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(54) Title: ANTIGENIC INFLUENZA-FERRITIN POLYPEPTIDES

(57) Abstract: This disclosure relates to antigenic influenza-ferritin polypeptides and their use in eliciting antibodies against influenza.

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ANTIGENIC INFLUENZA-FERRITIN POLYPEPTIDES

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/652,204, filed April 3, 2018, the entire contents of which is incorporated herein by reference.

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 18, 2019, is named 2019-03-18_01121-0034-00PCT_SL.txt and is 285,731 bytes in size.

[0003] Even with many successes in the field of vaccinology, new breakthroughs are needed to protect humans against many life-threatening infectious diseases. Many currently licensed vaccines rely on decade-old technologies to produce live-attenuated or inactivated killed pathogens, which carry inherent safety concerns and in many cases, stimulate only short-lived, weak immune responses that require the administration of multiple doses. While advances in genetic and biochemical engineering have made it possible to develop therapeutic agents to challenging disease targets, these applications to the field of vaccinology have not been fully realized. Recombinant protein technologies now allow the design of optimal antigens. Additionally, nanoparticles have increasingly demonstrated the potential for optimal antigen presentation and targeted drug delivery. Nanoparticles with multiple attached antigens have been shown to have increased binding avidity afforded by the multivalent display of their molecular cargos, and an ability to cross biological barriers more efficiently due to their nanoscopic size. *Helicobacter pylori* (*H. pylori*) ferritin nanoparticles fused to influenza virus haemagglutinin (HA) protein has allowed improved antigen stability and increased immunogenicity in mouse influenza models (*see* Kanekiyo et al., Nature 499:102-106 (2013)). This fusion protein self-assembled into an octahedrally-symmetric nanoparticle and presented 8 trimeric HA spikes to give a robust immune response in various pre-clinical models when used with an adjuvant.

[0004] Influenza remains a significant global health threat, infecting 5-10% of adults annually and killing 250,000-500,000 people each year. Because of long manufacturing times, vaccine strains must be picked half a year or more before flu season and often suffer from mismatch due to genetic shift and drift and the emergence of new strains. Even when properly matched, current vaccines only have a 50-60% efficacy. Thus, there is a substantial need to improve vaccine efficacy by increasing the breadth, potency and/or durability of vaccination, and to introduce modern manufacturing practices.

[0005] A substantial factor underlying the low efficacy of influenza vaccines, observed in many seasons, lies in the tendency of the virus to mutate through error-prone replication, which allows the virus to evade the binding of neutralizing antibodies in vaccinated individuals. The existence of conserved epitopes on the influenza virus suggests a path towards a broadly neutralizing influenza vaccine that can resist viral drift. However, in order to elicit antibodies against these conserved epitopes, recombinant protein engineering is required to present these epitopes in a favorable manner to the immune system. For example, when full-length hemagglutinin (HA) is used as an immunogen, the antibodies elicited are predominantly those that bind to the highly-variable head domain, which is said to be immunodominant. A strategy that has been devised to induce antibodies to the sub-dominant highly-conserved stem region involved "chopping" the head of HA and tying up the remaining protein strands through protein engineering (*see* Yassine, Nature Medicine 21(9):1065-1071 (2015)).

[0006] Here, a set of new advances is presented in vaccine therapeutics for influenza. Antigenic influenza-ferritin polypeptides were developed in which an engineered cysteine replaced a surface-exposed amino acid in the ferritin. Immune-stimulatory moieties, such as adjuvants, were conjugated to the antigenic polypeptide via the cysteine. Self-adjuvanting ferritin particles assembled from the antigenic polypeptides were highly antigenic. The direct conjugation of an immune-stimulatory moiety to the ferritin particle combined with its fusion to an influenza polypeptide allowed for targeted co-delivery of the immune-stimulatory moiety and influenza polypeptide in a single macromolecular entity, which can greatly decrease the potential for systemic toxicity that is feared with more traditional vaccines that comprise antigens and adjuvants as separate molecules. Moreover, the co-delivery of immune-stimulatory moieties together with influenza polypeptides in a macromolecular entity and their multivalent presentation may also reduce the overall dose of vaccine needed, reducing manufacturing burdens and costs.

SUMMARY

[0007] It is an object of this disclosure to provide compositions, kits, methods, and uses that can provide one or more of the advantages discussed above, or at least provide the public with a useful choice. Accordingly, the following embodiments are disclosed herein.

[0008] Embodiment 1 is an antigenic influenza-ferritin polypeptide comprising (i) a ferritin protein comprising a mutation replacing a surface-exposed amino acid with a cysteine, and (ii) an influenza polypeptide.

[0009] Embodiment 2 is an antigenic influenza-ferritin polypeptide comprising (i) a ferritin protein comprising a mutation replacing a surface-exposed amino acid with a cysteine and an immune-stimulatory moiety conjugated to the cysteine; and (ii) an influenza polypeptide.

[0010] Embodiment 3 is the antigenic influenza-ferritin polypeptide of embodiment 1, further comprising an immune-stimulatory moiety conjugated to the ferritin protein via the cysteine.

[0011] Embodiment 4 is the antigenic influenza-ferritin polypeptide of any one of embodiments 1-3, wherein the influenza polypeptide comprises a hemagglutinin (HA) or neuraminidase (NA) polypeptide.

[0012] Embodiment 5 is the antigenic influenza-ferritin polypeptide of embodiment 4, wherein the HA polypeptide comprises a conserved region.

[0013] Embodiment 6 is the antigenic influenza-ferritin polypeptide of embodiment 5, wherein the conserved region comprises all or part of the stem region of the HA.

[0014] Embodiment 7 is the antigenic influenza-ferritin polypeptide of any one of the preceding embodiments, wherein the influenza antigen comprises an HA antigen comprising a Y98F mutation.

[0015] Embodiment 8 is the antigenic influenza-ferritin polypeptide of any one of the preceding embodiments, further comprising a mutation replacing an internal cysteine with a non-cysteine amino acid.

[0016] Embodiment 9 is the antigenic influenza-ferritin polypeptide of embodiment 8, wherein the internal cysteine is at position 31 of H. pylori ferritin, or a position that corresponds to position 31 of H. pylori ferritin as determined by pair-wise or structural alignment, optionally wherein the internal cysteine is mutated to serine.

[0017] Embodiment 10 is the antigenic influenza-ferritin polypeptide of any one of the preceding embodiments, further comprising a mutation replacing a surface-exposed asparagine with a non-asparagine amino acid, optionally wherein the non-asparagine amino acid is glutamine.

[0018] Embodiment 11 is the antigenic influenza-ferritin polypeptide of any one of the preceding embodiments, wherein the surface exposed amino acid is a mutation of E12, S26, S72, A75, K79, S100, or S111 of H. pylori ferritin or an analogous amino acid in a non-H. pylori ferritin as determined by pair-wise or structural alignment.

[0019] Embodiment 12 is the antigenic influenza-ferritin polypeptide of embodiment 11, wherein the mutation at the surface exposed amino acid is E12C, S26C, S72C, A75C,

K79C, S100C, or S111C of H. pylori ferritin or an analogous amino acid in a non-H. pylori ferritin as determined by pair-wise or structural alignment.

[0020] Embodiment 13 is the antigenic influenza-ferritin polypeptide of any one of the preceding embodiments, wherein the immune-stimulatory moiety is an agonist of TLR7 or TLR8.

[0021] Embodiment 14 is the antigenic influenza-ferritin polypeptide of any one of the preceding embodiments, wherein the immune-stimulatory moiety is an agonist of TLR9.

[0022] Embodiment 15 is the antigenic influenza-ferritin polypeptide of any one of embodiments 1 or 3-14, further comprising a linker between the immune-stimulatory moiety and the ferritin protein.

[0023] Embodiment 16 is the antigenic influenza-ferritin polypeptide of embodiment 15, wherein the linker comprises one, two, or three of a maleimide moiety, a polyethylene glycol (PEG) moiety, and a dibenzocyclooctyne (DBCO) moiety.

[0024] Embodiment 17 is the antigenic influenza-ferritin polypeptide of any one of the preceding embodiments, further comprising a peptide linker between the ferritin protein and the influenza polypeptide.

[0025] Embodiment 18 is a ferritin particle comprising the antigenic influenza-ferritin polypeptide of any one of the preceding embodiments.

[0026] Embodiment 19 is a composition comprising the antigenic influenza-ferritin polypeptide or ferritin particle of any one of the preceding embodiments and a pharmaceutically acceptable carrier.

[0027] Embodiment 20 is the composition of embodiment 19, which further comprises a second antigenic influenza-ferritin polypeptide comprising a ferritin protein and a different influenza polypeptide.

[0028] Embodiment 21 is the composition of embodiment 20, wherein the influenza polypeptide is from influenza type A and the influenza polypeptide of the second antigenic influenza-ferritin polypeptide is from influenza type B, or wherein the influenza polypeptide and the influenza polypeptide of the second influenza-ferritin polypeptide are from subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, H17, or H18, or wherein one or both of the influenza polypeptides comprise engineered stabilized stem antigens from subtypes H1, H3, H7 or H10.

[0029] Embodiment 22 is the antigenic influenza-ferritin polypeptide, ferritin particle, or composition of any one of the preceding embodiments for use in a method of eliciting an immune response to influenza or in protecting a subject against infection with influenza.

[0030] Embodiment 23 is a method of eliciting an immune response to influenza or protecting a subject against infection with influenza comprising administering any one or more antigenic influenza-ferritin polypeptide, ferritin particle, or composition of any one of the preceding embodiments to a subject.

[0031] Embodiment 24 is the antigenic influenza-ferritin polypeptide, ferritin particle, composition, or method of any one of the preceding embodiments, wherein the subject is human.

[0032] Embodiment 25 is a nucleic acid encoding the antigenic influenza-ferritin polypeptide of any one of embodiments 1-17, optionally wherein the nucleic acid is an mRNA.

[0033] Additional objects and advantages will be set forth in the description which follows, and/or will be obvious from the description, or may be learned by practice. The objects and advantages will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[0034] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the claims.

[0035] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate certain embodiments and together with the description, serve to explain the principles described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] **Figures 1A-1B**. Sequence comparison and homology of representative H1N1 influenza virus strains. **(FIG 1A)** Sequence alignment of candidate antigenic HA polypeptides using vector NTI AlignX software. Black on white: consensus residue derived from a completely conserved residue at a given position. White on black: consensus residue derived from the occurrence of greater than 50% of a single residue at a given position. Black on white underlined and bold: residue weakly similar to consensus residue at given position. Black on white with double underline and italics: consensus residue derived from a block of similar residues at a given position. Black on white bold: non-similar residues. Sequences shown are the HA portions of the following sequences plus the serine of a linker at the C-terminus: CA09 HA-Np = residues 1-519 of SEQ ID NO: 15. COBRA P1 HA-Np = residues 1-519 of SEQ ID NO: 27. COBRA X6 HA-Np = residues 1-518 of SEQ ID NO: 29. NC99

HA-Np = residues 1-518 of SEQ ID NO: 1. HK77 HA-Np = residues 1-519 of SEQ ID NO: 18. FM47 HA-Np= residues 1-519 of SEQ ID NO: 17. DV57 HA-Np = residues 1-518 of SEQ ID NO: 21. MAL54 HA-Np = residues 1-519 of SEQ ID NO: 16. (**FIG 1B**) A dendrogram generated with the Neighbor Joining Method (Vector NTI) for the HA protein sequences from the listed influenza strains. Arrows indicate strains selected as candidates for evaluation by generating HA-ferritin nanoparticles from these sequences and testing their immunogenicity in mice.

[0037] **Figure 2**. Engineered surface-exposed cysteine (Cys) on ferritin. The location of a cysteine resulting from a mutation replacing a surface-exposed amino acid is shown in the context of a ferritin nanoparticle.

[0038] **Figures 3A-3B**. Conjugation of toll-like receptor (TLR) agonists to ferritin. (**FIG 3A**) The result of conjugation of SM7/8a small molecule to a cysteine resulting from a mutation replacing a surface-exposed amino acid in ferritin via a PEG4 linker harboring a maleimide reactive group is illustrated in the context of a ferritin nanoparticle. (**FIG 3B**) The result of conjugation of CpG (SEQ ID NO: 230) to a cysteine resulting from a mutation replacing a surface-exposed amino acid in ferritin is illustrated in the context of a ferritin nanoparticle using 2-step click chemistry, with a maleimide-DBCO bifunctional linker and azide-functionalized CpG reagent.

[0039] **Figures 4A-4B**. Conjugations of toll-like receptor (TLR) agonists to ferritin. (**FIG 4A**) Model of a ferritin nanoparticle comprising an HA polypeptide and 3M-012 conjugated to a surface-exposed cysteine of ferritin using a 2-step click chemistry reaction via a maleimide-DBCO bifunctional linker and Azide-functionalized 3M-012 reagent. (**FIG 4B**) Model of a ferritin nanoparticle comprising an HA polypeptide and CpG (SEQ ID NO: 231) conjugated to a surface-exposed cysteine of ferritin using a 2-step click chemistry reaction, with a maleimide-DBCO bifunctional linker and Azide-functionalized CpG reagent.

[0040] **Figure 5**. Trypsin cut site within the ferritin portion of certain ferritin nanoparticle constructs. Some ferritin nanoparticles comprise a trypsin cut site in addition to an unpaired, surface-exposed cysteine for conjugation of an adjuvant. The amino acid sequence presented in FIG 5 is a "generalized" sequence showing a sequence common to multiple constructs. For example, residues 519-694 of SEQ ID NO: 14 comprise the "generalized" sequence presented in FIG 5. The "XXX" sequence represents an influenza polypeptide. The location of the ferritin S111C mutation present in this sequence is also indicated.

[0041] **Figures 6A and 6B**. Gel-shift and mass spectrometry (MS) results with or without conjugation. (**FIG 6A**) Gel-shift and mass spectrometry results for H1/Stem-Np (SEQ ID NO: 43) in the presence (+) or absence (-) of conjugation to maleimide-PEG4-SM7/8a. (**FIG 6B**) Gel-shift and mass spectrometry results for H5/hCobra2-Np (SEQ ID NO: 32) in the presence (+) or absence (-) of conjugation to maleimide-PEG4-SM7/8a.

[0042] **Figures 7A and 7B**. Further gel-shift results with or without conjugation. (**FIG 7A**) Gel-shift results for H1/Stem-Np following peptide N-glycosidase (PNGase) treatment with or without conjugation to either maleimide-PEG4-SM7/8a or by 2-step click chemistry with maleimide-PEG4-DBCO and Azide-CpG. (**FIG 7B**) Gel-shift results for H1/Stem-Np following trypsin treatment with or without conjugation to either maleimide-PEG4-SM7/8a or maleimide-PEG4-DBCO and Azide-CpG by 2-step click chemistry.

[0043] **Figures 8A and 8B**. Mass spectra with or without conjugation of maleimide-PEG4-SM7/8a to H1/Stem-Np. (**FIG 8A**) MS data after treatment with PNGase shows the mass of H1/Stem-Np before and after conjugation to maleimide-PEG4-SM7/8a. (**FIG 8B**) MS data after treatment of H1/Stem-Np with trypsin shows the mass of the cleaved ferritin before and after conjugation to maleimide-PEG4-SM7/8a.

[0044] **Figure 9**. Mass spectra with or without conjugation of a maleimide-PEG4-DBCO linker to H1/Stem-Np. Maleimide-PEG4-DBCO Linker addition to H1/Stem-Np was confirmed by mass change measured by MS after linker addition.

[0045] **Figure 10**. SDS-PAGE of H1/Stem-Np before and at various stages of conjugation of CpG by 2-step click chemistry. "After azide-CpG conjugation" refers to the final product of the 2-step click reaction.

[0046] **Figure 11**. Characterization of conjugation of a linker comprising 3M-012 to NC99 HA-TEV-Np constructs. The construct in the WT and +TEV lanes had the sequence of SEQ ID NO: 13; S26C refers to SEQ ID NO: 10; S72C refers to SEQ ID NO: 11; A75C refers to SEQ ID No: 12; and S111C refers to SEQ ID NO: 9. All samples except WT were treated with tobacco etch virus protease (TEV).

[0047] **Figures 12A-12E**. Mass spectra of various constructs with and without reduction or conjugation. (**FIG 12A**) H1/Stem-Np comprising S111C before reduction. (**FIG 12B**) H1/Stem-Np comprising S111C after reduction. The decrease in mass (115 Da) observed after reduction is consistent with the removal of a post-translational modification that inhibits the reactivity of cysteine. (**FIG 12C**) Mass spectra of NC99 HA-TEV-Np-S26C nanoparticle (SEQ ID NO: 10) with and without conjugation to 3M012. (**FIG 12D**) Mass spectra of NC99 HA-TEV-Np-A75C nanoparticle (SEQ ID NO: 12) with and without

conjugation to 3M012. (**FIG 12E**) Mass spectra of NC99 HA-TEV-Np-S111C nanoparticle (SEQ ID NO: 9) with and without conjugation to 3M012.

[0048] **Figures 13A-13F**. Negative stain electron microscopy (EM) images of nanoparticles of H1/Stem-Np or NC99 HA-Np with and without conjugation to SM7/8a, 3M012, or CpG. (**FIG 13A**) Unconjugated H1/Stem-Np. (**FIG 13B**) H1/Stem-Np-SM7/8a conjugate. (**FIG 13C**) H1/Stem-Np-CpG conjugate. (**FIG 13D**) NC99 HA-Np (SEQ ID NO: 9)]. (**FIG 13E**) NC99 HA-Np-3M012 conjugate. (**FIG 13F**). NC99 HA-Np-CpG conjugate.

[0049] **Figures 14A-14C**. Dynamic light scattering (DLS) analysis of H1/Stem-Np-SM7/8a conjugate (**FIG 14A**), H1/Stem-Np-CpG conjugate (**FIG 14B**), or unconjugated H1/Stem-Np (**14C**).

[0050] **Figure 15A-B**. Antibody response to H1/Stem-Np formulated with admixed adjuvants as indicated, or conjugated to TLR agonists such as SM7/8a or CpG via a PEG4 linker, as shown in Figures 3A-B. Serum was collected from mice (n = 5) at 5 weeks following immunization (week 0 and week 3), and antibody titers were measured by Enzyme-Linked Immunosorbent Assay (ELISA). This data demonstrates the self-adjuvanting property of the H1/Stem-Np-TLR-agonist conjugates. Figure 15A shows ELISA to H1/New Caledonia/20/1999 HA trimers. PAA = polyacrylic acid; mixed equimolar = 83.3 ng SM7/8a (equivalent to the dose administered with the SM7/8 conjugated ferritin nanoparticles); high dose = 21.84 μg (higher than the dose administered with the SM7/8 conjugated ferritin nanoparticles). Figure 15B shows ELISA to H1/Stem trimers. Mixed equimolar = 850 ng of CpG (equivalent to the dose administered with the CpG conjugated ferritin nanoparticles); high dose = 20 μg of CpG.

[0051] **Figures 16A-16C**. Comparison of titers obtained with NC99 HA-Np with or without conjugated or separate 3M-012 (SEQ ID NO: 9). The antibody response to NC99 HA-Np-3M012 conjugated nanoparticles (0.22 μg/dose) was tested in mice. Admix controls included a mix of HA-Np (0.22 μg/dose) and 10 μg of 3M012 (a typical literature dose) and a mix of HA-Np (0.22 μg/dose) and 1.7 ng of 3M012 (an equimolar match to the conjugate). Additional controls included unconjugated HA-Np and IIV, dosed with matched HA content (0.17 μg HA/dose). Based on ELISA endpoint titers (**FIG 16A**), Pseudo-virus (PsV) neutralization IC50 titers, and (**FIG 16B**) Hemagglutination Inhibition (HAI) titers (**FIG 16C**), the HA-Np-3M012 conjugate induced significantly stronger antibody responses than the equimolar admix control. Conjugated nanoparticles also induced stronger responses (ELISA) and a stronger neutralizing antibody response (PsV) than unconjugated nanoparticles and the IIV standard of care, although this result was not statistically significant

in the HAI assay. Assays are of serum from 2 weeks post boost. All samples were run in triplicate. Median +/- SEM is graphed. **** $p<0.0001$; *** $p<0.001$; **$p<0.01$; *$p<0.05$.

[0052] **Figures 17A-17C**. Comparison of titers obtained with NC99 HA-Np with or without conjugated or separate CpG (SEQ ID NO: 9). The antibody response to HA-Np-CpG conjugated nanoparticles (0.22 μg/dose) was tested in mice. Admix controls included a mix of HA-Np (0.22 μg/dose) and 20 μg of CpG (a typical therapeutic dose) and a mix of HA-Np (0.22 μg/dose) and 21 ng of CpG (an equimolar match to the conjugate). Additional controls included unconjugated HA-Np and IIV, dosed with matched HA content (0.17 μg HA/dose). Based on ELISA endpoint titers **(FIG 17A)** and Pseudo-virus neutralization IC50 titers **(FIG 17B)**, the conjugate induced stronger binding and neutralizing antibody responses than the matched admixture, the unconjugated particle, or IIV. The HA-Np-CpG conjugate also induced significantly stronger HAI titers **(FIG 17C)** than the equimolar admix control. Additionally, the HAI titers were 2.6- and 3.0-fold higher than unconjugated HA-Ferr and IIV, although these results were not significant. ELISA and PsV assays are of serum from 2 weeks post boost, and HAI assays were performed on serum 5 weeks post boost. All samples were run in triplicate and median +/- SEM are graphed. **** $p<0.0001$; *** $p<0.001$; **$p<0.01$; *$p<0.05$.

[0053] **Figures 18A-18C**. Characterization of self-assembling HA-ferritin nanoparticles derived from six evolutionarily divergent H1 hemagglutinin (HA) antigens and two computationally generated (COBRA) antigens. (**FIG 18A**) Nanoparticle size and polydispersity ("Dispersity") were measured by dynamic light scattering (DLS). (**FIG 18B**) Purity of HA-nanoparticles was assessed by SDS-PAGE Coomassie staining. (**FIG 18C**) Nanoparticle integrity was visualized by negative stain electron microscopy at 80,000x magnification. FM47 = A/Fort Monmouth/1-JY2/1947; MAL54 = A/Malaysia/302/1954; DV57 = A/Denver/1957 (DV57); HK77 = A/Hong Kong/117/1977; NC99 = A/New Caledonia/20/99; CA09 = A/California/4/2009. COBRA P1 and COBRA X6 are computationally generated consensus from multiple sequences, described recently by Carter DM, et al., J Virol 90:4720-4734 (2016). *See* legend for Figures 1A-B for SEQ ID NOs.

[0054] **Figures 19A-19H**. Potency and breadth of immune response elicited by various HA-ferritin nanoparticles (*see* legend for Figures 1A-B for SEQ ID NOs.). Hemagglutination Inhibition (HAI) titers (log_2) of sera from mice 6 weeks after immunization with the indicated HA-Np vaccine were assayed against a panel of divergent H1N1 influenza viruses. Mice (n=5) were immunized with hemagglutinin nanoparticles (HA-Nps) at weeks 0 and 3. Dashed line indicates the assay limit of detection. (**FIGs 19A-19E**) Data with Ribi

adjuvant. (**FIGs 19F-19H**) Data with AF03 adjuvant. The x-axis indicates the panel of H1N1 influenza strains tested by reference year, from 1934 to 2013. Table 2 gives the complete strain designation corresponding to each year. Asterisks indicate matched strains.

[0055] **Figures 20A-20F**. HA antibody responses induced by HA-ferritin nanoparticles. (**FIG 20A**) Mice [n=5] were immunized with the specified nanoparticles or combinations of nanoparticles using Sigma Adjuvant System (catalog# S6322). IIV refers to Influenza Inactivated Vaccine. (**FIGs 20B-F**) Antibody response was measured by determining ELISA titers to HA timers from A/Fort Monmouth/1/1947 (**FIG 20B**); A/Malaysia/302/1954 (**FIG 20C**); A/Hong Kong/117/1977 (**FIG 20D**); A/New Caledonia/20/99 (**FIG 20E**); or A/California/4/2009 (**FIG 20F**). ELISA titers were measured 5 weeks after the first immunization. The lowest serum dilution tested sets the assay limit of detection as indicated by the dotted line. Open circles indicate a match between the strain of origin of a nanoparticle administered to mice and the strain assayed by ELISA.

[0056] **Figures 21A-21H**. Antibody response to HA-mixtures of multiple ferritin nanoparticles administered to mice as measured by assays of HAI titers. (**FIGs 21A-21B**) Bivalent combinations. (**FIGs 21C-21E**) Trivalent combinations. (**FIGs 21F-21H**) Quadrivalent combinations. HAI titers (log_2) for a panel of divergent H1N1 influenza viruses were assayed. Mice (n=5) were immunized with the indicated HA-nanoparticle combinations at weeks 0 and 3, with adjuvants. The bivalent combination COBRA X6 + COBRA P1 HA-Nps was tested with AF03 adjuvant, for consistency with the evaluation of the monovalent COBRA HA-Np. Likewise, the combinations of individual strain HA-ferritin nanoparticles used Ribi adjuvant for consistency with the evaluation of the monovalent individual strain HA-Nps. Asterisks indicate a match between the strain of origin of a nanoparticle administered to the mice and the strain assayed by ELISA. The x-axes indicate the panel of H1N1 influenza strains tested by reference year, from 1934 to 2013. Table 2 gives the complete strain designation for each year. Dashed line indicates the assay limit of detection.

[0057] **Figures 22A-22F**. Antibody response to compositions with Ribi or AF03 adjuvants as measured by assays of HAI titers. The results obtained with Ribi adjuvant (**FIGs 22A, 22C, and 22E**) compared to those obtained with AF03 adjuvant (**FIGs 22B, 22D, and 22F**) are similar for a bivalent combination of NC99 and CA09 HA-Nps (**FIGs 22A-22B**), a trivalent combination of NC99, CA09 and HK77 HA-Nps (**FIGs 22C-22D**), and a trivalent combination of NC99, CA09 and FM47 HA-Nps (**FIGs 22E-22F**). HAI titers were assayed in mouse serum 6 weeks after priming dose, with boost at week 3. Mice [n=5] were immunized with combinations of HA-Nps, as indicated, with either Ribi or AF03 adjuvants.

Asterisks indicate a match between the strain of origin of a nanoparticle administered to mice and the strain assayed by ELISA. The x-axes indicate the panel of H1N1 influenza strains tested by reference year, from 1934 to 2013. Table 2 gives the complete strain designation for each year. Dashed line indicates the assay limit of detection.

[0058] **Figures 23A-23C**. Comparative antibody response elicited by NC99 and CA09 inactivated influenza vaccines (IIV) produced in eggs as measured by assays of HAI titers. (**FIG 23A**) NC99 IIV. (**FIG 23B**) CA09 IIV. (**FIG 23C**) NC99+CA09 IIV. Hemagglutination Inhibition (HAI) titers (log_2) of sera from mice 6-weeks after immunization with the indicated IIV vaccines, as single components or co-administered, against a panel of divergent H1N1 influenza viruses. Mice (n=5) were immunized with 170 ng (HA content) of each vaccine at weeks 0 and 3, with Ribi adjuvant. The x-axis indicates the panel of H1N1 influenza strains tested by reference year, from 1934 to 2013 (*see also* Table 2). The x-axes indicate the panel of H1N1 influenza strains tested by reference year, from 1934 to 2013. Table 2 gives the complete strain designation for each year. Dashed line indicates the assay limit of detection.

[0059] **Figures 24A-24DE**. Antibody responses against various influenza viruses following immunization of ferrets using HA-ferritin nanoparticle compositions or IIV, as measured by assays of HAI titers. HAI titers (log_2) of serum from ferrets (n = 12 per group) following two immunizations with the following admixed with AF03 adjuvant: **FIG 24A**: PBS alone; **FIG 24B**: A/California/2009 IIV; **FIG 24C**: NC99+CA09+HK77 HA-Nps; and **FIG 24D**: COBRA-X6+P1+HK77 HA-Nps.

[0060] **Figure 24E.** Heterologous challenge of ferrets immunized with HA-ferritin nanoparticle compositions, IIV, or PBS. Ferrets were immunized as described for FIGs 24A-E and challenged with 1947 Fort Monmouth virus 4 weeks after the last immunization by intranasal inoculation with 1 mL of A/Fort Monmouth/1/1947 virus at $10^{4.65}$ times the 50% tissue culture infectious dose (TCID50). Virus titers were quantified in the nasal washes over a seven-day time-course after challenge. Dashed line indicates the assay limit of detection. Viral titers were significantly reduced at day 5 post-challenge in ferrets immunized with HA-Nps combinations, but not with CA09 IIV, as compared to vehicle (PBS) control by one-tailed unpaired t-test and by one-way ANOVA [$F(3,44) = 5.18$, $p = 0.00375$]. *** = $p \leq 0.001$, by Student's t-test.

[0061] **Figures 25A-C.** Antibody response of Cynomolgus macaques (*Macaca fascicularis*) following immunization with 50 μg of H1/Stem-Np formulated with admixed AF03 adjuvant, or 200 μg H1/Stem-Np (no adjuvant control), or 200 μg of H1/Stem-Np-

SM7/8a conjugate (shown in Figure 3A) or 200 μg of H1/Stem-Np-CpG conjugate (shown in Figure 3B). Figure 25A shows HA antibody titers measured by Enzyme-Linked Immunosorbent Assay (ELISA) on plates coated with H1/New Caledonia/20/1999 HA trimers at the indicated timepoints with immunizations at weeks 0, 4 and 10. Figure 25B shows neutralization IC50 of lentivirus pseudotyped with H1/New Caledonia/20/1999 HA and NA. Figure 25C shows neutralization IC50 of lentivirus pseudotyped with H5/Vietnam/1203/2004 HA and NA. This data demonstrates the self-adjuvanting property of the H1/Stem-Np-TLR-agonist conjugates in a primate model.

[0062] **Figures 26A-26B**. Comparison of titers obtained with NC99 HA-Np with or without conjugated or separate 3M-012 (SEQ ID NO: 9). The antibody response to NC99 HA-Np-3M012 conjugated nanoparticles four doses: 0.1 μg, 0.5 μg, 2.5 μg, and 12.5 μg. Admixture controls and unconjugated nanoparticle were included for comparison. The amount of TLR agonist used for the "equimolar" admix control and the "high dose" admix control were calculated to maintain the ratio of antigen to adjuvant used in the initial conjugation study (0.22 μg HA-NP with either 1.7 ng 3M012 for "equimolar" or 10 μg for "high dose"). Serum neutralization was measured in the NC99 lentivirus reporter assay (left, **FIG 26A**) and NC99 HAI assay (right, **FIG 26B**). Arrows highlight the 2.5 μg dose, wherein the HA-NP-3M012 conjugate induced similar antibody titers to HA-NP + 3M012, despite containing over 5000-fold less 3M012 adjuvant. Mean ± SEM is graphed.

DETAILED DESCRIPTION

[0063] Provided herein are antigenic influenza-ferritin polypeptides for immunization against influenza. The antigenic polypeptides comprise an influenza polypeptide and a ferritin, and can be used to vaccinate subjects against influenza. The ferritin allows for conjugation (e.g., via a mutation replacing a surface-exposed amino acid with a cysteine) to immune-stimulatory molecules, such as adjuvants, to eliminate the need for separately administered adjuvant, and to potentially reduce the amount of total adjuvant needed to elicit an immune response to the influenza polypeptide. Also provided are particles, compositions, and kits comprising the antigenic influenza-ferritin polypeptides, and uses thereof. Nucleic acids that encode the polypeptides described herein are also provided.

A. Definitions

[0064] "Hemagglutinin," or "HA," as used herein refers to the glycoprotein of any influenza virus responsible for binding to sialic acid on host cell membranes (an exemplary

hemagglutinin is UniProt Accession No: P03451). HA encompasses synthetic polypeptides that are recognized by or can elicit anti-HA antibodies, such as COBRA P1, COBRA X6 and COBRA X3 described below.

[0065] "HA stem," as used herein, refers to an engineered influenza polypeptide designed from the conserved region of HA within the HA ectodomain, which lacks an intact HA head. By conserved, it is meant that the region maintains a significantly higher sequence identity between HA from different strains of influenza with different HA subtypes than the sequence identity of HA as a whole. HA stem antigens are discussed in detail, for example, in Impagliazzo et al., Science 2015 Sep 18, 349(6254):1301-6; Valkenburg et al., Sci Rep. 2016 Mar 7, 6:22666; Mallajosyula et al., Front Immunol. 2015, 6: 329.

[0066] "Neuraminidase," or "NA," as used herein refers to the glycoprotein of any influenza virus responsible for catalyzing the removal of terminal sialic acid residues from viral and cellular glycoconjugates (an exemplary Neuraminidase is UniProt Accession No: P03472).

[0067] A "Y98F mutation," as used herein, refers to the replacement of a tyrosine in a wild-type HA sequence that makes a direct contact to sialic acid with a phenylalanine. The location of the phenylalanine resulting from this mutation is shown in Figure 1. Although the exact location may differ in some HA subtypes, it can be identified by sequence alignment or structural analysis. The presence of a Y98F mutation in an HA sequence implies that the corresponding wild-type HA is a subtype comprising a tyrosine that makes a direct contact to sialic acid.

[0068] "Ferritin" or "ferritin protein," as used herein, refers to a protein with detectable sequence identity to *H. pylori* ferritin (SEQ ID NO: 208 or 209) or another ferritin discussed herein, such as *P. furiosus* ferritin, *Trichoplusia ni* ferritin, or human ferritin, that serves to store iron, e.g., intracellularly or in tissues or to carry iron in the bloodstream. Such exemplary ferritins, including those that occur as two polypeptide chains, known as the heavy and light chains (e.g., *T. ni* and human ferritin), are discussed in detail below. In some embodiments, a ferritin comprises a sequence with at least 15%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identity to a ferritin sequence disclosed herein, e.g., in Table 1 (Sequence Table). A ferritin may be a fragment of a full-length naturally-occurring sequence.

[0069] "Wild-type ferritin," as used herein, refers to a ferritin whose sequence consists of a naturally-occurring sequence. Ferritins also include full-length ferritin or a

fragment of ferritin with one or more differences in its amino acid sequence from a wild-type ferritin.

[0070] As used herein, a "ferritin monomer" refers to a single ferritin molecule (or, where applicable, a single ferritin heavy or light chain) that has not assembled with other ferritin molecules. A "ferritin multimer" comprises multiple associated ferritin monomers. A "ferritin protein" includes monomeric ferritin and multimeric ferritin.

[0071] As used herein, "ferritin particle," refers to ferritin that has self-assembled into a globular form. Ferritin particles are sometimes referred to as "ferritin nanoparticles" or simply "nanoparticles". In some embodiments, a ferritin particle comprises 24 ferritin monomers (or, where applicable, 24 total heavy and light chains).

[0072] "Hybrid ferritin," as used herein, refers to ferritin comprising *H. pylori* ferritin with an amino terminal extension of bullfrog ferritin. An exemplary sequence used as an amino terminal extension of bullfrog ferritin appears as SEQ ID NO: 217. In hybrid ferritin, the amino terminal extension of bullfrog ferritin can be fused to *H. pylori* ferritin such that immune-stimulatory moiety attachment sites are distributed evenly on the ferritin particle surface. "Bullfrog linker" as used herein is a linker comprising the sequence of SEQ ID NO: 217. Hybrid ferritin is also sometimes referred to as "bfpFerr" or "bfp ferritin." Any of the constructs comprising a bullfrog sequence can be provided without the bullfrog sequence, such as, for example, without a linker or with an alternative linker. Exemplary bullfrog linker sequences are provided in Table 1. Where Table 1 shows a bullfrog linker, the same construct may be made without a linker or with an alternative linker.

[0073] "N-glycan," as used herein, refers to a saccharide chain attached to a protein at the amide nitrogen of an N (asparagine) residue of the protein. As such, an N-glycan is formed by the process of N-glycosylation. This glycan may be a polysaccharide.

[0074] "Glycosylation," as used herein, refers to the addition of a saccharide unit to a protein.

[0075] "Immune response," as used herein, refers to a response of a cell of the immune system, such as a B cell, T cell, dendritic cell, macrophage or polymorphonucleocyte, to a stimulus such as an antigen or vaccine. An immune response can include any cell of the body involved in a host defense response, including for example, an epithelial cell that secretes an interferon or a cytokine. An immune response includes, but is not limited to, an innate and/or adaptive immune response. As used herein, a "protective immune response" refers to an immune response that protects a subject from infection (e.g., prevents infection or prevents the development of disease associated with infection). Methods of measuring immune

responses are well known in the art and include, for example, by measuring proliferation and/or activity of lymphocytes (such as B or T cells), secretion of cytokines or chemokines, inflammation, antibody production and the like. An "antibody response" is an immune response in which antibodies are produced.

[0076] As used herein, an "antigen" refers to an agent that elicits an immune response, and/or an agent that is bound by a T cell receptor (e.g., when presented by an MHC molecule) or to an antibody (e.g., produced by a B cell) when exposed or administered to an organism. In some embodiments, an antigen elicits a humoral response (e.g., including production of antigen-specific antibodies) in an organism. Alternatively, or additionally, in some embodiments, an antigen elicits a cellular response (e.g., involving T-cells whose receptors specifically interact with the antigen) in an organism. A particular antigen may elicit an immune response in one or several members of a target organism (e.g., mice, rabbits, primates, humans), but not in all members of the target organism species. In some embodiments, an antigen elicits an immune response in at least about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the members of a target organism species. In some embodiments, an antigen binds to an antibody and/or T cell receptor, and may or may not induce a particular physiological response in an organism. In some embodiments, for example, an antigen may bind to an antibody and/or to a T cell receptor in vitro, whether or not such an interaction occurs in vivo. In some embodiments, an antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. Antigens include antigenic influenza-ferritin polypeptides as described herein.

[0077] An "immune-stimulatory moiety," as used herein, refers to a moiety that is covalently attached to a ferritin or antigenic ferritin polypeptide and that can activate a component of the immune system (either alone or when attached to ferritin or antigenic ferritin polypeptide). Exemplary immune-stimulatory moieties include agonists of toll-like receptors (TLRs), e.g., TLR 4, 7, 8, or 9. In some embodiments, an immune-stimulatory moiety is an adjuvant.

[0078] "Adjuvant," as used herein, refers to a substance or vehicle that non-specifically enhances the immune response to an antigen. Adjuvants can include, without limitation, a suspension of minerals (e.g., alum, aluminum hydroxide, or phosphate) on which antigen is adsorbed; a water-in-oil or oil-in-water emulsion in which antigen solution is emulsified in mineral oil or in water (e.g., Freund's incomplete adjuvant). Sometimes killed mycobacteria is included (e.g., Freund's complete adjuvant) to further enhance antigenicity.

Immuno-stimulatory oligonucleotides (e.g., a CpG motif) can also be used as adjuvants (for example, *see* U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199). Adjuvants can also include biological molecules, such as Toll-Like Receptor (TLR) agonists and costimulatory molecules. An adjuvant may be administered as a separate molecule in a composition or covalently bound (conjugated) to an antigenic influenza-ferritin polypeptide.

[0079] An "antigenic influenza-ferritin polypeptide" is used herein to refer to a molecule comprising a ferritin and an influenza polypeptide, wherein the molecule is antigenic with respect to the influenza polypeptide. Antigenicity may be a feature of the influenza polypeptide as part of the larger construct. That is, it is sufficient that the construct can serve as an antigen that generates antibodies against the influenza polypeptide, regardless of whether the influenza polypeptide without the ferritin could do so. In some embodiments, the influenza polypeptide and ferritin are genetically fused as a fusion protein. In some embodiments, the influenza polypeptide and ferritin are non-genetically linked, for example, by chemical conjugation.

[0080] "Self-adjuvanting," as used herein, refers to a composition or polypeptide comprising a ferritin and an immune-stimulatory moiety directly conjugated to the ferritin so that the ferritin and immune-stimulatory moiety are in the same molecular entity. An antigenic influenza-ferritin polypeptide may be conjugated to an immune-stimulatory moiety to generate a self-adjuvanting polypeptide.

[0081] A "surface-exposed" amino acid, as used herein, refers to an amino acid residue in a protein (e.g., a ferritin) with a side chain that can be contacted by solvent molecules when the protein is in its native three-dimensional conformation after multimerization, if applicable. Thus, for example, in the case of ferritin that forms a 24-mer, a surface-exposed amino acid residue is one whose side chain can be contacted by solvent when the ferritin is assembled as a 24-mer, e.g., as a ferritin multimer or ferritin particle.

[0082] As used herein, a "subject" refers to any member of the animal kingdom. In some embodiments, "subject" refers to humans. In some embodiments, "subject" refers to non-human animals. In some embodiments, subjects include, but are not limited to, mammals, birds, reptiles, amphibians, fish, insects, and/or worms. In certain embodiments, the non-human subject is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, a subject may be a transgenic animal, genetically-engineered animal, and/or a clone. In certain embodiments of the present invention the subject is an adult, an adolescent or an infant. In some

embodiments, terms "individual" or "patient" are used and are intended to be interchangeable with "subject".

[0083] As used herein, the term "vaccination" or "vaccinate" refers to the administration of a composition intended to generate an immune response, for example to a disease-causing agent. Vaccination can be administered before, during, and/or after exposure to a disease-causing agent, and/or to the development of one or more symptoms, and in some embodiments, before, during, and/or shortly after exposure to the agent. In some embodiments, vaccination includes multiple administrations, appropriately spaced in time, of a vaccinating composition.

[0084] The disclosure describes nucleic acid sequences and amino acid sequences having a certain degree of identity to a given nucleic acid sequence or amino acid sequence, respectively (a references sequence).

[0085] "Sequence identity" between two nucleic acid sequences indicates the percentage of nucleotides that are identical between the sequences. "Sequence identity" between two amino acid sequences indicates the percentage of amino acids that are identical between the sequences.

[0086] The terms "% identical", "% identity" or similar terms are intended to refer, in particular, to the percentage of nucleotides or amino acids which are identical in an optimal alignment between the sequences to be compared. Said percentage is purely statistical, and the differences between the two sequences may be but are not necessarily randomly distributed over the entire length of the sequences to be compared. Comparisons of two sequences are usually carried out by comparing said sequences, after optimal alignment, with respect to a segment or "window of comparison", in order to identify local regions of corresponding sequences. The optimal alignment for a comparison may be carried out manually or with the aid of the local homology algorithm by Smith and Waterman, 1981, Ads App. Math. 2, 482, with the aid of the local homology algorithm by Needleman and Wunsch, 1970, J. Mol. Biol. 48, 443, with the aid of the similarity search algorithm by Pearson and Lipman, 1988, Proc. Natl Acad. Sci. USA 88, 2444, or with the aid of computer programs using said algorithms (GAP, BESTFIT, FASTA, BLAST P, BLAST N and TFASTA in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.).

[0087] Percentage identity is obtained by determining the number of identical positions at which the sequences to be compared correspond, dividing this number by the number of

positions compared (e.g., the number of positions in the reference sequence) and multiplying this result by 100.

[0088] In some embodiments, the degree of identity is given for a region which is at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or about 100% of the entire length of the reference sequence. For example, if the reference nucleic acid sequence consists of 200 nucleotides, the degree of identity is given for at least about 100, at least about 120, at least about 140, at least about 160, at least about 180, or about 200 nucleotides, in some embodiments in continuous nucleotides. In some embodiments, the degree of identity is given for the entire length of the reference sequence.

[0089] Nucleic acid sequences or amino acid sequences having a particular degree of identity to a given nucleic acid sequence or amino acid sequence, respectively, may have at least one functional property of said given sequence, e.g., and in some instances, are functionally equivalent to said given sequence. One important property includes the ability to act as a cytokine, in particular when administered to a subject. In some embodiments, a nucleic acid sequence or amino acid sequence having a particular degree of identity to a given nucleic acid sequence or amino acid sequence is functionally equivalent to said given sequence.

[0090] As used herein, the term "kit" refers to a packaged set of related components, such as one or more compounds or compositions and one or more related materials such as solvents, solutions, buffers, instructions, or desiccants.

B. Antigenic influenza-ferritin polypeptides

[0091] Provided herein are antigenic polypeptides comprising an influenza polypeptide and a ferritin comprising a mutation replacing a surface-exposed amino acid with a cysteine. The polypeptides can be antigenic when administered with adjuvant as a separate molecule and/or as part of a nanoparticle (e.g., ferritin particle), which can be self-adjuvanting.

1. HA and NA polypeptides

[0092] In some embodiments, the influenza polypeptide is an HA or NA polypeptide comprising a full or partial length HA or NA. Any HA or NA polypeptide may be used. The HA or NA polypeptide may be naturally occurring or altered from nature. In some embodiments the HA is from any one of H1-H18. In some embodiments the NA is from any one of N1-N11.

[0093] In some embodiments, the HA polypeptide comprises an HA ectodomain. The HA ectodomain may be from any subtype of influenza, including H1-H18.

[0094] In some embodiments, the HA polypeptide comprises a stem region of HA. The stem region of HA may be from any subtype of influenza, including H1-H18.

[0095] In some embodiments, the HA polypeptide is from a Type A influenza virus. The Type A influenza virus may be A/Puerto Rico/1934, A/Weiss/1/1943, A/Fort Monmouth/1/1947 (FM47), A/Malaysia/302/54 (MAL54), A/Denver/1/1957 (DV57), A/New Jersey/8/1976, A/USSR/90/1977, A/Hong Kong/117/1977 (HK77), A/Brazil/11/1978, A/Chile/1/1983, A/Taiwan/1/1986, A/Texas/36/1991, A/Beijing/262/1995, A/New Caledonia/20/1999 (NC99), A/Solomon Islands/6/2006, A/Brisbane/59/2007, A/California/07/2009 (CA09), A/Bangladesh/2021/2012, or A/Vietnam/3050/2013.

[0096] In some embodiments, the HA polypeptide is from an H1 influenza virus. In some embodiments, the H1 virus is A/South Carolina/1/18.

[0097] In some embodiments, the HA polypeptide is from an H2 influenza virus. In some embodiments, the H2 virus is the 1957 pandemic H2N2 influenza A virus.

[0098] In some embodiments, the HA polypeptide is from an H3 influenza virus. In some embodiments, the H3 influenza virus is an H3N8 virus. In some embodiments, the H3N8 virus is Equine Ohio 2003. In some embodiments, the H3N8 virus is Equine Bari 2005. In some embodiments, the H3N8 virus is Equine Aboyne 2003. In some embodiments, the H3 influenza virus is an H3N2 virus. In some embodiments, the H3N2 virus is Perth 2009. In some embodiments, the H3N2 virus is Victoria 2011.

