in an

Amicon (Millipore) stirred cell apparatus to a final concentration of ~1mg/ml. Protein purity was assessed by SDS-PAGE and its identity confirmed by ESI-MS.

[00249] While we have carried out the process of production of the polypeptides in *E.coli*, a person ordinarily skilled in the art can readily produce the polypeptides as described herein or substantially the same polypeptides in other expression systems such as yeast, plant, and animal using expression system specific promoters and codon optimized DNA sequences that encode the polypeptides as described herein.

[00250] Circular Dichroism: Circular dichroism (CD) spectra for all proteins were recorded on a Jasco J-715C spectropolarimeter flushed with nitrogen gas. The concentration for all proteins was ~5-10μM. Measurements were done at 25°C in a 1mm path length quartz cuvette with a scan rate of 50nm/min, response time of 4s, and a bandwidth of 2nm. Each spectrum was an average of five scans. Mean residue ellipticity (MRE) was calculated as described previously (Ganesh C *et al.*, *Biochemistry*, 1997, *36(16)*, 5020-5028). The protein spectrum was corrected for buffer (PBS, pH 7.4) signals. While we have carried out the measurements for CD spectra for a subset of the polypeptides as described herein, we reasonably expect similar CD spectra for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00251] For thermal unfolding experiments, the CD-signal was monitored as a function of temperature. Thermal denaturation of H1HA10-Foldon was monitored at 208nm, and for H1N1 A/Puerto Rico/8/34 rHA at 215nm. The buffer used was PBS (pH 7.4). The data was collected between 15°C-90°C with a 1°C/min gradient and a data pitch of 0.2°C. Band width was 2nm and response time was 4s. A quartz cuvette of 1-mm path length was used. The scan was repeated after cooling the samples back-to 15°C to measure the reversibility of thermal unfolding. While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00252] Fluorescence spectroscopy: All fluorescence spectra were recorded at 25°C on a Jasco FP-6300 spectrofluorimeter. For intrinsic fluorescence measurements, the protein concentration used was in the range of 1-3μM. The protein samples were excited at 280nm, and emission was monitored from 300-400nm. The excitation and emission slit widths were 3nm and 5nm, respectively. Each spectrum was an average of five scans. The data was analyzed after buffer

correction. Fluorescence measurements were carried out under native conditions in PBS (pH 7.4) or under denaturing conditions of 7M guanidine hydrochloride (GdnCl) in PBS (pH 7.4). While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00253] NMR spectroscopy: One-dimensional ^{1}H NMR spectra were recorded on an Agilent 600 MHz NMR spectrometer using a triple resonance probe fitted with a z-axis only pulsed field gradient accessory. Spectra were recorded at 25°C. Chemical shifts were referenced to external DSS. A spectral width of 9615.4 Hz was sampled. Solvent suppression was achieved using the excitation sculpting pulse scheme (Hwang TL *et al.*, *J Magn Reson.*, 1995, A 112, 275-279). H1HA10-Foldon protein samples for NMR were prepared in PBS (pH 7.4) (90% H_2O:10% D_2O). A total of 2048 scans were recorded with a 1s relaxation delay. Protein sample (H1HA10-Foldon) was prepared in PBS (pH 7.4) (80% D_2O:20% H_2O) for the hydrogen exchange studies. One-dimensional ^{1}H NMR spectra of H1HA10-Foldon were recorded as a function of time over 24hours at every 2hour interval. While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00254] Gel-Filtration chromatography: The oligomeric status of purified proteins was analyzed under non-denaturing conditions by gel filtration chromatography at room temperature on a Superdex-200 analytical gel filtration column (GE HealthCare). The column was equilibrated with PBS (pH 7.4) and calibrated using broad range molecular weight markers (GE HealthCare). While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00255] Proteolysis: Proteolytic digestion of the designed immunogens was carried out using a protease (trypsin)/substrate molar ratio of 1:50. Reduced and carboxamidated ribonuclease A (RCAM-RNase A) was used as a positive control for trypsin activity. Proteolysis was carried out in the presence of 50mM HEPES (pH 8.0) and 2mM $CaCl_2$ on ice. Aliquots were collected at various time-points and quenched for trypsin activity with 0.1% formic acid. Samples collected at different time points were analyzed on 12% SDS-PAGE followed by staining with Coomassie Brilliant Blue R250 (Sigma). While we have carried out the measurements for a subset of the

polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00256] Binding affinity studies using Surface Plasmon Resonance (SPR): Binding affinity of the designed immunogens (H1HA10, H1HA10-IZ and H1HA10-Foldon) and full-length H1N1 A/Puerto Rico/8/34 recombinant HA (rHA) (Sino Biological Inc., Beijing, China) to the stem-directed bnAb (CR6261 IgG) or their single-chain variable fragment (scFv) derivatives (F10-scFv and FI6v3-scFv) was determined by SPR experiments performed with a Biacore3000 optical biosensor (Biacore, Uppsala, Sweden) at 25°C. Recombinant CR6261 IgG was produced in 293T cells as described previously (Bommakanti G, *et al.*, *J Virol.*, 2012, *86(24)*, 13434-13444). Plasmids encoding F10-scFv and FI6v3-scFv were synthesized (GenScript, USA) based on the published sequence (Sui J, *et al.*, *Nat Struct Mol Biol.*, 2009, *16(3)*, 265-273; Corti D, *et al.*, *Science*, 2011, *333(6044)*, 850-856) and expressed in *E.coli*. 500-750 response units (RU) of the ligand (CR6261 IgG, F10-scFv or FI6v3-scFv) was immobilized by standard amine coupling to the surface of a research-grade CM5 chip (GE HealthCare, Uppsala, Sweden). Ovalbumin immobilized sensor channel served as a negative-control for each binding interaction. Multiple concentrations of the analyte were passed over each channel in a running buffer of PBS (pH 7.4) with 0.05% P20 surfactant. Both binding and dissociation events were measured at a flow rate of 30µl/min. The sensor surface was regenerated after every binding event by repeated washes with 4M $MgCl_2$. Each binding curve was analyzed after correcting for non-specific binding by subtraction of signal obtained from the negative-control flow channel. The concentration of the monomeric fraction of H1HA10 was used for obtaining the kinetic parameters, while for H1HA10-IZ, H1HA10-Foldon, and H1N1 A/PR/8/34 rHA, concentration of the trimeric fraction were used. The kinetic parameters were obtained by globally fitting the data to a simple 1:1 Langmuir interaction model using BIA EVALUATION 3.1 software. Trace-1 was omitted from global fitting while obtaining the kinetic parameters for H1HA10-IZ, since it could not be fitted to a 1:1 interaction model. While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00257] Thermal tolerance of H1HA10-Foldon was assessed by its ability to bind CR6261 after heat stress. The protein sample was incubated at 40°C, 60°C and 80°C for 1h in a PCR cycler (BioRad) with heated lid to prevent evaporation. The samples were cooled to 25°C and binding

affinity to CR6261 was determined by SPR experiments as described above. While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00258] Pull down assay: The ability of H1HA10-Foldon to form a stable complex with the bnAb CR6261 IgG was further confirmed in a pull-down assay. CR6261 and H1HA10-Foldon were mixed together at different molar ratios and incubated for 2h at 4°C. Buffer (PBS, pH 7.4) equilibrated Protein-G (GE HealthCare) beads were added to the complex and incubated for 1h to specifically pull down CR6261 IgG. The beads were spun down at 3000g for 15mins at 4°C. The unbound supernatant was separated and the beads were washed with PBS (pH 7.4). The antibody bound to the beads was eluted with 100mM Glycine HCl (pH 3.0). The eluted fractions were neutralized with 1M Tris HCl (pH 9.0). The unbound and eluted fractions were subsequently analyzed by SDS-PAGE. While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00259] Immunization and challenge studies: Female BALB/c mice (4-5 weeks old) (Taconic Farms, Inc., Germantown, NY) were maintained at the animal facilities of Merck Research Laboratories. The study design was approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Mice (10/group) were immunized intramuscularly with 20μg of test immunogen along with 100μg CpG7909 adjuvant (TriLink BioTechnologies, San Diego, CA) at days 0 (prime) and 28 (boost). Naïve (buffer only) mice and adjuvant-treated mice were used as controls. Serum sample obtained from tail vein venipuncture were collected in Microtainer serum separator tubes (BD Biosciences, Franklin Lakes, NJ) 21 days after the prime and 14 days post boost from all the mice. 21 days after the secondary immunization, mice were anesthetized with ketamine/xylazine and challenged intranasally with $1LD_{90}$ of mouse-adapted H1N1 A/PR/8/34 virus in 20μL of PBS. In order to test for protection against a higher dose of the virus, one group of mice primed and boosted with H1HA10-Foldon was challenged with $2LD_{90}$ of homologous A/PR/8/34 virus. The ability of NCH1HA10-Foldon, pH1HA10-Foldon and H5HA10-Foldon to confer cross-protection was evaluated against a stringent $2LD_{90}$ heterologous H1N1 A/PR/8/34 virus challenge. Another group of mice (n=10) immunized with pH1HA10-Foldon were challenged with $2LD_{90}$ heterologous, mice-adapted H3N2 A/HK/68

virus. Survival and weight change of the challenged mice were monitored daily for 14 days post challenge. At each time point, all surviving mice of a group were weighed together and the mean weight calculated. Errors in the mean weight were estimated from three repeated measurements of the mean weight of the same number of healthy mice. While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00260] Determination of serum antibody titers: Antibody-titers against test immunogens were determined by ELISA. Briefly, test immunogens were coated on 96-well plates (Thermo Fisher Scientific, Rochester, NY) at 4μg/ml in 50μl PBS at 4°C overnight. Plates were then washed with PBS containing 0.05%Tween-20 (PBST) and blocked with 3% skim milk in PBST for 1h. 100μl of the antisera raised against the test immunogens was diluted in a 4-fold series in milk-PBST and added to each well. Plates were incubated for 2h at room temperature followed by washes with PBST. 50μl of HRP-conjugated goat anti-mouse IgG (H+L) secondary antibody in milk-PBST was added to each well at a predetermined dilution (1:5000) and incubated at room temperature for 1h. Plates were washed with PBST followed by development with 100μl per well of the substrate 3, 3',5,5'-tetramethylbenzidine (TMB) solution and stopped after 3-5 min of development with 100μl per well of the stop solution for TMB. OD at 450nm was measured and the antibody titer was defined as the reciprocal of the highest dilution that gave an OD value above the mean plus 2 standard deviations of control wells. While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00261] Binding of antisera to recombinant polypeptides: Binding of antisera raised against the test immunogens to several rHA proteins was determined by ELISA. Briefly, mammalian-expressed rHA proteins (H1N1 A/Puerto Rico/8/34, H1N1 A/California/04/2009, H1N1 A/Brisbane/59/2007, H5N1 A/Viet Nam/1203/2004, H3N2 A/Aichi/2/68, H3N2 A/Brisbane/10/07 from Sino Biological Inc., Beijing, China) were coated on 96-well plates at 2.5μg/ml in 50μl PBS at 4°C overnight. Ovalbumin (125ng/well) coated wells were used as a negative control. Plates were washed with PBST (PBS containing 0.05%Tween-20) and blocked with 1% BSA in PBST (PBSB). Antisera were then added to each well at a starting dilution of

