ALKYLATED INFLUENZA VACCINES

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application 62/038,753 filed August 18, 2014, which is incorporated by reference in its entirety.

BACKGROUND

[0002] Epidemic and pandemic influenza occurs annually and is a cause of significant morbidity and mortality worldwide. Influenza viruses are highly pleomorphic particles composed of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The HA mediates attachment of the virus to the host cell and viral-cell membrane fusion during penetration of the virus into the cell. Therefore, an influenza vaccine typically contains an effective amount of HA that matches the diseases strain to induce production of neutralizing antibodies against the disease strain.

[0003] Single-radial-immunodiffusion (SRID) assays have been used to determine the Hemagglutinin (HA) content in the influenza vaccine and to measure potency of influenza virus vaccines licensed by the Food and Drug Administration for use in the United States since 1978. Specifically, SRID determines HA content in an influenza vaccine by using specific anti-HA antibodies. Samples of vaccines are applied onto an agar plate containing a strain-specific antiserum. The plates are typically incubated in a moist chamber at room temperature to allow diffusion of the antigen. Reaction of the antigen with the antibody produces a zone of precipitation (which is in form of precipitation ring). The amount of HA in the vaccine samples can be quantified by comparing the ring diameters of samples with the diameters of known concentrations of the reference HA protein. A potency value for the vaccine tested can be obtained based on the amount of HA.

SUMMARY

[0004] The present disclosure provides, among other things, improved influenza vaccines having a remarkable ability to retain potency as measured by SRID upon storage. The present

disclosure is based in part on the surprising discovery that exposing HA antigens to alkylating agents (e.g., 2-Iodoacetamide (IOAM)) results in significantly reduced loss of HA potency as measured by SRID after storage. For example, as described in the Examples section, the present inventors have demonstrated that some IOAM treated HA antigens showed only approximately 1-5% loss in potency as measured by SRID assays after 6 months of storage. Without wishing to be bound by any particular theory, it is contemplated that a probable mechanism for the loss of HA potency over time is formation of intermolecular cysteine bonds. These covalent bonds prevent dissociation of HA rosette macrostructure upon zwittergent pretreatment and thereby restricting migration through the gel matrix, resulting in reduced interaction with the antisera. Treatment with alkylating agents results in alkylation of reactive cysteine groups of HA antigens, which prevents intermolecular disulfide bond formation. As a result, alkylated HA antigens provided by the present disclosure may better retain potency as measured by SRID after storage. Since SRID is one of the mostly commonly used measurements for determining the HA potency in an influenza vaccine and the only assay currently accepted by the FDA and other major health authorities, the present disclosure thus permits production of vaccines that ensure retention of required potency during the life of the vaccines.

[0005] In one embodiment, the present disclosure provides an influenza vaccine comprising a hemagglutinin containing one or more alkylated cysteine residues. In some embodiments, the hemagglutinin contains 1-5, 1-4, 1-3, 2-5, 2-4, 2-3, 3-5, 3-4, or 4-5 alkylated cysteine residues. In some embodiments, the hemagglutinin contains 1, 2, 3, 4, or 5 alkylated cysteine residues. In some embodiments, the one or more alkylated cysteine residues constitute about 1-50%, 1-40%, 1-30%, 1-20% or 1-10% of total cysteine residues present in the hemagglutinin. In some embodiments, the one or more alkylated cysteine residues constitute about 10%, 20%, 30%, 40% or 50% of total cysteine residues present in the hemagglutinin. In some embodiments, substantially all free cysteine residues are alkylated. In some embodiments, the alkylated cysteine residues comprise methylated cysteine residues. In some embodiments, the hemagglutinin does not have non-specific alkylation and/or ubiquitous methylation. In some embodiments, the alkylated cysteine residues are determined by mass spectrometry.

