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(71) Applicants: **CZ BIOHUB SF, LLC** [US/US]; 499 Illinois Street, San Francisco, California 94158 (US). **THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY** [US/US]; Office of the General Counsel, Bldg. 170, Third Floor, Main Quad, P. O. Box 20386, Stanford, California 94305-2038 (US).

(72) Inventors: **XU, Duo**; c/o The Board of Trustees of the Leland Stanford Junior University, Office of the General Counsel, Bldg. 170, Third Floor, Main Quad, P.O. Box 20386, Stanford, California 94305-2038 (US). **KIM, Peter S.**; c/o The Board of Trustees of the Leland Stanford Junior University, Office of the General Counsel, Bldg. 170, Third Floor, Main Quad, P.O. Box 20386, Stanford, California 94305-2038 (US).

(74) Agent: **MAO, Yifan** et al.; Kilpatrick Townsend & Stockton LLP, Mailstop: IP Docketing - 22, 1100 Peachtree Street, Suite 2800, Atlanta, Georgia 30309 (US).

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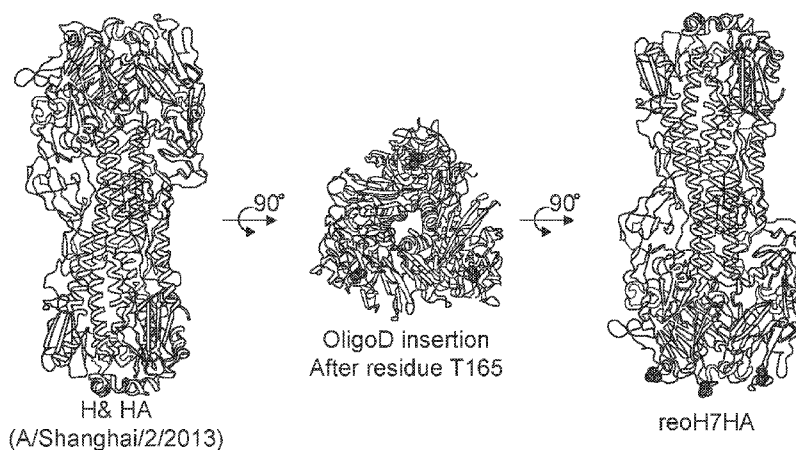


FIG. 1

(57) Abstract: New vaccine compositions comprising a modified antigen H7 HA bound to the surface of an adjuvant or carrier by electrostatic interactions are disclosed. The antigen of the vaccine composition is presented in a defined orientation on an adjuvant surface such that epitope accessibility is altered, and an immune response is redirected toward specific epitopes. In some embodiments the vaccine composition comprises one or more recombinant antigen polypeptides adsorbed to an alum particle. In some embodiments, the recombinant antigen polypeptide comprises a Region of Repetitive Carboxylic Groups (RRC) or a Region of Repetitive Lysyl/Guani-dino Groups (RRL).



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PCT Patent Application**DESIGN OF UNIVERSAL INFLUENZA VACCINE CANDIDATES VIA ANTIGEN REORIENTATION**

[0001] This application claims priority to US Provisional Application No. 63/510, 395, filed on June 27, 2023. The entire content of said provisional application is herein incorporated by reference for all purposes.

RELATED APPLICATION

[0002] The invention finds application in the fields of human and veterinary medicine.

FIELD OF THE INVENTION

[0003] The invention finds application in the fields of human and veterinary medicine.

STATEMENT OF GOVERNMENT SUPPORT

[0004] This invention was made with Government support under contract AI158125 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

[0005] Vaccines are among the most profound accomplishments of biomedical science in combating infectious diseases. The majority of current vaccines protect against infections by eliciting neutralizing antibody responses. Compared to traditional vaccines consisting of entire pathogens (inactivated or attenuated), subunit vaccines, particularly recombinant protein vaccines, are safer and easier to produce but often less immunogenic. Adjuvants are often co-administered with subunit vaccines to enhance the magnitude and durability of host immune responses. The most widely used adjuvant is aluminum hydroxide (alum), which has been safely used in various vaccines since the 1930s. The molecular mechanism of alum/protein interactions is complex. Alum is thought to create a 'depot effect' that enables the slow release of antigens from the immunization site while activating antigen-presenting

cells and inducing cytokine secretion. Despite its safety and prevalence, alum usually induces relatively ‘weak’ immune responses compared to other adjuvants (e.g., lipid-based adjuvants or cytosine phosphoguanosine oligodeoxynucleotides), potentially because antigens desorb from alum in the presence of interstitial fluid or serum and encounter in vivo clearance. Alum has an isoelectric point of 11 and a positive surface charge at physiological pH (7.4), which allows its attraction of negatively charged antigens through electrostatic interactions. Aluminum has a higher affinity for phosphate than hydroxyls, and phosphates can displace hydroxyls on the surface of alum. This ligand exchange reaction affords a stronger force for antigen binding to alum. Antigens with terminal phosphate groups have a high affinity for alum through such ligand exchange reactions.

[0006] Recently, Moyer et al. designed a peptide containing repeating units of phosphoserine (pSer) that can be conjugated to antigens with C-terminal cysteine residues (reduced). Compared to standard antigen-alum combinations, this conjugation led to a stable antigen-alum association, control of antigen-accessibility via C-terminal insertion of pSer and increased humoral antibody titers against antigens. Nonetheless, synthesis of pSer using solid-phase methods is at high cost and requires additional peptide purification processes, and the difficulties in the coupling and deprotection steps may lead to reduced yield. Additionally, the C-terminal introduction of cysteine residues anchored the antigen on alum and shifted antibody responses away from the base of the antigen. See Moyer et al., 2020, “Engineered immunogen binding to alum adjuvant enhances humoral immunity” *Nature Medicine* 26:430–440; Irvine et al., 2019, “Antigen-adjuvant coupling reagents and methods of use, US Patent No. US11224648.

[0007] Additional and better vaccine compositions are needed. This application incorporates the entire content of WO2023064631 by reference.

BRIEF SUMMARY OF THE INVENTION

[0008] In one aspect, provided herein is a recombinant antigen polypeptide comprises a Region of Repetitive Carboxylic Groups (RRC) or a Region of Repetitive Lysyl/Guanidino Groups (RRL) inserted after an amino acid residue in an amino acid sequence with at least 95% amino acid sequence identity to H7 SH HA (SEQ ID NO: 1) over the full-length protein, wherein the amino acid residue corresponds to an amino acid residue selected from the group consisting of amino acid residues 142-152 with reference to SEQ ID NO: 1. In some embodiments, the Region of Repetitive Carboxylic Groups (RRC) or a Region of Repetitive

Lysyl/Guanidino Groups (RRL) is inserted after an amino acid residue in H7 SH HA (SEQ ID NO: 1), and the amino acid residue is selected from the group consisting of 142W, 143L, 144L, 145S, 146N, 147T, 148D, 149N, 150A, 151A, and 152A. In some embodiments, the recombinant antigen polypeptide comprises from N terminus to C terminus continuously: a first antigen fragment having an amino acid sequence with at least 95% sequence identity to SEQ ID NO: 3, an RRC or an RRL, and a second antigen fragment having an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 4.

[0009] In another aspect, provided herein is an influenza antigen-adjuvant complex comprising the recombinant antigen polypeptide of any of the claims 1-3 and an adjuvant. In some embodiments, the influenza antigen-adjuvant complex is formed by an electrostatic interaction between the RRC or the RRL and an adjuvant. In some embodiments, the antigen polypeptide comprises the RRC and wherein the adjuvant is alum (aluminum hydroxide). In some embodiments, the antigen polypeptide comprises an RRL, and the influenza antigen-adjuvant complex comprises an aluminum-based adjuvant selected from aluminum phosphate and amorphous aluminum hydroxyphosphate sulfate (AAHS). In some embodiments, the RRC comprises a) [D]_N wherein N is 8-18; b) [E]_N wherein N is 8-18; c) a D-E copolymer [(D)_X, (Glu)_Y] where X is 1-17, Y is 1-17, and X + Y = 8-18; or d) a region having a high density of D and/or E. In some embodiments, the RRC comprises 8 to 12 amino acids selected from aspartic acid and glutamic acid. In some embodiments, the RRC is D8, D9, D10, D11, or D12. In some embodiments, the RRL comprises a) [K]_N wherein N is 8-18; b) [R]_N wherein N is 8-18; c) a K-R copolymer [(K)_X, (R)_Y] where X is 1-17, Y is 1-17, and X + Y = 8-18 or [(K)_X, (R)_Y] where X is 1-17, Y is 1-17, and X + Y = 8-18; or d) a region having a high density of K and/or R. In some embodiments, N is 11 or X + Y is 11. In some embodiments, H7 SH HA is presented as a trimer adsorbed to alum. In some embodiments, the influenza antigen-adjuvant comprises an alum particle and a plurality of copies of the recombinant antigen polypeptide; the antigen polypeptide comprises an RRC; and the plurality of copies of the antigen polypeptide is associated with the alum particle by electrostatic interaction between the alum particle and the RRC. In some embodiments, the recombinant antigen polypeptide comprises one or more auxiliary elements. In some embodiments, the one or more auxiliary elements are selected from the group consisting of a polyhistidine tag and a trimerization domain.

[0010] In another aspect, provided herein is a polynucleotide encoding a polypeptide comprising the recombinant antigen polypeptide described above.

[0011] In another aspect, provided herein is a cell comprising the polynucleotide disclosed herein.

[0012] In another aspect, provided herein is a vaccine composition comprising a plurality of the influenza antigen-adjuvant complexes of the above.

[0013] In another aspect, provided herein is a method for eliciting an immune response in a mammal comprising administering the vaccine composition disclosed herein to the mammal.

[0014] In another aspect, provided herein is a method of preparing a recombinant vaccine composition comprising (a) expressing a nucleic acid sequence encoding a recombinant antigen polypeptide, wherein the recombinant antigen polypeptide comprises a Region of Repetitive Carboxylic Groups (RRC), or a Region of Repetitive Lysyl/Guanidino Groups (RRL) inserted after an amino acid residue of 144L, 145S, 146N, 147T, 148D, 149N, or 150A of H7 SH HA (SEQ ID NO: 1); and (b) adsorbing the recombinant antigen polypeptide to alum.

[0015] In another aspect, provided herein is a method of preparing a recombinant vaccine composition comprising (a) introducing a nucleic acid sequence encoding a recombinant antigen polypeptide to a host cell, wherein the recombinant antigen polypeptide comprises from N terminus to C terminus continuously: a first antigen fragment having an amino acid sequence with at least 95% sequence identity to SEQ ID NO: 3, an RRC or an RRL, and a second antigen fragment having an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 4, thereby producing the recombinant antigen polypeptide; and (b) adsorbing the recombinant antigen polypeptide to alum. In some embodiments, the recombinant antigen polypeptide comprises one or more auxiliary elements. In some embodiments, wherein the one or more auxiliary elements are selected from the group consisting of a polyhistidine tag and a trimerization domain.

[0016] In another aspect, provided herein is a recombinant vaccine composition produced by the method disclosed above.

BRIEF SUMMARY OF THE DRAWINGS

[0017] FIG. 1 is a schematic representation of the reorientation of H7 HA by oligoD (poly-Asp) insertion into the head region of H7 HA. H7 HA sequence is based on H7N9 A/Shanghai/2/2013, also referred to as H7 SH - A/Shanghai/2/2013 in this application).

[0018] FIG. 2A and 2B show the results of the biochemical characterization of H7 HA and reoriented H7 HA (“reoH7HA”). FIG. 2A shows the results of size exclusion chromatography coupled with multi-angle light scattering analysis. FIG. 2B shows the results of protein gel electrophoresis.

[0019] FIG. 3A and 3B show the thermal melting profiles and the melting temperatures of H7 HA and reoH7HA in solution and in the presence of alum, respectively.

[0020] FIG. 4A and 4B show the epitope accessibility of reoH7HA on alum by ELISA. FIG. 4A shows the binding of head-directed mAbs (“FluA-20”) and stem-directed mAbs (“MEDI8852” and “FI6v3”) to the reoH7HA on streptavidin-coated plates. FIG. 4B shows the binding of monoclonal antibodies to the reoH7HA on alum-coated plates.