1:100 followed by a 4-fold dilution series and incubated for 2h. Plates were washed with PBST. Alkaline phosphatase (ALP)-conjugated goat anti-mouse secondary antibody in PBSB was added to each well at a predetermined dilution (1:10000) and incubated at room temperature for 2h. Plates were washed and developed using the chromogenic substrate p-nitrophenyl phosphate (Sigma). Plates were read at 405nm (SPECTRAmax Plus 384, Molecular Devices, USA). Antibody titer was defined as the reciprocal of the highest dilution that gave an OD value above the mean plus 2 standard deviations of control wells. While we have carried out the measurements for a subset of the polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00262] Competition ELISA: Competition ELISA between the antisera raised against the test immunogens and the bnAb CR6261 IgG was carried out as described elsewhere (Shembekar N, *et al.*, *PLoS One*, 2013, *8(1)*, e55516). Briefly, 96-well half area plates (Corning Incorporated, NY) were coated with 3µg of pandemic H1N1 A/California/04/2009 rHA (Sino Biological Inc., Beijing, China) in 50µl PBS and kept overnight at 4°C. Ovalbumin (3µg) coated wells were used as negative control for antisera binding. Plates were washed with PBST and blocked for 1h with PBSB. 25µl of antisera were added to each well starting at 1:100 dilution followed by a 3-fold serial dilution in PBSB. As a control for non-specific competition, a previously characterized head-specific neutralizing monoclonal antibody (MAb) IgG MA2077 was used. 25µl of MAb MA2077 was added to each well starting at a concentration of 2mg/ml followed by 3-fold serial dilutions. After 2h of incubation, the plates were washed and blocked with PBSB for 15mins. CR6261 was then added to each well at a fixed concentration (300ng/ml) as determined from the titration curve of CR6261 with pandemic H1N1 rHA. After 2h of incubation with CR6261, the plates were washed with PBST. The wells were then probed with 25µl of ALP-conjugated goat anti-human antibody (Sigma) at a predetermined dilution (1:10000) to detect the bound CR6261. The plates were washed and developed using the chromogenic substrate p-nitrophenyl phosphate (Sigma). The optical density was measured at 405nm (SPECTRAmax Plus 384, Molecular Devices, USA). Percent competition was calculated as follows: % Competition = [(A-P)/A] ×100, where A is the signal of CR6261 binding to rHA in the absence of anti-serum and P is the binding signal of CR6261 to rHA in the presence of anti-serum (Bommakanti G, *et al.*, *J Virol.*, 2012, *86(24)*, 13434-13444). While we have carried out the measurements for a subset of the

polypeptides as described herein, we reasonably expect similar results for all polypeptides as described herein or for variants of the polypeptides with similar characteristics.

[00263] Statistical analysis: Differences in antibody titers and mean fractional body weights of surviving mice between different groups were analyzed by analysis of variance and Student's t-test. The fractional body weight of mice is calculated relative to their starting body weight. Differences in survival were calculated by Kaplan-Meier survival analysis with the log rank significance test.

Example 2

Results (Immunogen/polypeptide design)

[00264] Antigenic differences between the HA surface glycoprotein of various influenza A viruses provides the basis for classification into 18 subtypes (H1-H18) (Tong S, *et al.*, *PLoS Pathog.*, 2013, *9(10)*, e1003657). Wide diversity and rapid antigenic variation of HA remains the principal challenge to the development of potent vaccines. Targeting conserved regions of HA offers a promising strategy to combat viral evolution and escape. We analyzed a large dataset (H1N1: 4241 and H5N1: 182 sequences) of Group-1 influenza virus sequences to identify conserved targets on HA. Consistent with previous results (Ellebedy AH *et al.*, *Front Immunol.*, 2012, *3*, 5), the HA stem is more conserved as opposed to the highly-variable globular head domain. The residue conservation across all full-length, human isolates of H1 HA and Group-1 HA was mapped onto the crystal structure of H1N1 A/PR/8/34 HA (PDB ID: 1RU7(Gamblin SJ, *et al.*, *Science*, 2004, *303(5665)*, 1838-1842). The HA stem comprising the epitope of bnAbs is therefore a plausible target for developing a broadly protective vaccine (Harris AK, *et al.*, *Proc Natl Acad Sci U S A*, 2013, *110(12)*, 4592-4597).

[00265] Mimicking the epitope of these stem-directed bnAbs in a native, pre-fusion conformation in a 'headless' stem immunogen is challenging because of the metastable conformation of HA. The HA2-subunit when expressed independently adopts the low-pH conformation spontaneously (Chen J, *et al.*, *Proc Natl Acad Sci U S A*, 1995, *92(26)*, 12205-12209). Extensive rearrangement at low-pH displaces the A-helix by over 100Å (Skehel JJ *et al.*, *Annu Rev Biochem.*, 2000, *69*, 531-569) disrupting the conformation-specific epitope of these bnAbs.

[00266] In order to enhance the immune response to the epitope of the HA stem-directed bnAbs like CR6261, F10 and FI6v3 (Ekiert DC, *et al.*, *Science*, 2009, *324(5924)*, 246-251; Sui J, *et al.*,

Nat Struct Mol Biol., 2009, *16(3)*, 265-273; Corti D, *et al ., Science*, 2011, *333(6044)*, 850-856), a protein-minimization approach to refine the previously reported HA stem immunogens, H1HA6 and H1HA0HA6 was adopted. These HA stem immunogens consisted of the entire stem region of HA and were 258 (H1HA6) and 243 (H1HA0HA6) residues in length. Smaller 'headless' HA stem immunogens that contained the above epitope were designed from influenza A (H1N1) A/Puerto Rico/8/34 subtype. We analyzed the interaction network of residues in the antibody-footprint of these bnAbs with the rest of HA using the in-house software PREDBURASA as described previously (Sharma D, *et al., Biochemistry*, 2005, *44(49)*, 16192-16202). Briefly, the accessible surface area (ASA) of every residue in HA was calculated in the absence and presence of at least 3-residue long stretches of the CR6261 antibody footprint (32_1-36_1, 292_1-294_1, 18_2-21_2, 36_2-56_2), covering ~95% of the CR6261-epitope. Residues belonging to either HA1 or HA2 subunits are distinguished by subscripts 1 or 2 respectively. All residues of HA which had a total side-chain ASA difference of $\geq 5Å^2$ in the aforementioned calculations were identified as interacting 'network' residues. We repeated the PREDBURASA calculations now including the 'network' residues to identify HA stem fragments defined by stable breakpoints with optimal termini distances, having minimalistic interactions with the rest of HA. Residue fragments 18_1-41_1 (24 amino acid residues), 290_1-323_1 (34 amino acid residues) and 41_2-113_2 (73 amino acid residues) were included in H1HA10 (Figure 1). H1HA10 includes ~80% of the CR6261 antibody-footprint. H1HA10 is 139 residues in length and is therefore ~46% smaller than the full-length HA stem immunogen described previously (Bommakanti G, *et al., J Virol.*, 2012, *86(24)*, 13434-13444). The newly generated exposed hydrophobic patches in H1HA10 due to interactions lost with the rest of HA were mutated using the software ROSETTA DESIGN (Version 3.0) to minimize potential protein aggregation. A similar approach has been previously used by us to design stable influenza and HIV-immunogens and inhibitors (Bommakanti G, *et al., J Virol.*, 2012, *86(24)*, 13434-13444; Bhattacharyya S, *et al., J Biol Chem.*, 2013, *288(14)*, 9815-9825; Saha P, *et al., Biochemistry*, 2011, *50(37)*, 7891-7900). The following mutations were incorporated to mask the hydrophobic patch: $I298_1T$, $V301_1T$, $I303_1N$, $V66_2T$ and $F110_2A$ (Figure 1). $Cys281_1$ and $Cys306_1$ form an intramolecular disulfide bond in full-length HA. Since $Cys281_1$ was not incorporated in our design, we mutated $Cys306_1$ to Ser to prevent incorrect, intermolecular disulfide bond formation. Low-pH conformation destabilizing mutations: $F63_2D$ and $L73_2D$ which were previously characterized (Bommakanti G, *et al., J Virol.*, 2012, *86(24)*,

13434-13444), were also incorporated in the design. Residues $S54_2$ and $N82_2$ present in the PR8 HA crystal structure (PDB ID: 1RU7) were replaced in H1HA10 with the most commonly occurring residue ($T54_2$ and $K82_2$) at that position among all the available H1N1 A/Puerto Rico/8/34 sequences deposited with the NCBI-Flu Database. None of the introduced mutations were in the epitopes of the previously reported stem-directed bnAbs CR6261, F10 or FI6v3. These independent HA fragments were connected by flexible, soluble linkers of appropriate length as described previously (Varadarajan R, *et al.*, *J Virol.*, 2005, *79(3)*, 1713-1723). Also to promote the formation of trimer derivatives of H1HA10 with C-terminal trimerization motifs connected by flexible linkers were made. The parallel, coiled-coil trimerization motif Ile-zipper (IZ) (Suzuki K *et al.*, *Protein Eng.*, 1998, *11(11)*, 1051-1055) was used in H1HA10-IZ. H1HA10-Foldon had the globular, β-rich trimerization motif 'Foldon' (Guthe S, *et al.*, *J Mol Biol.*, 2004, *337(4)*, 905-915).

[00267] In order to assess the ability of stem immunogens to confer cross-protection, constructs similar to H1HA10-Foldon were designed from unmatched, highly drifted influenza strains and tested against heterologous PR8-virus challenge in mice. Constructs from other strains [H1N1 A/New Caledonia/20/99 (NCH1HA10-Foldon), H1N1 A/California/04/2009 (pH1HA10-Foldon), and H5N1 A/Viet Nam/1203/2004 (H5HA10-Foldon)] were designed using a facile strategy. A simplistic, pair-wise sequence alignment which can guide immunogen design emphasizes the utility of the design. The hydrophobic residues mutated in H1HA10 to mask the newly generated hydrophobic patches are identical/similar within a subtype; therefore, analogous mutations can be included in H1HA10-like designs from other strains.

[00268] Table 1 represents the residue conservation within influenza A H1N1 subtype (representative protein sequence)

Strain	% identity with full-length A/Puerto Rico/8/34 HA	% identity with H1HA10 (A/Puerto Rico/8/34)	% identity within H1HA10-fragments (A/Puerto Rico/8/34)		
			HA1 (18_1-41_1)	HA1 (290_1-323_1)	HA2 (41_2-113_2)
A/South Carolina/1/1918	89	95	100	94	93

A/WSN/1933	91	93	83	94	96
A/Bellamy/1942	94	97	96	97	97
A/CHR/157/83	91	96	96	94	97
A/Taiwan/4845/1999	89	95	86	97	96
A/Oklahoma/03/2008	86	95	92	97	96
A/Texas/45034157/2009 [a]	82	89	96	79	92
A/Alabama/03/2010 [a]	82	89	96	79	92
A/Kenya/151/2011 [a]	81	89	96	79	92

Example 3

Results (Protein purification and biophysical characterization of HA stem immunogens)

[00269] Protein solubility is a coarse indicator of proper folding and remains a crucial problem in heterologous expression systems. HA fragments expressed previously in *E.coli* formed inclusion body aggregates and required refolding (Bommakanti G, *et al., J Virol.*, 2012, *86(24)*, 13434-13444; Song L, *et al., PLoS One*, 2008, *3(5)*, e2257). In contrast, all the designed immunogens/polypeptides as described herein, expressed in *E.coli* BL21(DE3) cells were purified from the soluble fraction of the cell culture lysate, suggesting proper folding and validation of the design protocol. The protein yields were about 10-15mg/liter culture using unoptimized shake-flask cultures and were purified using a single, affinity-purification step.

[00270] CD-spectra indicated that all the proteins were folded and predominantly α-helical as expected. The trimerization motifs assist in the folding of H1HA10. H1HA10-IZ and H1HA10-Foldon are more helical than the parent construct H1HA10 as observed from the double minima at 208nm and 222nm (Figure 2). The thermal stability of H1HA10-Foldon was monitored using CD. H1HA10-Foldon showed a reversible and co-operative unfolding thermal melt profile with an apparent transition mid-point (T_m) of ~323K (50°C) at a protein concentration (in monomer units) of ~15μM. Since the folded protein is a trimer and the unfolded protein is a likely to be a monomer, the T_m is expected to be concentration dependant. Consecutive scans recorded after cooling the sample back-to 15°C overlapped well with each other. (Figure 10A). In contrast, the

full-length H1N1 A/Puerto Rico/8/34 rHA showed a broad transition without clear baselines and it was therefore not possible to estimate a T_m. The rescan showed no transition, indicating that thermal denaturation for rHA is irreversible (Figure 10B). Intrinsic tryptophan fluorescence measurements also affirmed a folded, native structure for H1HA10 and its derivatives. All proteins showed a significant red-shift in the emission maxima upon denaturation with GdnCl (Figure 11A-C).

