Culture methods for virally-infected cells

Cross-reference to related applications

The present application claims priority from United States Provisional Patent Application No. 63/562,853 filed on 8 March 2024, the contents of which is incorporated herein by reference in its entirety.

Technical field

The present disclosure relates to the field of methods for culturing virally-infected cells. More particularly, this disclosure relates to methods of culturing cells with an influenza virus.

Background

More recent vaccine production methods may include the culturing of viruses for vaccines in cell culture, such as in Madin Darby Canine Kidney (MDCK) cells. However, such methods may suffer from several drawbacks, including low yields of viral proteins and high levels of contaminating proteins, such as host cell proteins. Accordingly, there remains a need for cell culture methods for propagating viruses for vaccine production that can generate high yields of viral proteins, whilst minimising the inclusion of contaminating proteins.

Summary

The present disclosure is based on the surprising finding that modifying a number of cell culture conditions, such as pH, virus dose and trypsin concentration, can be utilised to improve yield and/or purity of influenza viruses cultured on MDCK cells, as determined by haemagglutinin levels at the end of infection.

In a first aspect, the present disclosure provides a method of determining culture conditions for cells infected with an influenza virus, said method including the steps of:

(a) incubating the influenza virus-infected cells in a plurality of test culture media in which one or more culture conditions thereof are varied between the respective plurality of test culture media, wherein the one or more culture conditions are selected from a pH, a trypsin concentration and a combination thereof; and

(b) selecting the one or more culture conditions that optimise yield and/or purity of the influenza virus.

In particular examples, varying the one or more culture conditions between the respective plurality of the test culture media comprises:

(i) increasing the trypsin concentration from a baseline level to a maximum level thereof; and/or

(ii) decreasing the pH from a baseline level to a minimum level thereof;

in the plurality of the test culture media.

For some examples, the trypsin concentration is increased to the maximum level of 10000 U/L and/or the pH is decreased to the minimum level of 6.0 in the plurality of the test culture media.

In other examples, the pH is varied within a pH range of between about 6.0 to about 8.0. More particularly, the pH can be varied within a pH range of about 6.5 to about 7.5. Even more particularly, the pH can be varied within a pH range of about 6.7 to about 7.3.

According to certain examples, the trypsin concentration is varied within a concentration range of between about 2000 U/L to about 10000 U/L. More particularly, the trypsin concentration can be varied within a concentration range of between about 4000 U/L to about 8000 U/L.

Suitably, the cells are incubated with a virus dose of between about 10^{-8} and about 10.

For various examples of the present method, step (b) comprises selecting the one or more culture conditions that optimise yield and/or purity of a protein produced by the influenza virus. In such examples, the protein is suitably a haemagglutinin protein.

In a second aspect, the present disclosure provides a method of culturing cells infected with an influenza virus, said method including the step of incubating the influenza virus-infected cells at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of at least about 5000 U/L.

The method of the present aspect may further include the step of harvesting the influenza virus and/or an influenza virus protein from the culture media.

In a third aspect, the present disclosure provides a method of producing an influenza virus and/or an influenza virus protein, said method of including the steps of:

(a) incubating cells infected with an influenza virus at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of at least about 5000 U/L; and

(b) harvesting the influenza virus and/or the influenza virus protein produced thereby from the culture media.

Referring to the methods of the second and third aspects, the influenza virus-infected cells are suitably cultured at a pH of about 6.8 to about 7.1.

For the methods of the second and third aspects, the culture media suitably contains trypsin at a concentration of between about 5000 U/L to about 10000 U/L.

In certain examples of the second and third aspects, the cells can be incubated or infected with a virus dose of between about 10^{-8} and about 10.

In other examples of the second and third aspects, the method may further include the step of purifying the influenza virus and/or the influenza virus protein harvested from the culture media.

Suitably, the influenza virus protein of the two aforementioned aspects is a haemagglutinin protein. In such examples, the haemagglutinin protein harvested from the culture media can have a purity level of at least about 10% per total protein concentration thereof. More particularly, the haemagglutinin protein can be present in the culture media at a concentration of at least about 10 mg/L.

Suitably, the influenza virus of the above aspects is an influenza A virus, such as an influenza A virus of a H1 or H3 subtype, or more particularly a H3N2 strain or a H1N1 strain.

Suitably, the cells of the above aspects are or comprise MDCK cells.

Suitably, the incubating step of the above aspects is performed for about 48 hours to about 96 hours.

In a fourth aspect, the present disclosure provides an influenza virus or influenza virus protein produced by the method of the second or third aspects.

In a fifth aspect, the present disclosure provides a vaccine composition comprising the influenza virus protein of the fourth aspect and a pharmaceutically acceptable carrier, diluent or excipient.

In a sixth aspect, the present disclosure provides a method of eliciting an immune response in a subject, said method including the step of administering a therapeutically effective amount of the influenza virus protein of the fourth aspect or the vaccine composition of the fifth aspect to the subject to thereby elicit the immune response in the subject.

In a seventh aspect, the present disclosure provides a method of preventing and/or treating an influenza-associated disease, disorder or condition in a subject, said method including the step of administering a therapeutically effective amount of the influenza virus protein of the fourth aspect or the vaccine composition of the fifth aspect to the subject to thereby prevent and/or treat the influenza-associated disease, disorder or condition.

In an eighth aspect, the present disclosure provides a method of culturing cells infected with an influenza virus, said method including the step of culturing the influenza virus-infected

cells in a culture media containing trypsin at the one or more culture conditions selected in step (b) of the method of the first aspect.

Brief description of the drawings

The following figures form part of the present specification and are included to further demonstrate certain aspects of the present disclosure. The disclosure may be better understood by reference to one or more of these figures in combination with the detailed description of specific embodiments presented herein. It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the above-described embodiments, without departing from the broad general scope of the present disclosure. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Figure 1. Model prediction for A/Florida/50/2022.