DETAILED DESCRIPTION OF THE INVENTION

I. TERMS AND DEFINITIONS

[0021] As will be apparent from context, “vaccine” or “vaccine polypeptide” refers to the antigen (polypeptide) portion of a vaccine preparation, and “vaccine composition” refers to the antigen (polypeptide) in combination with an adjuvant (alum) and optionally other excipients.

[0022] As used herein, a “recombinant subunit vaccine” or “recombinant subunit vaccine polypeptide” refers to a recombinantly produced polypeptide intended for administration to a subject to elicit a protective immune response. Other terms used interchangeably with recombinant subunit vaccine include “recombinant polypeptide vaccine” “biosynthetic polypeptide vaccine,” and “genetically engineered polypeptide vaccine.”

[0023] As used herein, an “antigen polypeptide” or “antigenic portion” is a polypeptide or portion of a polypeptide that encodes a pathogen protein or portion of a pathogen protein (“pathogen antigen polypeptide”), or encodes a disease antigen or portion of disease antigen (“disease antigen polypeptide”), and elicits a desired protective immune response against the pathogen or disease antigen.

[0024] As used herein, a “disease antigen” refers to an antigen that is a target of a therapeutic vaccine, such as a cancer antigen. See Tagliamonte et al., 2014, “Antigen-specific vaccines for cancer treatment” *Hum Vaccin Immunother.* 10(11):3332-3346. doi:10.4161/21645515.

[0025] As used herein, a “subject” to which a vaccine is administered may be a human or may be a non-human animal (e.g., a pet, such as a cat or dog, livestock, such as cows, sheep, pigs, goats, fish, and poultry).

[0026] As used herein, “Region of Repetitive Carboxylic Groups” (RRC) has the meaning set forth hereinbelow.

[0027] As used herein, “RRC-encoding sequence” is a nucleic acid sequence that encodes an RRC.

[0028] As used herein, an “aspartate residue” is an amino acid residue in a polypeptide, having the side chain CH_2COOH . Aspartate is an α -amino-acid residue anion resulting from the deprotonation of the carboxy group of an aspartic acid residue and is generally the form found in physiological conditions. The terms “aspartic acid,” “aspartate,” “aspartic acid residue,” and “aspartate residue,” are often used interchangeably in the literature and are equivalent terms as used herein. Aspartate is represented as “D” or “Asp.”

[0029] As used herein, a “poly-Asp sequence” (or, equivalently, an “[Asp]_N sequence”) refers to 6 or more contiguous aspartate residues in an RRC portion of a recombinant polypeptide vaccine made as disclosed herein.

[0030] As used herein, a “poly-Asp encoding sequence” is a nucleic acid sequence that encodes multiple contiguous aspartate residues. In most systems aspartate is encoded by the codons GAT and GAC. In some embodiments, a poly-Asp encoding DNA sequence comprises [GAY]_N where G is guanine, A is adenine, Y is pyrimidine (C or T), and N = 6-40 or 8 to 20.

[0031] As used herein, a “glutamate residue” is an amino acid residue in a polypeptide, having the side chain $\text{CH}_2\text{CH}_2\text{COOH}$. Glutamate is an α -amino-acid residue anion resulting from the deprotonation of the carboxy group of a glutamic acid residue and is generally the form found in physiological conditions. The terms “glutamic acid,” “glutamate,” “glutamic acid residue,” and “glutamate residue,” are often used interchangeably in the literature and are equivalent terms as used herein. Glutamate is represented as “E” or “Glu.”

[0032] As used herein, a “poly-Glu sequence” (or, equivalently, a “[Glu]_N sequence”) refers to 6 or more contiguous glutamate residues in an RRC portion of a recombinant polypeptide.

[0033] As used herein, a “poly-Glu encoding sequence” is a nucleic acid sequence that encodes multiple contiguous aspartate residues. In most systems aspartate is encoded by the codons CAG and CAA. In some embodiments, a poly-Glu encoding DNA sequence comprises $[CAR]_N$ where G is cytidine, A is adenine, R is purine (A or G), and $N = 6$ to 40 or $N = 8$ to 20 or 8 to 18 .

[0034] An “RRC-containing polypeptide” is an antigenic polypeptide that can be used as a component of a vaccine and contains an RRC.

[0035] As used herein, in the context of an RRC, “introduction,” “installation,” and “insertion” are used interchangeably to refer to addition to and/or modification of a nucleic acid sequence encoding an RRC, e.g., poly-Asp or poly-Glu, for expression of an RRC-containing polypeptide (antigen). A polypeptide expressed from such a nucleic acid (i.e., a polypeptide having an RRC inserted) can be referred to as a polypeptide or antigen having an “inserted,” “installed,” or “introduced” RRC. Introduction of RRC-encoding codons is carried out using any suitable method, including molecular cloning and *de novo* synthesis of a polynucleotide. For ease of references, an insertion can be characterized as a “terminal insertion” or an “intervening insertion.”

[0036] As used herein, in one aspect, a “terminal” poly-Asp/poly-Glu/RRC sequence refers to a sequence found at the amino- or carboxy-terminus of a recombinant protein vaccine polypeptide. However, a poly-Asp/poly-Glu/RRC sequence that is at the amino-terminal but for an immediately preceding a single methionine can be considered a terminal RRC. As used herein, in cases in which an RRC-containing polypeptide is processed (e.g., by removal of a signal peptide) the amino- or carboxy-terminus of a recombinant protein refers to a terminus of a mature or processed protein or protein fragment as combined with alum and incorporated into the vaccine composition. In cases in which a recombinant protein is translated as a pre-protein (including a signal peptide), the terminal RRC can be positioned at the terminus of the mature protein. It will be understood that in a polynucleotide encoding pre-proteins, the terminal RRC encoding sequence can be positioned between codon corresponding to the C-terminus of the signal peptide and the codon corresponding to the N-terminus of mature polypeptide. Similarly, in the case of a pro-protein antigen (a protein that undergoes post-translational processing) the terminal RRC may be positioned such that it is located at the N- or C- terminus of the processed mature protein. Similarly, in the case of a protein antigen that is processed (e.g., cleaved) *ex vivo* after isolation from cells, the terminal RRC may be

positioned such that it is located at the N- or C- terminus of the processed (e.g., cleaved) protein. In short, a terminal RRC may be positioned at the terminus of the polypeptide product that is combined with alum.

[0037] As used herein, “in the form immobilized on alum” refers to the antigen polypeptide associated with alum in a vaccine composition. For example, the form immobilized on alum may refer to a mature polypeptide after removal of a signal peptide and cleavage. In some cases the antigen polypeptide presented on alum is member of a multimer (e.g., trimer). In embodiments, the multimer may be a homomultimer or a heteromultimer.

[0038] As used herein, an RRC “at” the amino- or carboxy-terminus of a polypeptide, means that in the form immobilized on alum the RRC is a terminal sequence. As used herein, an RRC “near” the amino- or carboxy-terminus of a polypeptide, means that in the form immobilized on alum the RRC within twenty-five (25) residues of a polypeptide terminus, i.e., the twenty-fifth residue from the polypeptide terminus is part of the RRC. In some embodiments the RRC near a terminus is within 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 residues of a polypeptide terminus.

[0038] As used herein, an “intervening” RRC sequence refers to an RRC that is not at the amino- or carboxy-terminus of an antigen polypeptide in the form immobilized on alum. An intervening RRC can be an insertion at a position within an antigen polypeptide (a “contiguous intervening” sequence) or may be a substitution that replaces residues of the unmodified antigen. In either case, additional amino acid flanking one or both ends of the RRC sequence may be included in the introduced sequence.

[0039] As used here, a “contiguous intervening” poly-Asp/poly-Glu/RRC sequence refers to a poly-Asp/poly-Glu/RRC sequence within a polypeptide, where the poly-Asp/poly-Glu/RRC separates and is contiguous with two sequences that are contiguous in the unmodified antigen (e.g., a pathogen protein found in nature).

[0040] As used herein, “sequence identity” in reference to similarity of two proteins (a target protein and a reference protein) or two nucleic acids (a target nucleic acid and a reference nucleic acid) is a quantification of identity of amino acids or nucleobases when the reference and target sequence are optimally aligned. Software tools that can be used for performing sequence alignment are well known in the art, including those for performing pairwise sequence alignment (for example, SSEARCH) or multiple sequence alignment (for example, ClustalW). Sequence identity can be determined manually by inspection, especially

when the target and reference have greater than 90% identity. Alternatively, for nucleotide sequences, percent identity to a reference nucleic acid sequence can be determined using a BLAST or BLAST 2.0 comparison program (described in Altschul et al. (1990) *J. Mol. Biol.* 215: 403-410 and Altschul et al. (1977) *Nucleic Acids Res.* 25: 3389-3402, respectively) with default parameters. BLASTP with default parameters can be used to determine percent to a reference polypeptide sequence. The BLASTN program uses as default parameters a word size (W) of 28, an expectation (E) of 10, M=1, N=-2, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word size (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)). Software for BLAST analyses is publicly available through the National Center for Biotechnology Information (NCBI) website.

[0041] As used herein, promoters or other regulatory elements, such as enhancers, is "operably linked" to a nucleic acid sequence when they affect to the expression of RNA from the nucleic acid sequence.

[0042] As used herein, an RRC-containing antigenic polypeptide can be described as a "derivative" of a non-RRC polypeptide (e.g., a naturally occurring pathogen protein, candidate subunit in development) when the RRC-containing antigenic polypeptide ("parental polypeptide") shares sequence identity with at least a portion of the non-RRC antigenic polypeptide and elicits an immune response specific for the non-RRC polypeptide. Exclusive of an RRC(s) and auxiliary elements a derivative of a polypeptide may have at least about 50% sequence identity, at least about 60% sequence identity, at least about 70% sequence identity, at least about 80% sequence identity, or at least about 90% sequence identity with a corresponding "parental" polypeptide.

[0043] As used herein, the term "substantially similar," when referring to sequence similarity, refers to that an amino acid sequence shares at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity with the reference amino acid sequence.

[0044] The terms "corresponding to," "determined with reference to," or "numbered with reference to" when used in the context of the identification of a given amino acid residue in a polypeptide sequence, refers to the position of the residue of a specified reference sequence when the given amino acid sequence is maximally aligned and compared to the reference

sequence. The polypeptide that is aligned to the reference sequence need not be the same length as the reference sequence.

II. INTRODUCTION

[0045] This disclosure describes new vaccine compositions comprising a modified antigen bound to the surface of an adjuvant or carrier by electrostatic interactions. The presentation of an antigen in a defined orientation on an adjuvant surface can be used to alter epitope accessibility and redirect an immune response toward specific epitopes. For example, a targeted immune response can be directed toward less dominant but more desirable epitopes of the antigen than is possible using conventional adsorption methods. In one implementation a targeted immune response can be directed to an epitope(s) that is conserved among members of a family of related antigens. An example of such a related antigen family are influenza hemagglutinin (HA) proteins, in which dominant immunogenic epitopes are not conserved among family members, and less dominant epitopes are conserved. By using a vaccine that directs an immune response to conserved epitopes, it is possible to elicit an immune response effective across multiple strains of a pathogen. For example, a vaccine described herein can elicit antibodies that are cross-reactive and protective against diverse influenza strains.

[0046] In one aspect of the present invention, the H7 HA protein is genetically engineered to introduce an amino acid sequence rich in charged residues (e.g., poly(aspartic acid), poly(glutamic acid), poly(lysine), and poly(arginine) at a defined location in the target protein. In some embodiments, the defined location is the amino acid residue corresponding to an amino acid residue selected from the group consisting of amino acid residues 142-152 with reference to SEQ ID NO: 1. In some embodiments, the amino acid residue is selected from the group consisting of 142W, 143L, 144L, 145S, 146N, 147T, 148D, 149N, 150A, 151A, and 152A with reference to SEQ ID NO: 1.

