centers. Second, to allow sufficient time for vaccine manufacture and distribution, strains must be selected approximately six months prior to the initiation of the influenza season. Often, the predictions of the vaccine strain selection committee are inaccurate, resulting in a substantial drop in the efficacy of vaccination.

**[0008]** The possibility of a novel subtype of influenza A virus entering the human population also presents a significant challenge to current vaccination strategies. Since it is impossible to predict what subtype and strain of influenza virus will cause the next pandemic, current, strain-specific approaches cannot be used to prepare a pandemic influenza vaccine.

## 3. SUMMARY

**[0009]** Provided herein are flu hemagglutinin (HA) polypeptides that induce a cross-protective immune response against the conserved HA stem domain (sometimes referred to herein as the "stalk" domain) of influenza viruses. The subject matter of the present invention is as defined in the attached set of claims. In one ~~aspect~~example, the ~~invention~~ disclosure concerns the design and construct of chimeric influenza virus hemagglutinin polypeptides having a stable HA stalk that displays a globular HA head heterologous to the stalk (i.e. chimeric influenza virus hemagglutinin polypeptides described herein). The HA immunogens designed for vaccination share the HA stalk region but are highly divergent in their globular heads. Such constructs are engineered into vaccine formulations such as live influenza viruses, killed influenza viruses, virus/viral-like particles ("VLPs"), subunit vaccines, split vaccines, etc., that elicit highly potent and broadly neutralizing antibodies against the conserved HA stalk. Such "universal" vaccines can be used to induce and/or boost cross-protective immune responses across influenza virus subtypes.

**[0010]** By way of background, neutralizing antibodies against influenza viruses target the HA glycoprotein and prevent either the binding or the fusion step involved in viral entry. Two basic subsets of neutralizing antibodies are elicited by exposure to influenza viruses: those directed to the strain-specific globular head (a domain that is non-conserved across the various strains and subtypes of influenza virus), and those directed to the highly conserved stem of the HA glycoprotein. The non-conserved HA globular head carries the immunodominant epitopes. Without being bound by theory, the strain-specific anti-globular head antibodies are thought to be more potent than anti-stem antibodies, thus explaining the largely strain-specific immunity conferred by infection with current vaccines.

**[0011]** The invention is based, in part, on the inventors' rational design strategies for influenza virus vaccines that elicit highly potent and broadly neutralizing antibodies against

the HA stem. In this regard, the chimeric HA immunogen is designed to share a relatively well conserved stalk domain from previous exposures/vaccinations, but contain a heterologous HA globular head – preferably one to which the intended vaccinate is naïve. Exposure to this construct should mainly boost antibodies directed to the conserved HA stem. Repeated immunizations with the conserved HA stem and changing the globular head should induce robust cross-neutralizing antibodies against the common stem region of HA.

**[0012]** When designing the chimeric HA constructs, care should be taken to maintain the stability of the resulting protein. In this regard it is recommended that the cysteine residues identified as Ap and Aq in Fig. 1 be maintained since they contribute to the stability of the HA stalk as discussed in more detail in Section 5.1 infra. For the best stability, it is preferred to "swap" the HA globular domain as a whole (between the Ap and Aq cysteine residues as shown in Fig. 1) since the resulting conformation would be closest to the native structure. In other words the "linker" referred to in Section 5.1.2 can be the entire globular head domain of a heterologous HA.

**[0013]** Instead of "swapping out " the native globular head of the HA stalk, the globular head can be made heterologous to the conserved stalk by altering the loops that contribute to the HA globular head epitopes. This approach may not work as well for generating the desired immune response against the conserved stalk, unless the altered globular head is designed to be vastly different from the native globular HA head – especially when using an HA to which the population has been exposed. Nevertheless, such alterations can be accomplished, e.g., by altering a majority of the five loops that contribute to the HA globular head epitopes. In one useful approach, all five loops can be altered. Alternatively, or in addition, the epitopes in the five loops can be masked by introducing glycosylation sites into the globular head domain.