[0099] In some embodiments, the HA polypeptide is from an H5 influenza virus. In some embodiments, the H5 influenza virus is an H5/N1 virus. In some embodiments, the H5/N1 virus is Indonesia 2005. In some embodiments, the H5/N1 virus is Bar Headed Goose 2005. In some embodiments, the H5/N1 virus is Whooper Swan 2005. In some embodiments, the H5/N1 virus is Mallard/Huadong 2003.

[00100] In some embodiments, the HA polypeptide is from an influenza Type B virus. In some embodiments, the Type B virus is Wisconsin 2010. In some embodiments, the Type B virus is Massachusetts 2012. In some embodiments, the Type B virus is Phuket 2013. In some embodiments, the Type B virus is Brisbane 2008. In some embodiments, the Brisbane 2008 sequence comprises a D197N mutation. This mutation was found to improve expression of this nanoparticle and it is a naturally occurring mutation in other strains, such as B/Brisbane/2009 and B/Phuket/2013. This amino acid may be involved in contacting sialic acid receptors.

[00101] In some embodiments, the HA polypeptide comprises a Computationally Optimized Broadly Reactive Antigen (COBRA) generated following examples of Giles BM and Ross TM Vaccine 29(16):3043-54 (2011) or Carter DM, et al., J Virol 90:4720-4734 (2016).

[00102] In some embodiments, a COBRA sequence is generated from human H1N1 influenza sequences. In some embodiments, a COBRA sequence is generated from human H1N1 influenza sequences spanning 1999-2012. An exemplary COBRA sequence generated from human H1N1 influenza sequences spanning 1999-2012 is COBRA X6, comprised in SEQ ID NO: 29. In some embodiments, a COBRA sequence is generated from human H1N1 strains spanning 1933-1957 and 2009-2011 plus swine H1N1 influenza strains from 1931-1998. An exemplary COBRA sequence generated from human H1N1 strains spanning 1933-1957 and 2009-2011 plus swine H1N1 influenza strains from 1931-1998 is COBRA P1, comprised in SEQ ID NO: 27.

[00103] In some embodiments, the COBRA sequence is X3. In some embodiments, the COBRA sequence is hCOBRA-2 generated from H5N1.

[00104] A mutation to eliminate the HA receptor binding site (Y98F) was described in Whittle et al. Journal of Virology 11(8):4047-4057 (2014). In some embodiments, the HA polypeptide comprises a Y98F mutation. Any of the HAs noted above can be modified to comprise a Y98F mutation. In some embodiments, the HA is from a H1/New Caledonia/1999 (NC99) virus and comprises a Y98F mutation.

2. Ferritin

[00105] Ferritin protein self-assembles into a globular protein complex comprising multiple individual monomers. The self-assembled ferritin complex may be referred to as a ferritin particle or nanoparticle.

[00106] Ferritin genes are found in many species and generally show a conserved highly alpha-helical structure despite sequence variation. As such, any ferritin can be used in the invention, including bacterial, insect, and human ferritin, despite its sequence identity to any particularly described ferritin.

[00107] In some embodiments, the ferritin is bacterial, insect, fungal, bird, or mammalian. In some embodiments, the ferritin is human. In some embodiments, the ferritin is bacterial. In some embodiments, the ferritin is *H. pylori* ferritin.

[00108] In some embodiments, the ferritin is a light chain and/or heavy chain ferritin. In some embodiments, the ferritin is human heavy chain ferritin (FTH1, GENE ID No: 2495)

or human light chain ferritin (FTL, GENE ID No: 2512), optionally with one or more modifications described herein. In some embodiments, the ferritin is *Trichoplusia ni* heavy chain ferritin (GenBank: AY970291.1) or *Trichoplusia ni* light chain ferritin (AY970292.1), optionally with one or more mutations described herein. In some embodiments, a ferritin nanoparticle comprises 24 total subunits of heavy chain ferritin and light chain ferritin, e.g., 12 heavy chain subunits and 12 light chain subunits. In some embodiments, a ferritin comprises a mutation replacing a surface-exposed amino acid with a cysteine.

[00109] In some embodiments, an antigenic influenza-ferritin polypeptide is provided comprising an influenza polypeptide of sufficient length that the molecule is antigenic with respect to the influenza polypeptide.

[00110] In some embodiments, the antigenic ferritin polypeptide comprises a light chain ferritin and an influenza polypeptide. In some embodiments, the antigenic ferritin polypeptide comprises a heavy chain ferritin and an influenza polypeptide. In some embodiments, a light chain ferritin and an influenza polypeptide can assemble with a heavy chain ferritin without an influenza polypeptide. In some embodiments, a heavy chain ferritin and an influenza polypeptide can assemble with a light chain ferritin without an influenza polypeptide. A ferritin without a non-ferritin polypeptide (e.g., influenza polypeptide) may be referred as a "naked ferritin."

[00111] In some embodiments, the antigenic influenza-ferritin polypeptide comprises a heavy chain ferritin and an influenza polypeptide, or a light chain ferritin and an influenza polypeptide. Such polypeptides can be combined to allow expression of two of the same or different influenza polypeptides on a single ferritin multimer or particle. In some embodiments, two different influenza polypeptides are obtained from two different infectious agents, e.g., different strains or types of influenza, and attached to heavy and light chain ferritins for assembly into a nanoparticle.

[00112] In some embodiments, the antigenic ferritin polypeptide comprises a heavy chain ferritin and a first influenza polypeptide assembled with a light chain ferritin and a second influenza polypeptide to produce a bivalent vaccine. In some embodiments, the ferritin is *H. pylori* ferritin (*see* SEQ ID NOS: 208 or 209 for exemplary *H. pylori* ferritin sequences) with one or more mutations described herein. In some embodiments, the lower sequence homology between *H. pylori* ferritin (or other bacterial ferritins) and human ferritin may decrease the potential for autoimmunity when used as a vaccine platform (*see* Kanekiyo et al., Cell 162, 1090–1100 (2015)).

[00113] In some embodiments, the ferritin is *Pyrococcus furiosus* ferritin (NCBI seq WP_011011871.1) with one or more mutations described herein.

[00114] In some embodiments, the ferritin comprises a sequence having greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 97%, greater than 98%, or greater than 99% identity to a wild-type ferritin.

a) **Ferritin mutations**

[00115] In some embodiments, the ferritin comprises one or more mutations are disclosed herein. In some embodiments, the one or more mutations comprise changes to the amino acid sequence of a wild-type ferritin and/or an insertion, e.g., at the N- or C-terminus. In some embodiments, one, two, three, four, five, or more different amino acids are mutated in the ferritin as compared to wild-type ferritin (in some embodiments, in addition to any N-terminal insertion). The one or more mutations can change functional properties of the ferritin, e.g., as discussed in detail below. In general, a mutation simply refers to a difference in the sequence (such as a substituted, added, or deleted amino acid residue or residues) relative to the corresponding wild-type ferritin.

(1) Cysteine for conjugation

[00116] In some embodiments, ferritin is mutated to provide a chemical handle for conjugation of an immune-stimulatory moiety and/or influenza polypeptide. This can be achieved with a mutation replacing a surface-exposed non-cysteine amino acid with a cysteine. For the avoidance of doubt, language such as "replacing a surface-exposed amino acid with a cysteine" necessarily implies that the surface-exposed amino acid in the wild-type or pre-mutation sequence is not cysteine. Another approach for providing a chemical handle for conjugation of an immune-stimulatory moiety or influenza polypeptide is to include a segment of amino acids, such as a linker, N- or C-terminal to the ferritin, wherein the segment of amino acids comprises a cysteine. In some embodiments, this cysteine (whether replacing a surface-exposed amino acid or in an N- or C-terminal linker) is unpaired, which means that it does not have an appropriate partner cysteine to form a disulfide bond. In some embodiments, this cysteine does not change the secondary structure of ferritin. In some embodiments, this cysteine does not change the tertiary structure of ferritin.

[00117] In some embodiments, this cysteine can be used to conjugate agents, such as immune-stimulatory moieties, to ferritin. In some embodiments, this cysteine provides a free thiol group that is reactive. In some embodiments, agents conjugated to this cysteine on ferritin are exposed on the surface of an assembled ferritin particle. In some embodiments,

this cysteine can interact with molecules and cells of the subject after administration while the ferritin particle is assembled.

[00118] In some embodiments, the presence of this cysteine allows conjugation of one or more immune-stimulatory moieties, e.g., adjuvants. In some embodiments, conjugation of the immune-stimulatory moiety would not occur in the absence of this cysteine.

[00119] In some embodiments, the non-cysteine amino acid that is replaced with a cysteine is selected from E12, S72, A75, K79, S100, and S111 of *H. pylori* ferritin. Thus, in some embodiments, the surface-exposed amino acid that is replaced in favor of cysteine is an amino acid residue that corresponds to E12, S26, S72, A75, K79, S100, or S111 of *H. pylori* ferritin. Analogous amino acids can be found in non-*H. pylori* ferritin by pair-wise or structural alignment. In some embodiments, the non-cysteine amino acid that is replaced with a cysteine can be selected from an amino acid that corresponds to S3, S19, S33, I82, A86, A102, and A120 of human light chain ferritin. In some embodiments, the surface-exposed amino acid to be replaced with a cysteine is selected based on the understanding that if the native amino acid were replaced with cysteine, it would be reactive in an assembled ferritin multimer or particle and/or that this cysteine does not disrupt the stability of the ferritin multimer or particle and/or that this cysteine does not lead to reduction in expression levels of ferritin.

[00120] In some embodiments, the ferritin comprises an E12C mutation. In some embodiments, the E12C residue can be used to conjugate agents (e.g., immune-stimulatory moieties and/or influenza polypeptides) to ferritin. In some embodiments, the E12C residue provides a free thiol group that is reactive. In some embodiments, agents conjugated to the E12C residue on ferritin monomers are expressed on the surface on an assembled ferritin multimer or particle. In some embodiments, twenty-four E12C residues (one from each monomer) are present on the surface of a ferritin multimer or particle.

[00121] In some embodiments, the ferritin comprises an S26C mutation. In some embodiments, the S26C residue can be used to conjugate agents (e.g., immune-stimulatory moieties and/or influenza polypeptides) to ferritin. In some embodiments, the S26C residue provides a free thiol group that is reactive. In some embodiments, agents conjugated to the S26C residue on ferritin monomers are expressed on the surface on an assembled ferritin multimer or particle. In some embodiments, twenty-four S26C residues (one from each monomer) are present on the surface of a ferritin multimer or particle.

[00122] In some embodiments, the ferritin comprises an S72C mutation. In some embodiments, the S72C residue can be used to conjugate agents (e.g., immune-stimulatory

moieties and/or influenza polypeptides) to ferritin. In some embodiments, the S72C residue provides a free thiol group that is reactive. In some embodiments, agents conjugated to the S72C residue on ferritin monomers are expressed on the surface on an assembled ferritin multimer or particle. In some embodiments, twenty-four S72C residues (one from each monomer) are present on the surface of a ferritin multimer or particle.

[00123] In some embodiments, the ferritin comprises an A75C mutation. In some embodiments, the A75C residue can be used to conjugate agents (e.g., immune-stimulatory moieties and/or influenza polypeptides) to ferritin. In some embodiments, the A75C residue provides a free thiol group that is reactive. In some embodiments, agents conjugated to the A75C residue on ferritin monomers are expressed on the surface on an assembled ferritin multimer or particle. In some embodiments, twenty-four A75C residues (one from each monomer) are present on the surface of a ferritin multimer or particle.

[00124] In some embodiments, the ferritin comprises an K79C mutation. In some embodiments, the K79C residue can be used to conjugate agents (e.g., immune-stimulatory moieties and/or influenza polypeptides) to ferritin. In some embodiments, the K79C residue provides a free thiol group that is reactive. In some embodiments, agents conjugated to the K79C residue on ferritin monomers are expressed on the surface on an assembled ferritin multimer or particle. In some embodiments, twenty-four K79C residues (one from each monomer) are present on the surface of a ferritin multimer or particle.

[00125] In some embodiments, the ferritin comprises an S100C mutation. In some embodiments, the S100C residue can be used to conjugate agents (e.g., immune-stimulatory moieties and/or influenza polypeptides) to ferritin. In some embodiments, the S100C residue provides a free thiol group that is reactive. In some embodiments, agents conjugated to the S100C residue on ferritin monomers are expressed on the surface on an assembled ferritin multimer or particle. In some embodiments, twenty-four S100C residues (one from each monomer) are present on the surface of a ferritin multimer or particle.

[00126] In some embodiments, the ferritin comprises an S111C mutation. In some embodiments, the S111C residue can be used to conjugate agents (e.g., immune-stimulatory moieties and/or influenza polypeptides) to ferritin. In some embodiments, the S111C residue provides a free thiol group that is reactive. In some embodiments, agents conjugated to the S111C residue on ferritin monomers are expressed on the surface on an assembled ferritin multimer or particle. In some embodiments, twenty-four S111C residues (one from each monomer) are present on the surface of a ferritin multimer or particle.

(2) Removal of internal cysteine

[00127] In some embodiments, the ferritin comprises a mutation replacing an internal cysteine with a non-cysteine amino acid. Removal of a native internal cysteine residue can ensure that there is only one unpaired cysteine per ferritin monomer and avoid undesired reactions such as disulfide formation and may result in a more stable and efficient result (e.g., adjuvant presentation). In some embodiments, C31 of *H. pylori* ferritin is replaced with a non-cysteine amino acid. In some embodiments, C31 of *H. pylori* ferritin is replaced with a serine (C31S), although any non-cysteine residue may be used, e.g., alanine, glycine, threonine, or asparagine. Analogous amino acids can be found in non-*H. pylori* ferritin by pair-wise or structural alignment. Thus, in some embodiments, the internal cysteine that is replaced in favor of non-cysteine is an amino acid residue that aligns with C31 of *H. pylori* ferritin. Exemplary ferritin sequences showing a C31S mutation are shown in SEQ ID NOS: 201-207. In some embodiments, when more than one internal cysteine is present in ferritin, two or more (e.g., each) internal cysteine is replaced with a non-cysteine amino acid, such as serine or an amino acid selected from serine, alanine, glycine, threonine, or asparagine.

(3) Glycosylation

[00128] Human-compatible glycosylation can contribute to safety and efficacy in recombinant drug products. Regulatory approval may be contingent on demonstrating appropriate glycosylation as a critical quality attribute (*see* Zhang et al., Drug Discovery Today 21(5):740-765 (2016)). N-glycans can result from glycosylation of asparagine side chains and can differ in structure between humans and other organisms such as bacteria and yeast. Thus, it may be desirable to reduce or eliminate non-human glycosylation and/or N-glycan formation in ferritin according to the disclosure. In some embodiments, controlling glycosylation of ferritin improves the efficacy and/or safety of the composition, especially when used for human vaccination.

[00129] In some embodiments, ferritin is mutated to inhibit formation of an N-glycan. In some embodiments, a mutated ferritin has reduced glycosylation as compared to its corresponding wild type ferritin.

[00130] In some embodiments, the ferritin comprises a mutation replacing a surface-exposed asparagine with a non-asparagine amino acid. In some embodiments, the surface-exposed asparagine is N19 of *H. pylori* ferritin or a position that corresponds to position 31 of *H. pylori* ferritin as determined by pair-wise or structural alignment In some embodiments, mutating such an asparagine, e.g., N19 of *H. pylori* ferritin, decreases glycosylation of ferritin. In some embodiments, the mutation replaces the asparagine with a glutamine. In

some embodiments, the ferritin is an *H. pylori* ferritin comprising an N19Q mutation. SEQ ID NOS: 201-207 are exemplary ferritin sequences comprising N19Q mutations.

[00131] A mammal exposed to a glycosylated protein produced in bacteria or yeast may generate an immune response to the glycosylated protein, because the pattern of glycosylation of a given protein in bacterial or yeast could be different from the pattern of glycosylation of the same protein in a mammal. Thus, some glycosylated therapeutic proteins may not be appropriate for production in bacteria or yeast.

[00132] In some embodiments, decreased glycosylation of ferritin by amino acid mutation facilitates protein production in bacteria or yeast. In some embodiments, decreased glycosylation of ferritin reduces the potential for adverse effects in mammals upon administration of mutated ferritin that is expressed in bacteria or yeast. In some embodiments, the reactogenicity in a human subject of a mutated ferritin produced in bacteria or yeast is lower because glycosylation is decreased. In some embodiments, the incidence of hypersensitivity responses in human subjects is lower following treatment with a mutated ferritin with reduced glycosylation compared to wildtype ferritin.

[00133] In some embodiments, degradation in a subject of a composition comprising a mutated ferritin with reduced glycosylation is slower compared with a composition comprising a wild-type ferritin, or a composition comprising a corresponding ferritin with wild-type glycosylation. In some embodiments, a composition comprising a mutated ferritin with reduced glycosylation has reduced clearance in a subject compared with a composition comprising a wild-type ferritin, or a composition comprising a corresponding ferritin with wild-type glycosylation. In some embodiments, a composition comprising a mutated ferritin with reduced glycosylation has a longer-serum half-life compared to wild-type ferritin, or a composition comprising a corresponding ferritin with wild-type glycosylation.

(4) Combinations of mutations

[00134] In some embodiments, a ferritin comprises more than one type of mutation described herein. In some embodiments, the ferritin comprises one or more mutations independently selected from: a mutation to decrease glycosylation, a mutation to remove an internal cysteine, and a mutation to generate a surface-exposed cysteine. In some embodiments, the ferritin comprises a mutation to decrease glycosylation, a mutation to remove an internal cysteine, and a mutation to generate a surface-exposed cysteine.

[00135] In some embodiments, the ferritin comprises an N19Q mutation, a C31S mutation, and a mutation to generate a surface-exposed cysteine. In some embodiments, the ferritin comprises an N19Q mutation, a C31S mutation, and an E12C mutation. In some

embodiments, the ferritin comprises an N19Q mutation, a C31S mutation, and an S72C mutation. In some embodiments, the ferritin comprises an N19Q mutation, a C31S mutation, and an A75C mutation. In some embodiments, the ferritin comprises an N19Q mutation, a C31S mutation, and an K79C mutation. In some embodiments, the ferritin comprises an N19Q mutation, a C31S mutation, and an S100C mutation. In some embodiments, the ferritin comprises an N19Q mutation, a C31S mutation, and an S111C mutation. In some embodiments, the ferritin comprises mutations corresponding to any of the foregoing sets of mutations, wherein the corresponding mutations change an N to a Q, a C to an S, and a non-cysteine surface-exposed amino acid to a cysteine at positions determined by pair-wise alignment of the ferritin amino acid sequence to an *H. pylori* ferritin amino acid sequence (SEQ ID NO: 208 OR 209).

[00136] Exemplary ferritins comprising more than one type of mutation are provided in SEQ ID NOS: 201-207.

3. Linker

[00137] The influenza polypeptide and ferritin may be connected via a linker to provide an antigenic influenza ferritin polypeptide. In some embodiments, a linker separates the amino acid sequence of the influenza polypeptide from the amino acid sequence of ferritin. Any linker may be used. In some embodiments, the ferritin is joined to the HA polypeptide via a peptide linker. This can facilitate expression of the antigenic ferritin polypeptide as a fusion protein (e.g., from a single open reading frame). In some embodiments, the linker is a glycine-serine linker. In some embodiments, the glycine-serine linker is GS, GGGS (SEQ ID NO: 226), 2XGGGS (SEQ ID NO: 227) (i.e., GGGSGGGS (SEQ ID NO: 227)), or 5XGGGS (SEQ ID NO: 228). The linker may be N- or C- terminal to ferritin.

[00138] In some embodiments, the linker is 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in length. In some embodiments, the linker is about 2-4, 2-6, 2-8, 2-10, 2-12, or 2-14 amino acids in length. In some embodiments, the linker is at least 15 amino acids in length. In some embodiments, the linker is at least 25 amino acids in length. In some embodiments, the linker is at least 30 amino acids in length. In some embodiments, the linker is at least 35 amino acids in length. In some embodiments, the linker is at least 40 amino acids in length. In some embodiments, the linker is less than or equal to 60 amino acids in length. In some embodiments, the linker is less than or equal to 50 amino acids in length. In some embodiments, the linker is about 16, 28, 40, 46, or 47 amino acids in length. In some

embodiments, the linker is flexible. In some embodiments, the linker comprises a cysteine, e.g., for use as a site for conjugation of an immune-stimulatory moiety (e.g., adjuvant); an exemplary linker comprising a cysteine is provided as SEQ ID NO: 225. In some embodiments, the linker comprises a sequence with at least 75%, 80%, 85%, 90%, or 95% identity to SEQ ID NO: 225, and further comprises a cysteine corresponding to the cysteine in SEQ ID NO: 225. In some embodiments, the linker comprises at least 25 amino acids (e.g., 25 to 60 amino acids), wherein a cysteine is located at a position ranging from the 8th amino acid from the N-terminus to the 8th amino acid from the C-terminus, or within 10 amino acids of the central residue or bond of the linker.

[00139] In some embodiments, the linker comprises glycine (G) and/or serine (S) amino acids. In some embodiments, the linker comprises or consists of glycine (G), serine (S), asparagine (N), and/or alanine (A) amino acids, and optionally a cysteine as discussed herein. In some embodiments, the linker comprises an amino acid sequence with at least 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 222. In some embodiments, the linker is GGGGSGGGGSGGGGSG (SEQ ID NO: 220), GGSGSGSNSSASSGASSGGASGGSGGSG (SEQ ID NO: 221), GGSGSASSGASASGSSNGSGSGSGSNSSASSGASSGGASGGSGGSG (SEQ ID NO: 222), or GS. In some embodiments, the linker is FR1 (SEQ ID NO: 223) or FR2 (SEQ ID NO: 224).

[00140] In some embodiments, the ferritin comprises *H. pylori* ferritin with the amino terminal extension of bullfrog ferritin (which will be referred to as hybrid ferritin). In some embodiments, this hybrid ferritin forms multimers with influenza polypeptide-attachment sites distributed evenly on the surface (*see* Kanekiyo 2015). In some embodiments, N-terminal fusion proteins with hybrid ferritin allow presentation of an influenza polypeptide on the ferritin nanoparticle surface. In some embodiments, a ferritin comprises a glutamate at a position corresponding to position 13 of SEQ ID NO: 208 (hybrid ferritin, which comprises this glutamate) or position 6 in SEQ ID NO: 209 (wild-type *H. pylori* ferritin, in which position 6 is isoleucine). In combination with a bullfrog linker, this glutamate is thought to preserve the conserved salt bridge found in human and bullfrog ferritins (6R and 14E in both human light chain and bullfrog lower-subunit ferritins). *See* Kanekiyo et al., Cell 162, 1090–1100 (2015)).

[00141] In some embodiments, an influenza polypeptide is linked to ferritin via a cysteine-thrombin-histidine linker. In some embodiments, this linker is used to directly conjugate a moiety (e.g., immune-stimulatory moiety or influenza polypeptide) to ferritin via

click chemistry. An exemplary sequence comprising a cysteine-thrombin-histidine linker is SEQ ID NO: 218**.** Click chemistry suitable for conjugation reactions involving the cysteine-thrombin-histidine linker is discussed herein.

[00142] In some embodiments, a linker comprising a cysteine as a conjugation site for an immune-stimulatory moiety such as an adjuvant is used in a construct comprising a ferritin molecule lacking an unpaired, surface-exposed cysteine, or in a construct comprising a ferritin molecule comprising an unpaired, surface-exposed cysteine.

[00143] In some embodiments, a construct does not comprise a linker. In some embodiments, a construct comprises one linker. In some embodiments, a construct comprises two or more than two linkers.

4. Structural alignment

[00144] As discussed herein, positions of mutations corresponding to those described with respect to a given polypeptide (e.g, *H. pylori* ferritin) can be identified by pairwise or structural alignment. Structural alignment is relevant to large protein families such as ferritin where the proteins share similar structures despite considerable sequence variation and many members of the family have been structurally characterized, and can also be used to identify corresponding positions in different versions of other polypeptides described herein, such as influenza polypeptides (e.g., hemagglutinin). The protein databank (PDB) comprises 3D structures for many ferritins, including those listed below with their accession numbers.

[00145] 2jd6, 2jd7 – PfFR - Pyrococcus furiosus. 2jd8 – PfFR+Zn. 3a68 – soFR from gene SferH4 – soybean. 3a9q - soFR from gene SferH4 (mutant). 3egm, 3bvf, 3bvi, 3bvk, 3bvl – HpFR – Heliobacter pylori. 5c6f – HpFR (mutant) + Fe. 1z4a, 1vlg – FR – Thermotoga maritime. 1s3q, 1sq3, 3kx9 – FR – Archaeoglubus fulgidus. 1krq – FR – Campylobacter jejuni. 1eum - EcFR – Escherichia coli. 4reu – EcFR + Fe. 4xgs – EcFR (mutant) + Fe2O2. 4ztt – EcFR (mutant) + Fe2O + Fe2 + Fe + O2. 1qgh – LiFR - Listeria innocua. 3qz3 - VcFR – Vibrio cholerae. 3vnx – FR – Ulva pertusa. 4ism, 4isp, 4itt, 4itw, 4iwj, 4iwk, 4ixk, 3e6s – PnmFR – Pseudo-nitschia multiseries. 4zkh, 4zkw, 4zkx, 4zl5, 4zl6, 4zlw, 4zmc – PnmFR (mutant) + Fe. 1z6o – FR – Trichoplusia ni. 4cmy – FR + Fe – Chlorobaculum tepidum. Ferritin light chain (FTL). 1lb3, 1h96 – mFTL – mouse. 1rcc, 1rcd, 1rci – bFTL+tartrate+Mg. 1rce, 1rcg - bFTL+tartrate+Mn. 3noz, 3np0, 3np2, 3o7r – hoFTL (mutant) - horse. 3o7s, 3u90 - hoFTL. 4v1w – hoFTL – cryo EM. 3rav, 3rd0 – hoFTL + barbiturate. Ferritin light+heavy chains: 5gn8 – hFTH + Ca.

[00146] Structural alignment involves identifying corresponding residues across two (or more) polypeptide sequences by (i) modeling the structure of a first sequence using the known structure of the second sequence or (ii) comparing the structures of the first and second sequences where both are known, and identifying the residue in the first sequence most similarly positioned to a residue of interest in the second sequence. Corresponding residues are identified in some algorithms based on alpha-carbon distance minimization in the overlaid structures (e.g., what set of paired alpha carbons provides a minimized root-mean-square deviation for the alignment). When identifying positions in a non-*H. pylori* ferritin corresponding to positions described with respect to *H. pylori* ferritin, *H. pylori* ferritin can be the "second" sequence. Where a non-*H. pylori* ferritin of interest does not have an available known structure, but is more closely related to another non-*H. pylori* ferritin that does have a known structure than to *H. pylori* ferritin, it may be most effective to model the non-*H. pylori* ferritin of interest using the known structure of the closely related non-*H. pylori* ferritin, and then compare that model to the *H. pylori* ferritin structure to identify the desired corresponding residue in the ferritin of interest. There is an extensive literature on structural modeling and alignment; representative disclosures include US 6859736; US 8738343; and those cited in Aslam et al., Electronic Journal of Biotechnology 20 (2016) 9–13. For discussion of modeling a structure based on a known related structure or structures, *see, e.g.*, Bordoli et al., Nature Protocols 4 (2009) 1–13, and references cited therein.

5. Immune-stimulatory moieties; adjuvants; conjugated influenza polypeptides

[00147] In some embodiments, an influenza polypeptide and/or an immune-stimulatory moiety, such as an adjuvant, is attached to a surface-exposed amino acid of ferritin or a linker. In some embodiments, the surface-exposed amino acid is a cysteine, e.g., resulting from a mutation discussed above. In some embodiments, the surface-exposed amino acid is a lysine, aspartate, or glutamate. Conjugation procedures using glutaraldehyde (for conjugation of a lysine with an amino-bearing linker or moiety) or a carbodiimide (e.g., 1-Cyclohexyl-3-(2-morpholin-4-yl-ethyl) carbodiimide or 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC; EDAC) for conjugating an aspartate or glutamate to an amino-bearing linker or moiety, or a lysine to a carboxyl-bearing linker or moiety) are described in, e.g., Chapter 4 of Holtzhauer, M., Basic Methods for the Biochemical Lab, Springer 2006, ISBN 978-3-540-32785-1, available from the world wide web at springer.com.

[00148] In some embodiments, an immune-stimulatory moiety, such as an adjuvant, is attached to a surface-exposed amino acid of ferritin. In some embodiments, more than one immune-stimulatory moiety, such as an adjuvant, is attached to a surface-exposed amino acid of ferritin. In some embodiments, twenty-four immune-stimulatory moieties are attached to a ferritin multimer or particle (e.g., one moiety for each monomer in the *H. pylori* ferritin particle). In some embodiments with multiple immune-stimulatory moieties attached to a ferritin nanoparticle, all of the immune-stimulatory moieties are identical. In some embodiments with multiple immune-stimulatory moieties attached to a ferritin nanoparticle, all of the immune-stimulatory moieties are not identical.

a) Types of immune-stimulatory moieties; adjuvants

[00149] Any immune-stimulatory moiety that can be attached to a surface-exposed amino acid (e.g., cysteine) can be used in ferritins according to this disclosure. In some embodiments, the immune-stimulatory moiety is a B cell agonist.

[00150] In some embodiments, the immune-stimulatory moiety is not hydrophobic. In some embodiments, the immune-stimulatory moiety is hydrophilic. In some embodiments, the immune-stimulatory moiety is polar. In some embodiments, the immune-stimulatory moiety is capable of hydrogen bonding or ionic bonding, e.g., comprises a hydrogen bond donor, hydrogen bond acceptor, cationic moiety, or anionic moiety. A moiety is considered cationic or anionic if it would be ionized in aqueous solution at a physiologically relevant pH, such as pH 6, 7, 7.4, or 8.

[00151] In some embodiments, the immune-stimulatory moiety is an adjuvant. In some embodiments, the adjuvant comprises a pathogen associated molecular pattern (PAMP). In some embodiments, the adjuvant is a toll-like receptor (TLR) agonist or stimulator of interferon genes (STING) agonist. In some embodiments, the adjuvant activates TLR signaling in B and/or T cells. In some embodiments, the adjuvant regulates the adaptive immune response.

(1) TLR2 agonists

[00152] In some embodiments, the immune-stimulatory moiety is a TLR2 agonist. In some embodiments, the immune-stimulatory moiety stimulates TLR2 signaling. In some embodiments, the immune-stimulatory moiety is a synthetic small molecule ligand of TLR2. In some embodiments, the immune-stimulatory moiety is a synthetic small molecule agonist of TLR2 signaling.

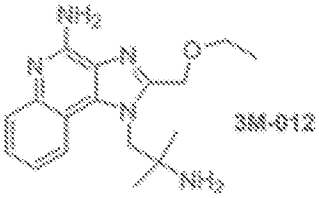
[00153] In some embodiments, the TLR2 agonist is PAM2CSK4, FSL-1, or PAM3CSK4.

(2) TLR7/8 agonists

[00154] In some embodiments, the immune-stimulatory moiety is a TLR7 and/or TLR8 agonist (i.e., an agonist of at least one of TLR7 and TLR8). In some embodiments, the immune-stimulatory moiety stimulates TLR7 and/or TLR8 signaling. In some embodiments, the immune-stimulatory moiety is a synthetic small molecule ligand of TLR7 and/or TLR8. In some embodiments, the immune-stimulatory moiety is a synthetic small molecule agonist of TLR7 and/or TLR8 signaling.

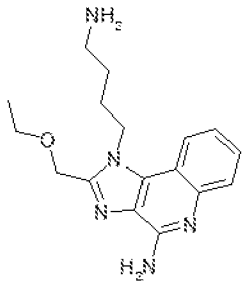
[00155] In some embodiments, the TLR7 and/or TLR8 agonist is single-stranded (ssRNA). In some embodiments, the TLR7 and/or TLR8 agonist is an imidazoquinoline. In some embodiments, the TLR7 and/or TLR8 agonist is a nucleoside analog.

[00156] In some embodiments, the TLR7 and/or TLR8 agonist is an imidazoquinolinamine Toll-like receptor (TLR) agonist, such as 3M-012 (3M Pharmaceuticals). The structure of free 3M-012 is:



. It is understood that an immune-stimulatory moiety such as 3M-012 or any moiety discussed herein can be conjugated to a ferritin by substituting an appropriate peripheral atom of the moiety (e.g., a hydrogen) with a bond to a ferritin described herein, e.g., at the sulfur of a surface-exposed cysteine or a linker attached to such a sulfur. Thus, when conjugated to a ferritin, the structure of the immune-stimulatory moiety will differ slightly from the structure of the free molecule.

[00157] In some embodiments the TLR7 and/or TLR8 agonist is SM 7/8a. The structure of free SM 7/8a is:



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[00158] *See, e.g.*, Nat Biotechnol. 2015 Nov;33(11):1201-10. doi: 10.1038/nbt.3371.

(3) TLR9 agonists

[00159] In some embodiments, the immune-stimulatory moiety is a TLR9 agonist. In some embodiments, the immune-stimulatory moiety stimulates TLR9 signaling. In some embodiments, the immune-stimulatory moiety is a synthetic small molecule ligand of TLR9. In some embodiments, the immune-stimulatory moiety is a synthetic small molecule agonist of TLR9 signaling.

[00160] In some embodiments, the TLR9 agonist is a CpG oligodeoxynucleotide (ODN). In some embodiments, the TLR9 agonist is an unmethylated CpG ODN. In some embodiments, the CpG ODN comprises a partial or complete phosphorothioate (PS) backbone instead of the natural phosphodiester (PO) backbone found in ordinary DNA.

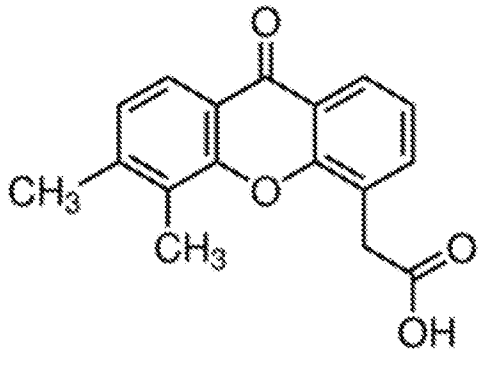
[00161] In some embodiments, the CpG ODN is a Class B ODN, which comprises one or more 6mer CpG motif comprising 5' Purine (Pu)-Pyrimidine (Py)-C-G-Py-Pu 3'; has a fully phosphorothioated (i.e., PS-modified) backbone; and has a length of 18-28 nucleotides. In some embodiments, the CpG ODN comprises the sequence of SEQ ID NO: 210, optionally comprising phosphorothioate linkages in the backbone.

[00162] In some embodiments, the TLR9 agonist comprises an immune-stimulatory sequence (ISS). In some embodiments the TLR9 agonist is ISS-1018 (Dynavax) (SEQ ID NO: 210).

(4) STING agonists

[00163] In some embodiments, the immune-stimulatory moiety is a STING (Stimulator of Interferon Genes Protein, also known as Endoplasmic Reticulum IFN Stimulator) agonist. In some embodiments, the immune-stimulatory moiety stimulates STING signaling. In some embodiments, the immune-stimulatory moiety is a synthetic small molecule ligand of STING. In some embodiments, the immune-stimulatory moiety is a synthetic small molecule agonist of STING signaling.

[00164] In some embodiments the STING agonist is a cyclic dinucleotide (CDN). *See, e.g.*, Danilchanka et al., Cell 154:962-970 (2013). Exemplary CDNs include cdA, cdG, cAMP-cGMP, and 2'-5',3'-5' cGAMP (see Danilchanka et al. for structures). STING agonists also include synthetic agonists such as DMXAA



b) Conjugated Influenza Polypeptides

[00165] In some embodiments, an influenza polypeptide is conjugated to a surface-exposed amino acid of ferritin. In some embodiments, the influenza polypeptide is antigenic alone, whereas in some embodiments, the influenza polypeptide is antigenic because of its association with ferritin. The influenza polypeptide can be any one of the influenza polypeptides described herein.

c) Conjugation

[00166] In some embodiments, a surface-exposed cysteine (e.g., resulting from a mutation described herein) or a cysteine in a peptide linker attached to ferritin (e.g., N-terminally to ferritin) is used to conjugate an immune-stimulatory moiety, such as an adjuvant, or an influenza polypeptide to a ferritin. In some embodiments, a linker is conjugated to such a cysteine, which linker can be subsequently conjugated to an immune-stimulatory moiety, such as an adjuvant, or an influenza polypeptide. In some embodiments, such a cysteine creates a chemical handle for conjugation reactions to attach an adjuvant, linker, or an influenza polypeptide. In some embodiments, bioconjugates are produced, wherein an immune-stimulatory moiety, such as an adjuvant, or an influenza polypeptide is linked to a ferritin after reduction of such a cysteine. In some embodiments, the cysteine is an unpaired surface-exposed cysteine, i.e., that lacks a partner cysteine in an appropriate position to form a disulfide bond. In some embodiments, the cysteine is an unpaired cysteine that comprises a free thiol side chain.

(1) Types of conjugation chemistries

[00167] Any type chemistry can be used to conjugate the immune-stimulatory moiety, such as an adjuvant, or an influenza polypeptide to the ferritin, e.g., via reaction a surface-exposed amino acid such as cysteine or another amino acid such as Lys, Glu, or Asp.

[00168] In some embodiments, the conjugation is performed using click chemistry. As used herein, "click chemistry" refers to a reaction between a pair of functional groups that rapidly and selective react (i.e., "click") with each other. In some embodiments, the click chemistry can be performed under mild, aqueous conditions. In some embodiments, a click chemistry reaction takes advantage of a cysteine on the surface of the ferritin, such as a cysteine resulting from mutation of a surface-exposed amino acid, to perform click chemistry using a functional group that can react with the cysteine.

[00169] A variety of reactions that fulfill the criteria for click chemistry are known in the field, and one skilled in the art could use any one of a number of published methodologies (*see, e.g.*, Hein et al., Pharm Res 25(10):2216-2230 (2008)). A wide range of commercially available reagents for click chemistry could be used, such as those from Sigma Aldrich, Jena Bioscience, or Lumiprobe. In some embodiments, conjugation is performed using click chemistry as described in the Examples below.

[00170] In some embodiments, the click chemistry reaction occurs after reduction of the ferritin.

[00171] In some embodiments, the click chemistry may be a 1-step click reaction. In some embodiments, the click chemistry may be a 2-step click reaction.

[00172] In some embodiments, the reaction(s) comprises metal-free click chemistry. In some embodiments, the reaction(s) comprise thiol-maleimide and/or disulfide exchange.

Metal-free click chemistry

[00173] Metal-free click chemistry can be used for conjugation reactions to avoid potential oxidation of proteins. Metal-free click chemistry has been used to form antibody conjugates (*see* van Geel et al., Bioconjugate Chem. 2015, 26, 2233−2242).

[00174] In some embodiments, metal-free click chemistry is used in reactions to attach adjuvant to ferritin. In some embodiments, copper-free conjugation is used in reactions to attach adjuvant to ferritin. In some embodiments, the metal-free click chemistry uses bicyclo[6.1.0]nonyne (BCN). In some embodiments, the metal-free click chemistry uses dibenzoazacyclooctyne (DBCO). In some embodiments BCN or DBCO reacts with an azide group.

[00175] DBCO has high specificity for azide groups via a strain-promoted click reaction in the absence of a catalyst, resulting in high yield of a stable triazole. In some embodiments, DBCO reacts with azide in the absence of copper catalyst.

[00176] In some embodiments, metal-free click chemistry is used in a 1-step click reaction. In some embodiments, metal-free click chemistry is used in a 2-step click reaction.

Thiol-maleimide and disulfide exchange

[00177] Ferritins described herein can comprise a cysteine comprising a thiol, also known as a sulfhydryl, which is available for reaction with sulfhydryl-reactive chemical groups (or which can be made available through reduction). Thus, the cysteine allows chemoselective modification to add an immune-stimulatory moiety, such as an adjuvant, to the ferritin. Under basic conditions, the cysteine will be deprotonated to generate a thiolate nucleophile, which can react with soft electrophiles, such as maleimides and iodoacetamides. The reaction of the cysteine with a maleimide or iodoacetamide results in a carbon-sulfur bond.

[00178] In some embodiments, a sulfhydryl-reactive chemical group reacts with the surface-exposed cysteine or cysteine in the linker of the ferritin. In some embodiments, the sulfhydryl-reactive chemical group is a haloacetyl, maleimide, aziridine, acryloyl, arylating agent, vinylsulfone, pyridyl disulfide, or TNB-thiol.

[00179] In some embodiments, the sulfhydryl-reactive chemical group conjugates to the sulfhydryl of the cysteine by alkylation (i.e., formation of a thioether bond)). In some embodiments, the sulfhydryl-reactive chemical group conjugates to the sulfhydryl of the cysteine by disulfide exchange (i.e., formation of a disulfide bond).

[00180] In some embodiments, the reaction to conjugate an immune-stimulatory moiety, such as an adjuvant, to the ferritin is a thiol-maleimide reaction.

[00181] In some embodiments, the sulfhydryl-reactive chemical group is a maleimide. In some embodiments, reaction of a maleimide with the cysteine results in formation of a stable thioester linkage, e.g., that is not reversible. In some embodiments, the maleimide does not react with tyrosines, histidines, or methionines in the ferritin. In some embodiments, unreacted maleimides are quenched at the end of the reaction by adding a free thiol, e.g., in excess.

[00182] In some embodiments, the reaction to conjugate an immune-stimulatory moiety, such as an adjuvant, to the ferritin is a thiol-disulfide exchange, also known as a disulfide interchange. In some embodiments, the reaction involves formation of a mixed disulfide comprising a portion of the original disulfide. In some embodiments, the original disulfide is the cysteine introduced in the ferritin by mutation of a surface-exposed amino acid or addition of an N-terminal linker.

[00183] In some embodiments, the sulfhydryl-reactive chemical group is a pyridyl dithiol. In some embodiments, the sulfhydryl-reactive chemical group is a TNB-thiol group.

(2) Linkers

[00184] In some embodiments, an immune-stimulatory moiety, such as an adjuvant, or an influenza polypeptide is attached to the ferritin via a linker that is covalently bound to a surface-exposed amino acid such as a cysteine. In some embodiments, the linker comprises a polyethylene glycol, e.g., a PEG linker. In some embodiments, the polyethylene glycol (e.g., PEG) linker increases water solubility and ligation efficiency of the ferritin linked to the immune-stimulatory moiety, such as an adjuvant. The PEG linker is between 2 and 18 PEGs long, e.g., PEG4, PEG5, PEG6, PEG7, PEG8, PEG9, PEG10, PEG11, PEG12, PEG13, PEG14, PEG15, PEG16, PEG17, and PEG18.

[00185] In some embodiments, the linker comprises a maleimide. In some embodiments, the linker comprises the components of immune-stimulatory moiety (ISM)-linker-maleimide. In some embodiments, the ISM-linker-maleimide is conjugated to ferritin in a 1-step click chemistry reaction by reaction of the maleimide with a cysteine of the ferritin. In some embodiments, the ISM of the adjuvant-linker-maleimide is SM7/8a. In some embodiments, the linker of the ISM-linker-maleimide is PEG4. In some embodiments, the ISM-linker-maleimide is SM7/8a-PEG4-maleimide.

[00186] In some embodiments, a 2-step click chemistry protocol is used with a linker comprising a sulfhydryl-reactive chemical group at one end and an amine-reactive group at the other end. In such a 2-step click chemistry protocol, a sulfhydryl-reactive chemical group reacts with a cysteine of the ferritin, while the amine-reactive group reacts with a reagent attached to the ISM. In this way, the ISM is conjugated to the ferritin via a set of 2 click chemistry reagents.

[00187] In some embodiments of the 2-step click chemistry protocol, the sulfhydryl-reactive chemical group is maleimide. In some embodiments of the 2-step click chemistry protocol, the maleimide reacts with the cysteine introduced in the ferritin by mutation of a surface-exposed amino acid or addition of an N-terminal linker.

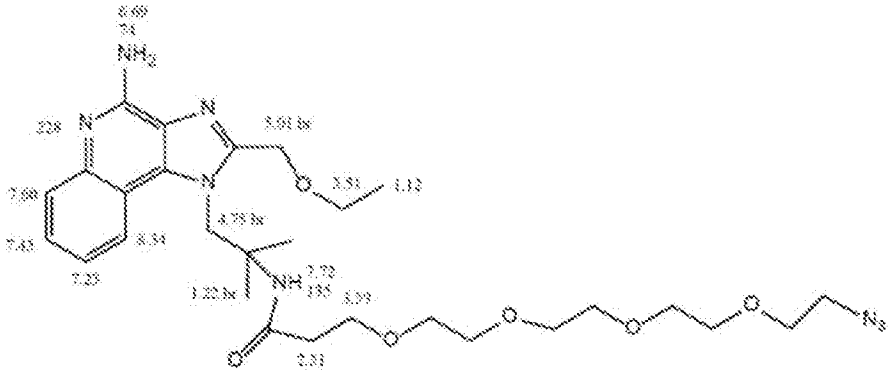
[00188] In some embodiments of the 2-step click chemistry protocol, the amine-reactive group is DBCO. In some embodiments of the 2-step click chemistry protocol, the DBCO reacts with an azide group attached to an ISM.

[00189] In some embodiments, a maleimide-linker-DBCO is used. In some embodiments, the maleimide-linker-DBCO is conjugated to ferritin after the ferritin is reduced. In some embodiments, the maleimide-linker-reagent is conjugated to ferritin by reaction of the maleimide with the cysteine of the ferritin in a first step. In some embodiments, the DBCO is used to link to an ISM attached to azide. In some embodiments,

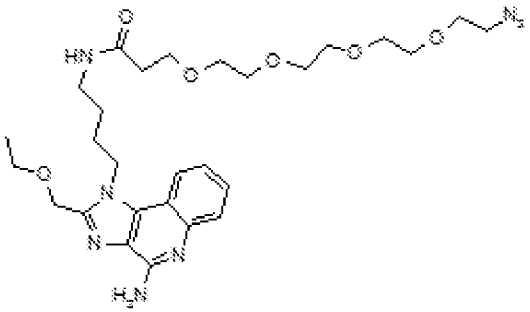
the ISM coupled to azide is ISS-1018. In some embodiments, the adjuvant coupled to azide is 3M-012 or CpG.

[00190] In some embodiments, a linker with a reactive group is added to the ISM. In some embodiments, the linker is a PEG4-azide linker or a PEG4-maleimide linker.

