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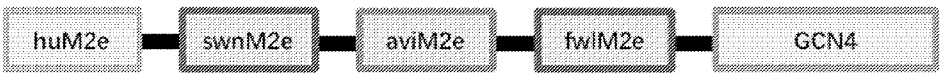
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Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

(54) **Title:** HEADLESS HEMAGGLUTIN INFLUENZA VACCINE



4M2e-GCN4 amino acids sequence:

MSLLTEVETPIRNEWGSRSNDSSDPGGSSGGSSSLLTEVETPTRSEWESRSSD
SSDPGGSSGGSSSLLTEVETPTRNGWESKSSGSSDPGSGSGSGSSLLTEVETPT
RNGWESNSSDSSDPGGGGSSSSLELKQIEDKLEEILSKLYHIENELARIKKLLGE

FIG. 1A

(57) **Abstract:** Disclosed are universal influenza based on a truncated influenza hemagglutin (HA) protein lacking a head domain (hrHA). Also disclosed is a composition comprising a nanoparticle coated with a disclosed hrHA polypeptide. Also disclosed is a composition comprising a virus like particle (VLP) expressing on its surface a disclosed hrHA polypeptide.

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HEADLESS HEMAGGLUTIN INFLUENZA VACCINE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of U.S. Provisional Application No. 62/567,936, filed October 4, 2017, which is hereby incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government Support under Grant No. AI101047 awarded by the National Institutes of Health. The Government has certain rights in the invention.

SEQUENCE LISTING

This application contains a sequence listing filed in electronic form as an ASCII.txt file entitled "220702-2450 Sequence Listing_ST25" created on October 4, 2018. The content of the sequence listing is incorporated herein in its entirety.

BACKGROUND

Seasonal influenza epidemics cause an estimated 250,000 deaths worldwide each year. The global burden of human influenza infection is huge. It was estimated that in 2008 seasonal influenza viruses cause 90 million new infections worldwide in children below 5 years of age and were responsible for up to 20% of all pediatric acute lower respiratory infections. The adaptive immune response that is built up in the human population due to influenza infection creates immune selection pressure on the circulating viruses. Mutant viruses acquire ability to escape from prevailing herd immunity by antigenic drift and shift, which necessitates the yearly update of composition of human influenza vaccines to match the newly circulating viruses. However, the protection efficacy of updated vaccines does not always live up to expectation, for instance the outbreak of pandemic Mexican flu H1N1 in 2009 caused 200,000 deaths during the first 12 months of its circulation. Low vaccine effectiveness was also observed recently during 2012-2013 and 2014-2015 epidemic. In addition, the threat from fatal zoonotic influenza viruses like H5N1 and H7N9 adds further necessity for the development of universal influenza vaccines with broad and long-term protection.

SUMMARY

Disclosed herein are influenza vaccines capable of providing broad cross-protection. Conserved epitopes from influenza virus are promising targets to develop universal

vaccines. Notably, the membrane proximal stalk domain of hemagglutinin (HA) is more conserved than the highly variable head domain. Disclosed herein are vaccine compositions and methods based on a truncated influenza HA protein lacking a head domain. For example, disclosed herein is a polypeptide comprising a truncated influenza HA protein lacking at least a portion of the HA head domain, also referred to herein as a head-removed HA (hrHA). Also disclosed is a nanoparticle coated with a hrHA polypeptide. Also disclosed is a virus like particle (VLP) expressing on its surface a hrHA polypeptide.

Also disclosed is a nanoparticle formed by desolvating the fusion protein with a desolvating agent and/or crosslinking a fusion protein with a crosslinking agent, wherein the fusion protein comprises a series of 2 to 8 influenza virus matrix protein 2 extracellular (M2e) domains linked to a multimerization domain. This nanoparticle can in some embodiments be coated with an antigen, such as the disclosed hrHA polypeptide.

Also disclosed are pharmaceutical compositions comprising a polypeptide or nanoparticle disclosed herein and an adjuvant.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

Figures 1A to 1C show schematic and sequence designs of recombinant 4M2e-GCN4 (SEQ ID NO:38) and structural designs of original the H1 and and H3 HA proteins as well as the head-removed H1 and head-removed H3 that have been engineered using the originals as a starting structure.

Figures 2A and 2B show western blot characterization of example crosslinked tetramer and monomer recombinant 4M2e-GCN4, head-removed H1 and head-removed H3. Figures 2C and 2D display sandwich ELISA data that indicate that the H3 and H1 hrHA proteins maintain well-preserved tertiary structural conformations compared to full-length HA proteins.

Figures 3A to 3E demonstrate the fabrication and characterization of PNP. Figure 3A schematically illustrates the PNP formation process by ethanol desolvation with or without DTSSP crosslinking. Figure 3B illustrates the effect of differing doses of DTSSP crosslinker on PNP size by zeta-sizer with Figure 3C showing a peak size of 141nm in the absence of DTSSP altogether. Figure 3D shows well-maintained primary structure of the fusion protein even after PNP formation and the ability to completely denature the PNP back into

monomers. Figure 3E shows the particulate and nearly-spherical spatial displacement of the PNP by scanning electron microscopy (SEM).

Figures 4A to 4C show design of head-removed HA coated PNPs, Uni4C1 and Uni4C3. Figure 4A schematically illustrates the fabrication process while Figures 4B and 4C show hydrophobic and amine-reactive groups present in the hrHA proteins.

Figures 5A to 5D show fabrication and characterization of example head-removed HA coated PNPs, Uni4C1 and Uni4C3. Uni3C1 has a peak size of 181nm (Figure 5A) while Uni4C3 has a peak size of 211nm (Figure 5B). Figure 5C demonstrates that the hrHA proteins successfully coated the core PNP while Figure 5D demonstrated PNP spatial charactertistics by SEM.

Figures 6A to 6G show humoral immune responses of mice immunized with PNPs as measured by serum antibody endpoint titers against the delineated target antigens. Figure 6E summarizes these data in a five-axis radar chart.

Figure 7 shows immune protection conferred against lethal homologous and heterologous influenza virus challenge as demonstrated by maintenance of body weight and survival after challenge.

Figure 8 shows results of histological analysis of lung sections after viral challenge in either vaccinated or mock-vaccinated mice.

Figure 9 shows the results of HAI assays of sera from the various immunization regimens either before or after challenge, using numerous influenza virus strains for the inhibition assays.

Figure 10 shows virus titers in the lung 5 days after after 1 x mLD50 viral challenge.

Figure 11A shows sequence alignment of hrH3 with A/Aichi/2/1968 H3. Figure 11B shows sequence alignment of hrH1 with A/Puerto Rico/8/1934 H1.

Figure 12 shows protein structures for three different strategies of HA head-removal.

Figure 13 shows Western blot analysis of expressed hrH3 that were constructed by different strategies. One lane of the gel was used for each strategy, as follows: (Lane 1), strategy 1 (Str1) combines with 4 Glycine linker (4G), Str1-4G; (Lane 2), Str2-4G; (Lane 3), Str3-4G; (Lane 4), Str1-3G; (Lane 5), Str2-3G; (Lane 6), Str3-3G; (Lane 7), Str1-5G; (Lane 8), Str2-5G; (Lane 9), Str3-5G; (Lane 10), Str1-4GC; (Lane 11), Str2-4GC; (Lane 12), Str3-4GC.

Figure 14 shows a schematic diagram of tetrabrachion motif screwed tetramer protein construct.

Figures 15A and 15B shows example structure of 4M2e-NA nanoparticles. Figure 15A shows combination of 4M2e and 4M2e-NA fusion proteins. GS linker: glycine and serine

GGSGGG (SEQ ID NO:20); M2e: combined M2e sequence from human, swine, wild bird, and domestic fowl with tetrabrachion together; M2e + NA: combined stalk and head of neuraminidase 1 (from A/ Vietnam/1203/2004/) with M2e above. Figure 15B shows schematic formation of M2e-NA nano-particle. 4M2e-NA was covered on the 4M2e core.

Figures 16A to 16F show the formation of M2e-NA nanoparticle. Figure 16A shows M2e-NA fusion protein crosslinked by by 12.5, 7.5, 2.5, and 1mM of BS3, as in line 1, 2, 3, 4, respectively. Line 5: soluble M2e + NA fusion protein. Figure 16B shows Coomassie blue staining of M2e-NA nanoparticle. Figures 16C and 16D show Western blot assay of M2e-NA nanoparticle. C: M2e antibody; D: NA antibody. Figure 16E shows the size of formed M2e+NA nanoparticle: 198.2 +/- 62.41nm. Figure 16F shows results of a neuraminidase activity test. The concentration of each protein: M2e+NA nano particle: 1mg/ml; soluble M2e+NA: 1mg/ml; H5N1 virus: 2.5mg/ml; BSA: 2mg/ml.

Figure 17 shows example tetramer constructs.

Figure 18 displays the sequence alignment of B/hrHA (SEQ ID NO:40) with the HA sequence from Influenza B strain B/Brisbane/60/2008 (SEQ ID NO:39). The glycine-rich linkers are marked in bold letters and the GCN4 trimerization domain in the C terminal tail is marked in italics.

Figure 19A shows PCR and restriction digest data from construction of the plasmid for expression of B/hrHA. Figure 19B displays Western blot data evidence of productive expression of B/hrHA into the supernatant of Sf9 cells that have been transfected with the plasmid encoding B/hrHA.

DETAILED DESCRIPTION

Current influenza vaccines provide limited protection against seasonal strains of influenza A viruses owing to the ability to escape from immune pressure through rapid antigenic drift and re-assortment. The conserved epitopes from influenza virus are promising targets to develop universal vaccine. Hemagglutinin (HA) is the predominant protein on the surface of influenza viruses, is highly antigenic, and is the dominant target for humoral immune responses that give rise to anti-influenza antibodies. On the exterior of the virus, HA is divided into two domains: the head domain, which is highly variable in sequence, and the membrane proximal stalk domain that is more conserved. Antibodies formed against the head domain can often neutralize the virus while antibodies against the stalk domain typically do not. The humoral immune response to influenza is therefore commonly dominated by antibodies against the head domain, potentially resulting in neutralization of that strain of virus, but providing very narrowly-limited or no immunity against other strains.

An immune response against a more conserved domain of HA could provide immunity against numerous strains and variants of the virus. Given these considerations, a vaccine was designed and produced using an HA that has had the variable head removed while keeping the conserved stalk domain. To increase immunogenicity of this head-removed HA (hrHA), a nanotechnology approach was used to produce a nanocluster protein nanoparticle (PNP) vaccine displaying the hrHA protein as a coat on the surface of the PNP. These vaccines can fully protect against influenza viruses expressing divergent HA.

Definitions

It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a peptide" includes a plurality of such peptides, reference to "the peptide" is a reference to one or more peptides and equivalents thereof known to those skilled in the art, and so forth.

In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings:

"Optional" or "optionally" means that the subsequently described event or circumstance can or cannot occur, and that the description includes instances where the event or circumstance occurs and instances where it does not. For example, the phrase "optionally a signal peptide" means that the signal peptide may or may not be included.

The term "universal influenza vaccine" refers to vaccine capable of providing cross-protection against at least two, including three, four, five or more, strains or subtypes of influenza.

The term "individual," "host," "subject," and "patient" are used interchangeably to refer to any individual who is the target of administration, treatment, or vaccination. The subject can be a vertebrate, for example, a mammal. Thus, the subject can be a human or veterinary patient.

The term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio.

The term "carrier" means a compound, composition, substance, or structure that, when in combination with a compound or composition, aids or facilitates preparation, storage, administration, delivery, effectiveness, selectivity, or any other feature of the compound or composition for its intended use or purpose. For example, a carrier can be

selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject.

The terms “peptide,” “protein,” and “polypeptide” are used interchangeably to refer to a natural or synthetic molecule comprising two or more amino acids linked by the carboxyl group of one amino acid to the alpha amino group of another.

The term “protein domain” refers to a portion of a protein, portions of a protein, or an entire protein showing structural integrity; this determination may be based on amino acid composition of a portion of a protein, portions of a protein, or the entire protein.

The term “nucleic acid” refers to a natural or synthetic molecule comprising a single nucleotide or two or more nucleotides linked by a phosphate group at the 3’ position of one nucleotide to the 5’ end of another nucleotide. The nucleic acid is not limited by length, and the nucleic acid can include deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).

The term “variant” refers to an amino acid sequence having conservative amino acid substitutions, non-conservative amino acid substitutions (i.e. a degenerate variant), or a peptide having 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%$, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the recited sequence.

The term “percent (%) sequence identity” is defined as the percentage of nucleotides or amino acids in a candidate sequence that are identical with the nucleotides or amino acids in a reference nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods. For purposes herein, the % sequence identity of a given nucleotides or amino acids sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given sequence C that has or comprises a certain % sequence identity to, with, or against a given sequence D) is calculated as follows:

100 times the fraction W/Z,

where W is the number of nucleotides or amino acids scored as identical matches by the sequence alignment program in that program’s alignment of C and D, and where Z is the total number of nucleotides or amino acids in D. It will be appreciated that where the length

of sequence C is not equal to the length of sequence D, the % sequence identity of C to D will not equal the % sequence identity of D to C.

"Homolog" and "homologous" as used herein refer to sequences that derive from a recent common ancestor and therefore maintain a level of percent sequence identity that is commonly understood in the art.

"Heterologous" refers to sequences that encode for the same protein (in the case of nucleic acids) or that are demonstrably the same protein (in the case of polypeptides), yet in which such sequences are not derived from a recent common ancestor and maintain a level of percent sequence identity that is less than 100%.

A "fusion protein" refers to a polypeptide formed by the joining of two or more polypeptides through a peptide bond formed between the amino terminus of one polypeptide and the carboxyl terminus of another polypeptide. The fusion protein can be formed by the chemical coupling of the constituent polypeptides or it can be expressed as a single polypeptide from nucleic acid sequence encoding the single contiguous fusion protein. A single chain fusion protein is a fusion protein having a single contiguous polypeptide backbone. Fusion proteins can be prepared using conventional techniques in molecular biology to join the two genes in frame into a single nucleic acid, and then expressing the nucleic acid in an appropriate host cell under conditions in which the fusion protein is produced.

A "spacer" as used herein refers to a peptide that joins the proteins of a fusion protein. Generally a spacer has no specific biological activity other than to join the proteins or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of a spacer may be selected to influence some property of the molecule, such as the folding, net charge, or hydrophobicity of the molecule.

An "antigen" refers to a substance that can act as the target of an immune response. As used herein, "antigen" refers to a biological substance, and may consist of a peptide, a polypeptide or protein, a glycoprotein, a saccharide or polysaccharide, a lipid, a nucleic acid, or another biological or bioactive compound, amongst other biological substances.

The "ectodomain" of a protein refers to the domain of a membrane protein that extends into the extracellular space or is found on the exterior surface of a viral particle.

The "transmembrane" domain of a protein refers to the domain of a membrane protein that passes through the biological membrane and directly interfaces laterally with the phospholipid bilayer of the membrane.

The “cytoplasmic” domain, also known as an “intracellular” domain, is the domain of an integral membrane protein that extends into the intracellular space or is found in the interior space of a virus.

A “strain” of influenza as used herein refers to a lineage of the virus with defined and durable antigenic parameters. Each type of influenza (Influenza A, B, C, and D) consists of numerous strains that differ from one another in their antigen sequences.

A ”subtype” of influenza A virus refers to the division of influenza A into categories based on the sequence of the viral HA and neuraminidase (NA) proteins. For example, an influenza A virus can have an H1 sequence of its HA protein and an N1 sequence of its NA protein and would therefore be categorized as an H1N1 subtype of influenza. Other subtype examples include H3N2, H5N1, H7N9, and H9N2.

Headless HA Polypeptide

Disclosed herein is a polypeptide comprising a truncated influenza hemagglutinin (HA) protein lacking a head domain (a head-removed HA (hrHA) polypeptide). The HA protein can be of any subtype and from any strain. The following is the amino acid sequence for the influenza A H3 stalk ectodomain:
MKTIIALSYIFCLPLGQDLPGNDNSTATLCLGHHAVPNGTLVKTITDDQIEVTNATELVQSSST
GKICNNPHRILDGIDCTLIDALLGDPHCDVFQNETWDLFVERSKAFSNCYPYDVPDYASLRS
LVASSGTLEFITEGFTWTGVTQNGGSNACKRGPGSGFFSRLNWLTKSGSTYPVLNVTMPN
NDNFDKLYIWGIHHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGLSSRISIY
WTIVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIDTCISECITPNGSIPNDKPFQN
VNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLFGAIAGFIENGWEGMIDGWYGFRHQ
NSEGTGQAADLKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKID
LWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQLRENAEEMGNGCFKIYHKCDNACIESI
RNGTYDHDVYRDEALNNRFQIKGVELKSGYK (SEQ ID NO:15). The corresponding amino acid sequences for other HA subtypes are known. Therefore, reference to specific amino acids within SEQ ID NO:15 is also a reference to the corresponding amino acids in the known amino acid sequences for the other subtypes.

In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 65 to 320 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 62 to 322 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 62 to 320 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 62 to 321 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA

amino acids corresponding to residues 63 to 320 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 63 to 321 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 63 to 322 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 64 to 320 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 64 to 321 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 64 to 322 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 65 to 321 of SEQ ID NO:15. In some embodiments, the disclosed polypeptide lacks at least the HA amino acids corresponding to residues 65 to 322 of SEQ ID NO:15.

Methods and compositions disclosed herein for the sequence of any particular HA are applicable to the sequences of other HA. Methods and compositions from any of the particular HA delineated in this specification may be adapted routinely to another HA for those skilled in the art. Thus, in one embodiment, a sequence for influenza A HA is used for production of hrHA. In one embodiment, a sequence for influenza B HA is used for production of hrHA. In one embodiment, a sequence for influenza A H1 HA is used for production of hrHA.In one embodiment, a sequence for influenza A H1, H3, H5, H7 or H9 HA is used for production of hrHA.

In some embodiments, the disclosed polypeptide is a fusion protein. Fusion proteins, also known as chimeric proteins, are proteins created through the joining of two or more genes which originally coded for separate proteins. Translation of this fusion gene results in a single polypeptide with functional properties derived from each of the original proteins. Recombinant fusion proteins can be created artificially by recombinant DNA technology for use in biological research or therapeutics.

The functionality of fusion proteins is made possible by the fact that many protein functional domains are modular. In other words, the linear portion of a polypeptide which corresponds to a given domain, such as a tyrosine kinase domain, may be removed from the rest of the protein without destroying its intrinsic enzymatic capability. Thus, any of the herein disclosed functional domains can be used to design a fusion protein. For example, the head domain of HA has a function in the binding to proteins on a cell's surface and in fusion of the viral particle with the cell, while the stalk domain of HA has a structural role in placing the head domain in space away from the viral membrane.

A recombinant fusion protein is a protein created through genetic engineering of a fusion gene. This typically involves removing the stop codon from a cDNA sequence coding for the first protein, then appending the cDNA sequence of the second protein in frame through ligation or overlap extension PCR. That DNA sequence will then be expressed by a cell as a single protein. The protein can be engineered to include the full sequence of both original proteins, or only a portion of either, or only a portion of both.

To create an antigenic HA that consists of the conserved stalk domain in the absence of the variable head domain, the head domain is removed from the HA sequence, resulting in hrHA. In order to maintain the overall structural integrity of HA in the absence of the head domain, the head domains in HA are preferably replaced with linkers (or "spacers") that do not affect the secondary structure of the HA protein. For example, the head domain can be replaced by a linker 3 to 5 amino acids in length that do not form a fixed secondary structure. In some embodiments, the linker comprises 3 to 5 amino acids selected from glycine, alanine, and serine. In particular embodiments, the linker is selected from the group consisting of GGG, GGGG (SEQ ID NO:16), GGGGG (SEQ ID NO:17), and GGGGC (SEQ ID NO:18), GGGSS (SEQ ID NO:19).

In some embodiments, the hrHA polypeptide comprises the transmembrane and cytoplasmic domains of HA. In some embodiments, the polypeptide lacks the HA transmembrane and/or cytoplasmic domains of HA, and instead the polypeptide comprises a heterologous membrane-anchoring sequence. For example, the heterologous membrane-anchoring sequence can be a glycosylphosphatidylinositol (GPI) membrane-anchoring sequence. In some embodiments, the hrHA polypeptide lacks the HA transmembrane and/or cytoplasmic domain and consists only of the ectodomain or some proportion of the ectodomain.

In some embodiments, the hrHA polypeptide is a stand-alone protein. In some embodiments, the hrHA polypeptide is a stand-alone protein that is not fused to another protein or protein domain. In some embodiments, the hrHA polypeptide is a domain that is fused together with another protein or protein domain to form a fusion protein. In some embodiments, the hrHA polypeptide forms a fusion protein with an oligomerization domain. In some embodiments, the hrHA polypeptide forms a fusion protein with a GCN4 oligomerization domain. In some embodiments, the hrHA polypeptide forms a fusion protein with a GCN4 oligomerization domain that forms trimers. In some embodiments, the hrHA polypeptide forms a fusion protein with a GCN4 oligomerization domain that forms dimers or tetramers.

Also disclosed is a nanoparticle that is coated with a disclosed hrHA polypeptide. In some cases, the hrHA polypeptide is crosslinked to a polymer nanoparticle surface. In emboidments, the hrHA polypeptide is absorbed onto the nanoparticle surface. In some embodiments, the hrHA polypeptide is absorbed onto the nanoparticle surface and then crosslinked to the nanoparticle surface. In some embodiments, the hrHA polypeptide is encapsulated into the nanoparticle. In particular embodiments, the nanoparticle is formed from a biocompatible polymer. Examples of biocompatible polymers include polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, or polyamines, or combinations thereof. In some cases, the nanoparticle is formed from a polyethylene glycol (PEG), poly(lactide-co-glycolide) (PLGA), polyglycolic acid, poly-beta-hydroxybutyrate, polyacrylic acid ester, or a combination thereof. In embodiments, the nanoparticle is formed from polypeptides. In some embodiments, the nanoparticle is formed by desolvation of polypeptides. In some embodiments, the nanoparticle is formed by desolvation of polypeptides into protein aggregates. In some embodiments, the nanoparticle is formed by desolvation of polypeptides into protein aggregates with defined physicochemical characteristics that are directly determined by the parameters of the treatment methods used for desolvation.

Virus Like Particles (VLPs)

Also disclosed is a composition comprising a virus like particle (VLP) expressing on its surface a disclosed hrHA polypeptide. For example, the disclosed fusion proteins may be incorporated into virus-like particles (VLPs) by including within the fusion protein a membrane anchor domain, such as a transmembrane domain and optional cytoplasmic domain of a viral envelope protein.

Non-replicating VLPs resemble infectious virus particles in structure and morphology, and contain immunologically relevant viral structural proteins. VLPs have been produced from both non-enveloped and enveloped viruses. Envelopes of VLPs are derived from the host cells similar to the way as enveloped viruses such as influenza A virus obtain their lipid envelopes from their host cells. Therefore, membrane-anchored proteins on the surfaces of enveloped viruses will be expressed in a native-like conformation if they are expressed in a membrane-anchored form.

Influenza VLPs involve lipid bilayers and host cell membrane proteins (Song, J. M., et al. J Proteome Res 2011 10:3450-3459). For example, Influenza VLPs containing the wild type M2 protein have been described (Song, J. M., et al. Proc Natl Acad Sci U S A 2011

108:757-761; Song, J. M., et al. PLoS One 2011 6:e14538). Enveloped VLPs may be composed of influenza matrix 1 (M1) protein as a particle forming core. These VLPs are produced, for example, by coinfecting insect cells with one or more recombinant baculoviruses co-expressing M1 proteins and the disclosed fusion proteins, culturing the insect cells under physiological conditions, and purifying the VLPs from insect cell culture supernatants.

Nanoparticle

A nanoparticle is a particle between 1 and 100s of nanometers in size. A nanoparticle can be natural or synthetic. A nanoparticle can be created from biological molecules or from abiological molecules. A nanoparticle has a core containing the material of which the nanoparticle consists. A nanoparticle has a surface that is the interface between the core and the space and/or solution outside of the core. A protein nanoparticle (PNP) is a particle in which the core material consists of protein. PNP can be synthesized in numerous ways. For example, PNP can be synthesized by desolvation of a solution of soluble protein, resulting in precipitation of particulate PNP. PNP can be synthesized by crosslinking proteins to form the particle. PNP can be synthesized by biochemical aggregation based on autonomouns protein-protein interactions. PNP can be formed by a combination of any of these PNP synthesis methods.

Disclosed herein is a nanotechnology approach to produce a nanocluster PNP. In one embodiment, the PNP consists of a protein antigen that can act as a target of an immune response. In one embodiment, the PNP can be used as an active pharmaceutical ingredient in a vaccine. In one embodiment, the PNP is an uncoated particle. In one embodiment, the PNP is coated with another protein antigen on its surface. In one embodiment, the PNP formed by desolvating the core protein with a desolvating agent and/or crosslinking a core protein with a crosslinking agent. Both or either of these procedures can be used to make double-layered nanoparticles. In some cases, the nanoparticle contains a core structure formed by desolvation and a coating formed by crosslinking of a surface antigen. Suitable desolvating agents include, for example, ethanol, acetone, or combinations thereof.

A humoral immune response is preferentially mounted against proteins, domains, and epitopes that are displayed in a repetitive and/or iterative fashion. Forming a PNP from a protein results in a particle with many repetitive epitopes on the surface of the PNP. In this way, formation of a PNP from a soluble protein results in a substance that is much more highly antigenic for initiating and/or promoting an immune response than the soluble protein alone and yet consists of biological substituents that are identical or nearly-identical to the

soluble protein alone. Thus formation of PNP from a soluble protein results in a composition that much more readily promotes humoral immune responses to that protein.

It is possible to modify a PNP consisting of a core protein by adding a coat of a second protein onto the surface of the PNP. This method leverages the same principle of promoting effective humoral immune responses toward repetitive epitopes yet has the benefit of directing an additional humoral immune response against the second protein that coats the PNP.

The core protein is preferably a protein with a strong T cell epitope. In some embodiments, the core protein comprises conserved T cell epitopes from influenza NP, M1, or a combination thereof. In some embodiments, the core protein comprises one or more influenza virus matrix protein 2 (M2) domains. In some embodiments, the core protein comprises one or more influenza virus matrix protein 2 extracellular (M2e) domains. In some embodiments, the core protein is a fusion protein comprising a series of 2, 3, 4, 5, 6, 7, or 8 M2e domains, optionally linked to a multimerization domain.