**[0014]** The constructs used for vaccination can advantageously be designed for the particular subjects/population to be vaccinated. There are three influenza subtypes to which human beings living today have been exposed: subtypes H1, H2, and H3. Influenza viruses of the H2 subtype disappeared from the population in 1968, whereas influenza viruses of the H1 and H3 subtypes persist in the population to the present day. As a result, adults living today that were born before 1968 have likely been exposed to each of the H1, H2, and H3 subtypes. In contrast, adults living today that were born after 1968 have likely only been exposed to the H1 and H3 subtypes.

**[0015]** Thus, in preferred ~~embodiments~~ examples for vaccination of adults, the chimeric influenza hemagglutinin polypeptides do not possess a globular head domain from the HA of an

influenza virus of subtype H1, H2, or H3, but do possess a stem domain from the HA of one of these three subtypes. The heterologous globular head can be selected from the HA of any non-H1, non-H2, or non-H3 subtype. Also, separate chimeric constructs made using H1/H2 stems on the one hand, and H3 stems on the other may beneficially be used in a vaccination program -- the H1 and H2 subtypes are Group 1 HA subtypes that share a conserved stalk domain; whereas H3 is a Group 2 subtype that has a stalk domain that is structurally different from the Group1 stalk. The use of H1 and H3 constructs would ensure generating/boosting an immune response against each stem domain. Immunization of adult subjects with such chimeric influenza hemagglutinin polypeptides will boost the memory immune response of the subject, resulting in the large scale production of cross-reactive, broadly neutralizing anti-stem domain antibodies that provide long-lasting immunity to influenza virus in the subject.

**[0016]** Infants who have not been exposed, of course, are naïve to all influenza virus subtypes. As a result, a wide range of HA stem/globular head combinations can be constructed for use in vaccines for infants. In a preferred ~~embodiment~~example, naïve infants can be vaccinated with constructs made using the HA stalk of a Group 1 (H1 or H2) or Group 2 (H3) strain, and a globular head from a heterologous strain; i.e., non-H1, non-H2, and/or non-H3 strains. Three different chimeric HA constructs for each HA stalk can be used advantageously in three sequential vaccinations to induce a cross-protective response.

**[0017]** The chimeric influenza hemagglutinin polypeptides used for vaccination can also advantageously be designed to effectively elicit highly potent and broadly neutralizing antibodies against the HA stem domain in a subject by the addition or modification of glycosylation sites in these polypeptides. It is believed that glycosylation of the HA globular head and stem domain can mask antigenic sites, thereby allowing an influenza virus to evade an immune response within a subject. Within the context of an influenza virus HA polypeptide, however, glycosylation within the stem domain of the chimeric influenza hemagglutinin polypeptide can hinder or prevent desired immune responses against antigenic regions within this domain that are shielded by glycosylation. Therefore, in certain preferred ~~embodiments~~examples, the chimeric influenza hemagglutinin polypeptide comprises one or more modified glycosylation sites that disrupts the binding of glycan to the stem domain, thereby making the stem domain more accessible for eliciting an immune response. To further increase the immunogenicity of the stem domain, the constructs can further comprise non-naturally occurring glycosylation sites in the globular head domain that, when glycosylated, shield immunodominant antigenic regions found in the globular head domain

from eliciting an immune response. One example of a non-naturally occurring glycosylation sites is the

addition of a glycosylation site to the globular head domain of an influenza virus HA of one subtype, wherein the glycosylation site is naturally found in the globular head domain of an influenza virus HA of another subtype. Another example of a non-naturally occurring glycosylation site is the addition of a glycosylation site to the globular head domain of an influenza virus HA from one strain, wherein the glycosylation site is naturally found in the globular head of an HA from another strain of influenza virus. Yet another example of a non-naturally occurring glycosylation site is the addition of a glycosylation site to the globular head of an HA from one strain, wherein the glycosylation site is not naturally found in the globular head of an HA from another subtype or strain of influenza virus. While not being bound by any particular theory of operation, it is believed that the additional glycosylation within the globular head domain will increase the immune response to the conserved stem domain, while reducing the immune response to the globular head domain.

