

SUMMARY

[0004] The present disclosure provides improved modified recombinant HA polypeptides with broadened immunogenic profile that extends coverage (i.e., the capability of eliciting a immunogenic response against the HA polypeptide) to antigenically distinct influenza strains, for example to new or additional pandemic and/or seasonal influenza strains. The present disclosure is, in part, based on modifications deduced from *in silico* analysis of sequence variation among circulating influenza strains, mapping of the antigenic region of HA, and/or epitope patterns and structural analyses of the HA peptide. Targeted modifications can be subsequently introduced at various amino acid residue locations and/or specific regions of an HA polypeptide with a known immune profile to yield novel HA polypeptides with improved and more balanced immune profiles. As described in detail below including the Examples section, the present inventors have developed -among other things- various distinct strategies for engineering HA polypeptides to extend a seasonal response profile to cover pandemic strains, or vice versa. These strategies extend the immune profile across clusters of sequences (or clades) of antigenically distinct strains; they can be applied to an engineered recombinant HA molecule over time so that it continues to elicit an immune response against antigenically drifted circulating seasonal strains. In all cases, the strategy is designed to generally preserve specific residues of the receptor binding site (RBS) of a host HA polypeptide with modifications engineered in the region near the RBS. Similar strategies may be used to extend a pandemic response profile to cover seasonal strains. The compositions and methods of the present disclosure are applicable to a wide variety of recombinant HA polypeptides, including those engineered by a variety of methods and those comprising substantially wild-type sequences.

[0004a] In one aspect, the present disclosure provides a method of altering the immunogenic profile of an engineered hemagglutinin (HA) polypeptide, comprising:

- a) identifying the presence or absence of one or more putative N-linked glycosylation sites in a head region of an engineered HA polypeptide, wherein the head region is a segment of the engineered HA polypeptide at positions corresponding to residues 63-278, 125-277 or 135-269 of SEQ ID NO: 1 according to California 09 Numbering (CA09 Numbering);
- b) introducing into the head region of the engineered HA polypeptide one or more amino acid substitutions, deletions or insertions to disrupt the one or more putative N- linked glycosylation sites or insert additional N-linked glycosylation sites, thereby generating a re-

engineered HA polypeptide with altered immunogenic profile as compared to the engineered HA polypeptide; and

c) introducing one or more amino acid substitutions in the region of or adjacent to the Receptor Binding Site (RBS) region of the engineered HA polypeptide, and/or introducing an insertion of a Lysine (K) or Arginine (R) residue into a loop or loops bounding the RBS of the engineered HA polypeptide.

[0004b] In another aspect, the present disclosure provides a re-engineered HA polypeptide generated by the method as described herein.

[0005] Thus, in one example, the present disclosure provides a recombinant hemagglutinin (HA) polypeptide comprising an engineered head region or segment thereof derived from an engineered HA polypeptide with a predominantly seasonal immune profile and a stem region derived from a pandemic strain. In some examples, the engineered head region or segment thereof comprises a sequence at least 95%, 96%, 97%, 98%, 99% or more identical to amino acids corresponding to positions 135 – 269, 125 – 277, or 63 – 278 of SEQ ID NO:1. In some examples, the engineered head region or segment thereof comprises a sequence identical to amino acids corresponding to positions 135 – 269, 125 – 277, or 63 – 278 of SEQ ID NO:1 (SMART_DO2a sequence).

[0006] In some examples, the stem region is derived from a naturally-occurring or wild-type pandemic strain. In some examples, a suitable naturally-occurring pandemic strain is selected from A/California/07/2009, A/New Jersey/10/1976, or A/South Carolina/1/1918. In some examples, the stem region is derived from an engineered HA polypeptide that has a pandemic immune profile.

[0007] In some examples, the engineered HA polypeptide that has a pandemic immune profile is engineered by a computationally optimized broadly reactive antigens (COBRA) technology, a mosaic technology, consensus-based combinations of influenza strains, deletion and/or rearrangement of structural domains, domain swapping, or combinations of neutralizing or cross-reactive epitopes among multiple influenza strains.

[0008] In another example, the present disclosure provides a recombinant influenza hemagglutinin (HA) polypeptide comprising an engineered head region derived from an HA polypeptide with a predominantly seasonal immune profile and comprising one or more amino acid substitutions, deletions or insertions at one or more putative N-linked

glycosylation sites defined by a consensus sequence of NxS/Ty, wherein x and y are not Proline (P), such that the one or more putative N-linked glycosylation sites are disrupted. In particular examples, each of the one or more amino acid substitutions, deletions or insertions is derived from a corresponding sequence in a pandemic strain.