[00191] In some embodiments, a PEG4-azide linker is conjugated to 3M-012. An exemplary structure of 3M-012 conjugated to a PEG4-azide linker is:

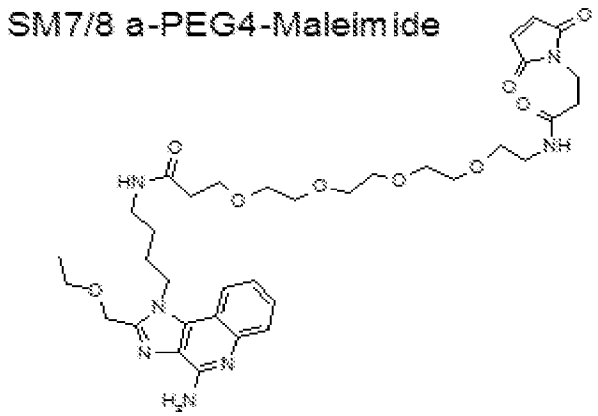


[00192] In some embodiments, a PEG4-azide linker is conjugated to SM7/8a. An exemplary structure of SM7/8a conjugated to a PEG4-azide linker is:

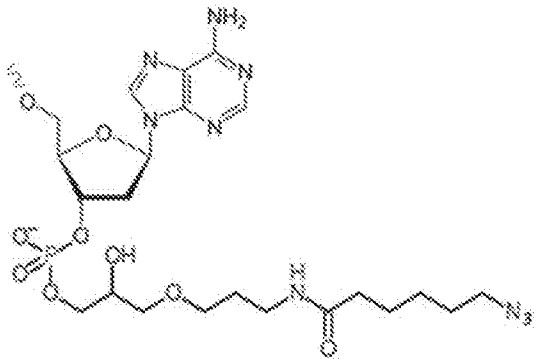


SM7/8a -PEG4-Azide

[00193] In some embodiments, a PEG4-maleimide linker is conjugated to SM7/8a. An exemplary structure of SM7/8a conjugated to a PEG4-maleimide linker is:



[00194] In some embodiments, an azide group is conjugated to ISS-1018. An exemplary structure of ISS-1018 conjugated to an NHS ester-azide linker is:



C. Exemplary compositions, kits, nucleic acids, uses, and methods

[00195] In some embodiments, a composition comprising any one or more of the antigenic polypeptides described herein and a pharmaceutically acceptable vehicle, adjuvant, or excipient is provided.

[00196] In some embodiments, the present invention provides methods of immunizing a subject against infection with influenza. The present invention further provides methods of eliciting an immune response against influenza in a subject. In some embodiments, the present methods comprise administering to the subject an effective amount of a pharmaceutical composition described herein to a subject. In some embodiments, the present methods comprise administering to the subject an effective amount of an antigenic influenza-ferritin polypeptide or nanoparticle described herein to a subject.

[00197] In some embodiments, the antigenic polypeptides or compositions described herein are administered to a subject, such as a human, to produce a protective immune

response against future infection with influenza. In some embodiments, an antigenic polypeptide comprising any one of SEQ ID NOS: 1-43 is administered.

[00198] In some embodiments, the protective immune response decreases the incidence of hospitalization. In some embodiments, the protective immune response decreases the incidence of laboratory-confirmed influenza infection.

[00199] In some embodiments, more than one antigenic polypeptide may be administered to an individual to immunize against multiple strains of influenza, wherein the HA or NA polypeptide portion of the antigenic polypeptide differs. The administrations may be at the same time or sequential. In some embodiments, two HA or NA polypeptides of different influenza strains are administered. In some embodiments, three HA or NA polypeptides of different influenza strains are administered. In some embodiments, four HA or NA polypeptides of different influenza strains are administered. In some embodiments, five HA or NA polypeptides of different influenza strains are administered. In some embodiments, more than five HA or NA polypeptides of different influenza strains are administered.

1. Subjects

[00200] In some embodiments, the subject is a mammal. In some embodiments, the subject is a human.

[00201] In some embodiments, the subject is an adult (greater than or equal to 18 years of age). In some embodiments, the subject is a child or adolescent (less than 18 years of age). In some embodiments, the subject is elderly (greater than 60 years of age). In some embodiments, the subject is a non-elderly adult (greater than or equal to 18 years of age and less than or equal to 60 years of age).

[00202] In some embodiments, more than one administration of the composition is administered to the subject. In some embodiments, a booster administration improves the immune response.

[00203] In some embodiments, any one or more of the antigenic polypeptides, or compositions described herein are for use in a mammal, such as a primate (e.g., non-human primate, such as a monkey (e.g., a macaque, such as rhesus or cynomolgus) or ape), rodent (e.g., mouse or rat), or domesticated mammal (e.g., dog, rabbit, cat, horse, sheep, cow, goat, camel, or donkey). In some embodiments, any one or more of the antigenic polypeptides, or compositions described herein are for use in a bird, such as a fowl (e.g., chicken, turkey, duck, goose, guineafowl, or swan).

2. Adjuvants

[00204] As described herein, adjuvants may be conjugated to ferritin via a surface exposed amino acid, e.g., a cysteine. Non-conjugated adjuvant may also be administered together with the antigenic ferritin polypeptides described herein to a subject. In some embodiments, administration of adjuvant together with the antigenic ferritin polypeptide produces a higher titer of antibodies against the influenza polypeptide in the subject as compared to administration of the influenza polypeptide alone, or antigenic ferritin polypeptide alone, without the adjuvant. An adjuvant may promote earlier, more potent, or more persistent immune response to the antigenic polypeptide.

[00205] In some embodiments, a composition comprises one adjuvant. In some embodiments, a composition comprises more than one adjuvant. In some embodiments, a composition does not comprise an adjuvant.

[00206] In some embodiments, an adjuvant comprises aluminum. In some embodiments, an adjuvant is aluminum phosphate. In some embodiments, an adjuvant is Alum (Alyhydrogel '85 2%; Brenntag – Cat# 21645-51-2).

[00207] In some embodiments, an adjuvant is an organic adjuvant. In some embodiments, an adjuvant is an oil-based adjuvant. In some embodiments, an adjuvant comprises an oil-in-water nanoemulsion.

[00208] In some embodiments, an adjuvant comprises squalene. In some embodiments, the adjuvant comprising squalene is Ribi (Sigma adjuvant system Cat #S6322-1vl), Addavax™ MF59, AS03, or AF03 (*see* US9703095). In some embodiments, the adjuvant comprising squalene is a nanoemulsion.

[00209] In some embodiments, an adjuvant comprises a polyacrylic acid polymer (PAA). In some embodiments, the adjuvant comprising PAA is SPA09 (*see* WO 2017218819).

[00210] In some embodiments, an adjuvant comprises non-metabolizable oils. In some embodiments, the adjuvant is Incomplete Freund's Adjuvant (IFA).

[00211] In some embodiments, an adjuvant comprises non-metabolizable oils and killed Mycobacterium tuberculosis. In some embodiments, the adjuvant is Complete Freund's Adjuvant (CFA).

[00212] In some embodiments, an adjuvant is a lipopolysaccharide. In some embodiments, an adjuvant is monophosphoryl A (MPL or MPLA).

3. Pharmaceutical Compositions

[00213] In various embodiments, a pharmaceutical composition comprising an antigenic ferritin polypeptide described herein and/or related entities is provided. In some embodiments, the pharmaceutical composition is an immunogenic composition (e.g., a vaccine) capable of eliciting an immune response such as a protective immune response against a pathogen.

[00214] For example, in some embodiments, the pharmaceutical compositions may comprise one or more of the following: (1) an antigenic ferritin protein comprising (i) a mutation replacing a surface-exposed amino acid with a cysteine and (ii) an influenza polypeptide; (2) an antigenic ferritin protein comprising (i) a mutation replacing a surface exposed amino acid with a cysteine and an immune-stimulatory moiety linked to the cysteine; and (ii) an influenza polypeptide; (3) antigenic ferritin protein comprising (i) a surface-exposed cysteine, (ii) a peptide linker N- terminal to the ferritin protein, and (iii) an influenza polypeptide N-terminal to the peptide linker; (4) an antigenic ferritin protein comprising: (i) a mutation replacing a surface exposed amino acid with a cysteine and an immune-stimulatory moiety linked to the cysteine, (ii) a mutation replacing the internal cysteine at position 31 of *H. pylori* ferritin, or a mutation of an internal cysteine at a position that is analogous to position 31 of a non-*H. pylori* ferritin as determined by pair-wise or structural alignment, with a non-cysteine amino acid, (iii) a mutation replacing a surface-exposed asparagine with a non-asparagine amino acid, and (iv) an influenza polypeptide; or (5) a ferritin particle comprising any of the foregoing ferritin proteins.

[00215] In some embodiments, the present invention provides pharmaceutical compositions comprising antibodies or other agents related to the antigenic polypeptides described herein. In an embodiment, the pharmaceutical composition comprises antibodies that bind to and/or compete with an antigenic polypeptide described herein. Alternatively, the antibodies may recognize viral particles or bacteria comprising the influenza polypeptide component of an antigenic polypeptide described herein.

[00216] In some embodiments, the pharmaceutical compositions as described herein are administered alone or in combination with one or more agents to enhance an immune response, e.g., an adjuvant described above. In some embodiments, a pharmaceutical composition further comprises an adjuvant described above.

[00217] In some embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier or excipient. As used herein, the term “carrier” refers to

a diluent, adjuvant, excipient, or vehicle with which a pharmaceutical composition is administered. In exemplary embodiments, carriers can include sterile liquids, such as, for example, water and oils, including oils of petroleum, animal, vegetable, or synthetic origin, such as, for example, peanut oil, soybean oil, mineral oil, sesame oil and the like. In some embodiments, carriers are or include one or more solid components. Pharmaceutically acceptable carriers can also include, but are not limited to, saline, buffered saline, dextrose, glycerol, ethanol, and combinations thereof. As used herein, an excipient is any non-therapeutic agent that may be included in a pharmaceutical composition, for example to provide or contribute to a desired consistency or stabilizing effect. Suitable pharmaceutical excipients include, but are not limited to, starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. In various embodiments, the pharmaceutical composition is sterile.

[00218] In some embodiments, the pharmaceutical composition contains minor amounts of wetting or emulsifying agents, or pH buffering agents. In some embodiments, the pharmaceutical compositions of may include any of a variety of additives, such as stabilizers, buffers, or preservatives. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents can be included.

[00219] In various embodiments, the pharmaceutical composition may be formulated to suit any desired mode of administration. For example, the pharmaceutical composition can take the form of solutions, suspensions, emulsion, drops, tablets, pills, pellets, capsules, capsules containing liquids, gelatin capsules, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, lyophilized powder, frozen suspension, desiccated powder, or any other form suitable for use. General considerations in the formulation and manufacture of pharmaceutical agents may be found, for example, in Remington's Pharmaceutical Sciences, 19th ed., Mack Publishing Co., Easton, PA, 1995; incorporated herein by reference.

[00220] The pharmaceutical composition can be administered via any route of administration. Routes of administration include, for example, oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, mucosal, epidural, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by intratracheal instillation, bronchial instillation, inhalation, or topically. Administration can be local or systemic. In some embodiments, administration is carried out orally. In another embodiment, the administration is by parenteral injection. In some instances, administration results in the

release of the antigenic ferritin polypeptide described herein into the bloodstream. The mode of administration can be left to the discretion of the practitioner.

[00221] In some embodiments, the pharmaceutical composition is suitable for parenteral administration (e.g. intravenous, intramuscular, intraperitoneal, and subcutaneous). Such compositions can be formulated as, for example, solutions, suspensions, dispersions, emulsions, and the like. They may also be manufactured in the form of sterile solid compositions (e.g. lyophilized composition), which can be dissolved or suspended in sterile injectable medium immediately before use. For example, parenteral administration can be achieved by injection. In such embodiments, injectables are prepared in conventional forms, i.e., either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. In some embodiments, injection solutions and suspensions are prepared from sterile powders, lyophilized powders, or granules.

[00222] In a further embodiment, the pharmaceutical composition is formulated for delivery by inhalation (e.g., for direct delivery to the lungs and the respiratory system). For example, the composition may take the form of a nasal spray or any other known aerosol formulation. In some embodiments, preparations for inhaled or aerosol delivery comprise a plurality of particles. In some embodiments, such preparations can have a mean particle size of about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, or about 13 microns. In some embodiments, preparations for inhaled or aerosol delivery are formulated as a dry powder. In some embodiments, preparations for inhaled or aerosol delivery are formulated as a wet powder, for example through inclusion of a wetting agent. In some embodiments, the wetting agent is selected from the group consisting of water, saline, or other liquid of physiological pH.

[00223] In some embodiments, the pharmaceutical composition in accordance with the invention are administered as drops to the nasal or buccal cavity. In some embodiments, a dose may comprise a plurality of drops (e.g., 1-100, 1-50, 1-20, 1-10, 1-5, etc.).

[00224] The present pharmaceutical composition may be administered in any dose appropriate to achieve a desired outcome. In some embodiments, the desired outcome is the induction of a long-lasting adaptive immune response against a pathogen, such as the source of an influenza polypeptide present in an antigenic ferritin polypeptide present in the composition. In some embodiments, the desired outcome is a reduction in the intensity, severity, frequency, and/or delay of onset of one or more symptoms of infection. In some embodiments, the desired outcome is the inhibition or prevention of infection. The dose required will vary from subject to subject depending on the species, age, weight, and general

condition of the subject, the severity of the infection being prevented or treated, the particular composition being used, and its mode of administration.

[00225] In some embodiments, pharmaceutical compositions in accordance with the invention are administered in single or multiple doses. In some embodiments, the pharmaceutical compositions are administered in multiple doses administered on different days (e.g., prime-boost immunization strategies). In some embodiments, the pharmaceutical composition is administered as part of a booster regimen.

[00226] In various embodiments, the pharmaceutical composition is co-administered with one or more additional therapeutic agents. Co-administration does not require the therapeutic agents to be administered simultaneously, if the timing of their administration is such that the pharmacological activities of the additional therapeutic agent and the active ingredient(s) in the pharmaceutical composition overlap in time, thereby exerting a combined therapeutic effect. In general, each agent will be administered at a dose and on a time schedule determined for that agent.

4. Nucleic acid/mRNA

[00227] Also provided is a nucleic acid encoding an antigenic influenza-ferritin polypeptide described herein. In some embodiments, the nucleic acid is an mRNA. Any nucleic acid capable of undergoing translation resulting in a polypeptide is considered an mRNA for purposes of this disclosure.

5. Kits

[00228] Also provided herein are kits comprising one or more antigenic polypeptides, nucleic acids, antigenic ferritin particles, compositions, or pharmaceutical compositions described herein. In some embodiments, a kit further comprises one or more of a solvent, solution, buffer, instructions, or desiccant.

* * *

[00229] This description and exemplary embodiments should not be taken as limiting. For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing quantities, percentages, or proportions, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term “about,” to the extent they are not already so modified. “About” indicates a degree of variation that does not substantially affect the properties of the described subject matter, e.g., within 10%, 5%, 2%, or 1%. Accordingly, unless indicated to the contrary, the numerical

parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed considering the number of reported significant digits and by applying ordinary rounding techniques.

[00230] It is noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the," and any singular use of any word, include plural referents unless expressly and unequivocally limited to one referent. As used herein, the term "include" and its grammatical variants are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items. The term "or" is used in the inclusive sense, i.e., equivalent to "and/or," unless the context dictates otherwise.

Table 1 (Sequence Table): Description of Sequences

Description	Sequences Key for SEQ ID NOs: 1-44: • leader sequences are underlined • HA – **Bold** • TEV – *wavy underline and Italicized* • Linker – double underline • Bf Ferr sequence – normal text • pFerr sequence – wavy underline • *Cys for conjugation is boxed, bold, italicized and wavy underline • F of ***Y98F mutation is bold, underlined and italicized*** • PQRET (SEQ ID NO: 229) (monobasic mutation) for H5 nanoparticles – *italicized*	SEQ ID NO
SIB 6001: HA (NC99 Y98F) pFerr N19Q/C31S/S111C	MKAKLLVLLCTFTATYAD**TICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTC*F*PGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKID**SGGDIIKLLNEQVNKEMQSSNLYMSMSSW SYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK*C*KDHATFNFLQ WYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKSGS	1
SIB 6243: HA (NC99 Y98F) bfpFerr N19Q/C31S/S111C	MKAKLLVLLCTFTATYA**DTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTC*F*PGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKID**GSESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK*C*K DHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	2

SIB 6244: HA (NC99 Y98F) bfpFerr N19Q/C31S	3
MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKSK DHATFNFTLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKSK DHATFNFTLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	4
SIB 6245: HA (NC99 Y98F) bfpFerr N19Q/C31S/E12C	
MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNCQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKSK DHATFNFTLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNCQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKSK DHATFNFTLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	5
SIB 6246: HA (NC99 Y98F) bfpFerr N19Q/C31S/S26C	
MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMCMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKSK DHATFNFTLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTQISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKSK DHATFNFTLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	6
SIB 6247: HA (NC99 Y98F) bfpFerr N19Q/C31S/S72C	
MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTCISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKSK DHATFNFTLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTCISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKSK DHATFNFTLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	6

SIB 6248: HA (NC99 Y98F) bfpFerr N19Q/C31S/A75C	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISCPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKSK DHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	7
SIB 6249: HA (NC99 Y98F) bfpFerr N19Q/C31S/S100C	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISECINNIVDHAIKSK DHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	8
SIB 6250: HA (NC99 Y98F) bfpFerr N19Q/C31S TEV-S111C	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKID*ENLYFQ*GSESQVRQQFSKDIEKLLNEQVN KEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVD HAIKCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	9
SIB 6252: HA (NC99 Y98F) bfpFerr N19Q/C31S TEV-S26C	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKID*ENLYFQ*GSESQVRQQFSKDIEKLLNEQVN	10

	KEMQSSNLYMCMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVD HAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	
SIB 6253: HA (NC99 Y98F) bfpFerr N19Q/C31S TEV-S72C	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTC*FPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKID*ENLYFQG*SESQVRQQFSKDIEKLLNEQVN KEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTCISAPEHKFEGLTQIFQKAYEHEQHISESINNIVD HAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	11
SIB 6254: HA (NC99 Y98F) bfpFerr N19Q/C31S TEV-A75C	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTC*FPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKID*ENLYFQG*SESQVRQQFSKDIEKLLNEQVN KEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSIS*C*PEHKFEGLTQIFQKAYEHEQHISESINNIVD HAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	12
SIB 6255: HA (NC99-Y98F) TEV bfpFerr-N19Q/C31S/S100C	MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTC*FPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYV NNKEKEVLVLWGVHHPPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKID*ENLYFQG*SESQVRQQFSKDIEKLLNEQVN KEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISE*C*INNIVD HAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	13
SIB9063: HA (South Carolina 1918, Y98F) bfpFerr	MEARLLVLLCAFAATNADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCKLKGIAPLQLGKCNIAGWLLGNPECDLLLTAS SWSYIVETSNSENGTC*FPGDFIDYEELREQLSSVSSFEKFEIFPKTSSWPNHETTKGVTAACSYAGASSFYRNLLWLTKKGSSYPKLSKSY VNNKGKEVLVLWGVHHPPTGTDQQSLYQNADAYVSVGSSKYNRRFTPEIAARPKVRDQAGRMNYYWTLLEPGDTITFEATGNLIAPWYAFA LNRGSGSGIITSDAPVHDCNTKCQTPHGAINSSLPFQNIHPVTIGECPKYVRSTKLRMATGLRNIPSIQSRGLFGAIAGFIEGGWTGMIDG	14

N19Q/C31S/S111C	WYGYHHQNEQGSGYAADQKSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNNLERRIENLNKKVDDGFLDIWTYNAELLVLLENERTLDFH DSNVRNLYEKVKSQLKNNAKEIGNGCFEFYHKCDDACMESVRNGTYDYPKYSEESKLNREEIDSGGESQVRQQFSKDIEKLLNEQVNKEMQ SSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK CKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	
SIB9065: HA (California 2009, Y98F) bfpFerr N19Q/C31S/S111C	MKAILVVLLYTFATANADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDKHNGKLCKLRGVAPLHLGKCNIAGWILGNPECESLSTAS SWSYIVETPSSDNGTCFPGDFIDYEELREQLSSVSSFERFEIFPKTSSWPNHDSNKGVTAACPHAGAKSFYKNLIWLVKKGNSYPKLSKSY INDKGKEVLVLWGIHHPSTSADQQSLYQNADTYVFVGSSRYSKKFKPEIAIRPKVRDQEGRMNYYWTLVEPGDKITFEATGNLVVPRYAFA MERNAGSGIIISDTPVHDCNTTCQTPKGAINTSLPFQNIHPITIGKCPKYVKSTKLRLATGLRNIPSIQSRGLFGAIAGFIEGGWTGMVDG WYGYHHQNEQGSGYAADLKSTQNAIDEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWTYNAELLVLLENERTLDYH DSNVKNLYEKVRSQLKNNAKEIGNGCFEFYHKCDNTCMESVKNGTYDYPKYSEEAKLNREEIDSGGESQVRQQFSKDIEKLLNEQVNKEMQ SSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK CKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	15
SIB9066: HA (Malaysia 1954, Y98F) bfpFerr N19Q/C31S/S111C	MKARLLILLCALSATDADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCRLKGIAPLQLGKCNIAGWILGNPECESLLSNR SWSYIAETPNSENGICFPGDFADYEELREQLSSVSSFERFEIFPKESSWPKHNITRGVTVACSHAKKSSFYKNLLWLTEANGLYPSLSKSY VNDREKEVLVLWGVHHPSNIEDQRTLYRKENAYVSVVSSNYNRRFTPEIAERPKVRGQPGRMNYYWTLLEPGDKIIFEANGNLIAPWYAFA LSRGPGSGIITSNASMDECDTKCQTPQGAINSSLPFQNIHPVTIGECPKYVRSTKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTGMVDG WYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLEKRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFH DSNVKNLYEKVKNQLRNNAKEIGNGCFEFYHKCDNECMESVKNGTYDYPKYSEESKLNRAKIDSGGESQVRQQFSKDIEKLLNEQVNKEMQ SSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK CKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	16
SIB9067: HA (Fort Monmouth 1947, Y98F) bfpFerr N19Q/C31S/S111C	MKAKLLILLCALTATDADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCRLKGIAPLQLGKCNIAGWILGNPECESLLSKR SWSYIAETPNSENGACFPGDFADYEELREQLSSVSSFERFEIFPKERSWPKHNITRGVTAACSHAGKSSFYKNLLWLTETDGSYPKLSKSY VNNKEKEVLVLWGVHHPSNIEDQKTLYRKENAYVSVVSSNYNRRFTPEIAERPKVRGQAGRINYYWTLLEPGDTIIFEANGNLIAPWYAFA LSRDFGSGIITSNASMDECDTKCQTPQGAINSSLPFQNIHPVTIGECPKYVKSTKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTGMIDG WYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLEKRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFH DSNVKNLYEKVKNQLRNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDSGGESQVRQQFSKDIEKLLNEQVNKEMQ SSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK CKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	17
SIB9068: HA (Hong Kong	MKAKLLVLLCALSATDADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCRLKGIAPLQLGKCSIAGWILGNPECESLFSKK SWSYIAETPNSENGTCFPGYFADYEELREQLSSVSSFERFEIFPKERSWPKHNVTRGVTASCSHKGKSSFYRNLLWLTEKNGSYPNLSKSY	18

1977, Y98F) bfpFerr N19Q/C31S/S111C	**VNNKEKEVLVLWGVHHPSNIEDQKTIYRKENAYVSVVSSNYNRRFTPEIAERPKVRGQAGRINYYWTLLEPGDTIIFEANGNLIAPWYAFA LSRGFGSGIITSNASMDECDTKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSTKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTGMIDG WYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLEKRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFH DSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKID**SGGESQVRQQFSKDIEKLLNEQVNKEMQ SSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK CKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	
SIB9070: HA (H2, 1957, Y98F) bfpFerr N19Q/C31S/S111C	MAIIYLILLFTAVRG**DQICIGYHANNSTEKVDTILERNVTVTHAKDILEKTHNGKLCKLNGIPPLELGDCSIAGWLLGNPECDRLLSVPEW SYIMEKENPRDGLC*F*PGSFNDYEELKHLLSSVKHFEKVKILPKDRWTQHTTTGGSRACAVSGNPSFFRNMVWLTKKESNYPVAKGSYNNTS GEQMLIIWGVHHPNDETEQRTLYQNVGTYVSVGTSTLNKRSTPDIATRPKVNGLGSRMEFSWTLLDMWDTINFESTGNLIAPEYGFKISKR GSSGIMKTEGTLENCETKCQTPLGAINTTLPFHNVHPLTIGECPKYVKSEKLVLATGLRNVPQIESRGLFGAIAGFIEGGWQGMVDGWYGY HHSNDQGSGYAADKESTQKAFDGITNKVNSVIEKMNTQFEAVGKEFSNLERRLENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNV KNLYDKVRMQLRDNVKELGNGCFEFYHKCDDECMNSVKNGTYDYPKYEEESKLNRNEIK**SGGESQVRQQFSKDIEKLLNEQVNKEMQSSNL YMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCKDH ATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	19
SIB9072: HA (H5N1, Indonesia 2005, Y98F, monobasic) bfpFerr N19Q/C31S/S111C	MEKIVLLLAIVSLVKS**DQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPE WSYIVEKANPTNDLC*F*PGSFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSSACPYLGSPSFFRNVVWLIKKNSTYPTIKKSYNN TNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYISIGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIV KKGDSAIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNS*PQRETR***GLFGAIAGFIEGGWQGMVDGWY GYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDS NVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESIRNGTYNYPQYSEEARLKREEIS**SGGESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCKK DHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	20
SIB9097: HA (Denver 1957, Y98F) bfpFerr N19Q/C31S/S111C	MKAKLLILLCALSATDA**DTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCRLKGKAPLQLGNCNIAGWVLGNPECESLLSNR SWSYIAETPNSENGTC*F*PGDFADYEELREQLSSVSSFERFEIFPKERSWPNHTTRGVTAACPHARKSSFYKNLVWLTEANGSYPNLSRSYV NNQEKEVLVLWGVHHPSNIEEQRALYRKDNAYVSVVSSNYNRRFTPEIAKRPKVRDQSGRMNYYWTLLEPGDTIIFEATGNLIAPWYAFAL SRGPGSGIITSNAPLDECDTKCQTPQGAINSSLPFQNIHPVTIGECPKYVRSTKLRMVTGLRNIPSVQSRGLFGAIAGFIEGGWTGMMDGW YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLEKRMENLNKKVDDGFMDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKNQLRNNAKELGNGCFEFYHKCDNECMESVKNGTYDYPKYSEESKLNREKID**SGGESQVRQQFSKDIEKLLNEQVNKEMQS SNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKC KDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	21

SIB9109: HA (H3N8, Equine Ohio 2003, Y98F) bfpFerr N19Q/C31S/S111C	MKTTIILILLTHWAYSQNPISGNNTATLCLGHHAVANGTLVKTISDDQIEVTNATELVQSISMGKICNNSYRILDGRNCTLIDAMLGDPHC DAFQYENWDLFIERSSAFSNCFPYDIPDYASLRSIVASSGTLEFTAEGFTWTGVTQNGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVT MPNNKNFDKLYIWGIHHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPWVRGQSGRISIYWTIVKPGDILMINSNGNLVAPRGYF KLKTGKSSVMRSDVPIDICVSECITPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQIRGIFGAIAGFIENGWEGMVDG WYGFRYQNSEGTGQAADLKSTQAAIDQINGKLNRVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLT DAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIGSIRNGTYDHYIYRDEALNNRFKIDSGGESQVRQQFSKDIEKLLNEQVNKEMQ SSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK CKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	22
SIB9110: HA (H3N8, Equine Bari 2005, Y98F) bfpFerr N19Q/C31S/S111C	MKTTIIFIFILLTHWAYSQNPISDNNTATLCLGHHAVANGTLVKTISDDQIEVTNATELVQSISMGKICNNSYRILDGRNCTLIDAMLGDP HCDVFQYENWDLFIERSSAFSNCFPYDIPDYASLRSIVASSGTLEFTAEGFTWTGVTQNGRSGACKRGSADSFFSRLNWLTKSGNSYPTLN VTMPNNKNFDKLYIWGIHHPSSNQEQTKLYIQESGRVTVSTKRSQQTMIPNIGSRPWVRGQSGRISIYWTIVKPGDILMINSNGNLVAPRG YFKLKTGKSSVMRSDVPIDICVSECITPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQIRGIFGAIAGFIENGWEGMV DGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLNRVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTID LTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIGSIRNGTYDHYIYRDEALNNRFKIDSGGESQVRQQFSKDIEKLLNEQVNKE MQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHA IKCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	23
SIB9111: HA (H3N8, Equine Aboyne 2003, Y98F) bfpFerr N19Q/C31S/S111C	METTIILILLTHWVYSQNPISGNNTATLCLGHHAVANGTLVKTITDDQIEVTNATELVESISMGKICNNSYRVLDGRNCTLIDAMLGDPHC DDFQYESWDLFIERSSASSNCFPYDIPDYASLRSIVASSGTLEFTAEGFTWTGVTQNGRSGACKRGSADSFFSRLNWLTKSGNSYPTLNVT MPNNKNFDKLYIWGIHHPSSNKEQTKLYIQESGRVTVSTERSQQTVIPNIGSRPWVRGQSGRISIYWTIVKPGDVLMINSNGNLVAPRGYF KLRTGKSSVMRSDALIDTCVSECITPNGSIPNDKPFQNVNKITYGRCPKYIRQNTLKLATGMRNVPEKQIRGIFGAIAGFIENGWEGMVDG WYGFRYQNSEGTGQAADLKSTQAAIDQINGKLNRVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLT DAEMNKLFERTRRQLRENAEDMGGGCFKIYHKCDNACIGSIRNGTYDHYIYRDEALNNRFKIDSGGESQVRQQFSKDIEKLLNEQVNKEMQ SSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK CKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	24
SIB9132: HA (B, Massachusetts 2012, Y98F) bfpFerr N19Q/C31S/S111C	MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSYFANLKGTKTRGKLCPDCLNCTDLDVALGRPMCVGTT PSAKASILHEVRPVTSGCFPIMHDRTKIRQLANLLRGYENIRLSTQNVIDAEKAPGGPYRLGTSGSCPNATSKSGFFATMAWAVPKDNNKN ATNPLTVEVPYICAEGEDQITVWGFHSDNKTQMKNLYGDSNPQKFTSSANGVTTHYVSQIGGFPDQTEDGGLPQSGRIVVDYMMQKPGKTG TIVYQRGVLLPQKVWCASGRSKVIKGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFF GAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISS QIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSLNITASGGESQVRQQF	25

	SKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEH EQHISESINNIVDHAIKCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	
SIB9147: HA (B, Phuket 2013) bfpFerr N19Q/C31S/S111C	MKAIIVLLMVVTSNA**DRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSYFANLKGTRTRGKLCPDCLNCTDLDVALGRPMCVGTT PSAKASILHEVRPVTSGCFPIMHDRTKIRQLPNLLRGYEKIRLSTQNVIDAEKAPGGPYRLGTSGSCPNATSKIGFFATMAWAVPKDNYKN ATNPLTVEVPYICTEGEDQITVWGFHSDNKTQMKSLYGDSNPQKFTSSANGVTTHYVSQIGDFPDQTEDGGLPQSGRIVVDYMMQKPGKTG TIVYQRGVLLPQKVWCASGRSKVIKGSLPLIGEADCLHEEYGGLNKSKPYYTGKHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFF GAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISS QIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSLNITAAS**SGGESQVRQ QFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAY EHEQHISESINNIVDHAIKCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	26
SIB9158: HA (H1N1 COBRA-P1, Y98F) bfpFerr N19Q/C31S/S111C	MKARLLVLLCALAATDA**DTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCKLKGIAPLQLGKCNIAGWLLGNPECESLLSAR SWSYIVETPNSENGTCFPGDFIDYEELREQLSSVSSFERFEIFPKESSWPNHNTTKGVTAACSHAGKSSFYRNLLWLTKKGGSYPKLSKSY VNNKGKEVLVLWGVHHPSTSTDQQSLYQNENAYVSVVSSNYNRRFTPEIAERPKVRGQAGRMNYYWTLLEPGDTIIFEATGNLIAPWYAFA LSRGSGSGIITSNASMHECNTKCQTPQGAINSSLPFQNIHPVTIGECPKYVRSTKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTGMIDG WYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNNLEKRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFH DSNVKNLYEKVKSQLRNNAKEIGNGCFEFYHKCDNECMESVKNGTYDYPKYSEESKLNREKID**SGGESQVRQQFSKDIEKLLNEQVNKEMQ SSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK CKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	27
SIB9160: HA (H1N1 COBRA-X3, Y98F) bfpFerr N19Q/C31S/S111C	MEARLLVLLCAFAATNA**DTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCRLKGIAPLQLGNCSVAGWILGNPECESLFSKE SWSYIAETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTKGVTASCSHNGKSSFYRNLLWLTEKNGLYPNLSKSY VNNKEKEVLVLWGVHHPSNIGDQRAIYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFA LSRGFGSGIITSNASMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSTKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTGMIDG WYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFH DSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKID**SGGESQVRQQFSKDIEKLLNEQVNKEMQ SSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK CKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	28
SIB9162: HA (H1N1 COBRA-X6, Y98F) bfpFerr	MEARLLVLLCAFAATNA**DTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKE SWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYA NNKEKEVLVLWGVHHPPNIGDQRALYHTENAYVSVVSSHYSRKFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPRYAFAL SRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTGMVDGW**	29

N19Q/C31S/S111C	YGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHD SNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDSGGESQVRQQFSKDIEKLLNEQVNKEMQS SNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCC KDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	
SIB9164: HA (H3N2, Perth 2009) bfpFerr N19Q/C31S/S111C	MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQSSSTGEICDSPHQILDGKNCTLIDALLGDPQ CDGFQNKKWDLFVERSKAYSNCYPYDVPDYASLRSLVASSGTLEFNNESFNWTGVTQNGTSSACIRRSKNSFFSRLNWLTHLNFKYPALNV TMPNNEQFDKLYIWGVHHPGTDKDQIFLYAQASGRITVSTKRSQQTVSPNIGSRPRVRNIPSRISIYWTIVKPGDILLINSTGNLIAPRGY FKIRSGKSSIMRSDAPIGKCNSECITPNGSIPNDKPFQNVNRITYGACPRYVKQNTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGMVD GWYGFRHQNSEGRGQAADLKSTQAAIDQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDL TDSEMNKLFEKTKKQLRENAEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKSGGESQVRQQFSKDIEKLLNEQVNKEM QSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAI KCCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	30
SIB9167: HA (H3N2 Victoria 2011) bfpFerr N19Q/C31S/S111C	MKTIIALSHILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVQNSSIGEICDSPHQILDGENCTLIDALLGDPQ CDGFQNKKWDLFVERSKAYSNCYPYDVPDYASLRSLVASSGTLEFNNESFNWTGVTQNGTSSACIRRSNNSFFSRLNWLTQLNFKYPALNV TMPNNEQFDKLYIWGVHHPVTDKDQIFLYAQSSGRITVSTKRSQQAVIPNIGYRPRIRNIPSRISIYWTIVKPGDILLINSTGNLIAPRGY FKIRSGKSSIMRSDAPIGKCNSECITPNGSIPNDKPFQNVNRITYGACPRYVKQSTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGMVD GWYGFRHQNSEGRGQAADLKSTQAAIDQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDL TDSEMNKLFEKTKKQLRENAEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKSGGESQVRQQFSKDIEKLLNEQVNKEM QSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAI KCCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	31
SIB9320: HA (H5N1, hCOBRA-2, Y98F, monobasic) bfpFerr N19Q/C31S/S111C	MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPE WSYIVEKANPANDLCFPGNFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSSACPYQGSPSFFRNVVWLIKKNNTYPTIKRSYNN TNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYISVGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIV KKGDSAIMKSELEYGNCNTKCQTPIGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNS*PQRETR*GLFGAIAGFIEGGWQGMVDGWY GYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDS NVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISSGGESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCK DHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	32
SIB9321: HA (H5N1, Bar	MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFLNVPE WSYIVEKINPANDLCFPGNFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSSACPYQGRSSFFRNVVWLIKKNNAYPTIKRSYNN	33

Headed Goose 2005, Y98F, monobasic) bfpFerr N19Q/C31S/S111C	**TNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYISVGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPENAYKIV** **KKGDSTIMKSELEYGNCNTKCQTPIGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNS***PQIETR***GLFGAIAGFIEGGWQGMVDGWY** **GYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDS** **NVKNLYDKVRLQLRDNAKELGNGCFEFYHRCDNECMESVRNGTYDYPQYSEEARLKREEIS**SGGESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCK DHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	
SIB9329: HA (H5N1, Whooper Swan 2005, Y98F, monobasic) bfpFerr N19Q/C31S/S111C	MEKIVLLLAIVSLVKS**DQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFLNVPE** **WSYIVEKINPANDLC*F*PGNFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSSACPYQGRSSFFRNVVWLIKKDNAYPTIKRSYNN** **TNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYISVGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPENAYKIV** **KKGDSTIMKSELEYGNCNTKCQTPIGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNS***PQGETR***GLFGAIAGFIEGGWQGMVDGWY** **GYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDS** **NVKNLYDKVRLQLRDNAKELGNGCFEFYHRCDNECMESVRNGTYDYPQYSEEARLKREEIS**SGGESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCK DHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	34
SIB9330: HA (H5N1, Mallard/Huadong 2003, Y98F, monobasic) bfpFerr N19Q/C31S/S111C	MEKIVLLLAIVSLVKS**DQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPE** **WSYIVEKANPANDLC*F*PGNFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSSACPYQGRSSFFRNVVWLIKKNSTYPTIKRSYNN** **TNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYISVGTSTLNQRLAPKIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIV** **KKGDSAIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNS***PQRET***RGLFGAIAGFIEGGWQGMVDGWY** **GYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDS** **NVKNLYDKVRLQLRDNAKELGNGCFEFYHRCDNECMESVRNGTYDYPQYSEEARLKREEIS**SGGESQVRQQFSKDIEKLLNEQVNKEMQSS NLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCK DHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	35
SIB9332: HA (B, Brisbane 2008, D197N) pFerr N19Q/C31S/S111C	MDTLLLWVLLLWVPGSTG**DRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSHFANLKGTETRGKLCPKCLNCTDLDVALGRPKCT** **GKIPSARVSILHEVRPVTSGCFPIMHDRTKIRQLPNLLRGYEHIRLSTHNVINAENAPGGPYKIGTSGSCPNITNGNGFFATMAWAVPKND** **KNKTATNPLTIEVPYICTEGEDQITVWGFHSD*N*ETQMAKLYGDSKPQKFTSSANGVTTHYVSQIGGFPNQTEDGGLPQSGRIVVDYMVQKS** **GKTGTITYQRGILLPQKVWCASGRSKVIKGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKE** **RGFFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRAD** **TISSQIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVEIGNGCFETKHKCNQTCLDRIAAGTFDAGEFSLPTFDSLNITAA**SSGRSS GGDIIKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEH EQHISESINNIVDHAIKCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKSGS	36

SIB9334: HA (B, Phuket 2013) pFerr N19Q/C31S/S111C	MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSYFANLKGTRTRGKLCPDCLNCTDLDVALGRPMCVGTT PSAKASILHEVRPVTSGCFPIMHDRTKIRQLPNLLRGYEKIRLSTQNVIDAEKAPGGPYRLGTSGSCPNATSKIGFFATMAWAVPKDNYKN ATNPLTVEVPYICTEGEDQITVWGFHSDNKTQMKSLYGDSNPQKFTSSANGVTTHYVSQIGDFPDQTEDGGLPQSGRIVVDYMMQKPGKTG TIVYQRGVLLPQKVWCASGRSKVIKGSLPLIGEADCLHEEYGGLNKSKPYYTGKHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFF GAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISS QIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSLNITAASSGGDLIKLL NEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESI NNIVDHAIKCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKSGS	37
SIB9337: HA (B, Brisbane 2008, D197N) pFerr N19Q/C31S/S111C	MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSHFANLKGTETRGKLCPKCLNCTDLDVALGRPKCTGKI PSARVSILHEVRPVTSGCFPIMHDRTKIRQLPNLLRGYEHIRLSTHNVINAENAPGGPYKIGTSGSCPNITNGNGFFATMAWAVPKNDKNK TATNPLTIEVPYICTEGEDQITVWGFHSDNETQMAKLYGDSKPQKFTSSANGVTTHYVSQIGGFPNQTEDGGLPQSGRIVVDYMVQKSGKT GTITYQRGILLPQKVWCASGRSKVIKGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGF FGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTIS SQIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVEIGNGCFETKHKCNQTCLDRIAAGTFDAGEFSLPTTDSLNITSGGDIIKLLNE QVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINN IVDHAIKCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	38
SIB9347: HA (B, Wisconsin 2010, Y98F) pFerr N19Q/C31S/S111C	MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVTGVIPLTTTPTKSYFANLKGTRTRGKLCPDCLNCTDLDVALGRPMCVGTT PSAKASILHEVRPVTSGCFPIMHDRTKIRQLPNLLRGYENIRLSTQNVIDAEKAPGGPYRLGTSGSCPNATSKIGFFATMAWAVPKDNYKN ATNPLTVEVPYICTEGEDQITVWGFHSDNKTQMKSLYGDSNPQKFTSSANGVTTHYVSQIGDFPDQTEDGGLPQSGRIVVDYMMQKPGKTG TIVYQRGVLLPQKVWCASGRSKVIKGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWVKTPLKLANGTKYRPPAKLLKERGFF GAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLRADTISS QIELAVLLSNEGIINSEDEHLLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSLNITASGGDIIKLLNE QVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINN IVDHAIKCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKSGS	39
SIB9379: (H3-stem) bfpFerr N19Q/C31S/S111C	MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATELVFPGCGVLKLATGMRNVPEKQTRGIFGAIAGFI ENGWEGMVDGWYGFRHQNSEGIGQAADLKSTQAAINQINGMVNRVIELMEQGGPDCYLAELLVALLNQHVIDLTDSEMRKLFERTKKQLRE NAEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQIKSGGESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSL DGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCKDHATFNFLQWYVAEQ HEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	40

SIB9380: (H7-Stem) bfpFerr N19Q/C31S/S111C	MNTQILVFALIAIIPTNA**DKICLGHHAVSNGTKVNTLTERGVEVVNATELVFPGCGVLKLATGMKNVPEIPKGRGLFGAIAGFIENGWEGL IDGWYGFRHQNAQGEGTAADYKSTQSAIDQITGMVNRVIELMEQGGPDCYLAELLVAMLNQHVIDLADSEMDKLYERVKRQLRENAEEDGT GCFEIFHKCDDDCMASIRNNTYDHSKYREEAMQNRIQID**SGGESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFL FDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCKDHATFNFLQWYVAEQHEEEVLF KDILDKIELIGNENHGLYLADQYVKGIAKSRKS	41
SIB9408: (H10-Stem) bfpFerr N19Q/C31S/S111C	MYKIVVIIALLGAVKGL**DKICLGHHAVANGTIVKTLTNEQEEVTNATELVFPGCGVLMLATGMRNVPELIQGRGLFGAIAGFLENGWEGMV DGWYGFRHQNAQGTGQAADYKSTQAAIDQITGMVNRVVELMEQGGPDCYLAELLVAMLNQHVIDMADSEMRNLYERVRKQLRQNAEEDGKG CFEIYHACDDSCMESIRNNTYDHSQYREEALLNRLNIN**SGGESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLF DHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCKDHATFNFLQWYVAEQHEEEVLFK DILDKIELIGNENHGLYLADQYVKGIAKSRKS	42
SIB9148: (H1-Stem) bfpFerr N19Q/C31S/S111C	MKAKLLVLLCTFTATYA**DTICIGYHANNSTDTVDTVLEKNVTVTHSVNLGSGLRMVTGLRNIPQRETRGLFGAIAGFIEGGWTGMVDGWYG YHHQNEQGSGYAADQKSTQNAINGITNMVNSVIEKMGSGGSGTDLAELLVLLLNERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFEFY HKCNNECMESVKNGTYDYPKYSEESKLNREKID**SGGESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAE EYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIKCKDHATFNFLQWYVAEQHEEEVLFKDILDK IELIGNENHGLYLADQYVKGIAKSRKS	43
TEV cleavage site	*ENLYFQG*	44
CpG (phosphoro-thioate modifica-tions where * is shown)	T*G*A*C*T*G*T*G*A*A*C*G*T*T*C*G*A*G*A*T*G*A	45
	Not Used	46-200
bfpFerritin-N19Q/C31S/S26C	ESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMCMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQ IFQKAYEHEQHISESINNIVDHAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	201

bfpFerritin-N19Q/C31S/S72C	ESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTCISAPEHKFEGLTQ IFQKAYEHEQHISESINNIVDHAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	202
bfpFerritin-N19Q/C31S/A75C	ESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISCPEHKFEGLTQ IFQKAYEHEQHISESINNIVDHAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	203
bfpFerritin-N19Q/C31S/K79C	ESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHCFEGLTQ IFQKAYEHEQHISESINNIVDHAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	204
bfpFerritin-N19Q/C31S/S100C	ESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQ IFQKAYEHEQHISECINNIVDHAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	205
bfpFerritin-N19Q/C31S/S111C	ESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQ IFQKAYEHEQHISESINNIVDHAIKCKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	206
bfpFerritin-N19Q/C31S/E12C	ESQVRQQFSKDIEKLLNCQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQ IFQKAYEHEQHISESINNIVDHAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	207
Exemplary H. pylori Ferritin with bullfrog linker	ESQVRQQFSKDIEKLLNEQVNKEMNSSNLYMSMSSWCYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQ IFQKAYEHEQHISESINNIVDHAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	208
Exemplary wild-type H. pylori ferritin (GenBank Accession AAD06160.1) (without bullfrog	LSKDIIKLLNEQVNKEMNSSNLYMSMSSWCYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNVPVQLTSISAPEHKFEGLTQIFQKAYE HEQHISESINNIVDHAIKSKDHATFNFLQWYVAEQHEEEVLFKDILDKIELIGNENHGLYLADQYVKGIAKSRKS	209

linker or N-terminal Met)		
CpG (ISS-1018)	TGACTGTGAACGTTCGAGATGA	210
Trichoplusia ni heavy chain ferritin	TQCNVNPVQIPKDWITMHRSCRNSMRQQIQMEVGASLQYLAMGAHFSKDVVNRPGFAQLFFDAASEEREHAMKLIEYLLMRGELTNDVSSL LQVRPPTRSSWKGGVEALEHALSMESDVTKSIRNVIKACEDDSEFNDYHLVDYLTGDFLEEQYKGQRDLAGKASTLKKLMDRHEALGEF IFDKKLLGIDV	211
Trichoplusia ni light chain ferritin	ADTCYNDVALDCGITSNSLALPRCNAVYGEYGSHGNVATELQAYAKLHLERSYDYLLSAAYFNNYQTNRAGFSKLFKKLSDEAWSKTIDII KHVTKRGDKMNFDQHSTMKTERKNYTAENHELEALAKALDTQKELAERAFYIHREATRNSQHLHDPEIAQYLEEEFIEDHAEKIRTLAGHT SDLKKFITANNGHDLSLALYVFDEYLQKTV	212
Pyrococcus furiosus ferritin	MLSERMLKALNDQLNRELYSAYLYFAMAAYFEDLGLEGFANWMKAQAEEEIGHALRFYNY IYDRNGRVELDEIPKPPKEWESPLKAFEAAYEHEKFISKSIYELAALAEEEKDYSTRAFL EWFINEQVEEEASVKKILDKLKFAKDSPQILFMLDKELSARAPKLPGLLMQGGE	213
human heavy chain ferritin	MTTASTSQVRQNYHQDSEAAINRQINLELYASYVYLSMSYYFDRDDVALKNFAKYFLHQSHEEREHAEKLMKLQNQRGGRIFLQDIKKPDC DDWESGLNAMECALHLEKNVQQSLLELHKLATDKNDPHLCDFIETHYLNEQVKAIKELGDHVTNLRKMGAPESGLAEYLFDKHTLGDSDQE S	214
human light chain ferritin (signal peptide is underlined)	MDSKGSSQKGSRLLLLLVVSNLLLPQGVLASSQIRQNYSTDVEAAVNSLVNLYLQASYTYLSLGFYFDRDDVALEGVSHFFRELAEEKREG YERLLKMQNQRGGRALFQDIKKPAEDEWGKTPDAMKAAMALEKKLNQALLDLHALGSARTDPHLCDFLETHFLDEEVKLIKKMGDHLTNLH RLGGPEAGLGEYLFERLTLKHD	215
	Not used	216
bullfrog linker	ESQVRQQF	217
Cysteine-Thrombin-His Linker (cysteine is	CLVPRGSLEHHHHHH	218

double underlined)		
	Not Used	219
16 amino acid linker	GGGGSGGGGSGGGGSG	220
28 amino acid linker	GGSGSGSNSSASSGASSGGASGGGSGGGSG	221
46 amino acid linker	GGSGSASSGASASGSSNGSGSGSGSNSSASSGASSGGASGGSGGSG	222
FR1	GGSGSASAEAAAKEAAAKAGGSGGSG	223
FR2	GGSGSASAEAAAKEAAAKEAAAKAGGSGGSG	224
47 amino acid linker comprising a C for conjugation	SGGGSGSASSGASASGSSCSGSGSGSSSASSGASSGGASGGGSGGSG	225

EXAMPLES

[00231] The following examples are provided to illustrate certain disclosed embodiments and are not to be construed as limiting the scope of this disclosure in any way.