[00271] Furthermore, the one-dimensional ^{1}H-NMR spectrum of H1HA10-foldon exhibits solution properties characteristic of a well-folded protein molecule. The presence of resolved resonance lines that appear in the downfield (9–11ppm) and the upfield (0.5 – -1.0ppm) regions of the spectrum are clear indicators that the molecule adopts a stable tertiary structure (Figure 3). The upfield shifted signals are those of methyl protons that are spatially proximal to aromatic rings in the interior of the protein (hydrophobic core). The conformational stability of the folded state of H1HA10-Foldon was further probed by hydrogen exchange studies. The slow exchange of the amide protons in the downfield (9-11ppm) region of the one-dimensional ^{1}H-NMR spectrum is suggestive of a well-packed molecule.

Example 4

Results (H1HA10-Foldon is a homogenous trimer in solution and resistant to proteolysis)

[00272] The long central α-helices (LAH) located in the HA stem assemble together into a parallel, trimeric coiled-coil promoting oligomerization. The oligomeric state of the designed immunogens was probed by analytical gel-filtration chromatography under native conditions. H1HA10 eluted predominantly as a monomer and a minor trimeric peak. This was probably because in the absence of the trans-membrane (TM) domain of HA, the trimer is not stable (Copeland CS *et al.*, *J Cell Biol.*, 1986, *103(4)*, 1179-1191). It has previously been demonstrated that trimerization motifs facilitate oligomerization in the absence of the HA TM domain (Stevens J, *et al.*, *Science*, 2006, *312(5772)*, 404-410). Both IZ and Foldon trimerization sequences aided in trimerization of H1HA10. H1HA10-IZ formed a mixture of stable trimeric (~65%) and monomeric (~35%) conformers in solution which did not re-equilibrate when partitioned. H1HA10-Foldon eluted exclusively as a trimer in solution (Figure 4). In contrast to previously designed immunogens H1HA6 and H1HA0HA6 which contain the entire stem domain (Bommakanti G, *et al.*, *J Virol.*, 2012, *86(24)*, 13434-13444), none of the proteins characterized

in the present dislcosure were aggregation prone. Gel-filtration studies indicate that we have effectively resurfaced exposed hydrophobic patches in our designed constructs.

[00273] Misfolded proteins having disordered sectors are subjected to increased proteolysis. H1HA10 showed limited resistance to proteolysis (trypsin digestion) compared to a control unfolded protein (RCAM-RNaseA). Proteolytic stability increased in the order H1HA10 < H1HA10-IZ < H1HA10-Foldon. Proteolysis confirmed a compact, folded conformation for H1HA10-Foldon (Figure 5).

Example 5

Results ("Headless" stem immunogens bind confirmation specific bnAbs)

[00274] Stem-directed bnAbs like CR6261, F10 and FI6v3 bind the native, neutral-pH conformation of HA with high affinity (Ekiert DC, *et al.*, *Science*,2009, *324(5924)*, 246-251; Sui J, *et al.*, *Nat Struct Mol Biol.*, 2009, *16(3)*, 265-273; Corti D, *et al.*, *Science*, 2011, *333(6044)*, 850-856). Epitopes of these bnAbs are disrupted in the low-pH, fusion-competent conformation of HA. Therefore, the ability of stem-derived immunogens to bind these bnAbs offers a robust validation of their conformation.

[00275] Binding of the designed 'headless' stem immunogens to the bnAbs was determined by SPR. H1HA10 bound IgG CR6261 with sub-micromolar affinity (315.4±14.5nM) (Table 2, Figure 12A).

Table 2

Immunogen	Ligand[a]	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
H1HA10	CR6261-IgG	$2.49\pm0.08\times10^{4}$	$7.85\pm0.09\times10^{-3}$	315.4±14.5
H1HA10-IZ	CR6261-IgG	$6.55\pm0.51\times10^{4}$	$4.84\pm0.11\times10^{-3}$	73.9±2.3
H1HA10-Foldon	CR6261-IgG	$3.72\pm0.22\times10^{4}$	$1.95\pm0.19\times10^{-3}$	52.4±1.8[b]
	F10-scFv	$1.54\pm0.14\times10^{5}$	$1.50\pm0.20\times10^{-3}$	9.8±2.1
	FI6v3-scFv	$1.69\pm0.53\times10^{5}$	$2.05\pm0.12\times10^{-3}$	12.1±3.4
H1 A/PR/8/34 rHA	CR6261-IgG	$2.89\pm0.02\times10^{5}$	$2.58\pm0.08\times10^{-3}$	8.9±0.3
	F10-scFv	$4.15\pm0.61\times10^{5}$	$1.21\pm0.21\times10^{-3}$	2.9±0.9
	FI6v3-scFv	$2.45\pm0.06\times10^{5}$	$2.16\pm0.27\times10^{-3}$	8.8±1.1

[00276] As previously mentioned, H1HA10 has ~80% of the antibody-footprint. H1HA10 though folded, is monomeric and not particularly compact as implied from proteolysis. These factors may contribute to the high k_{off} which would decrease the binding affinity (Table 2). Derivatives of H1HA10, which had improved biophysical and biochemical properties had considerably tighter binding to CR6261. H1HA10-IZ had an equilibrium dissociation constant (K_D) of 73.9±2.3nM. The conformational heterogeneity of H1HA10-IZ in solution may possibly lead to the observed biphasic binding to CR6261 at higher concentrations (Figure 12B) as observed previously with the HA stem immunogen H1HA6 (Bommakanti G, *et al., J Virol.*, 2012, *86(24)*, 13434-13444). H1HA10-Foldon, which assembles into a homogenous trimer in solution, bound CR6261 with the highest affinity among the designed stem immunogens (52.4±1.8nM) (Figure 12C). Although H1HA10-Foldon binds CR6261 with ~6-fold weaker affinity than full-length PR8 rHA (8.9±0.3nM) (Figure 12D) (Table 2), it is a significant improvement over the previously reported stem immunogen H1HA6 (~260nM) which comprised the entire HA stem with the complete CR6261-epitope.

[00277] Binding of H1HA10-Foldon to the scFv-derivatives of other stem-directed bnAbs (F10 and FI6v3) was also determined to confirm the native, pre-fusion HA-like conformation of H1HA10-Foldon. The slower off-rates for both H1HA10-Foldon and PR8 rHA result in a higher affinity to F10-scFV in comparison to IgG CR6261 (Table 2). The K_D of H1HA10-Foldon binding to F10-scFv was 9.8±2.1nM, about ~3-fold weaker than full-length PR8 rHA (2.9±0.9nM). H1HA10-Foldon also bound FI6v3 (the pan-influenza binding antibody) with a low K_D of 12.1±3.4nM (Table1).

[00278] Thermal tolerance, a pharmaceutically relevant parameter was assessed by determining the K_D of H1HA10-Foldon binding to CR6261 after prolonged heat stress. H1HA10-Foldon bound CR6261 with a K_D of 71.6±0.5nM even after incubating the protein at 80°C for 1h.

[00279] The specificity of H1HA10-Foldon binding to IgG CR6261 was also confirmed in a pull-down assay. Protein-G beads specific for human IgG CR6261 were used to pull down the antibody-antigen complex.

Example 6

Results (HA stem immunogens elicit broadly cross-reactive, anti-influenza antibodies)

[00280] During natural infection, the immunodominant head domain steers the immune response away from the conserved stem Kwong PD *et al., Nat Immunol.*, 2009, *10(6)*, 573-578).

This is probably the reason why there are low/undetectable cross-reactive antibodies in the anti-PR8 convalescent sera. In contrast, high titers of cross-reactive antibodies are elicited by the stem domain immunogens of the present disclosure (Figure 6) confirming that the immunogens have adopted a native-like, neutral-pH conformation. H1HA10-Foldon elicited the highest cross-reactive antibody titers (in the range of 409,600 – 1,638,400) with Group-1 HAs (Figure 6A-D). H1HA10-Foldon bound the pan-influenza neutralizing antibody FI6v3 with high affinity, which prompted us to examine the ability of anti-H1HA10-Foldon sera to bind Group-2 HAs. Interestingly, H1HA10-Foldon elicited moderate titers (25,600) of cross-reactive antibodies against (Group-2) H3 HAs (Figure 6E, F). However, additional design optimization incorporating the sequence variation information amongst the stem of all HA-subtypes will be essential to formulate an immunogen that can elicit a truly 'universal' response.

[00281] Antibodies elicited by the HA stem immunogens probably mediate virus neutralization by inhibiting virus-host cell membrane fusion as inferred from their ability to compete with the stem-directed bnAb IgG-CR6261 for binding to H1N1 A/California/04/2009 rHA (Figure 7). The sera elicited against H1HA10-Foldon showed maximum competition with CR6261, in accordance with the improved biophysical/ biochemical properties of the immunogen. The competition assay demonstrates the presence of CR6261-like antibodies following immunization with 'headless' stem immunogens. As a control, the nAb MA2077 which binds at the 'Sa' antigenic site on H1N1 A/California/04/2009 HA 9(Shembekar N, *et al., PLoS One*, 2013, *8(1)*, e55516) failed to compete with CR6261.

[00282] The sera were not tested in a hemagglutinin inhibition (HI) assay because the stem-directed bnAbs mediate neutralization by inhibiting membrane-fusion and not by blocking the virus from binding to receptors on the host cells (Okuno Y *et al., J Virol.*, 1994, *68(1)*, 517-520).

Example 7

Results (H1HA10-Foldon completely protects against a lethal homologous virus challenge)

[00283] All the 'headless' constructs elicited a robust immune response in mice with high serum antibody self-titers (>1,638,400). 21-days post-secondary immunization, mice were challenged intranasally with a lethal dose ($1LD_{90}$) of homologous PR8-virus. The challenged mice showed significant weight recovery by the end of the observation period after initial weight loss (Figure 8B). The monomeric immunogen H1HA10 conferred 50% protection, while its compact, trimeric-derivative H1HA10-Foldon protected mice completely from a lethal homologous virus

challenge (Figure 8A). Notably, immunization with H1HA10-Foldon gave improved protection in comparison to a previously reported stem-immunogen, H1HA6 (Bommakanti G, *et al., J Virol.*, 2012, *86(24)*, 13434-13444). The extent of weight loss was also slightly lower for mice immunized with H1HA10-Foldon relative to H1HA6. Immunization with H1HA10-Foldon also provided significant protection against a higher challenge dose ($2LD_{90}$) of the virus (Figure 9A).

Example 8

Results (HA stem immunogens confer robust subtype-specific protection)

[00284] Immunogens designed from unmatched, highly drifted influenza strains also elicited a robust immune response in mice with high serum antibody self-titers (≥1,638,400). The ability of stem-immunogens to provide cross-protection was tested against a heightened challenge dose ($2LD_{90}$) of heterologous PR8-virus in mice. All the immunogens significantly delayed viral infection (Figure 9A, C). NCH1HA10-Foldon conferred robust protection (Figure 9A), emphasizing the protective ability of stem immunogens across decades of genetic drift. Impressively, the stem fragment NCH1HA10-Foldon designed from a drifted strain (H1N1 A/New Caledonia/20/1999) had greater efficacy relative to the full length stem domain H1HA6 (designed from H1N1 A/PR/8/34) against PR8-virus challenge. H5HA10-Foldon, designed from an H5-subtype influenza strain (H5N1 A/Viet Nam/1203/2004) also provided partial protection and the surviving mice showed significant weight recovery (Figure 9B).. Hence, the protective ability of pH1HA10-Foldon against a Group-2 H3N2 HK68-virus challenge ($2LD_{90}$) was also tested. The immunogen delayed infection and conferred weak protection (Figure 9C). Surviving mice showed significant weight recovery (Figure 9D). The 'headless' stem fragment immunogens of the present disclosure confer robust subtype-specific, and weak cross-group protection *in vivo*.