**[0018]** It should be understood that use of the chimeric influenza hemagglutinin polypeptides described herein is advantageous because (i) said polypeptides are highly stable (by virtue of possessing an intact globular head domain) and (ii) the immune systems of the subjects to which said polypeptides are administered have not previously been exposed to the globular head domains of the chimeric influenza hemagglutinin, but have been exposed to the conserved epitopes of the stem domains of the chimeric influenza hemagglutinin.

**[0019]** In another ~~aspect~~example, provided herein is a flu HA polypeptide (e.g., influenza virus hemagglutinin stem domain polypeptides and non-chimeric influenza virus hemagglutinin polypeptides) that comprises a stem domain comprising one or more modified glycosylation sites that disrupt the binding of glycan to the stem domain.

**[0020]** In another ~~aspect~~example, provided herein is a flu HA polypeptide (e.g., influenza virus hemagglutinin stem domain polypeptides and non-chimeric influenza virus hemagglutinin polypeptides) that comprises a globular head domain comprising one or more non-naturally occurring glycosylation sites.

**[0021]** In yet another ~~aspect~~example, provided herein, is a flu HA polypeptide (e.g., influenza virus hemagglutinin stem domain polypeptides and non-chimeric influenza virus hemagglutinin polypeptides) that comprises (1) a stem domain comprising one or more modified glycosylation sites that disrupt the binding of glycan to the stem domain; and (2) a globular head domain comprising one or more non-naturally occurring glycosylation sites.

**[0022]** In a specific ~~embodiment~~example, provided herein is a chimeric influenza virus hemagglutinin (HA) polypeptide comprising an HA stem domain and an HA globular head domain, wherein: (1) the HA globular head domain is heterologous to the HA stem domain;

and (2) the HA stem domain comprises one or more modified glycosylation site(s), wherein the modified glycosylation site(s) comprises a modification to a glycosylation site that disrupts/interferes with binding of a glycan to the glycosylation site in the stem domain. In specific ~~embodiments~~example, the modified glycosylation site comprises a modification of a naturally occurring glycosylation site having an amino acid sequence Asn-Xaa-Ser/Thr/Cys, and wherein Xaa is any amino acid. In certain ~~embodiments~~example, the HA globular head domain further comprises one or more non-naturally occurring glycosylation sites having an amino acid sequence Asn-Xaa-Ser/Thr/Cys, wherein Xaa is any amino acid.

**[0023]** In another ~~embodiment~~example, provided herein is a non-chimeric influenza virus hemagglutinin (HA) polypeptide comprising an HA stem domain and an HA globular head domain, wherein: (1) the HA globular head domain is homologous to the HA stem domain, and (2) the HA stem domain comprises one or more modified glycosylation site(s), wherein the modified glycosylation site(s) comprises a modification to a glycosylation site that disrupts/interferes with binding of a glycan to the glycosylation site in the stem domain. In specific ~~embodiments~~examples, the modified glycosylation site comprises a modification of a naturally occurring glycosylation site having an amino acid sequence Asn-Xaa-Ser/Thr/Cys, wherein the modification disrupts the ability of a glycan to attach to the modified glycosylation site, wherein Xaa is any amino acid.

**[0024]** In another ~~embodiment~~example, provided herein is a an influenza virus hemagglutinin (HA) stem domain polypeptide comprising: an influenza hemagglutinin HA1 domain that comprises an HA1 N-terminal stem segment covalently linked to a linker of 1 to 50 heterologous residues that is in turn covalently linked to an HA1 C-terminal short stem segment; said HA1 domain in tertiary or quaternary association with an influenza hemagglutinin HA2 domain, wherein the influenza virus HA stem domain polypeptide domain further comprises one or more modified glycosylation site(s), wherein the modified glycosylation site(s) comprises a modification to a glycosylation site that disrupts/interferes with binding of a glycan to the glycosylation site in the stem domain. In specific ~~embodiments~~examples, the modified glycosylation site comprises a modification of a naturally occurring glycosylation site having an amino acid sequence Asn-Xaa-Ser/Thr/Cys, where the modification disrupts the ability of a glycan to attach to the modified glycosylation site, and wherein Xaa is any amino acid.