[0009] In yet another example, the present disclosure provides a recombinant influenza hemagglutinin (HA) polypeptide comprising an engineered head region derived from an HA polypeptide with a predominantly pandemic immune profile into which has been inserted one or more engineered putative N-linked glycosylation sites defined by a consensus sequence of NxS/Ty, wherein x and y are not P. In particular examples, each of the one or more engineered putative N-linked glycosylation sites is engineered by amino acid substitutions, deletions or insertions based on a corresponding sequence in a seasonal strain.

[0010] In some examples, the hemagglutinin corresponds to type A influenza. In some examples, the type A influenza is subtype H1N1.

[0011] In some examples, the one or more putative N-linked glycosylation sites correspond to positions 142-145 and/or 177-179 (normalized sequence alignment to A/California/07/2009 HA (SEQ ID NO: 2); “CA09 Numbering”). In some examples, the one or more putative N-linked glycosylation sites are within 15 angstroms of the Receptor Binding Site (RBS), wherein the RBS is defined as all amino acid residues within 15 angstroms of a position corresponding to W167 (CA09 Numbering) in a three-dimensional (3-D) structure. Alternatively, the RBS may be defined as the epitope bound by the paratope of monoclonal antibody CH65, and the one more amino acid substitutions occur adjacent to (e.g., within 100 amino acid residues, within 75 amino acid residues, within 50 amino acid residues, within 40 amino acid residues, within 30 amino acid residues, within 25 amino acid residues, within 20 amino acid residues, within 15 amino acid residues, within 10 amino acid residues, within 5 amino acid residues, etc.) the epitope of CH65. In some examples, the one or more amino acid substitutions, deletions or insertions are selected from lists or tables provided herein, e.g., Table 4, or Table 5. In some examples, the one or more amino acid substitutions, deletions or insertions comprise modifying the consensus sequence NxS/Ty to $z^1z^2z^3z^4$ wherein z^1 is N, D, K or S; z^2 is Y or is unchanged; z^3 is E, D or N; and z^4 is I, L, P, S or T, or is unchanged.

[0012] In some examples, the pandemic or seasonal strain from which one or more amino acid substitutions, deletions or insertions are derived is a circulating influenza strain. In some examples, the circulating influenza strain is selected from the group consisting of A/California/07/2009 and A/South Carolina/1/1918. In some examples, the amino acid substitutions, deletions or insertions comprise insertion of a Lysine (K) or Arginine (R) residue within 1-5 amino acids (e.g., within 1-4, 1-3, 1-2 amino acids) of the NxS/Ty consensus sequence. In some examples, the Lysine (K) or Arginine (R) residue is within 1-5 amino acids (e.g., within 1-4, 1-3, 1-2 amino acids) 3' of the NxS/Ty consensus sequence. In some examples, the one or more amino acid substitutions, deletions or insertions comprise an insertion at a position corresponding to residue 147 (CA09 Numbering). In some examples, the insertion at the position corresponding to residue 147 comprises an insertion of Lysine (K) or Arginine (R).

[0013] In a further example, the present disclosure provides a recombinant influenza HA polypeptide comprising an engineered head region derived from an HA polypeptide with a predominantly seasonal immune profile and comprising one or more amino acid substitutions between positions corresponding to 60 and 291 of (CA09 Numbering), wherein each of the one or more amino acid substitutions is derived from a corresponding sequence in a pandemic strain. In some examples, the one or more amino acid substitutions are selected from lists or tables provided herein, e.g., Table 6. In some examples, the one or more amino acid substitutions are between positions corresponding to 137 and 262 (CA09 Numbering), and wherein the one or more amino acid substitutions are selected from Table 7. In some examples, the one or more amino acid substitutions comprise two, three, four, five, six, seven, eight, nine, ten or more amino acid substitutions selected from Table 3 or Table 4. In some examples, the one or more amino acid substitutions comprise at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 consecutive substitutions selected from Table 6 or Table 7. In some examples, the one or more amino acid substitutions occur at positions corresponding to 137, 144, 145, 154, 155, 156, 157, 158, 159, 177, 210, 211, 212, 213, 214, 244, 245, and/or 262 (CA09 Numbering).