[00285] Overall, these data indicate that the immunogens/polypeptides designed from fragments of HA1, and HA2 stem of influenza virus are surprisingly soluble, correctly folded, and can elicit a robust, broadly cross-reactive, anti-influenza antibody titer in animals, which offer protection from subtype-specific influenza infection *in-vivo*. Given the homology in the stem, the immunogens/polypeptide of the present disclosure also provides limited hetero-subtypic protection *in-vivo*. The polypeptides/immunogens as described herein may be used for vaccination against influenza.

[00286] It is within the domain of a person ordinarily skilled in the art to develop DNA based vaccines, based on the nucleotide sequences encoding the polypeptides as described herein.

[00287] More importantly, the biochemical and biophysical properties of the polypeptides/immunogens as described herein provide support for usage of the polypeptides in various functions, including use as vaccines that are a novel alternative to any attempts made previously. Further, the present disclosure also provides a template for designing of immunogens related to other strains of influenza virus. Lastly, the present disclosure provides a design that can facilitate scale up production of an effective immunogen against a variant of influenza virus in a relatively short period of time that is considerably quicker than current techniques.

SEQUENCES:

[00288] **SEQ ID NO: 1** DTVDTVLEKNVTVTHSVNLLEDSH.

[00289] **SEQ ID NO: 2** NSSLPYQNIHPVTIGECPKYVRSAKLRMVTGLRN.

[00290] **SEQ ID NO: 3** TQNAINGITNKVNTVIEKMNIQFTAVGKEFNKLEKRMENLNKK VDDGFLDIWTY NAELLVLLENERTLDFHDS.

[00291] **SEQ ID NO: 4** GACACTGTTGACACAGTACTCGAGAAGAATGTGACAGTGAC ACACTCTGTTAACCTGCTCGAAGA CAGCCAC.

[00292] **SEQ ID NO: 5** AACAGCAGTCTCCCTTACCAGAATATACACCCAGTCACAAT AGGAGAGTGCCCAAAATACGTCAGGAGTGCCAAATTGAGGATGGTTACAGGACTAA GGAAC.

[00293] **SEQ ID NO: 6** ACACAAAATGCCATTAACGGGATTACAAACAAGGTGAACAC TGTTATCGAGAAAATGAACATTCAATTCACAGCTGTGGGTAAAGAATTCAACAAATT AGAAAAAAGGATGGAAAATTTAAATAAAAAAGTTGATGATGGATTTCTGGACATTT GGACATATAATGCAGAATTGTTAGTTCTACTGGAAAATGAAAGGACTCTGGATTTCC ATGACTCA.

[00294] **SEQ ID NO: 7** DTVDTVLEKNVTVTHSVNLLEDSH.

[00295] **SEQ ID NO: 8** DTVDTVLEKNVTVTHSVNLLEDKH.

[00296] **SEQ ID NO: 9** EQVDTIMEKNVTVTHAQDILEKTH.

[00297] **SEQ ID NO: 10** NSSLPYQNTHPTTNGESPKYVRSAKLRMVTGLRN

[00298] **SEQ ID NO: 11** NSSLPFQNTHPTTNGESPKYVRSAKLRMVTGLRN.

[00299] **SEQ ID NO: 12** NTSLPFQNTHPTTNGKSPKYVKSTKLRLATGLRN.

[00300] **SEQ ID NO: 13** NSSMPFHNTHPNTTGESPKYVKSNRLVLATGLRN.

[00301] SEQ ID NO: 14 TQNAINGITNKVNTVIEKMNIQDTATGKEFNKDEKRMENLNK KVDDGFLDIWTYNAELLVLLENERTLDAHDS.

[00302] SEQ ID NO: 15 TQNAINGITNKVNSVIEKMNTQDTAVGKEFNKDERRMENLNK KVDDGFLDIWTYNAELLVLLENERTLDAHDS.

[00303] SEQ ID NO: 16 TQNAIDEITNKVNSVIEKMNTQDTAVGKEFNHDEKRIENLNKK VDDGFLDIWTYNAELLVLLENERTLDAHDS.

[00304] SEQ ID NO: 17 TQKAIDGVTNKVNSIIDKMNTQFEADGREFNNDERRIENLNKK MEDGFLDVWTYNAELLVLMENERTLDAHDS.

[00305] SEQ ID NO: 18 GATACGGTTGACACGGTCCTGGAAAAGAATGTGACGGTTA CGCACTC GGTTAATCTGCTGGAAGACTCGCAC.

[00306] SEQ ID NO: 19 GACACGGTGGATACGGTCCTGGAAAAGAATGTTACGGTCA CGCACTCAGTCAATCTGCTGGAAGACAAGCAC.

[00307] SEQ ID NO: 20 GAACAAGTGGACACGATTATGGAAAAGAACGTCACGGTTA CGCACGCCCAAGACATCCTGGAAAAAACGCAC.

[00308] SEQ ID NO: 21 AACAGCAGCCTGCCGTATCAGAACACCCATCCGACCACCAA CGGCGAAAGCCCGAAATATGTGCGTAGCGCGAAACTGCGTATGGTGACCGGCCTGC GTAAC.

[00309] SEQ ID NO: 22 AACTCCTCACTGCCGTTTCAGAACACCCATCCGACCACGAA TGGTGAAAGTCCGAAATATGTCCGTTCCGCAAAGCTGCGTATGGTTACCGGTCTGCG TAAT.

[00310] SEQ ID NO: 23 AATACGTCACTGCCGTTTCAGAACACCCATCCGACCACGAA TGGTAAAAGTCCGAAGTATGTTAAATCCACCAAGCTGCGTCTGGCAACCGGTCTGCG TAAT.

[00311] SEQ ID NO: 24 AACAGCTCAATGCCGTTTCATAACACCCACCCGAATACCAC GGGTGAAAGTCCGAAATATGTCAAGTCCAATCGTCTGGTGCTGGCAACCGGTCTGCG TAAT.

[00312] SEQ ID NO: 25 ACCCAGAACGCGATTAACGGCATTACCAACAAAGTGAACA CCGTGATTGAAAAAATGAACATTCAGGATACCGCGACCGGCAAAGAATTTAACAAA GATGAAAAACGTATGGAAAACCTGAACAAAAAAGTGGATGATGGCTTTCTGGATAT TTGGACCTATAACGCGGAACTGCTGGTGCTGCTGGAAAACGAACGTACCCTGGATG CGCATGATAGC.

[00313] SEQ ID NO: 26 ACCCAGAACGCAATTAATGGTATCACGAACAAGGTGAACT CGGTTATCGAAAAGATGAACACCCAAGATACGGCCGTGGGCAAAGAATTTAATAAG GACGAACGTCGCATGGAAAACCTGAATAAAAAGGTTGATGACGGTTTCCTGGATAT TTGGACCTATAACGCAGAACTGCTGGTCCTGCTGGAAAATGAACGTACCCTGGATGC TCACGACTCT.

[00314] SEQ ID NO: 27 ACCCAGAATGCAATTGATGAAATCACGAACAAAGTGAATT CGGTTATTGAAAAGATGAACACCCAAGATACGGCCGTCGGCAAGGAATTCAACCAT GACGAAAAGCGTATCGAAAACCTGAACAAGAAGGTCGATGACGGCTTCCTGGATAT CTGGACCTATAACGCAGAACTGCTGGTGCTGCTGGAAAATGAACGTACCCTGGATG CTCACGACTCT.

[00315] SEQ ID NO: 28 ACCCAGAAAGCAATTGATGGTGTG ACGAACAAGGTTAACT CGATCATCGATAAGATGAACACCCAATTTGAAGCCGATGGCCGTGAATTCAACAAT GACGAACGTCGCATCGAAAACCTGAATAAAAAGATGGAAGATGGTTTCCTGGACGT TTGGACCTATAACGCAGAACTGCTGGTCCTGATGGAAAATGAACGTACCCTGGATGC TCATGACTCT.

[00316] SEQ ID NO: 29 GGCAGCGCGGGCAGCGCG.

[00317] SEQ ID NO: 30 GSAGSA.

[00318] SEQ ID NO: 31 IKKEIEAIKKEQEAIKKKIEAIEKEIEA

[00319] SEQ ID NO: 32 GYIPEAPRDGQAYVRKDGEWVLLSTFL.

[00320] SEQ ID NO: 33 ATCAAAAAAGAAATCGAAGCGATCAAAAAAGAACAGGAAG CCATTAAAAAGAAAATTGAAGCAATCGAAAAAGAAATCGAAGCG.

[00321] SEQ ID NO: 34 GGCTATATTCCGGAAGCGCCGCGTGATGGTCAGGCCTACGT GCGTAAAGATGGCGAATGGGTTCTGCTGAGCACCTTTCTG.

[00322] SEQ ID NO: 35 DTVDTVLEKNVTVTHSVNLLEDSHGSANSSLPYQNTHPTTNG ESPKYVRSAKLRMVTGLRNGSAGSATQNAINGITNKVNTVIEKMNIQDTATGKEFNKDE KRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDAHDS.

[00323] SEQ ID NO: 36 DTVDTVLEKNVTVTHSVNLLEDSHGSANSSLPYQNTHPTTNG ESPKYVRSAKLRMVTGLRNGSAGSATQNAINGITNKVNTVIEKMNIQDTATGKEFNKDE KRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDAHDSQGTGGIKKEIEAIKKEQEAI KKKIEAIEKEIEA.

[00324] SEQ ID NO: 37 DTVDTVLEKNVTVTHSVNLLEDSHGSANSSLPYQNTHPTTNG ESPKYVRSAKLRMVTGLRNGSAGSATQNAINGITNKVNTVIEKMNIQDTATGKEFNKDE KRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDAHDSQGTGGGYIPEAPRDGQAYV RKDGEWVLLSTFL.

[00325] SEQ ID NO: 38 DTVDTVLEKNVTVTHSVNLLEDSHGSANSSLPFQNTHPTTNGE SPKYVRSAKLRMVTGLRNGSAGSATQNAINGITNKVNSVIEKMNTQDTAVGKEFNKDE RRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDAHDSQGTGGGYIPEAPRDGQAYV RKDGEWVLLSTFL.

[00326] SEQ ID NO: 39 DTVDTVLEKNVTVTHSVNLLEDKHGSANTSLPFQNTHPTTNG KSPKYVKSTKLRLATGLRNGSAGSATQNAIDEITNKVNSVIEKMNTQDTAVGKEFNHDE KRIENLNKKVDDGFLDIWTYNAELLVLLENERTLDAHDSQGTGGGYIPEAPRDGQAYV RKDGEWVLLSTFL.

[00327] SEQ ID NO: 40 EQVDTIMEKNVTVTHAQDILEKTHGSANSSMPFHNTHPNTTGE SPKYVKSNRLVLATGLRNGSAGSATQKAIDGVTNKVNSIIDKMNTQFEADGREFNNDER RIENLNKKMEDGFLDVWTYNAELLVLMENERTLDAHDSQGTGGGYIPEAPRDGQAYV RKDGEWVLLSTFL.

[00328] SEQ ID NO: 41 GATACCGTGGATACCGTGCTGGAAAAGAACGTGACCGTGA CCCATAGCGTGAACCTGCTGGAAGATAGCCATGGCAGCGCGAACAGCAGCCTGCCG TATCAGAACACCCATCCGACCACCAACGGCGAAAGCCCGAAATATGTGCGTAGCGC GAAACTGCGTATGGTGACCGGCCTGCGTAACGGCAGCGCGGGCAGCGCGACCCAGA ACGCGATTAACGGCATTACCAACAAAGTGAACACCGTGATTGAAAAAATGAACATT CAGGATACCGCGACCGGCAAAGAATTTAACAAAGATGAAAAACGTATGGAAAACCT GAACAAAAAAGTGGATGATGGCTTTCTGGATATTTGGACCTATAACGCGGAACTGCT GGTGCTGCTGGAAAACGAACGTACCCTGGATGCGCATGATAGCTAA.