[0005a] In one aspect, there is provided an influenza vaccine comprising a hemagglutinin containing one or more alkylated cysteine residues, wherein the influenza vaccine is substantially free of alkylating agent and retains at least about 90% potency as determined by Single Radial Immunodiffusion (SRID) assay upon storage of about 6 months at a temperature ranging from 1-30 °C.

[0006] In another embodiment, the present disclosure provides an influenza vaccine comprising a hemagglutinin treated with an alkylating agent. As used herein, an alkylating agent suitable for the present disclosure may be any compound capable of alkylating cysteine sulfhydryl groups. In

some embodiments, the alkylating agent is selected from the group consisting of 2-iodoacetamide (IOAM), iodoacetic acid, 2-bromoacetamide, glutathione bromoacetamide, bromoacetic acid, 1,3-propane sultone, methyl methanethiosulfonate, methoxycarbonylmethyl disulfide, maleamide, maleimide-PEG, vinylpyridine, N-ethylmaleimide, tosylacetamide, and combination thereof. In some embodiments, the alkylating agent is 2-iodoacetamide (IOAM).

[0006a] In another aspect, there is provided an influenza vaccine comprising a hemagglutinin treated with an alkylating agent, wherein the influenza vaccine is substantially free of the alkylating agent and retains at least about 90% potency as determined by Single Radial Immunodiffusion (SRID) assay upon storage of about 6 month at a temperature ranging from 1-30 °C.

[0007] In various embodiments, a hemagglutinin suitable for the present disclosure is an H1 protein present in a California or Solomon strain, a B protein present in a Brisbane, Florida, Ohio, Jiangsu or Hong Kong strain, and/or or a H3 protein present in a Victoria, Perth, Bristane or Wisconsin strain.

[0008] In some embodiments, an influenza vaccine provided by the present disclosure is a split influenza virus vaccine. In some embodiments, an influenza vaccine provided by the present disclosure is a recombinant influenza vaccine. In some embodiments, an influenza vaccine provided by the present disclosure is an egg based influenza vaccine. In some embodiments, an influenza vaccine provided by the present disclosure is produced in animal cells, plant cells, yeast cells, viral cells, fungus or algae, or synthetic cells. In some embodiments, suitable animal cells include, but are not limited to, insect cells, mammalian cells, and avian cells such as embryonated eggs, chichen embryo cells, and duck cell lines.

[0009] In some embodiments, an influenza vaccine provided by the present disclosure is a monovalent, divalent, trivalent or quadrivalent influenza vaccine.

[0010] In some embodiments, an influenza vaccine provided by the present disclosure retains at least about 90% (e.g., at least about 95%, 96%, 97%, 98%, 99%) potency as determined by Single Radial Immunodiffusion (SRID) assay upon storage of about 6 month at a temperature ranging from 1-30 °C.

[0011] In some embodiments, an influenza vaccine provided by the present disclosure is substantially free of the alkylating agent.

[0012] In yet another embodiment, the present disclosure provides a method of alkylating a hemagglutinin comprising a step of treating a purified hemagglutinin antigen or a hemagglutinin-containing viral particle with an alkylating agent. In some embodiments, a hemagglutinin-containing viral particle suitable for the present disclosure is a split virus. In some embodiments,

a purified hemagglutinin antigen suitable for the present disclosure is a purified surface antigen. In some embodiments, a purified hemagglutinin antigen suitable for the present disclosure is a purified recombinant hemagglutinin protein. In some embodiments, a hemagglutinin suitable for the present disclosure is selected from an H1 protein present in a California or Solomon strain, a B protein present in a Brisbane, Florida, Ohio, Jiangsu or Hong Kong strain, or a H3 protein present in a Victoria, Perth, Bristane or Wisconsin strain.

[0012a] In another aspect, there is provided a method of alkylating a hemagglutinin comprising a step of treating a purified hemagglutinin antigen or a hemagglutinin-containing viral particle with an alkylating agent, wherein: the hemagglutinin is selected from an H1 protein present in a California or Solomon strain, a B protein present in a Brisbane, Florida, Ohio, Jiangsu or Hong Kong strain, or an H3 protein present in a Victoria, Perth, Bristane or Wisconsin strain.