[0014] In a related example, the present disclosure provides a recombinant influenza HA polypeptide comprising an engineered head region derived from an HA polypeptide with a predominantly seasonal immune profile and comprising one or more amino acid substitutions within 15 angstroms of the Receptor Binding Site (RBS), wherein the RBS is

defined as all amino acid residues within 15 angstroms of a position corresponding to W167 (CA09 Numbering) in a three-dimensional (3-D) structure, wherein each of the one or more amino acid substitutions is derived from a corresponding sequence in a pandemic strain. Alternatively, the RBS may be defined as the epitope bound by the paratope of monoclonal antibody CH65, and the one or more amino acid substitutions occur adjacent to (e.g., within 100 amino acid residues, within 75 amino acid residues, within 50 amino acid residues, within 40 amino acid residues, within 30 amino acid residues, within 25 amino acid residues, within 20 amino acid residues, within 15 amino acid residues, within 10 amino acid residues, within 5 amino acid residues, etc.) the epitope of CH65.

[0015] In some examples, the pandemic strain is a circulating influenza virus. In some examples, the one or more amino acid substitutions are within 10 (e.g., within 9, 8, 7, 6, 5, etc.) angstroms of Receptor Binding Site (RBS). In some examples, the one or more amino acid substitutions comprise two, three, four, five, six, seven, eight, nine, ten or more amino acid substitutions selected from Table 8. In some examples, the one or more amino acid substitutions comprise at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 consecutive substitutions selected from Table 8. In some examples, the one or more amino acid substitutions occur at positions corresponding to 137, 144, 145, 154, 155, 156, 157, 158, 159, 177, 210, 211, 212, 213, and/or 214 (CA09 Numbering).

[0016] In still another example, the present disclosure provides a recombinant influenza HA polypeptide comprising an engineered head region derived from an HA polypeptide with a predominantly seasonal immune profile and comprising one or more amino acid modifications selected from Table 9 (e.g., two or more, three or more, four or more, five or more modifications selected from Table 9).

[0017] In some examples, the HA polypeptide with a predominantly seasonal immune profile or the HA polypeptide with a predominantly pandemic immune profile suitable for the present disclosure is engineered by a computationally optimized broadly reactive antigens (COBRA) technology, a mosaic technology, influenza consensus sequences based combinations of influenza strains, deletion and/or rearrangement of structural domains, domain swapping, or combinations of neutralizing or cross-reactive epitopes among multiple influenza strains.

[0018] In some examples, the recombinant HA polypeptide elicits neutralizing antibodies against both seasonal and pandemic strains of influenza virus. In particular examples, the recombinant HA polypeptide elicits neutralizing antibodies against one or more seasonal strains and one more pandemic strains of influenza virus. In some examples, the recombinant HA polypeptide is characterized by a more balanced immunogenic profile against both seasonal and pandemic strains of influenza virus.

[0019] Among other things, the present disclosure provides an isolated nucleic acid encoding a recombinant HA polypeptide described herein and a vector comprising such a nucleic acid. In some examples, the present disclosure provides a cell containing such a vector or a nucleic acid. In some examples, a suitable cell is a human cell. In particular examples, the human cell is a HEK-293 cell. In some examples, a suitable cell is a monkey cell. In particular examples, the monkey cell is a Vero cell.

[0020] In various additional examples, the present disclosure provides virus-like particles (VLP)s comprising a recombinant HA polypeptide as described herein. In some examples, a VLP according to the disclosure further comprises an influenza neuraminidase (NA) protein, a human immunodeficiency virus (HIV) gag protein, or both. The present disclosure also provides pharmaceutical compositions comprising a recombinant HA polypeptide or a VLP as described herein. Furthermore, the present disclosure provides methods of generating an immune response (e.g., immunizing or vaccinating) against seasonal and pandemic influenza virus in a subject by administration of a pharmaceutical composition described herein.

[0021] In another example, the present disclosure provides methods of altering the immunogenic profile of an engineered HA polypeptide. In some examples, a method of altering the immunogenic profile of an engineered HA polypeptide comprises: selecting a head region of the engineered HA polypeptide and substituting the selected head region of the engineered HA polypeptide for a corresponding head region of an HA polypeptide with a distinct immunogenic profile, thereby generating a re-engineered HA polypeptide with altered immunogenic profile. In some examples, the engineered HA polypeptide has a predominantly seasonal immune profile, and the HA polypeptide with a distinct immunogenic profile has a predominantly pandemic immune profile.