[00329] SEQ ID NO: 42: GATACCGTGGATACCGTGCTGGAAAAGAACGTGACCGTGA CCCATAGCGTGAACCTGCTGGAAGATAGCCATGGCAGCGCGAACAGCAGCCTGCCG TATCAGAACACCCATCCGACCACCAACGGCGAAAGCCCGAAATATGTGCGTAGCGC GAAACTGCGTATGGTGACCGGCCTGCGTAACGGCAGCGCGGGCAGCGCGACCCAGA ACGCGATTAACGGCATTACCAACAAAGTGAACACCGTGATTGAAAAAATGAACATT CAGGATACCGCGACCGGCAAAGAATTTAACAAAGATGAAAAACGTATGGAAAACCT GAACAAAAAAGTGGATGATGGCTTTCTGGATATTTGGACCTATAACGCGGAACTGCT

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AATTGAAGCAATCGAAAAAGAAATCGAAGCGTAG.

[00330] SEQ ID NO: 43 GATACCGTGGATACCGTGCTGGAAAAGAACGTGACCGTGA
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TATCAGAACACCCATCCGACCACCAACGGCGAAAGCCCGAAATATGTGCGTAGCGC
GAAACTGCGTATGGTGACCGGCCTGCGTAACGGCAGCGCGGGCAGCGCGACCCAGA
ACGCGATTAACGGCATTACCAACAAAGTGAACACCGTGATTGAAAAAATGAACATT
CAGGATACCGCGACCGGCAAAGAATTTAACAAAGATGAAAAACGTATGGAAAACCT
GAACAAAAAAAGTGGATGATGGCTTTCTGGATATTTGGACCTATAACGCGGAACTGCT
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GTGGCTATATTCCGGAAGCGCCGCGTGATGGTCAGGCCTACGTGCGTAAAGATGGC
GAATGGGTTCTGCTGAGCACCTTTCTGTAA.

[00331] SEQ ID NO: 44 GATACGGTTGACACGGTCCTGGAAAAGAATGTGACGGTTA
CGCACTCGGTTAATCTGCTGGAAGACTCGCACGGCTCGGCAAACTCCTCACTGCCGT
TTCAGAACACCCATCCGACCACGAATGGTGAAAGTCCGAAATATGTCCGTTCCGCA
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GCAATTAATGGTATCACGAACAAGGTGAACTCGGTTATCGAAAAGATGAACACCCA
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[00332] SEQ ID NO: 45 GACACGGTGGATACGGTCCTGGAAAAGAATGTTACGGTCAC
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CAATTGATGAAATCACGAACAAAGTGAATTCGGTTATTGAAAAGATGAACACCCAA
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CAAGAAGGTCGATGACGGCTTCCTGGATATCTGGACCTATAACGCAGAACTGCTGGT
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GCTACATCCCGGAAGCGCCGCGTGATGGTCAGGCGTATGTGCGTAAAGACGGCGAA TGGGTGCTGCTGTCCACGTTTCTGTGA.

[00333] SEQ ID NO: 46 GAACAAGTGGACACGATTATGGAAAAGAACGTCACGGTTA CGCACGCCCAAGACATCCTGGAAAAAACGCACGGCTCAGCGAACAGCTCAATGCCG TTTCATAACACCCACCCGAATACCACGGGTGAAAGTCCGAAATATGTCAAGTCCAAT CGTCTGGTGCTGGCAACCGGTCTGCGTAATGGTAGCGCCGGCTCTGCCACCCAGAAA GCAATTGATGGTGTGACGAACAAGGTTAACTCGATCATCGATAAGATGAACACCCA ATTTGAAGCCGATGGCCGTGAATTCAACAATGACGAACGTCGCATCGAAAACCTGA ATAAAAAGATGGAAGATGGTTTCCTGGACGTTTGGACCTATAACGCAGAACTGCTG GTCCTGATGGAAAATGAACGTACCCTGGATGCTCATGACTCTCAGGGCACGGGCGG TGGCTACATTCCGGAAGCGCCGCGTGACGGTCAGGCGTATGTCCGCAAGGATGGTG AATGGGTGCTGCTGTCCACGTTTCTGTGA.

[00334] SEQ ID NO: 47 QGTGG.

[00335] SEQ ID NO: 48 CAAGGTACCGGCGGT.

[00336] SEQ ID NO: 49 GGGGGG.

[00337] SEQ ID NO: 50 GSGSGS.

[00338] SEQ ID NO: 51 GSSGSS.

[00339] SEQ ID NO: 52 GGCGGCGGTGGTGGCGGC.

[00340] SEQ ID NO: 53 GGCTCCGGTTCTGGCTCT.

[00341] SEQ ID NO: 54 GGCTCTTCCGGTTCCTCT.

I/We claim:

1. A polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, wherein the amino acid sequence of the third subunit shares atleast 70%-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, and wherein each subunit is connected by a linker.

2. The polypeptide as claimed in claim 1, wherein each of the first, second, and third subunit is further modified.

3. The polypeptide as claimed in claim 1, wherein the second subunit having amino acid sequence atleast 70% similar to a sequence as set forth in SEQ ID NO: 2 is modified at amino acid residues selected from the group consisting of I9, V12, I14, and C17, and wherein the third subunit having amino acid sequence atleast 70% similar to a sequence as set forth in SEQ ID NO: 3 is modified at amino acid residues selected from the group consisting of V26, F70, F23, L33, S14, and N42.

4. The polypeptide as claimed in claim 1, wherein the nucleotide sequence encoding the first subunit shares atleast 70%-100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 4, wherein the nucleotide sequence encoding the second subunit shares atleast 70%-100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 5, and wherein the nucleotide sequence encoding the third subunit shares atleast 70%-100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 6.

5. The polypeptide as claimed in claim 1, wherein the amino acid sequence of the first subunit is selected from the group consisting of SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9, wherein the amino acid sequence of the second subunit is selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, and SEQ ID NO: 13, and wherein the amino acid sequence of the third subunit is selected from the group consisting of SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17.

6. The polypeptide as claimed in claim 5, wherein the nucleotide sequence encoding the first subunit is selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, and SEQ ID NO: 20, wherein the nucleotide sequence encoding the second subunit is selected from

the group consisting of SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, and wherein the nucleotide sequence encoding the third subunit is selected from the group consisting of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, and SEQ ID NO: 28.

7. The polypeptide as claimed in claim 1, wherein the linkers of variable length have amino acid sequence selected from the group consisting of GSA, SEQ ID NO: 47, and SEQ ID NO: 30.

8. The polynucleotide as claimed in claim 7, wherein the nucleotide sequence encoding the linkers is selected from the group consisting of GGCAGCGCG, SEQ ID NO: 48, and SEQ ID NO: 29.

9. The polynucleotide as claimed in claim 1, further comprising a C-terminal trimerization motif.

10. The polynucleotide as claimed in claim 9, wherein the C-terminal trimerization motif amino acid sequence selected from the group consisting of SEQ ID NO: 31, and SEQ ID NO: 32.

11. The polynucleotide as claimed in claim 10, wherein the nucleotide sequence encoding the C-terminal trimerization motif is selected from the group consisting of SEQ ID NO: 33, and SEQ ID NO: 34.

12. The polypeptide as claimed in claim 1, wherein the amino acid sequence of the polypeptide is selected from the group consisting of SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39 and SEQ ID NO: 40.

13. The polypeptide as claimed in claim 12, wherein the nucleotide sequence encoding the polypeptide is selected from the group consisting of SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45 and SEQ ID NO: 46.

14. A recombinant DNA construct comprising a polynucleotide fragment operably linked to a promoter, wherein said polynucleotide fragment encodes a polypeptide as claimed in claim 1.

15. A recombinant vector comprising a recombinant DNA construct as claimed in claim 14.

16. A recombinant host cell comprising a recombinant vector as claimed in claim 15, wherein the recombinant host cell is selected from the group consisting of a bacterial cell, fungal cell, and mammalian cell, preferably *E.coli*.

17. An influenza vaccine comprising a polypeptide as claimed in any of the claims 1-13.

18. A method to produce a vaccine against influenza, said method comprising:
    a. obtaining a polypeptide as claimed in any of the claims 1-13;
    b. expressing the polypeptide from(a)in a host cell as claimed in claim 16; and
    c. purifying the expressed polypeptide from (b).
19. An influenza vaccine comprising a polynucleotide fragment encoding a polypeptide as claimed in any of the claims 1-13.
20. An influenza vaccine comprising of a polypeptide as claimed in any of the claims 1-13.
21. The influenza vaccine as claimed in any of the claims 19-20, further comprising of pharmaceutically acceptable carriers, diluents, and excipients.
22. Use of a polypeptide as claimed in any of the claims 1-13 as a vaccine against influenza.
23. A method of administering a vaccine to a subject in need of creating an immune response against influenza in said subject cell tissue, said method comprising of:
    a. obtaining a therapeutically or prophylactically effective amount of an influenza vaccine as claimed in any of the claims 19-21 or a polypeptide as claimed in any of the claims 1-13; and
    b. administering said effective amount of influenza vaccine or polypeptide to said subject,

    wherein said method creates an immune response against influenza.
24. The method as claimed in claim 23, wherein said administration is oral, intramuscular, or intraperitoneal.

AMENDED CLAIMS
received by the International Bureau on 11 September 2015 (11.09.15)

1. A polypeptide comprising a first subunit, a second subunit, and a third subunit, wherein the amino acid sequence of the first subunit shares at least 70-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 1, wherein the amino acid sequence of the second subunit shares at least 70-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 2, wherein the amino acid sequence of the third subunit shares at least 70-100% sequence similarity to an amino acid sequence as set forth in SEQ ID NO: 3, and wherein each subunit is connected by a linker of variable length have amino acid sequence selected from the group consisting of GSA, SEQ ID NO: 47, and 30.

2. The polypeptide as claimed in claim 1, wherein each of the second, and third subunit is further modified, wherein the second subunit is modified at amino acid residues selected from the group consisting of I9, V12, I14, and C17, and wherein the third subunit is modified at amino acid residues selected from the group consisting of V26, F70, F23, L33, S14, and N42.

3. The polypeptide as claimed in claim 1, wherein the nucleotide sequence encoding the first subunit shares at least 70-100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 4, wherein the nucleotide sequence encoding the second subunit shares at least 70-100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 5, and wherein the nucleotide sequence encoding the third subunit shares at least 70-100% sequence similarity to a polynucleotide sequence as set forth in SEQ ID NO: 6.

4. The polypeptide as claimed in claim 1, wherein the amino acid sequence of the first subunit is selected from the group consisting of SEQ ID NO: 7, 8, and 9, wherein the amino acid sequence of the second subunit is selected from the group consisting of SEQ ID NO: 10, 11, 12, and 13, and wherein the amino acid sequence of the third subunit is selected from the group consisting of SEQ ID NO: 14, 15, 16, and 17.

5. The polypeptide as claimed in claim 4, wherein the nucleotide sequence encoding the first subunit is selected from the group consisting of SEQ ID NO: 18, 19, and 20, wherein the nucleotide sequence encoding the second subunit is selected from the group consisting of SEQ ID NO: 21, 22, 23, and 24, and wherein the nucleotide sequence encoding the third subunit is selected from the group consisting of SEQ ID NO: 25, 26, 27, and 28.

6. The polypeptide as claimed in claim 1, wherein the nucleotide sequence encoding the linkers is selected from the group consisting of GGCAGCGCG, SEQ ID NO: 48, and 29.