In some embodiments, the core protein comprises two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) different influenza M2e domains (i.e., heterologous M2e domains). In some embodiments, the core protein comprises two or more homologous influenza M2e domains. In some embodiments, the core protein comprises a mixture of two or more homologous and/or heterologous influenza M2e domains. The core protein can contain heterologous M2e domains to increase antigenicity and cross-protection. By taking into account the mechanisms of effective T cell responses against immunologically active antigens, the use of heterologous M2e is herein purposely designed to increase immune cross-protection. In one embodiment, the core protein can contain one or more M2e domains from a human influenza, one or more M2e domains from a swine influenza, and one or more M2e domains from an avian influenza. As an example, the core protein can contain the following five influenza M2e sequences: 2x Human, 1x Swine, 1x Avian Type I, and 1x Avian Type II.

In one embodiment, the core protein can contain one or more M2e domains from a human influenza A subtype, one or more M2e domains from a swine influenza A subtype, and one or more M2e domains from an avian influenza A subtype. As an example, the core protein can contain the following five influenza A virus subtype M2e sequences: 2x Human, 1x Swine, 1x Avian Type I, and 1x Avian Type II.

In one embodiment, the core protein can contain one or more M2e domains from a human influenza B subtype, one or more M2e domains from a swine influenza B subtype, and one or more M2e domains from an avian influenza B subtype. As an example, the core

protein can contain the following five influenza B virus subtype M2e sequences: 2x Human, 1x Swine, 1x Avian Type I, and 1x Avian Type II.

Methods and compositions disclosed herein for the sequence of any particular M2e are applicable to the sequences of other M2e. Methods and compositions from any of the particular M2e delineated in this specification may be adapted routinely to another M2e for those skilled in the art. Thus, in one embodiment, a sequence for influenza A M2e is used. In one embodiment, a sequence for influenza B M2e is used. In one embodiment, a combination of sequences for influenza A M2e and influenza B M2e are used.

In some embodiments, the human M2e sequence comprises the amino acid sequence PIRNEWGSRSN (SEQ ID NO:21), or a conservative variant thereof having at least about 70%, 80%, or 90% sequence identity to SEQ ID NO:21 (i.e., one, two, or three conservative amino acid substitutions). For example, human M2e isolates H1N1 (A/PR8, A/NC/99) and H3N2 (A/Phil/82) have the amino acid sequence SLLTEVET PIRNEWGSRSN DSSD (SEQ ID NO:22).

In some embodiments, the swine M2e sequence comprises the amino acid sequence PTRSEWESRSS (SEQ ID NO:23), or a conservative variant thereof having at least 70%, 80%, or 90% sequence identity to SEQ ID NO:23. For example, swine M2e isolates from the 2009 H1N1 pandemic (A/California/4/2009) have the amino acid sequence SLLTEVET PTRSEWESRSS DSSD (SEQ ID NO:24).

In some embodiments, the avian M2e sequence (referred to herein as “avian type I”) comprises the amino acid sequence PTRX_1X_2WESRSS (SEQ ID NO:25), wherein X_1 is N, H, or K, wherein X_2 is E or G, or a conservative variant thereof having at least 70%, 80%, or 90% sequence identity to SEQ ID NO:25. For example, avian type I M2e isolates from H5N1 (A/Vietnam/1203/04, A/Indonesia/05, A/mandarin/kr/2010, A/ck/kr/2006) have the amino acid sequence SLLTEVET PTRNEWESRSS DSSD (SEQ ID NO:26). Avian type I M2e isolates from H7N3 (A/dk/Kr/2007), H9N2 (A/ck/Kr/2012) have the amino acid sequence SLLTEVET PTRNGWECRCS DSSD (SEQ ID NO:27). Avian type I M2e isolates from H5N1 (A/ck/Kr/Gimje/2008) have the amino acid sequence SLLTEVET PTRHEWECRCS DSSD (SEQ ID NO:28). Avian type I M2e isolates from H5N1 (A/ck/Vietnam/2011) have the amino acid sequence SLLTEVET PTRKEWECRCS DSSD (SEQ ID NO:29).

In some embodiments, the avian M2e sequence (referred to herein as “avian type II”) comprises the amino acid sequence LTRNGWGCRCS (SEQ ID NO:30), or a conservative variant thereof having at least 70%, 80%, or 90% sequence identity to SEQ ID NO:30. For example, avian type II M2e isolates from H5N1 (A/HK/156/97), H9N2 (A/HK/1073/99) have the amino acid sequence SLLTEVET LTRNGWGCRCS DSSD (SEQ ID NO:31).

The core protein may further comprise a signal peptide at the N-terminus to facilitate secretion. For example, the core protein may contain a mellitin signal peptide. In some embodiments, the melittin signal peptide has the amino acid sequence MKFLVNVALVFMVVYISYIYADPINMT (SEQ ID NO:32), or a conservative variant thereof having at least 72%, 76%, 80%, 84%, 88%, 92%, or 96% sequence identity to SEQ ID NO:32. Alternatively, the fusion protein may contain a baculovirus gp64 signal peptide (MVSAIVLYVLLAAAAHSAFA, SEQ ID NO:33) or a chitinase signal peptide (MPLYKLLNVLWLVAVSNAIP, SEQ ID NO:34) (Wang, B., et al. J Virol 2007 81: 10869-10878), or a conservative variant thereof having at least 70%, 75%, 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:33 or SEQ ID NO:34.

Influenza M2 is naturally a homotetramer. The M2e domain itself lacks the sequences for naturally forming a homotetramer. Therefore, in some embodiments, the fusion protein containing M2e also contains a multimerization domain, such as a tetramerization domain. An example of a suitable tetramerization domain includes a GCN4 (a leucine zipper tetramerization motif found in yeast proteins). For example, the GCN4 domain can have the amino acid sequence GGLKQIEDKLEEILSKLYHIENELARIKKLLGE (SEQ ID NO:35), or a conservative variant thereof having at least 70%, 73%, 76%, 79%, 82%, 85%, 88%, 91%, 94%, or 97% sequence identity to SEQ ID NO:36. In particular embodiments, the M2e fusion protein contains a GCN4 tetramerization domain. Other identified tetramerization domains include tetrabrachion protein, tumor suppressor p53 tetramerization domain, C-terminal 40-residue peptide of the AChE (tryptophan amphiphilic tetramerization (WAT) domain), erythrocyte spectrin tetramerization domain, and Mnt repressor tetramerization domain. In some embodiments, the M2e fusion protein contains one or more of these other identified tetramerization domains.

In some embodiments, the series of M2e domains are linked to the N-terminus of the multimerization domain. In some embodiments, the series of M2e domains are linked to the C-terminus of the multimerization domain. In some embodiments, the core protein comprises a series of 2 to 8 M2e domains linked to the N-terminus of the multimerization domain and/or a series of 2 to 8 M2e domains linked to the C-terminus of the multimerization domain.

In some embodiments, the core protein further comprises influenza neuraminidase (NA) protein linked to the multimerization domain. In some embodiments, the series of 2 to 8 M2e domains are linked to the N-terminus of the multimerization domain and/or the NA protein is linked to the C-terminus of the multimerization domain.

The M2e domains can be linked to each other by a flexible linker. Likewise, in some embodiments, the multimerization domain is linked to the M2e domains, the NA protein, or

any combination thereof, by a flexible linker. Suitable flexible linkers can be, for example, a peptide having 3, 4, 5, 6, 7, 8, or 9 amino acid selected from glycine, alanine, and serine. For example, the flexible linker can have the amino acid sequence GGSGGG (SEQ ID NO:20).

The disclosed nanoparticle can be used by itself, or it can be coated with another antigen, such as an influenza antigen. This nanoparticle can in some embodiments be coated with the disclosed hrHA polypeptide. In some embodiments, the antigen is coated on the nanoparticle using a crosslinking agent. In some embodiments, the antigen is absorbed onto the nanoparticle surface. In some embodiments, the antigen is absorbed onto the nanoparticle surface followed by covalent crosslinking of the antigen to the nanoparticle surface using a crosslinking agent.

Crosslinking agents suitable for crosslinking the core protein to produce the nanoparticle, or to coat an antigen on the nanoparticle are known in the art, and include those selected from the group consisting of formaldehyde, formaldehyde derivatives, formalin, glutaraldehyde, glutaraldehyde derivatives, a protein cross-linker, a nucleic acid cross-linker, a protein and nucleic acid cross-linker, primary amine reactive crosslinkers, sulfhydryl reactive crosslinkers, sulfydryl addition or disulfide reduction, carbohydrate reactive crosslinkers, carboxyl reactive crosslinkers, photoreactive crosslinkers, cleavable crosslinkers, AEDP, APG, BASED, BM(PEO)3, BM(PEO)4, BMB, BMDB, BMH, BMOE, BS3, BSOCOES, DFDNB, DMA, DMP, DMS, DPDPB, DSG, DSP, DSS, DST, DTBP, DTME, DTSSP, EGS, HBVS, sulfo-BSOCOES, Sulfo-DST, and Sulfo-EGS.

Vaccine Compositions

Disclosed are vaccine compositions that comprise one or more of the polypeptides or nanoparticles described above. Although not required, the vaccine compositions optionally contain one or more immunostimulants. An immunostimulant refers to essentially any substance that enhances or potentiates an immune response (antibody or cell-mediated) to an exogenous antigen. One preferred type of immunostimulant is an adjuvant.

Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis*- or *Mycobacterium tuberculosis*-derived proteins. The adjuvant may be a submicron oil-in-water emulsion of a metabolizable oil and an emulsifying agent. For example, the adjuvant may comprise MF59™, which is a sub-micron oil-in-water emulsion of a squalene, polyoxyethylene sorbitan monooleate (Tween™ 80) and sorbitan trioleate. The adjuvant may also be a combination of the TLR4 agonist MPL (3-O-desacyl-4′-monophosphoryl lipid A) and aluminum salt, e.g., AS04 (GlaxoSmithKline, Philadelphia, Pa.).

Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Mich.); Merck Adjuvant 65 (Merck and Company, Rahway, N.J.); AS01, AS02, AS03, and AS04 (GlaxoSmithKline, Philadelphia, Pa.); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, may also be used as adjuvants.

The adjuvant composition can be a composition that induces an anti-inflammatory immune response (antibody or cell-mediated). Accordingly, high levels of anti-inflammatory cytokines are produced as a result of adjuvant administration. Anti-inflammatory cytokines may include, but are not limited to, interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 10 (IL-10), and transforming growth factor beta (TGFβ).

The adjuvant composition can be a composition that induces an inflammatory immune response (antibody or cell-mediate). Accordingly, high levels of inflammatory cytokines are produced as a result of adjuvant administration. Inflammatory cytokines may include, but are not limited to, interleukin 1 alpha (IL-α), interleukin 1 beta (IL-ß), interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα). Optionally, an inflammatory response would be mediated by CD4+ T helper cells. Bacterial flagellin has been shown to have adjuvant activity (McSorley *et al.*, J. Immunol. 169:3914-19, 2002). Also disclosed are polypeptide sequences that encode flagellin proteins that can be used in adjuvant compositions.

Optionally, the adjuvants increase lipopolysaccharide (LPS) responsiveness. Illustrative adjuvants include but are not limited to, monophosphoryl lipid A (MPL), aminoalkyl glucosaminide 4-phosphates (AGPs), including, but not limited to RC-512, RC-522, RC-527, RC-529, RC-544, and RC-560 (Corixa, Hamilton, Mont.).

In addition, the adjuvant composition can be one that induces an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (e.g., IFN-γ, TNFα, IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (e.g., IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a subject will support an immune response that includes Th1- and Th2-type responses. Optionally, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. Certain adjuvants for eliciting a predominantly Th1-type response include,

for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with an aluminum salt adjuvants are available from Corixa Corporation (Seattle, Wash.). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Another adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, Mass.); Escin; Digitonin; or Gypsophila or Chenopodium quinoa saponins.

Additional illustrative adjuvants for use in the disclosed vaccine compositions include Montamide ISA 720 (Seppic, France), SAF (Chiron, Calif., United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (e.g., SBAS-2 or SBAS-4, available from GlaxoSmithKline, Philadelphia, Pa.), Detox (Enhanzyn™) (Corixa, Hamilton, Mont.), RC-529 (Corixa, Hamilton, Mont.) and other aminoalkyl glucosaminide 4-phosphates (AGPs).

In some embodiments, the adjuvant is a virosome (e.g. Berna Biotech). In some embodiments, the adjuvant comprises a CpG 1018 and/or CpG 7909 oligonucleotide. In some embodiments, the adjuvant comprises a Imidazoquinoline. In some embodiments, the adjuvant comprises a Polyinosinic:polycytidylic acid (Polyl:C). In some embodiments, the adjuvant comprises a Pam3Cys. In some embodiments, the adjuvant comprises a ISCOMATRIX adjuvant. In some embodiments, the adjuvant comprises a CAF01 and/or IC31 adjuvant. In some embodiments, the adjuvant comprises a Sigma adjuvant system (MPL from Salmonella Minnesota, synthetic trehalose dicorynomycolate and squalene oil). In some embodiments, the adjuvant comprises TITERMAX (water-in-oil emulsion, consisting of squalene, sorbitan monooleate 80, a block copolymer and microparticulate silica).

In some embodiments, the adjuvant is incorporated into the VLP in a membrane-anchored form. For example, GM-CSF or a bacterial flagellin protein containing a membrane anchor can be incorporated into the disclosed VLPs.

Pharmaceutical Compositions

The disclosed vaccines can be used therapeutically in combination with a pharmaceutically acceptable carrier. By “pharmaceutically acceptable” is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). Suitable carriers and their formulations are described in Remington: The Science and Practice of Pharmacy (22nd ed.) eds. Loyd V. Allen, Jr., et al., Pharmaceutical Press, 2012. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of vaccines to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the vaccine. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like.

The disclosed vaccines are preferably formulated for delivery via intranasal, intramuscular, subcutaneous, transdermal or sublingual administration.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer’s dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Combinations

The disclosed vaccine can be used to supplement existing human vaccines to improve cross protection. Therefore, the disclosed vaccine can further include (or be administered in combination with) a whole inactivated virus, split viral vaccine, live attenuated influenza vaccine, or an influenza virus-like particle (VLP) vaccine. For example, the disclosed vaccine can be combined with a trivalent inactivated vaccine (TIV) (e.g., containing killed A/H1N1, A/H3N2, and B), trivalent live attenuated influenza vaccine, trivalent split vaccine, trivalent subunit influenza vaccine, trivalent recombinant protein vaccine, or trivalent VLP vaccine. The disclosed vaccine can be combined with a bivalent inactivated vaccine, bivalent live attenuated influenza vaccine, bivalent split vaccine, or bivalent subunit influenza vaccine, bivalent recombinant protein vaccine, or bivalent VLP vaccine. The disclosed vaccine can be combined with a monovalent inactivated vaccine, monovalent live attenuated influenza vaccine, monovalent split vaccine, or monovalent subunit influenza vaccine, monovalent recombinant protein vaccine, or monovalent VLP vaccine. The disclosed vaccine can be combined with a monovalent, bivalent, or trivalent vaccine directed against Influenza A. The disclosed vaccine can be combined with a monovalent, bivalent, or trivalent vaccine directed against Influenza B. The disclosed vaccine can be combined with a monovalent, bivalent, or trivalent vaccine directed against a combination of Influenza A and Influenza B.

The disclosed vaccine can include a PNP that incorporates sequences from a particular influenza, such as Influenza A or Influenza B. The disclosed vaccine can include a PNP that incorporates sequences from a particular strain of influenza. The disclosed vaccine can include a PNP that incorporates sequences from a subtype of influenza A. The disclosed vaccine can include a PNP that incorporates sequences from a subtype of influenza A HA, such as H1 HA, H3 HA, H5 HA, and others.

In one embodiment, the vaccine includes a single PNP that incorporates sequences from a particular influenza, such as Influenza A or Influenza B. The disclosed vaccine can

include a single PNP that incorporates sequences from a particular strain of influenza. The disclosed vaccine can include a single PNP that incorporates sequences from a subtype of influenza A. The disclosed vaccine can include a single PNP that incorporates sequences from a subtype of influenza A HA, such as H1 HA, H3 HA, H5 HA, and others.

In one embodiment, the vaccine includes two PNP each of which incorporates sequences from particular influenzas, such as Influenza A or Influenza B. The disclosed vaccine can include two PNP each of which incorporates sequences from a particular strain of influenza. The disclosed vaccine can include two PNP each of which incorporates sequences from a subtype of influenza A. The disclosed vaccine can include two PNP each of which incorporates sequences from a subtype of influenza A HA, such as H1 HA, H3 HA, H5 HA, and others.

In one embodiment, the vaccine includes three or more PNP each of which incorporates sequences from particular influenzas, such as Influenza A or Influenza B. The disclosed vaccine can include three or more PNP each of which incorporates sequences from a particular strain of influenza. The disclosed vaccine can include three or more PNP each of which incorporates sequences from a subtype of influenza A. The disclosed vaccine can include three or more PNP each of which incorporates sequences from a subtype of influenza A HA, such as H1 HA, H3 HA, H5 HA, and others.

The disclosed vaccine can further include (or be administered in combination with) one or more of classes of antibiotics, steroids, analgesics, anti-inflammatory agents, anti-histaminic agents, or any combination thereof. Antibiotics include Aminoglycosides, Cephalosporins, Chloramphenicol, Clindamycin, Erythromycins, Fluoroquinolones, Macrolides, Azolides, Metronidazole, Penicillins, Tetracyclines, Trimethoprim-sulfamethoxazole, and Vancomycin. Suitable steroids include andranes, such as testosterone. Narcotic and non-narcotic analgesics include morphine, codeine, heroin, hydromorphone, levorphanol, meperidine, methadone, oxydone, propoxyphene, fentanyl, methadone, naloxone, buprenorphine, butorphanol, nalbuphine, and pentazocine. Anti-inflammatory agents include alclofenac, alclometasone dipropionate, algestone acetonide, alpha amylase, amcinafal, amcinafide, amfenac sodium, amiprilose hydrochloride, anakinra, anirolac, anitrazafen, apazone, balsalazide disodium, bendazac, benoxaprofen, benzydamine hydrochloride, bromelains, broperamole, budesonide, carprofen, cicloprofen, cintazone, cliprofen, clobetasol propionate, clobetasone butyrate, clopirac, cloticasone propionate, cormethasone acetate, cortodoxone, decanoate, deflazacort, delatestryl, depo-testosterone, desonide, desoximetasone, dexamethasone dipropionate, diclofenac potassium, diclofenac sodium, diflorasone diacetate, diflumidone sodium, diflunisal,

difluprednate, diftalone, dimethyl sulfoxide, drocinonide, endrysone, enlimomab, enolicam sodium, epirizole, etodolac, etofenamate, felbinac, fenamole, fenbufen, fenclofenac, fenclorac, fendosal, fenpipalone, fentiazac, flazalone, fluazacort, flufenamic acid, flumizole, flunisolide acetate, flunixin, flunixin meglumine, fluocortin butyl, fluorometholone acetate, fluquazone, flurbiprofen, fluretofen, fluticasone propionate, furaprofen, furobufen, halcinonide, halobetasol propionate, halopredone acetate, ibufenac, ibuprofen, ibuprofen aluminum, ibuprofen piconol, ilonidap, indomethacin, indomethacin sodium, indoprofen, indoxole, intrazole, isoflupredone acetate, isoxepac, isoxicam, ketoprofen, lofemizole hydrochloride, lomoxicam, loteprednol etabonate, meclofenamate sodium, meclofenamic acid, meclorisone dibutyrate, mefenamic acid, mesalamine, meseclazone, mesterolone, methandrostenolone, methenolone, methenolone acetate, methylprednisolone suleptanate, momiflumate, nabumetone, nandrolone, naproxen, naproxen sodium, naproxol, nimazone, olsalazine sodium, orgotein, orpanoxin, oxandrolane, oxaprozin, oxyphenbutazone, oxymetholone, paranyline hydrochloride, pentosan polysulfate sodium, phenbutazone sodium glycerate, pirfenidone, piroxicam, piroxicam cinnamate, piroxicam olamine, pirprofen, prednazate, prifelone, prodolic acid, proquazone, proxazole, proxazole citrate, rimexolone, romazarit, salcolex, salnacedin, salsalate, sanguinarium chloride, seclazone, sermetacin, stanozolol, sudoxicam, sulindac, suprofen, talmetacin, talniflumate, talosalate, tebufelone, tenidap, tenidap sodium, tenoxicam, tesicam, tesimide, testosterone, testosterone blends, tetrydamine, tiopinac, tixocortol pivalate, tolmetin, tolmetin sodium, triclonide, triflumidate, zidometacin, and zomepirac sodium. Anti-histaminic agents include ethanolamines (e.g., diphenhydrmine carbinoxamine), Ethylenediamine (e.g., tripelennamine pyrilamine), Alkylamine (e.g., chlorpheniramine, dexchlorpheniramine, brompheniramine, triprolidine), other anti-histamines like astemizole, loratadine, fexofenadine, bropheniramine, clemastine, acetaminophen, pseudoephedrine, triprolidine).

Methods of Vaccinating a Subject

A method of vaccinating a subject for influenza is disclosed that involves administering the disclosed cross-protective influenza vaccine to a subject in need thereof. The disclosed vaccine may be administered in a number of ways. For example, the disclosed vaccine can be administered intramuscularly, intranasally, or by microneedle in the skin. The compositions may be administered orally, intravenously, subcutaneously, transdermally (e.g., by microneedle), intraperitoneally, ophthalmically, vaginally, rectally, sublingually, or by inhalation.

Parenteral administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or

suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained.

The exact amount of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. For example, effective dosages and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms disorder are affected. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. A typical dosage of the disclosed vaccine used alone might range from about 1 μg/kg to up to 100 mg/kg of body weight or more per vaccination, such as 10 μg/kg to 50 mg/kg, or 50 μg/kg to 10 mg/kg, depending on the factors mentioned above. In addition to dosing by the ratio of mass-of-vaccine to mass-of-patient, standardized vaccine doses for demarcated demographics can also be used. A typical dose for an adult patient may be 1μg to 1000μg, or 10μg to 150μg, or 15μg to 135μg per subject. A typical dose for a child patient may be 1μg to 1000μg, or 10μg to 150μg, or 15μg to 135μg per subject. A typical dose for an elderly patient may be 1μg to 1000μg, or 10μg to 150μg, or 15μg to 135μg per subject.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

EXAMPLES

Example 1:

Results

Design and construction of four tandem copies of M2e linked to tetramerization motif GCN4

Tetrameric four tandem copies of M2e construct was stabilized by introducing a foreign tetramerization motif GCN4 at C-terminal end (4M2e-GCN4), a modified version of leucine zipper domain of a yeast transcription factor (De Filette M, et al. The Journal of biological chemistry 2008; 283(17): 11382-11387; Harbury PB, et al. Science 1993; 262(5138): 1401-1407) (Fig. 1A). A signal peptide encoding sequence from the honeybee melittin protein was added at N-terminal to facilitate protein expression in insect cells *Spodoptera frugiperda* Sf9 (Sf9, ATCC, CRL-1711) (Wang BZ, et al. Journal of virology 2007; 81(20): 10869-10878). Hexa-Histidine tag were added following GCN4 motif sequence. In four types of full length M2e, two site-mutations C17S and C19S were made, the order of modified M2e sequences from N- to C- terminal in 4M2e-GCN4 construct were: human consensus M2e, swine consensus M2e, avian consensus M2e, domestic fowl consensus M2e (Table 1). The consensus M2e sequences were made based on 11732 human, 5920 swine, 6267 avian and 3270 domestic fowl M2 sequences deposited in the National Center for Biotechnology Information (NCBI) databank, and the software molecular evolutionary genetics analysis version 6.0 (MEGA6) was used to align and analyze sequences (Tamura K, et al. Molecular biology and evolution 2013; 30(12): 2725-2729).

Design and construction of head-removed HA constructs

Conserved stalk region of HA offers a promising target to develop cross-protective influenza A vaccines (Yassine HM, et al. Nature medicine 2015; 21(9): 1065-1070; Impagliazzo A, et al. Science 2015; 349(6254): 1301-1306). It was previously reported that HA stalk domain is more conserved compared to the highly variable globular head domain (Mallajosyula VV, et al. Proc Natl Acad Sci USA 2014; 111(25): E2514-2523; Ellebedy AH, et al. Frontiers in immunology 2012; 3: 53). The goal is to design stable and soluble trimerized head-removed H1 (hrH1) and head-removed H3 (hrH3) proteins with part of receptor-binding polypeptide chain (termed HA1) at stalk region and ectodomain of membrane anchoring polypeptide chain of HA (termed HA2) (Fig. 1B, 1C; sequences in Figs. 11A, 11B). Previous research demonstrated the metastable HA2 conformation in viral HA and also showed that the trimer HA2 polypeptide when expressed independently from HA1 subunit, adopted the low-pH conformation spontaneously (Chen J, et al. Proc Natl Acad Sci USA 1995; 92(26): 12205-12209). The influenza A subviral particles lacking the entire

HA1 subunit of HA showed limited protection in mice model (Graves PN, et al. Virology 1983; 126(1): 106-116). To generate one hrHA representative from each phylogenetic group of influenza A virus will be essential to formulate a vaccine that can elicit the real universal response. Starting with the HA sequences of H1N1 A/Puerto Rico/8/1934 and H3N2 A/Aichi/2/1968, changes were introduced using a series of rational designs. According to previous results, trimerization motifs facilitate HA oligomerization in the absence of the HA transmembrane domain (Mallajosyula VV, et al. Proc Natl Acad Sci USA 2014; 111(25): E2514-2523; Yassine HM, et al. Nature medicine 2015; 21(9): 1065-1070; Kanekiyo M, et al. Nature 2013; 499(7456): 102-106; Stevens J, et al. Science 2006; 312(5772): 404-410) . A C-terminal sequence containing G6S3 or PGS linker, tetramerization motif GCN4 and hexa-Histidine tag were added following hrH1 or hrH3 sequence for oligomerization and purification purposes. The globular head domain was removed at similar cutting off positions in three-dimensional structure as reported previously (Impagliazzo A, et al. Science 2015; 349(6254): 1301-1306). The coding sequence of the major head domain of H1 (amino acids S53-P320) and the major head domain of H3 (amino acids S61-P322) were replaced with a linker sequence encoding four glycines (G4) which is predicted to be a flexible linker and not disrupt the folding of the remainder of the molecule (Steel J, et al. mBio 2010; 1(1)). To interrupt the conformational transfer at low pH value, the residues between F61 and L89 in HA2 of H1 and the residues between F63 and T87 in HA2 of H3 were replaced with flexible non-hydrophobic G4S2 linker. The F63 and V73 hydrophobic residues in HA2 are largely responsible for stabilizing the coiled coil of the low pH structure (Bommakanti G, et al. Proc Natl Acad Sci USA 2010; 107(31): 13701-13706; Bajaj K, et al. Proc Natl Acad Sci USA 2005; 102(45): 16221-16226). In the neutral pH conformation, these residues form a part of loop B connecting helix A and helix C and are exposed in head removed HA structure. To further stabilize the hrH3 construct, a presumed intra-disulfide bond was introduced in H3 by site-mutagenesis at V325C in HA1 and S91C in HA2. Head removed HA constructs were made in the context of a H1 (representative of group 1) and a H3 (representative of group 2) HA, as the cross-reactivity of neutralizing antibodies targeting the stalk region appears to be limited to HA subtypes within the same major phylogenetic group (Wang TT, et al. PLoS pathogens 2010; 6(2): e1000796; Tan GS, et al. Journal of virology 2014; 88(23): 13580-13592; Okuno Y, et al. Journal of virology 1993; 67(5): 2552-2558; Krammer F, et al. Journal of virology 2013; 87(12): 6542-6550; Ekiert DC, et al. Science 2009; 324(5924): 246-251; Kashyap AK, et al. Proc Natl Acad Sci USA 2008; 105(16): 5986-5991; Sui J, et al. Nature structural & molecular biology 2009; 16(3): 265-273).