Figure 2. Model prediction for A/Michigan/41/2022.

Figure 3. Model prediction for A/Darwin/11/2021 CVR-88.

Figure 4. Model prediction for A/Canberra/407/2019.

Figure 5. Model prediction for A/NorthCarolina/01/2021.

Figure 6. Model prediction for A/Washington/19/2020.

Figure 7. Model prediction for B/Philippines/28/2019.

Figure 8. Model prediction for B/Connecticut/01/2021.

Figure 9. Model prediction for A/Sydney/5/2021.

Figure 10. Model prediction for A/Victoria/10/2020.

Figure 11. Model prediction for B/South Australia/06/2019.

Detailed description

General Techniques and Definitions

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in genomics, immunology, molecular biology, immunohistochemistry, biochemistry, oncology, and pharmacology).

The present disclosure is performed without undue experimentation using, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant

DNA technology and immunology. Such procedures are described, for example in Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Fourth Edition (2012), whole of Vols I, II, and III; DNA Cloning: A Practical Approach, Vols. I and II (D. N. Glover, Second Edition., 1995), IRL Press, Oxford, whole of text; Oligonucleotide Synthesis: A Practical Approach (M. J. Gait, ed, 1984) IRL Press, Oxford, whole of text, and particularly the papers therein by Gait, ppl-22; Atkinson *et al*, pp35-81; Sproat *et al*, pp 83-115; and Wu *et al*, pp 135-151; 4. Nucleic Acid Hybridization: A Practical Approach (B. D. Hames & S. J. Higgins, eds., 1985) IRL Press, Oxford, whole of text; Immobilized Cells and Enzymes: A Practical Approach (1986) IRL Press, Oxford, whole of text; Perbal, B., A Practical Guide to Molecular Cloning (1984) and Methods In Enzymology (S. Colowick and N. Kaplan, eds., Academic Press, Inc.), whole of series.

Those skilled in the art will appreciate that the present disclosure is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure includes all such variations and modifications. The disclosure also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

The present disclosure is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the disclosure, as described herein.

Each feature of any particular aspect or embodiment or embodiment of the present disclosure may be applied *mutatis mutandis* to any other aspect or embodiment or embodiment of the present disclosure.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e., one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

As used herein, the singular forms of "a", "and" and "the" include plural forms of these words, unless the context clearly dictates otherwise. For example, a reference to "a bacterium" includes a plurality of such bacteria, and a reference to "an allergen" is a reference to one or more allergens.

The term "and/or", e.g., "X and/or Y" shall be understood to mean either "X and Y" or "X or Y" and shall be taken to provide explicit support for both meanings or for either meaning.

Throughout the present specification, various aspects and components of the disclosure can be presented in a range format. The range format is included for convenience and should not be interpreted as an inflexible limitation on the scope of the present disclosure. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range, unless specifically indicated. For example, description of a range such as from 1 to 5 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 5, from 3 to 5 etc., as well as individual and partial numbers within the recited range, for example, 1, 2, 3, 4, 5, 5.5 and 6, unless where integers are required or implicit from context. This applies regardless of the breadth of the disclosed range. Where specific values are required, these will be indicated in the specification.

Throughout this specification, the word "comprise' or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

By "consisting essentially of", in the context of an amino acid sequence, is meant the recited amino acid sequence together with an additional one, two or three amino acids at the N- or C-terminus.

The term "substantially" does not exclude "completely" (e.g., a composition which is "substantially free" from Y may be completely free from Y).

The term "about" in relation to a numerical value x is optional and means, for example, any number within 1, 5 or 10% of the referenced number. In certain embodiments, the term "about" encompasses the exact number recited.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

For the present disclosure, the database accession number or unique identifier provided herein for a gene, protein or virus strain, as well as the gene and/or protein sequence or sequences associated therewith, are incorporated by reference herein.

Methods of determining optimal culture conditions for virally-infected cells

The inventors have surprisingly shown that modifying particular culture conditions, such as one or more of pH, a virus dose and a trypsin concentration, for cells infected with an influenza virus can be utilised to optimise (i.e., improve or enhance) yield and/or purity of said virus at the end of the infection or incubation period.

Accordingly, in one form, the present disclosure provides a method of determining culture conditions for cells infected with an influenza virus, said method including the steps of:

(a) incubating the influenza virus-infected cells in a plurality of test culture media in which one or more culture conditions thereof are varied between the respective plurality of test culture media, wherein the one or more culture conditions are selected from a pH, a trypsin concentration and a combination thereof; and

(b) selecting the one or more culture conditions that optimise yield and/or purity of the influenza virus.

In a related form, the present disclosure provides a method of culturing cells infected with an influenza virus, said method including the step of culturing the influenza virus-infected cells in a culture media containing trypsin at the one or more culture conditions selected in step (b) of the above method. Such a method, for example, may include one or more additional culturing steps provided herein, such as harvesting the influenza virus and/or a protein produced thereby from the culture media.

For particular examples, the one or more culture conditions to be varied with the plurality of test culture media includes a pH or pH level thereof. Accordingly, the culturing of the cells after infection with the influenza virus is suitably carried out at a specific pH that is regulated within each of the test culture media. According to some examples, for the plurality of test culture media, the pH thereof is varied within a pH range of between about 6.0 to about 8.0 (e.g., a pH of about 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0 or any range therein), more particularly between about 6.5 to about 7.5, even more particularly between about 6.7 to about 7.3, or yet even more particularly between about 6.8 to about 7.2. In this regard, at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 etc) of the plurality of test culture media suitably have or are cultured at a different pH relative to the other test culture media. By way of example, the pH of a first test culture media can be about 6.5, the pH of a second test culture media can be about 7.0 and the pH of a third test culture media can be about 7.5.