7. The polypeptide as claimed in claim 1, further comprising a C-terminal trimerization motif, wherein said C-terminal trimerization motif amino acid sequence is selected from the group consisting of SEQ ID NO: 31, and 32.

8. The polypeptide as claimed in claim 7, wherein the C-terminal trimerization motif nucleotide sequence is selected from the group consisting of SEQ ID NO: 33, and 34.

9. The polypeptide as claimed in claim 1, wherein the amino acid sequence of the polypeptide is selected from the group consisting of SEQ ID NO: 35, 36, 37, 38, 39, and 40.

10. The polypeptide as claimed in claim 9, wherein the nucleotide sequence encoding the polypeptide is selected from the group consisting of SEQ ID NO: 41, 42, 43, 44, 45, and 46.

11. A recombinant DNA construct comprising a polynucleotide fragment operably linked to a promoter, wherein said polynucleotide fragment encodes a polypeptide as claimed in claim 1.

12. A recombinant vector comprising a recombinant DNA construct as claimed in claim 11.

13. A recombinant host cell comprising a recombinant vector as claimed in claim 12, wherein the recombinant host cell is selected from the group consisting of a bacterial cell, fungal cell, and mammalian cell, preferably *E.coli*.

14. An influenza vaccine comprising a polypeptide as claimed in any of the claims 1-10.

15. A method to produce a vaccine against influenza, said method comprising:

a. obtaining a host cell as claimed in claim 13;

b. expressing a polypeptide as claimed in any of the claims 1-10 from said host cell; and

c. purifying said polypeptide.

16. An influenza vaccine comprising a polynucleotide fragment encoding a polypeptide as claimed in any of the claims 1-10.

17. The influenza vaccine as claimed in any of the claims 14 or16, further comprising pharmaceutically acceptable carriers, diluents, and excipients.

18. A polypeptide as claimed in any of the claims 1-10 for use as a vaccine against influenza.

19. A method of administering a vaccine to a subject in need of creating an immune response against influenza in said subject cell tissue, said method comprising:

a. obtaining a therapeutically or prophylactically effective amount of an influenza vaccine as claimed in any of the claims 14, 16, or-17, or a polypeptide as claimed in any of the claims 1-10; and

b. administering said effective amount of influenza vaccine or polypeptide to said subject,

wherein said method creates an immune response against influenza.

20. The method as claimed in claim 19, wherein said administration is oral, intramuscular, or intraperitoneal.

STATEMENT UNDER ARTICLE 19(1)

Applicant has gone through the written opinion of International Search Report and observes that:

a) Novelty of claims 4, 6-8, 11-13, and 19 are acknowledged

b) Inventive step of claims 7, 8, and 11-13 are acknowledged

c) Industrial applicability of claims 1-24 are acknowledged.

d) Novelty of claims 1-3, 5, 9, 10, 14-18, and 20-24 are NOT acknowledged

e) Inventive step of claims 1-6, 9, 10, and 14-24 are NOT acknowledged

With respect to the Written Opinion, the Applicant hereby amends the claims under Art. 19, in order to bring out the novelty and inventive step of the invention.

Summary of claim amendments:

Original claim	Amended claim	Remarks
1	1	Pending claim 7 incorporated into claim 1
2	2	Pending claim 3 incorporated into claim 2
3	-	Incorporated into claim 2
4	3	Renumbered

Original claim	Amended claim	Remarks
5	4	Renumbered
6	5	Renumbered and antecedent basis amended
7	-	Incorporated into claim 1
8	6	Renumbered and antecedent basis amended
9	7	Pending claim 10 incorporated into pending claim 9
10	-	Incorporated into pending claim 9
11	8	Renumbered and antecedent basis amended
12	9	Renumbered
13	10	Renumbered and antecedent basis amended
14	11	Renumbered
15	12	Renumbered and antecedent basis amended
16	13	Renumbered and antecedent basis amended
17	14	Renumbered and antecedent basis amended
18	15	Renumbered and antecedent basis amended
19	16	Renumbered and antecedent basis amended
20	-	Cancelled
21	17	Renumbered and antecedent basis amended
22	18	Amended and renumbered
23	19	Renumbered and antecedent basis amended
24	20	Renumbered and antecedent basis amended

In view of the amendments, the Applicant hereby requests the Examiners to acknowledge novelty and inventive step of the amended claims in view of the cited prior art documents.