Characterization of recombinant 4M2e-GCN4 and head-removed HA proteins

Recombinant 4M2e-GCN4 and head-removed HA proteins were expressed and purified from recombinant baculovirus protein expression system. BS3 crosslinked recombinant protein and uncrosslinked recombinant protein were run in SDS-PAGE and stained with Commassie blue. As shown in Fig 2A and 2B, the BS3 crosslinked 4M2e-GCN4 protein showed its tetramerization size, BS3 crosslinked hrHA protein showed its trimerization size. The antigenicity of hrH3 and hrH1 were analyzed using sandwich ELISA with monoclonal antibody 12D1 and C179, respectively. The data demonstrate that 12D1 recognizes a conformation epitope that is well presented on recombinant hrH3 protein and that the HA2 region in hrH3 is folded in a similar fashion to the corresponding region in the neutral pH structure of full length H3 protein (Fig 2C). The data also show that recombinant hrH1 protein is recognized well by C179, as evidenced by the binding results in sandwich ELISA (Fig 2D).

Characterization of fabricated protein nanoparticles

It was previously reported that protein nanoparticle vaccines were formed by a modified desolvation process (Wang L, et al. Nanomedicine : nanotechnology, biology, and medicine 2017; 13(1): 253-262; Wang L, et al. Nanomedicine : nanotechnology, biology, and medicine 2014; 10(2): 473-482; Chang TZ, et al. Biomaterials science 2017; 5(2): 223-233) and stabilized by DTSSP crosslinking. This approach generated protein nanoparticles entirely composing antigens of interest, and do not include any vehicles or vectors. We recently found that desolvated H7 PNP displayed low ability in hemagglutination of chicken red blood cells and long-time contact with solvent ethanol may result in partial loss of intact structure, however the surface coating of H7 protein played the major role in immunogenicity (Wang L, et al. Nanomedicine : nanotechnology, biology, and medicine 2017; 13(1): 253-262). M2e is regarded as a flexible linear epitope which is able to induce broadly protective immune responses. It is presumed that the conformational change of M2e does not dramatically alter its immunogenicity. To further improve the immunogenicity of hrHA, we generated 4M2e-GCN4 core PNPs by desolvation and coated core with hrHA. Fig 3A showed the schematic diagram of 4M2e-GCN4 core PNP fabrication process. We found the use of DTSSP to stabilize the PNP while desolvation could vary the size of PNP by different DTSSP amounts. The size of PNP become larger by increasing DTSSP amounts (Fig 3B). Therefore, to minimize the size of PNP, the core PNP were not crosslinked until the addition of hrHA in the system. The size distribution analysis using zeta-sizer showed the fabricated Uni4MC PNP peaked at 141 nm (Fig 3C). The soluble 4M2e-GCN4 and Uni4MC PNP were analyzed in Western Blot using mouse anti-M2e antiserum. The PNP can be completely denatured into monomer and all samples can be blotted (Fig 3D). Scanning electron

microscopy (SEM) of dehydrated nanoparticles showed that particulates were relatively spherical (Fig 3E).

The coating process is showed in schematic diagram in Fig 4A. As the binding is mainly mediated via hydrophobic residues interaction. The hydrophobic residues in hrH1 and hrH3 proteins were showed in red (Fig 4B). The DTSSP crosslinker reacted mainly with primary amine in protein, the Lys residues with outwards primary amine group in hrH1 and hrH3 were labelled as spherical molecule in red (Fig 4C). The analysis showed the theoretical feasibility in hrHA protein coating and stabilization by DTSSP crosslinking. Dynamic light scattering analysis indicated the presence of a single, reproducible population distribution with an average hydrodynamic diameter of 181 nm of hrH1 and 211 nm of hrH3 (Fig 5A and 5B). The Commassie blue staining showed that the Uni4MC core PNPs were successfully coated with hrH1 or hrH3 (Fig 5C). The protein band intensity analysis of Uni4C1 and Uni4C3 using GelQuant software showed the content percentages of hrH1 and hrH3 are 11.5% and 14%, respectively. SEM analysis showed that coated nanoparticles have relative spherical shape with irregular rough surface (Fig 5D).

Robust seroconversion of immunized mice

Except mock immunized group, all other immunized mice have strong seroconversion against 4M2e-GCN4 protein (Fig 6A). The induced M2e antibodies have strong cross-reactivity to the diverse M2e peptides (Fig 6B). Both Uni4C1 and Uni4C3 vaccines induced serum strongly bind to the HA from same phylogenetic group, and have weaker binding activity to the heterologous H3 and H1 respectively (Fig 6C). The boosting immunization significantly enhanced the specific antibody titers (Fig 6A and 6C). The PNP vaccines induced a balanced Th1/Th2 responses, as evidenced by the similar levels of induced serum IgG1 and IgG2a titers (Fig 6D). As shown in the radar diagram of inactivated influenza A virus coated ELISA results, Uni4C13 induced serum has broad binding activity to all subtypes of viruses, whereas the Uni4C1-induced and Uni4C3-induced antiserum have strong binding activity to phylogenetic group 1 and group 2 HA, respectively (Fig 6E). The antiserum staining of HA transfected MDCK cells also showed that H3 stalk antiserum can bind to various subtypes of membrane anchored HA proteins, including H3, H7 and H10 but not H5 (Fig 6F). Antibodies in sera that reacted specifically with M2e, H1 HA, and H3 HA were predominated by IgG over IgA (Fig 6G).

Immunization and challenge study in mice

Six- to eight-week old female BALB/c mice were intramuscularly immunized for two times at 4-week interval in hind legs with 10.5 μg Uni4MC, 12 μg Uni4C1, 12 μg Uni4C3 or 12 μg Uni4C13. Four weeks after boosting immunization, mice were challenged intranasally

with 5 × mouse lethal dose 50 (mLD50) of mouse adapted (m.a.) influenza A viruses, PR8 H1N1, p09 H1N1, Aic H3N2, Phi H3N2, rVn H5N1 and rSH H7N9, respectively. Uni4C1 and Uni4C3 immunized mice were fully protected against influenza A viruses expressing HA from the same phylogenetic group as demonstrated by both weight retention and survival in the vaccinated conditions (Fig 7A). Uni4C13 immunization conferred complete protection against lethal dose of heterologous influenza A virus challenge. Weight loss monitoring records showed that Uni4C13 immunized mice still experienced slightly weight loss after lethal dose of rVn H5N1 and rSH H7N9 challenge. All mock immunized mice died after lethal dose infection. Those mice received Uni4MC immunization were fully protected from lethal dose challenge with PR8 H1N1, p09 H1N1, Aic H3N2 and Phi H3N2 but partially protected from lethal dose infection with rVn H5N1 and rSH H7N9. Histological analysis showed that after sublethal dose infection, the lung immune cell infiltration from well protected Uni4C13 and Uni4MC groups of mice is much less than DPBS mock immunized mice (Fig 8). The lung virus titers from Uni4C13 immunized mice are also significantly lower than the other groups. Uni4MC immunization also reduced lung viral load faster than DPBS group (Fig 10).

In order to investigate the mechanism of protection conferred by PNPs, HAI assays were performed (Fig 9). It was found that no HAI activities of induced serum were detected. The convalescent serum samples collected from mice challenged with diverse influenza A viruses were used as positive controls. This implies that other antibody mediated mechanism(s) such as neutralizing antibodies, antibody-dependent cellular cytotoxicity (ADCC) or complement dependent cellular cytotoxicity may contribute to the protection.

Materials and methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All mouse studies were approved by Georgia State University Institutional Animal Care and Use Committee (IACUC) under protocol number 2003060. Female BALB/c mice and female C57BL/6 mice (six to eight-week old) were purchased from the Jackson Laboratory and housed in the animal facility at Georgia State University. Bleeding, infecting and sampling were performed under anesthesia that was induced by inhalation of isoflurane to reduce mouse suffering.

Expression and purification of recombinant proteins

Recombinant baculovirus expressing 4M2e-GCN4, hrH1 or hrH3 was generated using the Bac-to-Bac protein expression kit (Invitrogen, Grand Island, NY) according to the manufacturer's instructions and soluble 4M2e-GCN4, hrH1 and hrH3 recombinant proteins

were purified from the sf9 insect cell protein expression system as previously described (Weldon WC, et al. PloS one 2010 5(9)).

Bis [sulfosuccinimidyl] (BS3) crosslinking

The oligomeric status of purified 4M2e-GCN4, hrH1 and hrH3 proteins were determined using the soluble Bis [sulfosuccinimidyl] (BS3) crosslinker (Pierce-Rockford, IL) in a crosslinking reaction to fix the polymeric structures of proteins following with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (Weldon WC, et al. PloS one 2010 5(9)).

Protein nanoparticles fabrication

Protein nanoparticles (PNP) were made as previously described with modification (Wang L, et al. Nanomedicine : nanotechnology, biology, and medicine 2017; 13(1): 253-262; Wang L, et al. Nanomedicine : nanotechnology, biology, and medicine 2014; 10(2): 473-482; Chang TZ, et al. Biomaterials science 2017; 5(2): 223-233). To make the 4M2e-GCN4 core (4MC) PNP, the 4M2e-GCN4 protein solution in DPBS (Gibco) was desolvated with a 4:1 volume ratio of absolute ethanol to protein solution, herein 4.8 ml absolute ethanol was dripped at a constant rate at 1 ml/min to 1.2 ml 3.2 mg/ml 4M2e-GCN4 protein solution under constant stirring at 600 rpm at room temperature. The water-soluble thiol-cleavable primary amine-reactive crosslinker 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTSSP; Cat No.21578, Thermo Scientific) was used to stabilize the resulting PNP. PNPs were crosslinked with DTSSP at final concentration 5 mM while stirring at room temperature for one hour, followed by centrifugation at 15,000 × g for 15 min at room temperature to pellet the PNP. PNPs were resuspended by sonication in either 1 ml 2.8 mg/ml hrH1 in DPBS, 1 ml 3.1 mg/ml hrH3 in DPBS or 1 ml DPBS for coating or non-coating respectively, to generate hrH1 coated 4MC PNP (vaccine code: Uni4C1), hrH3 coated 4MtG core PNP (vaccine code: Uni4C3) and 4MC PNP (vaccine code: Uni4MC). Coating reactions were performed in 5 mM DTSSP for 2 hours while stirring at 4 °C. The coat crosslinking reactions were quenched with 30 mM Tris-HCl solution at pH 7.4 for 15 min. Following collection by centrifugation at 20,000 × g for 30 min at 4 °C, PNPs were resuspended by sonication in 1 ml DPBS.

PNP characterization

Nanoparticle size distribution and zeta potential were assessed by dynamic light scattering (DLS) and electrophoretic light scattering (ELS) respectively with a Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA). Protein concentration in the PNP solution was assessed with a BCA assay according to the manufacturer's instructions (Thermo Scientific, Waltham, MA). PNP were resuspended in water, air-dried, and sputter-

coated with carbon prior to visualization with a Zeiss LEO 1450vp scanning electron microscope (Carl Zeiss, Jena, Germany) at 5.0 kV.

Immunization and influenza A viruses challenge

All animal experiments were approved and performed in accordance with local legislation on animal experiments. Mice (BALB/c strain, female, 6-8 weeks old, n=5 per group) received intramuscular (i.m.) immunizations for two times at 4 week interval in hind legs with 50 µl vaccine mixture in DPBS containing 10.5 µg Uni4MC, 12 µg Uni4C1, 12 µg Uni4C3 or 12 µg Uni4C13 (formulation comprising physical mixture of 6 µg Uni4C1 and 6 µg Uni4C3 in DPBS). Blood samples were collected at 1 day prior to priming and 3 weeks after priming and boosting. Four weeks after boosting immunization, mice were challenged intranasally with 5 × mouse lethal dose 50 (mLD50) of mouse adapted (m.a.) influenza A virus in 50 µl DPBS, including PR8 H1N1 (A/Puerto Rico/08/1934), p09 H1N1 (A/California/07/2009), Aic H3N2 (A/Aichi/02/2968), Phi H3N2 (A/Philippines/02/1982), rVn H5N1 (reassortant A/Vietnam/1203/2004; HA and neuraminidase (NA) were derived derived from A/Vietnam/1203/2004, and the remaining backbone genes from A/Puerto Rico/08/1934) or rSH H7N9 (reassortant A/Shanghai/02/2013; HA and NA were derived from A/Shanghai/02/2013, and the remaining backbone genes from A/Puerto Rico/08/1934). Body weight loss and survival rates were monitored daily for 14 days post infection (dpi). Weight loss of ≥ 20% was used as the end point at which mice were euthanized according to IACUC guidelines.

Determination of lung virus titers

Three mice per immunization group were euthanized at 5th day post 1 × mLD50 m.a. H1N1 A/Puerto Rico/08/1934 or m.a. H3N2 A/Aichi/02/1968 infection. Lungs were excised and homogenized in 10% (w:v) DPBS. Lung homogenates were cleared from debris by centrifugation for 10 min at 16,000 × g at 4 °C. Madin Darby Canine Kidney (MDCK) cells were seeded in triplicate at 2 × 104 cells per well in a 96-well plate and infected with 50 µl of a 1/10-dilution series of the cleared lung homogenates. After 1 hour of incubation at 37°C, wash the culture with DPBS three times, then add fresh 100 µl serum-free medium containing DMEM (Gibco), 2 mM L-glutamine (Life technologies), 10 µg/ml streptomycin, 10 U/ml penicillin (Hyclone), 0.1 mM non-essential amino acid (Gibco) and 2 µg/ml TPCK-treated trypsin (Sigma-Aldrich). After five days of incubation at 37 °C, the presence of virus in the supernatant was assayed by measuring the hemagglutinating activity in the supernatant and using the method of Reed and Muench for calculation (Reed LJ, et al. Am J Epidemiol 1938; 27: 493-497).

Histological analysis

Three mice per immunization group were euthanized at 5th day post 1 × mLD50 m.a. H1N1 A/Puerto Rico/8/1934 or m.a. H3N2 A/Aichi/2/1968 infection. Lung tissues were isolated and fixed with 10% neutral buffered formalin. Fixed lung tissues were embedded in paraffin and processed for Haemotoxylin and Eosin (H&E) staining. Three sections with 10 μm thickness from three different parts of the lungs were stained with H&E and examined microscopically unbiased by three pathologists. The severity of the inflammation in the examined lung sections was scored on a scale of 0 to 5 (with 0.5 interval). Scores were given as absent (0), subtle (1), mild (2), moderate (3), severe (4), and massive (5).

Hemagglutination inhibition (HAI) assay

Mouse sera were pretreated with three volumes of receptor-destroying enzyme (Sigma-Aldrich, U.S.) and incubated overnight at 37 °C. Five volumes of filter-sterilized 1.5% sodium citrate solution was added and the mixture was inactivated at 56°C for 30 min. 1/20 volume of 5% turkey red blood cells were added to the sera followed by incubation overnight at 4°C to pre-clear the sera. Samples were then centrifuged for 10 min at 1,500 g, and twofold serial dilutions of the supernatant were prepared and tested for HAI activity against 8HA in 50 μl of PR8 H1N1, p09 H1N1, Aic H3N2, Phi H3N2, Vtn H5N1 or SH H7N9.

Lung virus titer determination

To determine lung viral load, PR8 H1N1 or Aic H3N2 infected mice from Uni4MC, Uni4C13 and DPBS immunization groups were sacrificed at the 5th day post infection. Their lungs were isolated and homogenized in DPBS at 10% (w/v) ratio. The lung homogenates were spun at 16,000 × g for 15 min to pellet tissue debris, and the supernatants were collected. Samples were stored at – 80 °C until titration using TCID50 measurement method.

Table 1. List of M2e sequences used in study

Present in	Codes	Sequence	SEQ ID	Origin
Peptide	huM2e	SLLTEVETPIRNEWGCRCNDSSD	SEQ ID NO:1	Human consensus
	p09M2e	SLLTEVETPT**R**S**E**WE**C**RC**S**DSSD	SEQ ID NO:2	A/California/07/2009 (H1N1)
	VtnM2e	SLLTEVETPT**T**RNE**W**E**C**RC**S**DSSD	SEQ ID NO:3	A/Vietnam/1203/2004 (H5N1)
	SHM2e	SLLTEVETPT**T**R**TGW**E**C**N**C**SG**SS**E**	SEQ ID NO:4	A/Shanghai/02/2013 (H7N9)
Influenza A	PR8M2e	SLLTEVETPIRNEWGCRCN**G**SSD	SEQ ID NO:5	A/Puerto Rico/08/1934 (H1N1)
virus	AicM2e	SLLTEVETPIRNEWGCRCNDSSD	SEQ ID NO:6	A/Aichi/02/1968 (H3N2)
	p09M2e	SLLTEVETPT**R**S**E**WE**C**RCSDSSD	SEQ ID NO:7	A/California/07/2009 (H1N1)
	PhiM2e	SLLTEVETPIRNEWGCRCNDSSD	SEQ ID NO:8	A/Philippines/02/1982 (H3N2)
	rVnM2e	SLLTEVETPIRNEWGCRCN**G**SSD	SEQ ID NO:9	A/Vietnam/1203/2004 (rgH5N1)
	rSHM2e	SLLTEVETPIRNEWGCRCN**G**SSD	SEQ ID NO:10	A/Shanghai/2/2013 (rgH7N9)
4MtG	huM2e	SLLTEVETPIRNEWG**S**R**S**NDSSD	SEQ ID NO:11	Human consensus
	swnM2e	SLLTEVETP**T**R**S**EW**ES**R**SS**DSSD	SEQ ID NO:12	Swine consensus
	aviM2e	SLLTEVETP**T**RN**G**W**ESKSSG**SSD	SEQ ID NO:13	Avian consensus
	fwlM2e	SLLTEVETP**T**RN**G**W**ESNSS**DSSD	SEQ ID NO:14	Domestic fowl consensus

Example 2:

The following is an Influenza A H3 stalk ectodomain sequence:

MKTIIALSYIFCLPLGQDLPGNDNSTATLCLGHHAVPNGTLVKTITDDQIEVTNATELVQSSST
GKICNNPHRILDGIDCTLIDALLGDPHCDVFQNETWDLFVERSKAFSNCYPYDVPDYASLRS
LVASSGTLEFITEGFTWTGVTQNGGSNACKRGPGSGFFSRLNWLTKSGSTYPVLNVTMPN
NDNFDKLYIWGIHHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGLSSRISIY
WTIVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIDTCISECITPNGSIPNDKPFQN
VNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLFGAIAGFIENGWEGMIDGWYGFRHQ
NSEGTGQAADLKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKID
LWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQLRENAEEMGNGCFKIYHKCDNACIESI
RNGTYDHDVYRDEALNNRFQIKGVELKSGYK (SEQ ID NO:15)

Head-removed H3 HA were generated by replacing head domain (amino acids S61-P322), (amino acids S62-K323) or (amino acids S62-V325) with 3G, 4G, 5G or 4GC linker (Fig. 12). The protein expression of these candidates was not largely affected by different mutation sites as evidenced by the similar intensity of bands in Western blot (Fig. 13). Too much truncation and retaining will lead to failure in protein expression and misfolding. Therefore, an truncation range with 3 amino acids distance from S61 and K323 may be optimal.

The tetrabrachion motif screwed tetramer protein retains native functions and can be stably expressed. In our study, tetrabrachion motif was fused with tandem copies of M2e at N-terminal and followed by N1 ectodomain including stalk and head (Fig. 14). Construct 2 and 3 are simulated tetrabrachion motif screwed tetramer constructs (Fig. 14). With flanked tandem copies of M2e or flanked N1 ectodomain at both sides of tetrabrachion motif, it is speculated that the tetramer proteins can be expressed and retains native protein function.

Example 3:

Coated M2e-Neuraminidase fusion protein nanoclusters display neuraminidase activity. Figures 15A and 15B shows example structure of 4M2e-NA nanoparticles. Figure 15A shows combination of 4M2e and 4M2e-NA fusion proteins. GS linker: glycine and serine GGSGGG (SEQ ID NO:20); M2e: combined M2e sequence from human, swine, wild bird, and domestic fowl with tetrabrachion together; M2e + NA: combined stalk and head of neuraminidase 1 (from A/ Vietnam/1203/2004/) with M2e above. Figure 15B shows schematic formation of M2e-NA nano-particle. 4M2e-NA was covered on the 4M2e core.

Figures 16A to 16F show the formation of M2e-NA nanoparticle. Figure 16A shows M2e-NA fusion protein crosslinked by by 12.5, 7.5, 2.5, and 1mM of BS3, as in line 1, 2, 3, 4,

respectively. Line 5: soluble M2e + NA fusion protein. Figure 16B shows Coomassie blue staining of M2e-NA nanoparticle. Figures 16C and 16D show Western blot assay of M2e-NA nanoparticle. C: M2e antibody; D: NA antibody. Figure 16E shows the size of formed M2e+NA nanoparticle: 198.2 +/- 62.41nm. Figure 16F shows results of a neuraminidase activity test. The concentration of each protein: M2e+NA nano particle: 1mg/ml; soluble M2e+NA: 1mg/ml; H5N1 virus: 2.5mg/ml; BSA: 2mg/ml.

Figure 17 shows example tetramer constructs.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

Example 4:

The following is an Influenza B HA stalk ectodomain sequence:

MKFLVNVALVFMVVYISYIYAAADRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTG GGGCVKTPLKLANGTKYRPPAKLLTEQGFFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVA VAADLKSTQEAINKITKNLNSLSELEGGGSSADTICSQIELAVLLSNEGIINSEDEHLLALERKL KKMLGPSAVEIGNGCFETKHKCNQTCLDRIAAGTFDAGEFSLPTPGSSGGSSGMKQIEDKI EEILSKIYHIENEIARIKKLDDDDKHHHHHH (SEQ ID NO:36)

This head-removed influenza B HA (B/hrHA) was designed by replacing head domain (amino acids P33-W321 and V404-R432) with a GGGGC (SEQ ID NO:18) or GGGSS (SEQ ID NO:19) linker (Fig. 18). Sequences at the N-terminal and C-terminal were also added for expression and functionalization of the protein. The DNA sequence encoding this construct (SEQ ID NO:37) was cloned into a pFastBac1 expression vector using NotI and BamHI restriction sites. This vector permits expression of the protein in Sf9 insect cells using a PH promoter. After ligation of the insert into the plasmid, the insert could be identified by PCR and by restriction digest analysis (Fig. 19A). To generate this data, 3 μg of the bacmid was digested using BamHI and NotI restriction enzymes to cut. For PCR, 0.1μL of the bacmid was used as template combined with the M13 forward and reverse primers for amplification.

The hrHA protein was efficiently expressed by Sf9 cells as evidenced by the robust mass of protein of correct size that is clear in Western blot analysis of the supernatant from transfected cells (Fig. 19B). Inappropriate modification and truncation leading to protein

misfolding would result in retention of the protein in the cells with a clear failure in secrete protein into the supernatant. To generate this data 2 µg of the B/hrHA bacmid (in 100 µl) was combinged with 10 µL Cellfectin II (in 100 µl) for 40 min at RT. Attached Sf9 cells were transfected in 6 well plate by adding 200 µL of the liposome mixture. Transfection was incubated for 5 days at 28°C. A 20 µL sample of supernatant was taken for analysis, separated by SDS-PAGE gel, and analyzed by Western.

WHAT IS CLAIMED IS:

1. A composition comprising a polypeptide comprising a truncated influenza hemagglutin (HA) protein lacking a head domain.
2. A composition comprising a polypeptide comprising a truncated influenza hemagglutin (HA) protein lacking a head domain and an adjuvant.
3. A composition comprising a nanoparticle coated with a polypeptide comprising a truncated influenza hemagglutin (HA) protein lacking a head domain.
4. A composition comprising a virus like particle (VLP) expressing on its surface a polypeptide comprising a truncated influenza hemagglutin (HA) protein lacking a head domain.
5. The composition of claim 4, wherein the polypeptide comprises the transmembrane and cytoplasmic domains of HA.
6. The composition of claim 4, wherein the polypeptide lacks the HA transmembrane and/or cytoplasmic domains of HA, and wherein the polypeptide comprises a heterologous membrane-anchoring sequence.
7. The composition of claim 6, wherein the heterologous membrane-anchoring sequence comprises a glycosylphosphatidylinositol (GPI) membrane-anchoring sequence.
8. The composition of any one of claims 1 to 7, where the polypeptide lacks at least the amino acids corresponding to residues 65 to 320 of amino acid sequence SEQ ID NO:15.
9. The composition of claims 8, wherein the polypeptide lacks at least the amino acids corresponding to residues 62 to 322 of SEQ ID NO:15.
10. The composition of any one of claims 1 to 9, wherein the head domain is replaced by a linker 3 to 5 amino acids in length that cannot form a fixed secondary structure.
11. The composition of claim 10, wherein the linker comprises 3 to 5 amino acids selected from glycine, alanine, and serine.
12. The composition of claims 11, wherein the linker is selected from the group consisting of GGGG (SEQ ID NO:16), GGGGG (SEQ ID NO:17), and GGGGC (SEQ ID NO:18), and GGGSS (SEQ ID NO:19).
13. The composition of any one of claims 1 to 12, wherein the influenza HA protein is an influenza subtype A HA protein.
14. The composition of any one of claims 1 to 12, wherein the influenza HA protein is an influenza subtype B HA protein.
15. A nanoparticle formed by crosslinking a fusion protein with a crosslinking agent and/or desolvating the fusion protein with a desolvating agent, wherein the fusion protein

comprises a series of 2 to 8 influenza virus matrix protein 2 extracellular (M2e) domains linked to a multimerization domain.