**[0025]** In another ~~embodiment~~example, provided herein is an influenza virus hemagglutinin (HA) stem domain polypeptide comprising: an influenza hemagglutinin HA1

domain that comprises an HA1 N-terminal long stem segment covalently linked to a linker of 1 to 50

heterologous residues that is in turn covalently linked to an HA1 C-terminal long stem segment; said HA1 domain in tertiary or quaternary association with an influenza hemagglutinin HA2 domain, wherein the influenza virus HA stem domain polypeptide domain further comprises one or more modified glycosylation site(s), wherein the modified glycosylation site(s) comprises a modification to a glycosylation site that disrupts/interferes with binding of a glycan to the glycosylation site in the stem domain. In specific ~~embodiments~~examples, the modified glycosylation site comprises a modification of a naturally occurring glycosylation site having an amino acid sequence Asn-Xaa-Ser/Thr/Cys, where the modification disrupts the ability of a glycan to attach to the modified glycosylation site, and wherein Xaa is any amino acid.

**[0026]** In another ~~embodiment~~example, provided herein is an influenza virus hemagglutinin (HA) stem domain polypeptide comprising: an influenza hemagglutinin HA1 domain that comprises an HA1 N-terminal stem segment covalently linked to a linker of 1 to 50 heterologous residues that is in turn covalently linked to an HA1 C-terminal stem segment; said HA1 domain in tertiary or quaternary association with an influenza hemagglutinin HA2 domain, wherein the influenza virus HA stem domain polypeptide domain further comprises one or more modified glycosylation site(s), wherein the modified glycosylation site(s) comprises a modification to a glycosylation site that disrupts/interferes with binding of a glycan to the glycosylation site in the stem domain. In specific ~~embodiments~~examples, the modified glycosylation site comprises a modification of a naturally occurring glycosylation site having an amino acid sequence Asn-Xaa-Ser/Thr/Cys, where the modification disrupts the ability of a glycan to attach to the modified glycosylation site, and wherein Xaa is any amino acid.

**[0027]** In another ~~embodiment~~example, provided herein is an influenza virus hemagglutinin (HA) stem domain polypeptide comprising: an influenza hemagglutinin HA1 domain that comprises, linked in the following order: an HA1 N-terminal stem segment, a first linker of 1 to 50 heterologous residues, an HA1 intermediate stem segment, a second linker of 1 to 50 heterologous residues and an HA1 C-terminal stem segment; said HA1 domain in tertiary or quaternary association with an influenza hemagglutinin HA2 domain, wherein the influenza virus hemagglutinin (HA) stem domain polypeptide domain further comprises one or more modified glycosylation site(s), wherein the modified glycosylation site(s) comprises a modification to a glycosylation site that disrupts/interferes with binding of a glycan to the glycosylation site in the stem domain. In specific ~~embodiments~~examples, the

modified glycosylation site comprises a modification of a naturally occurring glycosylation site having an amino acid

10. A nucleic acid encoding the polypeptide of any of claims 1 to 9.

11. A cell expressing the nucleic acid of claim 10.

12. A virus comprising a genome engineered to express the nucleic acid of claim 10.

13. A virus comprising the polypeptide of any one of claims 1 to 9.

14. The virus of claim 12, wherein the virus is an influenza virus.

15. The virus of claim 13, wherein the virus is an influenza virus.

16. The virus of claim 14, which is inactivated or split.

17. The virus of claim 15, which is inactivated or split.

18. A virus-like particle comprising the polypeptide of any one of claims 1 to 9.

19. An immunogenic composition comprising the polypeptide of any one of claims 1 to 9.

20. An immunogenic composition comprising the virus of claim 12.

21. An immunogenic composition comprising the virus of claim 13.

22. An immunogenic composition comprising the virus of claim 14.

23. An immunogenic composition comprising the virus of claim 15.

24. An immunogenic composition comprising the virus-like particle of claim 18.

25. Use of an immunogenic composition of claim 19 in the manufacture of a medicament for ~~A method of~~ immunizing a subject ~~comprising administering to the subject an effective amount of the immunogenic composition of claim 19~~.