[0022] In some examples, the selected head region of the engineered HA polypeptide with a predominantly seasonal immune profile correspond to residues 63-278, 125-277 or 135-269 (CA09 Numbering). In some examples, the corresponding head region of the HA polypeptide with a distinct immunogenic profile, which is predominantly pandemic, comprises an amino acid sequence selected from: residues of 63-277 of SEQ ID NO:2 [full-length wt CA09 (A/California/07/2009 HA sequence) sequence], residues of 63-277 of SEQ ID NO: 3 [full-length wt SC1918 sequence], residues 63-277 of SEQ ID NO: 4 [full-length wt NJ1976 sequence], residues 125-277 of SEQ ID NO: 2 [full-length wt CA09 sequence], residues 125-277 of SEQ ID NO:3 [full-length wt SC1918 sequence], residues 125-277 of SEQ ID NO: 4[full-length wt NJ1976 sequence], residues 135-269 of SEQ ID NO: 2 [full-length wt CA09 sequence], residues 135-269 of SEQ ID NO: 3 [full-length wt SC1918 sequence], or residues 135-269 of SEQ ID NO: 4 [full-length wt NJ1976 sequence].

[0023] In some examples, the HA polypeptide with a distinct immunogenic profile, which is predominantly pandemic, is an HA polypeptide from a wild-type influenza virus. In some examples, the HA polypeptide with a distinct immunogenic profile, which is predominantly pandemic is an engineered HA polypeptide. In some examples, the HA polypeptide with a distinct immunogenic profile, which is predominantly pandemic, comprises SEQ ID NO: 5 (full-length sequence for DO1A).

[0024] In some examples, the engineered HA polypeptide has a predominantly pandemic immune profile, and the HA polypeptide with a distinct immunogenic profile has a predominantly seasonal immune profile. In some examples, the selected head region of the engineered HA polypeptide with a predominantly pandemic immune profile is selected from the group consisting of: residues of 63-277 of SEQ ID NO:2 [full-length wt CA09 (A/California/07/2009 HA sequence) sequence]; residues of 63-277 of SEQ ID NO: 3 [full-length wt SC1918 sequence]; residues of 63-277 of SEQ ID NO: 4 [full-length wt NJ1976 sequence]; residues of 125-277 of SEQ ID NO: 2 [full-length wt CA09 sequence]; residues of 125-277 of SEQ ID NO: 3 [full-length wt SC1918 sequence]; residues of 125-277 of SEQ ID NO: 4 [full-length wt NJ1976 sequence]; residues of 135-269 of SEQ ID NO: 2 [full-length wt CA09 sequence]; residues of 135-269 of SEQ ID NO: 3 [full-length wt SC1918 sequence]; and residues of 135-269 of SEQ ID NO: 4 [full-length wt NJ1976 sequence]. In some examples, the corresponding head region of the HA polypeptide with a distinct

immunogenic profile, which is predominantly seasonal, comprises residues 63-278, 125-277 or 135-269 of SEQ ID NO: 2 (full-length sequence for SMARt_DO2A).

[0025] In some examples, the present disclosure provides methods of altering the immunogenic profile of an engineered HA polypeptide comprising steps of: identifying the presence or absence of one or more putative N-linked glycosylation sites in a head region of the engineered HA polypeptide as compared to the corresponding head region of an HA polypeptide with a distinct immunogenic profile; introducing into the head region of the engineered HA polypeptide one or more amino acid substitutions, deletions or insertions to disrupt the one or more putative N-linked glycosylation sites or insert additional N-linked glycosylation sites based on the corresponding sequence of the HA polypeptide with a distinct immunogenic profile, thereby generating a re-engineered HA polypeptide with altered immunogenic profile. In some examples, the one or more putative or additional N-linked glycosylation sites are defined by a consensus sequence of NxS/Ty, wherein x and y are not P. In some examples, the engineered HA polypeptide has a predominantly seasonal immune profile, and the HA polypeptide with a distinct immunogenic profile has a predominantly pandemic immune profile, wherein the one or more amino acid substitutions, deletions or insertions are introduced into the engineered HA polypeptide to disrupt the one or more putative N-linked glycosylation sites, and wherein the re-engineered HA polypeptide is altered to be more pandemic. In some examples, the engineered HA polypeptide has a predominantly pandemic immune profile, and the HA polypeptide with a distinct immunogenic profile has a predominantly seasonal immune profile, wherein the one or more amino acid substitutions, deletions or insertions are introduced into the engineered HA polypeptide to insert one or more putative additional N-linked glycosylation sites, and wherein the re-engineered HA polypeptide is altered to be more seasonal.