16. The nanoparticle of claim 15, wherein the multimerization domain comprises a tetramerization domain.

17. The nanoparticle of claim 16, wherein the tetramerization domain comprises a GCN4 or tetrabrachion protein.

18. The nanoparticle of any one of claims 15 to 17, wherein the M2e domains are linked to each other by a flexible linker comprising 3 to 9 amino acid selected from glycine, alanine, and serine.

19. The nanoparticle of claim 18, wherein the flexible linker comprises the amino acid sequence GGSGGG (SEQ ID NO:20).

20. The nanoparticle of any one of claims 15 to 19, wherein the series of M2e domains are linked to the N-terminus of the multimerization domain.

21. The nanoparticle of any one of claims 15 to 19, wherein the series of M2e domains are linked to the C-terminus of the multimerization domain.

22. The nanoparticle of any one of claims 15 to 19, wherein the fusion protein comprises a series of 2 to 8 M2e domains linked to the N-terminus of the multimerization domain and series of 2 to 8 M2e domains linked to the C-terminus of the multimerization domain.

23. The nanoparticle of any one of claims 15 to 22, wherein the fusion protein further comprises influenza neuraminidase (NA) protein linked to the multimerization domain.

24. The nanoparticle of claim 23, wherein the series of 2 to 8 M2e domains are linked to the N-terminus of the multimerization domain and the NA protein is linked to the C-terminus of the multimerization domain.

25. The nanoparticle of any one of claims 15 to 24, wherein the multimerization domain is linked to the M2e domains, the NA protein, or any combination thereof, by a flexible linker comprising 3 to 9 amino acid selected from glycine, alanine, and serine.

26. The nanoparticle of claim 25, wherein the flexible linker comprises the amino acid sequence GGSGGG (SEQ ID NO:20).

27. The nanoparticle of any one of claims 15 to 26, comprising four M2e (4M2e) domains in each series.

28. The nanoparticle of any one of claims 15 to 27, comprising two or more heterologous M2e domains.

29. The nanoparticle of claim 28, wherein the fusion protein comprises one or more M2e domains from a human influenza, one or more M2e domains from a swine influenza, and one or more M2e domains from an avian influenza.

30. The nanoparticle of claim 28, wherein the fusion protein comprises one or more M2e domains from a human influenza A subtype, one or more M2e domains from a swine influenza A subtype, and one or more M2e domains from an avian influenza A subtype.

31. The nanoparticle of claim 28, wherein the fusion protein comprises one or more M2e domains from a human influenza B subtype, one or more M2e domains from a swine influenza B subtype, and one or more M2e domains from an avian influenza B subtype.

32. The nanoparticle of any one of claims 15 to 31, wherein the fusion protein further comprises a signal peptide at the N-terminus.

33. The nanoparticle of claim 32, wherein the signal peptide comprises mellitin signal peptide.

34. The nanoparticle of any one of claims 15 to 33, wherein the desolvating agent comprises ethanol, acetone, or combinations thereof.

35. The nanoparticle of any one of claims 15 to 34, wherein the crosslinking agent is selected from the group consisting of formaldehyde, formaldehyde derivatives, formalin, glutaraldehyde, glutaraldehyde derivatives, a protein cross-linker, a nucleic acid cross-linker, a protein and nucleic acid cross-linker, primary amine reactive crosslinkers, sulfhydryl reactive crosslinkers, sulfydryl addition or disulfide reduction, carbohydrate reactive crosslinkers, carboxyl reactive crosslinkers, photoreactive crosslinkers, cleavable crosslinkers, AEDP, APG, BASED, BM(PEO)3, BM(PEO)4, BMB, BMDB, BMH, BMOE, BS3, BSOCOES, DFDNB, DMA, DMP, DMS, DPDPB, DSG, DSP, DSS, DST, DTBP, DTME, DTSSP, EGS, HBVS, sulfo-BSOCOES, Sulfo-DST, and Sulfo-EGS.

36. The nanoparticle of claim 35, wherein the crosslinking agent is a water-soluble crosslinker that contains amine-reactive NHS-ester ends and whose central disulfide bond can be cleaved with reducing agents.

37. The nanoparticle of claim 35, wherein the crosslinking agent can be cleaved with reducing agents.

38. The nanoparticle of claim 35, wherein the crosslinking agent is DTSSP.

39. The nanoparticle of any one of claims 15 to 38, wherein the nanoparticle is coated with an influenza antigen.

40. The nanoparticle of claim 39, wherein the antigen comprises a truncated influenza hemagglutin (HA) protein lacking a head domain.

41. The nanoparticle of claim 39 or 40, wherein the antigen is coated on the nanoparticle using a crosslinking agent.

42. The nanoparticle of claim 41, wherein the crosslinking agent is selected from the group consisting of formaldehyde, formaldehyde derivatives, formalin, glutaraldehyde,

glutaraldehyde derivatives, a protein cross-linker, a nucleic acid cross-linker, a protein and nucleic acid cross-linker, primary amine reactive crosslinkers, sulfhydryl reactive crosslinkers, sulfydryl addition or disulfide reduction, carbohydrate reactive crosslinkers, carboxyl reactive crosslinkers, photoreactive crosslinkers, cleavable crosslinkers, AEDP, APG, BASED, BM(PEO)3, BM(PEO)4, BMB, BMDB, BMH, BMOE, BS3, BSOCOES, DFDNB, DMA, DMP, DMS, DPDPB, DSG, DSP, DSS, DST, DTBP, DTME, DTSSP, EGS, HBVS, sulfo-BSOCOES, Sulfo-DST, and Sulfo-EGS.

43. The nanoparticle of claim 42, wherein the crosslinking agent is a water-soluble crosslinker that contains amine-reactive NHS-ester ends and whose central disulfide bond can be cleaved with reducing agents.

44. The nanoparticle of claim 42, wherein the crosslinking agent can be cleaved with reducing agents.

45. The nanoparticle of claim 42, wherein the crosslinking agent is DTSSP.

4M2e-GCN4 amino acids sequence:

MSLLTEVETPIRNEWGSRSNDSSDPGGSSGGSSSLLTEVETPTRSEWESRSSD
SSDPGGSSGGSSSLLTEVETPTRNGWESKSSGSSDPGSGGSGGSSLLTEVETPT
RNGWESNSSDSSDPGGGSSSLELKQIEDKLEEILSKLYHIENELARIKKLLGE

FIG. 1A

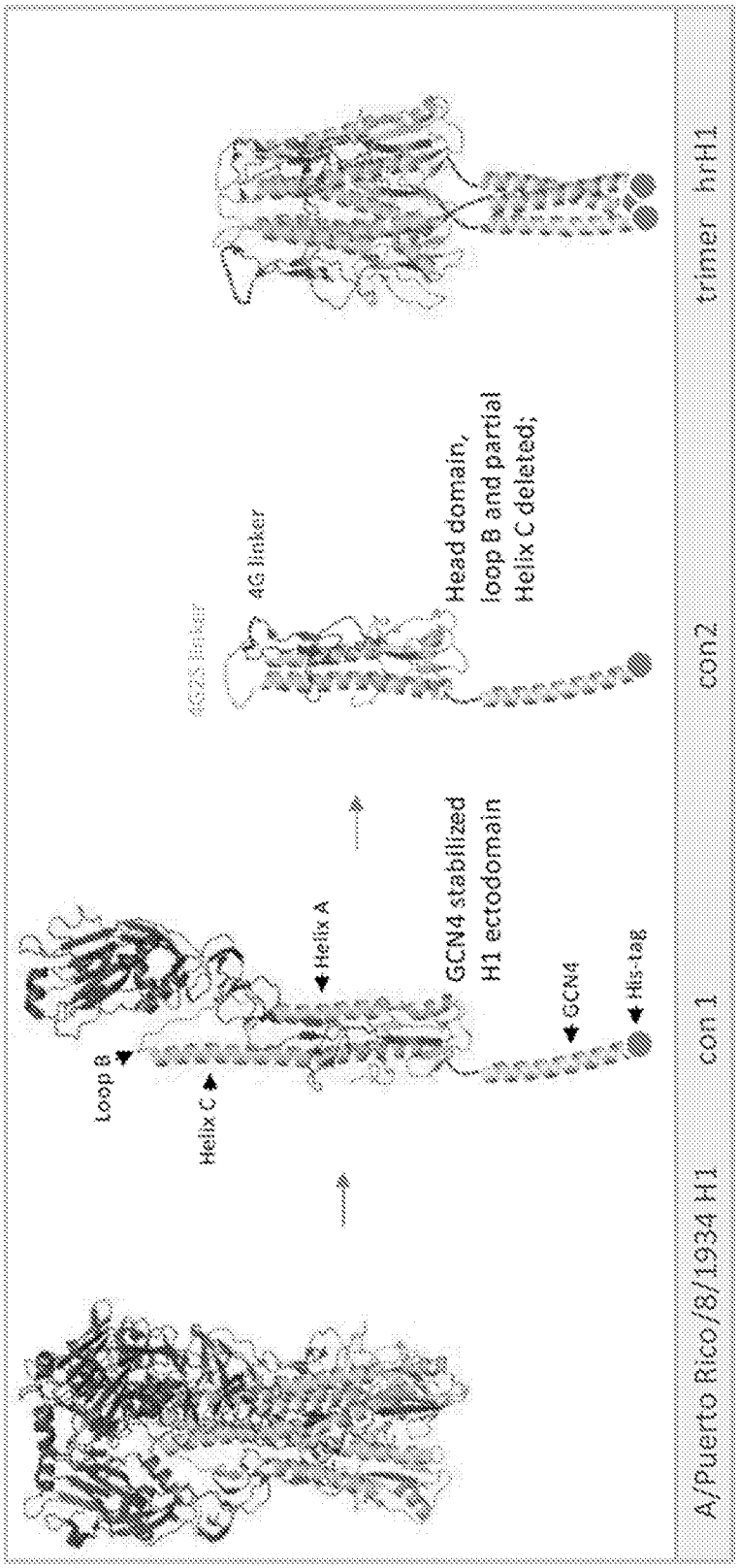

A/Puerto Rico/8/1934 H1
con 1
con2
trimer hrH1
Loop B
Helix C
Helix A
GCN4 stabilized
H1 ectodomain
GCN4
His-tag
4G linker
Head domain,
loop B and partial
Helix C deleted;

FIG. 1B

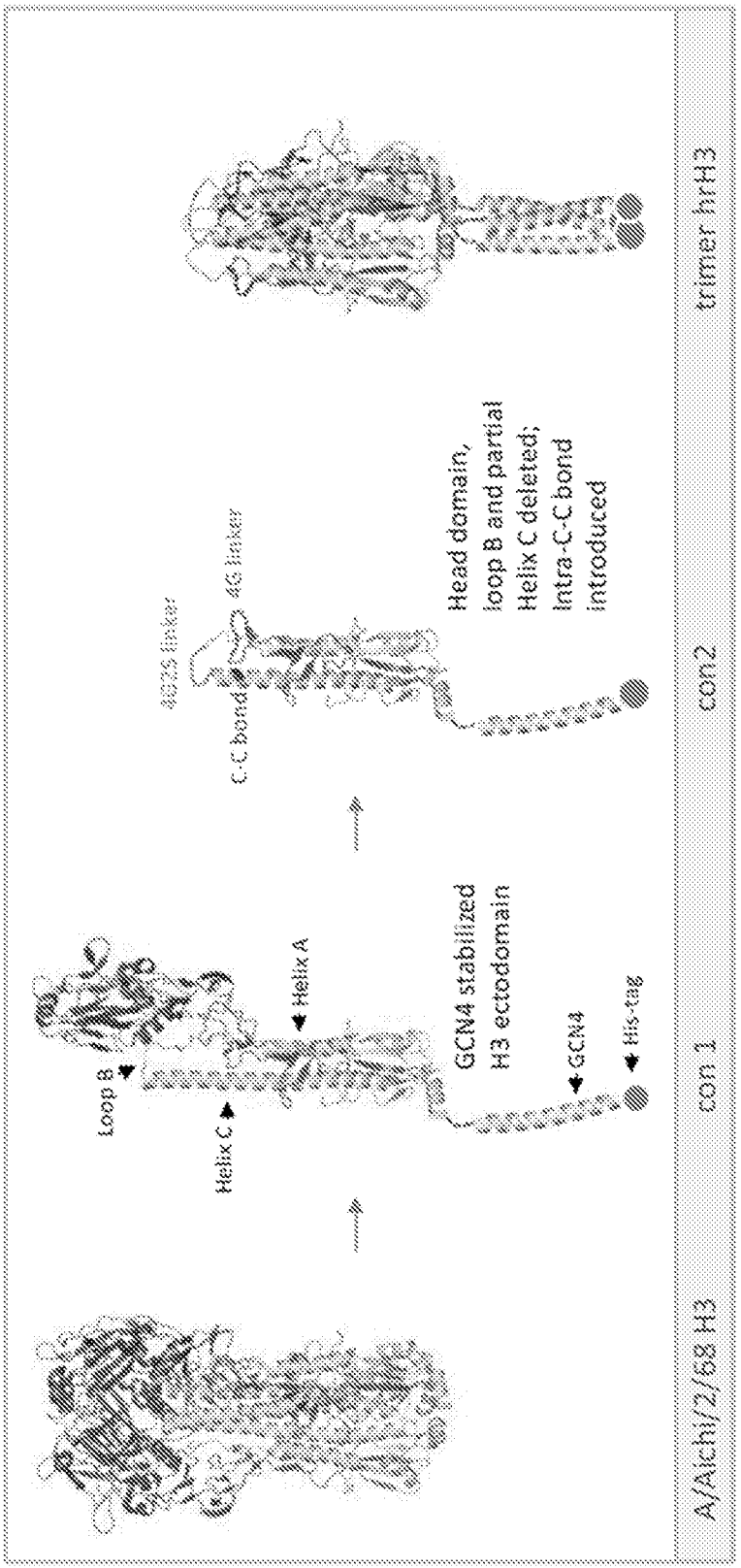

Loop B
Helix C
Helix A
GCN4 stabilized
H3 ectodomain
GCN4
His-tag
C-C bond
4G3S linker
4G linker
Head domain,
loop B and partial
Helix C deleted;
Intra-C-C bond
introduced
A/Aichi/2/68 H3
con 1
con2
trimer hrH3

FIG. 1C

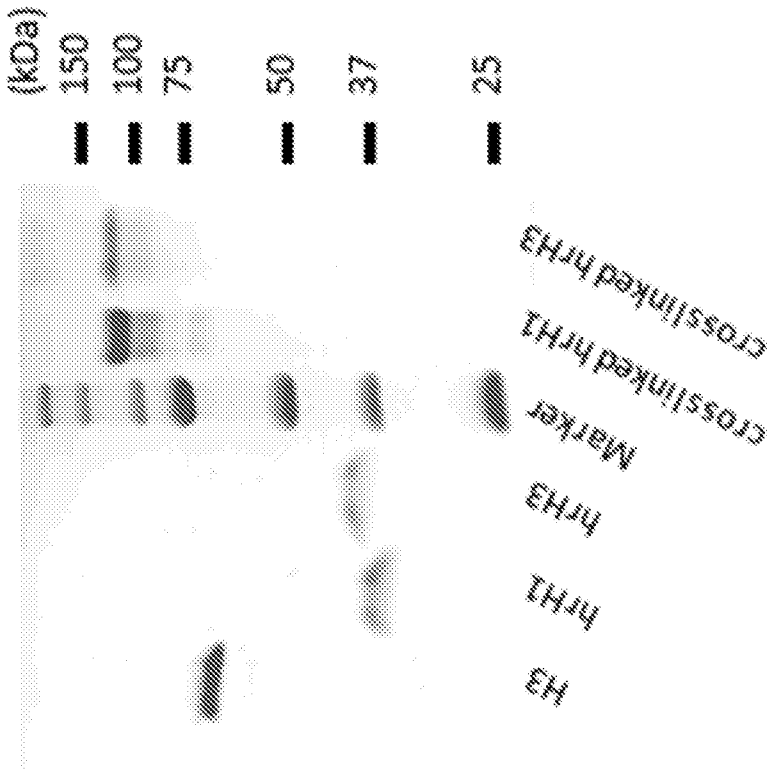

(kDa)
150
100
75
50
37
25
Crosslinked hrH3
Crosslinked hrH1
Marker
hrH3
hrH1
H3

FIG. 2B

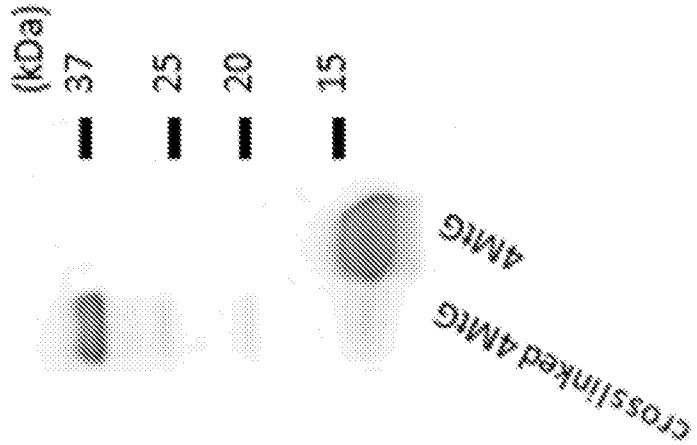

(kDa)
37
25
20
15
4MIG
Crosslinked 4MIG

FIG. 2A

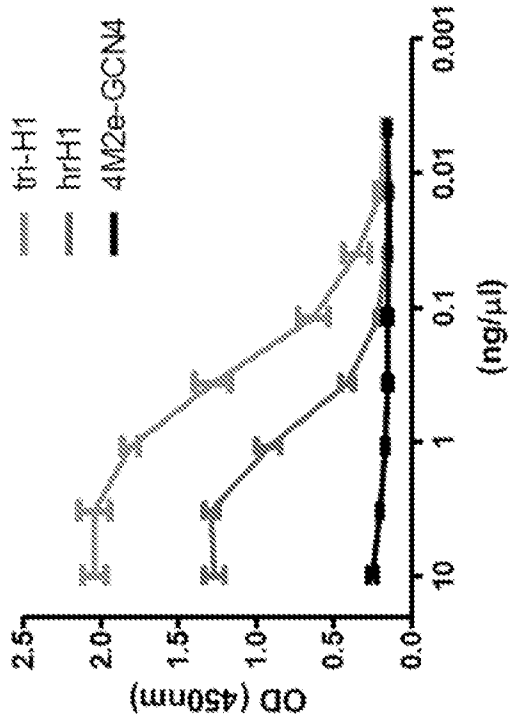

tri-H1
hrH1
4M2e-GCN4
OD (450nm)
2.5
2.0
1.5
1.0
0.5
0.0
10
1
0.1
0.01
0.001
(ng/μl)

FIG. 2D

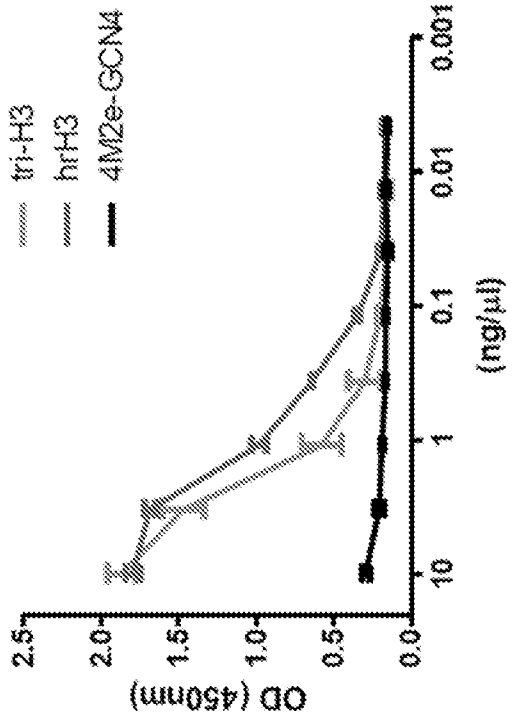

tri-H3
hrH3
4M2e-GCN4
OD (450nm)
2.5
2.0
1.5
1.0
0.5
0.0
10
1
0.1
0.01
0.001
(ng/μl)

FIG. 2C

Soluble
4M2e-GCN4
Ethanol Desolvalted
DTSSP crosslinked
Uni4MC
nanocluster

FIG. 3A

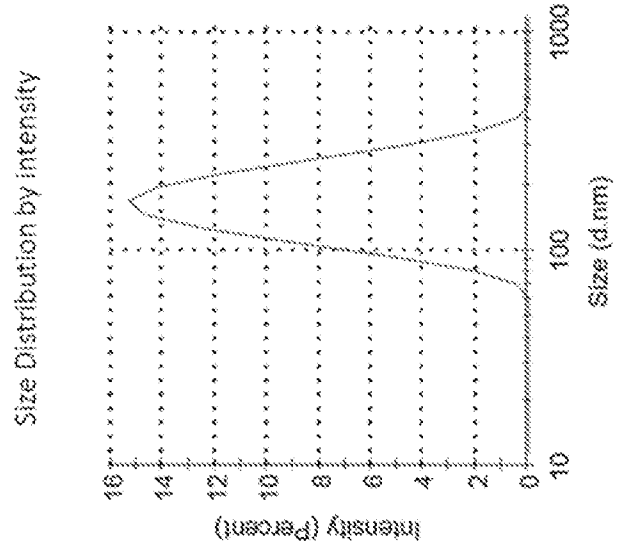

Size Distribution by Intensity
Intensity (Percent)
Size (d.nm)
0
2
4
6
8
10
12
14
16
10
100
1000

FIG. 3C

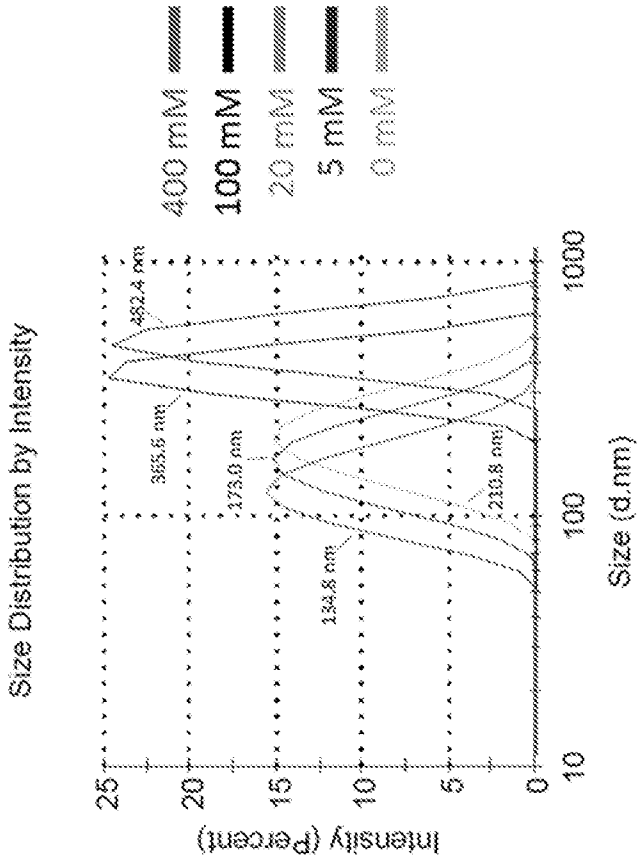

Size Distribution by Intensity
400 mM
100 mM
20 mM
5 mM
0 mM
492.4 nm
365.6 nm
173.0 nm
134.8 nm
210.8 nm
Intensity (Percent)
Size (d.nm)
0
5
10
15
20
25
10
100
1000

FIG. 3B

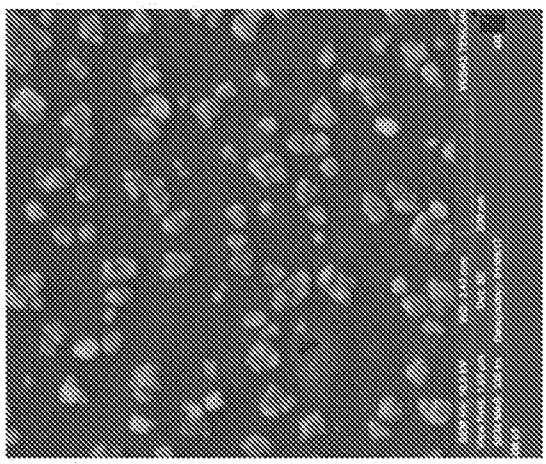


FIG. 3E

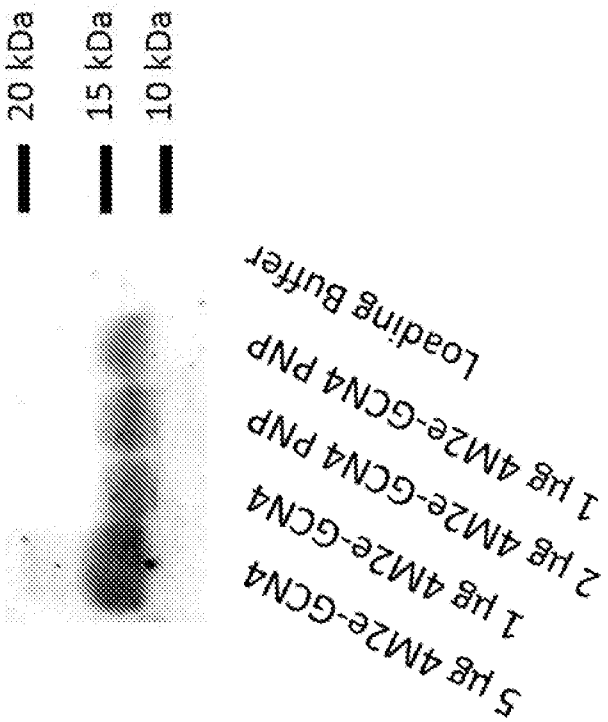

20 kDa
15 kDa
10 kDa
5 µg 4M2e-GCN4
1 µg 4M2e-GCN4
2 µg 4M2e-GCN4 PNP
1 µg 4M2e-GCN4 PNP
Loading Buffer