[0047] In some embodiments, the insertion is a poly(aspartic acid) $[D]_N$, wherein the N is 8-18. In some embodiments, the insertion is a poly(aspartic acid) $[E]_N$, wherein the N is 8-18. In some embodiments, the insertion is a D-E copolymer $[(D)_X, (Glu)_Y]$ where X is 1-17, Y is 1-17, and $X + Y = 8-18$. In some embodiments, a region having a high density of D and/or E. In some embodiments, the insertion is 11D. The introduced sequence (or “region”) forms an electrostatic association with the adjuvant such that the antigen is retained on the surface in an advantageous orientation.

[0048] For illustration and not limitation three categories of vaccine compositions can be described:

[0049] (a) Vaccine polypeptides modified by introduction of a Region of Repetitive Carboxylic Groups (RRC). In this approach the modification introduces a region rich in aspartate (D) and/or glutamate (E) causing the polypeptide to associate with a negatively charged region of an alum aggregate (comprising aluminum hydroxide).

[0050] (b) Vaccine polypeptides modified by introduction of a Region of Repetitive Lysyl/Guanidino Groups (RRL). In this approach the modification introduces a region rich in lysine (K) and/or arginine (R), causing the polypeptide to associate with a negatively charged region of an aluminum-based adjuvant, such as aluminum phosphate and amorphous aluminum hydroxyphosphate sulfate (AAHS). See Section XIV, below.

[0051] (c) Vaccine polypeptides modified by introduction of an RRC or RRL and adsorbed to lipid nanoparticles (LNPs) used as carriers or adjuvants (e.g., liposomal saponin, monophosphoryl lipid A). See US Pat. No. 10,434,167 (“Non-toxic adjuvant formulation comprising a monophosphoryl lipid A (MPLA)-containing liposome composition and a saponin”), Rao et al., 2021, “Liposome Formulations as Adjuvants for Vaccines” In Gill et al. (eds) Nanoparticles for Rational Vaccine Design. Current Topics in Microbiology and Immunology, vol 433, Springer; Alving et al., 2020, “Army Liposome Formulation (ALF) family of vaccine adjuvants” Expert Review of Vaccines, 19:279-292. The surface charge of LNPs can be fine-tuned by the lipid composition using art-known means. RRC-modified antigens will adsorb onto positively-charged LNPs and RRL-modified antigens will adsorb onto negatively-charged LNPs.

[0052] This disclosure describes exemplary recombinant protein vaccines developed against influenza viruses expressing H7 HA. In one approach these proteins may be modified by addition of an RRC or RRL and delivered using, for example but not limited to, an alum adjuvant.

III. INFLUENZA VACCINES AND ANTIGEN MODIFICATIONS

[0053] Influenza A viruses are the influenza viruses known to cause flu pandemics. Influenza A viruses are divided into subtypes based on two proteins on the surface of the virus: hemagglutinin (HA) and neuraminidase (NA). There are 18 known HA subtypes and 11 known NA subtypes. For example, H7N9 has an H7 HA protein and an N9 NA protein. Exemplary H7 HA proteins are shown in **Table 1**.

Table 1 Exemplary influenza H7 HA proteins:

Hemagglutinins	UniProt No.	Accession
H7 NT – A/FPV/Dutch/1927	Q82794	
H7 SH – A/Shanghai/2/2013	R4NN21	
H7N7-A/Netherlands/219/2003	Q6VMK1	
H7N9-A/Anhui/1-DEWH730/2013	A0A024E364	

[0054] Influenza Hemagglutinin (HA) is a glycoprotein found on the surface of influenza viruses. It is responsible for binding the virus to cell membranes, such as, cells in the upper respiratory tract or erythrocytes. HA is also responsible for the fusion of the viral envelope with the endosomal membrane, after the pH drops in the endosome. HA is a homotrimeric integral membrane glycoprotein. HA is expressed as a precursor protein (referred to as HA0) that trimerizes and then is cleaved into two smaller polypeptides — the HA1 and HA2 subunits, which remain complexed. The mature form of HA is thus a trimer of HA1-HA2 heterodimers. The HA1 subunit includes a globular head region containing the hemagglutinin receptor binding site that interacts with sialic acid on the surface of eukaryotic cells. The HA2 subunit includes a long, helical chain, a transmembrane region, and a cytoplasmic region. A portion of the HA1 subunit and the helical chain portion of the HA2 subunit are referred to as the stem region of the Hemagglutinin (HA) protein.

[0055] The head region of HA appears to be immunodominant, meaning that during viral infection or during vaccination, subjects often produce antibodies predominantly against the head region. The head region, however, has significantly higher sequence variability when compared to the stem region, and antibodies against it are often not protective against challenges with other viral isolates. The HA stem domain is highly conserved and appears to contain broadly neutralizing epitopes. As such, antibodies directed against the HA stem domain may protect against many strains of the virus.

[0056] Disclosed herein are the methods and compositions for preparing influenza vaccines effective against multiple related H7 influenza viruses. According to the present disclosure, regions of a polypeptide with a high density of carboxylic groups, known as a Region of Repetitive Carboxylic Groups or “RRC”, further discussed below, are introduced into a H7 influenza virus antigen polypeptide to produce an “enhanced antigen.” When administered, for example, as an antigen-alum complex, enhanced antigens increase humoral antibody responses and increase the neutralization potency of the antibody response relative to administration of an unmodified antigen-alum complex. Moreover, the antigenic-alum

complexes of the invention may be designed to present antigens in a predetermined orientation. In an aspect, the orientation directs the immune system to generate antibodies against a specific region, or epitope, of the antigen, a process referred to herein as “immunofocusing.” For example, the orientation can be used to elicit production of neutralizing antibodies.

[0057] As described in the Examples, below, we genetically engineered H7 HA proteins by site-specific introduction of repeating units of aspartate residues (“poly-Asp” or “[Asp]_N”). In studies using influenza hemagglutinin (HA) administered with alum, we have demonstrated that introduction of poly-Asp into antigens enhanced humoral antibody responses in a mouse model, resulted in minimal in-group variations among immunized individuals, and significantly increased the neutralization potency of the antibody response.

[0058] Without intending to be limited to a particular mechanism of action, we believe introduction of repetitive carboxylic groups from RRC not only increases the electrostatic interaction between antigens and alum but also provides RRCs that act as chelating ligands for aluminum that further enhances alum-binding. Without intending to be limited to a particular mechanism of action, we believe the modified interaction with alum surprisingly results in the advantageous properties of the antigen-alum complex described herein.

[0059] Previously, attempts were made to produce an engineered H7 HA, for example, H7 NT, H7 SH with the desired “upside down” configuration by inserting poly-Asp at various locations, such as T165 (with reference to UniProt Accession No. Q82794), G205 (with reference to UniProt Accession No. Q82794), S136 (with reference to UniProt Accession No. R4NN21), D166 (with reference to UniProt Accession No. R4NN21), G205 (with reference to UniProt Accession No. R4NN21), respectively. However, to produce an insertion of poly-Asp into various H7 HA proteins has caused problems in protein expression. Engineered H7 HA constructs comprising these poly-Asp insertions were not able to produce detectable amount of proteins; thus, they cannot be used to produce vaccines. **Table 2** lists a number of such engineered H7 HA constructs; no protein expression of these constructs was detected by Western blots.

Table 2 Certain poly-Asp insertion into the H7 HA proteins resulted in loss of expression in a cell culture system.

Hemagglutinin	UniProt Accession No.	poly-Asp insertion site	Expression
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H7 NT – A/FPV/Dutch/1927	Q82794	T165	-
H7 NT – A/FPV/Dutch/1927	Q82794	G205	-
H7 SH -A/Shanghai/2/2013	R4NN21	S136	-
H7 SH - A/Shanghai/2/2013	R4NN21	D166	-
H7 SH - A/Shanghai/2/2013	R4NN21	G205	-

[0060] An insertion of poly-Asp, into H7 HA (H7N9 A/Shanghai/2/2013) after residue T165 was possible; the resulting recombinant H7 HA proteins comprising these poly-Asp insertions were able to express in a reasonable level. See FIG. 2A and 2B. Further, the engineered H7 HA shows a configuration that is consistent with an upside-down orientation, such that the stem of the HA is more accessible to the immune system and can generate an immune response that is effective against multiple related pathogens. See Example 2 and FIG. 4A and 4B.

[0061] In some embodiments, provided herein is a recombinant antigen polypeptide comprises a Region of Repetitive Carboxylic Groups (RRC) or a Region of Repetitive Lysyl/Guanidino Groups (RRL) inserted into H7 SH HA (SEQ ID NO: 1) or an amino acid sequence with at least 90%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% amino acid sequence identity to H7 SH HA (SEQ ID NO: 1) over the full-length protein. The types of RRC and insertion location of the RRC may vary and they are further disclosed below in sections below entitled “RRC TYPES AND PROPERTIES” and “SITE OF RRC INSERTIONS.” In some embodiments, the RRC or RRL is inserted into an amino acid residue that is selected from the group consisting of amino acid residues 142-152 with reference to SEQ ID NO: 1. In some embodiments, the RRC or RRL is inserted after an amino acid residue in H7 SH HA (SEQ ID NO: 1), and the amino acid residue is selected from the group consisting of 142W, 143L, 144L, 145S, 146N, 147T, 148D, 149N, 150A, 151A, and 152A.

[0062] In some embodiments, the recombinant antigen polypeptide comprises from N terminus to C terminus continuously: a first antigen fragment having an amino acid sequence with at least 90%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 3, an RRC or an RRL, and a second antigen fragment having an amino acid sequence having at least 90%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% amino acid sequence identity to SEQ ID NO: 4. The term

“continuously” refers to that there is no intervening amino acid residue between any two of the 1) the first antigen fragment, 2) the RRC or RRL, and 3) the second antigen fragment.

IV. RRC TYPES AND PROPERTIES

[0063] RRCs are rich in glutamic acid and/or aspartic acid, both of which are acidic amino acids with a side chain containing a terminal carboxyl group. RRCs are sometimes categorized as TYPE 1, TYPE 2, TYPE 3, or TYPE 4 RRCs. Each type of RRC begins with a glutamic acid or aspartic acid residue and ends with a glutamic acid or aspartic acid residue. In some embodiments the RRC contains aspartic acid residues and does not contain glutamic acid residues. In some embodiments the RRC contains glutamic acid residues and does not contain aspartic acid residues. In a polypeptide, an RRC can be described as the region of contiguous amino acid residues having a D or E at the amino end of the RRC, a D or E at the carboxy end of the RRC and having the properties of a TYPE 1-4 RRC. It will be recognized that in some cases an RRC may be inserted adjacent a D or E containing sequence present in the unmodified antigen. For example, in SEQ ID NO: 6, a sequence DDDDDDDDDDD is inserted into the wild type sequence “. . . LLSNTDNAA.” resulting in “LLSNTDDDDDDDDDDNAA” In the resulting modified protein the RRC has the sequence DDDDDDDDDDD including the added [D]₁₁ sequence and the D from the wild-type H7 SH protein.

[0064] A TYPE 1 RRC contains a poly-Asp sequence (“poly-Asp” or “[Asp]_N” or “poly-D” or “oligoD”). Several RRCs described in Examples are TYPE 1 RRCs. In some embodiments N is 6-40 or 8-20 or 8-18.

[0065] A TYPE 2 RRC contains a poly-Glu sequence (“poly-Glu” or “[Glu]_N” or “poly-E”). In some embodiments N is 6-40 or 8-20 or 8-18.

[0066] A TYPE 3 RRC can be described as a copolymer of Asp and Glu comprising N contiguous residues each independently selected from D and E, where N is 6-40 (e.g., [(Asp)_x, (Glu)_y] where X is 1-39, Y is 1-39, and X + Y = 6-40 or where N is 6-20 [e.g., (Asp)_x, (Glu)_y where X is 1-19, Y is 1-19, and X + Y = 6-20]. The term “TYPE 3 RRC” includes such copolymers, as well as TYPE 1 and TYPE 2 RRCs. Residues in a TYPE 3 RRC can be described, without limitation, as a copolymer, a block copolymer, an alternating copolymer, or a random copolymer, such as DEDEDEDEDE (alternating copolymer), DDDEEEDDDEEE (block copolymer) or, e.g., EEEDEDDDEDEEEED (random copolymer).