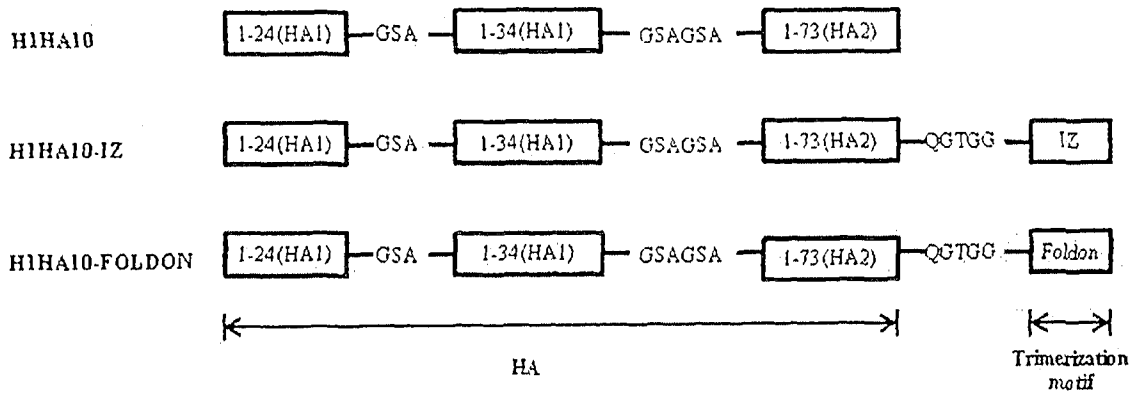

FIGURE 1

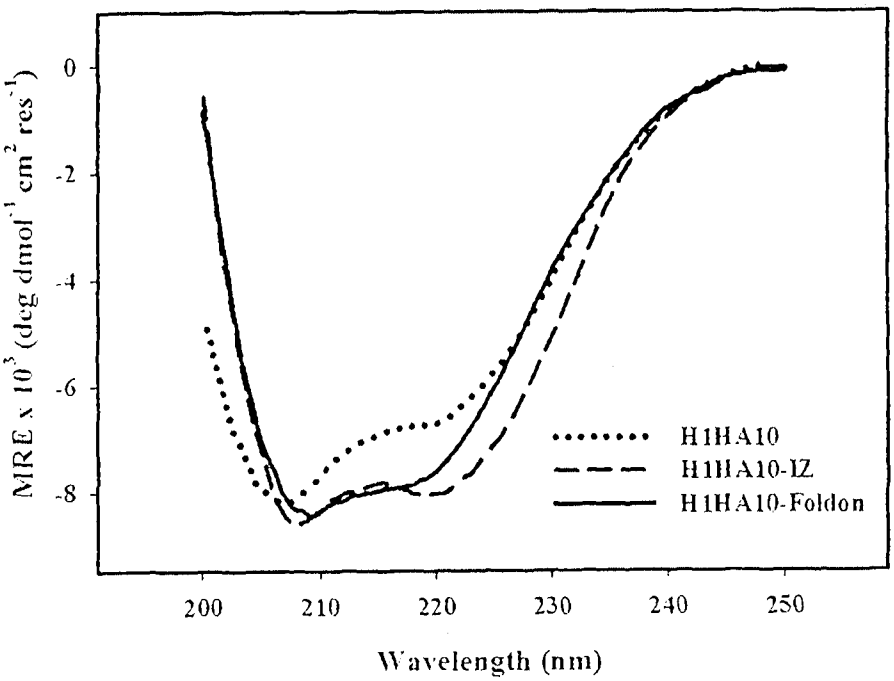

FIGURE 2

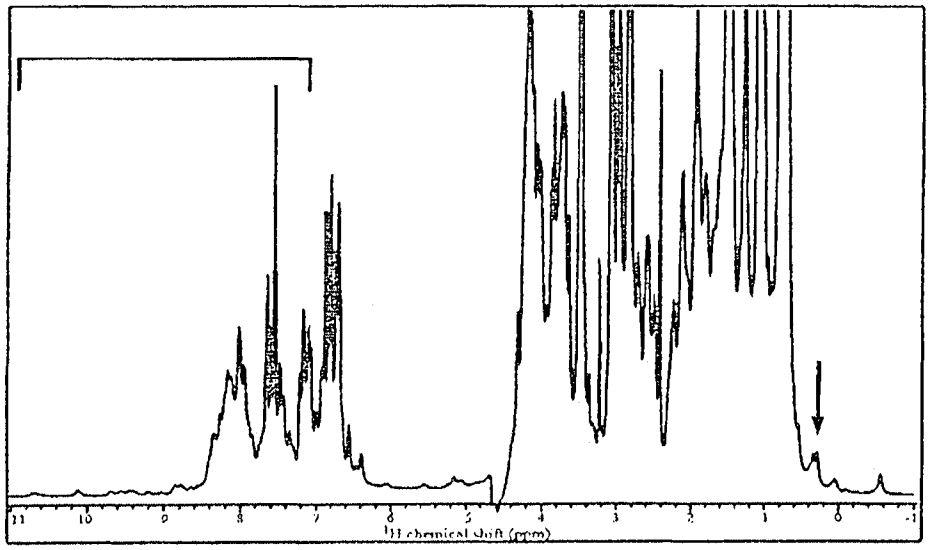

FIGURE 3

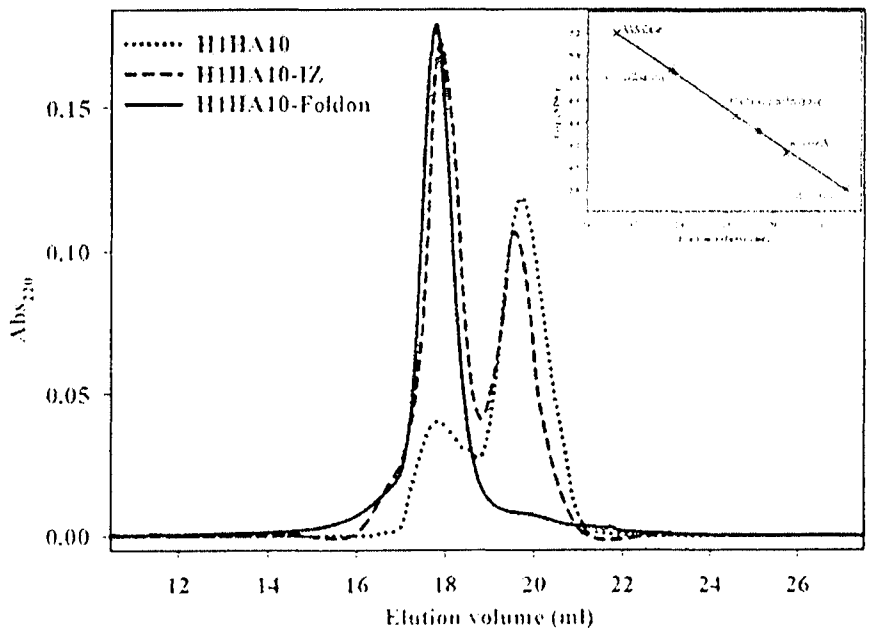

FIGURE 4

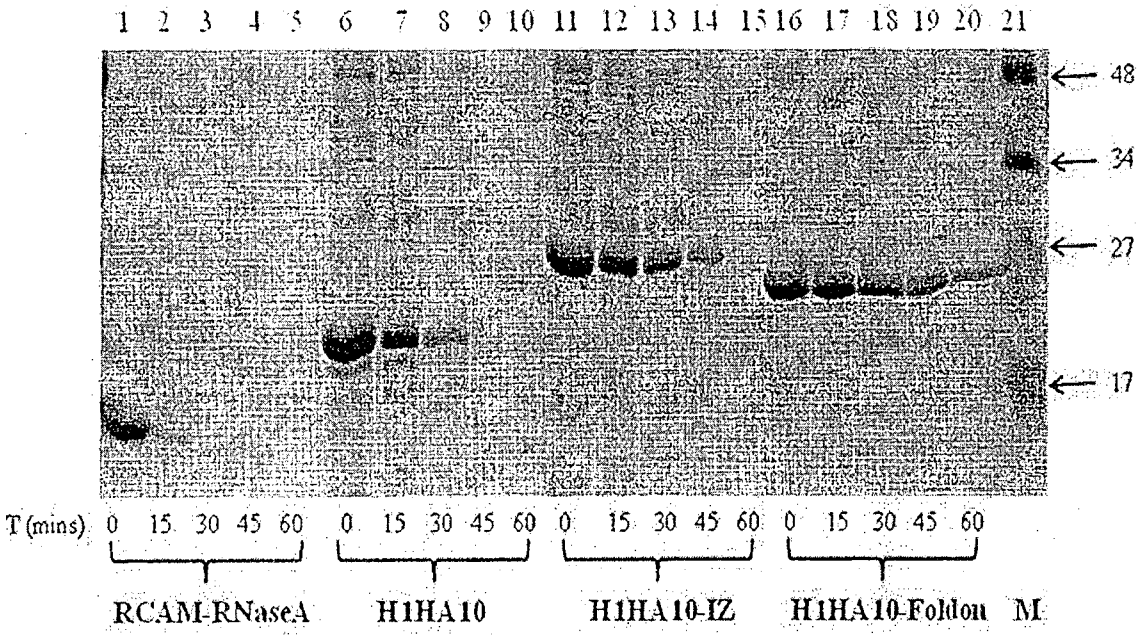

FIGURE 5

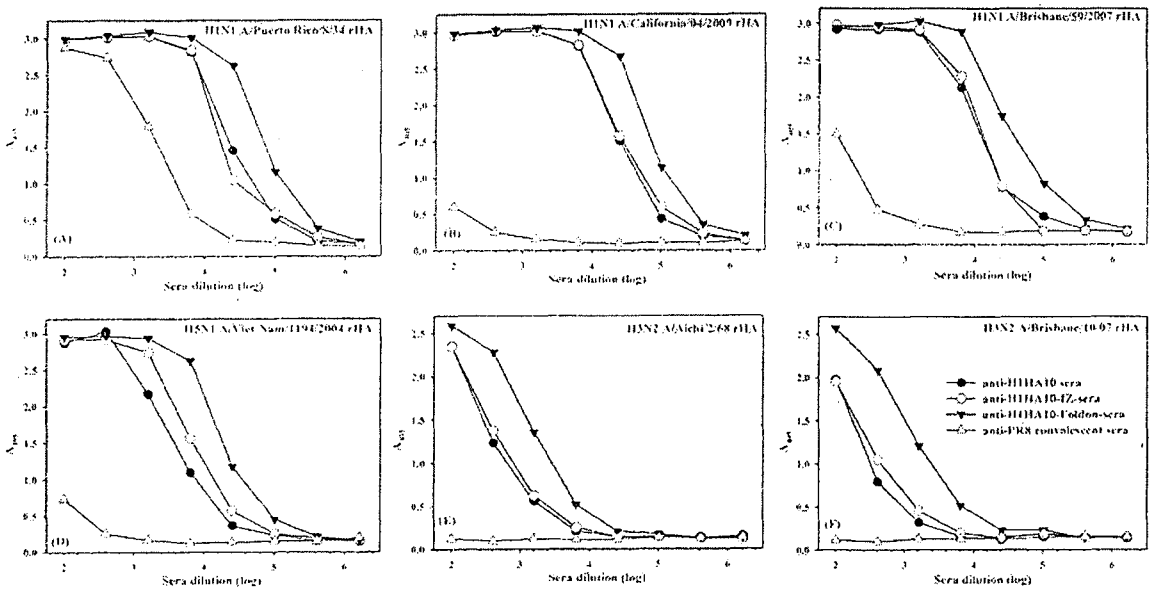

FIGURE 6

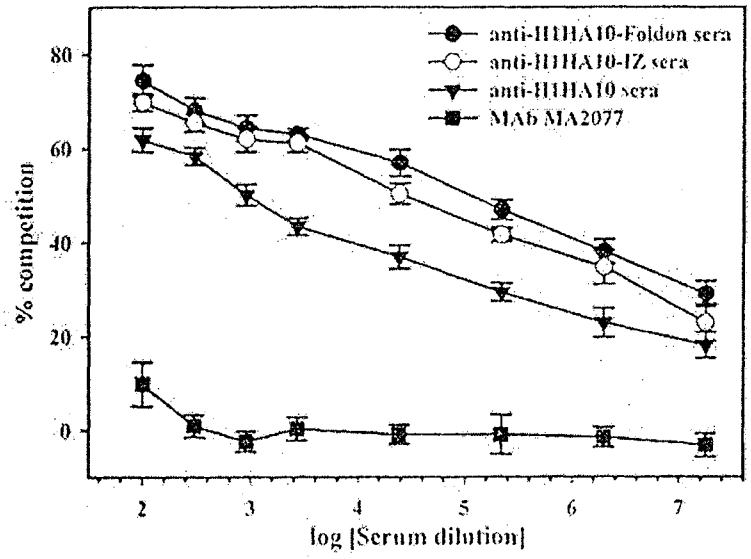
anti-HHHA10-Foldon sera
anti-HHHA10-IZ sera
anti-HHHA10 sera
MAb MA2077
% competition
80
60
40
20
0
2
3
4
5
6
7
log [Serum dilution]

FIGURE 7

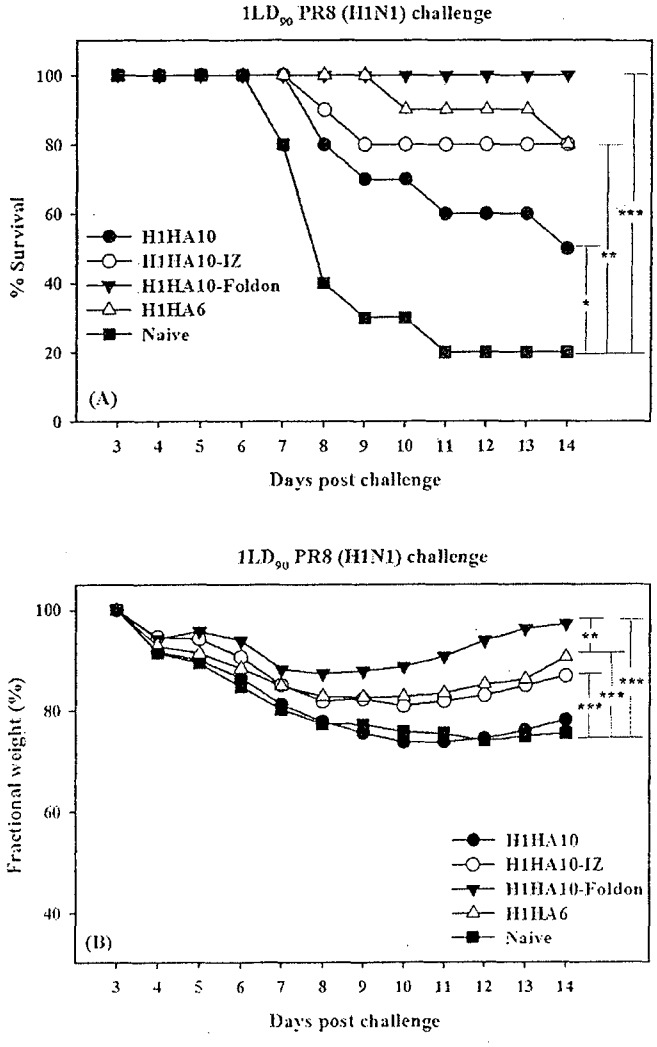
1LD90 PR8 (H1N1) challenge
% Survival
100
80
60
40
20
0
H1HA10
H1HA10-IZ
H1HA10-Foldon
H1HA6
Naive
(A)
3 4 5 6 7 8 9 10 11 12 13 14
Days post challenge
1LD90 PR8 (H1N1) challenge
Fractional weight (%)
100
80
60
40
H1HA10
H1HA10-IZ
H1HA10-Foldon
H1HA6
Naive
(B)
3 4 5 6 7 8 9 10 11 12 13 14
Days post challenge

FIGURE 8

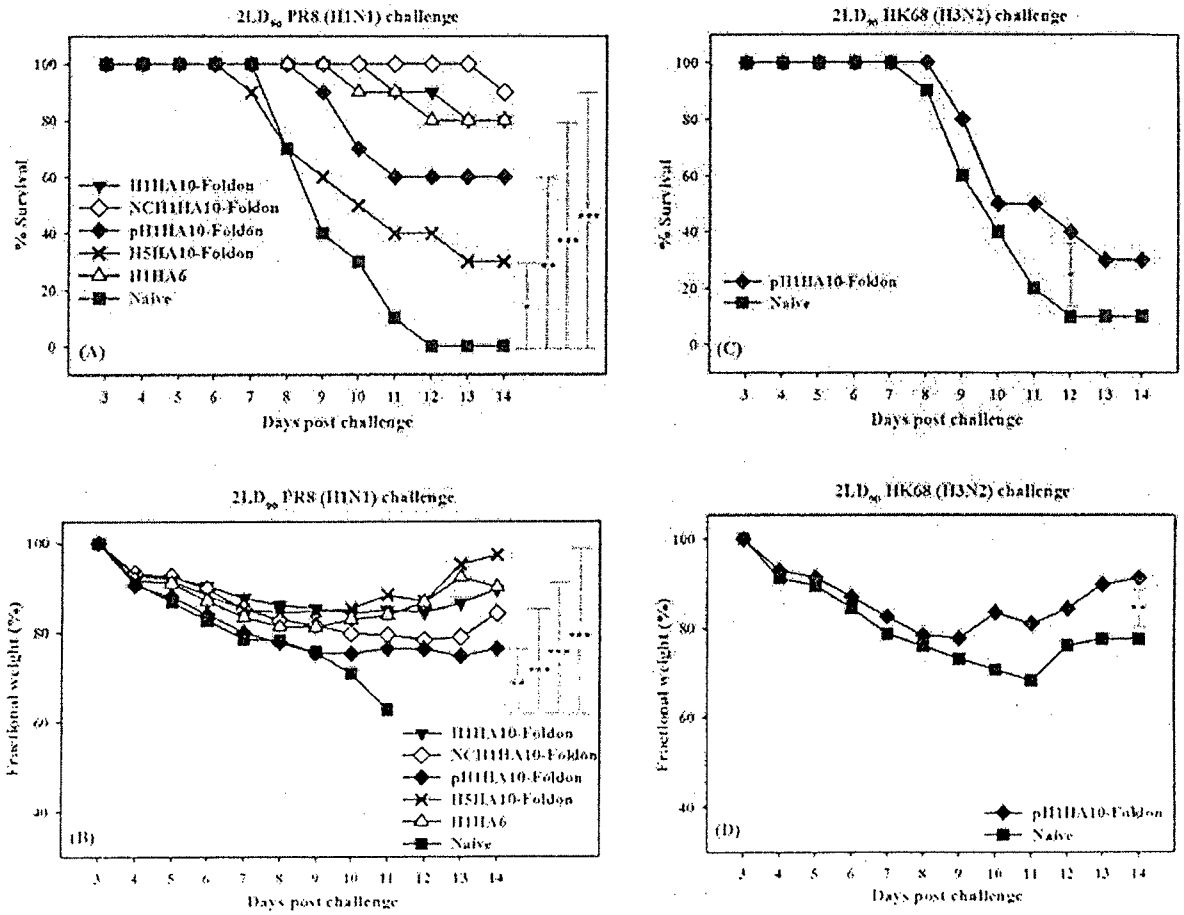
2LD50 PR8 (H1N1) challenge
% Survival
Days post challenge
H1HA10-Foldon
NCH1HA10-Foldon
pH1HA10-Foldon
H5HA10-Foldon
H1HA6
Naive
(A)
2LD50 HK68 (H3N2) challenge
pH1HA10-Foldon
Naive
(C)
2LD50 PR8 (H1N1) challenge
Fractional weight (%)
(B)
2LD50 HK68 (H3N2) challenge
(D)