1. Design, purification, and characterization of HA-ferritin nanoparticles

[00232] HA nanoparticles (HA-Nps) were generated by fusing HA ectodomain sequences (lacking 48 C-terminal transmembrane residues) to the N-terminus of a ferritin to produce nanoparticles that self-assembled in mammalian cells. The ferritins comprised a mutation replacing a surface-exposed amino acid with a cysteine (resulting from a S26C, A75C, or S111C mutation relative to the ferritin sequence of SEQ ID NO: 208) to allow conjugation of adjuvant onto to HA-Nps. A representation of such a cysteine is presented in Figure 2.

[00233] The cysteine resulting from the mutation described above can be used to conjugate an immune-stimulatory moiety. Figure 3A illustrates the result of an exemplary 1-step click chemistry reaction to conjugate a TLR agonist to ferritin using a maleimide-PEG4-SM7/8a click reagent. In this reaction, the maleimide reacts with the unpaired cysteine to conjugate the TLR7/8 agonist molecule covalently to the ferritin at surface-exposed cysteine via the intervening linker, yielding a covalent TLR7/8-agonist-ferritin conjugate. In this example, one cysteine on the surface of one monomer is indicated. Ferritin nanoparticles are multimers, e.g., consisting of 24 monomers. Thus, a ferritin nanoparticle can comprise a number of surface-exposed cysteines equal to the number of monomers, and each of the surface-exposed cysteines can be conjugated.

[00234] Figures 3B and 4A-4B illustrate exemplary 2-step click chemistries that can be used to conjugate adjuvants to ferritin nanoparticles and illustrate how CpG and 3M-012 (sometimes also referred to as 3M012) can also be conjugated to ferritin by 2-step click chemistry reactions via an intermediate bifunctional linker. An exemplary linker is Sigma PEG linker (catalog# 760676) which contains the maleimide and DBCO reactive groups. In this example, the TLR agonists are functionalized by a reactive azide group.

[00235] Mass spectrometry (MS) is used in experiments described below to characterize influenza-ferritin with and without conjugated immune-stimulatory moieties. The analyte subjected to MS was in some cases a trypsin digest of the influenza-ferritin. Trypsin generally cuts after lysine and arginine residues, except when followed by a proline. The structured nanoparticle (C-terminal to the indicated trypsin site) is resistant to

proteolysis, however. The most distal (C-terminal) trypsin site that can be cleaved by trypsin in bullfrog-*H. pylori* ferritin constructs, such as SEQ ID NO: 14, under native conditions is indicated in Figure 5. Thus, the trypsin digest releases a proteolysis-resistant ferritin particle suitable for MS analysis.

[00236] Thus, trypsin digestion followed by a simplified MS analysis regardless of the N-terminal antigen present in the uncleaved polypeptide can be used to evaluate conjugation to the nanoparticle, given that the linker sequence is accessible to Trypsin. This method was devised to overcome the complexity that glycoprotein antigens pose to MS analysis. For example, the H1/Stem-Np can otherwise be analyzed by MS only after PNGase-treatment (Fig. 8A).

[00237] HA sequences from the following influenza strains were genetically fused to the N-terminus of Helicobacter pylori-bullfrog hybrid ferritin to construct the HA-nanoparticles: A/Fort Monmouth/1-JY2/1947 (GenBank CY147342, amino acids 1-518, Y108F); A/Malaysia/302/1954 (GenBank CY009340.1, amino acids 1-518, Y108F); A/Denver/1957 (GenBank CY008988, amino acids 1-517, Y108F); A/Hong Kong/117/1977 (GenBank CY009292,amino acids 1-518, Y108F); A/New Caledonia/20/99 (GenBank AHJ09883.1, amino acids 1-518); A/California/4/2009 (GenBank AHJ09884.1, amino acids 1-518); COBRA P1 (SEQ ID NO: 2 in US patent publication US20150017196A1, amino acids 1-518, Y108F) and COBRA X6 (US patent publication US20140127248A1, amino acids 1-517, Y108F). COBRA P1 and COBRA X6 were generated through a computational method of hierarchical sequence averaging (Carter DM, et al., J Virol 90:4720-4734 (2016)). COBRA X6, was generated from human H1N1 influenza sequences spanning 1999-2012, and COBRA P1 from human H1N1 strains spanning 1933-1957 and 2009-2011 plus swine H1N1 influenza strains from 1931-1998 (Carter 2016). The HA-ferritin genes were cloned into the XbaI/BamHI sites of SIB002 vector for mammalian expression, with a gccacc kozak sequence in front of the ATG start codon. All sequences were codon-optimized for expression in human cell lines.

[00238] HA-Np plasmids were purified with the Powerprep kit (Origene catalog# NP100009) and used to transfect Expi293 cells (ThermoFisher catalog# A14635). The FectoPRO DNA transfection reagent (Polyplus #116-100) was used with standard conditions (0.5 µg of DNA/mL, 0.75 µl FectoPRO reagent/mL and 0.45 µl of enhancer/mL). Nanoparticles were harvested from Expi293 supernatant by centrifugation at 3,488 g for 15 min at 4 degrees, 4-6 days after transfection and filtered through a 0.45 µm vacuum-driven filter unit (Thermo Scientific catalog# 167-0045). HA-Nps were passed through a Q-

Sepharose Fast flow column (GE catalog#17051001) by gravity-flow and the flow-through was collected and diluted 3X with water, and pH was adjusted by adding Tris buffer pH 8.5 at a final concentration of 50 mM; or supernatants were diluted 5X with Buffer A (50 mM Tris pH 8.5, 5 mM NaCl) instead of using the initial Q-Sepharose column. The samples were then loaded to a Q-Sepharose column (HiTrap Q HP, GE catalog# 17115401) and proteins were eluted over a NaCl gradient, with 0-60% mixing of buffer A (50 mM Tris pH 8.5, 5 mM NaCl) and buffer B (50 mM Tris, pH 8.5, 1 M NaCl) over 30 column volumes. The HA-nanoparticle protein fractions were collected and concentrated with Amicon Ultra-15 centrifugal filter unit (Millipore catalog #UFC910024) and further purified by size exclusion chromatography with a Superose 6 column PG XK 16/70,60-65 cm (catalog# #:90100042) in phosphate buffered saline. The final fractions were concentrated and filter-sterilized through 0.22 μm filter (Millipore SLGV004SL). Endotoxin-free solutions were used, and final proteins were tested using the Charles Rivers Endosafe PTS instrument with LAL cartridges with 0.05 EU/ml limit of detection.

[00239] Trypsin digestions of H1/Stem-Np (SEQ ID NO: 43, Figure 6A) and H5/COBRA-Np (SEQ ID NO: 32, Figure 6B) are shown. In both cases, the shift in molecular weight with trypsin digestion (indicated by "+") corresponded to the molecular weight of SM7/8a (711 daltons). Thus, these data indicate successful conjugation of SM7/8a to ferritin nanoparticles comprising a surface-exposed cysteine.

[00240] Resolution of the change in weight of ferritin nanoparticles after conjugation to molecules of low molecular weight such as maleimide-PEG4-SM7/8a is not clear in gel-shift experiments (Figure 7A) using PNGase to remove glycosylation of H1/Stem-Np, without trypsin treatment. As shown in Figure 7B, trypsin treatment allows confirmation of conjugation of small molecules, such as SM7/8a, to H1/Stem-Np by gel-shift assay. Conjugations of molecules of higher molecular weight, such as CpG, can be observed by gel-shift assay of the deglycosylated H1/Stem-Np (Fig. 7A), and also by gel-shift assay of the ferritin nanoparticle released from H1/Stem-Np by trypsin digestion under native conditions. Thus, use of trypsin digestion of ferritin nanoparticles under native conditions provides a method for the characterization of the core fragment to which TLR agonists are conjugated, as a platform technology, that might otherwise be difficult to analyze with gel-shift or mass spectrometry (MS) experiments on a given antigen with properties can interfere with these analytical assays.

[00241] After conjugation of SM7/8a-PEG4-maleimide to the H1/Stem-Np, and PNGase-treatment for analytical purposes only, a conjugation product on MS was seen with a

mass of 42930 Da that was approximately 715 kDa heavier than the unconjugated H1/Stem-Np (Figure 8A). After trypsin digestion, the reduced ferritin nanoparticle had a weight of 19510 Da (based on cleavage of the H1/Stem portion of the nanoparticle) (Figure 8B). After conjugation, the weight of the trypsin-digested conjugated nanoparticle was also approximately 715 kDa heavier. Thus, both Figure 8A and Figure 8B support a conjugation efficiency of about 100% in conjugating a linker comprising adjuvant to the ferritin nanoparticle.

[00242] A maleimide-PEG4-DBCO linker was conjugated to H1/stem-Np in a 2-step click chemistry reaction, in which the maleimide reacted with the surface-exposed cysteine on the ferritin nanoparticle, an increase in weight consistent with the molecular weight of the linker addition, approximately 675 Da, was seen by MS analysis after trypsin digestion (Figure 9).

[00243] Next, an azide-CpG was added to the H1/Stem-Np-PEG4-DBCO intermediate (labeled "After Mal-PEG4-DBCO" in Figure 10), which was confirmed by gel-shift assay. Azide-CpG was ordered from IDT (SEQ ID NO: 45). Before SDS-PAGE, conjugates and controls were treated with PNGase. CpG conjugation caused an upward gel-shift due to the added mass (~7.5 kDa) in comparison to linker conjugation. An example of 50% conjugation efficiency quantified by densitometry of the two bands labeled "Stem-Np-CpG" and "Stem-Np" is shown in Figure 10. Stem-Np is H1/Stem-Np (SEQ ID NO: 43). Untreated refers to H1/Stem-Np before reduction. TCEP-Reduced refers to H1/Stem-Np treated with 3 mM TCEP for 1 hour at room temperature and dialyzed against PBS overnight at 4° Celsius.

[00244] A tobacco etch virus (TEV) protease cleavage site (SEQ ID NO: 44) was also used to evaluate conjugation. 3M-012 was conjugated to various NC99 HA-TEV-Np constructs (SEQ ID NOS: 9-12) using a 2-step click chemistry process. HA-TEV-Np constructs were reduced with 2 or 10 mM TCEP. TCEP was removed with dialysis (3 mL dialysis cassette with 10 kDa MWCO, Thermo #87730) into TRIS buffer (100 mM TRIS pH 8.0, 50 mM NaCl). The DBCO-PEG4-Maleimide linker (Sigma #760676) was added. Excess linker was removed with ultrafiltration while also buffer exchanged into PBS 7.4 (100 kDa MWCO, Millipore Amicon #UFC810024 or #UFC910096). Finally, the azide-fuctionalized TLR agonist (CpG or 3M-012) was clicked onto the molecule. Excess drug was removed by ultrafiltration (100 kDa MWCO, Millipore Amicon #UFC810024 or #UFC910096 or gel filtration. A TEV cleavage site in HA-TEV-Np allows selective cleavage by TEV for analysis of conjugation.

[00245] After conjugation to 3M-012 (~500 Da + ~600 Da linker) to HA-TEV-Np constructs, the constructs were treated with TEV. Following TEV cleavage, the resulting HA-band on gel-shift SDS-PAGE is about ~57kDa plus glycans and Ferr ~20 kDa (Figure 11, lane labelled "+TEV"). The double bands at ~20 and 21 kDa (lanes labelled "S26C," "S72C," "A75C,", and "S111C" (SEQ ID NOS: 10, 11, 12, and 9, respectively) indicate successful conjugation. The conjugation efficacy corresponds to the band intensity, where the lower band is unconjugated and the upper band is conjugated ferritin.

[00246] It was found that H1/Stem-Np comprising S111C has a post-translational modification of 109 Daltons (consistent with cysteinylation) when expressed in 293Expi cells (Figure 12A), which was removed by reduction with TCEP (tris(2-carboxyethyl)phosphine) at 3 mM concentration for 1 hour at room temperature to free the reactive thiol (Figures 12B). Thus, the modification can be reduced to expose the surface-exposed cysteine as a free thiol, which is highly reactive towards maleimide groups on commercial linkers or customized small molecules.

[00247] MS data also confirmed successful conjugation of 3M-012 to HA-Np. HA-TEV-Np-S26C (SEQ ID NO: 10; Figure 12C), A75C (SEQ ID NO: 12; Figure 12D), or S111C (SEQ ID NO: 9; Figure 12E). Nanoparticles comprising A75C and S111C ferritin modifications (SEQ ID NOS: 12 and 9, respectively) showed greater than 95% conjugation to 3M-012. Thus, the MS data indicates that adjuvants can be linked to various surface exposed cysteines resulting from mutations in ferritins to successfully generate antigenic ferritin polypeptides linked to adjuvants.

[00248] Negative stain electron microscopy (EM) confirmed that conjugations did not affect the nanoparticle integrity. Shown are H1/Stem-Np (SEQ ID NO: 43) alone (Figure 13A), H1/Stem-Np conjugated to maleimide-PEG4-SM7/8a (Figure 13B), H1/Stem-Np conjugated to CpG (Figure 13C), NC99 HA-Np (SEQ ID NO: 9) alone (Figure 13D), NC99 HA-Np conjugated to 3M-012 via the DBCO-maleimide linker (Figure 13E), and NC99 HA-Np conjugated to CpG via the DBCO-maleimide linker (Figure 13F). Further, conjugation of a TLR7/8 agonist (maleimide-PEG4-SM7/8a, Figure 14A) or TLR9 agonist (CpG, Figure 14B) via maleimide-PEG4-DBCO linker to H1/Stem-Np does not affect nanoparticle integrity as measured by dynamic light scattering (DLS) compared to unconjugated H1/Stem-Np (Figure 14C).

[00249] These data demonstrate the successful preparation of antigenic polypeptides comprising ferritin and HA polypeptides. Further, linkers comprising adjuvant can be successfully conjugated to these ferritin nanoparticles.

2. Characterization of immunogenicity of HA-ferritin nanoparticle compositions

[00250] The effect of various adjuvants and of conjugating TLR agonists to H1/Stem-Np on the immunogenicity of H1/Stem-Np compositions was assessed. Figure 15A shows IgG antibody titers to H1/New Caledonia/1999 (NC99) HA trimers induced by immunization with H1/Stem-Np TLR7/8-agonist conjugate. Dosing was at week 0 and week 3 with 10 μg of H1/Stem-Np, administered without adjuvant, with admixed equimolar amount of free SM7/8a adjuvant (83.3 ng), with admixed high dose free SM7/8a adjuvant (21.84 μg), or with a 1:1 volume ratio of either PAA or AF03 adjuvant. The H1/Stem-Np-SM7/8a conjugate was administered in the absence of any other adjuvants. Sera were assayed 2 weeks after a second dose. Figure 15B shows IgG antibody titers to H1/Stem trimers induced by immunization with H1/Stem-Np TLR9-agonist conjugate. Dosing was at week 0 and week 3 with 10 μg of H1/Stem-Np, administered without adjuvant, with admixed equimolar amount of free CpG adjuvant (850 ng), with admixed high dose free CpG adjuvant (20 μg), or with a 1:1 volume ratio of AF03 adjuvant. The H1/Stem-Np-CpG conjugate was administered in the absence of any other adjuvants. Sera were assayed 2 weeks after a second dose.

[00251] Data in Figure 15A indicated that an SM7/8a-conjugated H1/Stem-Np showed comparable immunogenicity as an unconjugated nanoparticle admixed with a polyacrylic acid (PAA) or AF03 adjuvant. The SM7/8a conjugated directly to the ferritin nanoparticles was more effective that an equimolar dose of SM7/8a administered as a separate compound from the ferritin nanoparticle ("mixed equimolar"). Further, the SM7/8 conjugated directly to the ferritin nanoparticles was more effective than a higher dose of SM7/8a administered as a separate compound from the ferritin nanoparticle ("mixed high"). Thus, conjugation of SM7/8a to the antigenic polypeptide comprising an HA polypeptide and ferritin rendered the compound immunogenic and self-adjuvanting. Administration of a self-adjuvanting composition could allow reduction or elimination of a separate adjuvant. Similarly, Figure 15B indicated that conjugation of the TLR9-agonist CpG to H1/Stem-Np improves its immunogenicity compared to unconjugated and equimolar admixed controls to levels that are comparable to administering a 23x higher dose of admixed CpG molecule.

[00252] Sera were also assessed for the ability to neutralize H1/New Caledonia/1999 (NC99) HA/NA pseudotyped lentivirus in vitro. The pseudotyped neutralization assay was performed as previously described (Kanekiyo et al., Nature 499:102-106 (2013)). Briefly, lentiviruses were packaged in HEK293T cells by transfection of 5 plasmids, 400 ng of HA, 100 ng of NA, 50 ng of TMPRSS2, 7 μg of CMVΔR8.2 and 7 μg of pHR'CMV-Luc with the

Profection Mammalian Transfection kit (Promega catalog# E1200). Viral particles were collected 48 and 72 hours post-transfection and filtered through a 0.45 μm filter.

[00253] Mice (n=5/group) were immunized twice at a three-week interval. NC99 HA-Np (SEQ ID NO: 9) conjugated to 3M-012 (HA-Ferr-3M-012, 0.22 μg/dose) was administered in parallel with admixed controls: a mix of NC99 HA-Np (0.22 μg/dose) and 10 μg of 3M-012 (a typical therapeutic dose), and separately, a mix of NC99 HA-Np (0.22 μg/dose) and 1.7 ng of 3M-012 (an equimolar match to the conjugate). Additional controls were unconjugated NC99 HA-Np and an inactivated influenza vaccine (IIV), dosed with matched HA content (0.17 μg HA/dose). At week 5, serial dilutions of the serum from these mice were assayed for neutralization activity towards lentiviruses pseudotyped with HA and neuraminidase (NA) genes from the strains indicated. Antisera at a range of dilutions were pre-incubated with a fixed amount of lentivirus and used to infect target 293A cells. Infection was quantified 72 hours later with the Promega Luciferase Assay System (catalog# E1500). The IC_{50} values were calculated with Graphpad Prism software from these neutralization curves, to determine the serum dilution factor that attains 50% neutralization of PsV. Endpoint titers (ELISA) and Hemagglutination Inhibition (HAI) titers were also determined at weeks 5 and 8, respectively. See the following example for exemplary ELISA and HAI procedures. All samples were run in triplicate.

[00254] Based on the ELISA endpoint titers (Figure 16A) or pseudo-virus neutralization IC_{50} titers (Figure 16B), NC99 HA-Np conjugated to 3M-012 induced stronger binding and neutralizing antibody responses than the matched admixture, the unconjugated particle, or IIV. The HA-Np-3M-012 conjugate also induced significantly stronger HAI titers (Figure 16C) than the equimolar admix control. Additionally, the HAI titers were 3.7- and 1.6-fold higher than unconjugated HA-Ferr and IIV, although these results were not significant.

[00255] The immunogenicity of NC99 HA-Np (SEQ ID NO: 9) conjugated to CpG (HA-Ferr-CpG, 0.22 μg/dose) was also assessed in comparison with admixed controls (a mix of HA-Np (0.22 μg/dose) and 20 μg of CpG (a typical therapeutic dose) and a mix of HA-ferr (0.22 μg/dose) and 21 ng of CpG (an equimolar match to the conjugate)). Additional controls were unconjugated HA-Np and IIV, dosed with match HA content (0.17 μg HA/dose). Dosing was at weeks 0 and 3 as described above. ELISA and PsV assays are of serum from 2-weeks post-boost, and HAI was performed on serum 5-weeks post-boost. All samples were run in triplicate.

[00256] Based on ELISA endpoint titers (Figure 17A) or pseudo-virus neutralization IC_{50} titers (Figure 17B), the conjugate induces stronger binding and neutralizing antibody responses than the matched admixture, the unconjugated particle, or IIV. The HA-Np-CpG conjugate induces significantly stronger HAI titers (Figure 17C) than the equimolar admix control. Additionally, the HAI titers were 2.6- and 3.0-fold higher than unconjugated HA-Ferr and IIV, although these results were not significant.

[00257] The immunogenicity of H1/Stem-Np-TLR agonist conjugates was also assessed in a non-human primate model. Twelve cynomolgus macaques (*Macaca fascicularis*) were housed and cared for by Bioqual Inc. in compliance with all federal regulations, including USDA regulations and the Animal Welfare Act. Animals were pre-screened and selected for lack of reactivity to 2016-2017 Fluzone Quadrivalent antigens (A/California/07/2009; A/HongKong/4801/2014 X-263B; B/Phuket/3073/2013; B/Brisbane/60/2008) by ELISA. NHP subjects (10 females, 2 males), 5 to 13 years old, and 3.5 kg to 7.8 kg in weight, were randomly-assigned to each immunization group (n = 3 animals/group). Each received three doses, at weeks 0, 4 and 10 of either 50 μg of H1-SS-np with AF03, or 200 μg of H1-SS-np (no adjuvant control), or 200 μg of H1-SS-np-SM7/8 conjugate, or 200 μg of H1-SS-np-CpG. Blood samples of 10 mLs were collected for serum isolation at weeks 0, 2, 4, 6, 8, 10 and 12. Blood samples of 10-16 mLs were collected at weeks 0, 2, 5, 6 and 12 for PBMC isolation. Serum and PBMCs were isolated and cryopreserved following standard operating procedures. Cynomolgus macaques were assigned with to be immunized at weeks 0, 4 and 10 with either 50 μg of H1/Stem-Np formulated with admixed AF03 adjuvant, or 200 μg H1/Stem-Np (no adjuvant control), or 200 μg of H1/Stem-Np-SM7/8a conjugate (shown in Figure 3A) or 200 μg of H1/Stem-Np-CpG conjugate (shown in Figure 3B). Serum, isolated at various timepoints, were assayed for anti-HA antibody titers by ELISA (Figure 25A), and for neutralization of lentivirus pseudotyped with H1-subtype HA and NA (Figure 25B) or with H5-subtype HA and NA (Figure 25C). Taken together, the results shown in Figures 25A-C demonstrate that conjugation of TLR-agonists improves the immunogenicity of H1/Stem-Np in a non-human primate model. In this model, the TLR7/8 agonist conjugate appears to give higher titers than the CpG conjugate and the AF03 admixed adjuvant elicited the highest titers at the conditions tested.

[00258] Next, it was determined if the immunogenicity of the 3M012 conjugate could be improved when given at a higher dose. To do this, the conjugate, an equimolar admixture, a higher dose admixture, and unconjugated nanoparticles were tested at 0.1 μg, 0.5 μg, 2.5

μg, and 12.5 μg doses of HA-NP (Figures 26A-B). At the 0.1 μg and 0.5 μg HA-NP dose, minimal response was seen in neutralization and HAI assays except for the high admixture dose. At the 2.5 μg dose, the unconjugated HA-NP and the equimolar admixture of HA-NP and 3M012 both induced minimum antibody responses. Compared to the high dose admixture group, the 3M012-conjugated HA-NP induced similar antibody responses despite containing >5000-fold less 3M012, documenting the efficacy of direct conjugation and targeted delivery of a TLR agonist.

3. Characterization of nanoparticles comprising candidate polypeptides by EM and DLS

[00259] HA-Nps comprising the flu polypeptides presented in Figure 1A were expressed and released into the culture media (sequence alignment of HA portions shown in Fig. 1A and dendogram shown in Figure 1B) using the processes outlined above. The arrows in Figure 1B indicate candidate polypeptides. HA-Nps listed in Figure 18A were purified from 293Expi cell culture supernatant by anion exchange and size exclusion chromatography. Coomassie staining results confirming successful product of nanoparticles are shown in Figure 18B.

[00260] Electron microscopy (EM) and dynamic light scattering (DLS) analyses were performed as follows.

[00261] Dynamic Light Scattering was measured on Wyatt's DynaPro Plate Reader II at 25°C. The purified HA-Nps displayed the expected size by dynamic light scattering, between 16 and 18 nanometers (Figure 18A, indicated by the "size" measure). The formation of nanoparticles was also confirmed by negative stain transmission electron microscopy (TEM) (Figure 18C). For EM, HA-ferritin nanoparticle samples were adsorbed for 1 minute to a carbon coated grid that had been made hydrophilic by a 30 second exposure to a glow discharge. Excess liquid was removed with a filter paper (Whatman #1) and the samples were stained with 0.75% uranyl formate for 30 seconds. After removing the excess uranyl formate with a filter paper the grids were examined in a TecnaiG² Spirit BioTWIN and images were recorded with an AMT 2k CCD camera.

4. Immunogenicity of single strain HA-ferritin nanoparticles against a representative panel of divergent H1N1 influenza viruses

[00262] To study the immunogenicity of HA-Nps from specific viral strains, mice were immunized twice with the nanoparticle from the strain of interest, and sera were assayed

3 weeks after the second immunization using hemagglutinin inhibition assay (HAI). All immunizations followed established guidelines for animal handling. Balb/C mice (5/group) were immunized at weeks 0 and 3 with 220 ng of HA-ferritin nanoparticles (170 ng of HA content), and where applicable mixed 1:1 with adjuvant immediately before intramuscular injection (50 µL per hind leg). Ribi (Sigma Adjuvant System, catalog# S6322-1vl) or AF03 (Sanofi Pasteur) were used as indicated in the figure legends. For bivalent, trivalent, and quadrivalent combinations, 220 ng of each nanoparticle was pre-mixed before injection. Sera were collected at 2 and 3 weeks post-boost injection.

[00263] HA inhibition assays (HAI) used influenza seed stocks were from the Centers for Disease Control and Prevention (Atlanta, Georgia, USA). The immune sera were pre-treated with receptor-destroying-enzyme (RDE) by diluting one-part serum with three-parts enzyme and incubated overnight in a 37°C water bath. The enzyme was inactivated by 30-minute incubation at 56°C followed by addition of six parts PBS to a final dilution of 1/10. HAI assays were performed in V-bottom 96-well microtiter plates with 4 HA units of virus (HAU) in 0.5% turkey red blood cells. The HAI titer was determined as the highest dilution of serum resulting in complete inhibition of hemagglutination.

[00264] For all data, error bars represent the standard error of the mean obtained from assaying samples from each animal in a given treatment group; n = 5 for mice, and n = 12 for ferrets. Student's T test was calculated with Microsoft Excel. ANOVA was calculated with VassarStats (available via web at vassarstats.net/anova1u.html).

[00265] A panel of 16 representative influenza strains was used spanning 78 years of viral evolution. Viruses in the panel are listed in Table 2.

Table 2: Panel of H1N1 Influenza strains used for HAI

H1N1 HAI Influenza Panel
A/Puerto Rico/1934
A/Weiss/1/1943
A/FM/1/1947
A/Denver/1/1957
A/New Jersey/8/1976
A/USSR/90/1977
A/Brazil/11/1978
A/Chile/1/1983
A/Taiwan/1/1986
A/Texas/36/1991
A/Beijing/262/1995

A/New Caledonia/20/1999
A/Solomon Islands/6/2006
A/Brisbane/59/2007
A/California/07/2009
A/Bangladesh/2021/2012
A/Vietnam/3050/2013

[00266] Potent neutralization of the matched strain was observed in all cases (CA09, NC99, FM47, and HK77, Figures 19A-19D).

[00267] Serological responses were also confirmed by ELISA against matched antigens (Figures 20A-20F). Immunogens administered to each group are listed in Figure 20A. Trimers were used for ELISAs, as indicated by Figures 20B-20F.

[00268] For the ELISA assay, Nunc MaxiSorp 96-well plates (catalog# 44-2404-21) were coated with 100 ng/well of trimeric HA or stabilized stem proteins overnight at 4ºC and blocked with 5% skim milk in PBST. Anti-sera were diluted as indicated and incubated for 1 hour at room temperature, and bound antibodies were detected with an anti-mouse-HRP antibody (catalog# NA931 at 1:5,000) or anti-monkey-HRP antibody (Southern Biotech catalog# 4700-05, Lot# A3814-P907, at 1:5,000) in 5% milk-PBST, also incubated for 1 hour at room temperature. After washing with PBST 5 times, HRP was developed with SureBlueTMB substrate (Catalog# 52-00-02) and stopped with 0.5 N sulfuric acid. Absorbance was read at 450 nm (Spectramax M5). Endpoint titers were calculated with Graphpad prism with a threshold value of 0.2 and the typical background level is 0.05.

[00269] Neutralization of HA/NA pseudotyped lentiviruses was also evaluated as described above and results are shown in Table 3. Strong neutralization activity was observed for the matched strains in all cases tested, and these values were used as thresholds. The combination of HA-Nps with complementary neutralization activities led to expanded cross-reactivity in an additive manner.

Table 3: Neutralization IC50 towards HA/NA pseudotyped lentiviruses (PsV) in mice after two immunizations with HA-ferritin nanoparticles administered as single components or in combination

Group #	Immunogens	FM 1947	Malaysia 1954	Hong Kong 1977	New Caledonia 1999
1	NC99 Np	<200	<200	1.8×10^3	**5.8×10^4**

2	CA09 Np	<200	<200	8.8×10^2	5.3×10^2
3	FM47 Np	*4.2×10^5*	**2.1×10^4**	**2.8×10^4**	4.8×10^2
4	HK77 Np	*1.2×10^5*	**1.7×10^4**	*4.6×10^5*	9.9×10^2
5	Mal54 Np	4.3×10^3	*5.7×10^5*	6.8×10^3	2.8×10^3
6	NC99+CA09 Np	<200	<200	2.1×10^3	**6.2×10^4**
7	NC99+CA09+FM47 Np	*4.4×10^5*	1.6×10^3	**2.3×10^4**	*2.9×10^5*
8	NC99+CA09+HK77 Np	*2.8×10^5*	8.9×10^3	*2.1×10^5*	*1.5×10^5*
9	NC99+CA09+Mal54 Np	1.7×10^3	*1.6×10^5*	2.2×10^3	*1.7×10^5*
10	NC99+CA09+HK77+FM47 Np	*4.1×10^5*	2.6×10^3	*2.1×10^5*	*1.4×10^5*
11	NC99+CA09+HK77+MAL54 Np	**7.4×10^4**	*1.5×10^5*	*1.6×10^5*	*2.7×10^5*
12	NC99+CA09+FM47+MAL54 Np	*3.7×10^5*	*1.1×10^5*	**3.1×10^4**	**9.8×10^4**
13	NC99 IIV	1.0×10^3	<200	1.6×10^3	**4.8×10^4**
14	CA09 IIV	<200	<200	1.6×10^3	1.0×10^3
15	NC99+CA09 IIV	1.0×10^3	<200	1.7×10^3	**3.0×10^4**

< 10,000 **>10,000** *>100,000*
Regular font **Bold font** *Italic font*

[00270] The CA09 HA-Np elicited strong immune responses, but these were limited to contemporary (post-2009) strains and a 1976 isolate that also originated from swine and has close homology to CA09 (Figure 19A) (*see* Gaydos et al. 2006. Emerg Infect Dis 12:23-28 (1976)). NC99 HA-Np elicited potent neutralization against influenza viruses from the late 1990s and early 2000s (Figure 19B). The immunological response to FM47 HA-Np extended primarily to the matched strain, but it also showed modest cross-reactivity to the 1977 strain (Figure 19C). This level of cross-reactivity has clinical relevance to the 1977 outbreak, which affected primarily people under 26 years of age, suggesting that exposure to influenza viruses from 1940-50 outbreaks conferred protection against the 1977 strains (Kilbourne 2006). Interestingly, among the HA sequences, the HK77 HA-Np stood out because its cross-reactivity extended to strains from 1947, 1978 and 1983 (Figure 19D). On the other hand, the immunological responses to MAL54 HA-Np and DV57 HA-Np were restricted to the matched strain (Fig. 19E, 19F, and Table 3).

[00271] The cross-reactivity observed with COBRA P1 and COBRA X6 nanoparticles were consistent with their virus-like particle (VLP) counterparts (*see* Carter DM, et al., J Virol 90:4720-4734 (2016)). The immune response elicited by COBRA P1 HA-Np was similar to CA09 HA-Np (Fig. 19A and 19G) and COBRA X6 HA-Np showed a similar immune profile to NC99 HA-Np (Fig. 19B and 19H).

5. Monovalent, bivalent, trivalent and quadrivalent formulation of HA-ferritin nanoparticles

[00272] The HAI cross-reactivity elicited by combinations of select HA-Nps was evaluated. Mice were immunized and tested as described above with bivalent, trivalent or quadrivalent formulations made by combining individual nanoparticles. The bivalent combination of NC99 and CA09 HA-Nps showed expanded cross-reactivity relative to either monovalent composition (Figure 21A). However, this bivalent combination did not elicit detectable antibody titers against the older divergent strains from 1934-1957 and 1977-1991. The immunogenicity of the COBRA X6 and COBRA P1 bivalent combination followed the same trend (Figure 21B). This combination showed increased breadth compared to the NC99/CA09 bivalent composition, although HAI titers against several strains were moderate. For the trivalent combinations, inclusion of a third component to NC99 and CA09 HA-Nps increased cross-reactivity when the third component was either FM47 HA-Np (Figure 21C) or HK77 HA-Np (Figure 21E), but MAL54 HA-Np (Figure 21D) did not enhance breadth. Addition of a fourth component in the quadrivalent formulations, resulted in no additional observable cross-reactivity compared to the trivalent combination of NC99, CA09, and HK77 HA-Nps (Figures 21F-21H).

[00273] Comparable results were observed when different adjuvants were used (i.e., Ribi versus AF03, Figures 22A-22F). Importantly, there was no evidence for antigenic competition by co-administration of different HA-Nps. These data suggest that cross-reactivity profiles are additive for cases in which there is a high degree of complementarity in their individual HAI profiles. HAI profiles obtained with NC99 and CA09 immunogens delivered as egg-produced inactivated influenza vaccines (IIV) using a normalized dose of HA were also measured (Figures 23A-23C).

6. Protection against challenge with nanoparticle compositions in ferrets

[00274] The efficacy of certain HA-Np combinations were tested in ferrets, an animal model relevant to human disease. Ferrets were immunized (n=12 per group) with either phosphate buffered saline (Figure 24A), CA09 inactivated influenza vaccine (IIV, Figure 24B), a trivalent combination of wildtype HA-Nps (NC99+CA09+HK77, Figure 24C) or a combination of COBRA P1+COBRA X6+HK77 HA-Nps (Figure 24D). Before intramuscular injection, these compositions were mixed 1:1 with AF03 adjuvant for a 1-ml final injection volume.

[00275] After two immunizations, significant HAI titers against the matched strains were observed (Figures 24B-24D). Both groups of ferrets immunized with nanoparticle combinations showed significant HAI titers against FM47, HK77 and NC99. CA09 IIV did not elicit cross-neutralizing titers against FM47 and HK77 strains in ferrets (Figure 24B), as observed in mice.

[00276] After immunization, the ferrets were challenged with an unmatched divergent strain, H1/Fort Monmouth/1947 virus. The influenza challenge was performed 4 weeks after the second immunization by intranasal inoculation with 1 ml of A/Fort Monmouth/1/1947 virus with $10^{4.65}$ times a 50% tissue culture infectious dose (TCID50). Clinical signs were followed daily for 2 weeks and nasal washes were collected daily for 7 days post challenge and tested for viral load by a standard $TCID_{50}$ assay. Viral titers were quantified from nasal washes following the challenge. The ferret cohorts that received either trivalent NC99+CA09+HK77 or COBRA-P1+X6+HK77 nanoparticle combinations cleared the virus faster than the control group, displaying significantly reduced viral titers at day 5 post-infection (Figure 24E, $p \leq 0.001$). In contrast, the CA09 IIV-immunized group did not clear virus significantly faster than the PBS-immunized control group. In all groups, the FM47 strain successfully replicated and was cleared no later than one week after infection. Thus, in an animal model of infection relevant to human disease, the trivalent combinations stimulated effective HAI responses that also protect against divergent viral challenge.

We claim:

1. An antigenic influenza-ferritin polypeptide comprising (i) a ferritin protein comprising a mutation replacing a surface-exposed amino acid with a cysteine, and (ii) an influenza polypeptide.
2. An antigenic influenza-ferritin polypeptide comprising (i) a ferritin protein comprising a mutation replacing a surface-exposed amino acid with a cysteine and an immune-stimulatory moiety conjugated to the cysteine; and (ii) an influenza polypeptide.
3. The antigenic influenza-ferritin polypeptide of claim 1, further comprising an immune-stimulatory moiety conjugated to the ferritin protein via the cysteine.
4. The antigenic influenza-ferritin polypeptide of any one of claims 1-3, wherein the influenza polypeptide comprises a hemagglutinin (HA) or neuraminidase (NA) polypeptide.
5. The antigenic influenza-ferritin polypeptide of claim 4, wherein the HA polypeptide comprises a conserved region.
6. The antigenic influenza-ferritin polypeptide of claim 5, wherein the conserved region comprises all or part of the stem region of the HA.
7. The antigenic influenza-ferritin polypeptide of any one of the preceding claims, wherein the influenza antigen comprises an HA antigen comprising a Y98F mutation.
8. The antigenic influenza-ferritin polypeptide of any one of the preceding claims, further comprising a mutation replacing an internal cysteine with a non-cysteine amino acid.
9. The antigenic influenza-ferritin polypeptide of claim 8, wherein the internal cysteine is at position 31 of *H. pylori* ferritin, or a position that corresponds to position 31 of *H. pylori* ferritin as determined by pair-wise or structural alignment, optionally wherein the internal cysteine is mutated to serine.
10. The antigenic influenza-ferritin polypeptide of any one of the preceding claims, further comprising a mutation replacing a surface-exposed asparagine with a non-asparagine amino acid, optionally wherein the non-asparagine amino acid is glutamine.
11. The antigenic influenza-ferritin polypeptide of any one of the preceding claims, wherein the surface exposed amino acid is a mutation of E12, S26, S72, A75, K79, S100, or S111 of *H. pylori* ferritin or an analogous amino acid in a non-*H. pylori* ferritin as determined by pair-wise or structural alignment.
12. The antigenic influenza-ferritin polypeptide of claim 11, wherein the mutation at the surface exposed amino acid is E12C, S26C, S72C, A75C, K79C, S100C, or S111C of *H.*

pylori ferritin or an analogous amino acid in a non-*H. pylori* ferritin as determined by pair-wise or structural alignment.

13. The antigenic influenza-ferritin polypeptide of any one of the preceding claims, wherein the immune-stimulatory moiety is an agonist of TLR7 or TLR8.
14. The antigenic influenza-ferritin polypeptide of any one of the preceding claims, wherein the immune-stimulatory moiety is an agonist of TLR9.
15. The antigenic influenza-ferritin polypeptide of any one of claims 1 or 3-14, further comprising a linker between the immune-stimulatory moiety and the ferritin protein.
16. The antigenic influenza-ferritin polypeptide of claim 15, wherein the linker comprises one, two, or three of a maleimide moiety, a polyethylene glycol (PEG) moiety, and a dibenzocyclooctyne (DBCO) moiety.
17. The antigenic influenza-ferritin polypeptide of any one of the preceding claims, further comprising a peptide linker between the ferritin protein and the influenza polypeptide.
18. A ferritin particle comprising the antigenic influenza-ferritin polypeptide of any one of the preceding claims.
19. A composition comprising the antigenic influenza-ferritin polypeptide or ferritin particle of any one of the preceding claims and a pharmaceutically acceptable carrier.
20. The composition of claim 19, which further comprises a second antigenic influenza-ferritin polypeptide comprising a ferritin protein and a different influenza polypeptide.
21. The composition of claim 20, wherein the influenza polypeptide is from influenza type A and the influenza polypeptide of the second antigenic influenza-ferritin polypeptide is from influenza type B, or wherein the influenza polypeptide and the influenza polypeptide of the second influenza-ferritin polypeptide are from subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, H17, or H18, or wherein one or both of the influenza polypeptides comprise engineered stabilized stem antigens from subtypes H1, H3, H7 or H10.
22. The antigenic influenza-ferritin polypeptide, ferritin particle, or composition of any one of the preceding claims for use in a method of eliciting an immune response to influenza or in protecting a subject against infection with influenza.
23. A method of eliciting an immune response to influenza or protecting a subject against infection with influenza comprising administering any one or more antigenic influenza-ferritin polypeptide, ferritin particle, or composition of any one of the preceding claims to a subject.

24. The antigenic influenza-ferritin polypeptide, ferritin particle, composition, or method of any one of the preceding claims, wherein the subject is human.
25. A nucleic acid encoding the antigenic influenza-ferritin polypeptide of any one of claims 1-17, optionally wherein the nucleic acid is an mRNA.

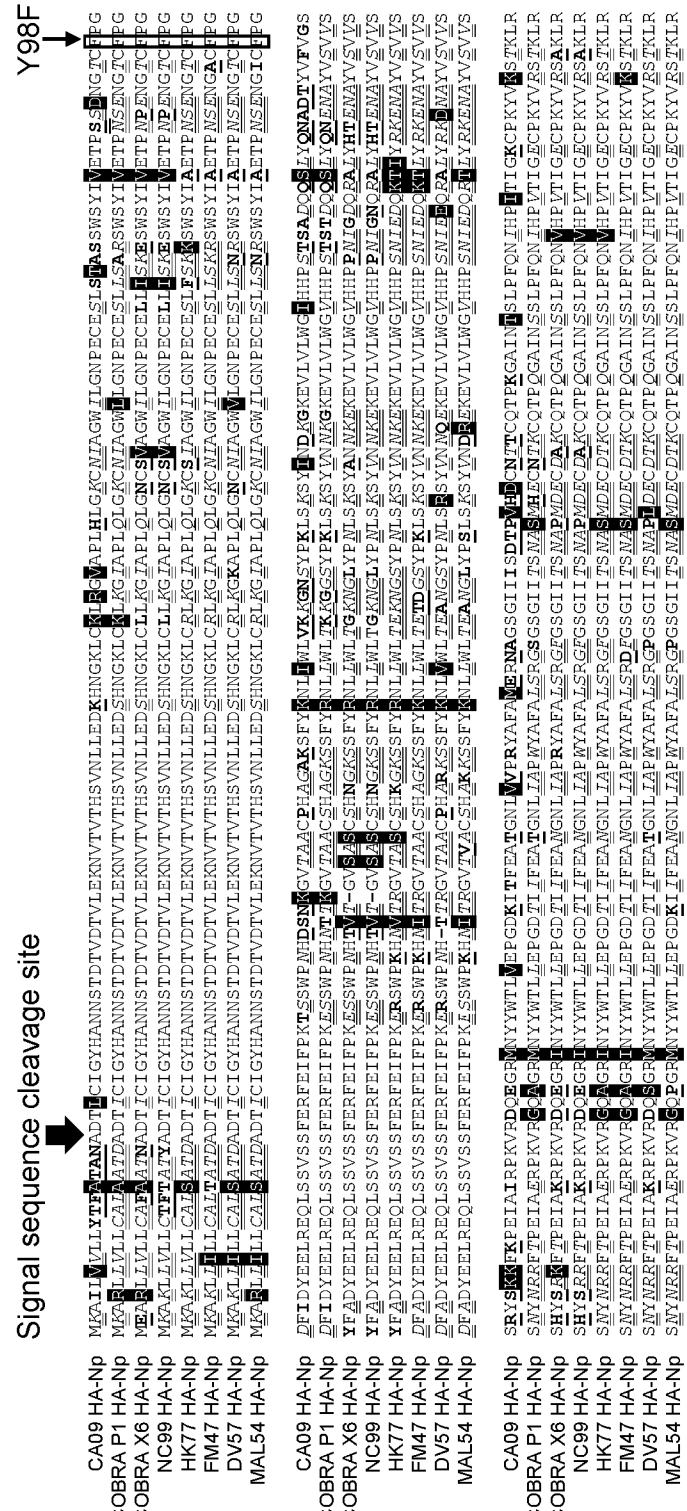

Signal sequence cleavage site
Y98F
CA09 HA-Np
COBRA P1 HA-Np
COBRA X6 HA-Np
NC99 HA-Np
HK77 HA-Np
FM47 HA-Np
DV57 HA-Np
MAL54 HA-Np

Fig. 1A

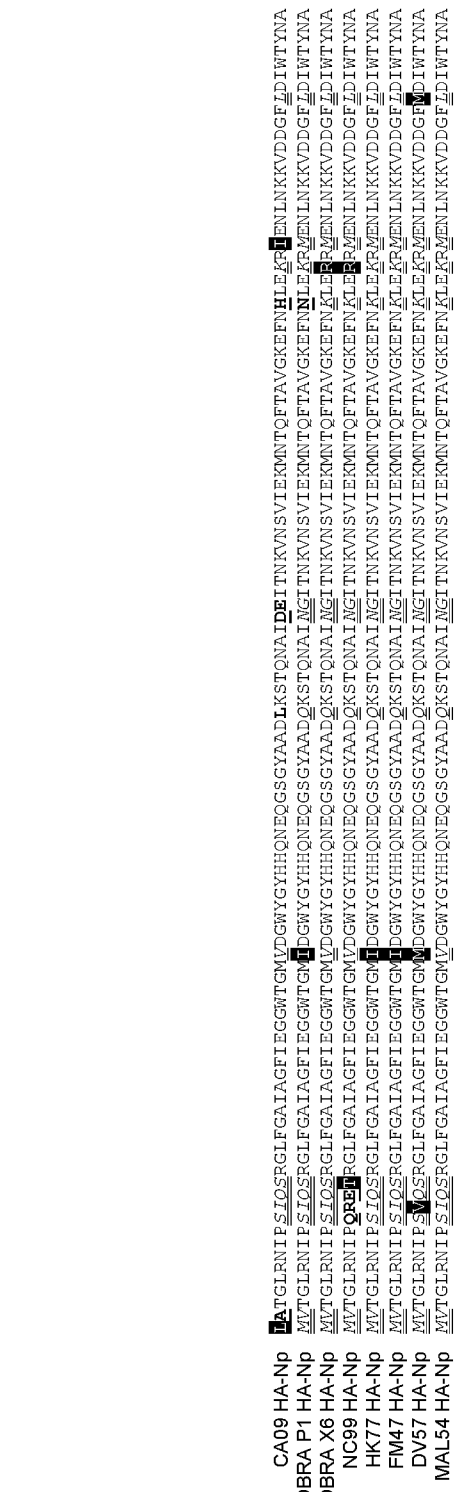

CA09 HA-Np
COBRA P1 HA-Np
COBRA X6 HA-Np
NC99 HA-Np
HK77 HA-Np
FM47 HA-Np
DV57 HA-Np
MAL54 HA-Np

Fig. 1A (cont.)