[0067] A TYPE 4 RRC, has a high density of Asp and/or Glu, but may include other residues as well. Examples of TYPE 4 RRCs are the sequences DDDDDLEEEEEE and DEDEDDLEGEED. “High density” means that at least 50% of residues in the RRC are Asp or Glu. See TABLES 1A and 1B. In other embodiments, “High density” means that at least 50%, at least 60%, at least 75% or at least 80% of residues in the RRC are Asp or Glu. It will be apparent that the first residue of a TYPE 4 RRC will be D or E and the last residue of a TYPE 4 RRC will be D or E. It will also be apparent that all TYPE 1-3 RRCs (each having 100% Asp or Glu) are also TYPE 4 RRCs. **Table 3** and **Table 4** list various types of RRCs.

Table 3 Exemplary RRCs which at least 80% of residues in the RRC are Asp or Glu

Length of RRC	Maximum Number of non E/D if at least 80% of residues in the RRC are D or E	Example
6-9	1	EEDLD
10-14	2	DDEEDDGED
15-19	3	DDEEDDEELDDLED
20	4	DDEELDDEGDDDGEDDLEED

Table 4 Exemplary RRCs which at least 50% of residues in the RRC are Asp or Glu

Length of RRC	Maximum Number of non E/D if at least 50% of residues in the RRC are D or E.	Example
9	4	DGDGDGDGD
14	7	DDGGDDGGDDGGDD

[0068] In some cases, the non-D non-E residues in a TYPE 4 RRC are small, non-polar and neutral amino acids such as glycine, leucine or alanine. In some cases, the RRC does not contain lysine or arginine (positively charged residues).

[0069] In some cases, the antigen protein is modified by introduction of two or more RRCs. In some cases, the RRCs are positioned in different flexible loops, but are close in three-dimensional space.

[0070] The number of amino acid residues in an RRC (i.e., the “length” of the RRC) is generally in the range of 6 to 40. Often the RRC has a length of 8 to 20 residues. Generally, an RRC contains at least six residues that are D or E, preferably at least eight residues that are

D or E. In some embodiments, the length of the RRC insertion sequence) is 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15. However, in some embodiments the RRC sequence comprises a greater or smaller number of Asp residues, such as 2 to 30 or 3 to 20 Asp residues.

[0071] In some cases the RRC is 8 residues (e.g., 8D). In some cases, the RRC is 11 residues (e.g., 11D). As shown below in Example 2 and FIG. 3B, FIG. 4A and 4B, 11D insertion into H7 HA (SEQ ID NO: 1) resulted in complete binding of the engineered H7 HA to alum and the desired “upside down” configuration.

V. ALUM

[0072] As used herein, “alum” refers to insoluble aluminum hydroxide (also called aluminum oxyhydroxide) suitable for use as an adjuvant in humans and nonhuman animals. See HogenEsch et al., 2018, “Optimizing the utilization of aluminum adjuvants in vaccines: you might just get what you want.” *npj Vaccines* 3, 51. doi.org/10.1038/s41541-018-0089-x; also see Baylor et al, 2002, “Aluminum salts in vaccines--US perspective” *Vaccine* 20 Suppl 3:S18-23. doi: 10.1016/s0264-410x(02)00166-4. Alum has been described as aggregates of aluminum hydroxide nanoparticles or microparticles. See Harris et al. 2012, *Alhydrogel(R) adjuvant, ultrasonic dispersion and protein binding: a TEM and analytical study*. *Micron* 43, 192–200; Li et al., 2017 “Aluminum (Oxy)Hydroxide Nanosticks Synthesized in Bicontinuous Reverse Microemulsion Have Potent Vaccine Adjuvant Activity” *ACS Appl Mater Interfaces*. 2017;9(27):22893-22901. doi:10.1021/acsami.7b03965; Orr et al., 2019, “Reprogramming the adjuvant properties of aluminum oxyhydroxide with nanoparticle technology” *npj Vaccines* 4, 1. doi.org/ 10.1038/s41541-018-0094-0. As used herein, the term “alum particles” is used to describe to alum of various sizes and shapes, provided the alum is suitable for use as an adjuvant. Alum is available from a variety of commercial sources. ALHYDROGEL® type adjuvant is commercially available (CRODA, Invivogen).

[0073] As discussed below, in some cases an aluminum-based material with a surface negative charge is used as an aluminum-based adjuvant. Examples include aluminum phosphate and amorphous aluminum hydroxyphosphate sulfate (AAHS). Vaccine antigen polypeptides modified by introduction of a Regions of Repetitive Lysyl/Guanidino Groups (“RRL”) may be combined with aluminum-based adjuvants to prepare vaccines of the invention.

VI. GENETIC ENGINEERING AND EXPRESSION OF RECOMBINANT SUBUNIT VACCINE POLYPEPTIDES

[0074] Manipulation and expression of RRC recombinant subunit vaccines according to the invention can be carried out as desired. For example, recombinant DNA methodology may be used to modify an antigen-encoding sequence by site-specific introduction of an RRC encoding sequence. See, e.g., Irwin et al., 2012, “In-Fusion® Cloning with Vaccinia Virus DNA Polymerase” In: Isaacs S. (eds) Vaccinia Virus and Poxvirology. *Methods in Molecular Biology* (Methods and Protocols), vol 890. Humana Press, Totowa, NJ. doi.org/10.1007/978-1-61779-876-4_2. As used herein, “introduction” of an RRC does not imply use of any particular methodology. Exemplary methods include insertion and ligation, homologous recombination, and introduction using a CRISPR/CAS system.

[0075] Recombinant subunit polypeptide vaccines can be produced using any suitable method, including expression as heterologous proteins in recombinant systems. Exemplary expression systems are well known and include bacteria, yeast, insect cell, mammalian cell, plant and transgenic animal platforms. See, e.g., Cid and Bolívar, 2021, “Platforms for Production of Protein-Based Vaccines: From Classical to Next-Generation Strategies” *Biomolecules* 11, 1072.doi.org/10.3390/ biom11081072; also see Man Wang et al., 2016, “Recent advances in the production of recombinant subunit vaccines in *Pichia pastoris*, *Bioengineered* 7:3,155-165, DOI:10.1080/ 21655979.2016.1191707.

VII. EXPRESSION OF RECOMBINANT SUBUNIT VACCINE POLYPEPTIDE

[0076] A recombinant subunit vaccine can be prepared by (a) obtaining a first polynucleotide comprising a sequence that encodes an antigen polypeptide; (b) introducing a RRC-encoding nucleic acid sequence into the sequence that encodes the antigen polypeptide, thereby producing a second polynucleotide encoding a chimeric protein sequence having (i) a RRC portion and (ii) an antigen polypeptide sequence portion(s). Typically, the nucleic acid sequence encoding the chimeric protein sequence linked to a promoter that drives transcription of the protein-encoding a sequence. The chimeric protein encoded by the second polynucleotide is expressed to produce a RRC containing vaccine antigen polypeptide. The polypeptide can be expressed using art-known methods, e.g., as discussed above, such as a cell based or cell-free expression system. The vaccine polypeptide can be purified using routine methods.

[0077] In an aspect, the invention provides vaccine polypeptides and vaccine compositions prepared using the methods described herein. In one approach, the method further includes

the step of adsorbing the polypeptide to alum to produce a protein-alum complex. See Section XII below, titled “*Adsorbing Antigen to Alum.*” In one approach, the method further includes the step of combining the protein-alum complex with excipients. Components (e.g., protein, alum, excipient) can be combined in any order.

VIII. SEQUENCE CHARACTERISTICS OF A RECOMBINANT SUBUNIT VACCINE POLYPEPTIDE

[0078] The recombinant subunit vaccine (or “antigen polypeptide”) comprises a sequence that elicits an immune response, such as an immune response against a pathogen. In one approach the antigen polypeptide has a sequence found in nature (e.g., a polypeptide expressed by the pathogen). Insertion of the RRC sequence results in a protein in which a pathogen sequence is close to or adjacent to a RRC in an arrangement not found in nature. In a related approach insertion of the RRC sequence results in a protein in which the RRC is adjacent to a pathogen sequence. A recombinant subunit vaccine containing a RRC or other RRC sequence can be recognized by reference to naturally occurring sequences identified in database such as Genbank or Uniprot. A hallmark of some vaccine polypeptides is an RRC adjacent to or near a known pathogen sequence. It will be understood that a characteristic of the recombinant subunit vaccine polypeptides is the presence of RRC near or adjacent to known or naturally occurring sequences (e.g., pathogen sequences), i.e., an arrangement not found in nature as can be readily deduced by reference to a sequence database.

[0079] In a related approach, the antigen polypeptide is a component of a vaccine that is approved or licensed by a regulatory agency, as is discussed in greater detail herein below. In this case the RRC is inserted to improve the properties of the known vaccine. In another related approach, the antigen polypeptide is a known (e.g., published) vaccine polypeptide candidate. In this case the RRC is inserted to improve the properties of the candidate vaccine. In general, the hallmarks a RRC-containing recombinant subunit vaccine can be recognized by reference to a sequence database, having the hallmark of RRC adjacent to or close a known sequence of a licensed vaccine polypeptide or vaccine polypeptide candidate.

[0080] For illustration and not limitation, the structure of the chimeric polypeptide produced by insertion of RRC can be described as shown in **Table 5**.

Table 5 Exemplary chimeric polypeptides produced by insertion of RRC

N-[A1]-[D] _N -[A2]-C, where A1 and A2 are contiguous amino acid sequences of an H7 HA protein. In one example, A1 comprises or consists of SEQ ID NO: 3 and A2 comprises or consists of SEQ ID NO: 4.
N-[A1]-[D] _N -[B2]-C, where A1 and B2 are contiguous amino acid sequences of H7 HA, or A1 is an amino acid sequence of an H7 HA protein and B2 is substantially similar to a sequence that is contiguous to A1 in the H7 HA. A1 is substantially similar to an amino acid sequence of an H7 HA protein that is contiguous to B2 in the protein or A1 is substantially similar to a first amino acid sequence of an H7 HA protein and B2 is substantially similar to a second amino acid sequence of the H7 HA protein and A1 and B2 are contiguous in the pathogen or vaccine protein.
N-[A1]-[D] _N -C, where A1 is an amino acid sequence of an H7 HA protein
N-[D] _N -[A1]-C, where A1 is an amino acid sequence of an H7 HA protein
N-[A1]-[X]-[D] _N -C, where A1 is an amino acid sequence of an H7 HA protein and X comprises one or more auxiliary elements.
N-[D] _N -[X] _P -[A1]-C, wherein P is 0 or 1
N-[D] _N -[X] _P -[A1]-[X] _Q -C, wherein P is 0 or 1 and Q is 0 or 1 and each X is individually selected
Each of the above-described examples where [D] _N is replaced with [E] _N .
Each of the above-described examples where [D] _N is replaced with another RCG insertion sequence.

[0081] In some embodiments, a recombinant antigen polypeptide disclosed herein comprises a Region of Repetitive Carboxylic Groups (RRC) or a Region of Repetitive Lysyl/Guanidino Groups (RRL) inserted after an amino acid residue in an amino acid sequence with at least 95% amino acid sequence identity to H7 HA (SEQ ID NO: 1) over the full-length protein. In some embodiments, the recombinant antigen polypeptide comprises SEQ ID NO: 1. In some embodiments, the RRC or RRL is inserted into the amino acid

sequence after an amino acid residue corresponding to amino acid residues 142-152 with reference to SEQ ID NO: 1.

[0082] In some embodiments, a recombinant antigen polypeptide disclosed herein comprises from N terminus to C terminus continuously: a first antigen fragment having an amino acid sequence with at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3, an RRC or an RRL, and a second antigen fragment having an amino acid sequence having at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4.

IX. SITE OF RRC INSERTION

[0083] Optimal sites of insertion were determined. The effects of RCC insertion were assessed by comparing thermal melting relative to a reference sequence. Retention of conformational epitopes were assessed by determining the effect of the insertion using panels of antibodies. Methods of determining the optimal sites of insertion are also disclosed in WO2023064631. The effect of alum binding by the RRC-containing antigen relative to the wild-type antigen were assessed in a variety of ways, including as described in Examples 1-2, FIG. 2A and 2B. The ability of adjuvanted antigen to elicit an immune response can be determined using art-known methods such as measuring antibody response including specific IgG production including. See Example 4 and FIG. 5, 11, 14, and 18 of WO2023064631. In one approach, the ability of vaccination to generate neutralizing antibodies is assessed. See Example 1. Methods of assessing ability of vaccination to generate neutralizing antibody is also described in WO2023064631, Example 4.