26. Use of an immunogenic composition of claim 20 in the manufacture of a medicament for ~~A method of~~ immunizing a subject ~~comprising administering to the subject an effective amount of the immunogenic composition of claim 20~~.

27. Use of an immunogenic composition of claim 21 in the manufacture of a medicament for ~~A method of~~ immunizing a subject ~~comprising administering to the subject an effective amount of the immunogenic composition of claim 21~~.

28. Use of an immunogenic composition of claim 22 in the manufacture of a medicament for ~~A method of~~ immunizing a subject ~~comprising administering to the subject an effective amount of the immunogenic composition of claim 22~~.

29. Use of an immunogenic composition of claim 23 in the manufacture of a medicament for ~~A method of~~ immunizing a subject ~~comprising administering to the subject an effective amount of the immunogenic composition of claim 23~~.

30. Use of an immunogenic composition of claim 24 in the manufacture of a medicament for ~~A method of~~ immunizing a subject ~~comprising administering to the subject an effective amount of the immunogenic composition of claim 24~~.

31. The ~~method~~ use of claim 25, wherein the subject is a human.

32. The use~~method~~ of claim 26, wherein the subject is a human.

33. The use~~method~~ of claim 27, wherein the subject is a human.

34. The use~~method~~ of claim 28, wherein the subject is a human.

35. The use~~method~~ of claim 29, wherein the subject is a human.

36. The use~~method~~ of claim 30, wherein the subject is a human.

37. The use~~method~~ of claim 25, wherein the immunogenic composition is to be administered intramuscularly or intranasally to the subject.

38. Use of an immunogenic composition of claim 25 in the manufacture of a medicament for ~~A method of~~ preventing an influenza virus disease ~~comprising administering to a subject an effective amount of the immunogenic composition of claim 25~~.

39. Use of an immunogenic composition of claim 25 in the manufacture of a medicament for~~A method of~~ treating an influenza virus infection or an influenza virus disease ~~comprising administering to a subject an effective amount of the immunogenic composition of claim 25~~.

40. Use of an immunogenic composition of claim 25 in the manufacture of a medicament for~~A method of~~ preventing an influenza virus disease ~~comprising administering to a subject an effective amount of the immunogenic composition of claim 25~~.

41. Use of an immunogenic composition of claim 25 in the manufacture of a medicament for ~~A method of~~ preventing an influenza virus disease ~~comprising administering to a subject an effective amount of the immunogenic composition of claim 25~~.

42. The ~~method~~ use of claim 26, wherein the immunogenic composition is to be administered intramuscularly or intranasally to the subject.

43. Use of an immunogenic composition of claim 26 in the manufacture of a medicament for~~A method of~~ preventing an influenza virus disease ~~comprising administering to a subject an effective amount of the immunogenic composition of claim 26~~.

44. Use of an immunogenic composition of claim 26 in the manufacture of a medicament for~~A method of~~ treating an influenza virus infection or an influenza virus disease ~~comprising administering to a subject an effective amount of the immunogenic composition of claim 26~~.

45. Use of an immunogenic composition of claim 26 in the manufacture of a medicament for~~A method of~~ preventing an influenza virus disease ~~comprising administering to a subject an effective amount of the immunogenic composition of claim 26~~.

46. Use of an immunogenic composition of claim 26 in the manufacture of a medicament for~~A method of~~ preventing an influenza virus disease ~~comprising administering to a subject an effective amount of the immunogenic composition of claim 26~~.