FIG. 3D

Uni4MC nanocluster
+
Soluble head-removed HA
hrHA coated
DTSSP crosslinked
Coated nanocluster

FIG. 4A

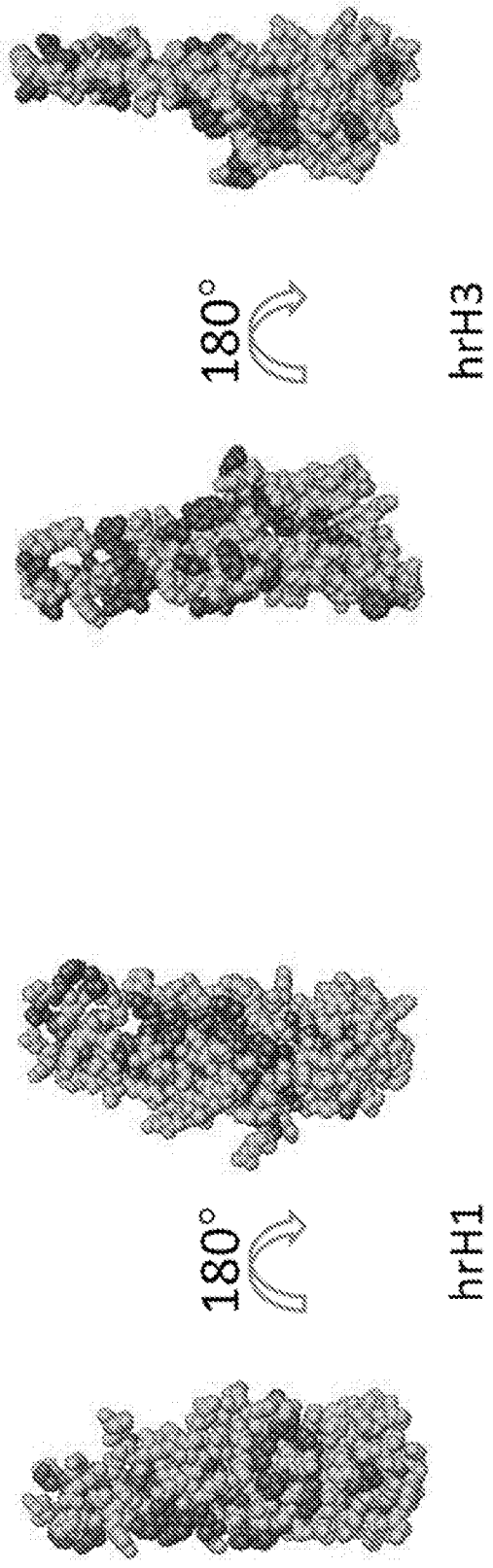
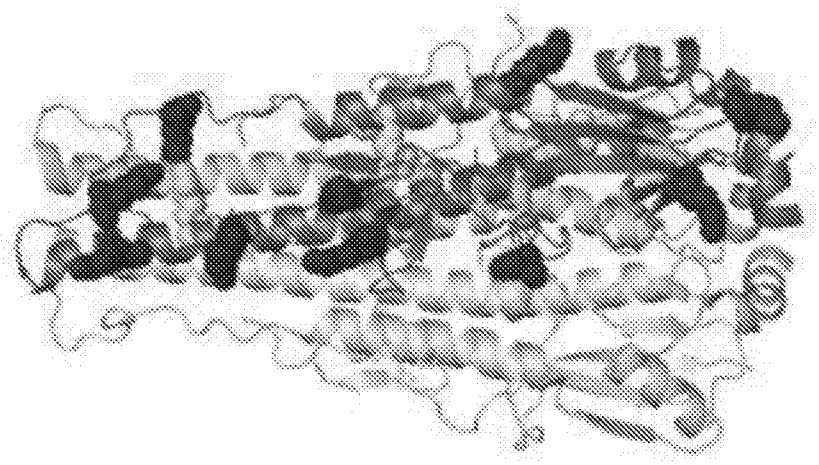


FIG. 4B



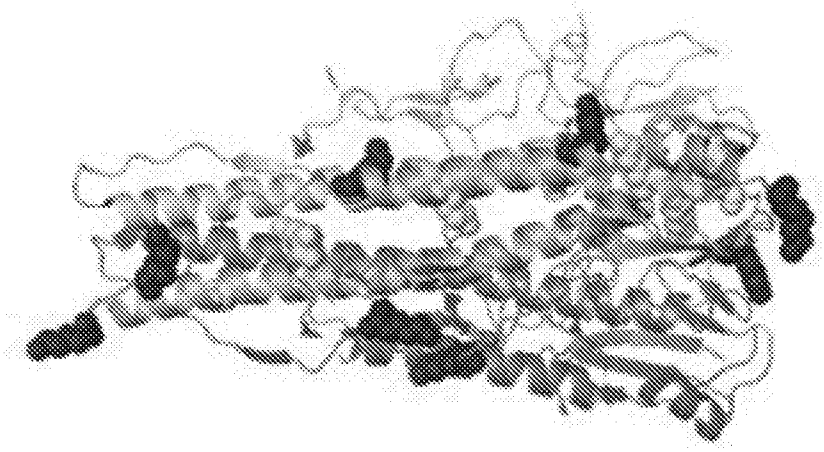


FIG. 4C

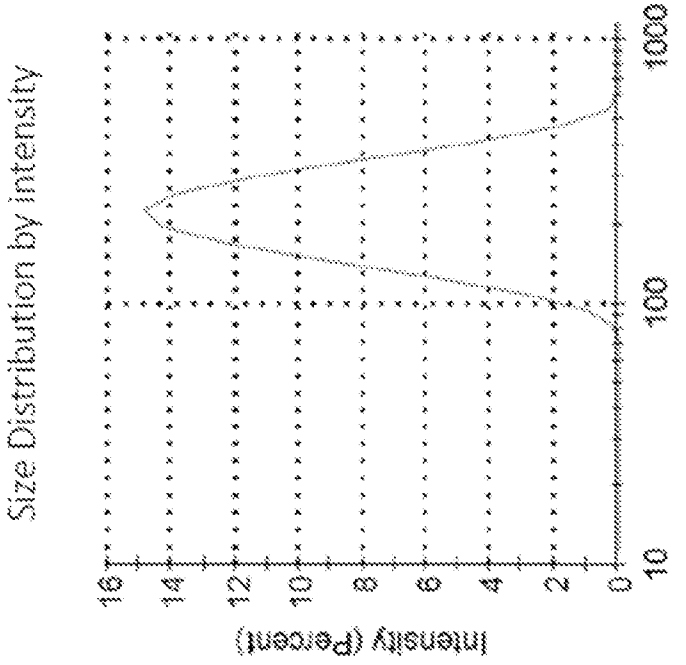

Size Distribution by intensity
Intensity (Percent)
Size (d.nm)
0
2
4
6
8
10
12
14
16
10
100
1000

FIG. 5B

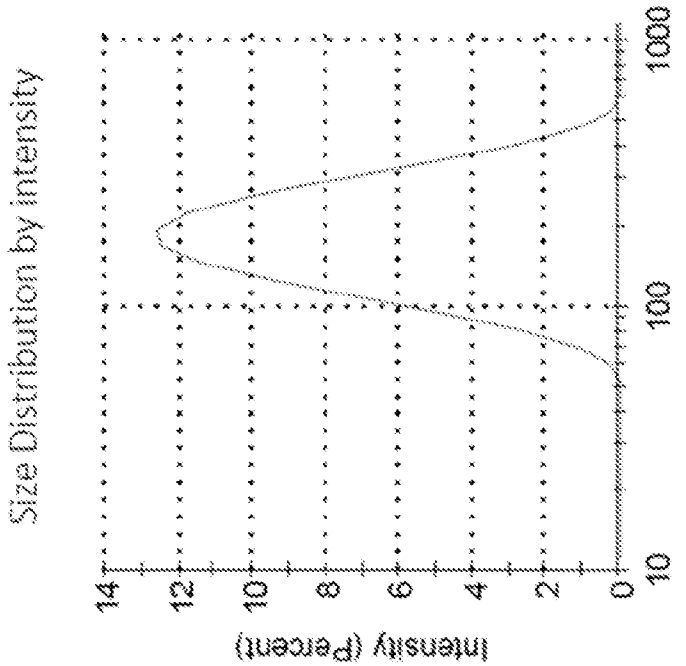

Size Distribution by intensity
Intensity (Percent)
Size (d.nm)
0
2
4
6
8
10
12
14
10
100
1000

FIG. 5A

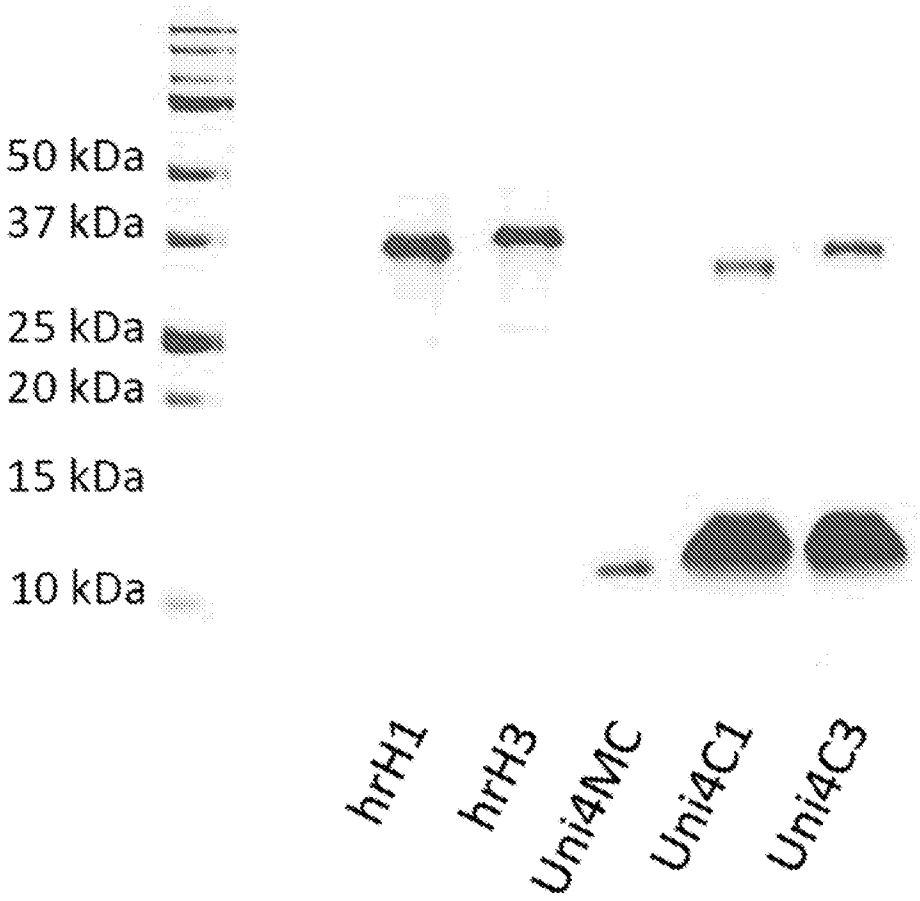

50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
hrH1
hrH3
Uni4MC
Uni4C1
Uni4C3

FIG. 5C

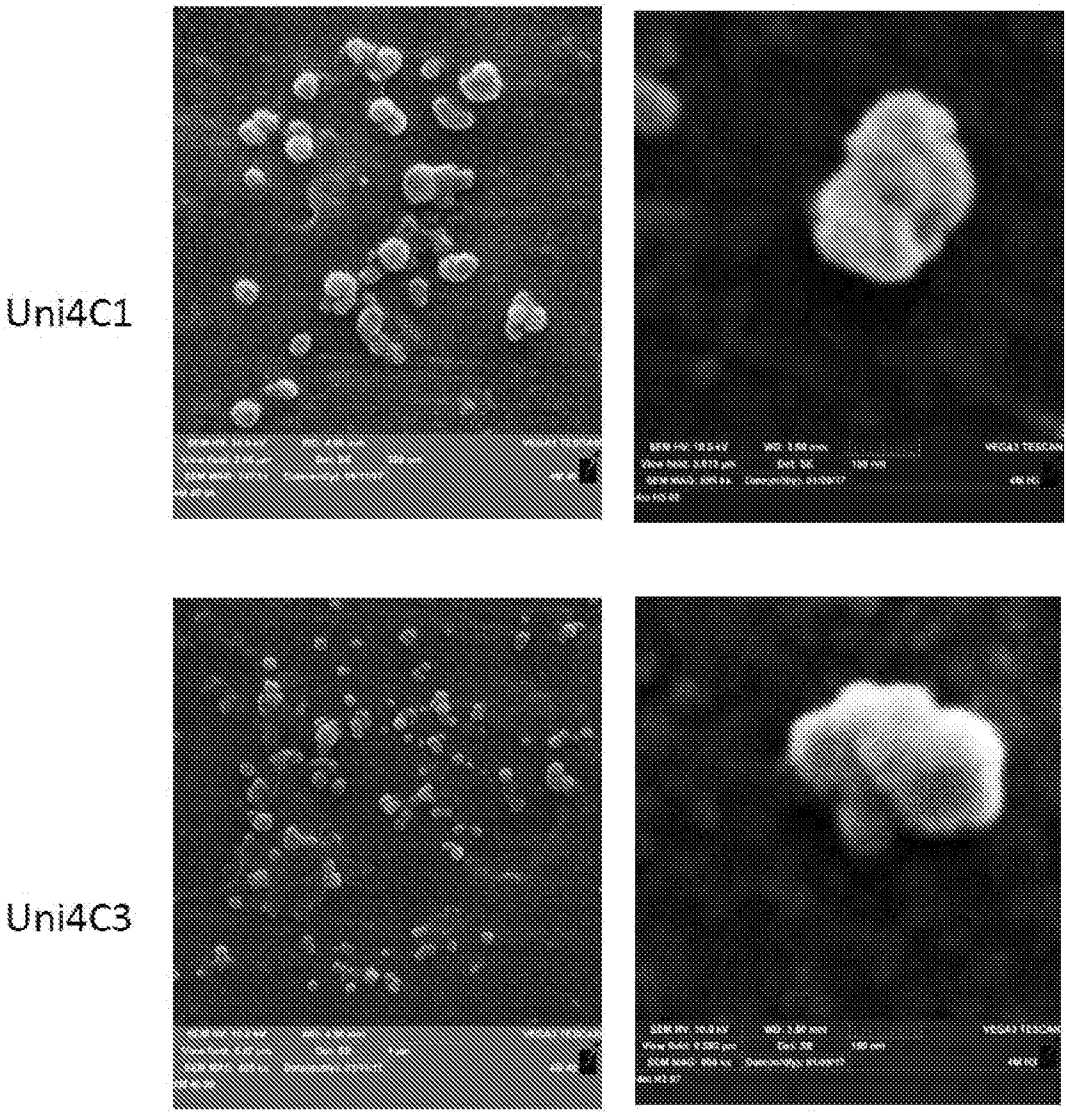


FIG. 5D

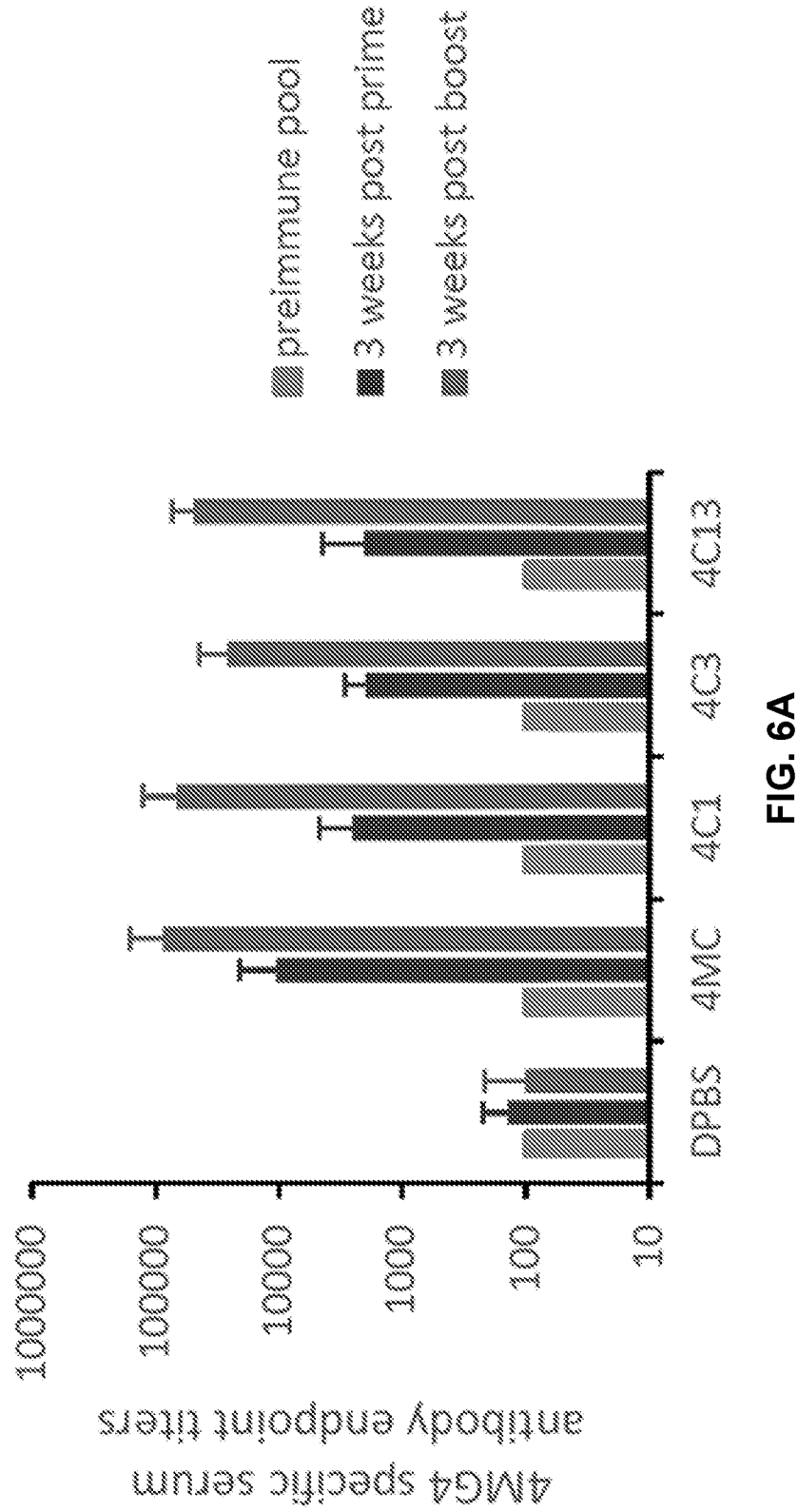

preimmune pool
3 weeks post prime
3 weeks post boost
4MG4 specific serum antibody endpoint titers
1000000
100000
10000
1000
100
10
DPBS
4MC
4C1
4C3
4C13

FIG. 6A

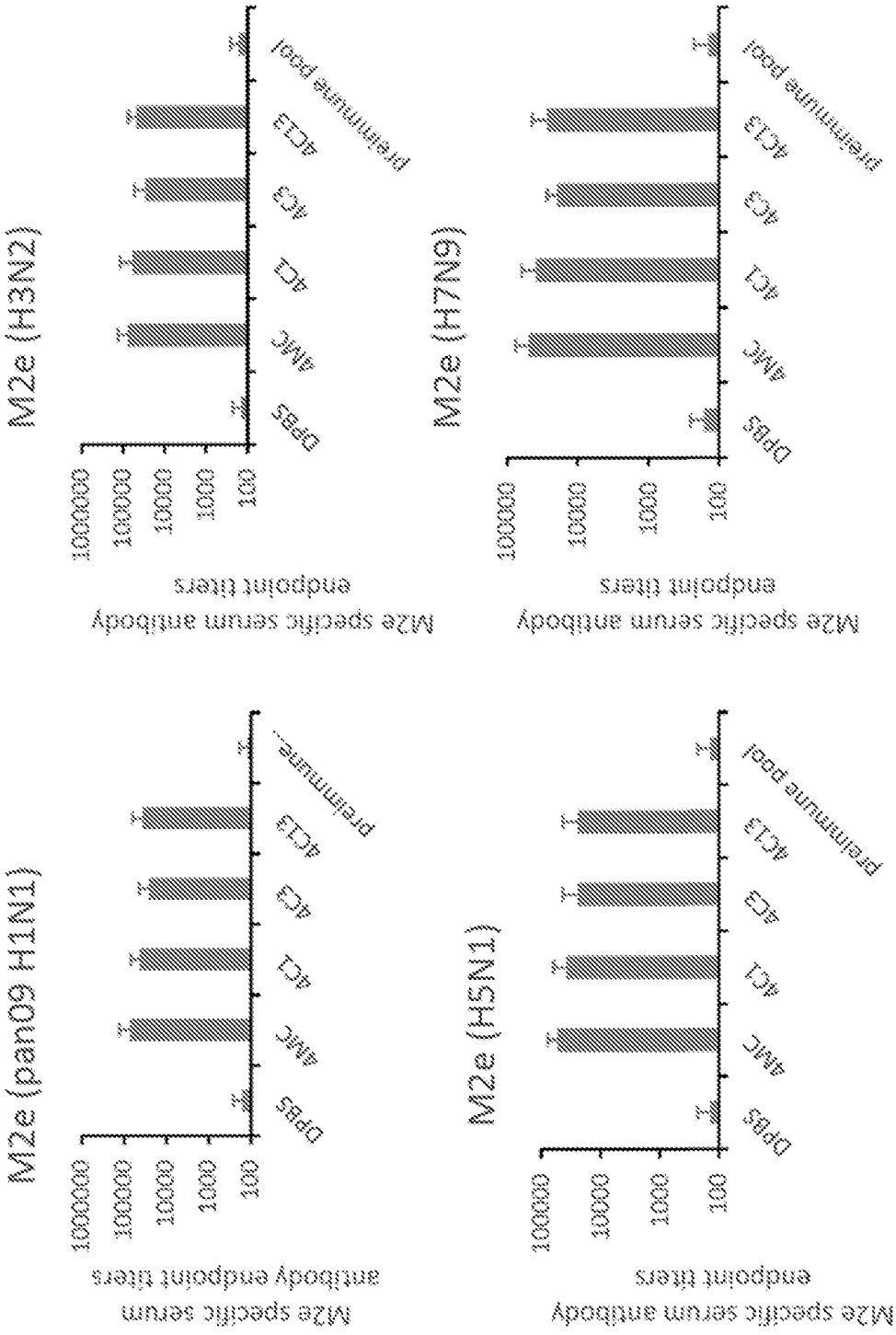

M2e (H3N2)
M2e specific serum antibody endpoint titers
1000000
100000
10000
1000
100
DPBS
4MC
4C1
4C3
4C13
preimmune pool
M2e (H7N9)
M2e specific serum antibody endpoint titers
1000000
10000
1000
100
DPBS
4MC
4C1
4C3
4C13
preimmune pool
M2e (pan09 H1N1)
M2e specific serum antibody endpoint titers
1000000
100000
10000
1000
100
DPBS
4MC
4C1
4C3
4C13
preimmune
M2e (H5N1)
M2e specific serum antibody endpoint titers
1000000
10000
1000
100
DPBS
4MC
4C1
4C3
4C13
preimmune pool

FIG. 6B

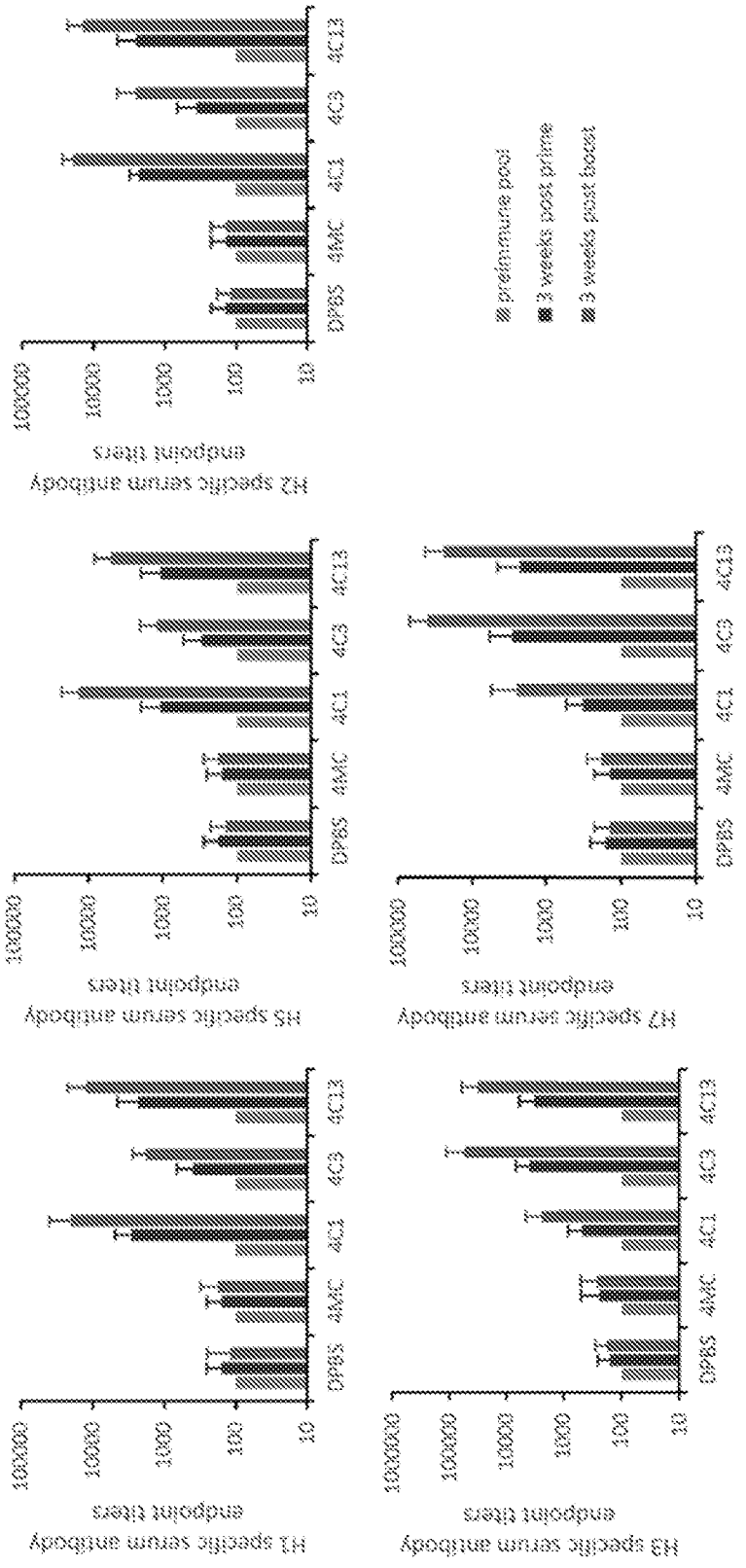

H1 specific serum antibody endpoint titers
H2 specific serum antibody endpoint titers
H3 specific serum antibody endpoint titers
H5 specific serum antibody endpoint titers
H7 specific serum antibody endpoint titers
DPBS
4MC
4C1
4C3
4C13
pre-immune post
3 weeks post prime
3 weeks post boost