X. IMMUNOFOCUSING

[0084] As noted above, the position of an inserted RRC can be used to control the orientation of the antigen polypeptide relative to the alum surface, providing methods for immunofocusing. The control of antigen orientation on alum by introducing RRC to different locations on antigen proteins has several advantages. For example, the RRC-containing antigenic proteins of this disclosure are useful as vaccine immunogens that can direct the immune system of a subject immunized with such vaccine immunogens to generate antibodies against a specific region, or epitope, of a protein that is known to be productive or neutralizing in the case of an infection See Weidenbacher and Kim, 2019, "Protect, Modify,

Deprotect (PMD): A strategy for creating vaccines to elicit antibodies targeting a specific epitope,” PNAS 116 (20): 9947-9952 and Weidenbacher and Kim, 2019, WO 2019/222674, both incorporated by reference for all purposes, for a discussion.

[0085] In one aspect the invention provides a vaccine composition comprising antigen protein molecules adsorbed to alum particles, wherein the antigen protein molecules comprises a Region of Repetitive Carboxylic Groups and wherein a majority of the of the antigen protein molecules in the composition that are adsorbed to alum have the same orientation relative to a surface of the alum particle to which it is adsorbed. Orientation can be determined as described above (in section captioned “Site of RRC Insertion”) and as described in Examples 2. In one approach, antigen proteins in antigen protein-alum complexes have the same orientation relative to the alum surface when a panel of 4, 5, or more monoclonal antibodies against the protein exhibit substantially similar binding patterns.

XI. AUXILIARY ELEMENTS INCLUDING SPACERS AND TRIMERIZATION DOMAINS

[0086] As used herein, “auxiliary element” refers to a functional element in an RRC-containing polypeptide sequence that are not present in the antigen sequence that is modified by insertion of the RRC into, e.g., naturally occurring sequence. Without limitation examples of auxiliary elements include tags for analysis or purification (e.g., a histidine tag or AviTag, and the like), spacer elements (e.g., a glycine-serine spacer having the structure [N]₃₋₅ where N is glycine or serine, e.g., GGS), and trimerization domains (e.g., foldon, GCN4, GCN4-pIqI). Exemplary auxiliary elements are shown in **Table 6**.

Table 6 Exemplary auxiliary elements

AviTag™	GLNDIFEAQKIEWHE	Biotin-acceptor peptide
[histidine] ₆ Tag	HHHHHH	Polyhistidine tag
FOLDON	GYIPEAPRDGQAYVRKDGEWVLLSTFL	T4 fibrin trimerization domain
GCN4	MKQIEDKIEEILSKIYHIENEIARIKKLIGEV	Trimerization domain
GCN4-pIqI	MKQIEDKIEEILSKQYHIENEIARIKKLIGER	Trimerization domain

[0087] Gly-Ser spacers provide flexibility in the polypeptide that allows the RRC (or an auxiliary element) to adopt orientations to facility binding to alum.

[0088] Trimerization domains may be included in the vaccine preparation. Influenza Hemagglutinin (HA) proteins are found in nature as trimers. In some embodiments a

trimerization domain is included in the RRC-containing polypeptide stabilize the trimeric structure of the antigen complex. For example, the RRC-containing polypeptides set forth in SEQ ID NOs: 7- 10. The trimerization domain foldon (or GCN4) in these polypeptides can be replaced with GCN4 (or foldon) or any other trimerization domain. It will be recognized that some antigen polypeptides will comprise a dimerization domain or other multimerization domain.

XII. ADSORBING ANTIGEN TO ALUM

[0089] Methods for combining a vaccine protein and alum to make a protein-alum complex are well known. For a general description see HogenEsch et al., 2018, “Optimizing the utilization of aluminum adjuvants in vaccines: you might just get what you want.” *npj Vaccines* 3, 51. doi.org/10. 1038/s41541-018-0089-x. Also see Example 1, below (protein antigens with alum (protein:alum, 1:10, w/w) for 30 min PBS at room temperature, followed by addition of naïve mouse serum to a final concentration of 10% (v/v).

XIII. Vaccine Compositions

[0090] In one aspect, the invention provides a first composition comprising (1) a polypeptide having a defined sequence and having a RRC insertion and (2) alum, wherein at least some of the polypeptides are adsorbed to alum particles.

[0091] In an embodiment, a majority of the polypeptides in the first composition that are associated with alum are associated in the same orientation.

[0092] In addition to protein and alum, the vaccine compositions may include one or more other vaccine reagents selected from citric acid monohydrate, trisodium citrate dihydrate, sugars (e.g., 2-hydroxypropyl- β -cyclodextrin), sodium chloride, thiomersal, antibiotics, MgCl₂ (for OPV), MgSO₄, lactose-sorbitol and sorbitol-gelatine.

Additional adjuvants

[0093] In general, the vaccine compositions may include other adjuvants. A list of approved adjuvants is included here: www.cdc.gov/vaccinesafety/concerns/adjuvants.html. In an embodiment, the composition comprises CpG oligonucleotides.

XIV. Antigen Modification to Introduce Regions of Repetitive Lysyl/Guanidino Groups (“RRL”)

[0094] As noted above in Section II entitled “INTRODUCTION,” antigenic vaccine polypeptides can be modified by introduction of a Region of Repetitive Lysyl/Guanidino Groups (“RRL”). In this approach, the modification introduces a region rich in lysine (K) and/or arginine (R), causing the polypeptide to associate with a negatively charged region of an adjuvant, such as an aluminum-based adjuvant. Exemplary aluminum-based adjuvants include aluminum phosphate and amorphous aluminum hydroxyphosphate sulfate (AAHS). Polypeptides modified by introduction of RRLs can also associate with lipid-based adjuvants.

[0095] RRL’s generally share the features of RRC’s, except that RRLs comprise lysine (K) and/or arginine (R) while RRCs comprise aspartic acid (D, Asp) and/or glutamic acid (E, Glu) and RRLs comprise lysine (K, Lys) and/or arginine (R, Arg). The description of RRLs is embodied in this disclosure: The reader is instructed to replace (except in working examples or otherwise clear from context) every reference to “aspartic acid” may be replaced with “lysine,” and every reference to “glutamic acid” may be replaced with “arginine” just as if the text had been duplicated and rewritten with these changes. As noted, references to alum in the context of RRCs will be understood to refer to adjuvants with a positive surface charge such as, but not limited to, those specifically listed herein).

RRCs	RRLs
Aspartic Acid (D, Asp, poly-Asp, 12D, etc.)	Lysine (K, Lys, Polylys, 12K, etc.)
Glutamic Acid (E, Glu, polyGlu, 12E, etc.)	Arginine (R, Arg, Polyarg, 12R, etc.)

XV. VACCINATION TARGETS

[0096] The methods and vaccine compositions disclosure herein may be used for any therapeutic or prophylactic treatment responsive to vaccination. Exemplary diseases, pathogens, pathogen polypeptides, and disease-associated polypeptides are known and additional targets will be identified in the future. Exemplary targets, for illustration and not limitation, are described below. See Cid and Bolivar, 2021, “Platforms for Production of Protein-Based Vaccines: From Classical to Next-Generation Strategies” *Biomolecules* 11(8), 1072.

XVII. EXEMPLARY EMBODIMENTS

[0097] Embodiment 1. A recombinant antigen polypeptide comprises a Region of Repetitive Carboxylic Groups (RRC) or a Region of Repetitive Lysyl/Guanidino Groups (RRL) inserted after an amino acid residue in an amino acid sequence with at least 95% amino acid sequence identity to H7 SH HA (SEQ ID NO: 1) over the full-length protein, wherein the amino acid residue corresponds to an amino acid residue selected from the group consisting of amino acid residues 142-152 with reference to SEQ ID NO: 1.

[0098] Embodiment 2. The recombinant antigen polypeptide of embodiment 1, wherein the Region of Repetitive Carboxylic Groups (RRC) or a Region of Repetitive Lysyl/Guanidino Groups (RRL) is inserted after an amino acid residue in H7 SH HA (SEQ ID NO: 1), wherein the amino acid residue is selected from the group consisting of 142W, 143L, 144L, 145S, 146N, 147T, 148D, 149N, 150A, 151A, and 152A.

[0099] Embodiment 3. The recombinant antigen polypeptide of any one of the preceding embodiments, wherein the recombinant antigen polypeptide comprises from N terminus to C terminus continuously: a first antigen fragment having an amino acid sequence with at least 95% sequence identity to SEQ ID NO: 3, an RRC or an RRL, and a second antigen fragment having an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 4.

[0100] Embodiment 4. An influenza antigen-adjuvant complex comprising the recombinant antigen polypeptide of any of the embodiments 1-3 and an adjuvant.

[0101] Embodiment 5. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein the influenza antigen-adjuvant complex is formed by an electrostatic interaction between the RRC or the RRL and an adjuvant.

[0102] Embodiment 6. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein the antigen polypeptide comprises the RRC and wherein the adjuvant is alum (aluminum hydroxide).

[0103] Embodiment 7. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein the antigen polypeptide comprises an RRL, wherein the influenza antigen-adjuvant complex comprises an aluminum-based adjuvant selected from aluminum phosphate and amorphous aluminum hydroxyphosphate sulfate (AAHS).

[0104] Embodiment 8. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein the RRC comprises

- a) [D]N wherein N is 8-18;
- b) [E]N wherein N is 8-18;
- c) a D-E copolymer [(D)X, (Glu)Y] where X is 1-17, Y is 1-17, and $X + Y = 8-18$; or
- d) a region having a high density of D and/or E.

[0105] Embodiment 9. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein the RRC comprises 8 to 12 amino acids, wherein each of the 8 to 12 amino acids is selected from the group consisting of aspartic acid and glutamic acid.

[0106] Embodiment 10. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein the RRC is D8, D9, D10, D11, or D12.

[0107] Embodiment 11. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein RRL comprises

- a) [K]N wherein N is 8-18;
- b) [R]N wherein N is 8-18;
- c) a K-R copolymer [(K)X, (R)Y] where X is 1-17, Y is 1-17, and $X + Y = 8-18$ or [(K)X, (R)Y] where X is 1-17, Y is 1-17, and $X + Y = 8-18$; or
- d) a region having a high density of K and/or R.

[0108] Embodiment 12. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein N is 11 or $X + Y$ is 11.

[0109] Embodiment 13. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein H7 SH HA is presented as a trimer adsorbed to alum.

[0110] Embodiment 14. The influenza antigen-adjuvant complex of any one of the preceding embodiments that comprises an alum particle and a plurality of copies of the recombinant antigen polypeptide, wherein the antigen polypeptide comprises an RRC, wherein the plurality of copies of the antigen polypeptide is associated with the alum particle by electrostatic interaction between the alum particle and the RRC.

[0111] Embodiment 15. The influenza antigen-adjuvant complex of any one of the preceding embodiments in which the recombinant antigen polypeptide comprises one or more auxiliary elements.

[0112] Embodiment 16. The influenza antigen-adjuvant complex of any one of the preceding embodiments, wherein the one or more auxiliary elements are selected from the group consisting of a polyhistidine tag and a trimerization domain.

[0113] Embodiment 17. A polynucleotide encoding a polypeptide comprising the recombinant antigen polypeptide described in any of any one of the preceding embodiments.

[0114] Embodiment 18. A cell comprising the polynucleotide of embodiment 17.

[0115] Embodiment 19. A vaccine composition comprising a plurality of the influenza antigen-adjuvant complexes of any of any one of the preceding embodiments.

[0116] Embodiment 20. A method for eliciting an immune response in a mammal comprising administering the vaccine composition of embodiment 19 to the mammal.