It is envisaged, however, that two or more of the plurality of test culture media suitably have or are cultured at the same or substantially the same pH, whilst one or more of the other culture conditions described herein, such as the virus dose and/or the trypsin concentration, are varied or different between these respective test culture media. For example, a first test culture media may have a pH of about 6.5 and a trypsin concentration of about 5000 U/L, whilst a second test culture media may have a pH of about 6.5 and a trypsin concentration of about 7500 U/L.

The pH or pH level of the respective test culture media may be controlled through CO_2 gas transfer from the test culture media or to the test culture media. In other examples, an acid or a base, such as a buffer (e.g., sodium bicarbonate, HEPES), is added to the test culture media so as to facilitate the adjustment and/or maintenance of the pH thereof to a desired pH value. The cell culture yields and/or purities are typically maximised at an optimum pH, which is desirable to maintain during cell seeding and cell culture. Suitably, the pH of the test culture media is maintained or substantially maintained (e.g., maintained within ± 1%, 2.5%, 5% or 10% of the desired pH) for the entire period of incubation or at least about 70%, 75%, 80%, 85%, 90% or 95% of the period of incubation of the influenza virus-infected cells.

According to certain examples, the one or more culture conditions to be varied with the plurality of test culture media includes a trypsin concentration thereof. The term "trypsin", as used herein, generally refers to a proteolytic enzyme, and more particularly a serine protease, classified as EC 3.4.21.4. Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, normally except when either is followed by proline. Trypsin may be derived or isolated from natural and/or recombinant/synthetic sources. In particular examples, the trypsin described herein is recombinant trypsin. Although, *in vivo*, trypsin is formed together with a pro-peptide (i.e., trypsinogen), the term "trypsin", as used herein suitably refers to a mature trypsin protein devoid of any pro-peptide. The term "trypsin" also includes reference to any type or isoform of trypsin known in the art, such as trypsin 1, trypsin 2, trypsin 3, trypsin 3, trypsin 4, trypsin 5 and trypsin 6. The use of trypsin for proteolytic cleavage can also be referred to as "trypsin proteolysis" or "trypsinization", and proteins that result from cleavage with trypsin are said to have been "trypsinized".

Similar to that for pH, trypsin can be added to each of the test culture media so as to achieve a trypsin concentration that is specific or regulated within each of the test culture media. According to certain examples, for the plurality of test culture media, the trypsin concentration is varied within a concentration range of between about 1000 U/L to about 12000 U/L (e.g., about 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500, 4750, 5000, 5250, 5500, 5750, 6000, 6250, 6500, 6750, 7000, 7250, 7500, 7750, 8000, 8250, 8500, 8750, 9000, 9250, 9500, 9750, 10000, 10250, 10500, 10750, 11000, 11250, 11500, 11750, 12000 U/L or any range therein), more particularly between about 2000 U/L to about 10000 U/L, even more particularly between about 3000 U/L to about 9000 U/L, yet even more particularly between about 4000 U/L to about 8000 U/L, still even more particularly between about 5000 U/L to about 10000 U/L, even more particularly between about 4000 U/L to about 9000 U/L or even more particularly between about 4000 U/L to about

10000 U/L. By extension, for the plurality of test culture media, the trypsin concentration can varied within a concentration range of between about 10 mg/L to about 100 mg/L (e.g., about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 mg/L or any range therein), more particularly between about 20 mg/L to about 75 mg/L, even more particularly between about 30 mg/L to about 70 mg/L, even more particularly between about 35 mg/L to about 65 mg/L, even more particularly between about 20 mg/L to about 80 mg/L or even more particularly between about 30 mg/L to about 80 mg/L. To this end, at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 etc) of the plurality of test culture media suitably have or are cultured at a different trypsin concentration relative to the other test culture media. By way of example, the trypsin concentration of a first test culture media can be about 3500 U/L, the trypsin concentration of a second test culture media can be about 5500 U/L and the trypsin concentration of a third test culture media can be about 7500 U/L.

During incubation of the infected cells according to the methods of the present disclosure, trypsin reactivation can be carried out by the addition of further trypsin in the case of a batch process or in the case of a perfusion system by continuous or intermittent addition of a trypsin solution. To this end, incubating the cells by the methods provided herein, may include a substitution of at least part of the cell culture medium with freshly prepared medium, medium concentrate or with defined constituents, such as amino acids, vitamins, lipid fractions, phosphates, trypsin etc. for optimizing the yield and/or purity of the viral protein.

Again, it is envisaged that two or more of the plurality of test culture media suitably have or are cultured at the same or substantially the same trypsin concentration, whilst one or more of the other culture conditions described herein, such as the virus dose and/or the pH, are varied or different between these respective test culture media. For example, a first test culture media may have a trypsin concentration of about 5000 U/L and a pH of about 6.5, whilst a second test culture media may have a trypsin concentration of about 5000 U/L and a pH of about 7.0.

The addition of the trypsin, which functions to cleave the precursor protein of haemagglutinin (HA) and thus the adsorption of the influenza viruses on the cells, can be carried out shortly before, simultaneously to or shortly after the infection of the cells with the influenza virus. If the addition is carried out simultaneously to the infection step, trypsin can either be added directly to the test culture media or, for example, as a concentrate together with the influenza virus inoculate.