[0026] In some examples, the present disclosure provides methods of altering the immunogenic profile of an engineered HA polypeptide comprising introducing one or more amino acid substitutions within 15 (e.g., within 10, 9, 8, 7, 6, 5, etc.) angstroms of the Receptor Binding Site (RBS), wherein the RBS is defined as all amino acids residues within 15 (e.g., within 10, 9, 8, 7, 6, or 5) angstroms of a position corresponding to residue W167 (CA09 Numbering) in a three-dimensional (3-D) structure; wherein each of the one or more amino acid substitutions comprises replacing an amino acid residue at a specific position with an amino acid residue observed at the corresponding position in an HA polypeptide with a

distinct immunogenic profile, thereby generating an re-engineered HA polypeptide with altered immunogenic profile. Alternatively, the RBS may be defined as the epitope bound by the paratope of monoclonal antibody CH65, and the one more amino acid substitutions occur adjacent to (e.g., within 100 amino acid residues, within 75 amino acid residues, within 50 amino acid residues, within 40 amino acid residues, within 30 amino acid residues, within 25 amino acid residues, within 20 amino acid residues, within 15 amino acid residues, within 10 amino acid residues, within 5 amino acid residues, etc.) the epitope of CH65. In some examples, the HA polypeptide with a distinct immunogenic profile is derived from a circulating seasonal or pandemic influenza strain. In some examples, the engineered HA polypeptide has a predominantly seasonal immune profile, and the HA polypeptide with a distinct immunogenic profile has a predominantly pandemic immune profile, and wherein the re-engineered HA polypeptide is altered to be more pandemic. In some examples, the engineered HA polypeptide has a predominantly pandemic immune profile, and the HA polypeptide with a distinct immunogenic profile has a predominantly seasonal immune profile, and wherein the re-engineered HA polypeptide is altered to be more seasonal.

[0027] In some examples, the present disclosure provides methods of altering the immunogenic profile of an engineered HA polypeptide comprising introducing one or more modifications selected from those shown in Table 4, Table 5, Table 6, Table 7, Table 8, or Table 9, into one or more corresponding positions of the engineered HA polypeptide, thereby generating a re-engineered HA polypeptide with altered immunogenic profile. In some examples, the one or more modifications occur at positions corresponding to 137, 144, 145, 154, 155, 156, 157, 158, 159, 177, 210, 211, 212, 213, 214, 244, 245, and/or 262 of the engineered HA polypeptide (CA09 numbering). In some examples, the one or more modifications occur at positions corresponding to 137, 144, 145, 154, 155, 156, 157, 158, 159, 177, 210, 211, 212, 213, and/or 214 of the engineered HA polypeptide (CA09 numbering). In some examples, the one or more modifications comprise two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, or ten or more modifications selected from those shown in Table 4, Table 5, Table 6, Table 7, Table 8, or Table 9. In some examples, the one or more modifications comprise at least 2, 3, 4, 5, or 10 consecutive substitutions selected from Table 4, Table 5, Table 6, Table 7, Table 8, or Table 9.

[0028] In some examples, the present disclosure provides methods of altering the immunogenic profile of an engineered HA polypeptide comprising selecting two or more modifications selected from the group consisting of:

(i) selecting a head region of the engineered HA polypeptide, and substituting the selected head region of the engineered HA polypeptide for a corresponding head region of an HA polypeptide with a distinct immunogenic profile,

(ii) identifying the presence or absence of one or more putative N-linked glycosylation sites in a head region of the engineered HA polypeptide as compared to the corresponding head region of an HA polypeptide with a distinct immunogenic profile, and introducing into the head region of the engineered HA polypeptide one or more amino acid substitutions, deletions or insertions to disrupt the one or more putative N-linked glycosylation sites or insert additional N-linked glycosylation sites in the engineered HA polypeptide based on the corresponding sequence of the HA polypeptide with a distinct immunogenic profile,