[0117] Embodiment 21. A method of preparing a recombinant vaccine composition comprising

(a) expressing a nucleic acid sequence encoding a recombinant antigen polypeptide, wherein the recombinant antigen polypeptide comprises a Region of Repetitive Carboxylic Groups (RRC), or a Region of Repetitive Lysyl/Guanidino Groups (RRL) inserted after an amino acid residue of 144L, 145S, 146N, 147T, 148D, 149N, or 150A of H7 SH HA (SEQ ID NO: 1); and

(b) adsorbing the recombinant antigen polypeptide to alum.

[0118] Embodiment 22. A method of preparing a recombinant vaccine composition comprising

(a) introducing a nucleic acid sequence encoding a recombinant antigen polypeptide to a host cell, wherein the recombinant antigen polypeptide comprises from N terminus to C terminus continuously: a first antigen fragment having an amino acid sequence with at least 95% sequence identity to SEQ ID NO: 3, an RRC or an RRL, and a second antigen fragment having an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 4, thereby producing the recombinant antigen polypeptide; and

(b) adsorbing the recombinant antigen polypeptide to alum.

[0119] Embodiment 23. The method of embodiment 22, where the recombinant antigen polypeptide comprises one or more auxiliary elements.

[0120] Embodiment 24. The method of embodiment 23, wherein the one or more auxiliary elements are selected from the group consisting of a polyhistidine tag and a trimerization domain.

[0121] Embodiment 25. A recombinant vaccine composition produced by the method of embodiments 22-24.

XVII. EXAMPLES

Example 1 Methods

[0122] *Antigen cloning.* DNA encoding influenza hemagglutinins (HAs of H5N1 A/Vietnam/1203/2004 or H7N9 A/Shanghai/2/2013) were cloned into the pADD2 vector with a foldon trimerization domain followed by an Avi-Tag and a hexahistidine tag on the C-terminus. OligoD was also inserted into the flexible loop regions on H5 HA and H7 HA in pADD2 expression plasmids. All plasmid sequences were confirmed by Sanger sequencing (Sequetech). For transfection purposes, plasmids were transformed into StellarTM cells, isolated by Maxiprep kits (NucleoBond Xtra Maxi kit, Macherey Nagel), filtered through a sterile 0.45- μ m membrane in a biosafety cabinet and stored at -20 °C.

[0123] *Protein expression and purification.* All antigens were expressed in Expi-293F cells. Expi-293F cells were cultured at 37 °C under constant shaking (120 rpm) in a humidified CO₂ (8%) incubator. Expi-293F cells were transfected at a density of $3-4 \times 10^6$ cells/mL. For 200 mL transfection of antigen proteins, the transfection mixture was made by adding 120 μ g plasmid DNA (from Maxiprep) to 20 mL expression media, followed by the dropwise addition of 260 μ L FectoPro transfection reagent (Polyplus) with vigorous mixing. Transfection mixtures were incubated at room temperature for 10 minutes before being transferred to Expi-293F cells. *D*-glucose (4 g/L, Sigma-Aldrich) and valproic acid (3 mM, Acros Organics) were added to the cells immediately post-transfection to increase recombinant protein production. Cells were boosted again with *D*-glucose three days post-transfection and harvested on day four by centrifugation at 7000 $\times$ g for five min. The supernatant was filtered through a 0.22- μ m membrane for subsequent purification processes.

[0124] Antigen proteins with hexahistidine tags were purified with HisPurTM Ni-NTA resin (Thermo Fisher). Briefly, the filtered supernatant from Expi-293F cells was mixed with Ni-

NTA resin (1 mL resin per liter supernatant) and incubated at 4 °C overnight. The mixture was then passed through a gravity-flow column, washed with 20 mM imidazole in HEPES buffer saline (HBS, 20 mM HEPES, pH 7.4, 150 mM NaCl), and then eluted with 250 mM imidazole in HBS. Elution was concentrated with centrifugal filters (30 or 50 kDa MWCO, Millipore Sigma) and buffer-exchanged to HBS for size-exclusion chromatography using a SuperoseTM 6 (Increase 10/300 GL, Cytiva) or SuperdexTM 200 column (Increase 10/300 GL, Cytiva) on an ÄKTA Protein Purification System (Cytiva). Peak fractions were pooled, concentrated, buffer-exchanged to HBS with 10% glycerol and filtered through a 0.22- μ m membrane.

[0125] The concentration of all proteins was determined by absorbance at 280 nm (A₂₈₀), and the purity was assessed by protein gel electrophoresis. Protein samples were flash-frozen in liquid nitrogen and stored at -20 °C.

[0126] *Size-exclusion chromatography–multi-angle light scattering (SEC-MALS) analysis.* SEC-MALS analysis of wild-type and oligoD-modified proteins was performed on a 1260 Infinity II high-performance liquid chromatography system (Agilent) coupled with a miniDAWN and Optilab detectors (Wyatt Technologies) for light scattering and refractive index analysis. Purified protein samples were loaded onto a SuperdexTM 200 column (Increase 3.2/300, Cytiva) sequentially for analysis. ASTRA software (Wyatt Technologies) was used for data analysis.

[0127] *Differential scanning fluorimetry.* Thermal melting profiles of proteins were measured by differential scanning fluorimetry on a Prometheus NT.48 instrument (NanoTemper). Protein samples (0.1 mg/mL) were loaded into glass capillaries (NanoTemper) and then subjected to a temperature gradient from 20 to 95 °C at a heating rate of 1 °C per min. Alternatively, protein samples (0.1 mg/mL) were pre-mixed with alum (10 mg/mL, Alhydrogel[®], InvivoGen) at a ratio of 1:10 (protein: alum, *w/w*) for 30 minutes at room temperature before loading into glass capillaries. HBS and alum (diluted to 1 mg/mL in HBS) were also loaded into glass capillaries and measured as controls. Intrinsic fluorescence (350 nm and 330 nm) was recorded as a function of temperature. Thermal melting curves were plotted using the first derivative of the ratio (350 nm/330 nm). Melting temperatures were calculated automatically by the instrument (PR.ThermControl software, version 2.3.1) and represented peaks in the thermal melting curves.

[0128] ***Streptavidin- and alum-based ELISA.*** To investigate antigen orientation on alum, we measured mAb binding in streptavidin- or alum-based ELISAs. For streptavidin-based ELISAs, Nunc 96-well MaxiSorp plates were coated with streptavidin (4 µg/mL in DPBS, 60 µL per well) for one hour at room temperature. These plates were washed three times with Milli-Q H₂O and then blocked with ChonBlock (120 µL per well) overnight at 4 °C. For subsequent steps, all dilutions were made in DPBS with 0.05% Tween-20 and 0.1% BSA, and ELISA plates were rinsed with PBST in between steps. Biotinylated HA or reoriented HA (2 µg/mL) were added to the plates and incubated for one hour at room temperature. mAbs were serially diluted (10-fold dilution starting from 20 nM) and then added to the ELISA plates for one-hour incubation at room temperature. Rabbit anti-human IgG, HRP-conjugated (1:4,000) was added for one-hour incubation before rinsing with PBST six times.

[0129] For alum-based ELISA, Nunc 96-well MaxiSorp plates were coated with ZsGreen-Avi-His-12D (4 µg/mL in DPBS, 60 µL per well) for one hour at room temperature. These plates were washed three times with Milli-Q H₂O and then blocked with ChonBlock overnight at 4 °C. For subsequent steps, all dilutions were made in DPBS with 0.05% Tween-20 and 0.1% BSA unless otherwise noted, and ELISA plates were rinsed with PBST in between steps. Alum (100 µg/mL in HBS) was added to the plates and incubated for one hour at room temperature. After rinsing, HA or reoriented HA (2 µg/mL) was added to the plates and incubated for one hour at room temperature. mAbs were serially diluted (10-fold dilution starting from 20 nM) and then added to the ELISA plates for one-hour incubation at room temperature. Rabbit anti-human IgG, HRP-conjugated (1:4,000) was added for one-hour incubation before rinsing with PBST six times. ELISA plates were developed with the TMB substrate for five minutes and terminated with sulfuric acid. Absorbance at 450 nm was recorded on a microplate reader.

[0130] ***Bio-layer interferometry (BLI).*** BLI experiments are performed on an OctetRed 96 system. All samples are diluted with Octet buffer (DPBS with 0.02% Tween-20 and 0.1% BSA), and assays are performed under agitation (1000 rpm). Monoclonal antibodies (200 nM) are loaded onto anti-human Fc sensors and then dipped into antigen solutions (100 nM GPΔmucin or 150 nM HA) for binding analysis, followed by dissociation into Octet buffer. Data are processed by Data Analysis software and then plotted.

[0131] **Statistical analyses.** Statistics are analyzed using GraphPad Prism software. Non-transformed data are presented as arithmetic mean ± s.d. Log-transformed data (ELISA titers

and NT₅₀) are presented as geometric mean $\pm$ s.d. P values of 0.05 or less are considered significant.

EXAMPLE 2 ALUM-BINDING H7 HA WITH 11D INSERTION

[0132] We succeeded in inserting oligoD (poly-Asp) into an H7 HA (A/Shanghai/2/2013) after residue T165, which also resides in a flexible loop region (FIG. 1). After purification, both wild-type and reoriented H7 HA (abbreviated as reoH7HA) appeared as homogenous and monodisperse populations on size exclusion chromatography coupled with multi-angle light scattering (FIG. 2A) and protein gel electrophoresis (FIG. 2B). Although the thermal melting profile of reoH7HA slightly shifted toward a lower temperature compared to wild-type H7 HA (FIG. 3A), reoH7HA shared an almost identical thermal melting profile and temperature with wild-type H7 HA in the presence of alum (FIG. 3B), suggesting that binding to alum may help stabilize reoH7HA.

[0133] To validate the “upside down” configuration of reoH7HA on alum, we also measured binding of head-directed mAbs (“FluA-20”) and stem-directed mAbs (“MEDI8852” and “FI6v3”) to reoH7HA on streptavidin- or alum-coated ELISA plates. As a control, all mAbs bound to wild-type H7 HA with high affinity in streptavidin-based ELISAs (FIG. 4A), whereas binding to H7 HA decreased dramatically in alum-based ELISAs due to its weak alum-binding (FIG. 4B). By comparison, both head-directed and stem-directed mAbs bound reoH7HA in streptavidin-based ELISAs, while there was a clear decrease in binding of head-directed mAb (FluA-20) when reoH7HA was adsorbed on alum, suggesting an “upside down” configuration where head epitopes were much less accessible.