[0013] In another aspect, there is provided a method of manufacturing an influenza vaccine comprising a step of treating a viral particle obtained from an influenza virus produced from an egg based or cell culture based production system with an alkylating agent. In some embodiments, a viral particle suitable for the present disclosure is obtained by splitting the influenza virus.

[0014] In a further embodiment, the present disclosure provides a method of manufacturing an influenza vaccine, comprising: harvesting influenza virus from an egg based or cell culture based production system; splitting the influenza virus; treating the split influenza virus with an alkylating agent; and inactivating the treated split influenza virus.

[0014a] In another aspect, there is provided a method of manufacturing an influenza vaccine, comprising

harvesting influenza virus from an egg based or cell culture based production system;

splitting the influenza virus, by treating the influenza virus with a detergent, selected from the group consisting of Triton®, sodium taurodeoxycholate, nonylphenol ethoxylate, cetyltrimethylammonium bromide (CTAB), and sodium deoxycholate;

treating the split influenza virus with an alkylating agent selected from the group consisting of 2-iodoacetamide (IOAM), iodoacetic acid, 2-bromoacetamide, glutathione bromoacetamide, bromoacetic acid, 1,3-propane sultone, methyl methanethiosulfonate, methoxycarbonylmethyl disulfide, maleamide, maleimide-PEG, vinylpyridine, N-ethylmaleimide, tosylacetamide, and combination thereof, and

inactivating the treated split influenza virus; and wherein the influenza vaccine is substantially free of the alkylating agent and retains at least about 90% potency as determined by Single Radial Immunodiffusion (SRID) assay upon storage of about 6 month at a temperature ranging from 1-30 °C.

[0015] In various embodiments, the splitting step comprises treating the influenza virus with a detergent. In some embodiments, a suitable detergent is selected from Triton® (a nonionic surfactant that has a hydrophilic polyethylene chain), sodium taurodeoxycholate, nonylphenol ethoxylate, cetyltrimethylammonium bromide (CTAB), and/or sodium deoxycholate. In some embodiments, the detergent is Triton® (e.g., Triton® X-100, Triton® N-101, Triton® 720 and/or Triton® X-200).

[0016] In some embodiments, a suitable alkylating agent is selected from the group consisting of 2-iodoacetamide (IOAM), iodoacetic acid, 2-bromoacetamide, glutathione bromoacetamide, bromoacetic acid, 1,3-propane sultone, methyl methanethiosulfonate, methoxycarbonylmethyl disulfide, maleamide, maleimide-PEG, vinylpyridine, N-ethylmaleimide, tosylacetamide, and combination thereof. In some embodiments, a suitable alkylating agent is 2-iodoacetamide (IOAM).

[0017] In some embodiments, an influenza virus suitable for the present disclosure is produced in the egg based production system. In some embodiments, a suitable influenza virus is produced in animal cells, plant cells, yeast cells, viral cells, fungus or algae. In some

treating with the alkylating agent results in alkylation of 1-5, 1-4, 1-3, 2-5, 2-4, 2-3, 3-5, 3-4, or 4-5 cysteine residues. In some embodiments, the step of treating with the alkylating agent results in alkylation of 1, 2, 3, 4, or 5 cysteine residues. In some embodiments, the step of treating with the alkylating agent results in alkylation of about 1-50%, 1-40%, 1-30%, or 1-20% total cysteine residues present in the hemagglutinin. In some embodiments, the step of treating with the alkylating agent results in alkylation of about 10%, 20%, 30%, 40% or 50% total cysteine residues present the hemagglutinin. In some embodiments, the step of treating with the alkylating agent results in alkylation of substantially all free cysteine residues present in the hemagglutinin. In some embodiments, treatment with the alkylating agent does not result in non-specific alkylation. In some embodiments, treatment with the alkylating agent does not result in ubiquitous methylation.