It will be appreciated by the skilled person that individual influenza virus strains may demonstrate a preferred or optimal virus dose at the time of infection of the cells. Determining

an optimal virus dose of an influenza virus strain can therefore be included as a standard step prior to commencing commercial vaccine production in cells, such as MDCK cells, based on the influenza virus strain in question. Accordingly, this step of determining an optimal virus dose may be incorporated into the methods described herein. As such, the one or more culture conditions in step (a) may further include a virus dose (e.g., a MOI value, "multiplicity of infection", which corresponds to the number of virus units per cell at the time of infection). As such, the one or more culture conditions of step (a) may be selected from a pH, a trypsin concentration, a virus dose and any combination thereof. To this end, the cells of each test culture media can suitably be infected with a virus dose or MOI of between about 10^{-10} and about 10 (e.g., a MOI of about 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 0.0001, 0.0005, 0.001, 0.005, 0,01, 0.05, 0.1, 0.5, 1, 5, 10 and any range therein). Moreover, at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 etc) of the plurality of test culture media suitably include a different virus dose relative to the other test culture media. By way of example, the virus dose of a first test culture media can be about 10^{-7}, the virus dose of a second test culture media can be about 10^{-6} and the virus dose of a third test culture media can be about 10^{-5}. In alternative examples, the virus dose of each of the test culture media is substantially the same or similar.

Similar to that for the culture conditions of pH and trypsin concentration, it is envisaged that two or more of the plurality of test culture media suitably have or are cultured at the same or substantially the same virus dose, whilst one or more of the other culture conditions described herein are varied or different between these respective test culture media. For example, a first test culture media may have a virus dose of about 10^{-6} and a pH of about 6.5, whilst a second test culture media may have a virus dose of about 10^{-6} and a pH of about 7.0.

In some examples, the one or more culture conditions to be varied with the plurality of test culture media includes a pH level and a trypsin concentration thereof. It is envisaged that this may include any combination of those examples of a pH level and a trypsin concentration (inclusive of ranges thereof) provided herein. For such examples, the pH is suitably varied within a pH range of between about 6.0 to about 8.0 and the trypsin concentration is suitably varied within a concentration range of between about 2000 U/L to about 10000 U/L. More particularly, the pH is suitably varied within a pH range of between about 6.5 to about 7.5 and the trypsin concentration is suitably varied within a concentration range of between about 3000 U/L to about 9000 U/L. Even more particularly, the pH is suitably varied within a pH range of between about 6.7 to about 7.3 and the trypsin concentration is suitably varied within a concentration range of between about 4000 U/L to about 8000 U/L.

In other examples, the one or more culture conditions to be varied with the plurality of

test culture media includes a pH level and a virus dose thereof. It is envisaged that this may include any combination of those examples of a pH level and a virus dose (inclusive of ranges thereof) provided herein. For such examples, the pH is suitably varied within a pH range of between about 6.0 to about 8.0 and the virus dose is suitably varied within a concentration range of between about 10^{-8} and about 10. More particularly, the pH is suitably varied within a pH range of between about 6.5 to about 7.5 and the virus dose is suitably varied within a concentration range of between about 10^{-7} and about 1. Even more particularly, the pH is suitably varied within a pH range of between about 6.7 to about 7.3 and the virus dose is suitably varied within a concentration range of between about 10^{-6} and about 0.1.

In various examples, the one or more culture conditions to be varied with the plurality of test culture media includes a trypsin concentration and a virus dose thereof. It is envisaged that this may include any combination of those examples of a trypsin concentration and a virus dose (inclusive of ranges thereof) provided herein. For such examples, the trypsin concentration is suitably varied within a concentration range of between about 2000 U/L to about 10000 U/L and the virus dose is suitably varied within a concentration range of between about 10^{-8} and about 10. More particularly, the trypsin concentration is suitably varied within a concentration range of between about 3000 U/L to about 9000 U/L and the virus dose is suitably varied within a concentration range of between about 10^{-7} and about 1. Even more particularly, the trypsin concentration is suitably varied within a concentration range of between about 4000 U/L to about 8000 U/L and the virus dose is suitably varied within a concentration range of between about 10^{-6} and about 0.1.

In particular examples, the one or more culture conditions to be varied with the plurality of test culture media includes a pH level, a trypsin concentration and a virus dose thereof. It is envisaged that this may include any combination of those examples of a pH level, a trypsin concentration and a virus dose (inclusive of ranges thereof) provided herein. For such examples, the pH is suitably varied within a pH range of between about 6.0 to about 8.0, the trypsin concentration is suitably varied within a concentration range of between about 2000 U/L to about 10000 U/L and the virus dose is suitably varied within a concentration range of between about 10^{-8} and about 10. More particularly, the pH is suitably varied within a pH range of between about 6.5 to about 7.5, the trypsin concentration is suitably varied within a concentration range of between about 3000 U/L to about 9000 U/L and the virus dose is suitably varied within a concentration range of between about 10^{-7} and about 1. Even more particularly, the pH is suitably varied within a pH range of between about 6.7 to about 7.3, the trypsin concentration is suitably varied within a concentration range of between about 4000 U/L to

about 8000 U/L and the virus dose is suitably varied within a concentration range of between about 10^{-6} and about 0.1.

As demonstrated in the Examples, the present inventors have surprisingly shown that lower pH levels and/or higher trypsin concentrations during incubation of virally-infected cells typically increased or maximised HA protein purity at end of infection. As such, and according to certain examples, varying the one or more culture conditions between the respective plurality of the test culture media comprises: (i) increasing the trypsin concentration from a baseline level (e.g., a first level) to a maximum level (e.g., a second level) thereof; and/or (ii) decreasing the pH from a baseline level (e.g., a first level) to a minimum level (e.g., a second level) thereof; in the plurality of the test culture media. Accordingly, the present methods may include a first test culture media having a baseline level of trypsin concentration, a second test culture media having a maximum level of trypsin concentration and optionally one or a plurality of further test culture media having a level of trypsin concentration between the baseline level and the maximum level thereof. Similarly, the present methods may include a first test culture media having a baseline level of pH, a second test culture media having a minimum level of pH and optionally one or a plurality of further test culture media having a level of pH between the baseline level and the minimum level thereof.