(iii) introducing one or more amino acid substitutions within 15 (e.g., within 10, 9, 8, 7, 6, or 5) angstroms of the Receptor Binding Site (RBS), wherein the RBS is defined as all amino acids residues within 15 angstroms of a position corresponding to W167 (CA09 Numbering) in a three-dimensional (3-D) structure, wherein each of the one or more amino acid substitutions comprises replacing an amino acid residue at a specific position with an amino acid residue observed at the corresponding position in an HA polypeptide with a distinct immunogenic profile. Alternatively, the RBS may be defined as the epitope bound by the paratope of monoclonal antibody CH65, and the one more amino acid substitutions occur adjacent to (e.g., within 100 amino acid residues, within 75 amino acid residues, within 50 amino acid residues, within 40 amino acid residues, within 30 amino acid residues, within 25 amino acid residues, within 20 amino acid residues, within 15 amino acid residues, within 10 amino acid residues, within 5 amino acid residues, etc.) the epitope of CH65, and

(iv) introducing one or more modifications selected from those shown Table 4, Table 5, Table 6, Table 7, Table 8, or Table 9, into one or more corresponding positions of the engineered HA polypeptide;

thereby generating a re-engineered HA polypeptide with altered immunogenic profile.

[0029] In various examples, a method according to the present disclosure further includes assessing expression and conformation of the re-engineered HA polypeptide.

[0030] In various examples, a method according to the present disclosure further includes a step of determining if the re-engineered HA polypeptide elicits neutralizing antibodies against seasonal and/or pandemic strains of influenza virus.

[0031] In some examples, generating a re-engineered HA polypeptide with altered immunogenic profile comprises increasing the binding of one or more anti-head monoclonal antibodies against seasonal and/or pandemic influenza strains. In some examples, generating a re-engineered HA polypeptide with altered immunogenic profile comprises increasing the breadth of binding of anti-head monoclonal antibodies against pandemic influenza strains. In some examples, generating a re-engineered HA polypeptide with altered immunogenic profile comprises increasing the breadth of binding of anti-stem monoclonal antibodies. In such examples, modifications in the head region induce an increase in binding to anti-stem monoclonal antibodies, demonstrating that substitutions at one place may exert long-range allosteric effects on a distant location. In some examples, increases in binding are determined by flow cytometry detection of the monoclonal antibodies bound to re-engineered HA polypeptides expressed on the surface of mammalian cells. In some examples, the level of monoclonal antibody bound to re-engineered HA polypeptides expressed on the surface of mammalian cells is quantified.

[0032] In this application, the use of “or” means “and/or” unless stated otherwise. As used in this application, the term “comprise” and variations of the term, such as “comprising” and “comprises,” are not intended to exclude other additives, components, integers or steps. As used in this application, the terms “about” and “approximately” are used as equivalents. Any numerals used in this application with or without about/approximately are meant to cover any normal fluctuations appreciated by one of ordinary skill in the relevant art.

[0033] Other features, objects, and advantages of the present disclosure are apparent in the detailed description, drawings and claims that follow. It should be understood, however, that the detailed description, the drawings, and the claims, while indicating examples of the present disclosure, are given by way of illustration only, not limitation. Various changes and modifications within the scope of the disclosure will become apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWING

[0034] The drawings are for illustration purposes only, not for limitation.

[0035] **Figure 1** shows three different regions differing in the specific residue sites at which the globular head was truncated.

[0036] **Figure 2** shows a schematic of the flow cytometry assay used to demonstrate proper folding, expression, and antibody binding capabilities of influenza HA polypeptides.

[0037] **Figure 3** shows flow cytometry assay results demonstrating the proper folding and expression of recombinant HA polypeptides generated by grafting globular head regions of the influenza RBS onto recipient HA stems.

[0038] **Figure 4** shows flow cytometry assay results demonstrating an improved seasonal immune profile (as demonstrated by increased mAb binding) of recombinant HA polypeptides generated by grafting globular head regions of the influenza RBS onto recipient HA stems.

[0039] **Figure 5** shows a numerical representation of the increased immunological breadth of recombinant HA polypeptides generated by grafting globular head regions of the influenza RBS onto recipient HA stems.