[0023] In various embodiments, treatment with the alkylating agent results in reduced potency loss of the influenza vaccine determined by Single Radial Immunodiffusion (SRID) as compared to an otherwise identical influenza vaccine without the treatment of the alkylating agent.

[0024] In some embodiments, treatment with the alkylating agent results in no more than about 50% (e.g., no more than about 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1%) potency loss determined by SRID when the influenza vaccine is stored at 1-30 °C for 30 days.

[0025] In some embodiments, the step of treating with the alkylating agent is performed prior to a step of inactivating the influenza virus. In other embodiments, the step of treating with the alkylating agent is performed concurrently with a step of inactivating the influenza virus.

[0026] In some embodiments, the inactivating step comprises treating the influenza virus with ultraviolet light or a chemical inactivating agent. In some embodiments, the inactivating step comprises treating the influenza virus with a chemical inactivating agent selected from beta-propiolactone, sodium deoxycholate, and/or formalin. In particular embodiments, the inactivating step comprises treating the influenza virus with formalin.

[0027] In some embodiments, a method according to the present disclosure further includes a step of removing the alkylating agent. In some embodiments, the alkylating agent is removed by diafiltration. In some embodiments, the alkylating agent is removed together with the chemical inactivating agent.

[0028] In another aspect, there is provided an influenza vaccine manufactured according to a method described herein. In various embodiments, an influenza vaccine provided by the present disclosure is a monovalent, divalent, trivalent or quadrivalent influenza vaccine. In various embodiments, an influenza vaccine of the present disclosure can be provided as a single-dose, multi-dose, adult-dose or pediatric-dose vaccine. In some embodiments, each individual dose may be presented in a vial, a syringe including a pediatric syringe, or other type of container. In various embodiments, an influenza vaccine of the present disclosure may be formulated for intradermal, intramuscular, subcutaneous, intravenous, and other mode of administration. Additional dosing, formulations, administration routes are described throughout the specification, for example, in the "Immunogenic Vaccine Compositions" section.

[0029] As used herein, "potency" or "stability" as determined by a Single Radial Immunodiffusion (SRID) assay encompasses "apparent potency" or "apparent stability", respectively.

[0030] Other features, objects, and advantages of the present invention are apparent in the detailed description, drawings and claims that follow. It should be understood, however, that the detailed description, the drawings, and the claims, while indicating embodiments of the present invention, are given by way of illustration only, not limitation. Various changes and modifications within the scope of the invention will become apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE FIGURES

[0031] The drawings are for illustration purposes only, not for limitation.

[0032] **Figure 1** depicts exemplary data illustrating the correlation of percent potency loss by SRID and free thiols in HA based on data from different strains.

[0033] **Figure 2** depicts exemplary data illustrating that cysteine residues are highly conserved among HA strains by bioinformatics analysis.

[0034] **Figure 3** depicts an exemplary scheme illustrating the involvement of cysteine in HA potency loss due to HA rosette formation.

We claim:

1. An influenza vaccine comprising a hemagglutinin containing one or more alkylated cysteine residues, wherein the influenza vaccine is substantially free of alkylating agent and retains at least about 90% potency as determined by Single Radial Immunodiffusion (SRID) assay upon storage of about 6 months at a temperature ranging from 1-30 °C.

2. The influenza vaccine of claim 1, wherein the hemagglutinin contains 1-5, 1-4, 1-3 or 1-2 alkylated cysteine residues.

3. The influenza vaccine of claim 1, wherein the one or more alkylated cysteine residues constitute about 1-50%, 1-40%, 1-30%, 1-20% or 1-10% of total cysteine residues present in the hemagglutinin.