FIG. 6C

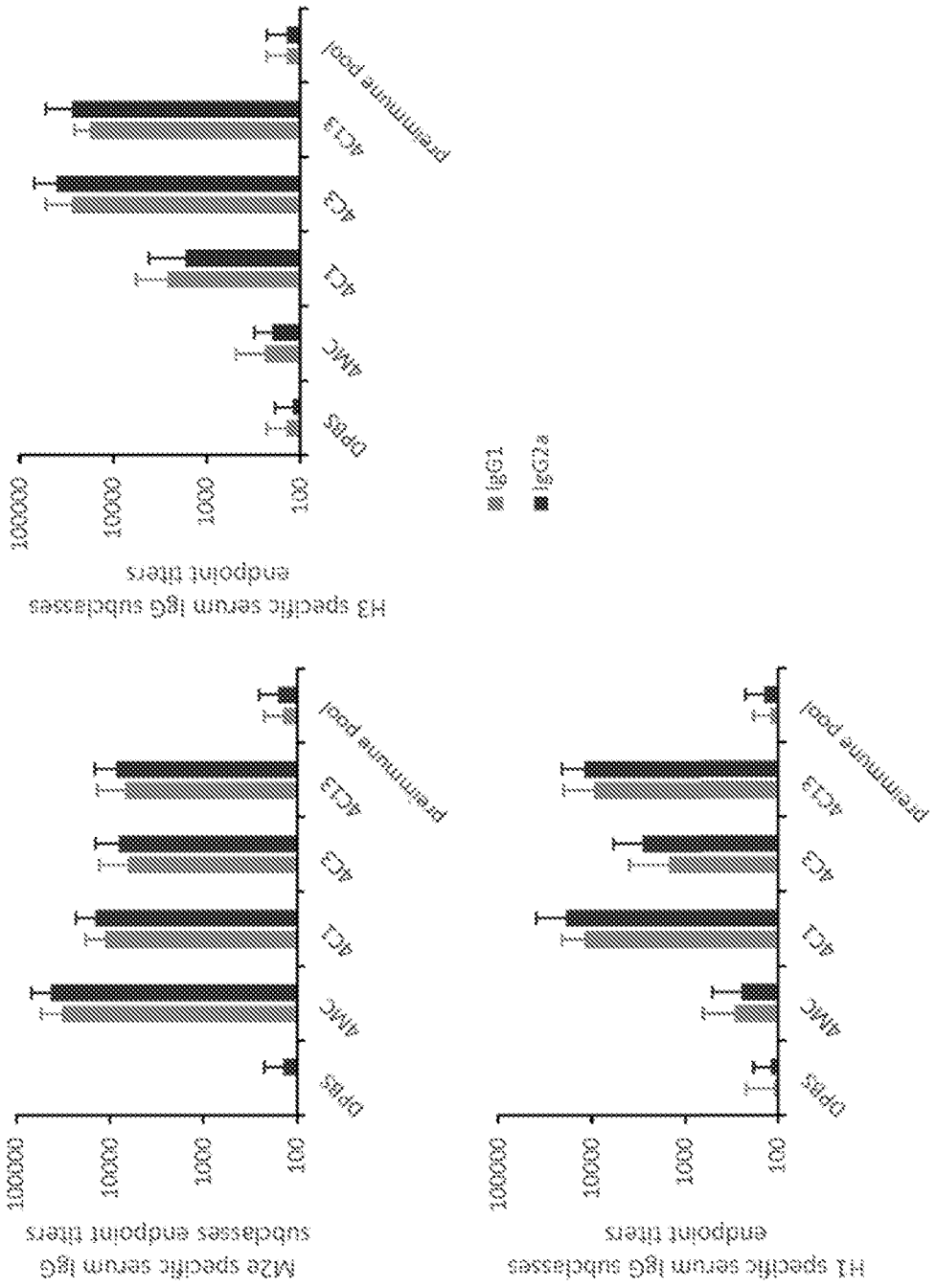

H3 specific serum IgG subclasses endpoint titers
M2e specific serum IgG subclasses endpoint titers
H1 specific serum IgG subclasses endpoint titers
IgG1
IgG2a
100000
10000
1000
100
DPBS
4MC
4C1
4C
4C13
Preimmune Pool

FIG. 6D

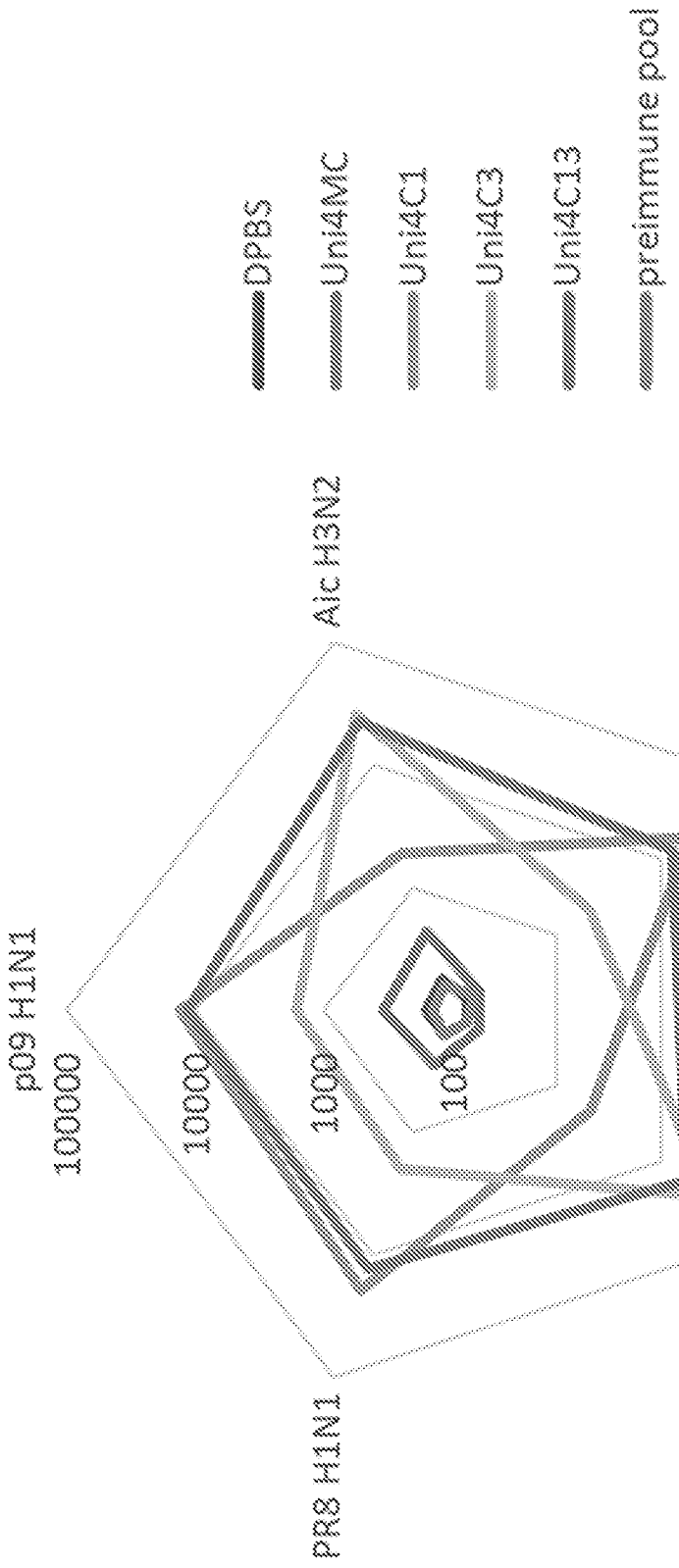

DPBS
Uni4MC
Uni4C1
Uni4C3
Uni4C13
preimmune pool
A/c H3N2
Vtn H5N1
SH H7N9
PR8 H1N1
p09 H1N1
100000
10000
1000
100

FIG. 6E

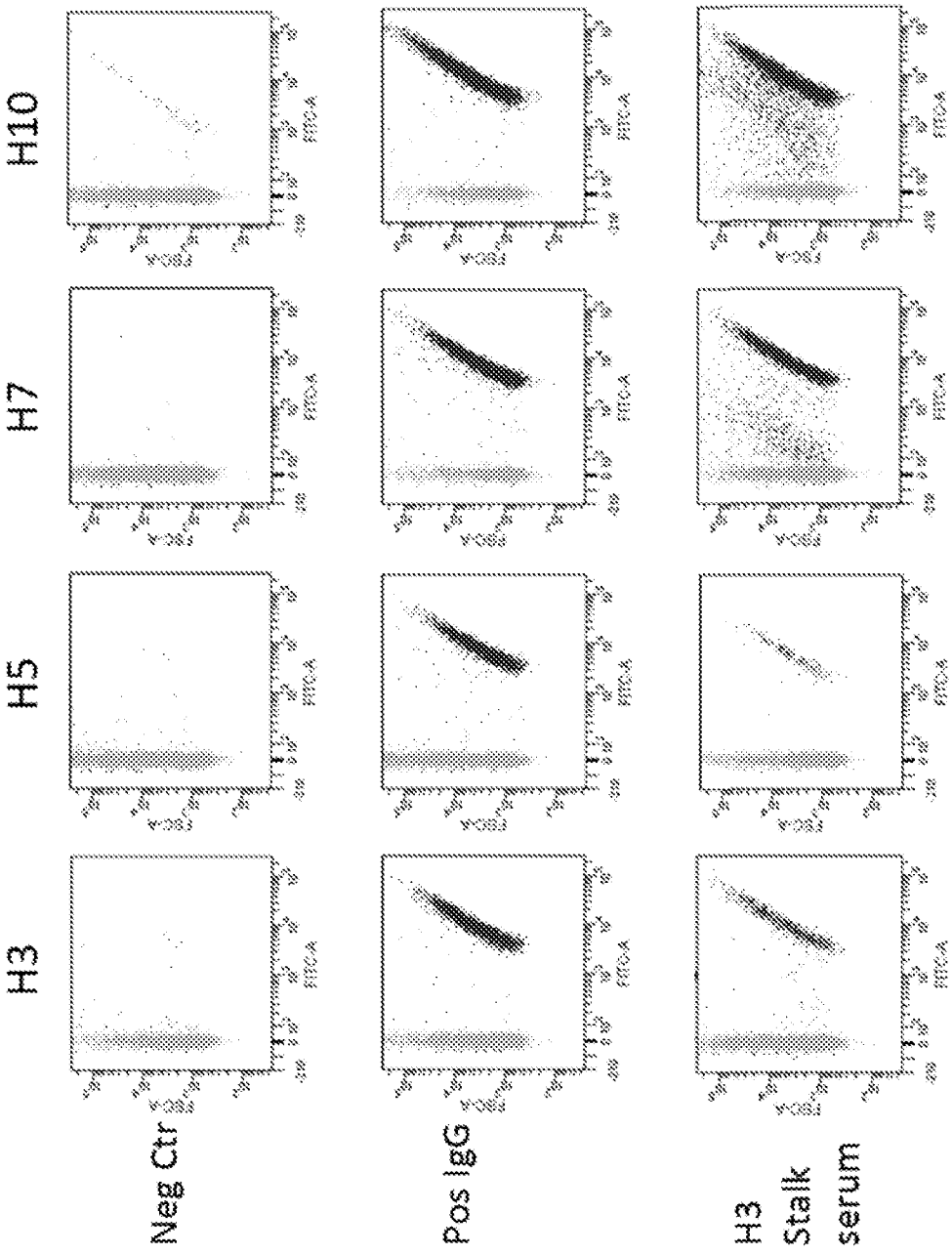


FIG. 6F

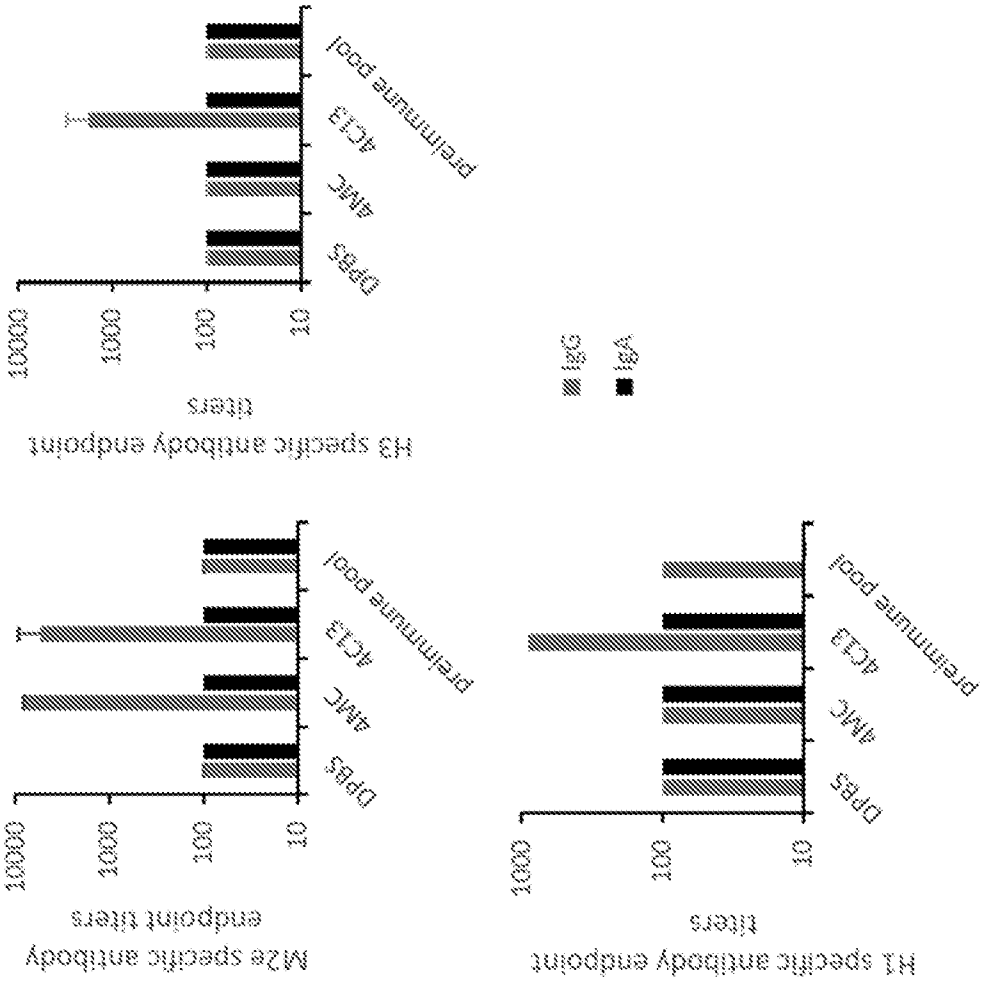

IgG
IgA
H1 specific antibody endpoint titers
M2e specific antibody endpoint titers
H3 specific antibody endpoint titers
10000
1000
100
10
DPBS
4MC
4C13
preimmune pool

FIG. 6G

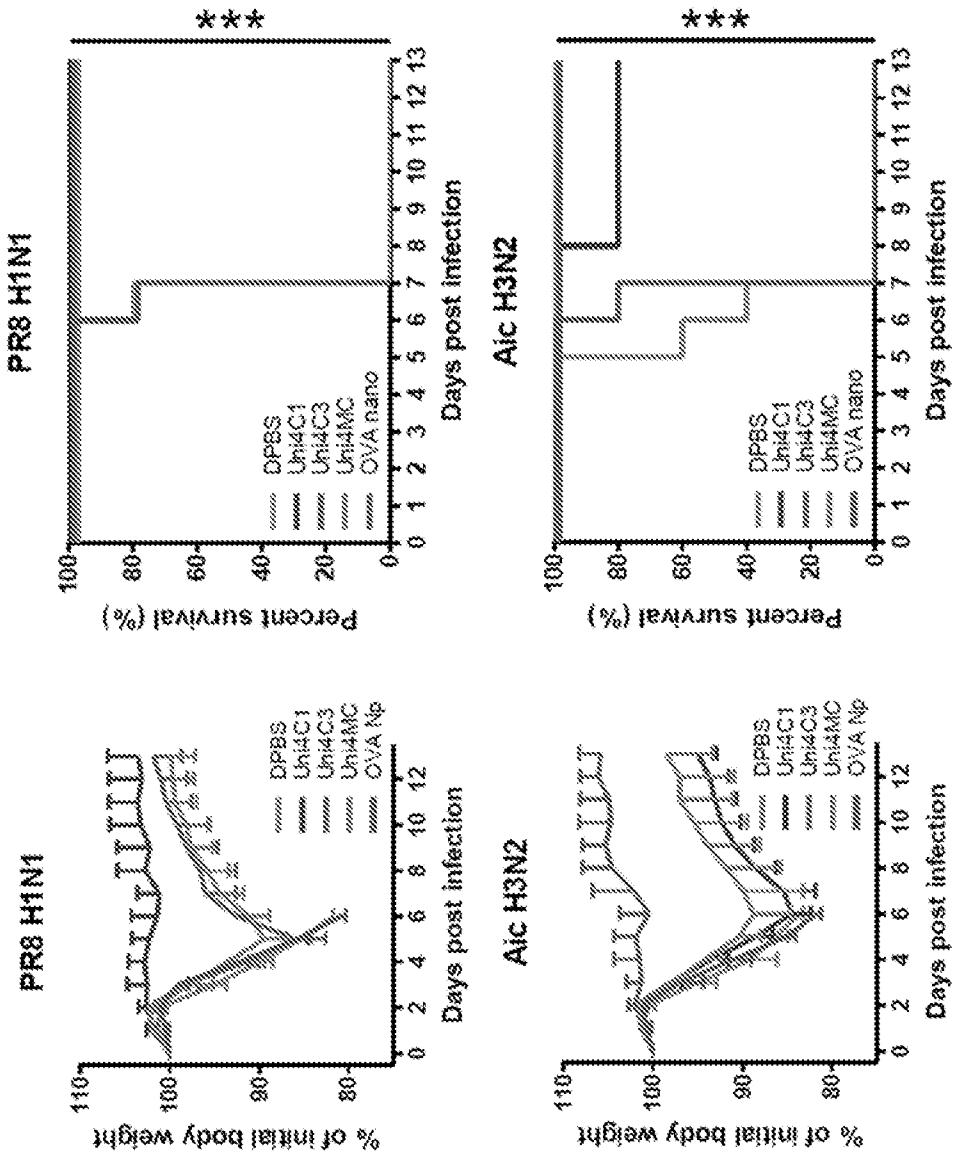

PR8 H1N1
Aic H3N2
Percent survival (%)
Days post infection
% of initial body weight
DPBS
Uni4C1
Uni4C3
Uni4MC
OVA nano
OVA Np
***

FIG. 7A

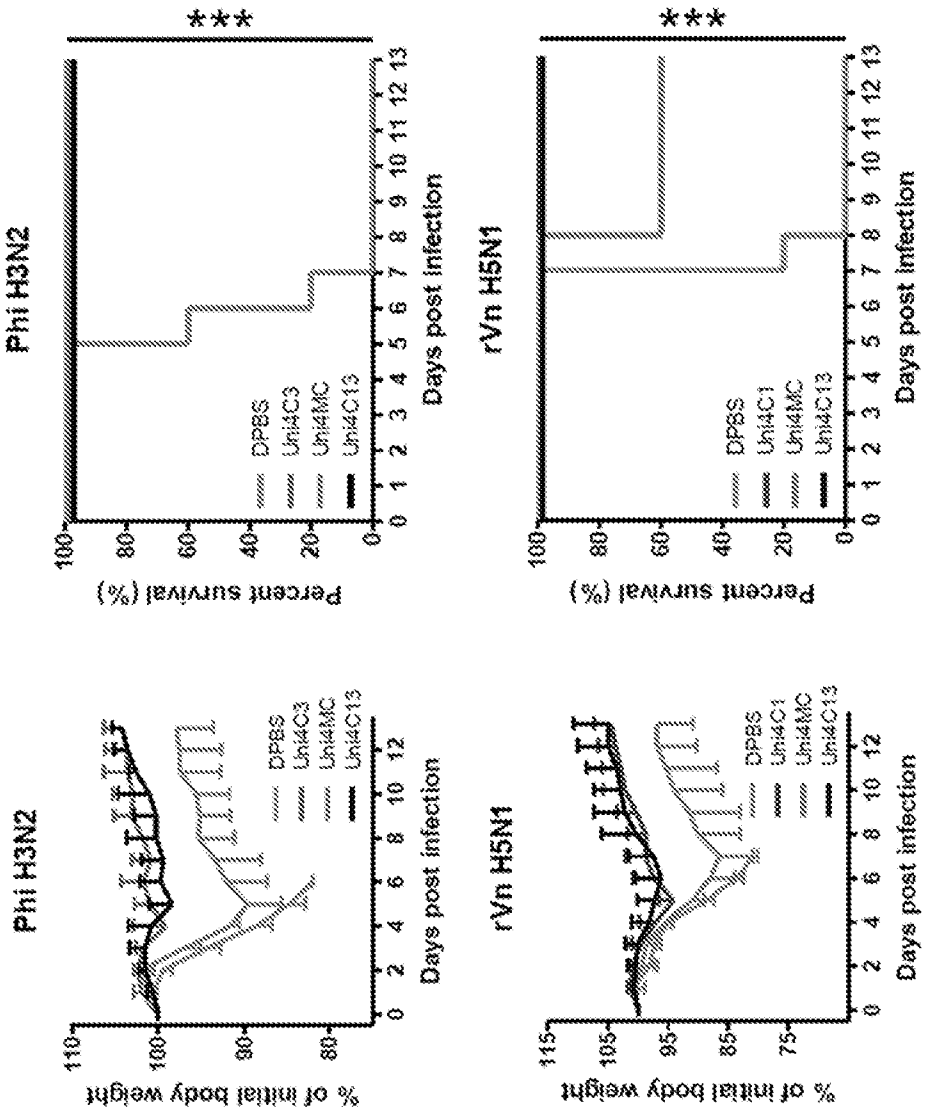

Phi H3N2
Percent survival (%)
Days post infection
DPBS
Uni4C3
Uni4MC
Uni4C13
***
rVn H5N1
Percent survival (%)
Days post infection
DPBS
Uni4C1
Uni4MC
Uni4C13
***
Phi H3N2
% of initial body weight
Days post infection
DPBS
Uni4C3
Uni4MC
Uni4C13
rVn H5N1
% of initial body weight
Days post infection
DPBS
Uni4C1
Uni4MC
Uni4C13

FIG. 7A (cont. . .)

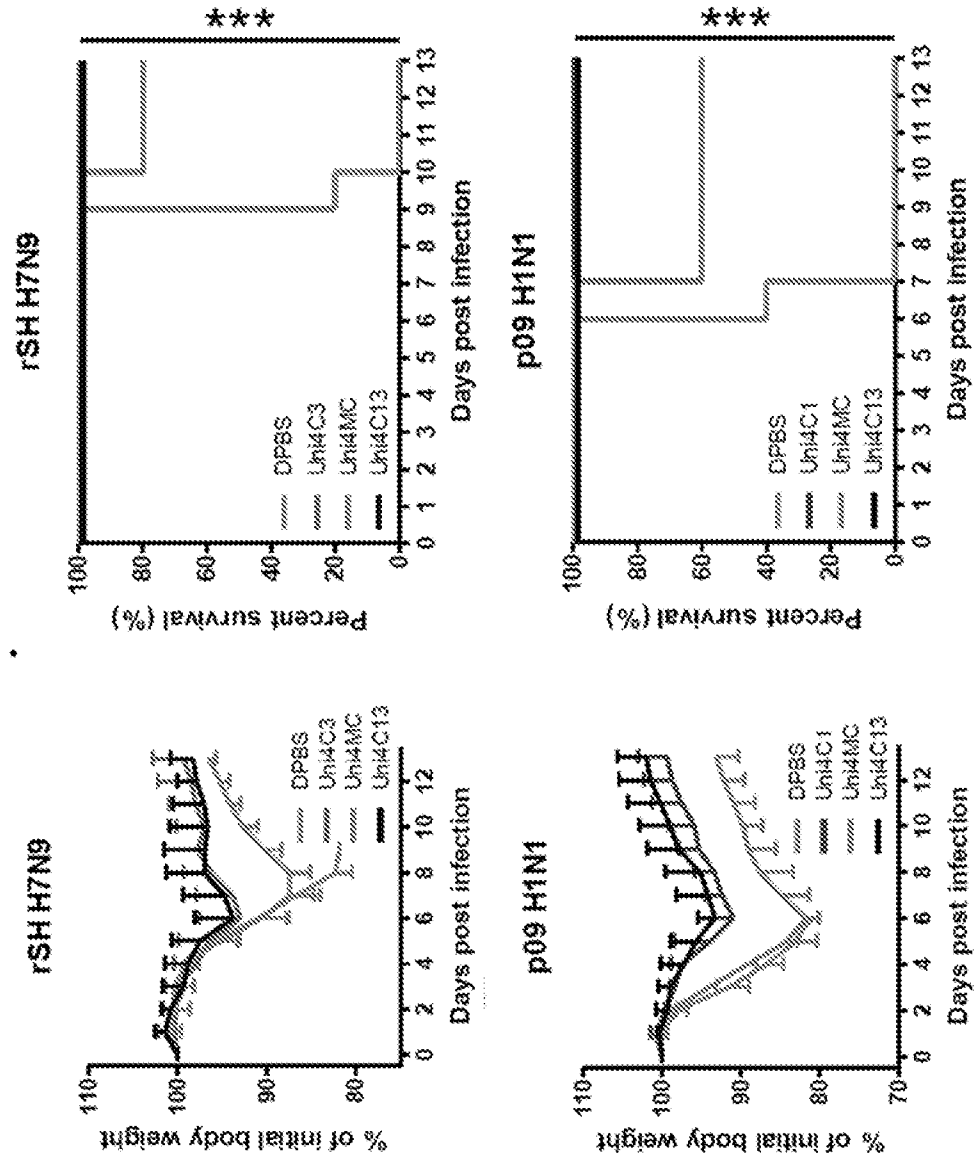

rSH H7N9
p09 H1N1
***
***
Percent survival (%)
% of initial body weight
Days post infection
DPBS
Uni4C3
Uni4C1
Uni4MC
Uni4C13

FIG. 7A (cont. . .)