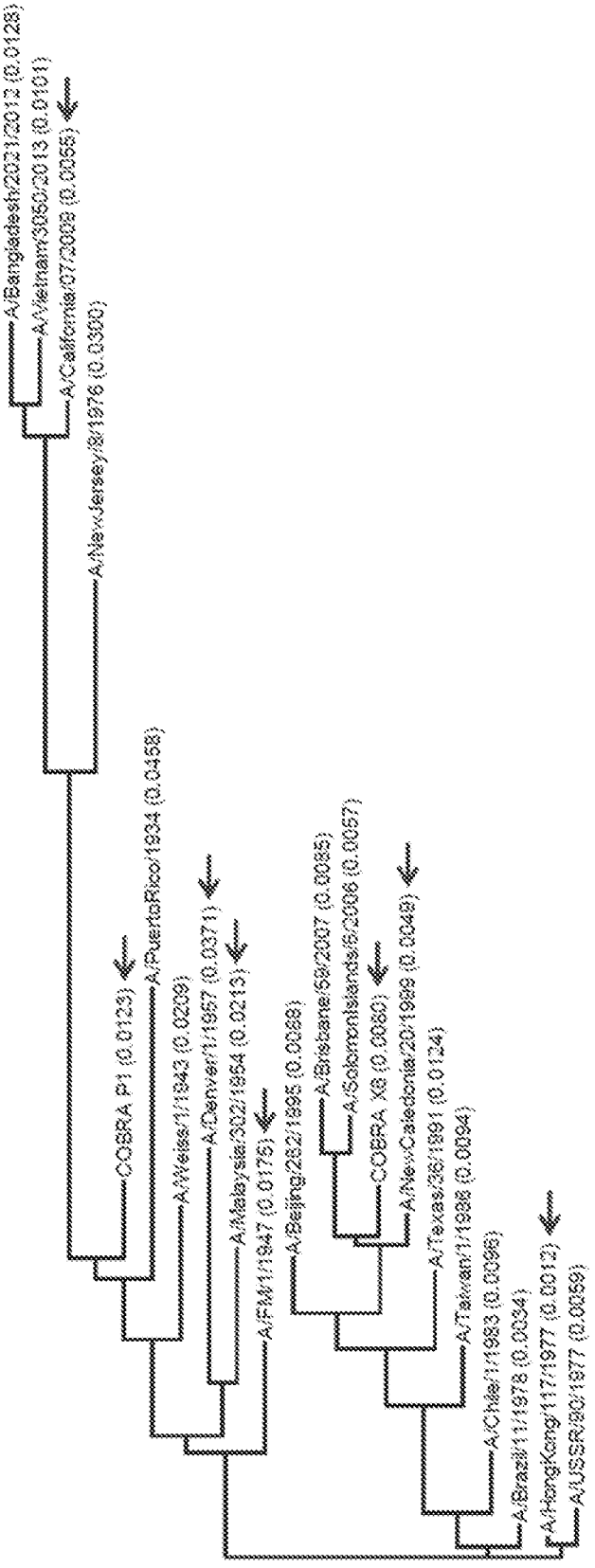

A/Bangladesh/2221/2012 (0.0128)
A/Vietnam/3055/2013 (0.0101)
A/California/07/2009 (0.0055)
A/NewJersey/8/1976 (0.0300)
COBRA P1 (0.0123)
A/PuertoRico/1934 (0.0458)
A/Weiss/1/1943 (0.0209)
A/Denver/1/1957 (0.0371)
A/Malaysia/302/1954 (0.0215)
A/FM/1/1947 (0.0176)
A/Beijing/262/1995 (0.0088)
A/Brisbane/59/2007 (0.0085)
A/Solomonislands/6/2006 (0.0057)
COBRA X8 (0.0080)
A/NewCaledonia/20/1999 (0.0049)
A/Texas/36/1991 (0.0124)
A/Taiwan/1/1986 (0.0094)
A/Chile/1/1983 (0.0096)
A/Brazil/11/1978 (0.0034)
A/HongKong/117/1977 (0.0012)
A/USSR/90/1977 (0.0059)

Fig. 1B

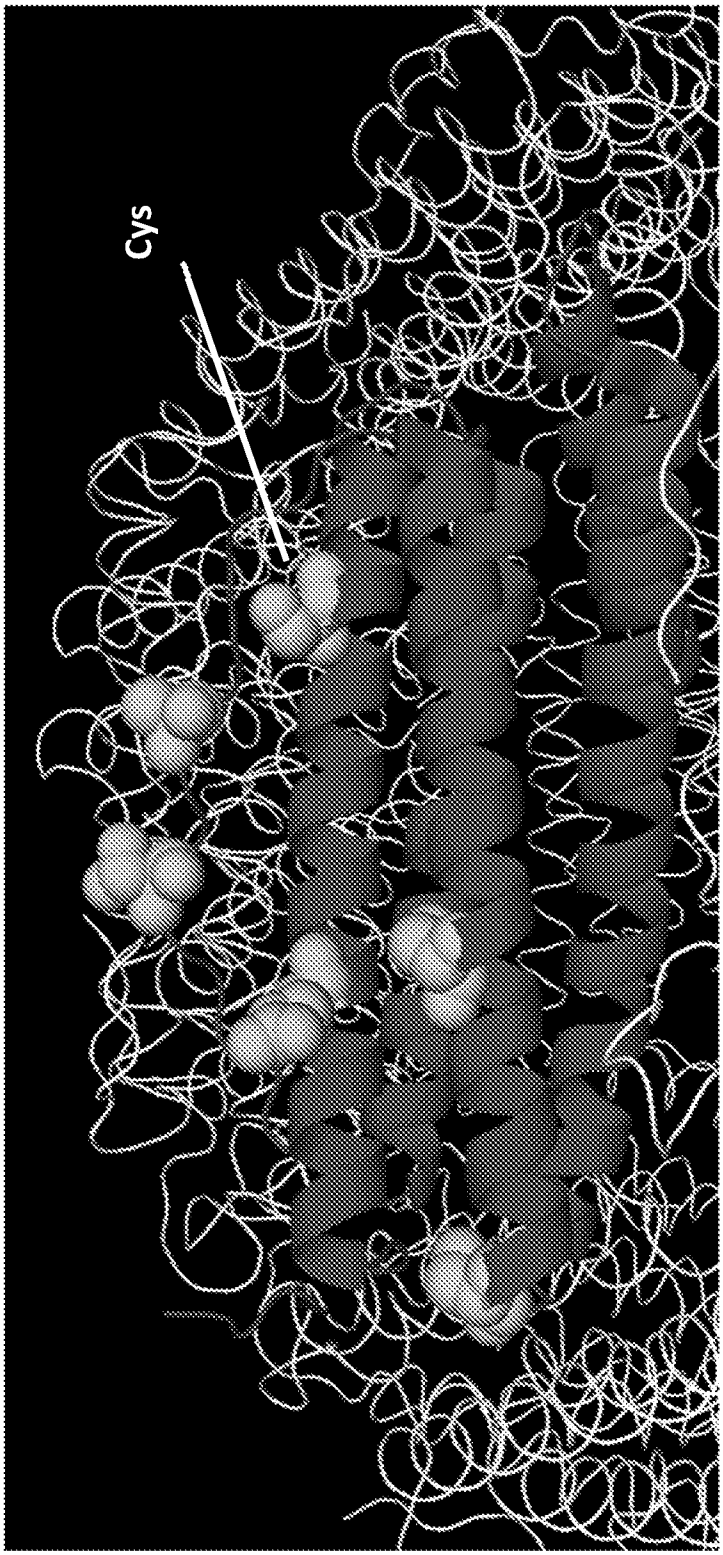


Fig. 2

1-step click chemistry: SM7/8a-PEG4-Maleimide is site-specifically conjugated to the unpaired cysteine (Cys 111) after reduction of H1/Stem-Np (N19Q, C31S, S111C).

Fig. 3A

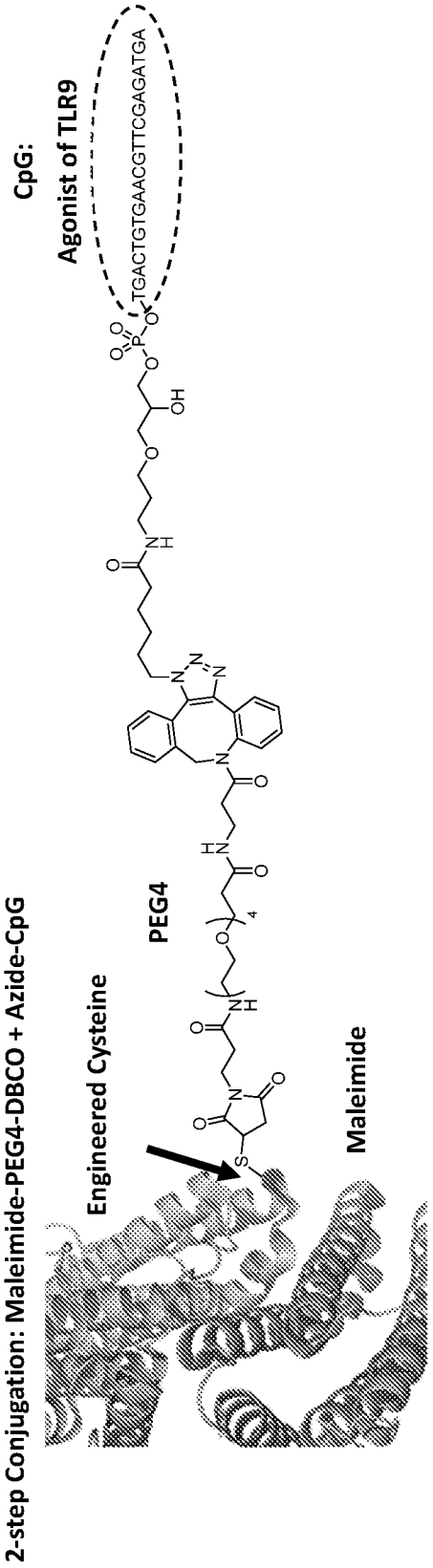

2-step Conjugation: Maleimide-PEG4-DBCO + Azide-CpG
Engineered Cysteine
PEG4
Maleimide
CpG:
Agonist of TLR9
TGACTGTGAACGTTCGAGATGA

Fig. 3B

2-step click chemistry: following conjugation of Maleimide-PEG4-DBCO linker to Cys 111, a 3M012-Azide is added to the linker on H1/Stem-Np(N19Q,C31S,S111C)

Fig. 4A

2-step click chemistry: following conjugation of Maleimide-PEG4-DBCO linker to Cys 111, a CpG-Azide is added to the linker on H1/Stem-Np(N19Q,C31S,S111C)
CpG:
Agonist of Toll-like Receptor 9
(TLR9)
Model of HA-Ferr-CpG
Not to scale
1 out of 24
conjugation sites is
shown

Fig. 4B

Trypsin cut site on bfpFerritin nanoparticles

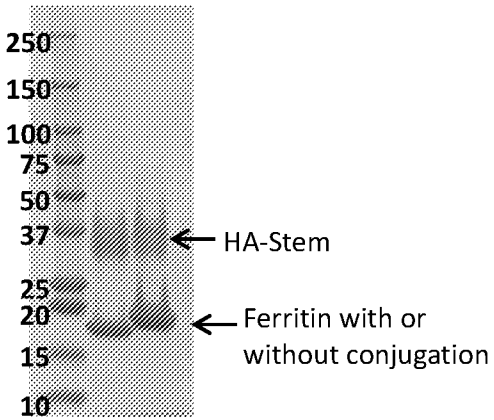
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXX(**NON-FERRITIN-POLYPEPTIDE SEQUENCE**)XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
SGGESQVRQQFSKDIEKLLNEQVNKEMQSSNLYMSMSSWSYTHSLDGAGLFLFDHAAEEYEHAKKLIIFLNENNV
PVQLTSISAPEHKFEGLTQIFQKAYEHEQHISESINNIVDHAIK**C**KDHATFNFLQWYVAEQHEEEVLFKDILDKIELIG
NENHGLYLADQYVKGIAKSRKS

Conjugation site: S111C

Fig. 5

Gel-Shift: **H1/Stem-Np**

SM7/8a conjugation: - +



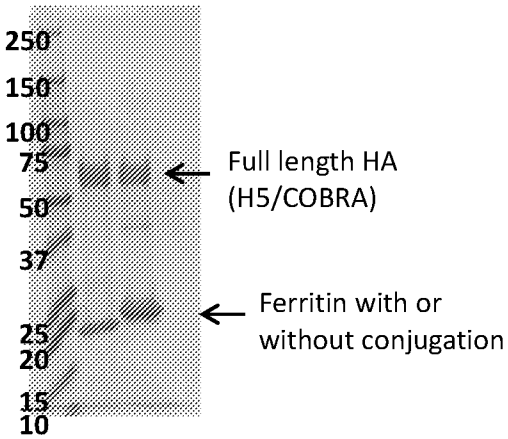
Mass Spec:

H1/Stem-Np:	before reduction:	19,629 Da
	after reduction:	19,511 Da
+SM7/8a-PEG4 conjugation:		20,223 Da
	Δmass:	712 Da
	(Expected):	711 Da

Fig. 6A

Gel-Shift: **H5/COBRA-Np**

SM7/8a conjugation: - +



Mass Spec:

H5/COBRA-Np:	before reduction:	19,631 Da
	after reduction:	19,511 Da
+SM7/8a-PEG4 conjugation:		20,222 Da
	Δmass:	711 Da
	(Expected):	711 Da

Fig. 6B

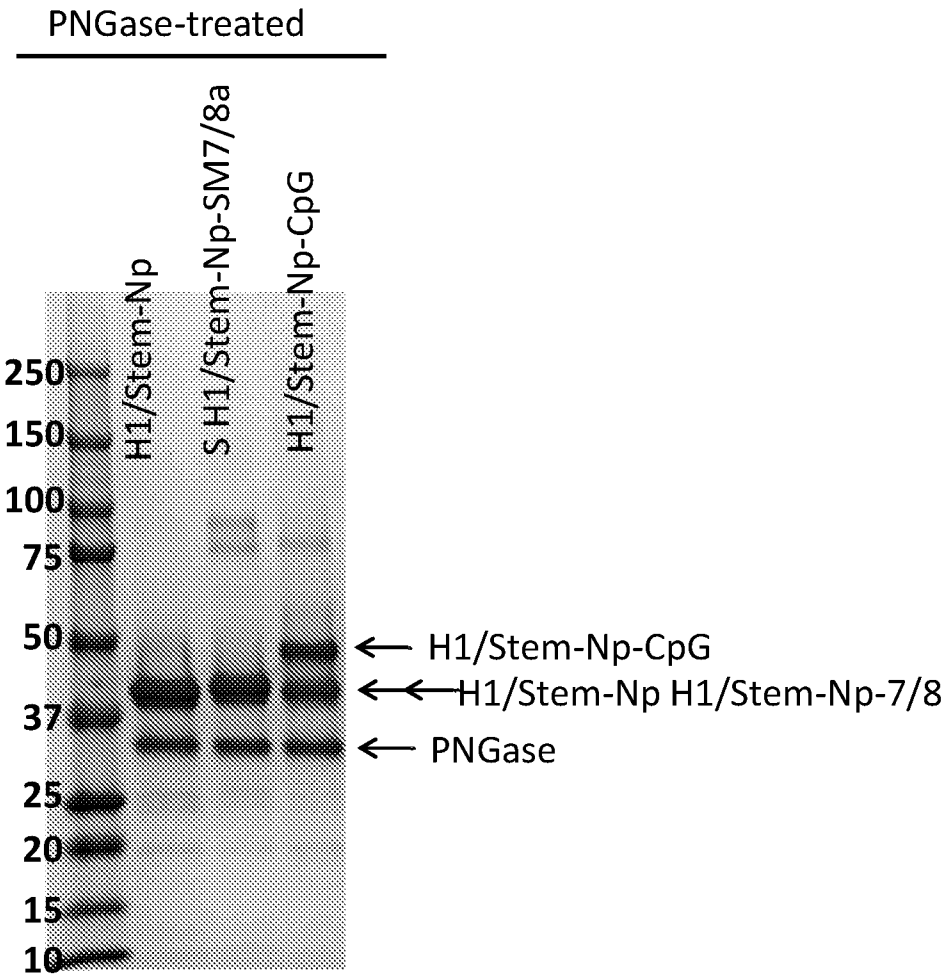

PNGase-treated
H1/Stem-Np
S H1/Stem-Np-SM7/8a
H1/Stem-Np-CpG
250
150
100
75
50
37
25
20
15
10
H1/Stem-Np-CpG
H1/Stem-Np H1/Stem-Np-7/8
PNGase

Fig. 7A

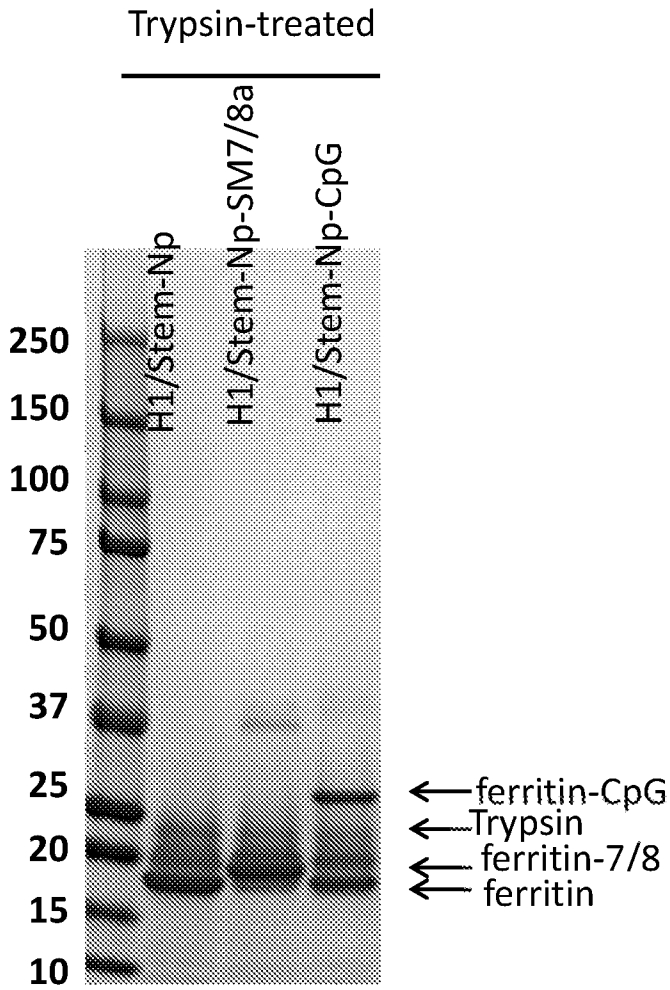
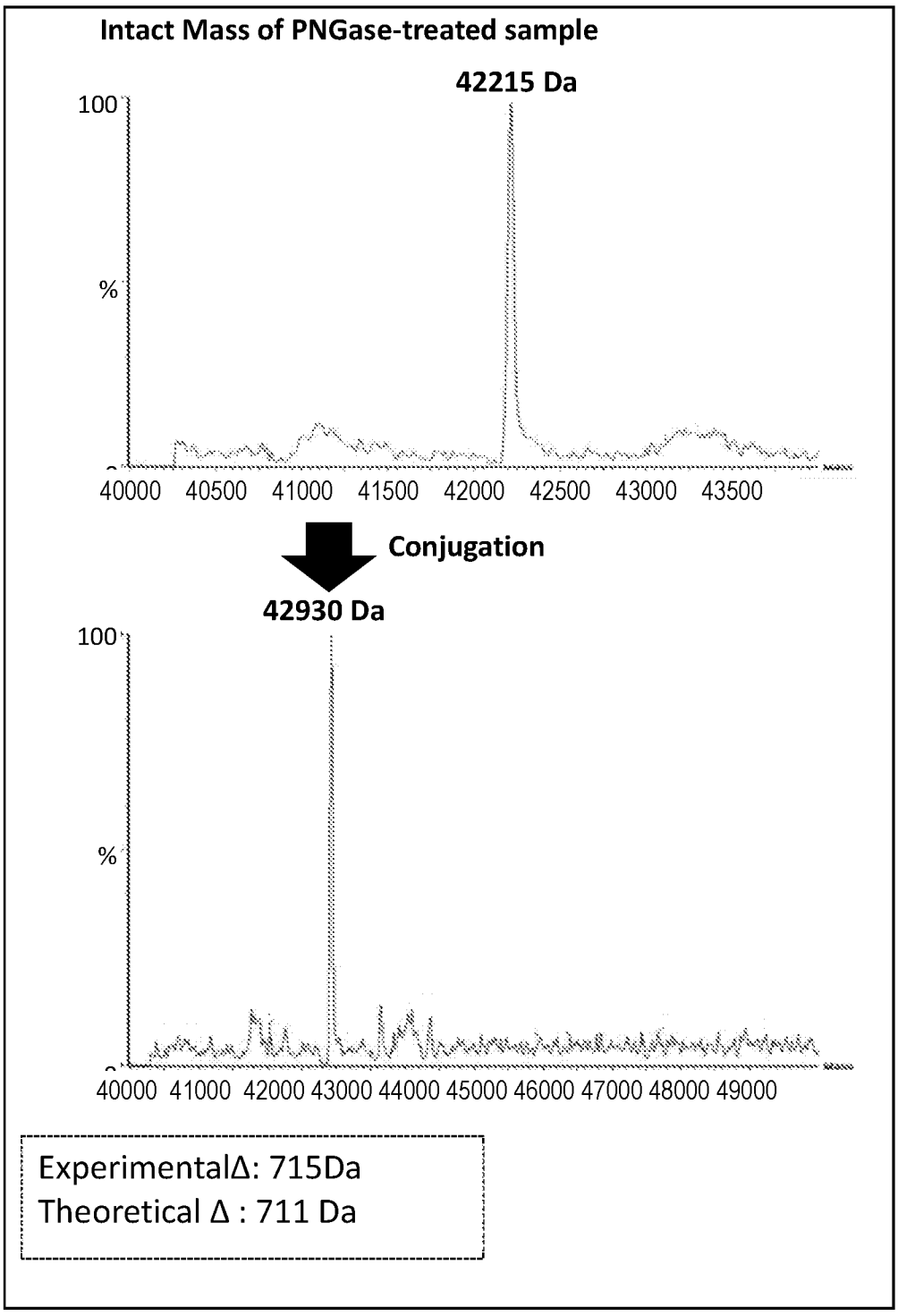
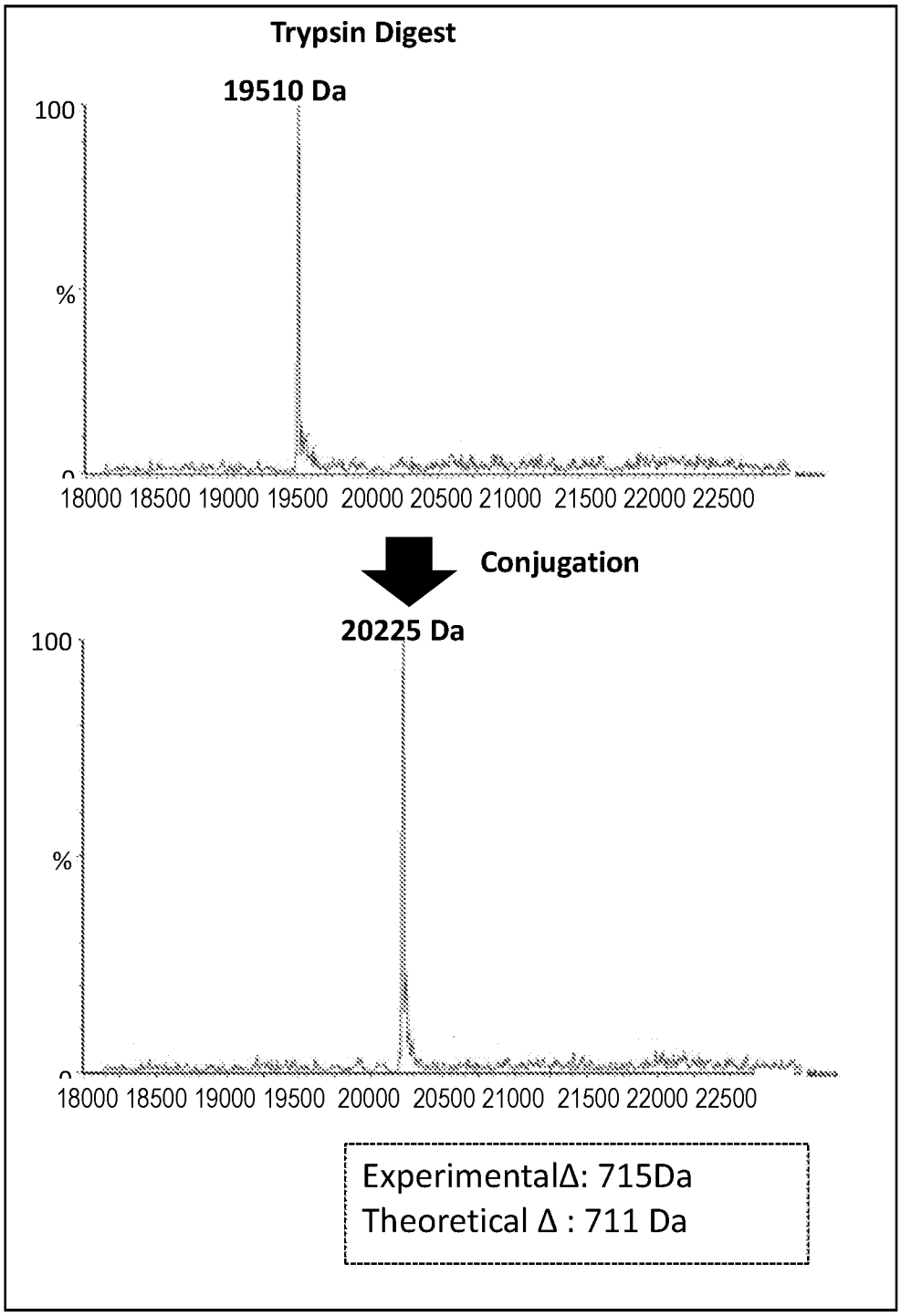

Trypsin-treated
H1/Stem-Np
H1/Stem-Np-SM7/8a
H1/Stem-Np-CpG
250
150
100
75
50
37
25
20
15
10
ferritin-CpG
Trypsin
ferritin-7/8
ferritin

Fig. 7B



Conjugation efficiency = 100%

Fig. 8A



Conjugation efficiency = 100%

Fig. 8B

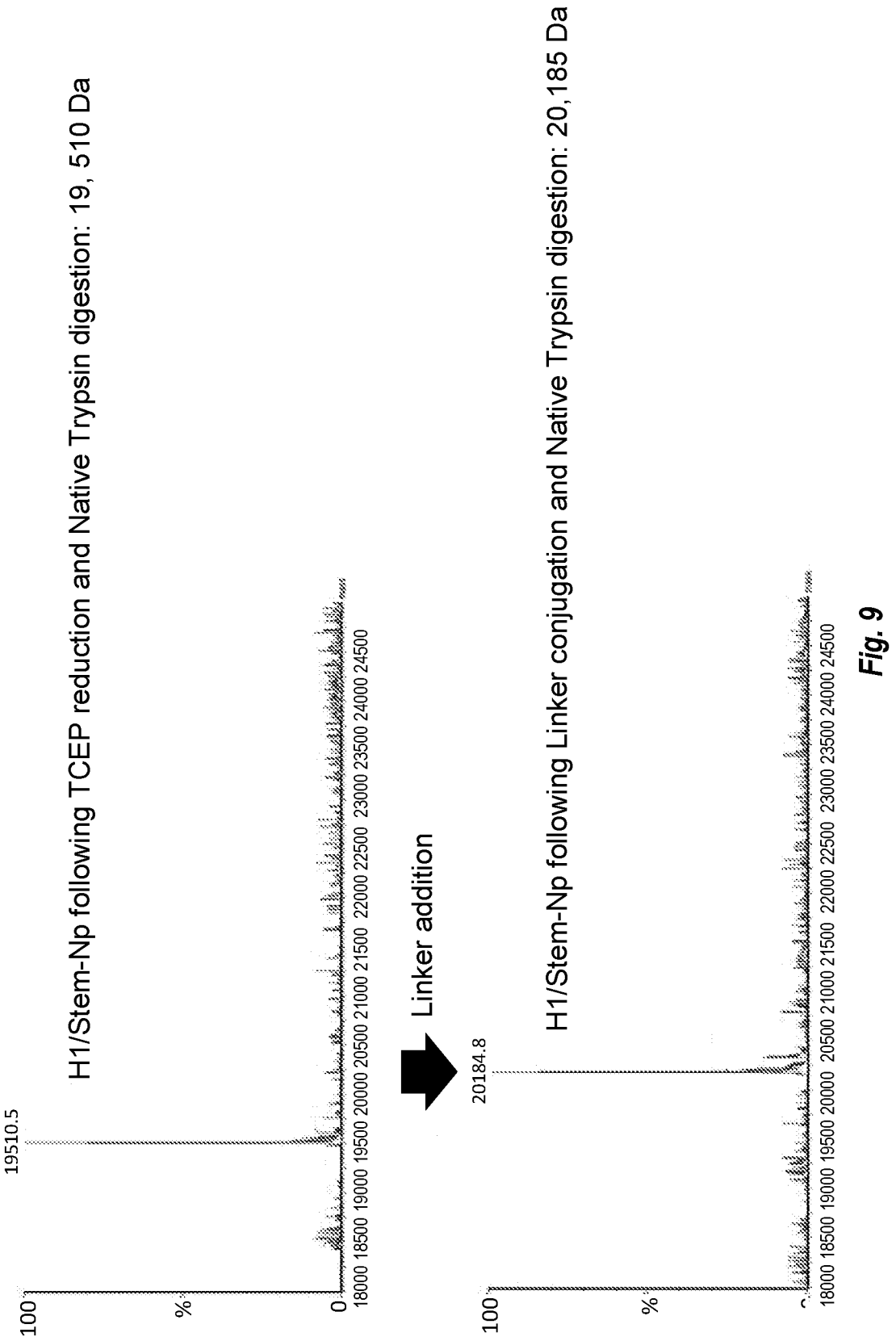

H1/Stem-Np following TCEP reduction and Native Trypsin digestion: 19, 510 Da
19510.5
100
%
0
18000 18500 19000 19500 20000 20500 21000 21500 22000 22500 23000 23500 24000 24500
Linker addition
H1/Stem-Np following Linker conjugation and Native Trypsin digestion: 20,185 Da
20184.8
100
%
0
18000 18500 19000 19500 20000 20500 21000 21500 22000 22500 23000 23500 24000 24500

Fig. 9

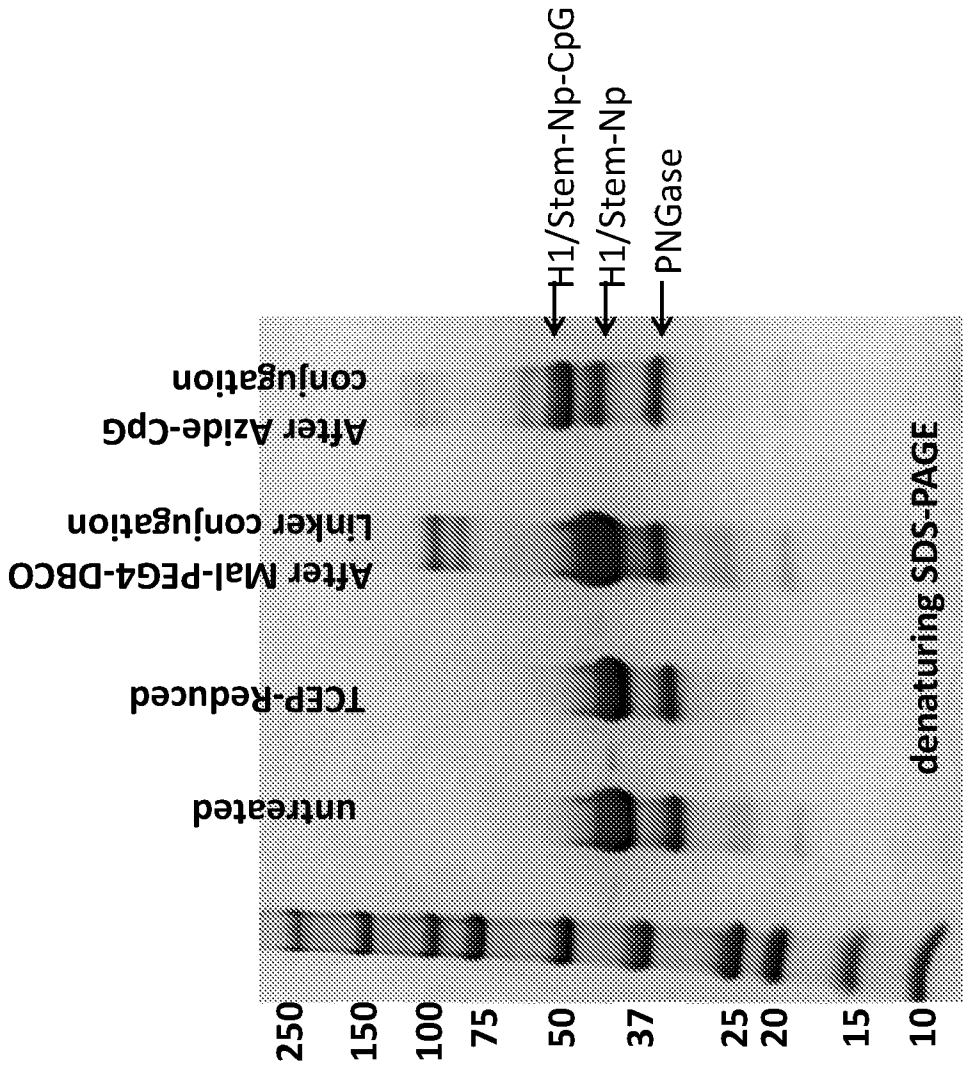

H1/Stem-Np-CpG
H1/Stem-Np
PNGase
After Azide-CpG conjugation
After Mal-PEG4-DBCO Linker conjugation
TCEP-Reduced
untreated
denaturing SDS-PAGE
250
150
100
75
50
37
25
20
15
10

Fig. 10

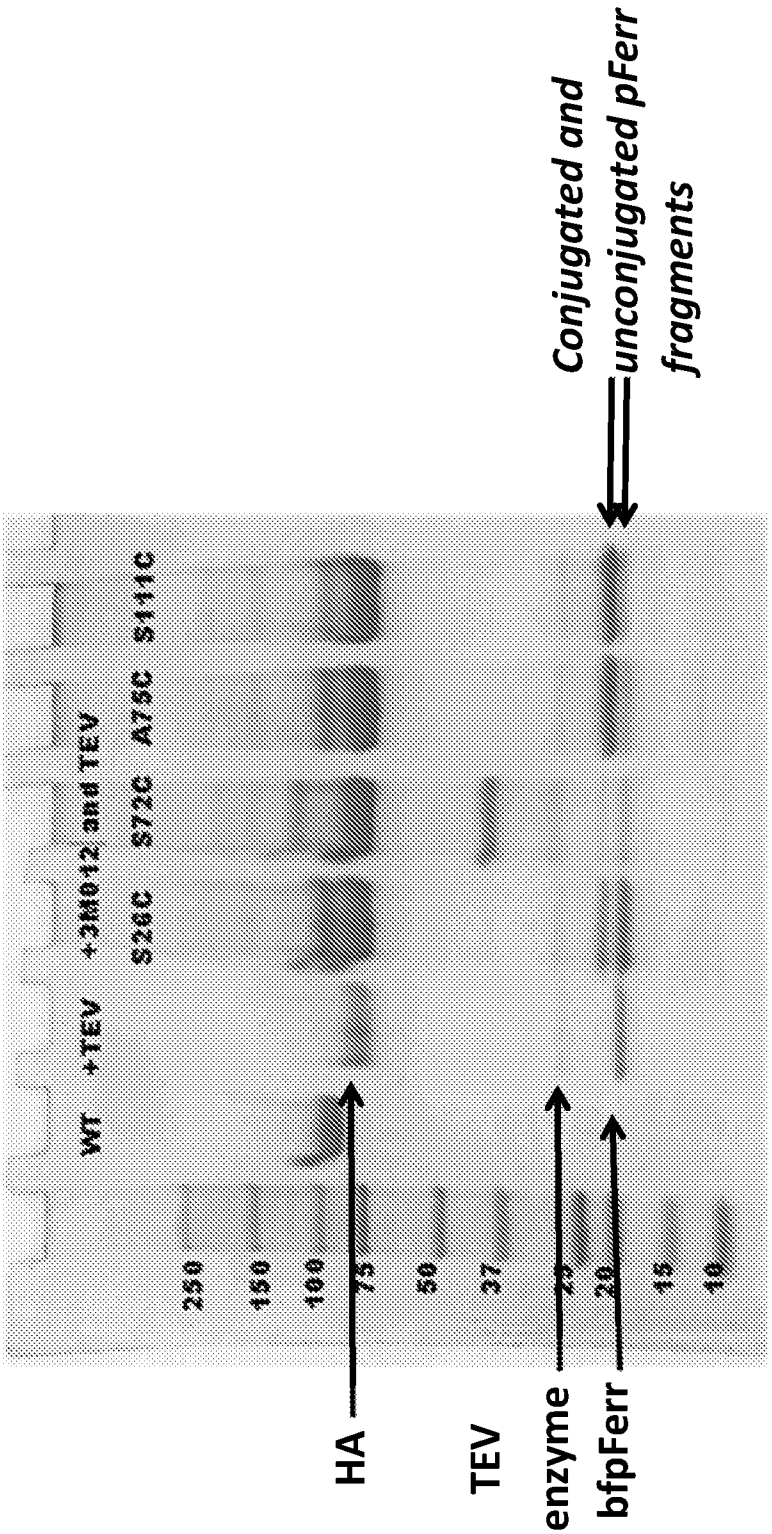

WT +TEV +3M012 and TEV
S26C S72C A75C S111C
250
150
100
75
50
37
25
20
15
10
HA
TEV
enzyme
bfpFerr
Conjugated and
unconjugated pFerr
fragments

Fig. 11

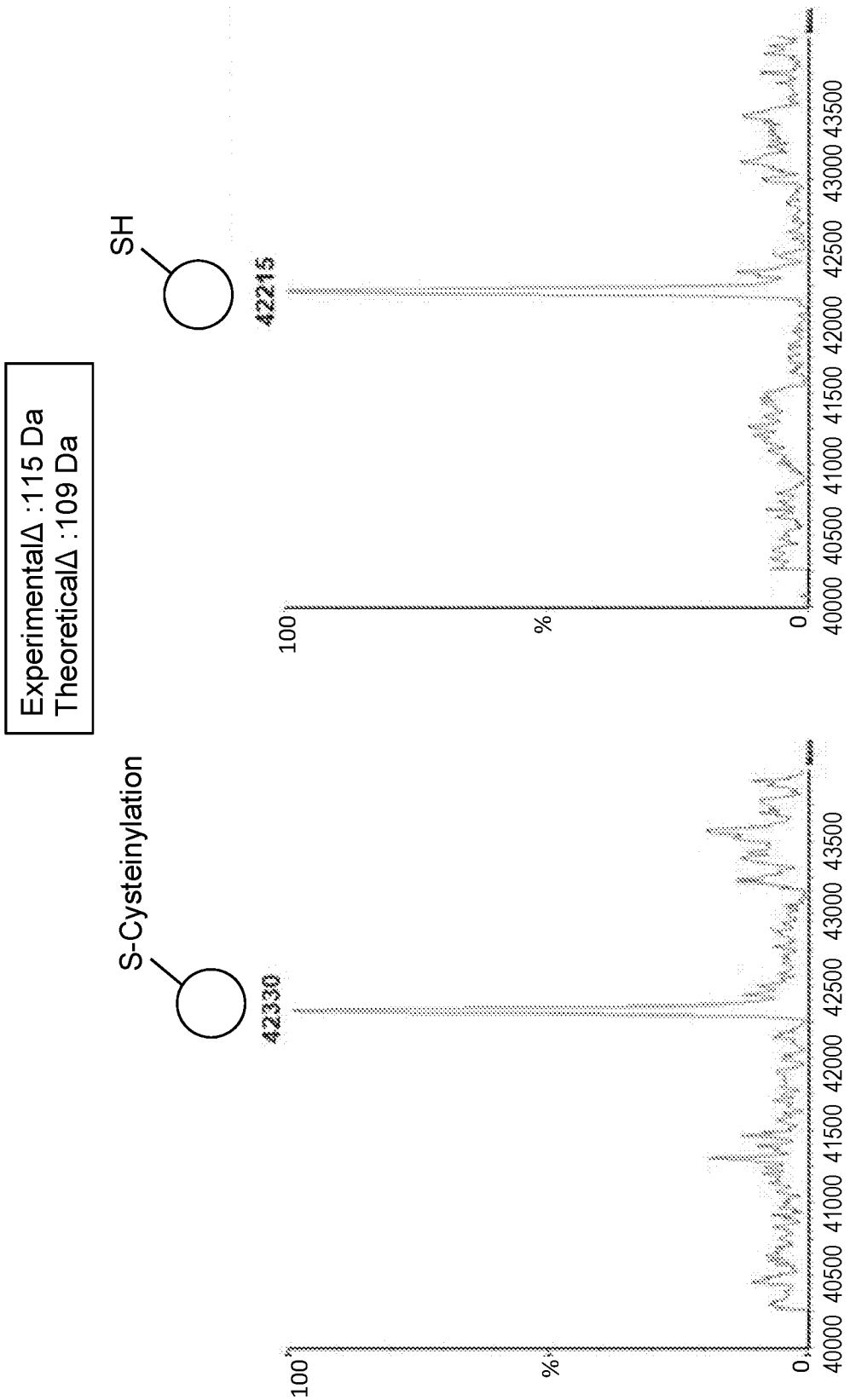


Fig. 12A

Fig. 12B

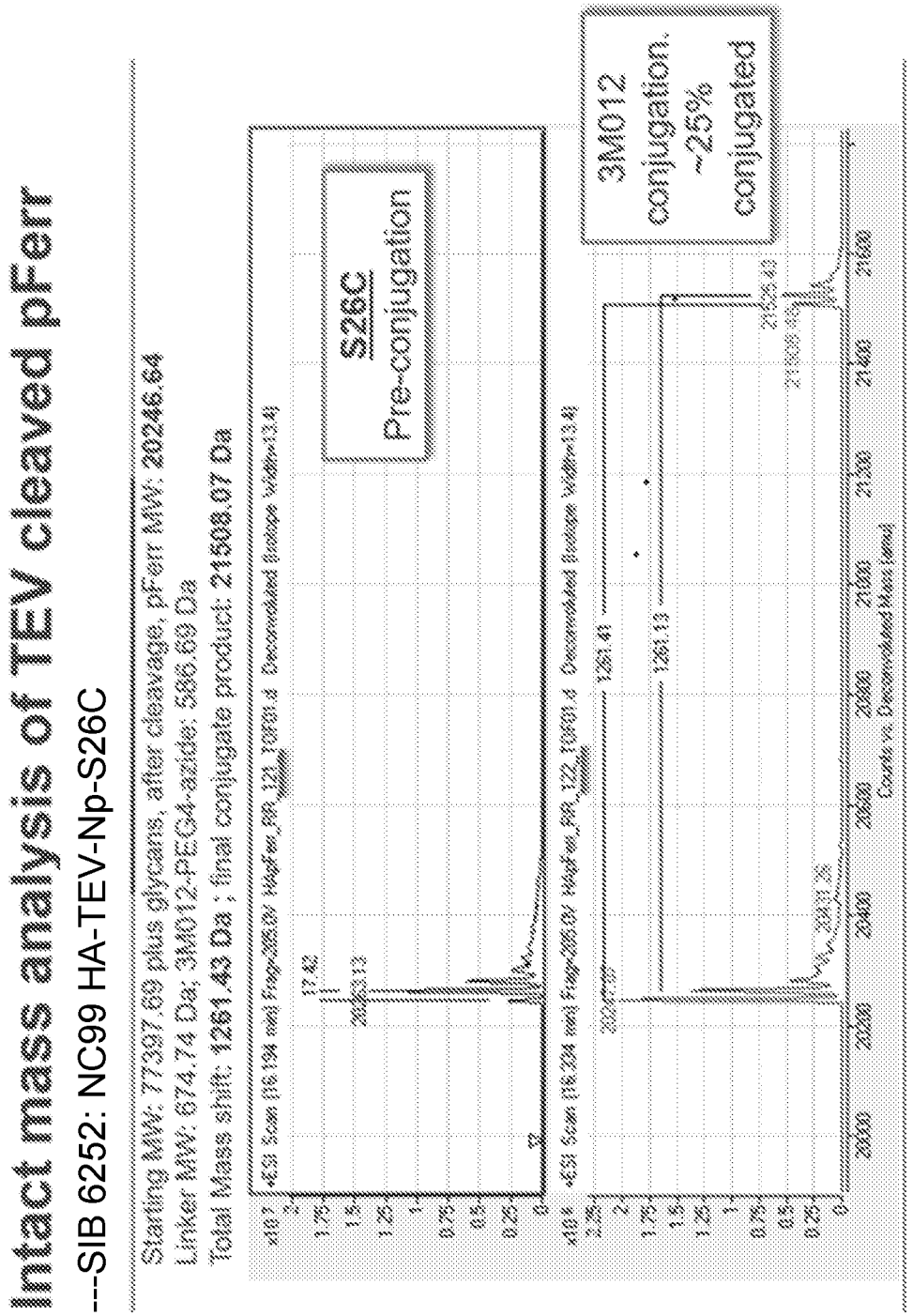

Intact mass analysis of TEV cleaved pFerr
---SIB 6252: NC99 HA-TEV-Np-S26C
Starting MW: 77397.69 plus glycans, after cleavage, pFerr MW: 20246.64
Linker MW: 674.74 Da; 3M012-PEG4-azide: 586.69 Da
Total Mass shift: 1261.43 Da ; final conjugate product: 21508.07 Da
S26C
Pre-conjugation
3M012
conjugation.
~26%
conjugated
Deconvoluted Mass [Da]