4. The influenza vaccine of claim 1, wherein substantially all free cysteine residues are alkylated.

5. The influenza vaccine of any one of the preceding claims, wherein the alkylated cysteine residues comprise methylated cysteine residues, and/or wherein the hemagglutinin does not have non-specific alkylation and/or ubiquitous methylation.

6. The influenza vaccine of any one of the preceding claims, wherein the number of alkylated cysteine residues is determined by mass spectrometry.

7. An influenza vaccine comprising a hemagglutinin treated with an alkylating agent, wherein the influenza vaccine is substantially free of the alkylating agent and retains at least about 90% potency as determined by Single Radial Immunodiffusion (SRID) assay upon storage of about 6 month at a temperature ranging from 1-30 °C.

8. The influenza vaccine of claim 7, wherein the alkylating agent is selected from the group consisting of 2-iodoacetamide (IOAM), iodoacetic acid, 2-bromoacetamide, glutathione bromoacetamide, bromoacetic acid, 1,3-propane sultone, methyl methanethiosulfonate, methoxycarbonylmethyl disulfide, maleamide, maleimide-PEG, vinylpyridine, N-ethylmaleimide, tosylacetamide, and combination thereof.

9. The influenza vaccine of claim 7 or 8, wherein

(a) the hemagglutinin is an H1 protein present in a California or Solomon strain, a B protein present in a Brisbane, Florida, Ohio, Jiangsu or Hong Kong strain, and/or an H3 protein present in a Victoria, Perth, Bristane or Wisconsin strain, and/or

(b) the vaccine is a split influenza virus vaccine or a recombinant influenza vaccine, and/or

(c) the influenza vaccine is an egg based influenza vaccine or the influenza vaccine is produced in insect cells, mammalian cells, yeast cells, viral cells, fungus or algae, and/or

(d) the influenza vaccine is a monovalent, divalent, trivalent or quadrivalent influenza vaccine.

10. A method of alkylating a hemagglutinin comprising a step of treating a purified hemagglutinin antigen or a hemagglutinin-containing viral particle with an alkylating agent, wherein:

the hemagglutinin is selected from an H1 protein present in a California or Solomon strain, a B protein present in a Brisbane, Florida, Ohio, Jiangsu or Hong Kong strain, or an H3 protein present in a Victoria, Perth, Bristane or Wisconsin strain.

11. A method of manufacturing an influenza vaccine comprising a step of treating a viral particle obtained from an influenza virus produced from an egg based or cell culture based production system with an alkylating agent.

12. The method of claim 11, wherein the viral particle is obtained by splitting the influenza virus.

13. A method of manufacturing an influenza vaccine, comprising

harvesting influenza virus from an egg based or cell culture based production system;

splitting the influenza virus, by treating the influenza virus with a detergent, selected from the group consisting of Triton®, sodium taurodeoxycholate, nonylphenol ethoxylate, cetyltrimethylammonium bromide (CTAB), and sodium deoxycholate;

treating the split influenza virus with an alkylating agent selected from the group consisting of 2-iodoacetamide (IOAM), iodoacetic acid, 2-bromoacetamide, glutathione bromoacetamide, bromoacetic acid, 1,3-propane sultone, methyl

methanethiosulfonate, methoxycarbonylmethyl disulfide, maleamide, maleimide-PEG, vinylpyridine, N-ethylmaleimide, tosylacetamide, and combination thereof, and

inactivating the treated split influenza virus; and

wherein the influenza vaccine is substantially free of the alkylating agent and retains at least about 90% potency as determined by Single Radial Immunodiffusion (SRID) assay upon storage of about 6 month at a temperature ranging from 1-30 °C.

14. The method of claim 13, wherein the alkylating agent is 2-iodoacetamide (IOAM).

15. The method of any one of claims 11-14, wherein the influenza virus is produced in the egg based production system or in animal cells, insect cells, mammalian cells, avian cells, plant cells, yeast cells, viral cells, fungus, algae, or synthetic cells.