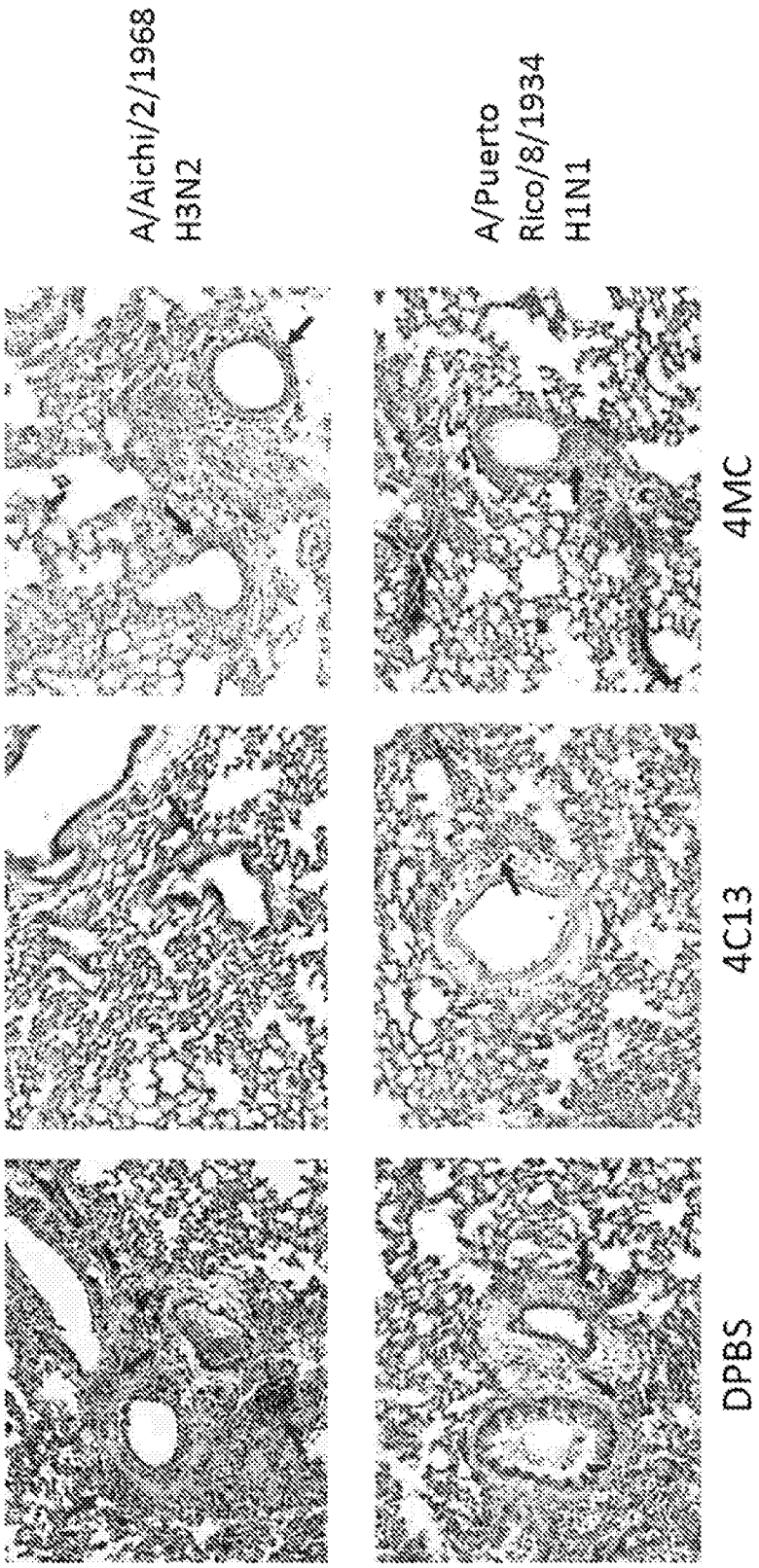


FIG. 8

Low titer High titer

Virus subtypes	Pre-challenge sera three weeks post boost						Subunit protein sera or convalescent sera			
	Pre-immune	DPBS	Uni4MC	Uni4C1	Uni4C3	Uni4C13	PR8 H1	Aic H3	rVn HSN1	rSH H7
PR8 H1N1	<10	<10	<10	<10	<10	<10	160	<10	40	<10
Aic H3N2	<10	<10	<10	<10	<10	<10	<10	640	<10	<10
rVn HSN1	<10	<10	<10	<10	<10	<10	<10	<10	160	<10
rSH H7N9	<10	<10	<10	<10	<10	<10	<10	<10	<10	80
D68 H3N2	<10	<10	<10	<10	<10	<10	<10	640	<10	<10

FIG. 9

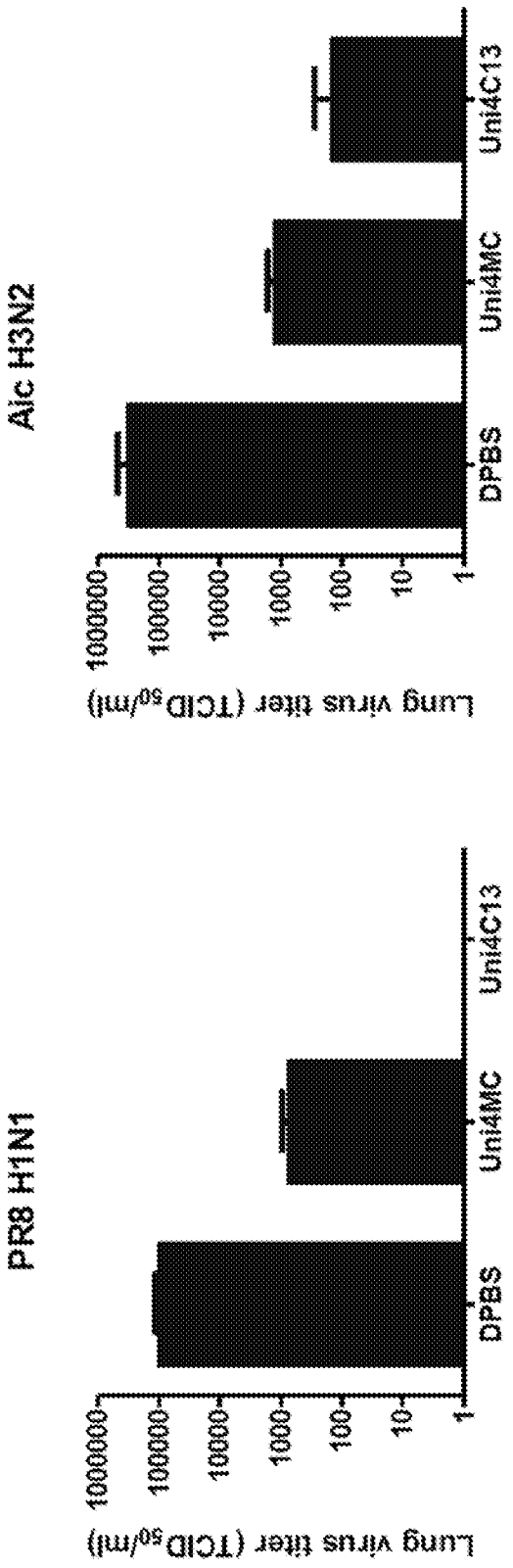

PR8 H1N1
Aic H3N2
Lung virus titer (TCID50/ml)
1000000
100000
10000
1000
100
10
1
DPBS
Uni4MC
Uni4C13

FIG. 10

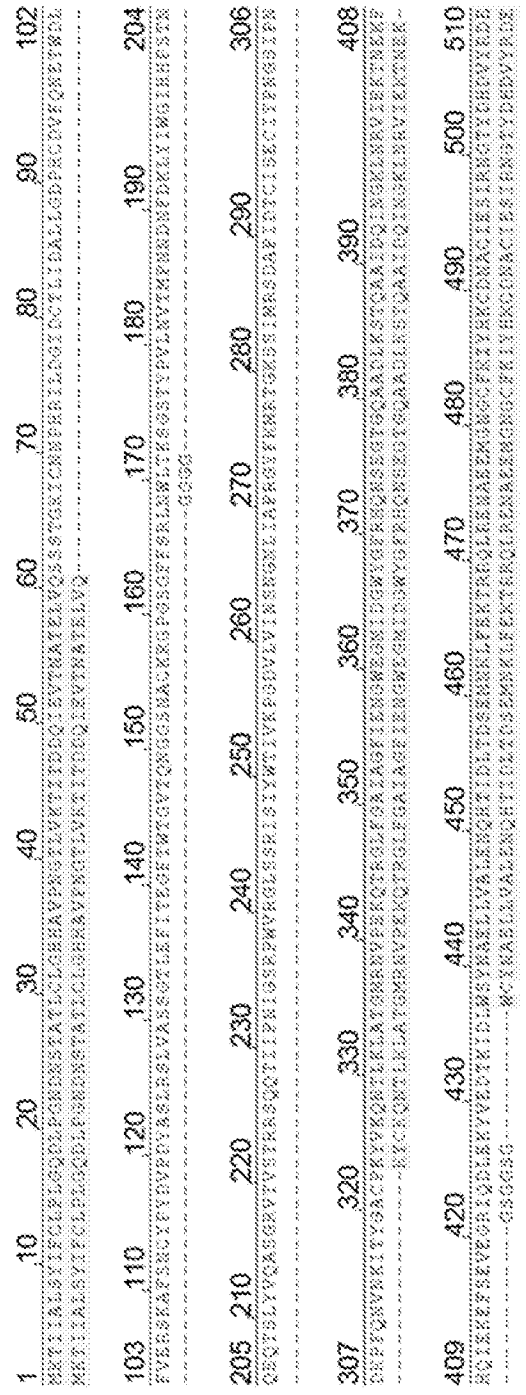


FIG. 11A

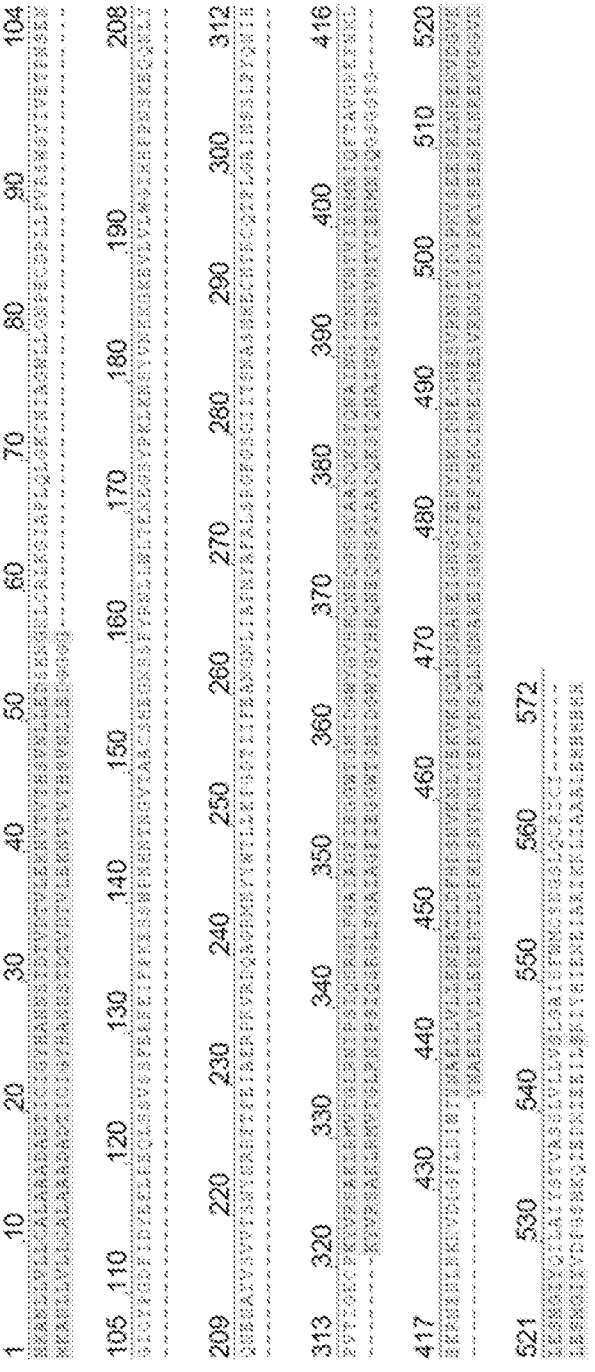


FIG. 11B

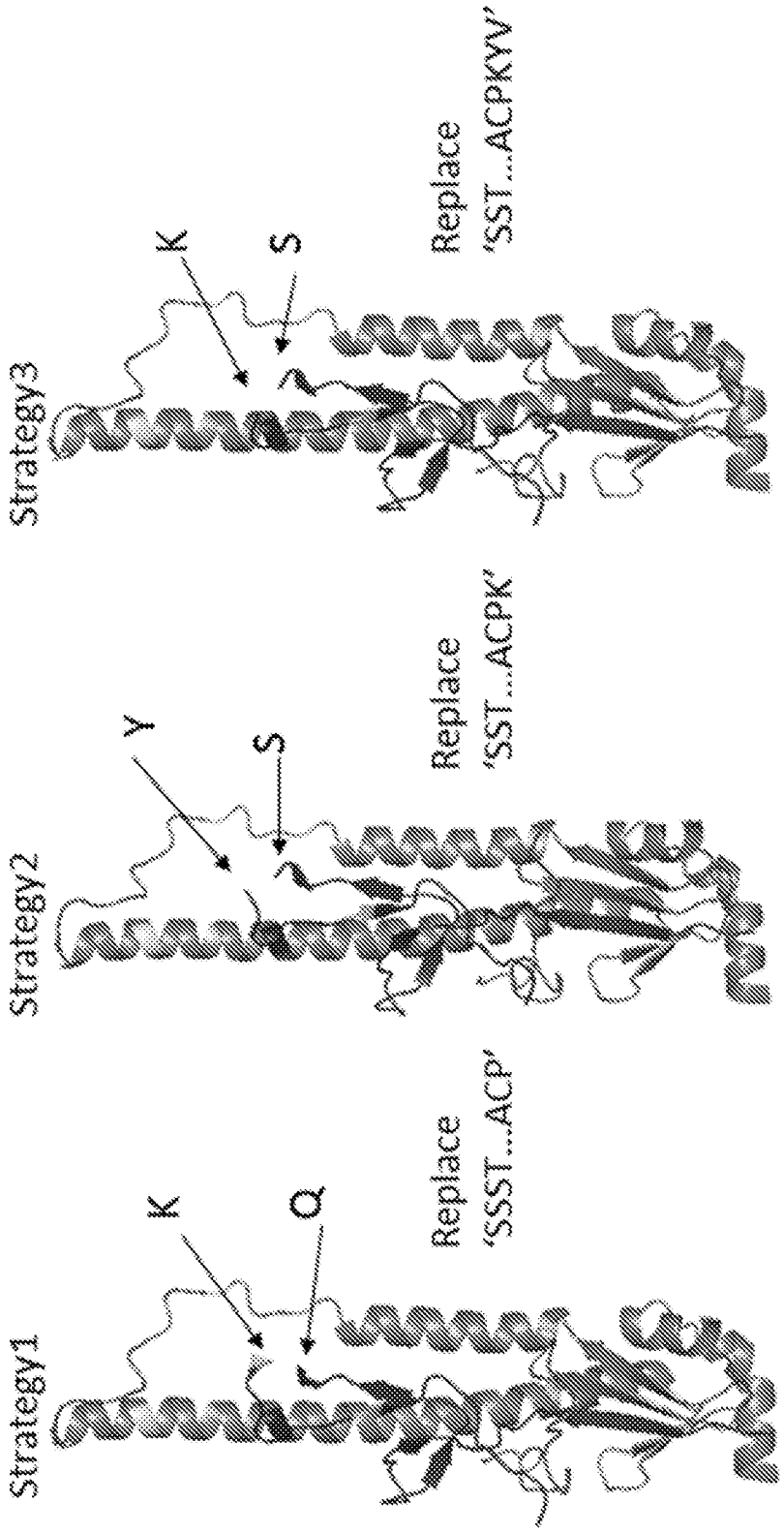
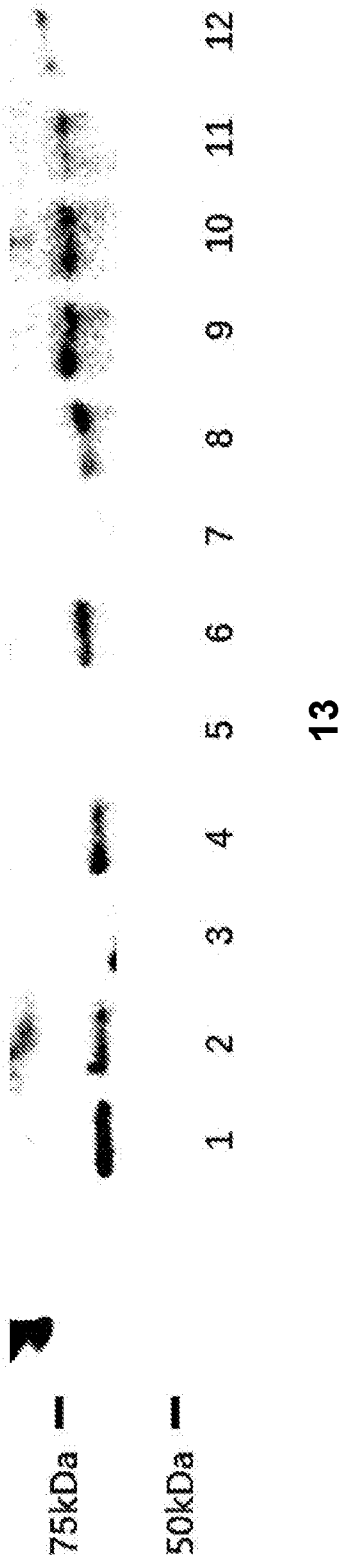

Strategy1
K
Q
Replace
'SSST...ACP'
Strategy2
Y
S
Replace
'SST...ACPK'
Strategy3
K
S
Replace
'SST...ACPKYV'

FIG. 12

FIG. 13



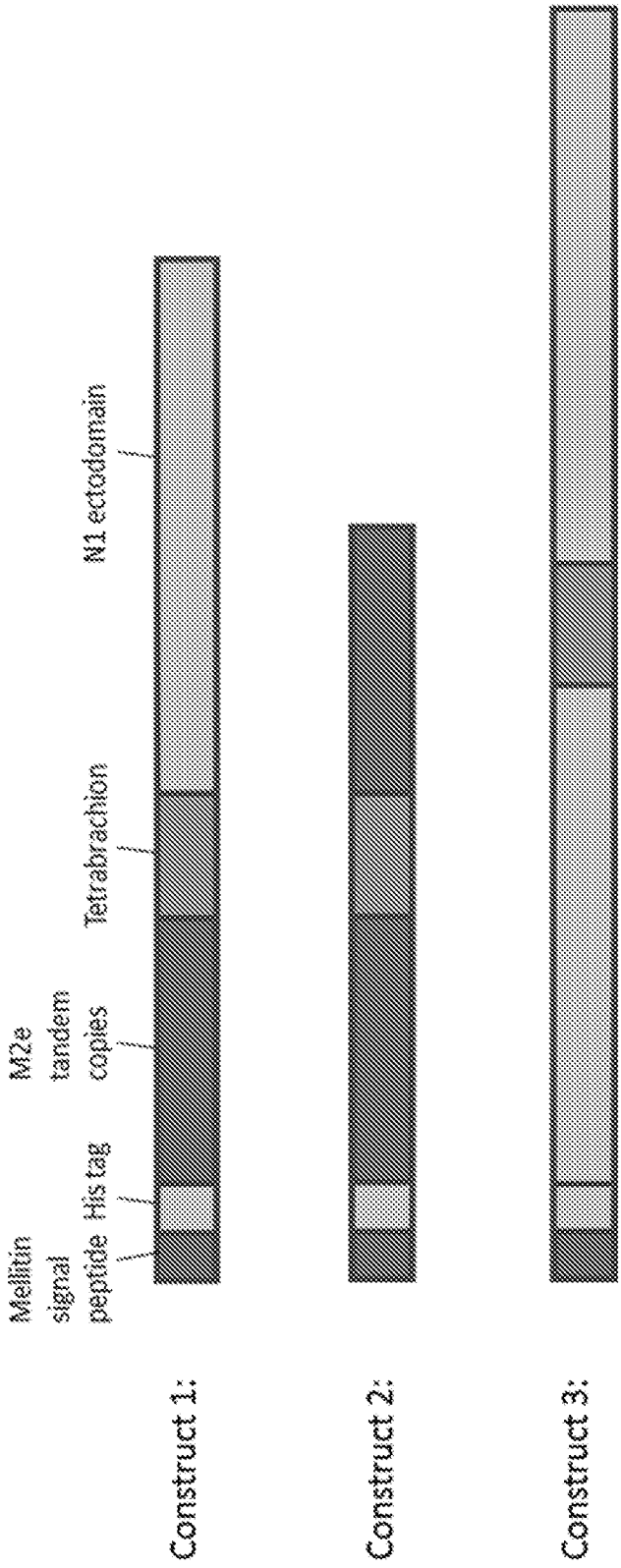

Mellitin signal peptide
His tag
M2e tandem copies
Tetrabrachion
N1 ectodomain
Construct 1:
Construct 2:
Construct 3:

FIG. 14

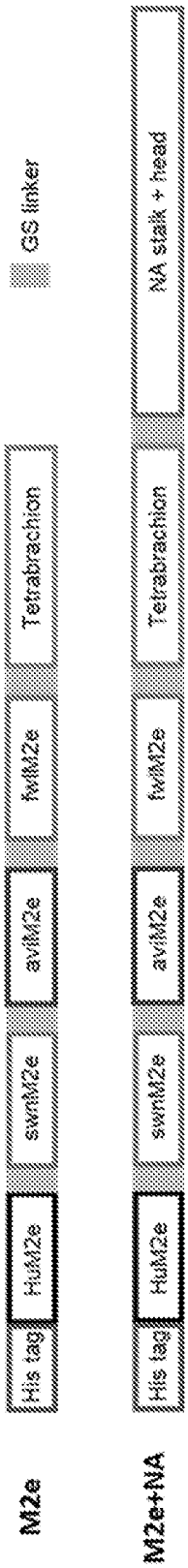

M2e
His tag
HuM2e
swnM2e
aviM2e
fwiM2e
Tetrabrachion
GS linker
M2e+NA
His tag
HuM2e
swnM2e
aviM2e
fwiM2e
Tetrabrachion
NA stalk + head