47. Use of an influenza virus hemagglutinin polypeptide to which a subject is naive in the manufacture of a medicament for~~A method of~~ preventing an influenza virus disease or infection in ~~a~~ the subject~~, comprising administering to said subject an influenza virus hemagglutinin polypeptide to which the subject is naive~~.

48. The ~~method~~ use of claim 47, wherein the influenza virus hemagglutinin polypeptide is from an influenza virus of subtype H2, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, and/or H17.

49. The usemethod of claim 48, comprising administering to said subjectwherein a second influenza virus hemagglutinin polypeptide to which the subject is naive is to be administered to said subject, wherein the second influenza virus hemagglutinin polypeptide is from an influenza virus of subtype H2, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, and/or H17, and wherein the influenza virus hemagglutinin polypeptides of the first and second administrations are from different influenza virus subtypes.

50. The usemethod of claim 49, comprising administering to said subjectwherein a third influenza virus hemagglutinin polypeptide to which the subject is naive is to be administered to said subject, wherein the third influenza virus hemagglutinin polypeptide is from an influenza virus of subtype H2, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, and/or H17, and wherein the influenza virus hemagglutinin polypeptides of the first, second, and third administrations are from different influenza virus subtypes.

51. Use of an influenza virus in the manufacture of a medicament for A method of preventing an influenza virus disease or infection in a subject, comprising administering to said subject an influenza virus, wherein said influenza virus comprises a hemagglutinin polypeptide to which the subject is naive.

52. The method use of claim 51, wherein the influenza virus hemagglutinin polypeptide is from an influenza virus of subtype H2, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, and/or H17.

53. The usemethod of claim 52, comprising administering to said subject a second influenza virus, wherein said second influenza virus comprises a hemagglutinin polypeptide to which the subject is naive; wherein the hemagglutinin polypeptide of the second influenza virus is from an influenza virus of subtype H2, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, and/or H17; and wherein the influenza virus hemagglutinin polypeptides of the first and second administrations are from different influenza virus subtypes.

54. The method use of claim 53, comprising administering to said subjectwherein a third influenza virus is to be administered to said subject, wherein said third influenza virus comprises a hemagglutinin polypeptide to which the subject is naive; wherein the hemagglutinin polypeptide of the third influenza virus is from an influenza virus of subtype H2, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13,

298, 410-412, and 481-483, according to the H3 numbering system, wherein the HA stem domain is an HA stem domain from an influenza virus of subtype H3, H4, H7, H10, H14, H15.

73. The HA polypeptide of any one of claims 1-9 or 55-72, wherein said polypeptide is soluble.

74. A nucleic acid encoding the polypeptide of any of claims 55-62.

75. A nucleic acid encoding the polypeptide of any of claims 63-66.

76. A cell expressing the nucleic acid of claim 74.

77. A cell expressing the nucleic acid of claim 75.

78. A virus comprising the chimeric influenza virus hemagglutinin (HA) polypeptide of any one of claims 55-62.

79. A virus comprising the chimeric influenza virus hemagglutinin (HA) polypeptide of any one of claims 63-66.

80. An immunogenic composition comprising the chimeric influenza virus hemagglutinin (HA) polypeptide of any one of claims 55-62.

81. An immunogenic composition comprising the chimeric influenza virus hemagglutinin (HA) polypeptide of any one of claims 63-66.

82. Use of an immunogenic composition of claim 80 in the manufacture of a medicament for ~~A method of~~ immunizing a subject ~~comprising administering to the subject an effective amount of the immunogenic composition of claim 80~~.

83. Use of an immunogenic composition of claim 81 in the manufacture of a medicament for~~A method of~~ immunizing a subject ~~comprising administering to the subject an effective amount of the immunogenic composition of claim 81~~.

84. The ~~method~~ use of claim 82, wherein the subject is human.

85. The ~~method~~ use of claim 83, wherein the subject is human.