Suitably, the baseline level, insofar as it relates to the trypsin concentration and the pH, is a standard, average, typical or accepted level of these cell culture conditions for incubating virally-infected cells. Similarly, in the context of the trypsin concentration and the pH, the maximum level is a level that is higher than the standard, average, typical or accepted level of these cell culture conditions for incubating virally-infected cells. Conversely, the minimum level is a level that is lower than the standard, average, typical or accepted level of these cell culture conditions for incubating virally-infected cells. By way of example, a baseline level for pH can be between about 7.0 to about 7.5 (e.g., about 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3, 7.35, 7.4, 7.45, 7.5 or any range therein) and a minimum level can be between about 6.0 to about 7.0 (e.g., about 6.0, 6.05, 6.1, 6.15, 6.2, 6.25, 6.3, 6.35, 6.4, 6.45, 6.5, 6.55, 6.6, 6.65, 6.7, 6.75, 6.8, 6.85, 6.9, 6.95, 7.0, or any range therein). Accordingly, varying the one or more culture conditions between the respective plurality of the test culture media comprises decreasing the pH from a baseline level of between about 7.0 to about 7.5, or more particularly between about 7.0 to about 7.3, to a minimum level of between about 6.0 to about 7.0, more particularly between about 6.5 to about 7.0, or even more particularly between about 6.5 to about 6.8, in the plurality of the test culture media. Additionally, a baseline level for the trypsin concentration can be between about 1000 U/L and about 5000 U/L (e.g., about 1000, 1500,

2000, 2500, 3000, 3500, 4000, 4500, 5000 U/L or any range therein) and a maximum level can be between about 5000 and about 12000 U/L (e.g., about 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, 10500, 11000, 11500, 12000 U/L or any range therein). Accordingly, varying the one or more culture conditions between the respective plurality of the test culture media comprises increasing the pH from a baseline level of between about 1000 U/L and about 5000 U/L, or more particularly between about 2000 U/L to about 4000 U/L, to a maximum level of between about 5000 U/L and about 12000 U/L, more particularly between about 5000 U/L and about 10000 U/L, or even more particularly between about 5000 U/L and about 8000 U/L, in the plurality of the test culture media.

With respect to assessing yield and/or purity of the influenza virus, this may be performed by any method or means known in the art. Suitably, step (b) of the present method includes selecting the one or more culture conditions that optimise (e.g., result in the highest level thereof) yield and/or purity of a protein produced by the influenza virus. For some examples, step (b) of the present method includes selecting the one or more culture conditions that optimise yield of a protein produced by the influenza virus. In these examples, the one or more culture conditions selected suitably also result in a suitable or appropriate level of purity of the protein, such as those levels provided herein. In other examples, step (b) of the present method includes selecting the one or more culture conditions that optimise purity of a protein produced by the influenza virus. For these examples, the one or more culture conditions selected suitably also result in a suitable or appropriate level of yield of the protein, such as those levels provided herein. In particular examples, the influenza virus protein to be assessed for yield and/or purity is a haemagglutinin protein.

The present method may therefore further include the step of measuring or assessing yield of an influenza virus protein for each of the one or more culture conditions tested thereby. Yield of the influenza virus protein may be measured by any means of protein quantification known in the art, such as by gel electrophoresis (e.g., Western Blot); by ELISA, or by chromatographic methods, such as HPLC (high performance liquid chromatograph), HPLC-UV or mass spectrometry methods, such as LC-MS (Liquid Chromatography-Mass Spectrometry).

Further to the above, a level of purity of the influenza virus and/or the influenza virus protein for each of the one or more culture conditions may be determined for the present method. To this end, an extract or isolate containing the isolated influenza virus protein will typically include impurities or contaminating proteins (e.g., non-viral proteins), such as host cell proteins derived from the cells. Purity of the isolated influenza virus protein may be

measured by any means known in the art. Exemplary methods may include calculating a ratio of a level, content or concentration of a particular influenza virus protein (e.g., haemagglutinin), such as determined by those methods described above, in the isolated influenza virus protein to a total protein content thereof. Alternatively, purity may be assayed by determining a ratio of a level, concentration or content of one or more host cell proteins in the isolated influenza virus protein to a total protein content thereof. The total protein content can be assessed, for example, by a bicinchoninic acid (BCA) assay, gel electrophoresis, such as by Bradford assay, SDS-PAGE and Western Blot, or Coomassie Stain; by ELISA; by chromatographic methods, such as HPLC; or by mass spectrometry methods. In particular examples, the purity of the isolated influenza virus protein is determined by the ratio of a level of a haemagglutinin protein thereof to a total protein content thereof. For such examples, the level of the haemagglutinin protein is suitably measured by HPLC and the total protein content is suitably measured by a BCA assay.

In various examples, purity of the isolated influenza virus and/or the isolated influenza virus protein is also assessed following harvesting, isolation, purification or separation thereof from the cells and/or the respective test culture media, such as by those methods described herein. To do this, for example, the cells or cell residues may be separated from the respective test culture media by methods known to the person skilled in the art, such as centrifugation, separators or filters. The influenza virus present in the test culture media may then be isolated, concentrated or harvested by methods known to the person skilled in the art, such as those described herein (e.g., gradient centrifugation, filtration, precipitation and the like). In particular examples, the influenza virus and/or one or more proteins produced thereby are isolated or harvested from the test culture media at least in part by gradient ultracentrifugation. Accordingly, a level of purity can be assessed in relation to the isolated influenza virus protein that has been at least partially purified or separated from the test culture media, such as by gradient ultracentrifugation.

In order to determine or select the one or more culture conditions that optimise yield and/or purity of the protein produced by the influenza virus, the present method may include comparing the obtained or measured levels of yield and/or purity of the respective culture conditions to one or more threshold levels thereof. In various examples, a level of yield and/or purity may be classified or selected as optimal if it exceeds a particular threshold level thereof. The nature and numerical value of the threshold level of purity or yield will typically vary based on the influenza virus protein in question and/or the method or means chosen to determine the level of purity or yield of that virus protein for the respective cell culture

conditions.