Informal Sequences:

SEQ ID NO: 1

H7-SH (without signal peptide)

DKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGKRTVDLGQCGLLGTTITGP
 PQCDQFLEFSADLIHERREGSDVCFPGKFVNNEALRQILRESGGIDKEAMGFTYSGIRTNGATS
 ACRRSGSSFYAEMKWLLSNTDNAAFQMTKSYKNTRKSPALIVWGIHHSVSTAEQTKLYGS
 GNKLVTVGSSNYQQSFVPSGARPVNGLSGRIDFHWLMLNPNDTVTFSFNGAFIAPDRASF
 LRGKSMGIQSGVQVDANCEGDCYHSGGTIISNLPFQNIDSRAVGKCPRYVKQRSLLLATGMK
 NVPEIPKGRGLFGAIAAGFIENGWEGLIDGWYGFRHQNAQEGTAADYKSTQSAIDQITGKLN
 RLIEKTNQFELIDNEFNEVEKQIGNVINWTRDSITEVWSYNAELLVAMENQHTIDLADSEMD
 KLYERVKRQLRENAEEDGTGCFEIFHKCDDDCMASIRNNTYDHSKYREEAMQNRIQIDGS

SEQ ID NO: 2

H7-SH pre-protein (including a signal peptide)

MNTQILVFALIAIPTNADKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGK
 RTVDLGQCGLLGTTITGPPQCDQFLEFSADLIHERREGSDVCFPGKFVNNEALRQILRESGGIDKE
 AMGFTYSGIRTNGATSACRRSGSSFYAEMKWLLSNTDNAAFQMTKSYKNTRKSPALIVWGI
 HHSVSTAEQTKLYGSGNKLVTVGSSNYQQSFVPSGARPVNGLSGRIDFHWLMLNPNDTV
 TFSFNGAFIAPDRASF LRGKSMGIQSGVQVDANCEGDCYHSGGTIISNLPFQNIDSRAVGKCPR
 YVKQRSLLLATGMKNVPEIPKGRGLFGAIAAGFIENGWEGLIDGWYGFRHQNAQEGTAADY
 KSTQSAIDQITGKLNRLEKTNQFELIDNEFNEVEKQIGNVINWTRDSITEVWSYNAELLVA
 MENQHTIDLADSEMDKLYERVKRQLRENAEEDGTGCFEIFHKCDDDCMASIRNNTYDHSKY
 REEAMQNRIQIDGS

SEQ ID NO: 3

H7-SH fragment 1, corresponding to amino acid residues 1-147 of H7-SH (SEQ ID NO: 1)

DKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGKRTVDLGQCGLLGTTITGP
 PQCDQFLEFSADLIHERREGSDVCFPGKFVNNEALRQILRESGGIDKEAMGFTYSGIRTNGATS
 ACRRSGSSFYAEMKWLLSNT

SEQ ID NO: 4

H7-SH fragment 2, corresponding to amino acid residues 148-497 of H7-SH (SEQ ID NO: 1)

DNAAFQMTKSYKNTRKSPALIVWGIHHSVSTAEQTKLYGSGNKLVTVGSSNYQQSFVPSG
 ARPQVNGLSGRIDFHWLMLNPNDTVTFSFNGAFIAPDRASF LRGKSMGIQSGVQVDANCEGD
 CYHSGGTIISNLPFQNIDSRAVGKCPRYVKQRSLLLATGMKNVPEIPKGRGLFGAIAAGFIENG
 WEGLIDGWYGFRHQNAQEGTAADYKSTQSAIDQITGKLNRLEKTNQFELIDNEFNEVEK
 QIGNVINWTRDSITEVWSYNAELLVAMENQHTIDLADSEMDKLYERVKRQLRENAEEDGTG
 CFEIFHKCDDDCMASIRNNTYDHSKYREEAMQNRIQIDGS

SEQ ID NO: 5

H7-SH with 11D insertion (without signal peptide)

DKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGKRTVDLGQCGLLGTTITGP
 PQCDQFLEFSADLIHERREGSDVCFPGKFVNNEALRQILRESGGIDKEAMGFTYSGIRTNGATS
 ACRRSGSSFYAEMKWLLSNT**DDDDDDDDDD**DNAAFQMTKSYKNTRKSPALIVWGIHHS
 VSTAEQTKLYGSGNKLVTVGSSNYQQSFVPSGARPVNGLSGRIDFHWLMLNPNDTVTFSF
 NGAFIAPDRASF LRGKSMGIQSGVQVDANCEGDCYHSGGTIISNLPFQNIDSRAVGKCPRYVK
 QRSLLLATGMKNVPEIPKGRGLFGAIAAGFIENGWEGLIDGWYGFRHQNAQEGTAADYKST
 QSAIDQITGKLNRLEKTNQFELIDNEFNEVEKQIGNVINWTRDSITEVWSYNAELLVAMEN

QHTIDLADSEMDKLYERVKRQLRENAEEDGTGCFEIFHKCDDDCMASIRNNTYDHSKYREE
AMQNRIQIDGS

SEQ ID NO: 6

H7-SH with 11D insertion (without signal peptide)

DKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGKRTVDLGQCGLLGTTITGP
PQCDQFLEFSADLIIRREGSDVCFPGKFVNNEALRQILRESGGIDKEAMGFTYSGIRTNGATS
ACRRSGSSFYAEMKWLLSNT**DDDDDDDDDDDD**DNAAFPQMTKSYKNTRKSPALIVWGIHHS
VSTAEQTKLYGSGNKLVTVGSSNYQQSFVPSGARPVNGLSGRIDFHWLMLNPNDTVTF
NGAFIAPDRASFLRGKSMGIQSGVQVDANCEGDCYHSGGTIISNLPFQNIDSRAVGKCPRYVK
QRSLLLATGMKNVPEIPKGRGLFGAIAAGFIENGWEGLIDGWYGFRHQNAQGEGTAADYKST
QSAIDQITGKLNRLIEKTNQQFELIDNEFNEVEKQIGNVINWTRDSITEVWSYNAELLVAMEN
QHTIDLADSEMDKLYERVKRQLRENAEEDGTGCFEIFHKCDDDCMASIRNNTYDHSKYREE
AMQNRIQIDGS

SEQ ID NO: 7

H7 SH-foldon-Avi-His (H7 SH)

MNTQILVFALIAIPTNADKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGK
RTVDLGQCGLLGTTITGPPQCDQFLEFSADLIIRREGSDVCFPGKFVNNEALRQILRESGGIDKE
AMGFTYSGIRTNGATSACRRSGSSFYAEMKWLLSNTDNAAFPQMTKSYKNTRKSPALIVWGI
HHSVSTAEQTKLYGSGNKLVTVGSSNYQQSFVPSGARPVNGLSGRIDFHWLMLNPNDTV
TFSFNGAFIAPDRASFLRGKSMGIQSGVQVDANCEGDCYHSGGTIISNLPFQNIDSRAVGKCP
RYVKQRSLLLATGMKNVPEIPKGRGLFGAIAAGFIENGWEGLIDGWYGFRHQNAQGEGTAADY
KSTQSAIDQITGKLNRLIEKTNQQFELIDNEFNEVEKQIGNVINWTRDSITEVWSYNAELLVA
MENQHTIDLADSEMDKLYERVKRQLRENAEEDGTGCFEIFHKCDDDCMASIRNNTYDHSKY
REEAMQNRIQIDGS-GYIPEAPRDGQAYVRKDGEWVLLSTFLGS-GLNDIFEAQKIEWHE-
GHHHHHH

SEQ ID NO: 8

H7 SH-foldon-Avi-His (H7 SH)

with 11D insertion after residue T165 of the H7 HA pre-protein; amino acid residues 1-18 constitute the signal peptide)

MNTQILVFALIAIPTNADKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGK
RTVDLGQCGLLGTTITGPPQCDQFLEFSADLIIRREGSDVCFPGKFVNNEALRQILRESGGIDKE
AMGFTYSGIRTNGATSACRRSGSSFYAEMKWLLSNT**DDDDDDDDDDDD**DNAAFPQMTKSYK
NTRKSPALIVWGIHHSVSTAEQTKLYGSGNKLVTVGSSNYQQSFVPSGARPVNGLSGRIDF
HWLMLNPNDTVTFSTFNGAFIAPDRASFLRGKSMGIQSGVQVDANCEGDCYHSGGTIISNLPF
QNIDSRAVGKCPRYVKQRSLLLATGMKNVPEIPKGRGLFGAIAAGFIENGWEGLIDGWYGFRH
QNAQGEGTAADYKSTQSAIDQITGKLNRLIEKTNQQFELIDNEFNEVEKQIGNVINWTRDSITE
VWSYNAELLVAMENQHTIDLADSEMDKLYERVKRQLRENAEEDGTGCFEIFHKCDDDCMA
SIRNNTYDHSKYREEAMQNRIQIDGS-GYIPEAPRDGQAYVRKDGEWVLLSTFLGS-
GLNDIFEAQKIEWHE-GHHHHHH

SEQ ID NO: 9

H7 SH-GCN4-Avi-His (H7 SH); H7 HA pre-protein; amino acid residues 1-18 constitute the signal peptide)

MNTQILVFALIAIPTNADKICLGHHAVSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGK
RTVDLGQCGLLGTTITGPPQCDQFLEFSADLIIRREGSDVCFPGKFVNNEALRQILRESGGIDKE

AMGFTYSGIRTNGATSACRRSGSSFYAEMKWLLSNTDNAAFPMQTKSYKNTRKSPALIVWGI
 HHSVSTAEQTKLYGSGNKLVTVGSSNYQQSFVPSPGARPQVNGLSGRIDFHWLMLNPNDTV
 TFSFNGAFIAPDRASFLRGKSMGIQSGVQVDANCEGDCYHSGGTIISNLPFQNIDSRAVGKCP
 YVKQRSLLLATGMKNVPEIPKGRGLFGAIAAGFIENGWEGLIDGWYGFRHQNAQGEGTAADY
 KSTQSAIDQITGKLNRLIEKTNQQFELIDNEFNEVEKQIGNVINWTRDSITEVWSYNAELLVA
 MENQHTIDLADSEMDKLYERVKRQLRENAEEDGTGCFEIFHKCDDDCMASIRNNTYDHSKY
 REEAMQNRIQIDGS-MKQIEDKIEEILSKIYHIENEIARIKKLIGEVASSS-
 GLNDIFEAQKIEWHE-GHHHHHH

SEQ ID NO: 10

H7 SH-GCN4-Avi-His (H7 SH)

(11D insertion after residue T165 of the H7 HA pre-protein; amino acid residues 1-18 constitute the signal peptide)

MNTQILVFALIAIPTNADKICLGHHA VSNGTKVNTLTERGVEVVNATETVERTNIPRICSKGK
 RTVDLGQCGLLGTITGPPQCDQFLEFSADLIERREGSDVCFPGKFVNEEALRQILRESGGIDKE
 AMGFTYSGIRTNGATSACRRSGSSFYAEMKWLLSNT**DDDDDDDDDDDD**DNAAFPMQTKSYK
 NTRKSPALIVWGIHHSVSTAEQTKLYGSGNKLVTVGSSNYQQSFVPSPGARPQVNGLSGRIDF
 HWLMLNPNDTVTFSFNGAFIAPDRASFLRGKSMGIQSGVQVDANCEGDCYHSGGTIISNLPF
 QNIDSRAVGKCPRYVKQRSLLLATGMKNVPEIPKGRGLFGAIAAGFIENGWEGLIDGWYGFRH
 QNAQGEGTAADYKSTQSAIDQITGKLNRLIEKTNQQFELIDNEFNEVEKQIGNVINWTRDSITE
 VWSYNAELLVAMENQHTIDLADSEMDKLYERVKRQLRENAEEDGTGCFEIFHKCDDDCMA
 SIRNNTYDHSKYREEAMQNRIQIDGS-MKQIEDKIEEILSKIYHIENEIARIKKLIGEVASSS-
 GLNDIFEAQKIEWHE-GHHHHHH

[0134] It is understood that the examples and embodiments described in the present disclosure are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited in the present disclosure are hereby incorporated by reference in their entirety for all purposes.

CLAIMS

1. A recombinant antigen polypeptide comprises a Region of Repetitive Carboxylic Groups (RRC) or a Region of Repetitive Lysyl/Guanidino Groups (RRL) inserted after an amino acid residue in an amino acid sequence with at least 95% amino acid sequence identity to H7 SH HA (SEQ ID NO: 1) over the full-length protein, wherein the amino acid residue corresponds to an amino acid residue selected from the group consisting of amino acid residues 142-152 with reference to SEQ ID NO: 1.
2. The recombinant antigen polypeptide of claim 1, wherein the Region of Repetitive Carboxylic Groups (RRC) or a Region of Repetitive Lysyl/Guanidino Groups (RRL) is inserted after an amino acid residue in H7 SH HA (SEQ ID NO: 1), wherein the amino acid residue is selected from the group consisting of 142W, 143L, 144L, 145S, 146N, 147T, 148D, 149N, 150A, 151A, and 152A.
3. The recombinant antigen polypeptide of claim 1, wherein the recombinant antigen polypeptide comprises from N terminus to C terminus continuously: a first antigen fragment having an amino acid sequence with at least 95% sequence identity to SEQ ID NO: 3, an RRC or an RRL, and a second antigen fragment having an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 4.
4. An influenza antigen-adjuvant complex comprising the recombinant antigen polypeptide of any of the claims 1-3 and an adjuvant.
5. The influenza antigen-adjuvant complex of claim 4, wherein the influenza antigen-adjuvant complex is formed by an electrostatic interaction between the RRC or the RRL and an adjuvant.
6. The influenza antigen-adjuvant complex of claim 5, wherein the antigen polypeptide comprises the RRC and wherein the adjuvant is alum (aluminum hydroxide).
7. The influenza antigen-adjuvant complex of claim 5, wherein the antigen polypeptide comprises an RRL, wherein the influenza antigen-adjuvant complex comprises an aluminum-based adjuvant selected from aluminum phosphate and amorphous aluminum hydroxyphosphate sulfate (AAHS).