Fig. 12C

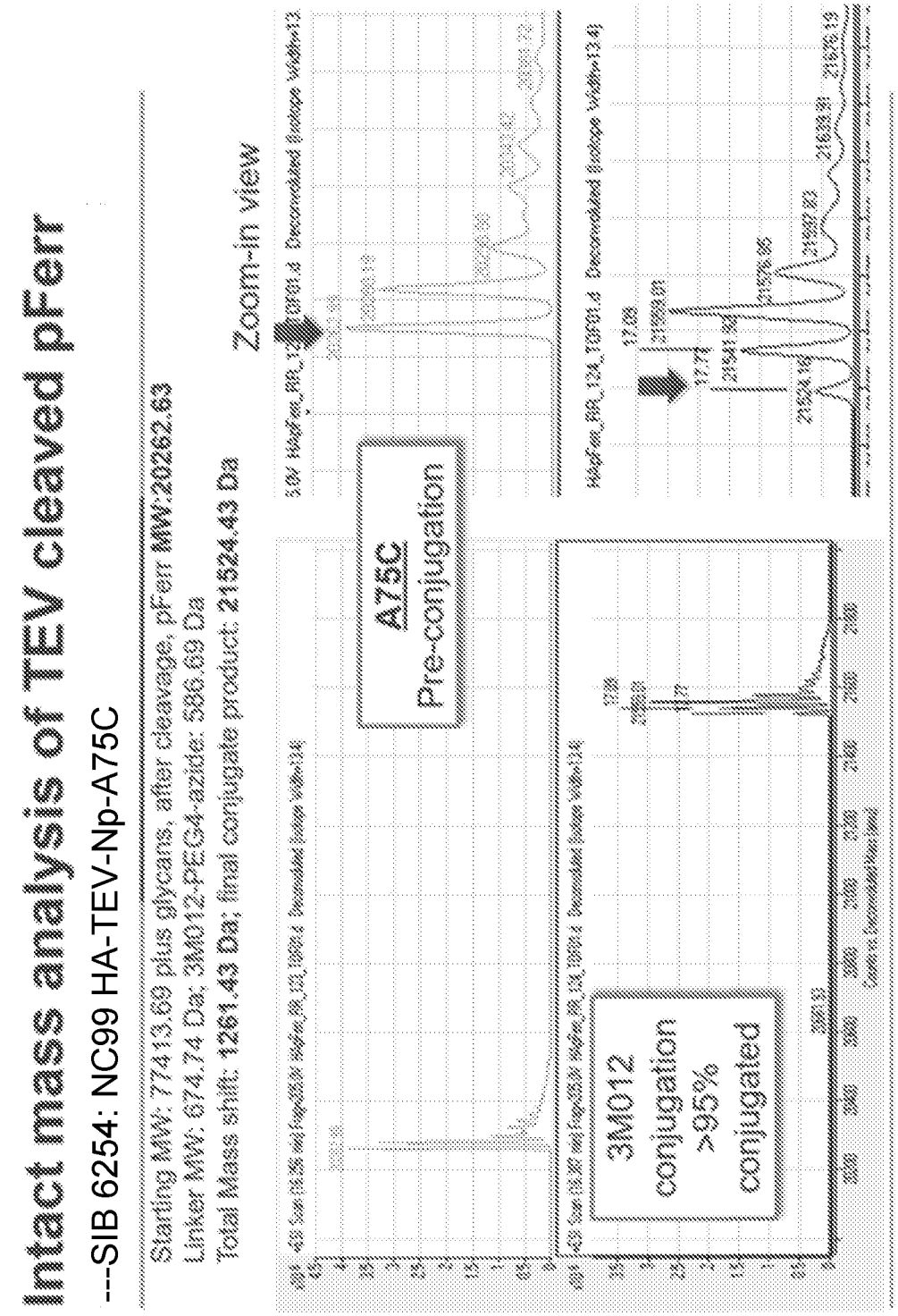

Intact mass analysis of TEV cleaved pFerr
---SIB 6254: NC99 HA-TEV-Np-A75C
Starting MW: 77413.69 plus glycans, after cleavage, pFerr MW:20262.63
Linker MW: 674.74 Da; 3M012-PEG4-azide: 586.69 Da
Total Mass shift: 1261.43 Da; final conjugate product: 21524.43 Da
Zoom-in view
A75C
Pre-conjugation
3M012
conjugation
>95%
conjugated

Fig. 12D

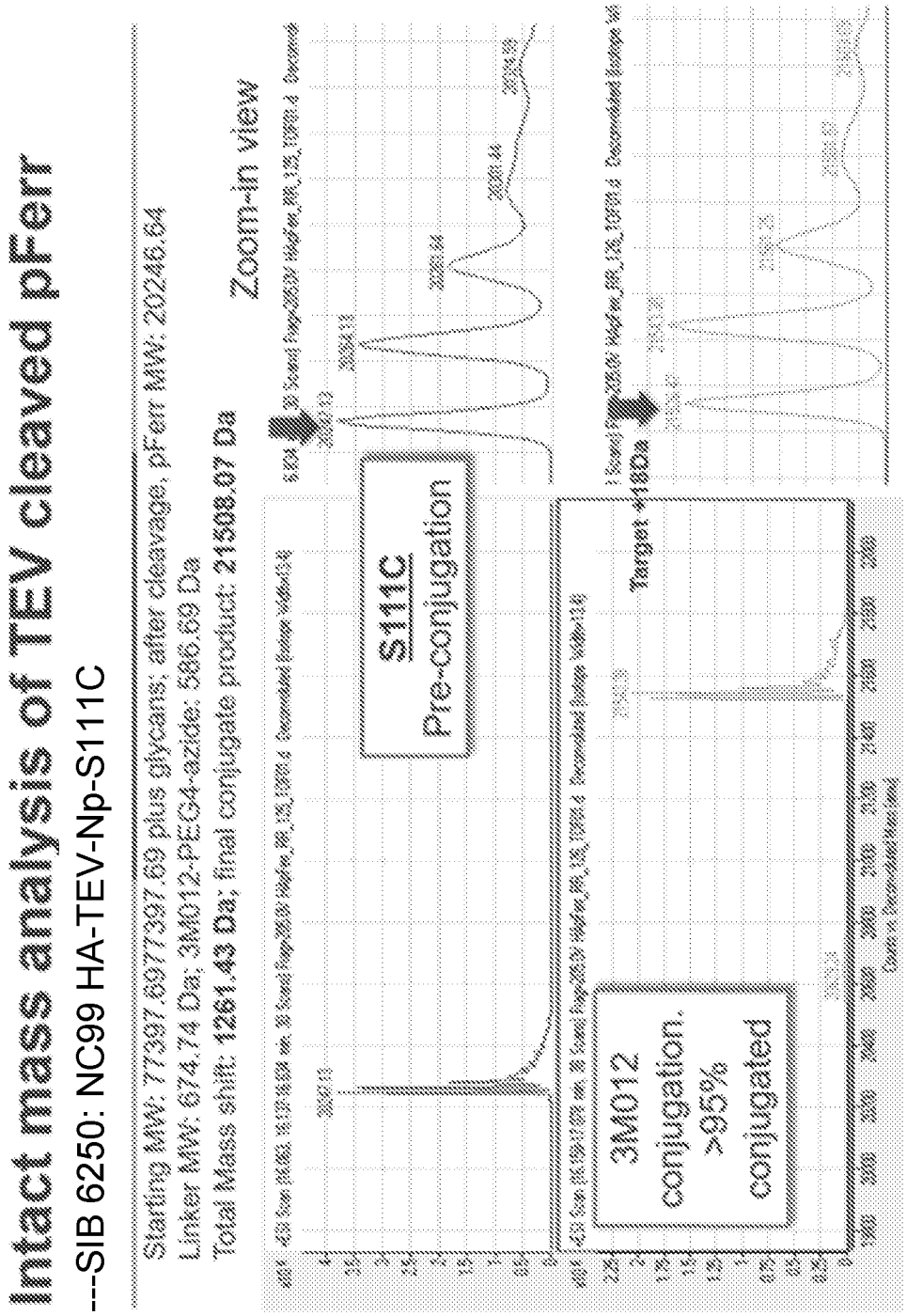

Intact mass analysis of TEV cleaved pFerr
---SIB 6250: NC99 HA-TEV-Np-S111C
Starting MW: 77397.6977397.69 plus glycans; after cleavage, pFerr MW: 20246.64
Linker MW: 674.74 Da; 3M012-PEG4-azide: 586.69 Da
Total Mass shift: 1261.43 Da; final conjugate product: 21508.07 Da
Zoom-in view
S111C
Pre-conjugation
3M012
conjugation.
>95%
conjugated
Target +1kDa

Fig. 12E

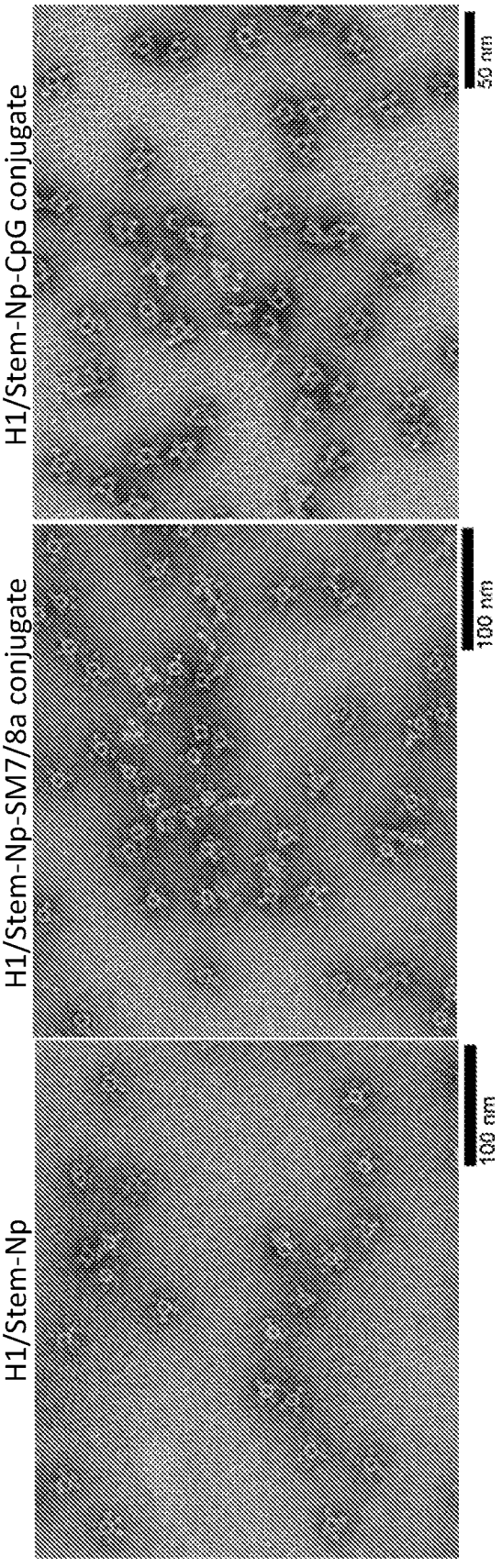


Fig. 13A

Fig. 13B

Fig. 13C

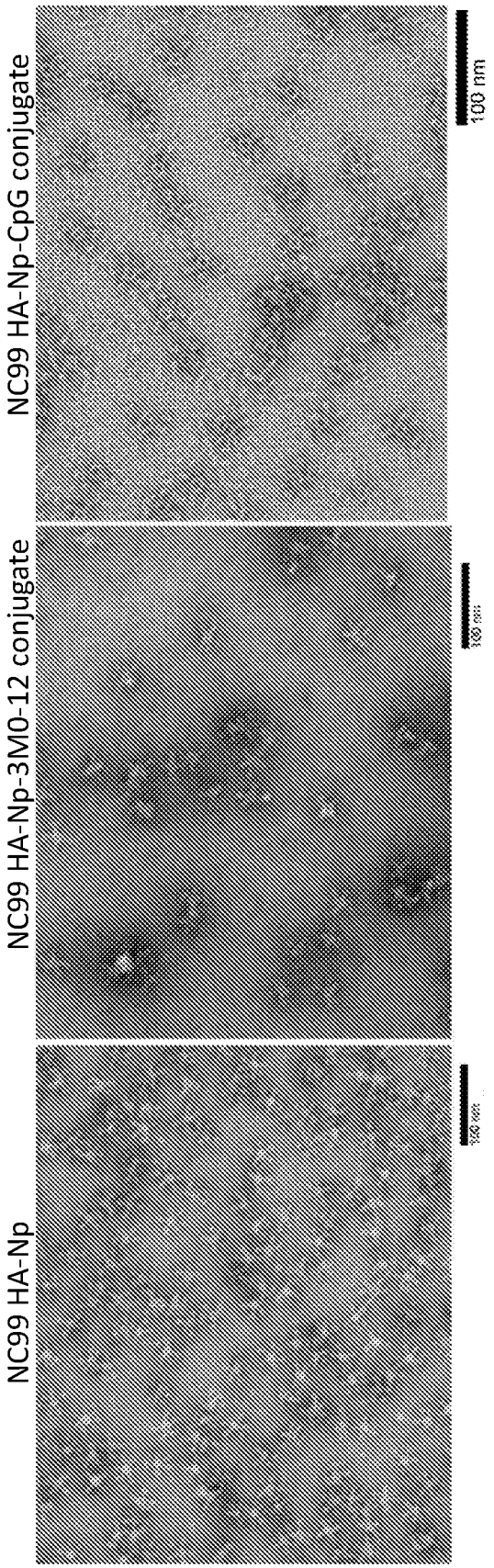


Fig. 13D

Fig. 13E

Fig. 13F

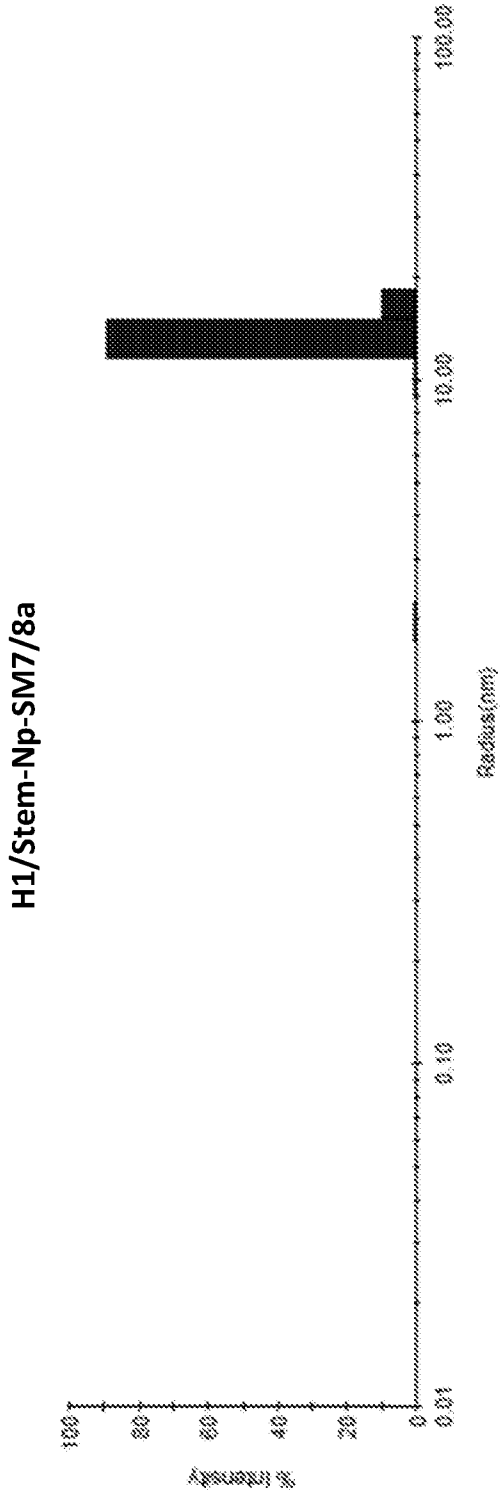

H1/Stem-Np-SM7/8a
% Intensity
100
80
60
40
20
0
0.01
0.10
1.00
10.00
100.00
Radius(nm)

Fig. 14A

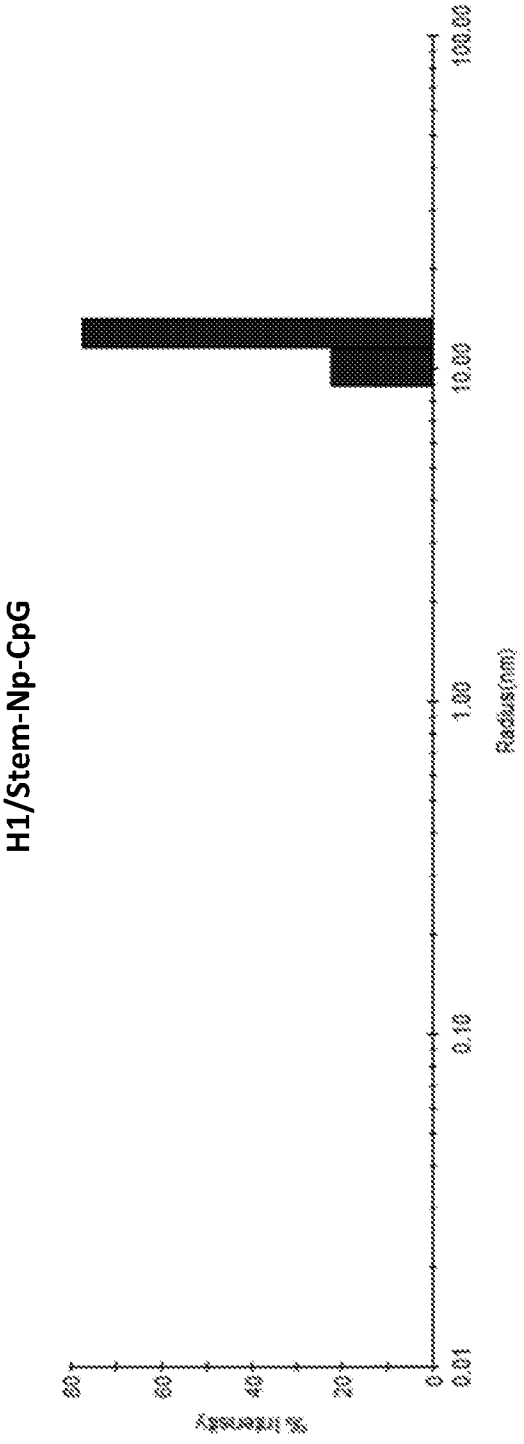

H1/Stem-Np-CpG
100.00
10.00
1.00
0.10
0.01
Radius(nm)
% Intensity
80
60
40
20
0

Fig. 14B

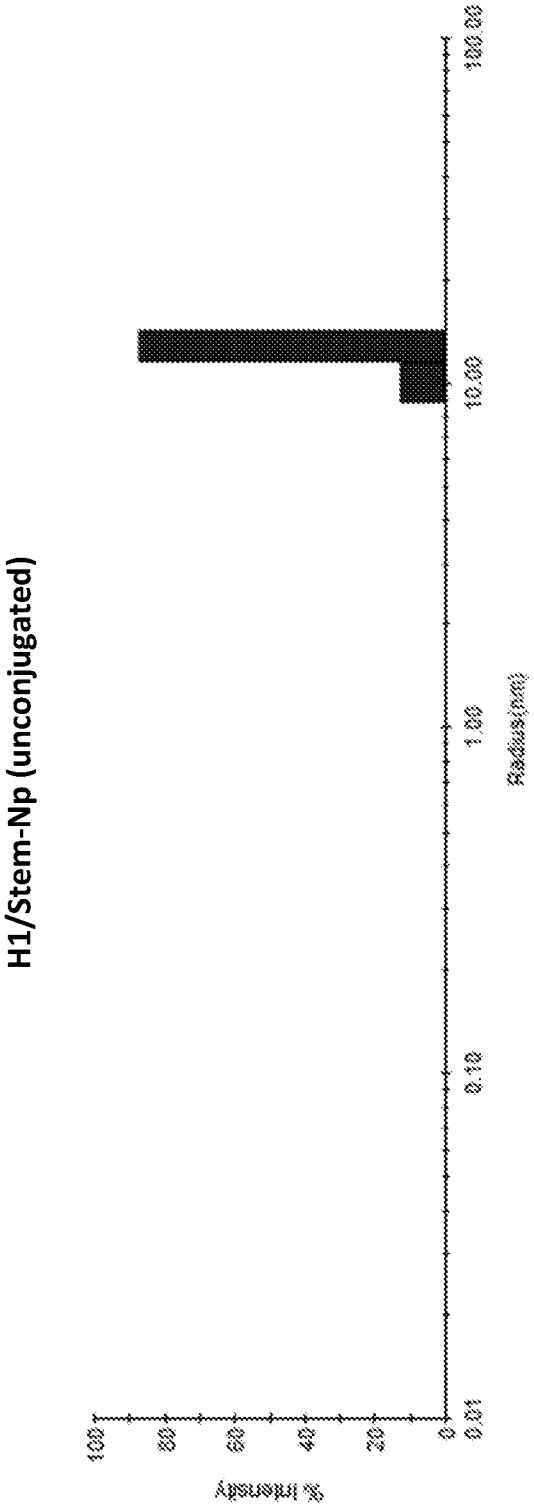
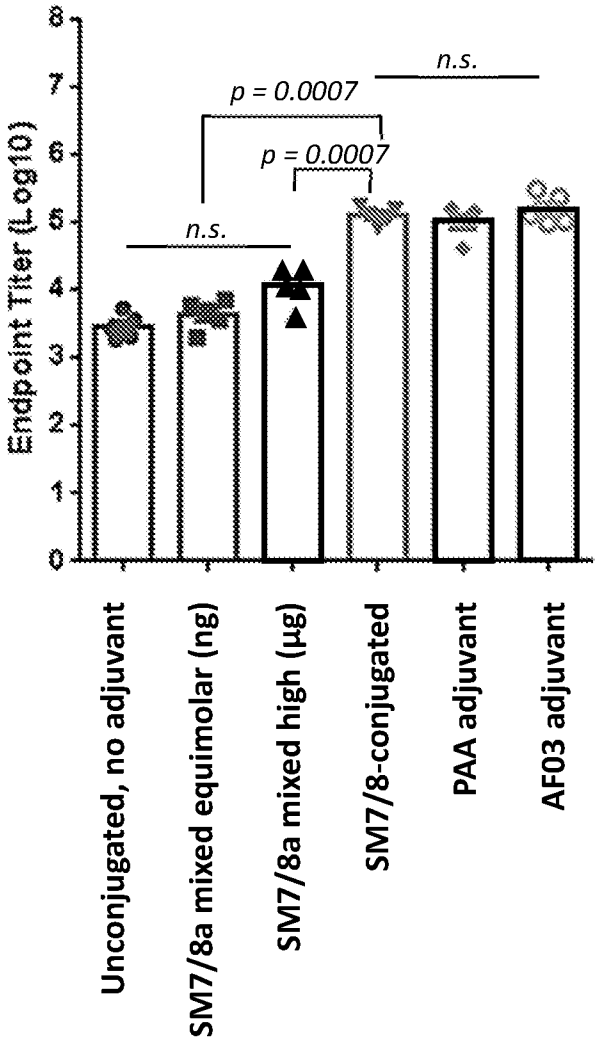

H1/Stem-Np (unconjugated)
% Intensity
100
80
60
40
20
0
0.01
0.10
1.00
10.00
100.00
Radius (nm)

Fig. 14C

ELISA: H1/New Caledonia/1999 HA trimers



H1/Stem-Np antigen admixed or conjugated

Fig. 15A

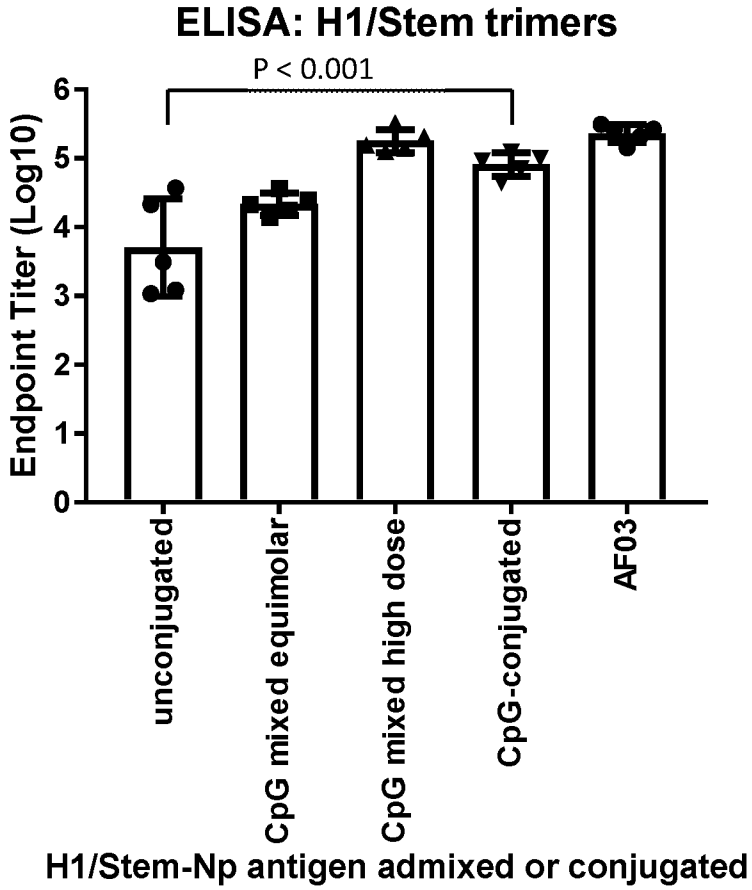

ELISA: H1/Stem trimers
P < 0.001
Endpoint Titer (Log10)
0
1
2
3
4
5
6
unconjugated
CpG mixed equimolar
CpG mixed high dose
CpG-conjugated
AF03
H1/Stem-Np antigen admixed or conjugated

Fig. 15B

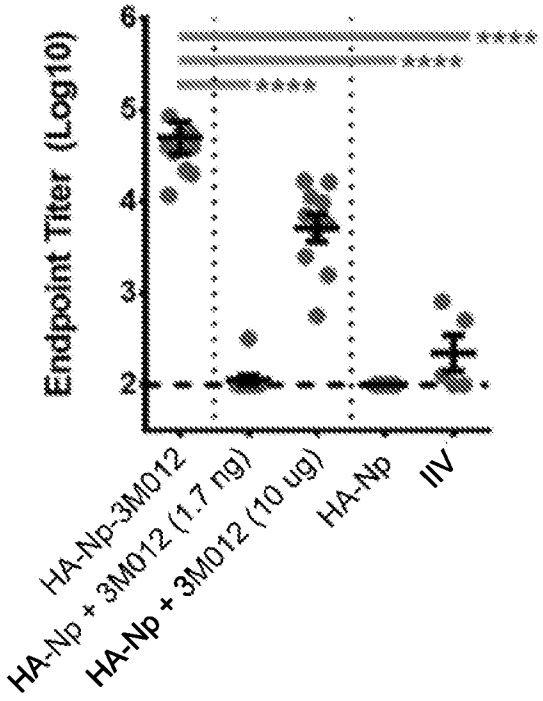


Fig. 16A

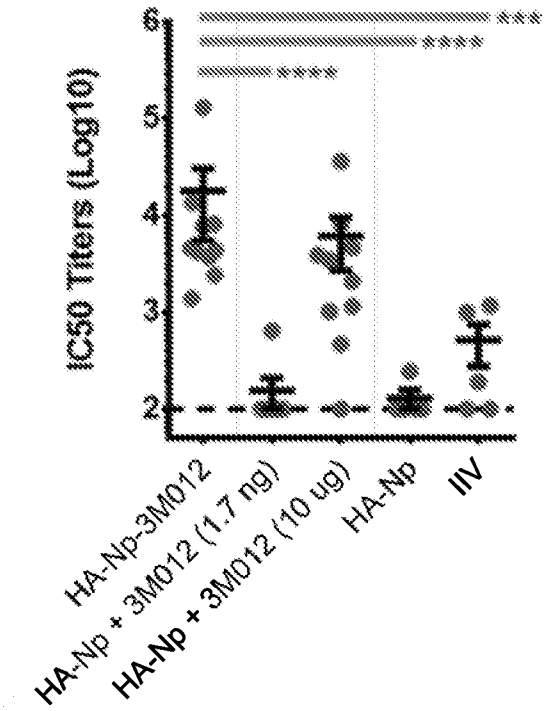


Fig. 16B

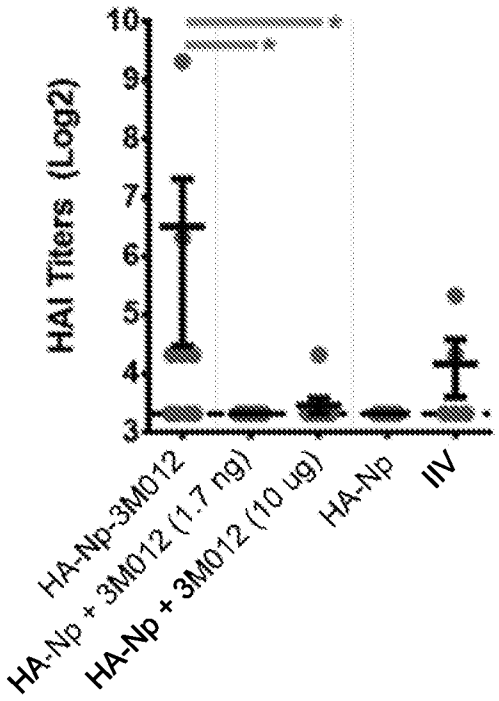

HAI Titers (Log2)
10
9
8
7
6
5
4
3
HA-Np-3M012
HA-Np + 3M012 (1.7 ng)
HA-Np + 3M012 (10 ug)
HA-Np
IIV

Fig. 16C

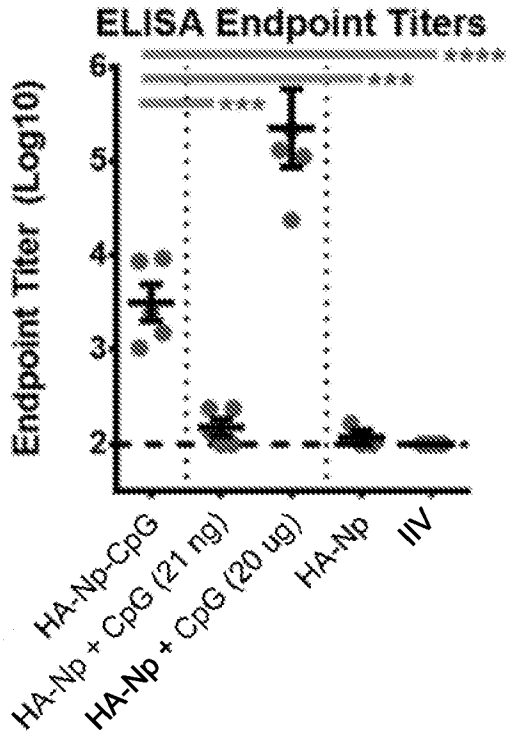


Fig. 17A

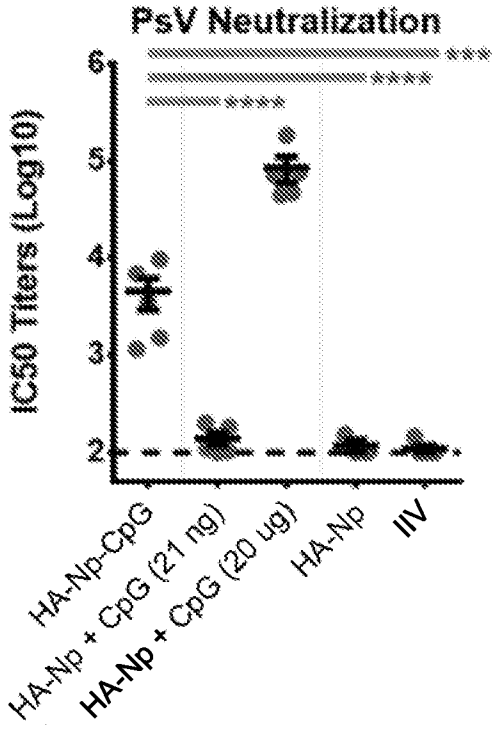


Fig. 17B

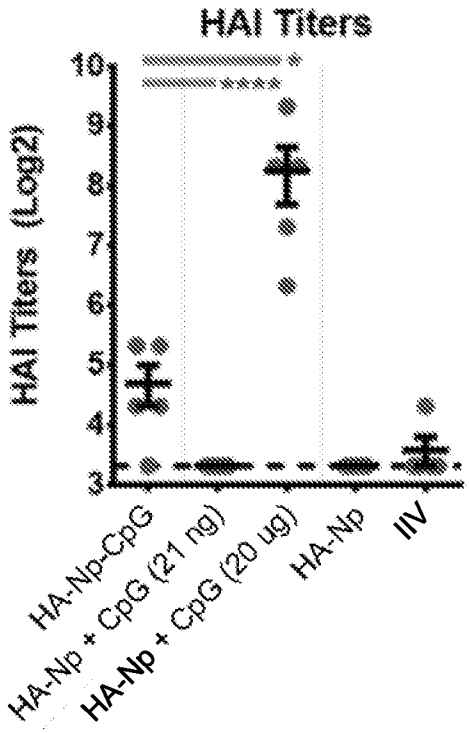

HAI Titers
HAI Titers (Log2)
10
9
8
7
6
5
4
3
****
HA-Np-CpG
HA-Np + CpG (21 ng)
HA-Np + CpG (20 ug)
HA-Np
IIV

Fig. 17C

Year	Nanoparticle	Size (nm)	Dispersity (%)
1947	FM47 HA-Np	16.8	8.9
1954	MAL54 HA-Np	17.9	8.9
1957	DV57 HA-Np	17.7	6.2
1977	HK77 HA-Np	17.6	10.9
1999	NC99 HA-Np	17.3	8.6
2009	CA09 HA-Np	16.8	9.3
1999-2012	COBRA X6 HA-Np	17.5	5.1
1933-1957 + 2009-2011 and swine 1931-1998	COBRA P1 HA-Np	17.1	7