Referring to haemagglutinin, suitable threshold levels of yield for this viral protein can be at least about 10 mg/L (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 mg/L or any range therein), at least about 15 mg/L, at least about 20 mg/L or at least about 25 mg/L in the test culture media at the end of the infection or incubation period. Accordingly, the one or more culture conditions that produce a yield of haemagglutinin of at least about 10 mg/L (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 mg/L or any range therein), at least about 15 mg/L, at least about 20 mg/L or at least about 25 mg/L in the test culture media can be selected in step (b) of the present method as optimising yield. In certain examples, the one or more culture conditions that produce a yield of haemagglutinin of between about 10 mg/L and about 50 mg/L, between about 15 mg/L and about 45 mg/L, or between about 20 mg/L and about 40 mg/L in the test culture media can be selected as optimising yield.

Again, in relation to haemagglutinin, suitable threshold levels of purity for this viral protein can be at least about 10% per total protein concentration (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 %), at least about 15% per total protein concentration, at least about 20% per total protein concentration or at least about 25% per total protein concentration. Accordingly, the one or more culture conditions that produce a purity of haemagglutinin of at least about 10% per total protein concentration (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 % or any range therein), at least about 15% per total protein concentration, at least about 20% per total protein concentration or at least about 25% per total protein concentration can be selected in step (b) of the present method as optimising purity. In certain examples, the one or more culture conditions that produce a purity of haemagglutinin of between about 10% and about 50% of total protein concentration, between about 15% and about 45% of total protein concentration, or between about 20% and about 40% of total protein concentration can be selected as optimising purity.

In certain examples, the one or more culture conditions that produce a yield of haemagglutinin of between about 20 mg/L and about 50 mg/L in the test culture media and a purity of haemagglutinin of between about 20% and about 50% of total protein concentration can be selected as optimising yield and purity of the influenza virus.

It is envisaged that the present method may also include one or more of the following steps of:

(i) proliferation of the cells, such as in a culture medium described herein (e.g., in a serum-free medium in suspension) prior to infection with the influenza virus;

(ii) infection of the cells with the influenza virus;

(iii) addition of trypsin shortly before, simultaneously to or shortly after infection of the cells with the influenza virus; and

(iv) harvesting and isolation of the replicated influenza virus or a virally-derived protein following culturing or incubation of the infected cells; such as by those methods described herein.

Methods of culturing virally-infected cells and producing viral proteins

Based on the aforementioned methods of optimising culture conditions for virally-infected cells, the inventors have further surprisingly shown that culturing influenza virus-infected cells under specific conditions of pH, trypsin concentration and optionally virus dose can optimise yield and/or purity of influenza viruses based on virally-derived proteins, such as haemagglutinin, at end of infection.

Accordingly, in one form, the present disclosure provides a method of culturing cells infected with an influenza virus, said method including the step of incubating the influenza virus-infected cells at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of at least about 5000 U/L.

Suitably, the present method further includes the step of harvesting the influenza virus and/or an influenza virus protein from the culture media.

In a related form, the present disclosure provides a method of producing an influenza virus and/or an influenza virus protein, said method of including the steps of:

(a) incubating cells infected with the influenza virus at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of at least about 5000 U/L; and

(b) harvesting or isolating the influenza virus and/or the influenza virus protein from the culture media.

In one particular form, the present disclosure relates to a method of improving yield and/or purity in the manufacture of an influenza vaccine, said method including the steps of:

(a) incubating influenza virus-infected cells in a plurality of test culture media in which one or more culture conditions thereof are varied between the respective plurality of test culture

media, wherein the one or more culture conditions are selected from a pH, a trypsin concentration and a combination thereof;

(b) selecting the one or more culture conditions that optimise yield and/or purity of the influenza virus;

(c) culturing suspension mammalian cells capable of influenza virus infection in a serum free media; and

(d) infecting the mammalian cells with an influenza virus; and

(e) incubating the influenza virus-infected mammalian cells at the one or more culture conditions selected in (b).

In another related form, the present disclosure provides an influenza virus or an influenza virus protein produced according to the aforementioned methods.

To this end, the present methods may include harvesting or isolating intact or whole virions from the cell culture media. Alternatively or additionally, the present methods can include harvesting or isolating split virions from the culture media. In such examples, the harvesting or isolating step suitably includes contacting the influenza virus with a splitting agent, such as a detergent. The present methods may alternatively or additionally include harvesting or isolating one or more particular influenza virus proteins, such as haemagglutinin, from the culture media and/or from the harvested influenza virus, such as by affinity chromatography and/or gradient ultracentrifugation.

For the purposes of this disclosure, by "isolated" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material, such as influenza viruses and virally-derived proteins like haemagglutinin, may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

In some examples, the methods described herein further include the step of multiplication or proliferation of the cells, such as in a culture medium described herein (e.g., in a serum-free medium in suspension) prior to infection with the influenza virus. Multiplication of the cells prior to viral infection can be conducted in accordance with methods known to those of skill in the art. For example, the cells can be cultivated in a perfusion system or a fed-batch system before infection. In the context of the present disclosure, a culture system is referred to as a fed-batch system in which the cells are initially cultured in a batch system and depleted at least partly of nutrients and then grown in a culture medium, which is

compensated by controlled feeding of concentrated nutrients. The pH value of the medium during multiplication of the cells before viral infection is suitably between pH 6.6 and pH 7.8 and more particularly in a range between pH 7.2 and pH 7.3. Following infection with the influenza virus, the cells are suitably cultured at a pH of about 6.7 to about 7.3 (e.g., about 6.7, 6.75, 6.8, 6.85, 6.9, 6.95, 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3 and any range therein) or more particularly at a pH of about 6.8 to about 7.1. Culturing of the cells to be infected with the influenza virus typically occurs at a temperature between about 30°C and about 37°C (e.g., 30, 31, 32, 33, 34, 35, 36, 37 °C and any range therein) and more particularly at a temperature of about 37°C. After infection with the influenza virus, the cells can then be cultured at a temperature described herein (e.g., between about 30°C and about 36°C or between about 32°C and about 34°C or at about 33°C).