FIG. 15A

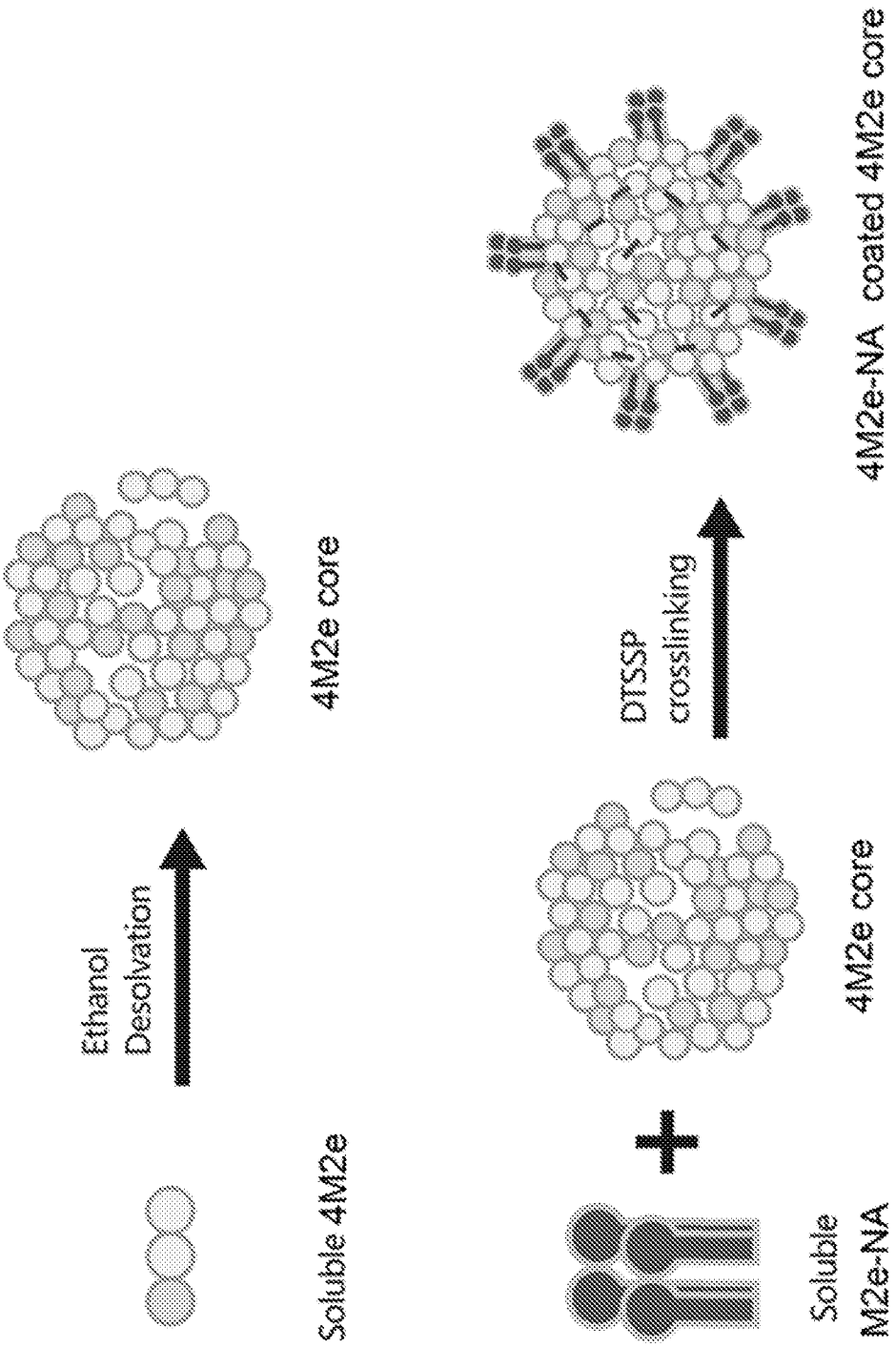

Soluble 4M2e
Ethanol Desolvation
4M2e core
Soluble M2e-NA
4M2e core
DTSSP crosslinking
4M2e-NA coated 4M2e core

FIG. 15B

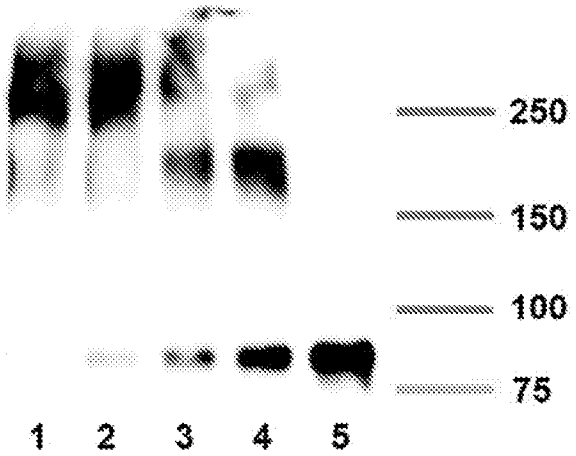


FIG. 16A

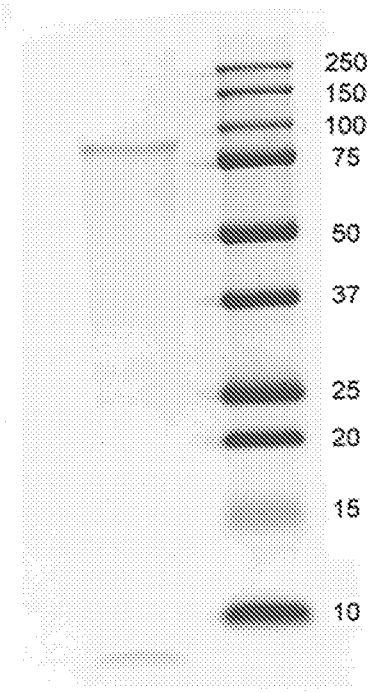


FIG. 16B

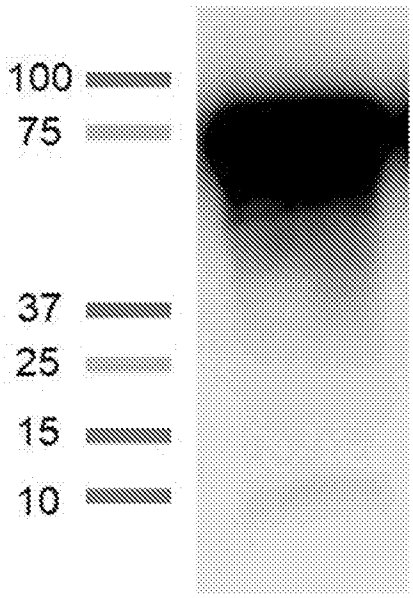


FIG. 16C

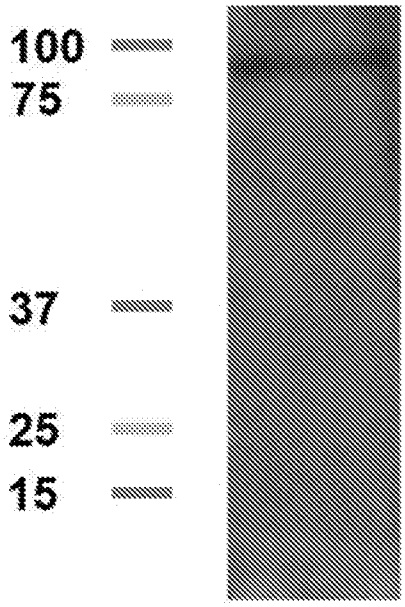


FIG. 16D

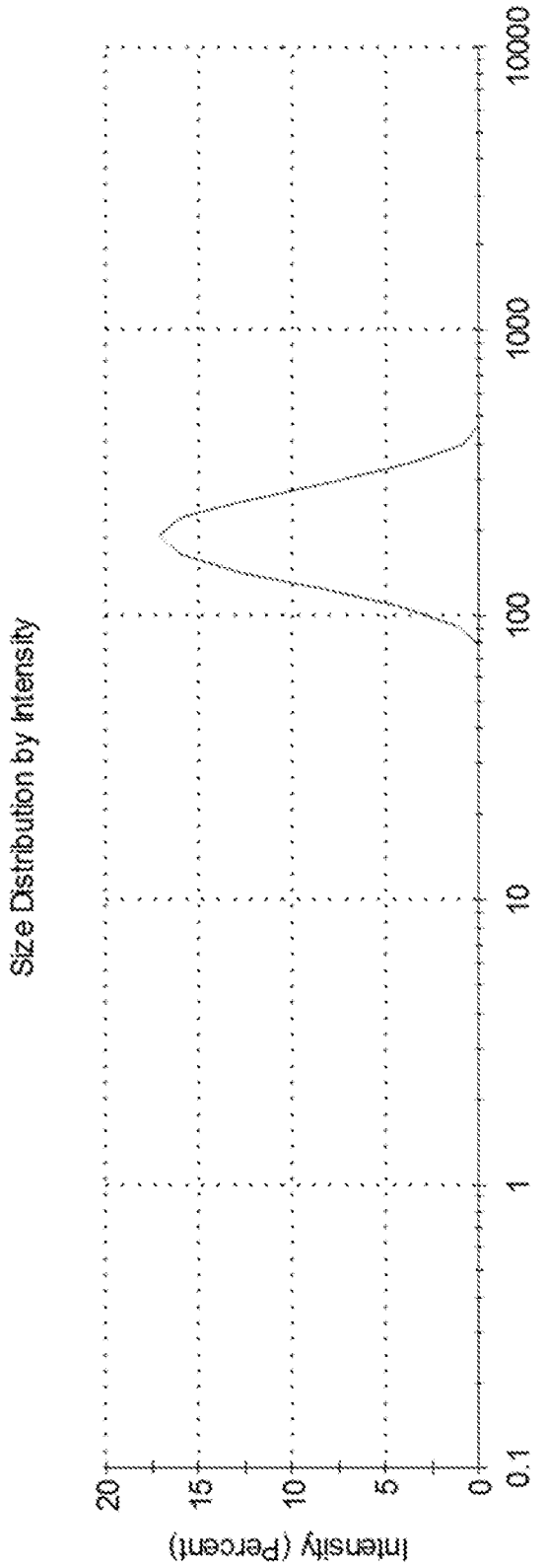

Size Distribution by Intensity
Intensity (Percent)
20
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Record 92: h2o 1

FIG. 16E

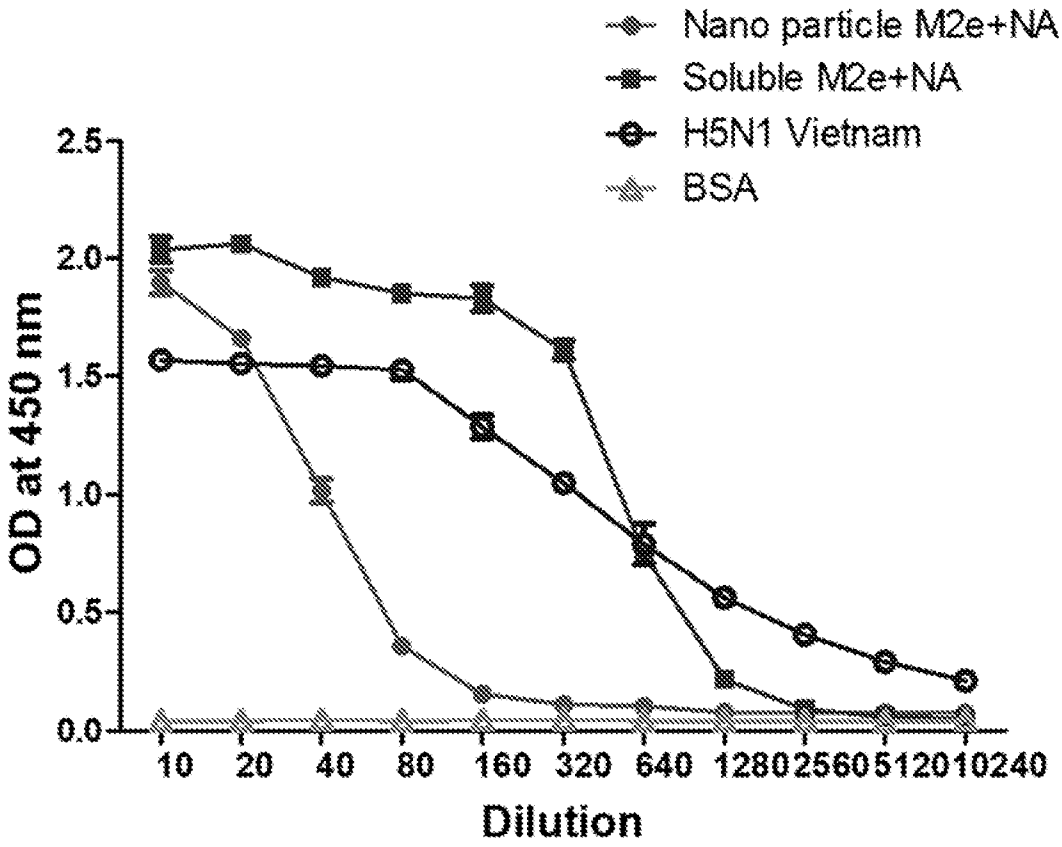

Nano particle M2e+NA
Soluble M2e+NA
H5N1 Vietnam
BSA
OD at 450 nm
2.5
2.0
1.5
1.0
0.5
0.0
10
20
40
80
160
320
640
1280
2560
5120
10240
Dilution

FIG. 16F

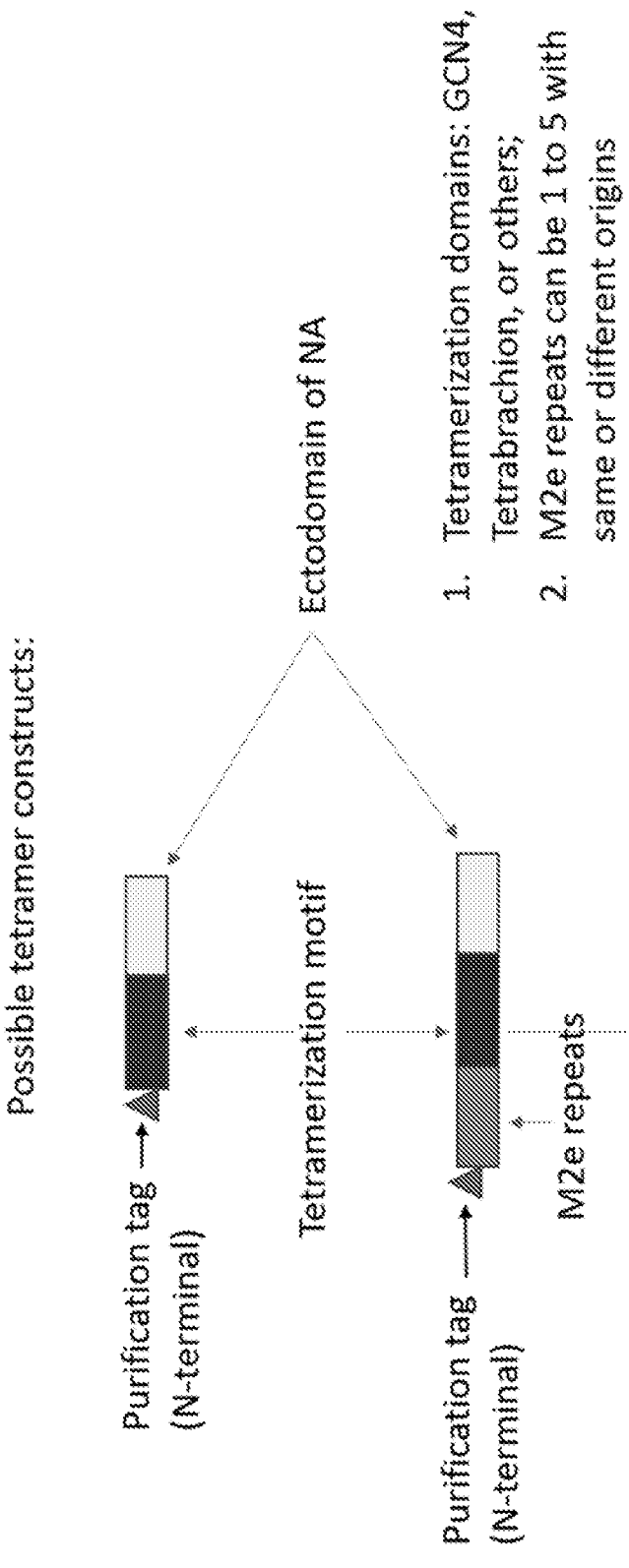

Possible tetramer constructs:
Purification tag
(N-terminal)
Tetramerization motif
Ectodomain of NA
Purification tag
(N-terminal)
M2e repeats
Purification tag
(N-terminal)
M2e repeats
(1 to 5)
1. Tetramerization domains: GCN4, Tetrabrachion, or others;
2. M2e repeats can be 1 to 5 with same or different origins

FIG. 17

```
B/Brisbane/60/2008:   1     CTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSHFANLKGTETRGKLCPKCLNCTDL  60
                            ||||||||||||||||||||||||||||||||
B/hrHA:               1     CTGITSSNSPHVVKTATQGEVNVTGVIPLTT TGGGGC----------------------  37

B/Brisbane/60/2008:   61    DVALGRPKCTGKIPSARVSILHEVRPVTSGCFPIMHDRTKIRQLPNLLRGYEHIRLSTHN  120
B/hrHA:               37    ---------------------------------------------- ------------  37

B/Brisbane/60/2008:   121   VINAENAPGGPYKIGTSGSCPNITNGNGFFATMAWAVPKNDKNKTATNPLTIEVPYICTE  180
B/hrHA:               37    ------------------------------------------------------------  37

B/Brisbane/60/2008:   181   GEDQITVWGFHSDNETQMAKLYGDSKPQKFTSSANGVTTHYVSQIGGFPNQTEDGGLPQS  240
B/hrHA:               37    ------------------------------------------------------------  37

B/Brisbane/60/2008:   241   GRIVVDYMVQKSGKTGTITYQRGILLPQKVWCASGRSKVIKGSLPLIGEADCLHEKYGGL  300
B/hrHA:               37    ------------------------------------------------------------  37

B/Brisbane/60/2008:   301   NKSKPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFFGAIAGFLEGGWEG  360
                                                  |||||||||||||||||| | ||||||||||||||||
B/hrHA:               38    ---------------------VKTPLKLANGTKYRPPAKLLTEQGFFGAIAGFLEGGWEG   76

B/Brisbane/60/2008:   36    MIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDELHNE  420
                            ||||||||||||||||||||||||||||||||||||||||||||||
B/hrHA:               77    MIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSEL EGGGSS------------  124

B/Brisbane/60/2008:   421   ILELDEKVDDLRADTISSQIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVEIGNG  480
                            ||||||||||||||| |||||||||||||||||||||||||||||| ||||||||||||
B/hrHA:               125   ------------ADTICSQIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVEIGNG  172

B/Brisbane/60/2008:   481   CFETKHKCNQTCLDRIAAGTFDAGEFSLPTFDSLNITAASLNDDGLDNHTILLYYSTAAS  540
                            ||||||||||||||||||||||||||||||
B/hrHA:               173   CFETKHKCNQTCLDRIAAGTFDAGEFSLPT PGSSGGSSGMKQIEDKIEEILSKIYHIEN E  231

B/Brisbane/60/2008:   541   S------  541

B/hrHA:               232   IARIKKL  239
```

FIG. 18

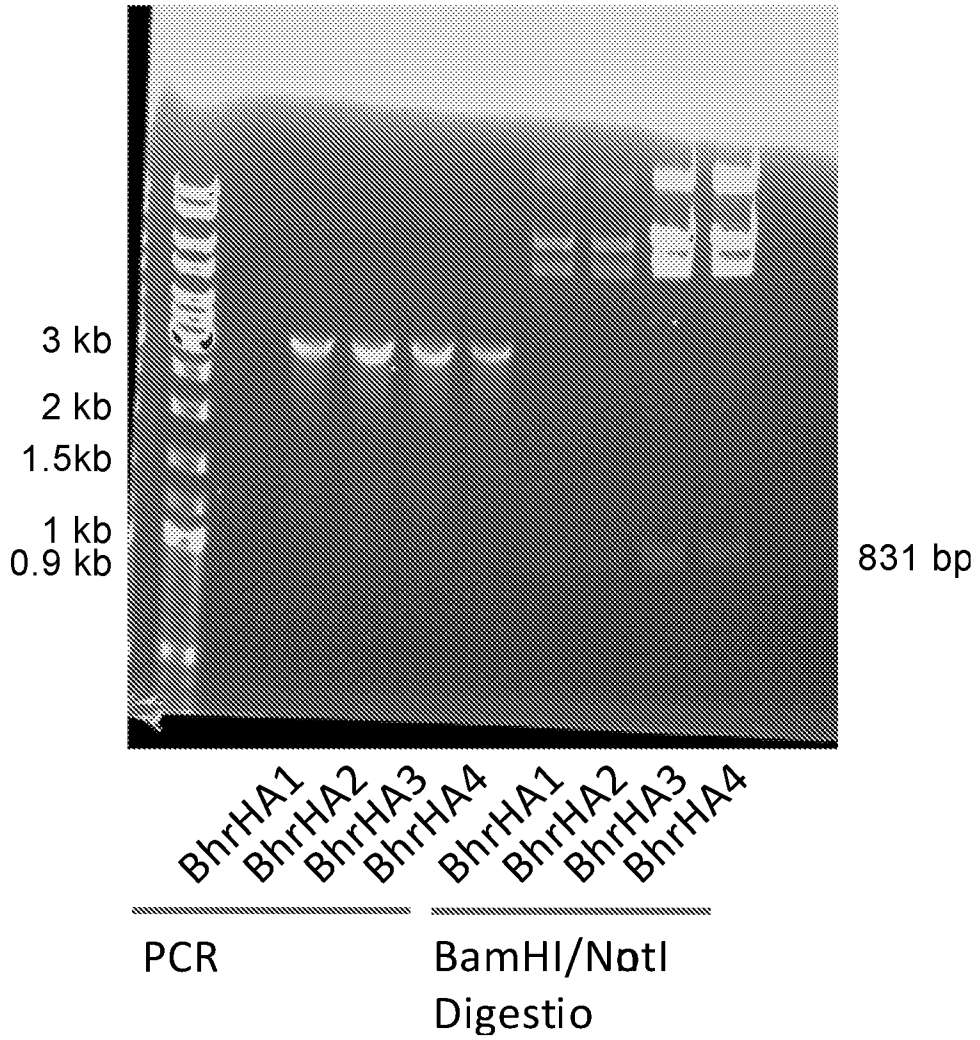

3 kb
2 kb
1.5kb
1 kb
0.9 kb
831 bp
BhrHA1
BhrHA2
BhrHA3
BhrHA4
BhrHA1
BhrHA2
BhrHA3
BhrHA4
PCR
BamHI/NotI
Digestio

FIG. 19A

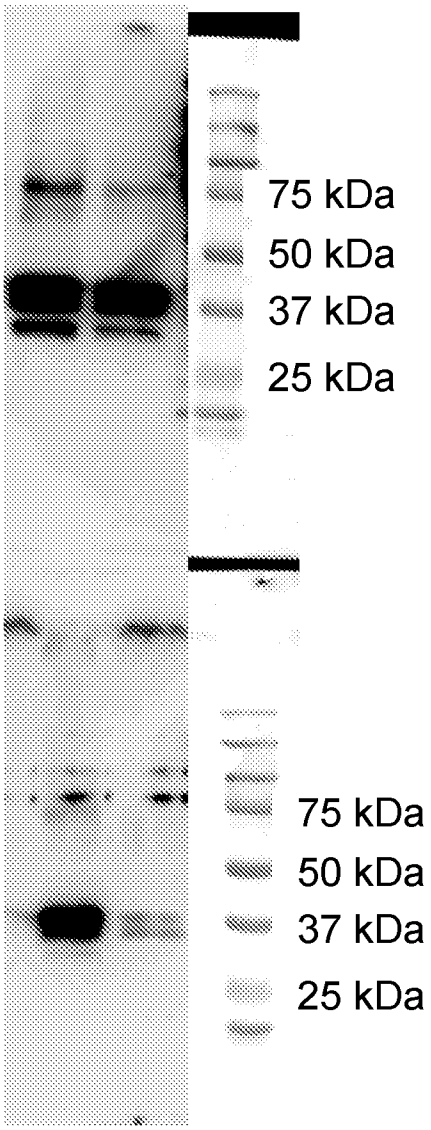


FIG. 19B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/54335

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/145, A61K 39/12, C07K 14/11 (2019.01)
CPC - A61K 39/145, A61K 39/12, C07K 14/005, C12N 2760/16123

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)
See Search History Document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	STEEL et al. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio. 18 May 2010. Vol 1, No 1, Article e00018-10 (pp 1-9). Especially Abstract; Fig 1; page 2, col 1, para 2; page 2, col 2, para 4; page 8, col 2, para 3	1-5 --- 6-9
Y	WO 2013/116656 Al (EMORY UNIVERSITY) 08 August 2013 (08.08.2013) Claim 1; Claim 7; page 3, ln 21-23; page 4, ln 24-25	6-7, (8-9)/(6-7)
Y	UniProtKB Accession No. HEMA_I68A0 "Pre-mRNA-splicing factor 38A" (21 July 1986) [online]. [Retrieved on 30 January 2019]. Retrieved from the internet:<https://www.uniprot.org/uniprot/P03437> Entire document	8-9
Y	US 2015/0273048 A1 (GEORGIA STATE UNIVERSITY RESEARCH FOUNDATION) 1 October 2015 (01.10.2015) Claim 1; Claim 11; Claim 12; Claim 13; Claim 21	15-19
Y	US 2016/0361411 A1 (GINDY et al.) 15 December 2015 (15.12.2015) Abstract; para [0136]; para [0144]	15-19
Y	CHEN et al. Fusion protein linkers: property, design and functionality. Advanced drug delivery reviews. 15 October 2013. Vol 65, No 10, pp1357-1369. Especially Abstract; page 4, para 2; page 4, para 3	18-19

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"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
29 January 2019	27 FEB 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/54335

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

3. ☒ Claims Nos.: 10-14, 20-45
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

--Please see extra sheet-

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/54335

Continuation of Box No. III. Observations where unity of invention is lacking

Group I: claims 1-9, drawn to a composition comprising a polypeptide comprising a truncated influenza hemagglutin (HA) protein lacking a head domain, and a nanoparticle or VLP coated with truncated HA protein.

Group II: claims 15-19, drawn to a process of forming a nanoparticle by crosslinking a influenza protein domains with a crosslinking agent.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I includes the special technical feature of a composition comprising a truncated influenza hemagglutin (HA) protein lacking a head domain, not required by Group II.

Group II includes the special technical feature of a fusion protein comprising a series of 2 to 8 influenza virus matrix protein 2 extracellular (M2e) domains linked to a multimerization domain, not required by Group I.

Common Technical Features

The inventions of Groups I and II share the technical feature of a nanoparticle coated with a polypeptide comprising an influenza protein.

However, these shared technical features do not represent a contribution over prior art in view of the article "Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection" by Yassine et al. (hereinafter 'Yassine') (Nature Medicine. September 2015, Vol 21, No 9, pp 1065-1079). Yassine discloses a nanoparticle coated with a polypeptide comprising an influenza protein (page 1, col 1, para 1-"We hypothesized that an immune response focused on the conserved HA stem could elicit broad heterosubtypic influenza protection and therefore used iterative structure-based design to develop HA stabilized-stem (HA-SS) glycoproteins that lack the immunodominant HA head region (Fig. 1 and Supplementary Fig. 1)."; page 2, col 2, para 1-"We therefore genetically fused Gen4, Gen5, Gen6' or Gen6 HA-SS through their respective HA C-termini (replacing the foldon) to the ferritin subunit from Helicobacter pylori to create self-assembling HA-SS nanoparticles (HA-SS-np)."; page 5, col 1, para 1-"Using structure-based design to circumvent immunodominant responses to the HA head domain, combined with a nanoparticle antigen-display platform, we successfully generated an HA stem-only nanoparticle vaccine immunogen that elicits antibody mediated heterosubtypic protective immunity against H5N1 disease in mice and ferrets").

As said technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Groups I and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.