Fig. 18A

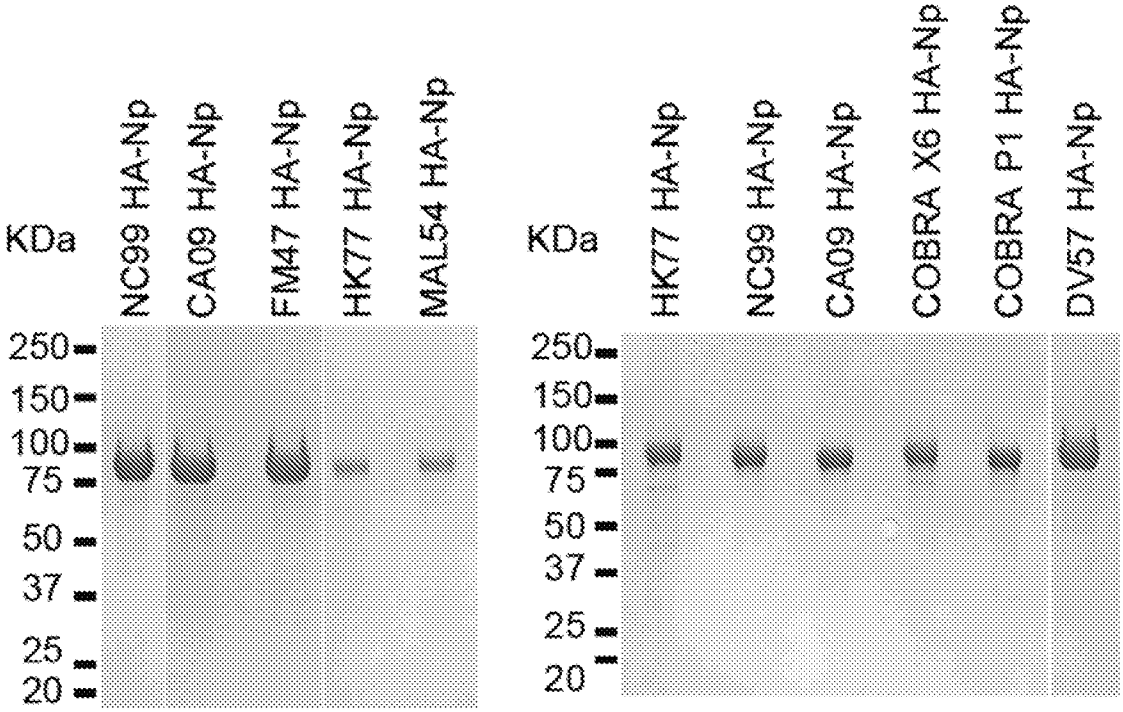


Fig. 18B

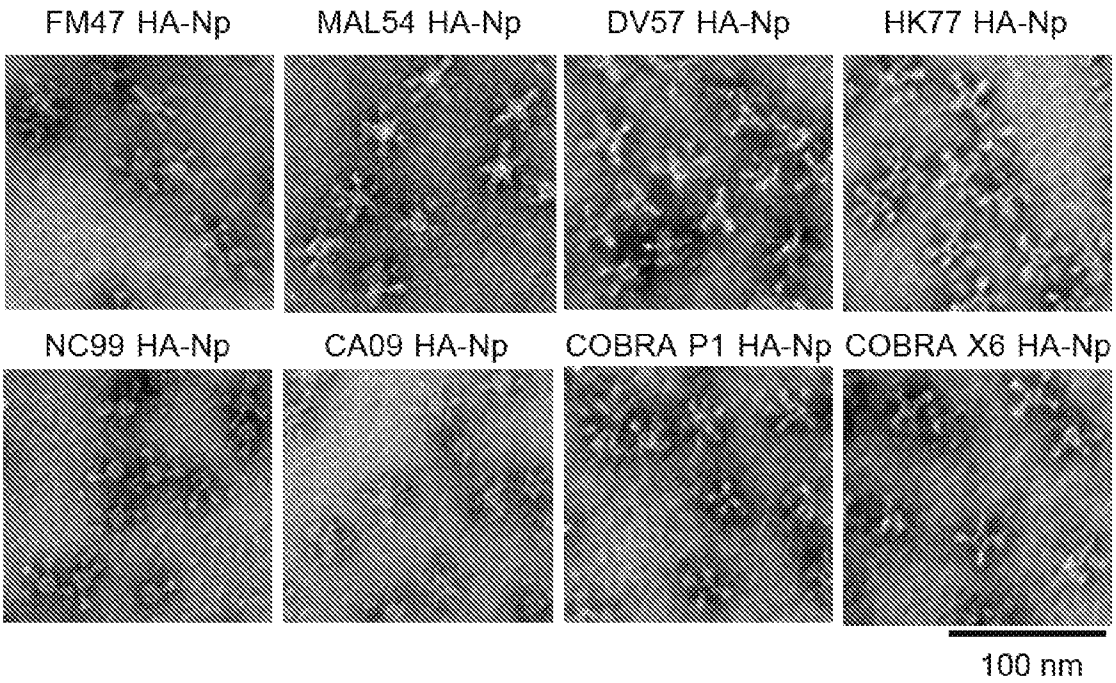

FM47 HA-Np
MAL54 HA-Np
DV57 HA-Np
HK77 HA-Np
NC99 HA-Np
CA09 HA-Np
COBRA P1 HA-Np
COBRA X6 HA-Np
100 nm

Fig. 18C

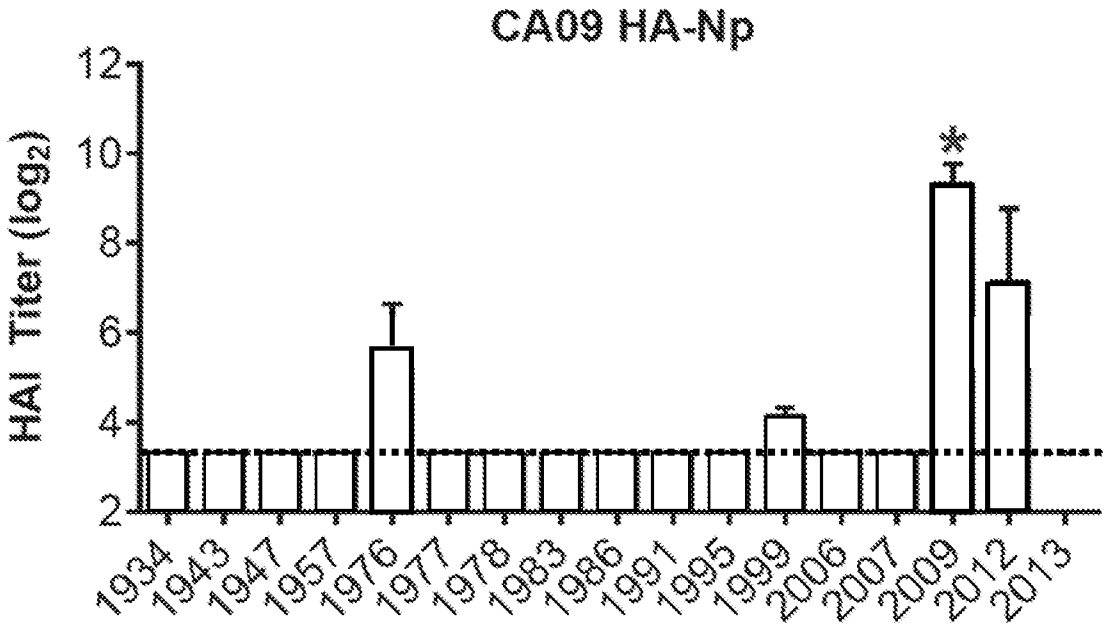


Fig. 19A

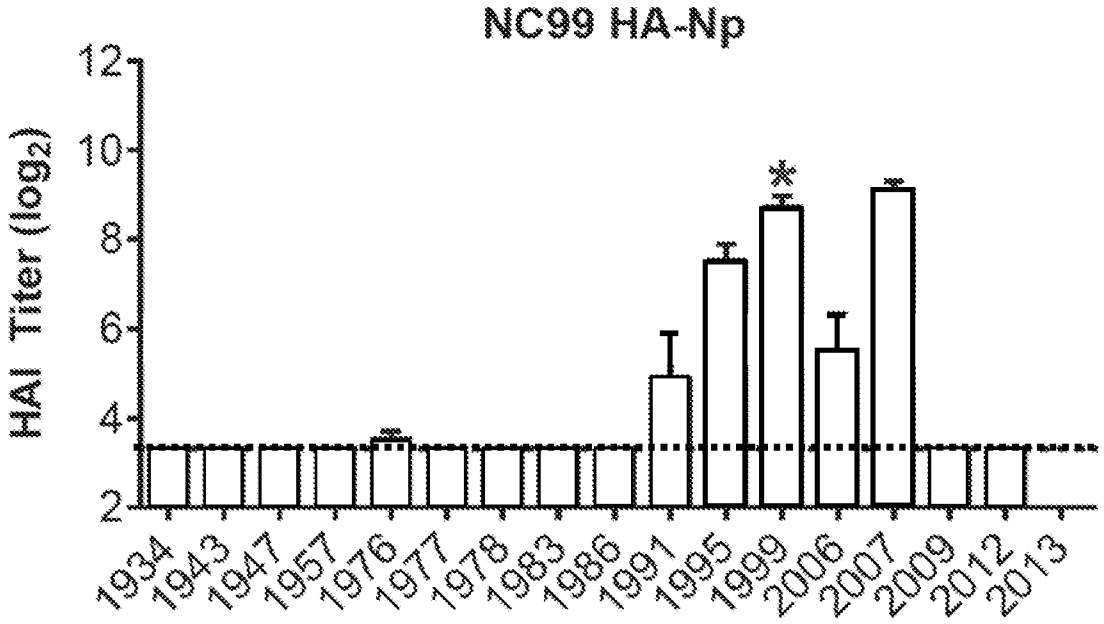


Fig. 19B

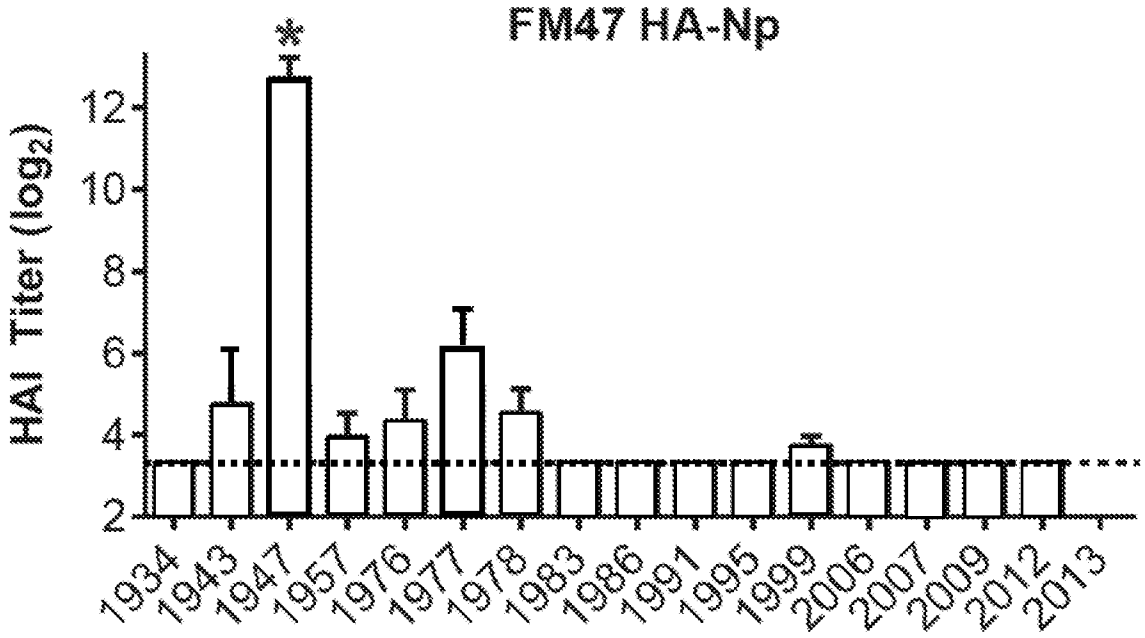


Fig. 19C

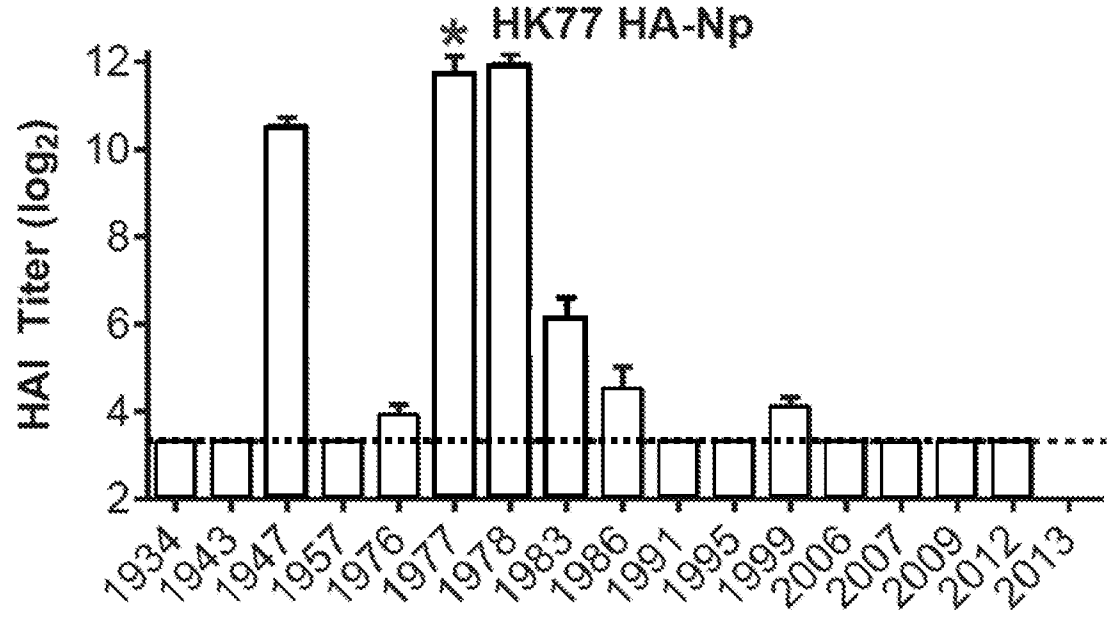


Fig. 19D

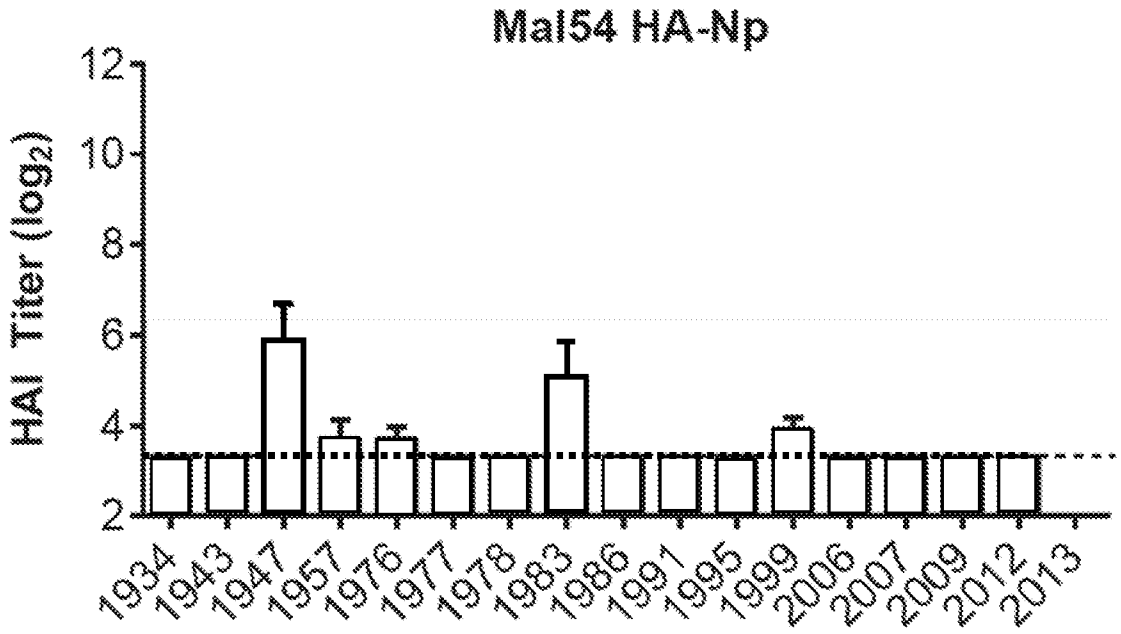


Fig. 19E

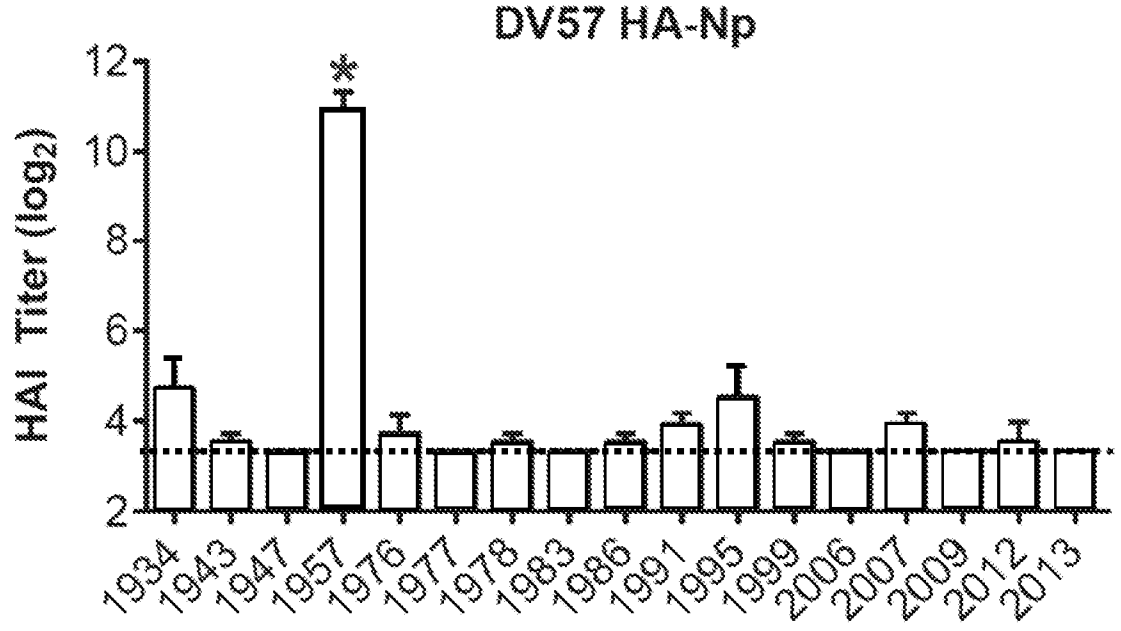


Fig. 19F

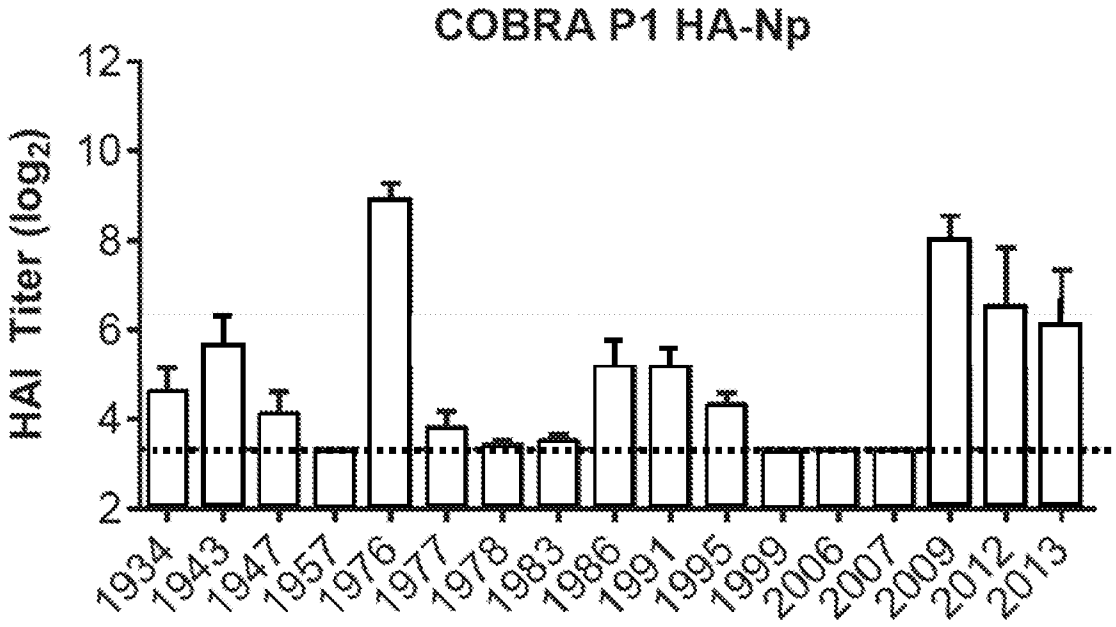


Fig. 19G

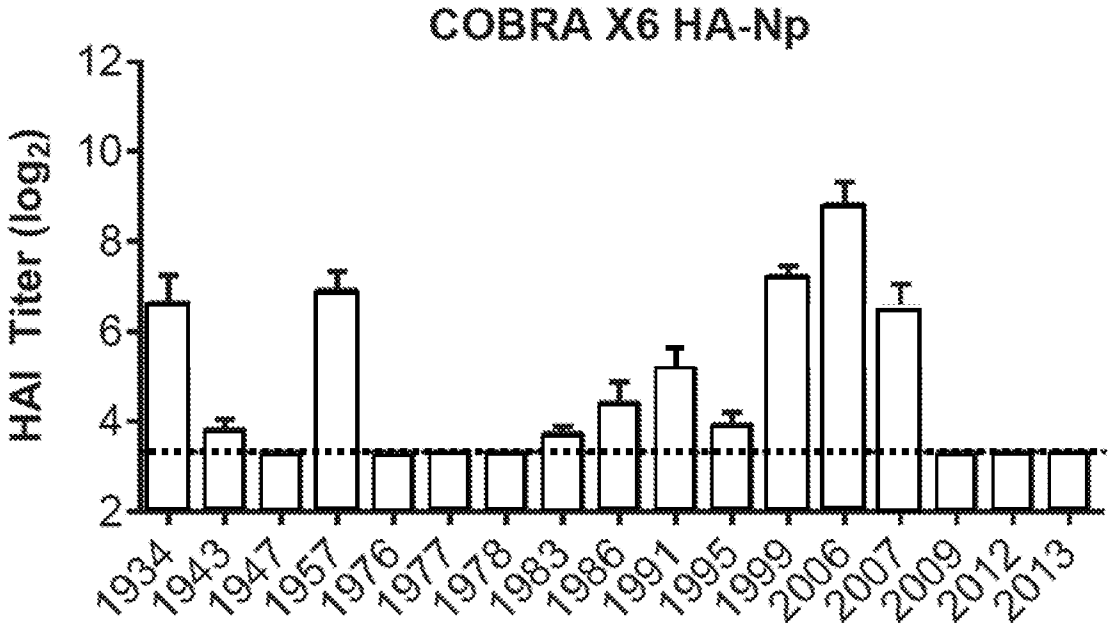


Fig. 19H

#	Immunogens
1	NC99 HA-Np
2	CA09 HA-Np
3	FM47 HA-Np
4	HK77 HA-Np
5	MAL54 HA-Np
6	NC99 + CA09 HA-Nps
7	NC99 + CA09 + FM47 HA-Nps
8	NC99 + CA09 + HK77 HA-Nps
9	NC99 + CA09 + MAL54 HA-Nps
10	NC99 + CA09 + HK77 + FM47 HA-Nps
11	NC99 + CA09 + HK77 + MAL54 HA-Nps
12	NC99 + CA09 + FM47 + MAL54 HA-Nps
13	NC99 IIV
14	CA09 IIV
15	NC99 + CA09 IIV