8. The influenza antigen-adjuvant complex of claim 4, wherein the RRC comprises
 - a) $[D]_N$ wherein N is 8-18;
 - b) $[E]_N$ wherein N is 8-18;
 - c) a D-E copolymer $[(D)_X, (Glu)_Y]$ where X is 1-17, Y is 1-17, and $X + Y = 8-18$; or
 - d) a region having a high density of D and/or E.
9. The influenza antigen-adjuvant complex of any of claims 4-8, wherein the RRC comprises 8 to 12 amino acids, wherein each of the 8 to 12 amino acids is selected from the group consisting of aspartic acid and glutamic acid.
10. The influenza antigen-adjuvant complex of claim 9, wherein the RRC is D₈, D₉, D₁₀, D₁₁, or D₁₂.
11. The influenza antigen-adjuvant complex of claim 4 wherein RRL comprises
 - a) $[K]_N$ wherein N is 8-18;
 - b) $[R]_N$ wherein N is 8-18;
 - c) a K-R copolymer $[(K)_X, (R)_Y]$ where X is 1-17, Y is 1-17, and $X + Y = 8-18$ or $[(K)_X, (R)_Y]$ where X is 1-17, Y is 1-17, and $X + Y = 8-18$; or
 - d) a region having a high density of K and/or R.
12. The influenza antigen-adjuvant complex of claim 8 or claim 9, wherein N is 11 or $X + Y$ is 11.
13. The influenza antigen-adjuvant complex of any one of claims 4-12, wherein H7 SH HA is presented as a trimer adsorbed to alum.
14. The influenza antigen-adjuvant complex of any of claims 4-13 that comprises an alum particle and a plurality of copies of the recombinant antigen polypeptide, wherein the antigen polypeptide comprises an RRC, wherein the plurality of copies of the antigen polypeptide is associated with the alum particle by electrostatic interaction between the alum particle and the RRC.

15. The influenza antigen-adjuvant complex of any one of claims 4-14 in which the recombinant antigen polypeptide comprises one or more auxiliary elements.
16. The influenza antigen-adjuvant complex of claim 15, wherein the one or more auxiliary elements are selected from the group consisting of a polyhistidine tag and a trimerization domain.
17. A polynucleotide encoding a polypeptide comprising the recombinant antigen polypeptide described in any of claims 4-15.
18. A cell comprising the polynucleotide of claim 17.
19. A vaccine composition comprising a plurality of the influenza antigen-adjuvant complexes of any of claims 4-16.
20. A method for eliciting an immune response in a mammal comprising administering the vaccine composition of claim 19 to the mammal.
21. A method of preparing a recombinant vaccine composition comprising
 - (a) expressing a nucleic acid sequence encoding a recombinant antigen polypeptide, wherein the recombinant antigen polypeptide comprises a Region of Repetitive Carboxylic Groups (RRC), or a Region of Repetitive Lysyl/Guanidino Groups (RRL) inserted after an amino acid residue of 144L, 145S, 146N, 147T, 148D, 149N, or 150A of H7 SH HA (SEQ ID NO: 1); and
 - (b) adsorbing the recombinant antigen polypeptide to alum.
22. A method of preparing a recombinant vaccine composition comprising
 - (a) introducing a nucleic acid sequence encoding a recombinant antigen polypeptide to a host cell, wherein the recombinant antigen polypeptide comprises from N terminus to C terminus continuously: a first antigen fragment having an amino acid sequence with at least 95% sequence identity to SEQ ID NO: 3, an RRC or an RRL, and a second antigen fragment having an amino acid sequence having at least 95% sequence identity to SEQ ID NO: 4, thereby producing the recombinant antigen polypeptide; and

(b) adsorbing the recombinant antigen polypeptide to alum.

23. The method of claim 22, where the recombinant antigen polypeptide comprises one or more auxiliary elements.

24. The method of claim 23, wherein the one or more auxiliary elements are selected from the group consisting of a polyhistidine tag and a trimerization domain.

25. A recombinant vaccine composition produced by the method of claims 22-24.

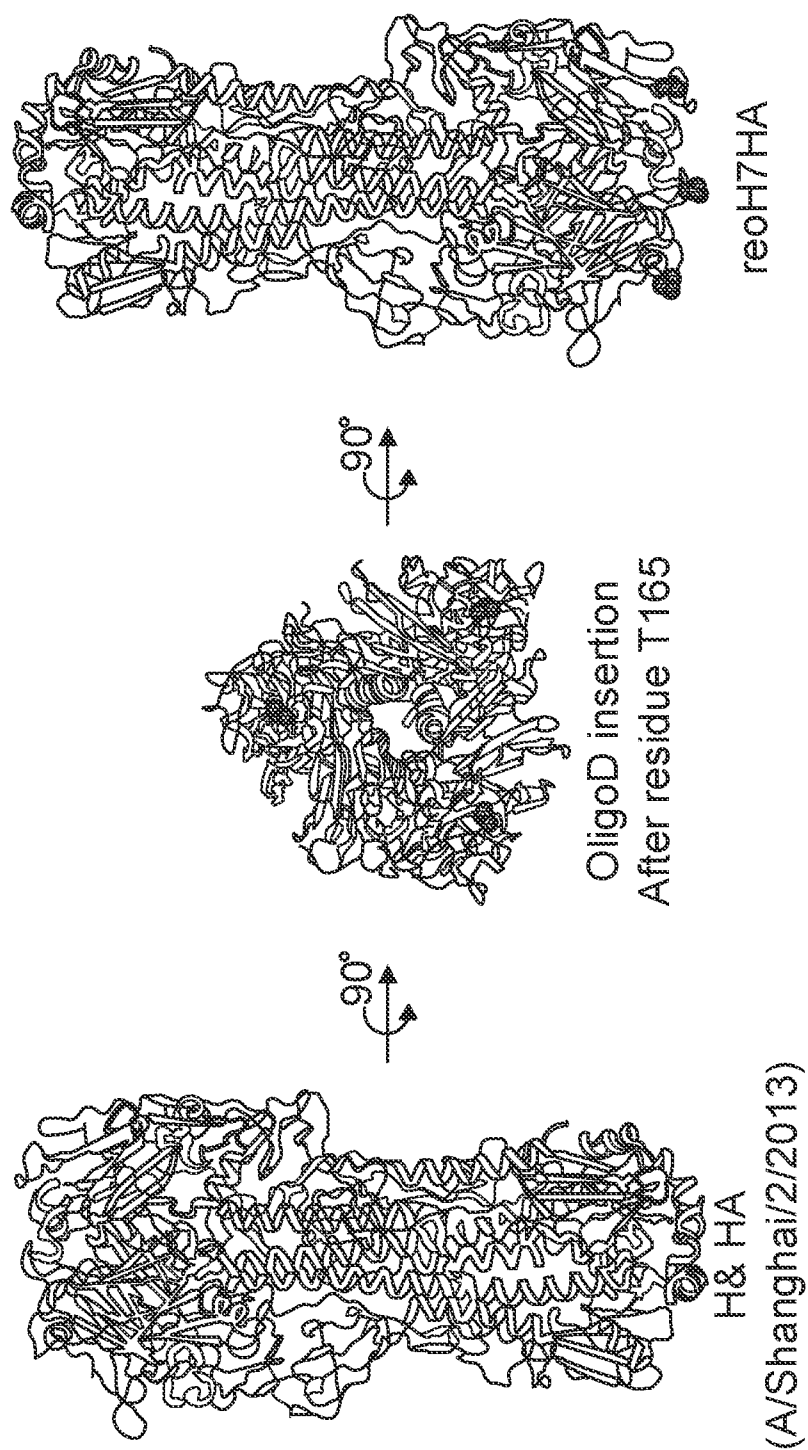
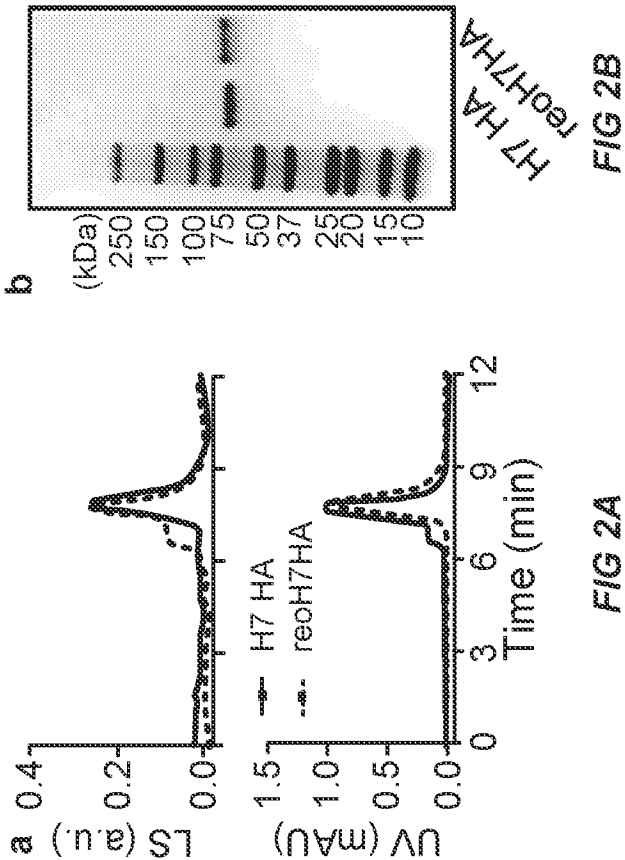


FIG. 1



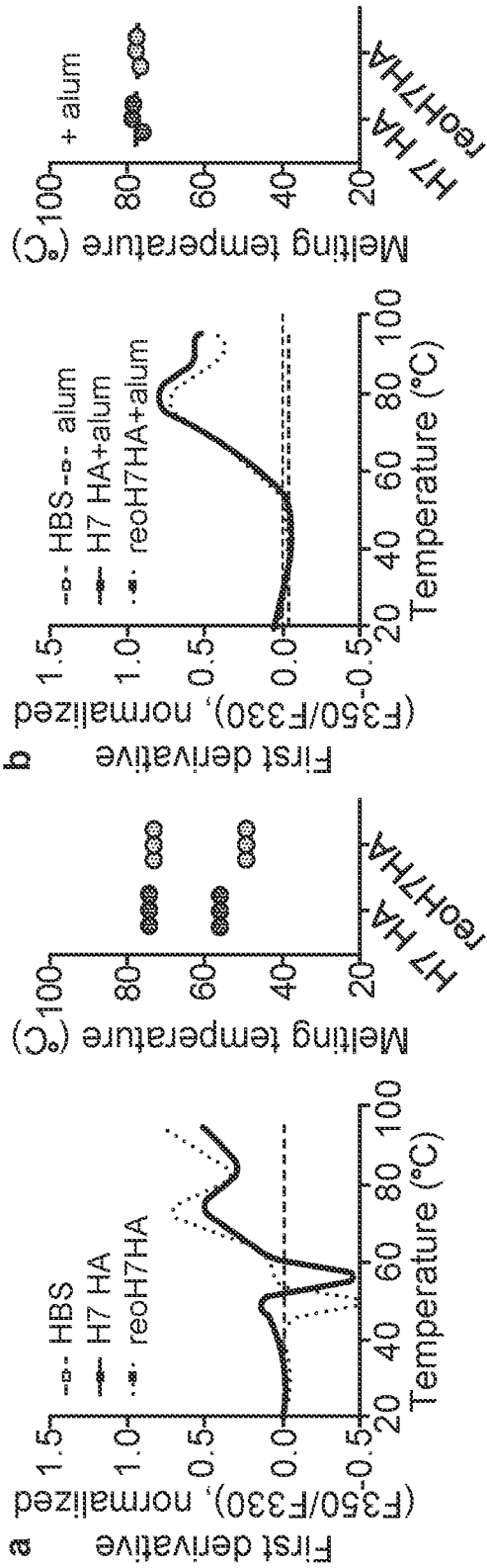


FIG 3B

FIG 3A