As noted above, it is known in the art that individual virus strains may demonstrate a preferred or optimal virus dose at the time of infection of the cells. Accordingly, step (a) may further include incubating or contacting the cells with a virus dose or MOI of between about 10^{-10} and about 10 (e.g., a MOI of about 10^{-10}, 10^{-9},10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 0.0001, 0.0005, 0.001, 0.005, 0,01, 0.05, 0.1, 0.5, 1, 5, 10 and any range therein). In particular examples, the cells are incubated with a virus dose or MOI of between about 10^{-7} and about 1 or more particularly between about 10^{-6} and about 0.1. As such, the present method may include the steps of contacting the cells with a virus dose or MOI of between about 10^{-8} and about 10 then incubating or culturing the infected cells at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of at least about 5000 U/L. In other examples, the present method includes the steps of contacting the cells with a virus dose or MOI of between about 10^{-8} and about 10 then incubating or culturing the infected cells at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of between about 5000 U/L and about 12000 U/L. In various examples, the present method includes the steps of contacting the cells with a virus dose or MOI of between about 10^{-8} and about 10 then incubating or culturing the infected cells at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of between about 5000 U/L and about 10000 U/L (e.g., about 5000, 5250, 5500, 5750, 6000, 6250, 6500, 6750, 7000, 7250, 7500, 7750, 8000, 8250, 8500, 8750, 9000, 9250, 9500, 9750 or 10000 U/L). More particularly, the present method may include the steps of contacting the cells with a virus dose or MOI of between about 10^{-7} and about 1 then incubating or culturing the infected cells at a pH of about 6.7 to about 7.2 and in a culture media containing trypsin at a concentration of between about 5000 U/L and about 8000 U/L. More particularly, the present method may include the steps of contacting the cells with a virus dose or MOI of

between about 10^{-6} and about 0.1 then incubating or culturing the infected cells at a pH of about 6.8 to about 7.1 and in a culture media containing trypsin at a concentration of between about 5000 U/L and about 7500 U/L

The infected cells are suitably incubated under conditions that permit replication of the influenza virus. In this regard, the oxygen partial pressure can also be adjusted during culturing before infection, such as at a value between about 25% and about 95% (e.g., about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or any range therein) and more particularly at a value between about 35% and about 60%. The values for these oxygen partial pressures are suitably based on saturation of air.

Similar to the aforementioned methods, trypsin can be added to the culture media so as to achieve a trypsin concentration that is specific or regulated within each of the test culture media, as previously described herein. Accordingly, in some examples, the present methods include the step of adding trypsin to the culture media shortly before, simultaneously to or shortly after infection of the cells with the influenza virus. Trypsin is suitably added to the culture media to achieve a concentration of between about 5000 U/L to about 12000 U/L (e.g., about 5000, 5250, 5500, 5750, 6000, 6250, 6500, 6750, 7000, 7250, 7500, 7750, 8000, 8250, 8500, 8750, 9000, 9250, 9500, 9750, 10000, 10250, 10500, 10750, 11000, 11250, 11500, 11750, 12000 U/L or any range therein) or between about 27.5 mg/L to about 100 mg/L (e.g., about 27.5, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 mg/L or any range therein). In some examples, trypsin is added to the culture media to achieve a concentration of at least about 27.5 mg/L. In particular examples, trypsin has been added or is present in the culture media at a concentration of between about 5000 U/L to about 10000 U/L, between about 5000 U/L to about 9000 U/L, between about 5000 U/L to about 8000 U/L, between about 5000 U/L to about 7000 U/L, between about 6000 U/L to about 10000 U/L, between about 6000 U/L to about 9000 U/L, between about 6000 U/L to about 8000 U/L or between about 6000 U/L to about 7000 U/L. In other examples, trypsin has been added or is present in the culture media at a concentration of between about 5000 U/L to about 6000 U/L. In some examples, trypsin has been added or is present in the culture media at a concentration of between about 7000 U/L to about 9000 U/L. In various examples, trypsin has been added or is present in the culture media at a concentration of about 5000 U/L. In certain examples, trypsin has been added or is present in the culture media at a concentration of about 7500 U/L.

As is evident from the examples herein, the trypsin concentration necessary to achieve optimal or suitable levels of haemagglutinin purity and/or yield may vary between viral strains and with other cell culture conditions (e.g., pH, virus dose, incubation period). According to

certain examples, step (a) of the present method includes incubating the cells infected with the influenza virus at a pH of about 6.7 to about 7.3 (e.g., about 6.7, 6.75, 6.8, 6.85, 6.9, 6.95, 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3 and any range therein) and in a culture media containing trypsin at a concentration of between about 5000 U/L and about 12000 U/L (e.g., about 5000, 5250, 5500, 5750, 6000, 6250, 6500, 6750, 7000, 7250, 7500, 7750, 8000, 8250, 8500, 8750, 9000, 9250, 9500, 9750, 10000, 10250, 10500, 10750, 11000, 11250, 11500, 11750, 12000 U/L or any range therein) or between about 27.5 mg/L to about 100 mg/L (e.g., about 27.5, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 mg/L or any range therein). More particularly, step (a) of the present method suitably includes incubating the cells infected with the influenza virus at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of between about 5000 U/L and about 10000 U/L. Even more particularly, step (a) of the present method suitably includes incubating the cells infected with the influenza virus at a pH of about 6.7 to about 7.2 and in a culture media containing trypsin at a concentration of between about 5000 U/L and about 8000 U/L. Yet even more particularly, step (a) of the present method suitably includes incubating the cells infected with the influenza virus at a pH of about 6.8 to about 7.1 and in a culture media containing trypsin at a concentration of between about 5000 U/L and about 7500 U/L.