Fig. 20A

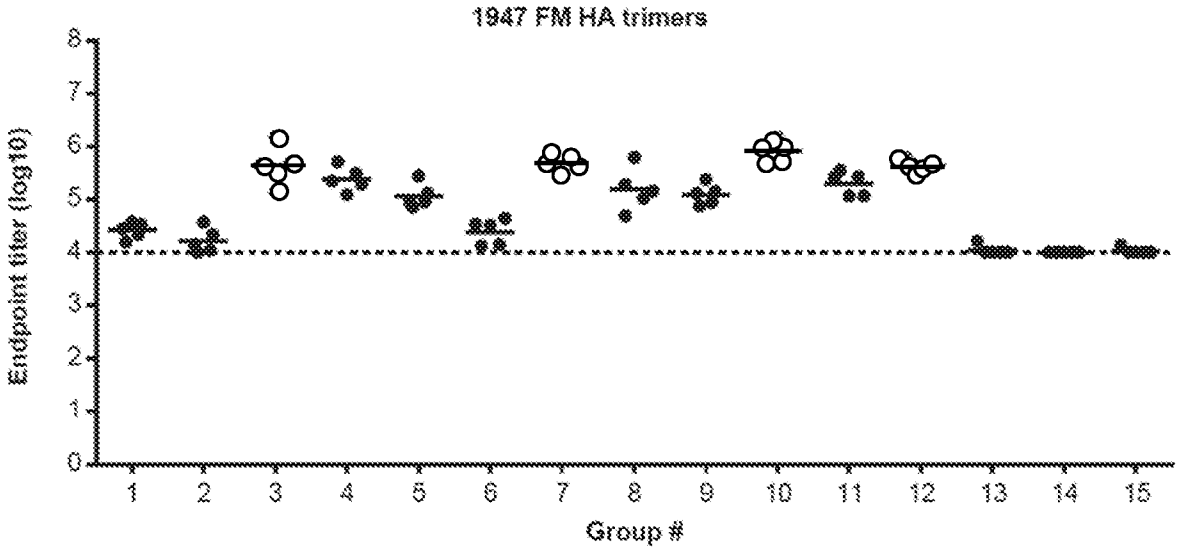


Fig. 20B

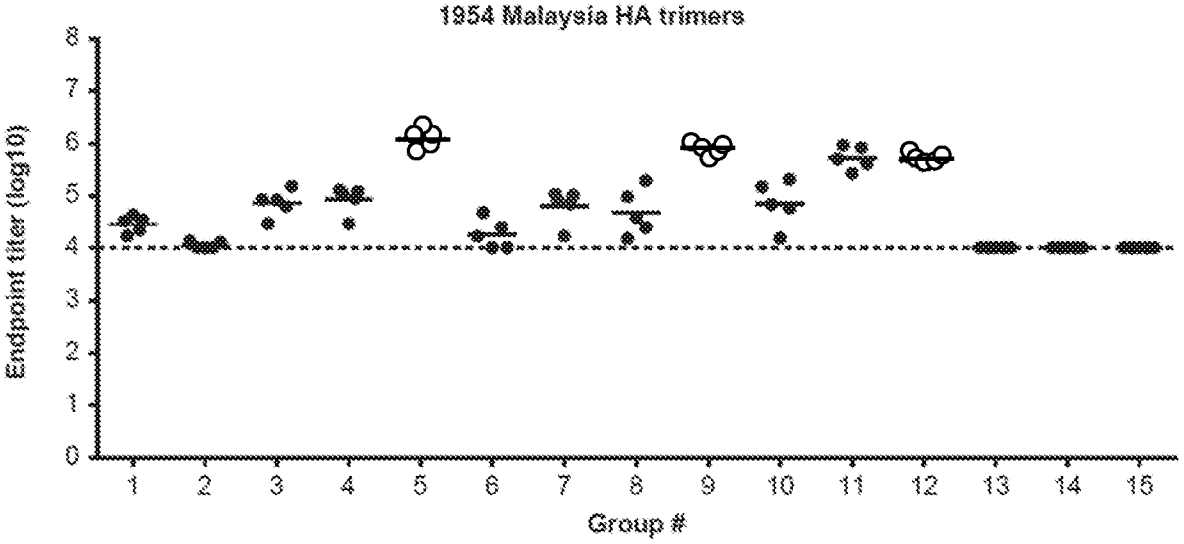


Fig. 20C

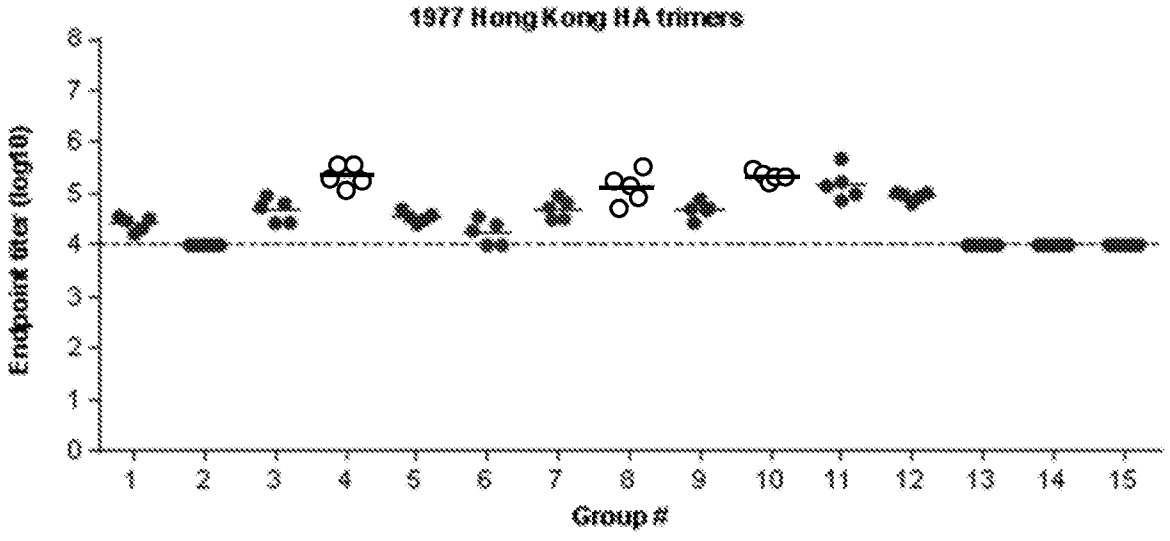


Fig. 20D

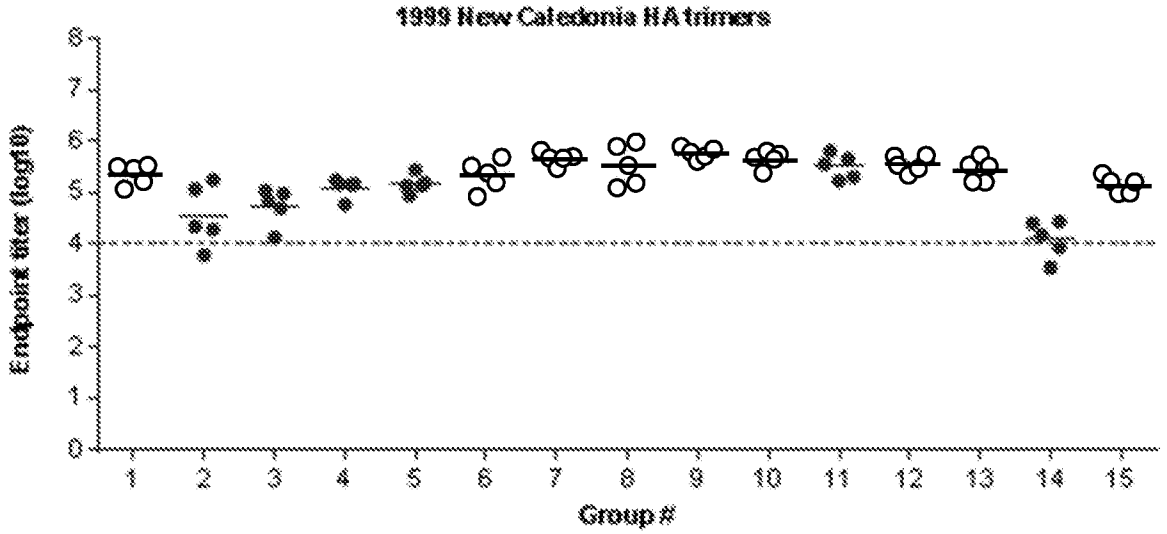


Fig. 20E

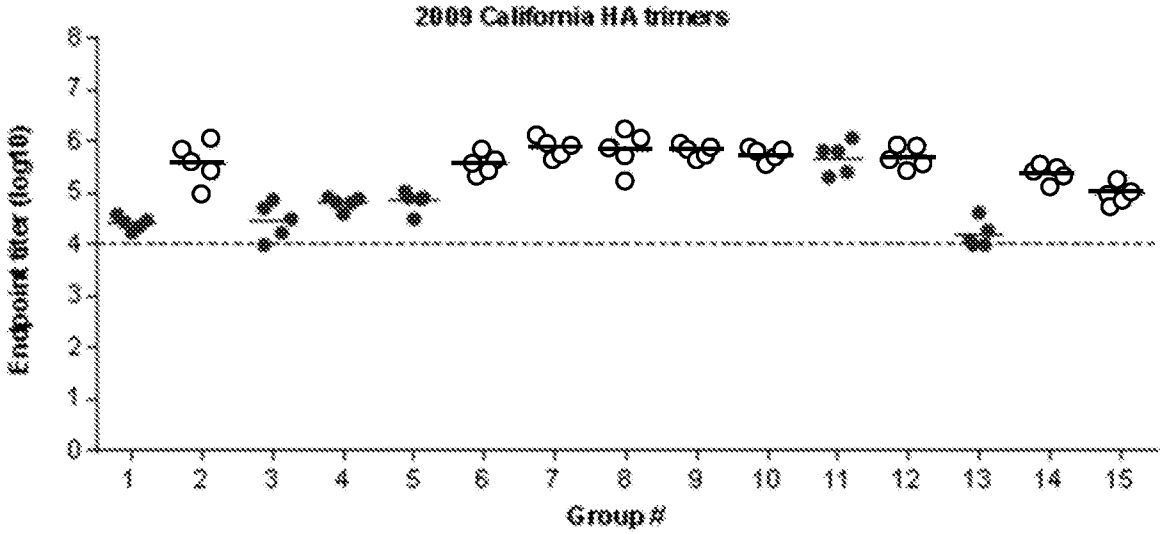


Fig. 20F

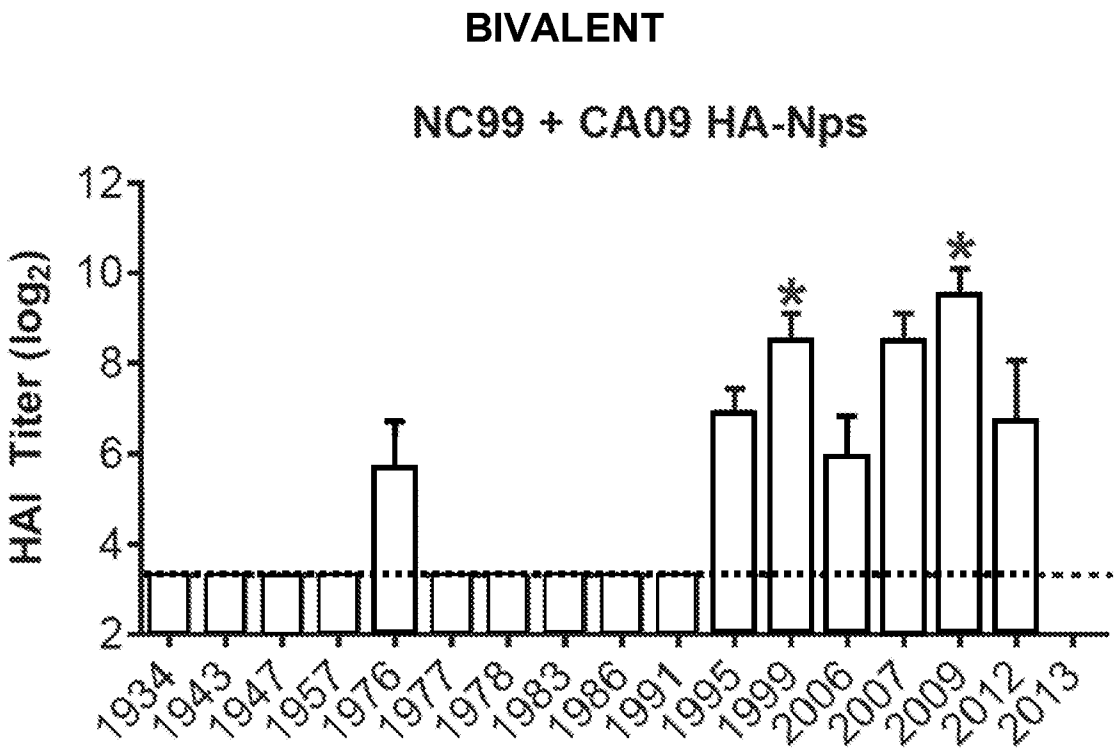


Fig. 21A

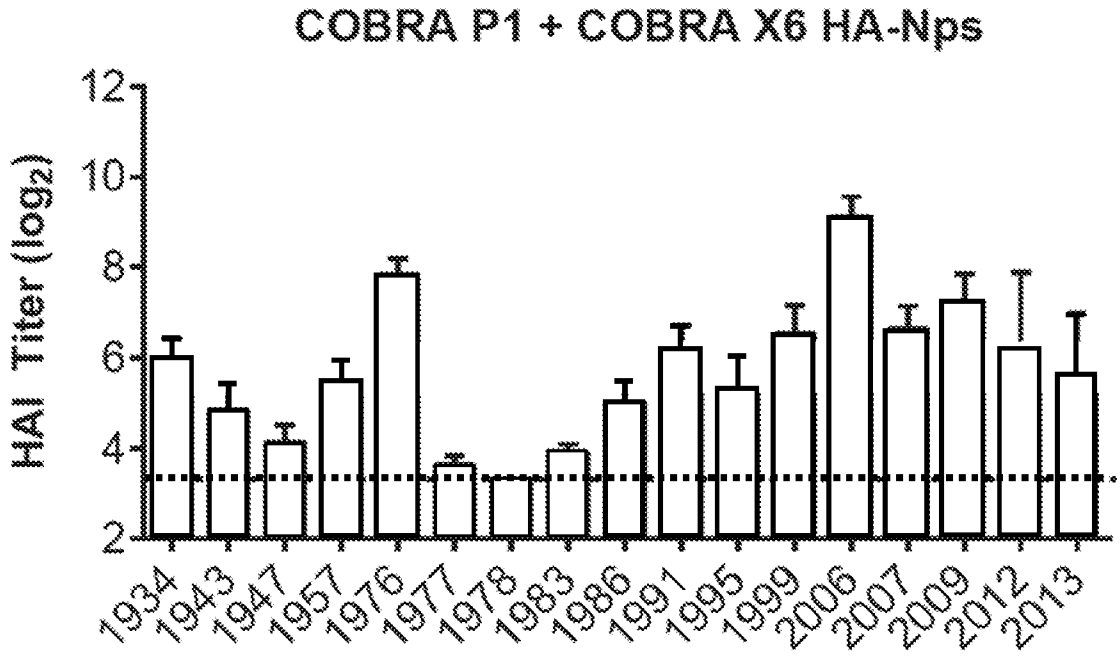


Fig. 21B

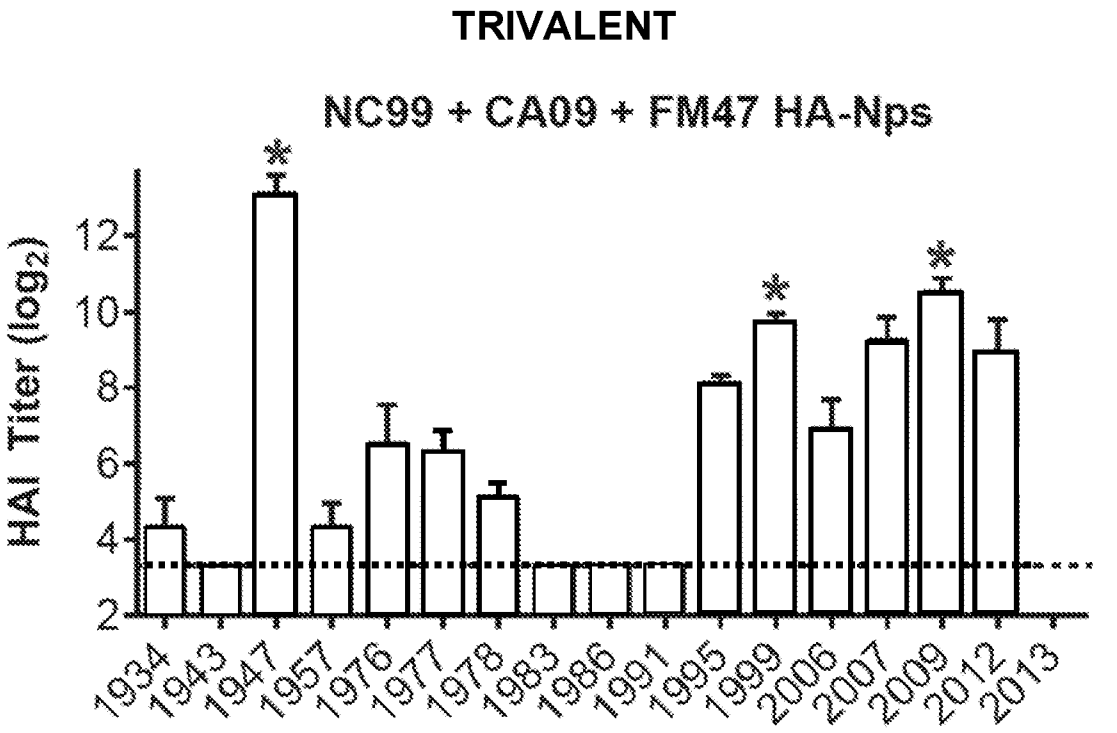


Fig. 21C

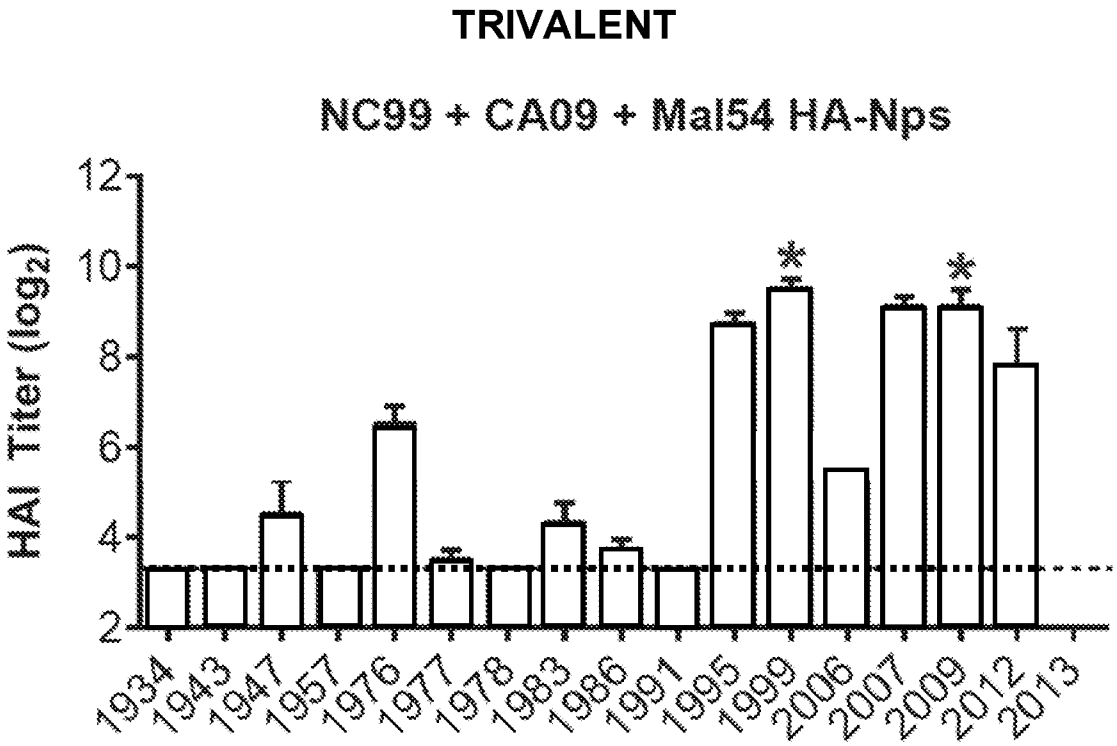


Fig. 21D

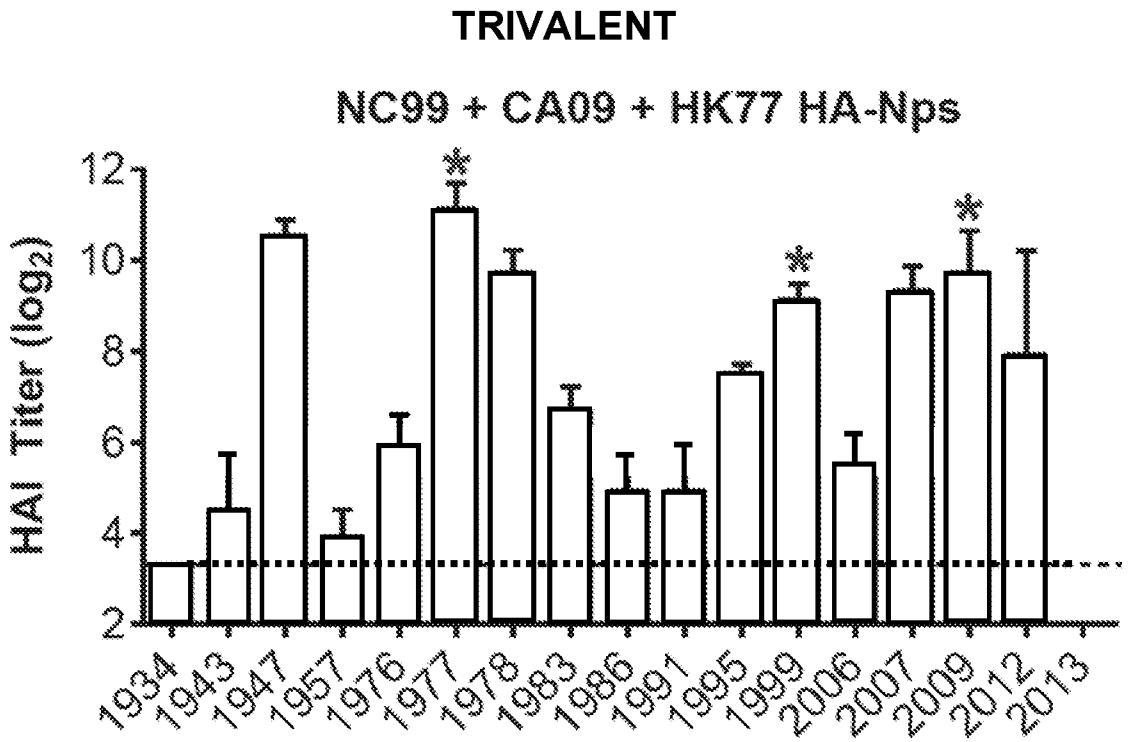


Fig. 21E

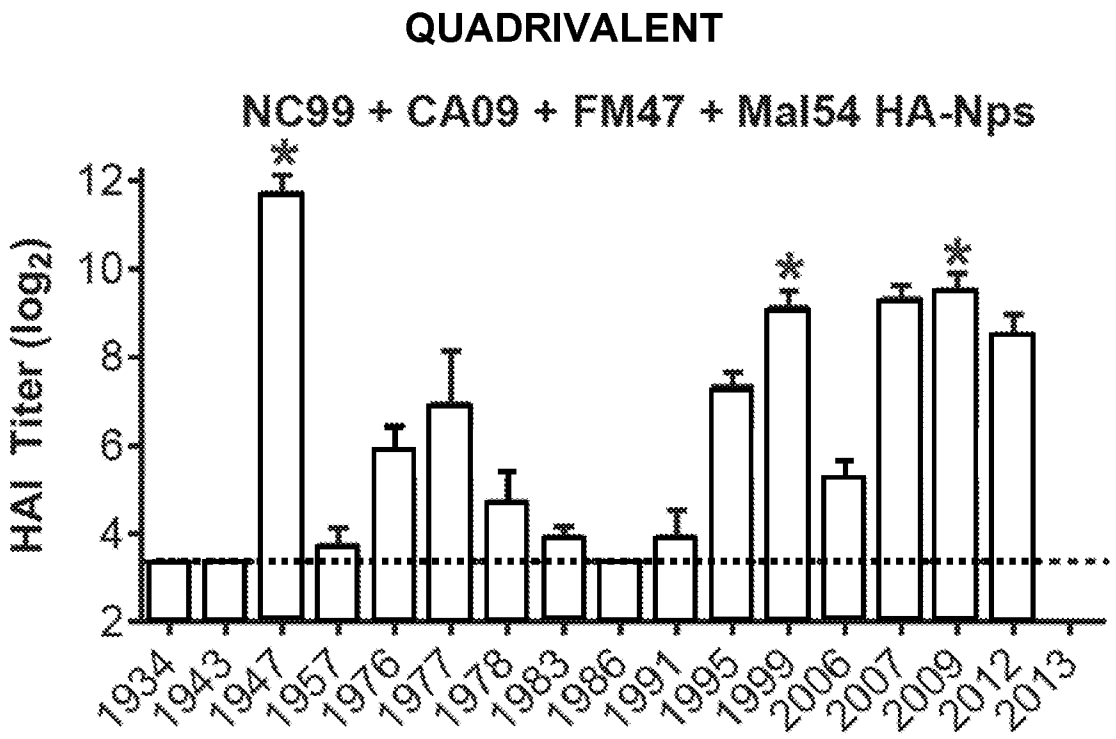


Fig. 21F

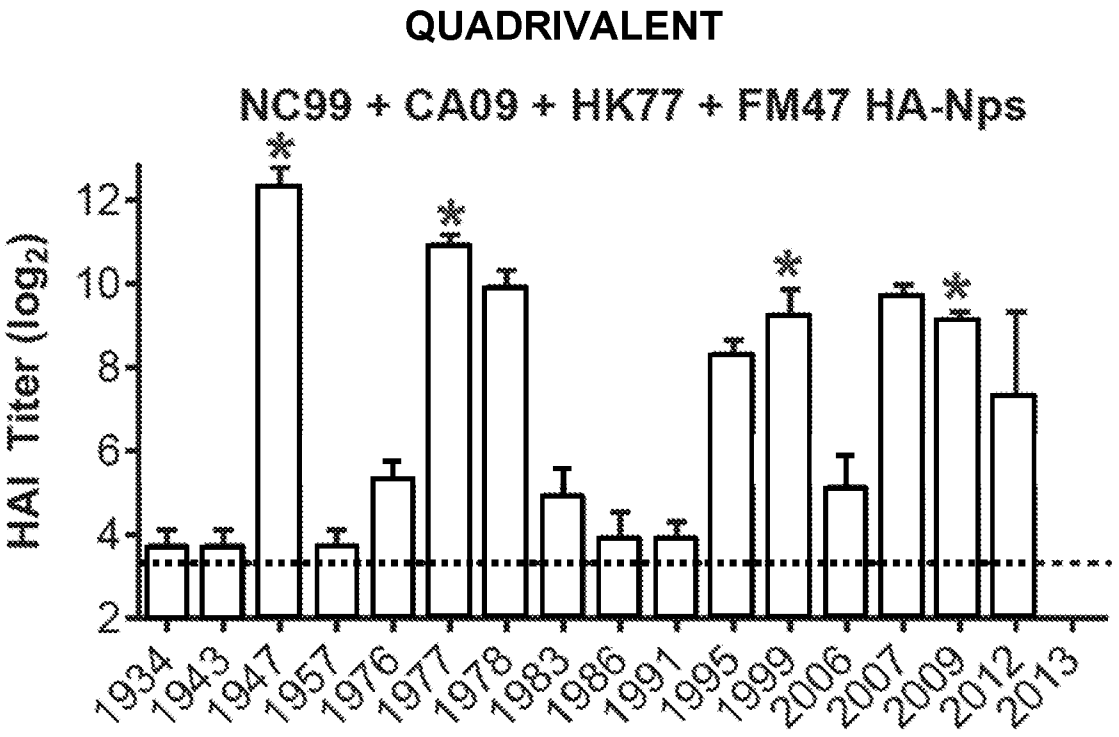


Fig. 21G

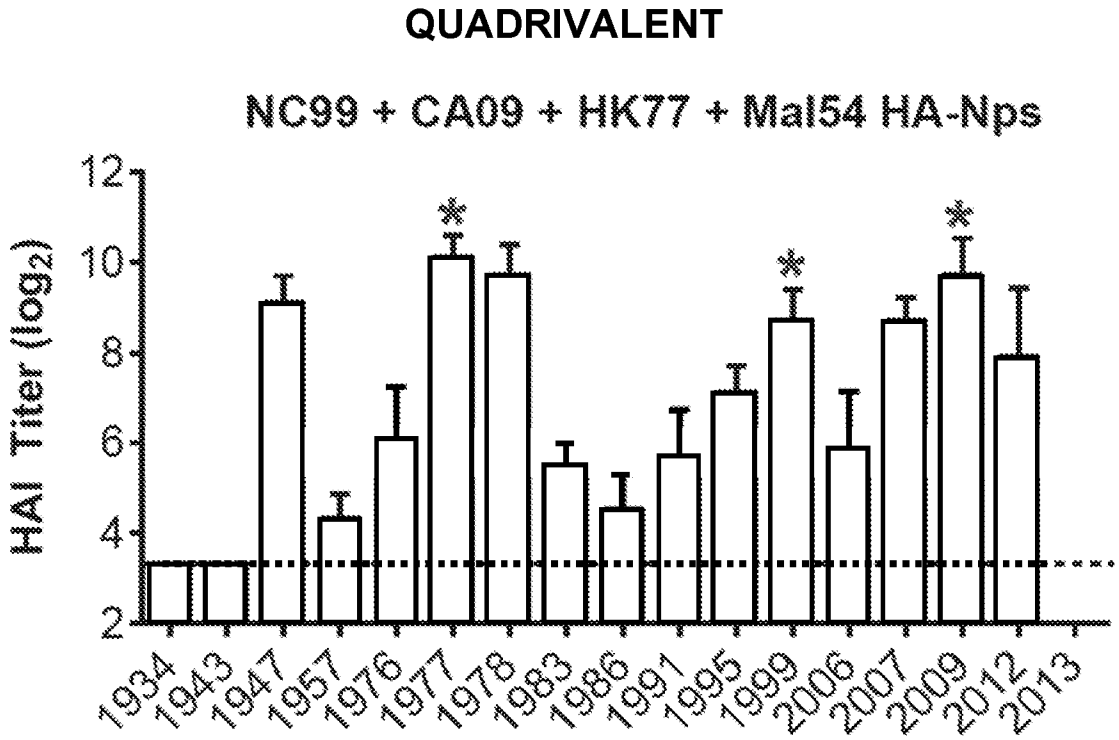


Fig. 21H

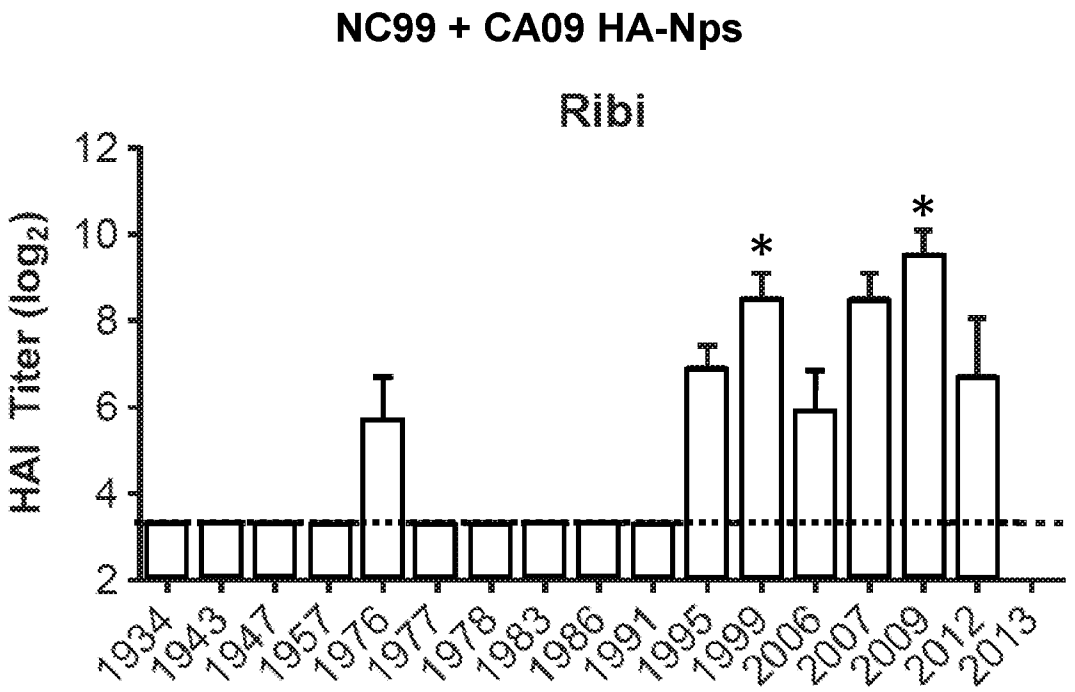


Fig. 22A

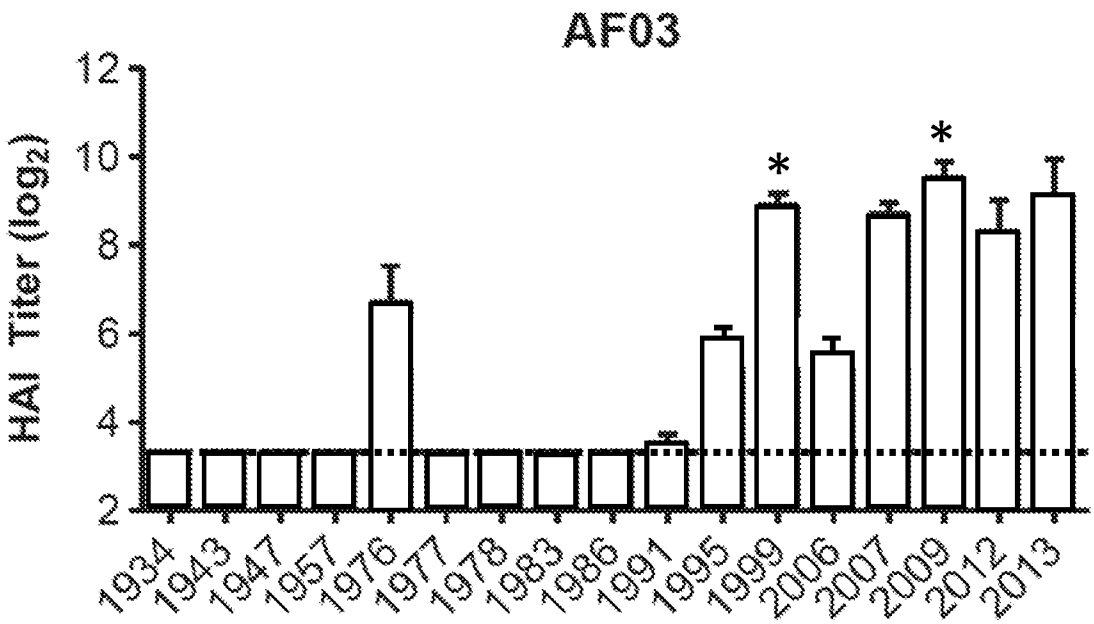


Fig. 22B

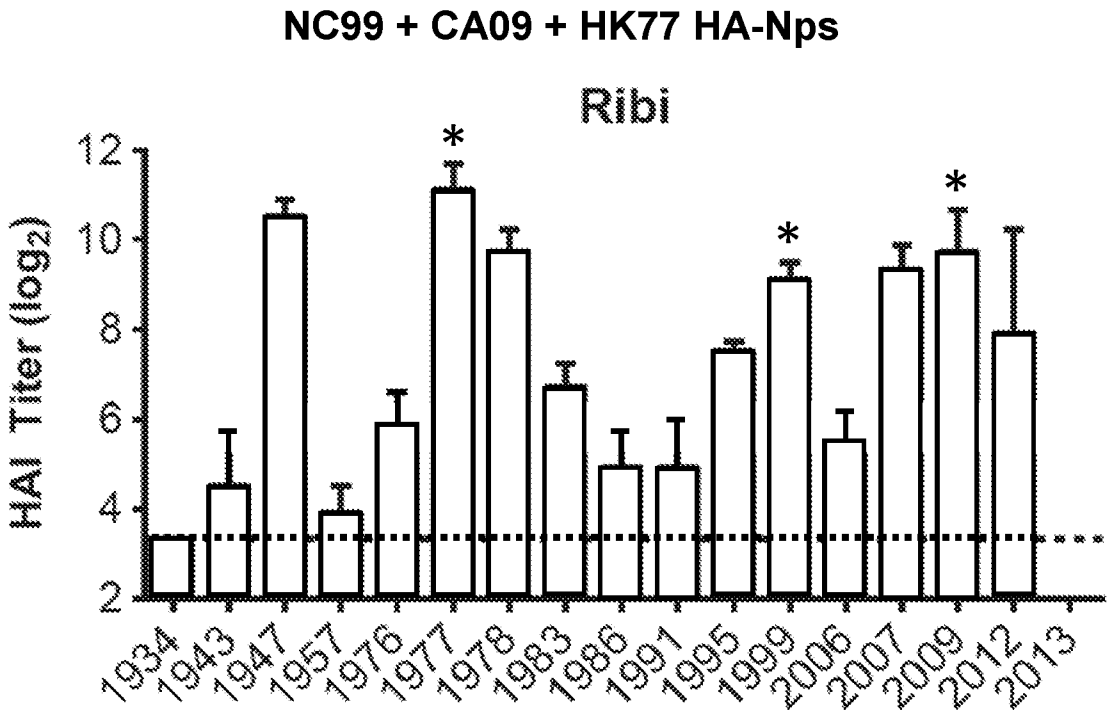


Fig. 22C

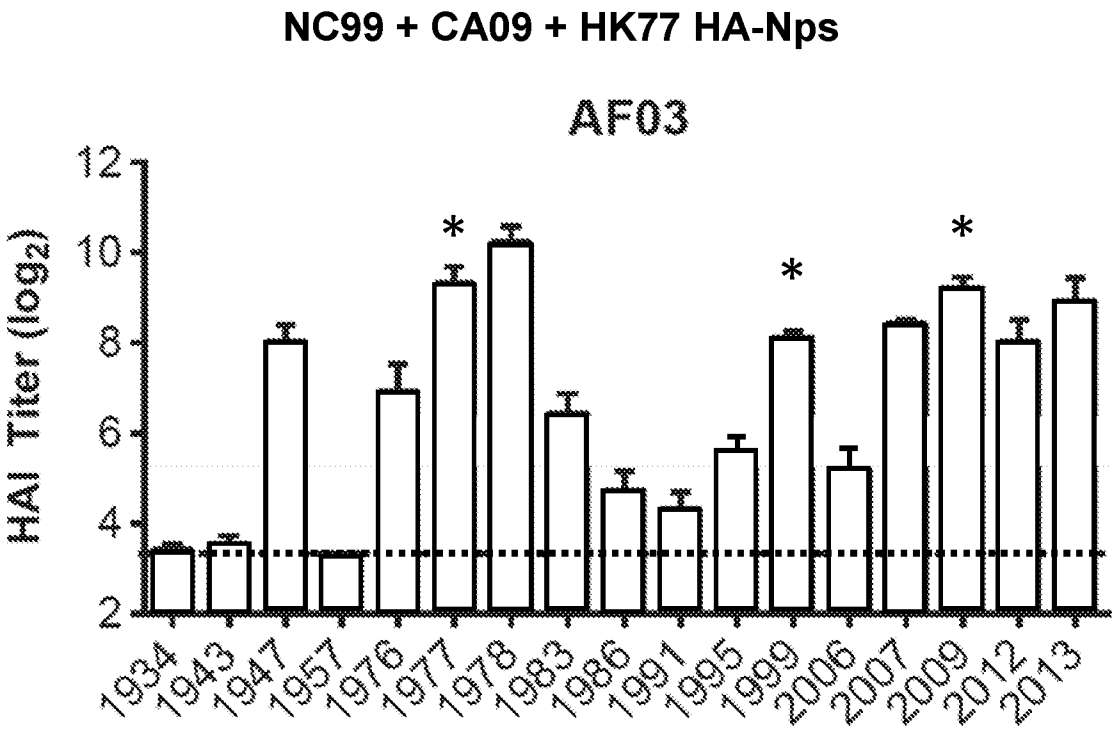


Fig. 22D

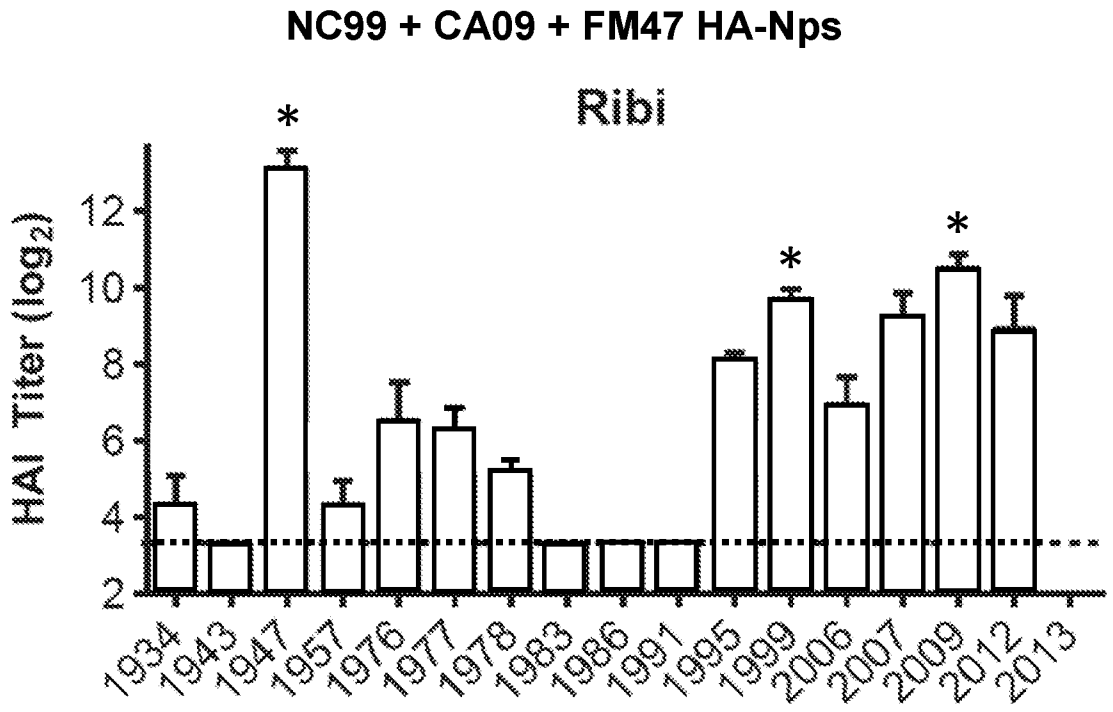


Fig. 22E

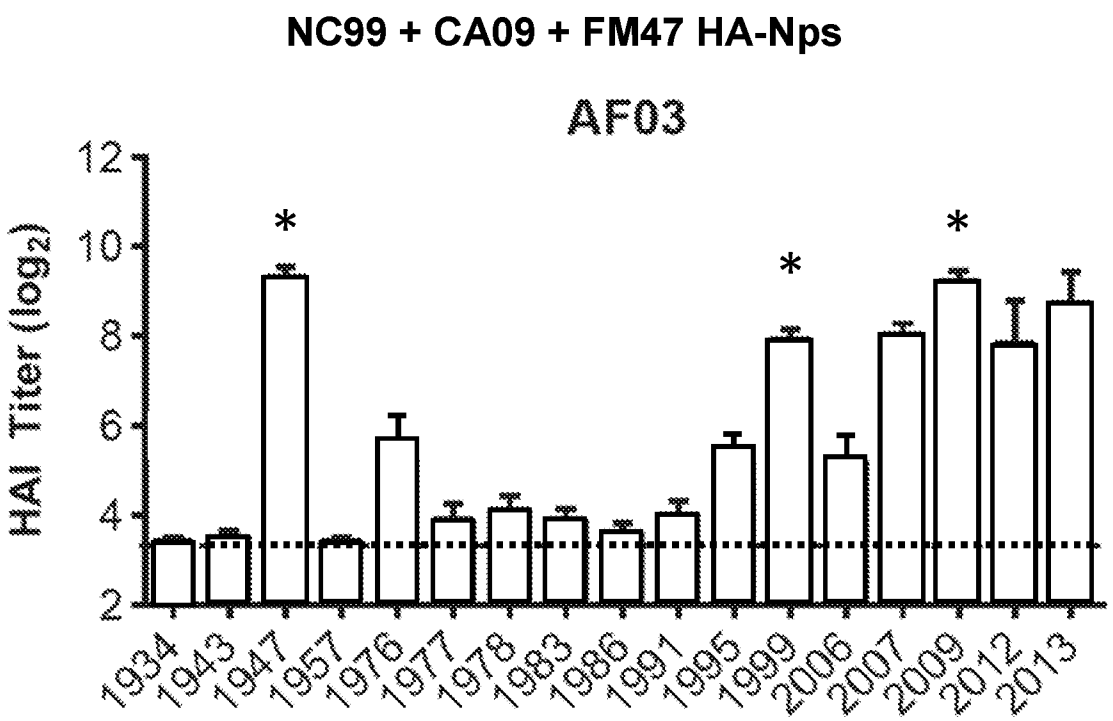


Fig. 22F

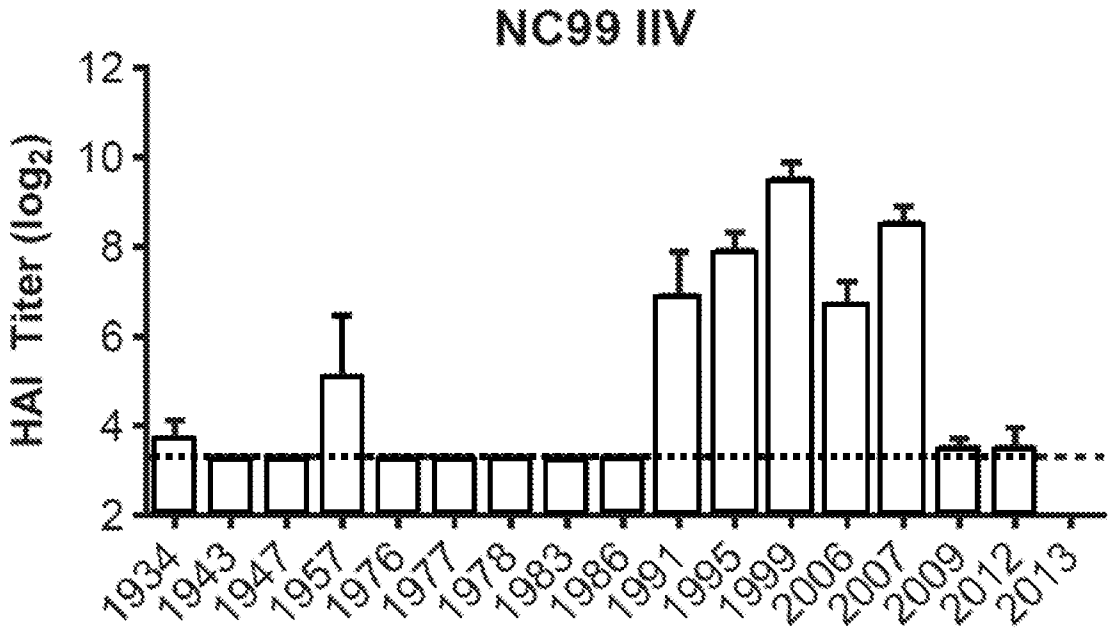


Fig. 23A

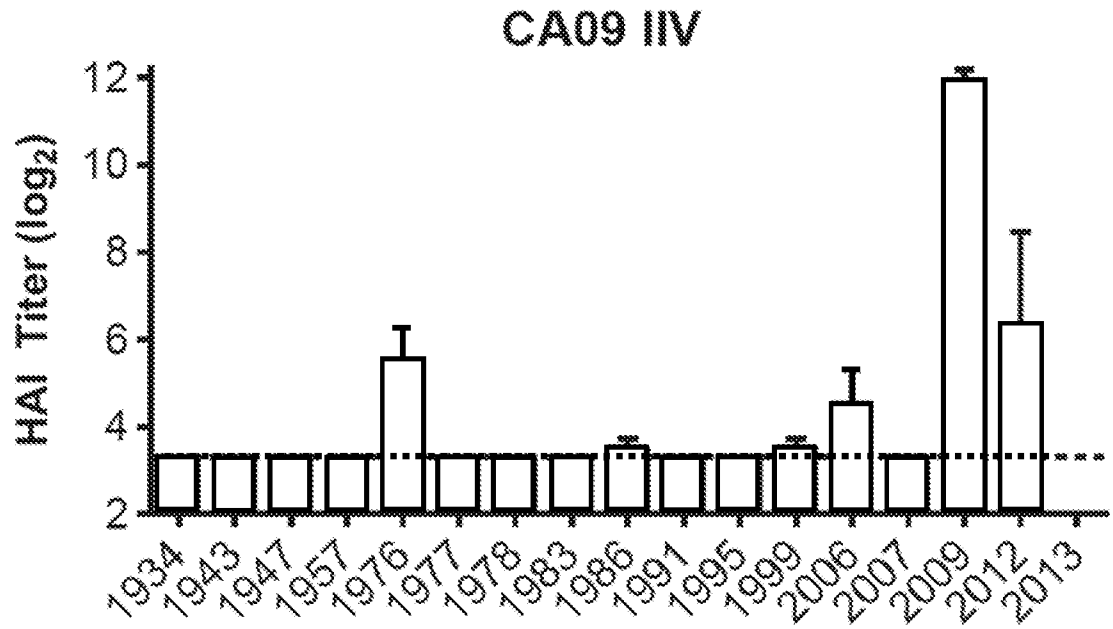


Fig. 23B

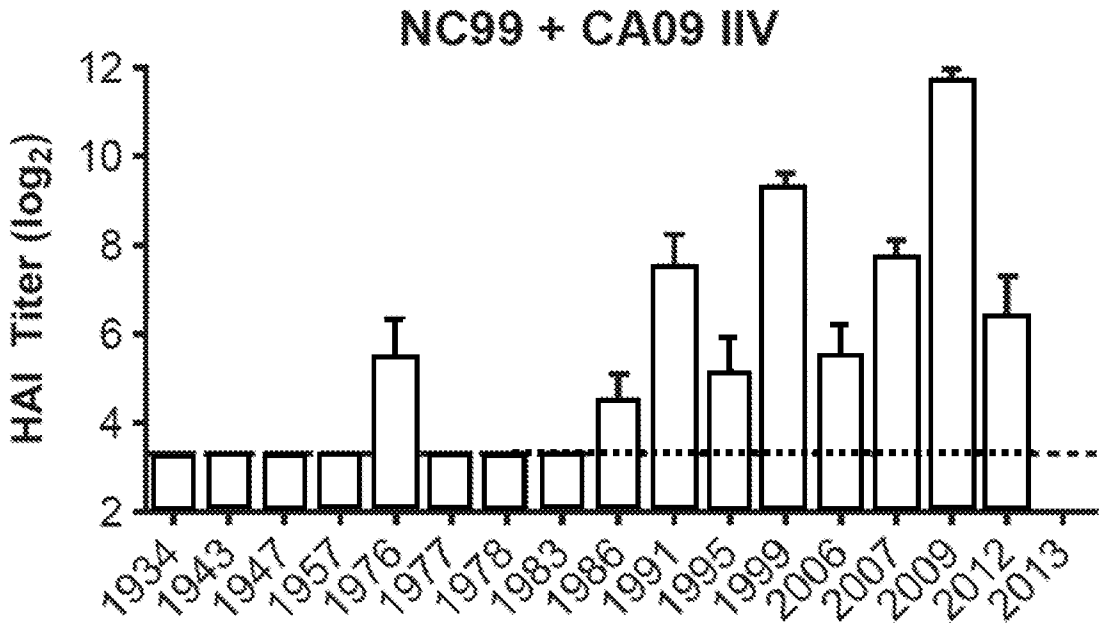

NC99 + CA09 IIV
HAI Titer (log2)
12
10
8
6
4
2
1934
1943
1947
1957
1976
1977
1978
1983
1986
1991
1995
1999
2006
2007
2009
2012
2013

Fig. 23C

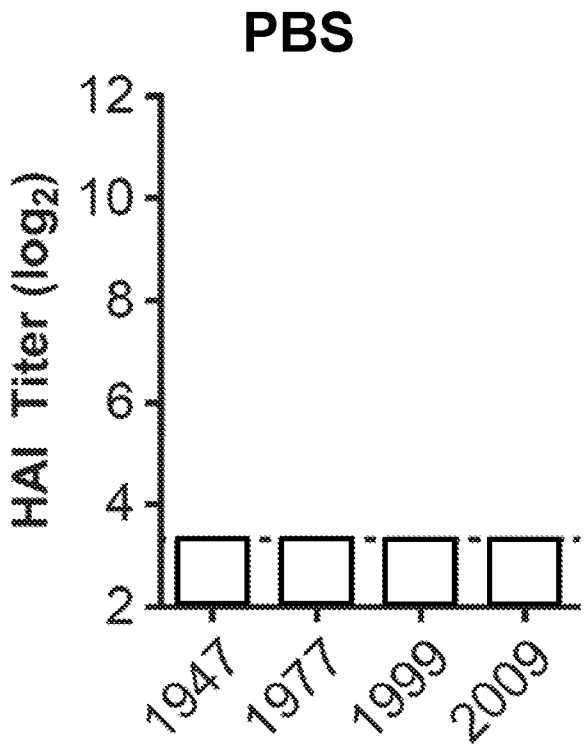


Fig. 24A

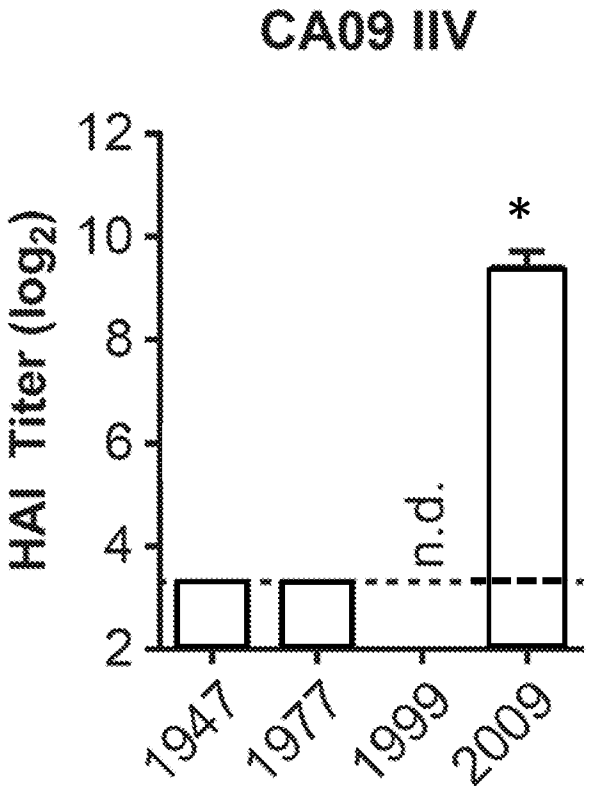


Fig. 24B

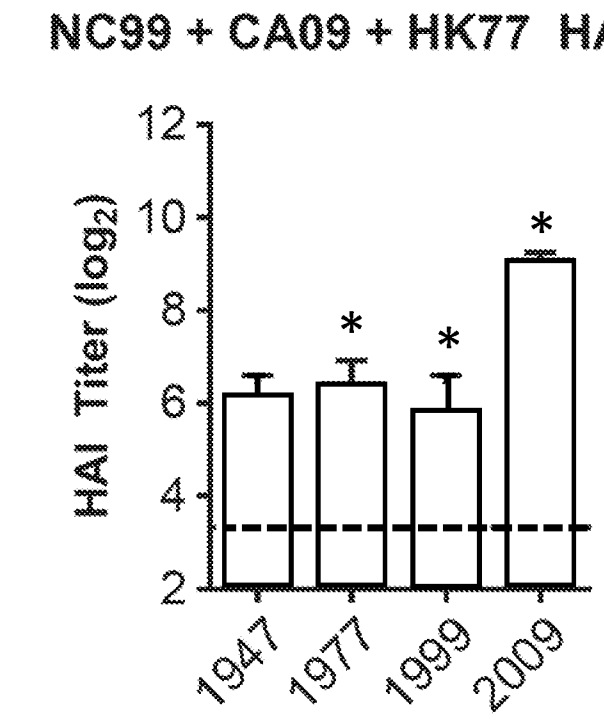


Fig. 24C

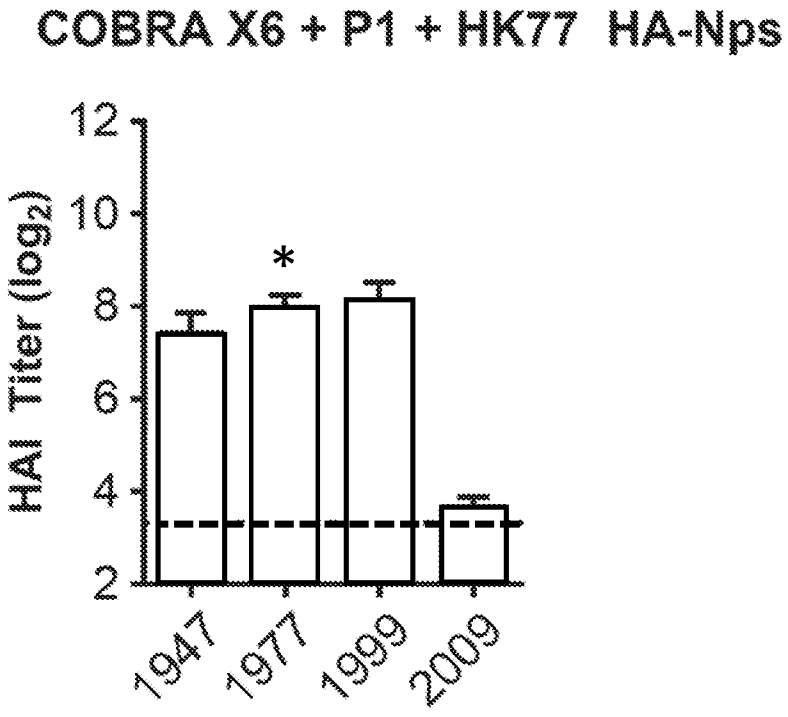


Fig. 24D

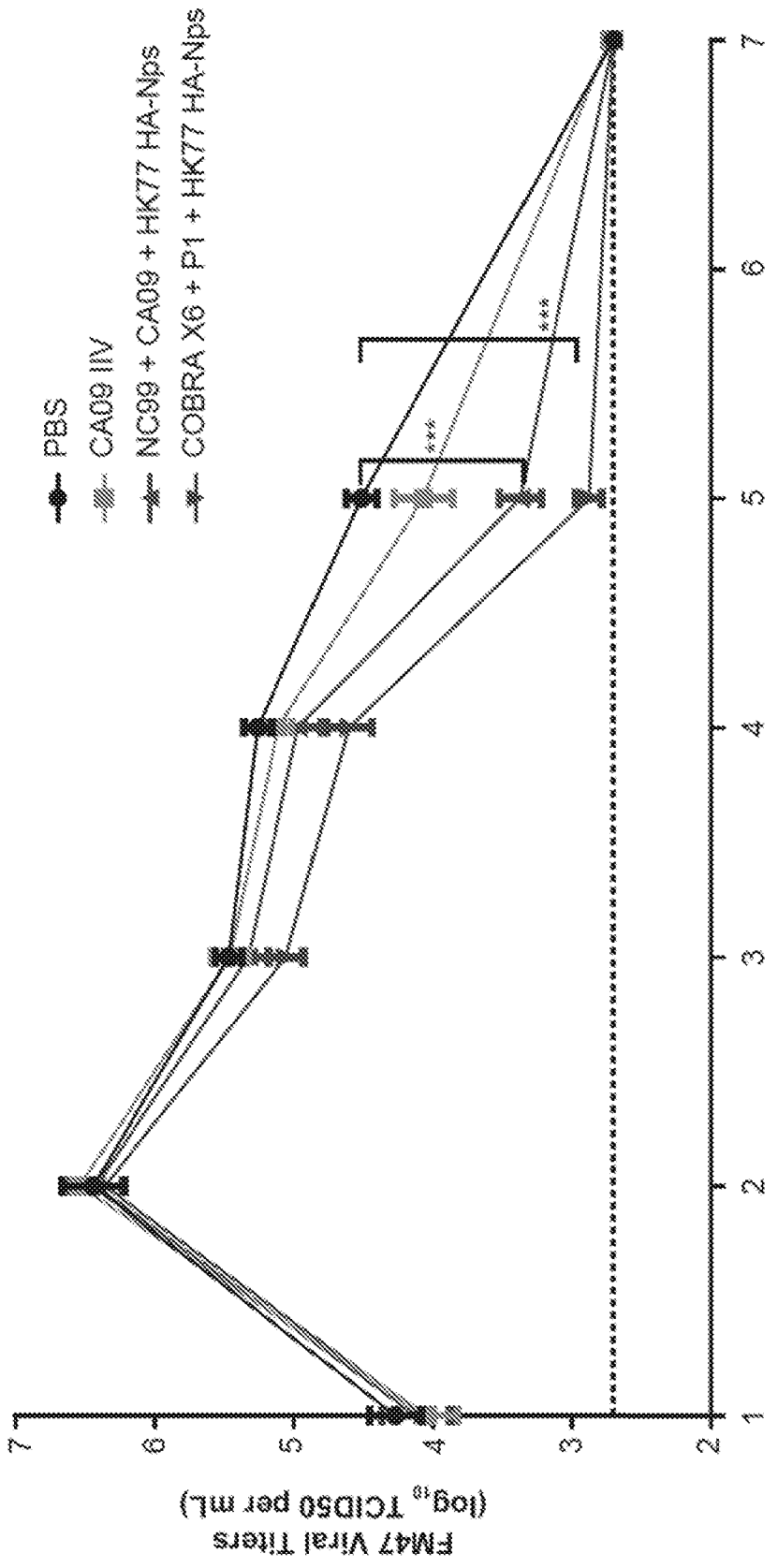

PBS
CA09 IIV
NC99 + CA09 + HK77 HA-Nps
COBRA X6 + P1 + HK77 HA-Nps
***
***
FM47 Viral Titers
(log10 TCID50 per mL)
Days after Challenge with H1/Fort Monmouth/1/1947
1
2
3
4
5
6
7

Fig. 24E

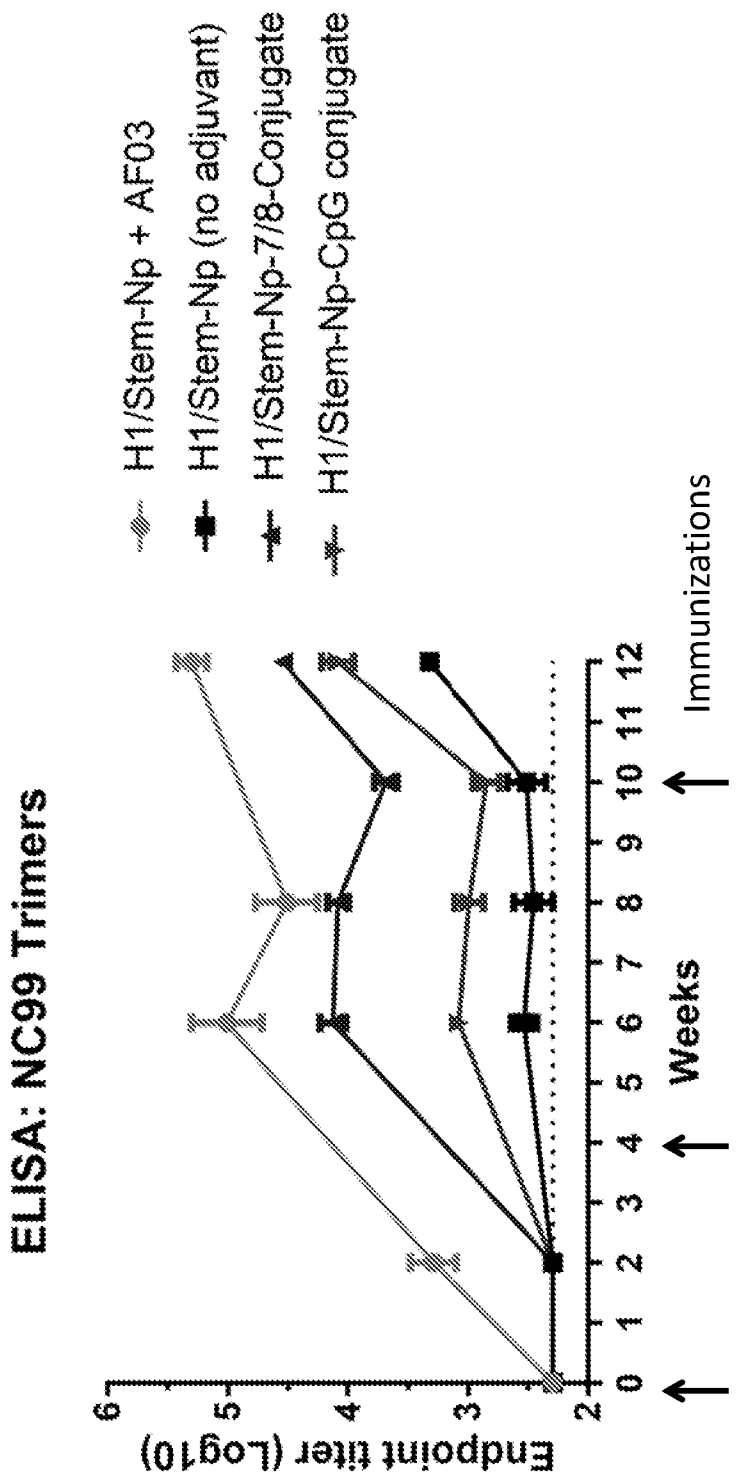

ELISA: NC99 Trimers
H1/Stem-Np + AF03
H1/Stem-Np (no adjuvant)
H1/Stem-Np-7/8-Conjugate
H1/Stem-Np-CpG conjugate
Endpoint titer (Log10)
6
5
4
3
2
0 1 2 3 4 5 6 7 8 9 10 11 12
Weeks
Immunizations

Fig. 25A

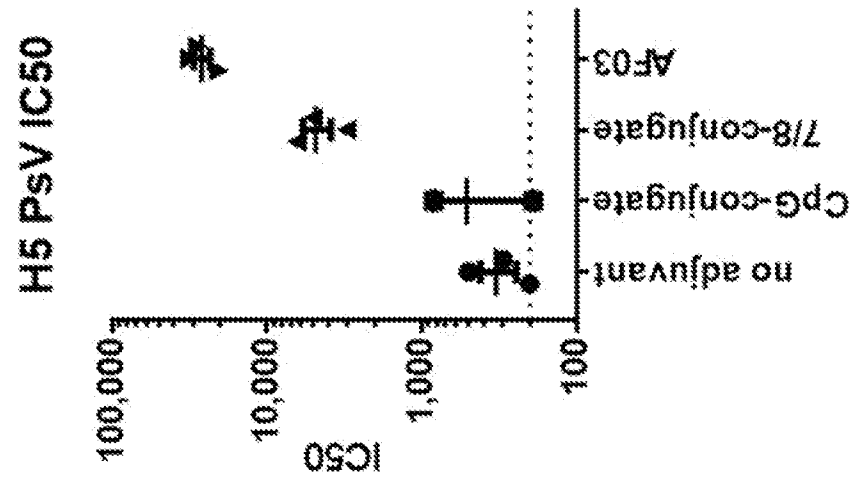


Fig. 25C

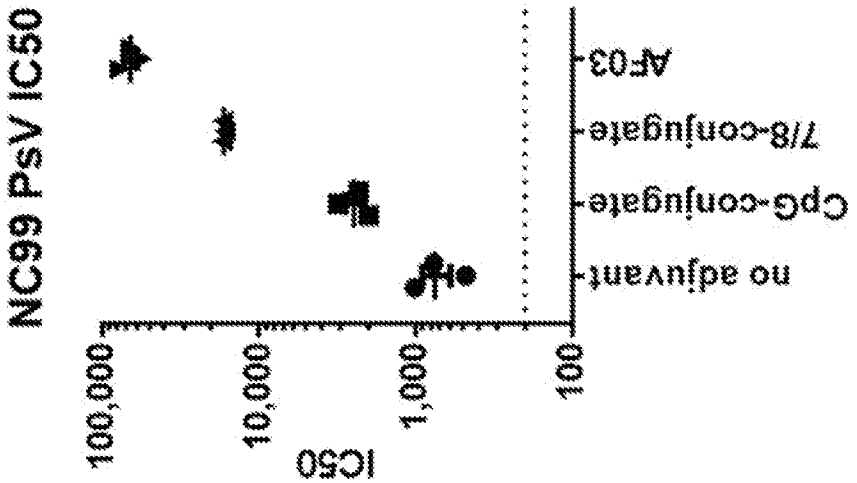


Fig. 25B

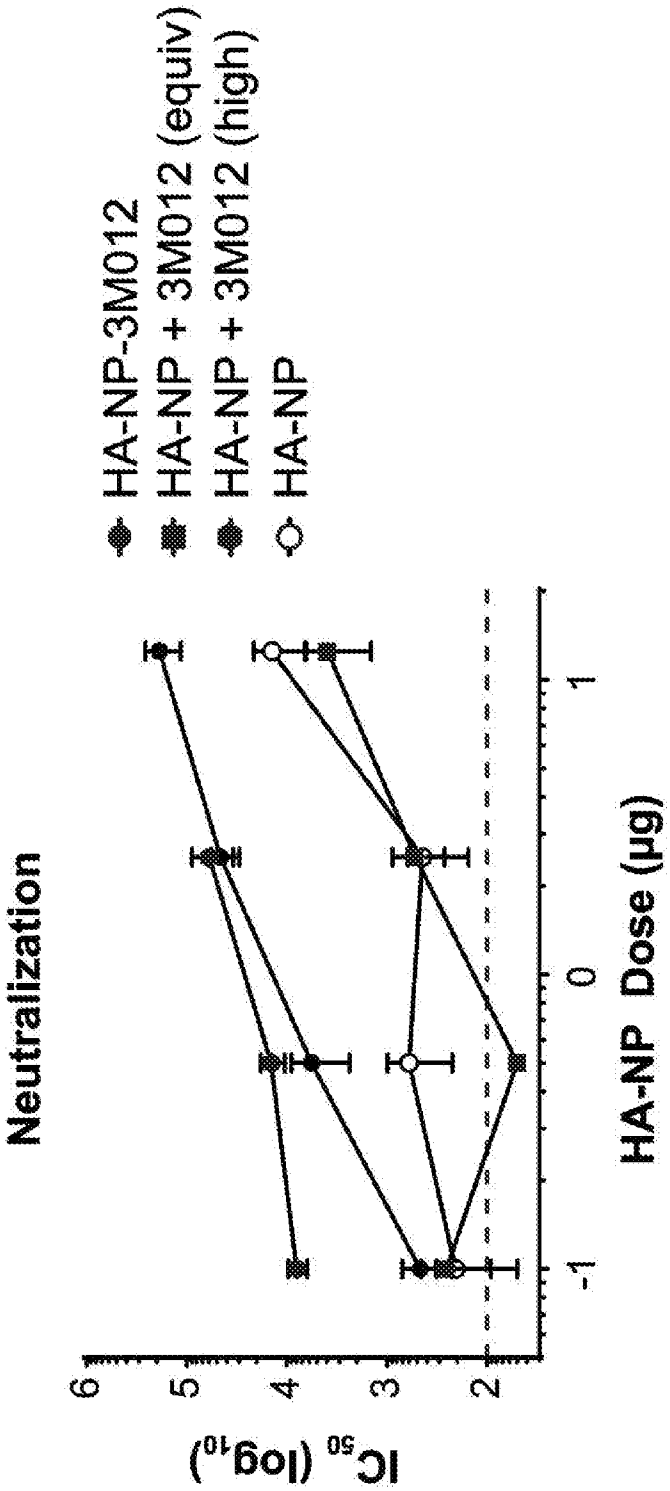

HA-NP-3M012
HA-NP + 3M012 (equiv)
HA-NP + 3M012 (high)
HA-NP
Neutralization
IC50 (log10)
6
5
4
3
2
-1
0
1
HA-NP Dose (µg)

Fig. 26A

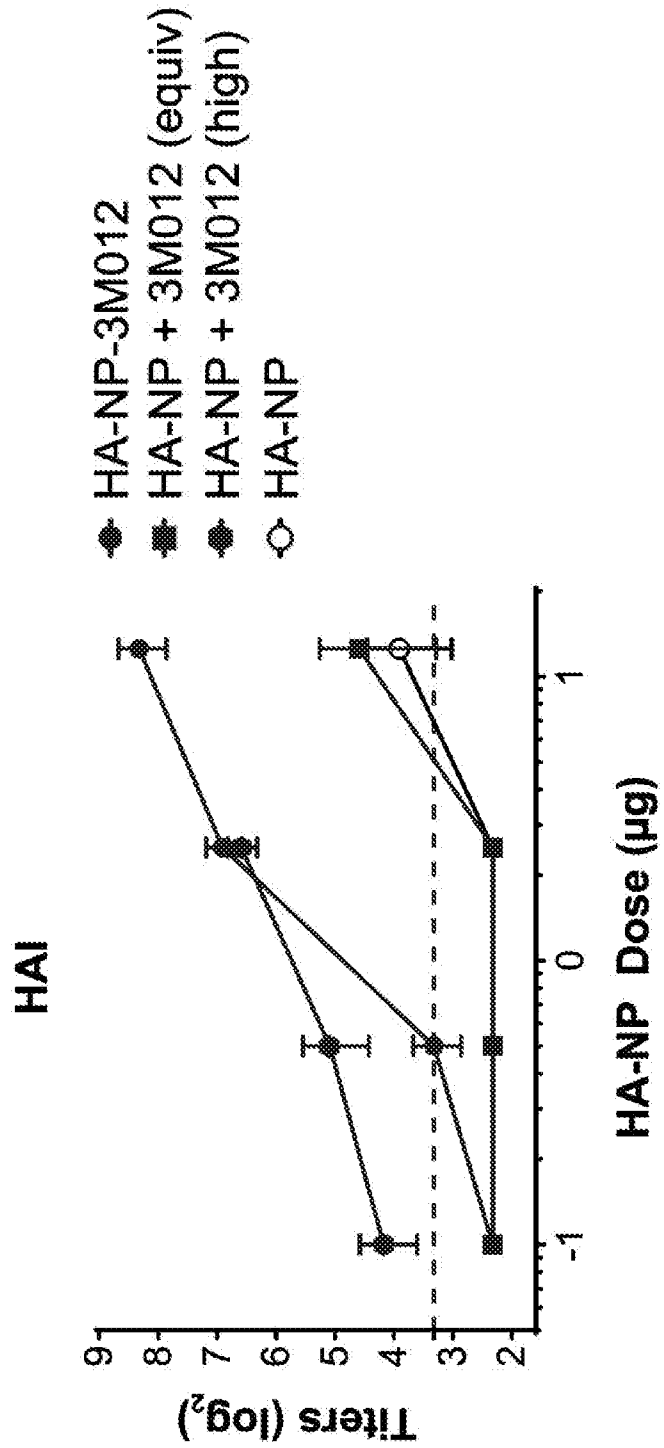

HAI
HA-NP-3M012
HA-NP + 3M012 (equiv)
HA-NP + 3M012 (high)
HA-NP
Titers (log2)
9
8
7
6
5
4
3
2
-1
0
1
HA-NP Dose (µg)

Fig. 26B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2019/025377

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12 A61P31/16 C07K14/205
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N A61P C12R C07K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MASARU KANEKIYO ET AL: "Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies", NATURE, MACMILLAN JOURNALS LTD, LONDON, vol. 499, no. 7456, 4 July 2013 (2013-07-04), pages 102-106, XP002735539, ISSN: 0028-0836, DOI: 10.1038/NATURE12202 [retrieved on 2013-05-22] abstract, page 102, right-hand col.; page 105, last paragraph; figures 1-4; methods, supplementary figures -----	1-25
Y	WO 2015/183969 A1 (USA AS REPRESENTED BY THE SECRETARY DEPT OF HEALTH AND HUMAN SERVICES) 3 December 2015 (2015-12-03) examples, figures, in particular figure 1 -----	1-25

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 June 2019	10/07/2019

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

1

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2013/044203 A2 (US HEALTH [US]) 28 March 2013 (2013-03-28) abstract, examples	1-25
Y	HU QI-YING ET AL: "Towards the next generation of biomedicines by site-selective conjugation", CHEMICAL SOCIETY REVIEWS,, vol. 45, no. 6, 21 March 2016 (2016-03-21), pages 1691-1719, XP002772944, DOI: 10.1039/C4CS00388H page 1695, left-hand col.	1-25
Y	YOUNGGYU KIM ET AL: "Efficient Site-Specific Labeling of Proteins via Cysteines", BIOCONJUGATE CHEMISTRY, vol. 19, no. 3, 1 March 2008 (2008-03-01), pages 786-791, XP055008636, ISSN: 1043-1802, DOI: 10.1021/bc7002499 abstract	1-25
Y	TOM Y.-H. WU: "Strategies for designing synthetic immune agonists", IMMUNOLOGY, vol. 148, no. 4, 11 July 2016 (2016-07-11), pages 315-325, XP055594767, GB ISSN: 0019-2805, DOI: 10.1111/imm.12622 page 316, left-hand col.	2-25
A	ARIANE GOMES ET AL: "Harnessing Nanoparticles for Immunomodulation and Vaccines", VACCINES, vol. 5, no. 1, 14 February 2017 (2017-02-14), page 6, XP055580035, DOI: 10.3390/vaccines5010006 the whole document in particular abstract, table 1, figure 1, pages 4-5	1-25

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WANG ZHANTONG ET AL: "Functional ferritin nanoparticles for biomedical applications", FRONTIERS OF CHEMICAL SCIENCE AND ENGINEERING, HIGHER EDUCATION PRESS, HEIDELBERG, vol. 11, no. 4, 15 February 2017 (2017-02-15), pages 633-646, XP036409437, ISSN: 2095-0179, DOI: 10.1007/S11705-017-1620-8 [retrieved on 2017-02-15] whole document, in particular abstract, figures 1, 2 pages 635-636	1-25
A	LI C Q ET AL: "Ferritin nanoparticle technology... A new platform for antigen presentation and vaccine development", INDUSTRIAL BIOTECHNOLOGY, MARY ANN LIEBERT, US, vol. 2, no. 2, 17 July 2006 (2006-07-17), pages 143-147, XP002739855, ISSN: 1550-9087, DOI: 10.1089/IND.2006.2.143 pages 143-147	1-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2019/025377

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015183969 A1	03-12-2015	CA 2950085 A1	03-12-2015
		CN 106715474 A	24-05-2017
		EP 3148578 A1	05-04-2017
		US 2017202946 A1	20-07-2017
		WO 2015183969 A1	03-12-2015
WO 2013044203 A2	28-03-2013	CA 2849822 A1	28-03-2013
		CN 103957891 A	30-07-2014
		EP 2758038 A2	30-07-2014
		US 2014072958 A1	13-03-2014
		US 2014302079 A1	09-10-2014
		US 2017189518 A1	06-07-2017
		WO 2013044203 A2	28-03-2013