16. The method of any one of claims 11-15, wherein

(a) the step of treating comprises incubating the purified hemagglutinin antigen, the hemagglutinin-containing viral particle, the viral particle, or the split influenza virus with the alkylating agent at room temperature, and/or a temperature ranging between 1-30 °C, 1-5 °C, 2-8 °C, 5-10 °C, 10-15 °C, 15-20 °C, 20-25 °C, or 25-30 °C, and/or

(b) the step of treating comprises incubating the purified hemagglutinin antigen, the hemagglutinin-containing viral particle, the viral particle, or the split influenza virus with the alkylating agent for up to about 24, 20, 18, 16, 14, 12, 10, 8, 6, 4, 2, 1.5 hours, 1 hour, 50 minutes, 40 minutes, 30 minutes, 20 minutes, or 10 minutes, and/or

(c) the alkylating agent is present at a concentration of more than 1X molar excess to hemagglutinin (HA)-cysteine concentration, or a concentration ranging from 1X to 100X, from 5X to 85X, from 1X to 50X, or from 5X to 50X molar excess to hemagglutinin (HA)-cysteine concentration, or a concentration ranging from 1 mM – 1 M, 1 mM – 500 mM, 1 mM – 400 mM, 1 mM – 300 mM, 1 mM – 200 mM, or 1 mM – 100 mM, or a concentration of about 1 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 100 mM, 200 mM, 300 mM, 400 mM, or 500 mM, or a concentration ranging from 1 µg/ml – 1,000 µg/ml, 1 µg/ml – 500 µg/ml, 1 µg/ml – 400 µg/ml, 1 µg/ml – 300 µg/ml, 1 µg/ml – 200 µg/ml, or 1 µg/ml – 100 µg/ml, or a concentration of about 1 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml, 70 µg/ml, 80 µg/ml, 90 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, or 500 µg/ml.

17. The method of any one of claims 11-16, wherein the step of treating with the alkylating agent results in alkylation of one of more cysteine residues of the hemagglutinin, or alkylation of 1-5, 1-4, 1-3 or 1-2 cysteine residues of the hemagglutinin, or alkylation of 1, 2, 3, 4, or 5 cysteine residues of the hemagglutinin, or alkylation of about 1-50%, 1-40%, 1-30%, or 1-20% of total cysteine residues present in the hemagglutinin, or alkylation of about 10%, 20%, 30%, 40% or 50% of total cysteine residues present in the hemagglutinin, or alkylation of substantially all free cysteine residues of the hemagglutinin, and/or

wherein treatment with the alkylating agent does not result in non-specific alkylation, and/or

wherein treatment with the alkylating agent does not result in ubiquitous methylation.

18. The method of any one of claims 13-17, wherein

(a) the step of treatment with the alkylating agent results in no more than 50% potency loss as determined by SRID when the influenza vaccine is stored at 1-30 °C for 30 days, no more than 30% potency loss as determined by SRID when the influenza vaccine is stored at 1-30 °C for 30 days, or no more than 10% potency loss as determined by SRID when the influenza vaccine is stored at 1-30 °C for 30 days, as compared to an otherwise identical influenza vaccine without the treatment of the alkylating agent, and/or

(b) the step of treating with the alkylating agent is performed prior to or concurrently with a step of inactivating the influenza virus, and/or

(c) the inactivating step comprises treating the influenza virus with ultraviolet light or a chemical inactivating agent, beta-propiolactone, sodium deoxycholate, and/or formalin.

19. The method of any one of claims 11-18, wherein the method further comprises a step of removing the alkylating agent by diafiltration, and/or the alkylating agent is removed together with the chemical inactivating agent.

20. The method of any one of claims 11-19, wherein the influenza vaccine is a monovalent, divalent, trivalent or quadrivalent influenza vaccine.

21. An influenza vaccine manufactured according to a method of any one of claims 11-20.