CLAIMS

We claim:

1. A method of altering the immunogenic profile of an engineered hemagglutinin (HA) polypeptide, comprising:
 - a) identifying the presence or absence of one or more putative N-linked glycosylation sites in a head region of an engineered HA polypeptide, wherein the head region is a segment of the engineered HA polypeptide at positions corresponding to residues 63-278, 125-277 or 135-269 of SEQ ID NO: 1 according to California 09 Numbering (CA09 Numbering);
 - b) introducing into the head region of the engineered HA polypeptide one or more amino acid substitutions, deletions or insertions to disrupt the one or more putative N-linked glycosylation sites or insert additional N-linked glycosylation sites, thereby generating a re-engineered HA polypeptide with altered immunogenic profile as compared to the engineered HA polypeptide; and
 - c) introducing one or more amino acid substitutions in the region of or adjacent to the Receptor Binding Site (RBS) region of the engineered HA polypeptide, and/or introducing an insertion of a Lysine (K) or Arginine (R) residue into a loop or loops bounding the RBS of the engineered HA polypeptide.
2. The method of claim 1, wherein the re-engineered HA polypeptide comprises a Lysine (K) or Arginine (R) residue inserted within 1-5 amino acids of the NxS/Ty consensus sequence.
3. The method of claim 1 or 2, wherein the RBS comprises all amino acids residues within 15 angstroms of a position corresponding to residue W167 according to CA09 Numbering in a three-dimensional (3-D) structure.
4. The method of claim 2 or 3, wherein the RBS comprises the epitope bound by the paratope of monoclonal antibody CH65.
5. The method of any one of claims 1-4, wherein the one or more putative N-linked glycosylation sites or additional N-linked glycosylation sites are defined by a consensus sequence of NxS/Ty, wherein x and y are not proline (P).

6. The method of claim 1, wherein the one or more putative N-linked glycosylation sites are at positions corresponding to positions 142-145 and/or 177-179 of SEQ ID NO: 1 according to CA09 Numbering.
7. The method of any one of claims 1-3, wherein the one or more amino acid substitutions, deletions or insertions to disrupt the one or more putative N-linked glycosylation sites comprise modifying the consensus sequence NxS/Ty to z1z2z3z4 wherein
- z1 is N, D, K or S;
 - z2 is Y or is unchanged;
 - z3 is E, D or N; and
 - z4 is I, L, P, S or T, or is unchanged.
8. The method of any one of claims 1-7, wherein the immunogenic profile of the re-engineered HA polypeptide is more pandemic than the engineered HA polypeptide.
9. The method of any one of claims 1-7, wherein the immunogenic profile of the re-engineered HA polypeptide is more seasonal than the engineered HA polypeptide.
10. The method of any one of claims 1-9, wherein the re-engineered HA polypeptide comprises one or more amino acid substitutions, deletions or insertions at positions corresponding to residues 137, 144, 145, 154, 155, 156, 157, 158, 159, 177, 210, 211, 212, 213, 214, 244, 245 and/or 262 according to CA09 Numbering.
11. The method of any one of claims 1-10, wherein the re-engineered HA polypeptide comprises one or more amino acid substitutions, deletions or insertions at positions corresponding to residue 137, 144, 145, 154, 155, 156, 157, 158, 159, 177, 210, 211, 212, 213 and/or 214 according to CA09 Numbering.
12. The method of any one of claims 1-11, further comprising assessing expression and conformation of the re-engineered HA polypeptide.
13. The method of any one of claims 1-12, further comprising determining if the re-engineered HA polypeptide elicits neutralizing antibodies against both seasonal and pandemic strains of influenza virus.
14. The method of any one of claims 1-13, wherein the altered immunogenic profile of the re-engineered HA polypeptide comprises increasing the binding of one or more anti-head monoclonal antibodies against seasonal and/or pandemic influenza strains.

15. The method of any one of claims 1-14, wherein the altered immunogenic profile of the re-engineered HA polypeptide comprises increasing the breadth of binding of anti-head monoclonal antibodies against pandemic influenza strains.
16. The method of claims 14 or 15, wherein increases in binding are determined by flow cytometry detection of the monoclonal antibodies bound to the re-engineered HA polypeptides expressed on the surface of mammalian cells.
17. The method of claim 16, further comprising quantitating the level of monoclonal antibody binding.
18. The method of claim 1, wherein the engineered HA polypeptide is engineered by a computationally optimized broadly reactive antigens (COBRA) technology, a mosaic technology, deletion and/or rearrangement of structural domains, domain swapping, or combinations of neutralizing or cross-reactive epitopes among multiple influenza strains.
19. A re-engineered HA polypeptide generated by the method of any one of claims 1-16.