FIGURE 9

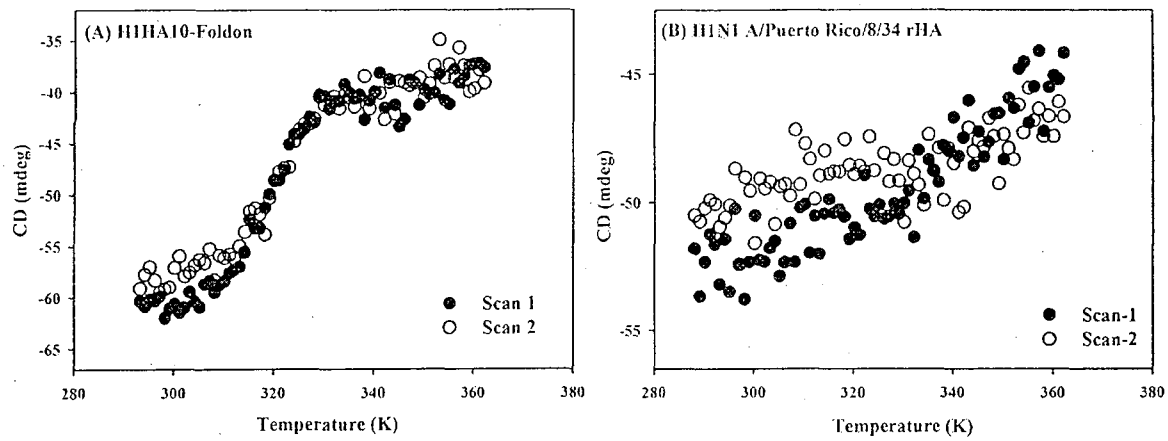
(A) H1HA10-Foldon
CD (mdeg)
Temperature (K)
Scan 1
Scan 2
(B) H1N1 A/Puerto Rico/8/34 rHA
CD (mdeg)
Temperature (K)
Scan-1
Scan-2

FIGURE 10

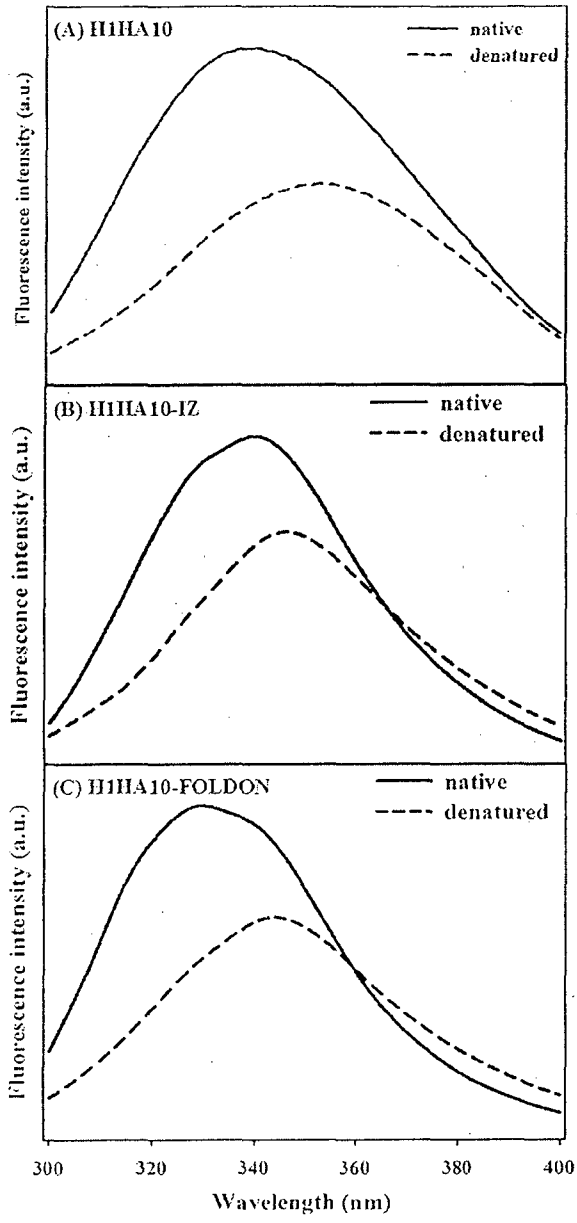
(A) H1HA10
native
denatured
Fluorescence intensity (a.u.)
(B) H1HA10-IZ
native
denatured
Fluorescence intensity (a.u.)
(C) H1HA10-FOLDON
native
denatured
Fluorescence intensity (a.u.)
300
320
340
360
380
400
Wavelength (nm)

FIGURE 11

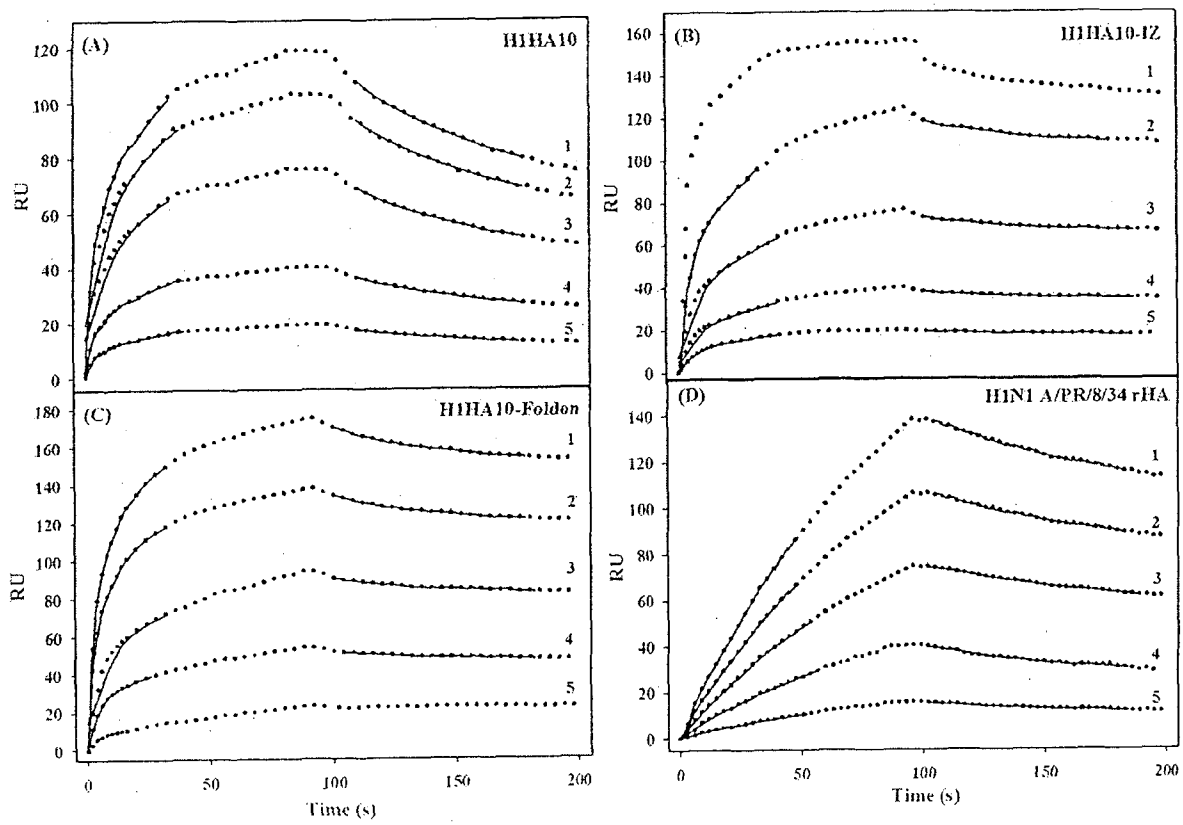
(A)
H1HA10
(B)
H1HA10-IZ
(C)
H1HA10-Foldon
(D)
H1N1 A/PR/8/34 rHA
RU
Time (s)
1
2
3
4
5

FIGURE 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2015/000589

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/005 C12N15/09 C12N15/70 C12N5/10 A61K39/145
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	G. BOMMAKANTI ET AL: "Design of Escherichia coli-Expressed Stalk Domain Immunogens of H1N1 Hemagglutinin That Protect Mice from Lethal Challenge", JOURNAL OF VIROLOGY, vol. 86, no. 24, 26 September 2012 (2012-09-26), pages 13434-13444, XP055100077, ISSN: 0022-538X, DOI: 10.1128/JVI.01429-12 cited in the application see the whole document, in particular item "H1HA0HA6" and its use for immunization; figure 2 ----- -/--	1-6, 14-24

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 2015	17/07/2015

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Y. LU ET AL: "Production and stabilization of the trimeric influenza hemagglutinin stem domain for potentially broadly protective influenza vaccines", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 1, 16 December 2013 (2013-12-16), page 125, XP055095011, ISSN: 0027-8424, DOI: 10.1073/pnas.1308701110 cited in the application see whole document, in particular "Supporting information" pages S3 to S5 for construct sequences	1-3,9, 10,14,15
X	WO 2013/177444 A2 (UNIV LELAND STANFORD JUNIOR [US]) 28 November 2013 (2013-11-28) cited in the application paragraphs [00117] - [00121], [00140] - [00141]	1-3,9, 10,14,15
X	JOHN STEEL ET AL: "Influenza virus vaccine based on the conserved hemagglutinin stalk domain", MBIO, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 1, no. 1, 18 May 2010 (2010-05-18), pages e00018-10, XP002676044, ISSN: 2150-7511, DOI: 10.1128/MBIO.00018-10 [retrieved on 2010-04-01] cited in the application see the full document, in particular Figure 1	1,14-18, 20-24
X	WO 2010/117786 A1 (SINAI SCHOOL MEDICINE [US]; LOWEN ANICE C [US]; STEEL JOHN [US]; GARCI) 14 October 2010 (2010-10-14) paragraphs [00364] - [00372], [00399] - [00409], [00412]; figures 3,10,17; sequences 170,172,174,176	1,14-18, 20-24
A	WO 2013/079473 A1 (CRUCELL HOLLAND BV [NL]) 6 June 2013 (2013-06-06) the whole document	1-24

-/--

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2015/000589

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	V. V. A. MALLAJOSYULA ET AL: "Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 25, 24 June 2014 (2014-06-24), pages E2514-E2523, XP055158737, ISSN: 0027-8424, DOI: 10.1073/pnas.1402766111 the whole document -----	1-24

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2015/000589

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013177444 A2	28-11-2013	CN 104582714 A	29-04-2015
		EP 2852396 A2	01-04-2015
		US 2015132331 A1	14-05-2015
		WO 2013177444 A2	28-11-2013
WO 2010117786 A1	14-10-2010	AU 2010234849 A1	17-11-2011
		CA 2787099 A1	14-10-2010
		EP 2413962 A1	08-02-2012
		JP 2012521786 A	20-09-2012
		US 2010297174 A1	25-11-2010
		WO 2010117786 A1	14-10-2010
WO 2013079473 A1	06-06-2013	AR 089005 A1	23-07-2014
		AU 2012343981 A1	10-07-2014
		CA 2857087 A1	06-06-2013
		CN 104066446 A	24-09-2014
		EA 201491051 A1	30-04-2015
		EP 2785372 A1	08-10-2014
		JP 2015502353 A	22-01-2015
		KR 20140099515 A	12-08-2014
		PH 12014501118 A1	04-08-2014
		US 2014357845 A1	04-12-2014
		WO 2013079473 A1	06-06-2013