It is envisaged that the step of harvesting or isolating the replicated influenza virus and/or one or more proteins produced thereby can be carried out about 24 hours to about 240 hours (e.g., about 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204, 216, 228, 240 hours or any range therein), more particularly about 36 hours to about 96 hours, even more particularly about 48 hours to about 72 hours, or yet even more particularly about 60 hours to about 72 hours, after infection or incubation of the cells with the influenza virus. In particular examples, the step of harvesting or isolating the replicated influenza virus is conducted about 60 hours after infection or incubation of the cells with the influenza virus. In other examples, the step of harvesting or isolating the replicated influenza virus is conducted about 72 hours after infection or incubation of the cells with the influenza virus.

During harvesting or isolation of the influenza virus and/or the influenza virus proteins in step (b), the cells may be separated from the culture medium by standard methods like centrifugation, separation, filtration or ultrafiltration. The influenza virus or the viral proteins produced therefrom can be then concentrated and/or purified according to methods known to those skilled in the art, such as gradient centrifugation (e.g., gradient ultracentrifugation (GUC)), filtration, precipitation, chromatography, and any combination thereof. In particular examples, the influenza virus and/or one or more proteins produced thereby are isolated or

harvested from the culture media by gradient ultracentrifugation. Suitably, the influenza viruses are inactivated during or after purification. Virus inactivation can occur, for example, by the contacting the influenza virus with an inactivating agent (e.g., addition of b-propiolactone or formaldehyde) at any point within the purification process.

Suitably, the current methods achieve particular levels (e.g., threshold levels) of yield and/or purity with respect to the harvested or isolated influenza virus and/or influenza virus protein. Accordingly, the present methods may further include the step of measuring or assessing yield and/or purity for the influenza virus and/or the influenza virus protein harvested or isolated from the culture media at end of infection. To this end, the assessment with respect to yield and/or purity of the isolated influenza virus can be based at least partly on a particular influenza virus protein, such as a haemagglutinin protein, as described in more detail herein.

In particular examples, the influenza virus harvested or isolated in step (b) has a purity level (%) based on a haemagglutinin protein of at least about 10% (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 % or any range therein), at least about 15%, at least about 20%, or at least about 25% per total protein concentration thereof. According to certain examples, the influenza virus harvested or isolated in step (b) has a purity level (%) of a haemagglutinin protein of between about 10% and about 50% of total protein concentration, between about 15% and about 45% of total protein concentration, or between about 20% and about 40% of total protein concentration.

Referring to yield and following the incubation step (a), a haemagglutinin protein is suitably present in the culture media at a concentration of at least about 10 mg/L (e.g., about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 mg/L or any range therein), at least about 15 mg/L, at least about 20 mg/L or at least about 25 mg/L at end of infection. As such, the incubation step (a) suitably produces a yield of a haemagglutinin protein of at least about 10 mg/L, at least about 15 mg/L, at least about 20 mg/L or at least about 25 mg/L in the culture media at end of infection. In various examples, the incubation step (a) produces a yield of a haemagglutinin protein of between about 10 mg/L and about 50 mg/L, between about 15 mg/L and about 45 mg/L, or between about 20 mg/L and about 40 mg/L in the culture media at end of infection.

Culture cells

Referring to the cells described herein, the present methods will typically use a cell line,

although primary cells may be used as an alternative. The cells will typically be mammalian. Suitable mammalian cells of origin include, but are not limited to, hamster, cattle, primate (including humans and monkeys) and dog cells. Various cell types may be used, such as kidney cells, fibroblasts, retinal cells and lung cells, as are known in the art. Examples of suitable hamster cells are the cell lines having the names BHK21 or HKCC. Suitable monkey cells include African green monkey cells, such as kidney cells as in the Vero cell line (Kistner et al. (1998) Vaccine 16:960-8; Kistner et al. (1999) Dev Biol Stand 98:101-110; Bruhl et al. (2000) Vaccine 19:1149-58). Suitable dog cells include canine kidney cells, as in the CLDK and MDCK cell lines (W097/37000; Brands et al. (1999) Dev Biol Stand 98:93-100; Halperin et al. (2002) Vaccine 20:1240-7; Tree et al. (2001) Vaccine 19:3444-50). Thus, suitable cell lines include, but are not limited to: MDCK; CHO; 293T; BHK; Vero; MRC-5; PER.C6 and WI-38 cell lines.

According to particular examples, the cell or cell line described herein for growing influenza viruses include: MDCK cells derived from Madin Darby canine kidney; Vero cells derived from the African green monkey (*Cercopithecus aethiops*) kidney; or PER.C6 cells derived from human embryonic retinoblasts (Pau et al. (2001) Vaccine 19:2716-21). These cell lines are widely available, such as from the American Type Cell Culture (ATCC) collection, the Coriell Cell Repositories and the European Collection of Cell Cultures (ECACC).

The influenza virus described herein can be grown on avian cell lines (see, e.g., WO2003/076601; WO2005/042728; WO2003/043415), including cell lines derived from ducks (e.g., duck retinal cells) or hens (e.g., chicken embryo fibroblasts (CEF)). Examples include avian embryonic stem cells, including the EBx cell line derived from chicken embryonic stem cells, EB45, EB14, EB14-074 and EB66.

Suitably, the cell or cell line for in the methods of the present disclosure are MDCK cells derived from Madin Darby canine kidney. The original MDCK cells are available from the ATCC as CCL-34. Derivatives of MDCK cells may also be used. For instance, the MDCK cell line may be adapted for growth in suspension culture (e.g., 'MDCK 33016', deposited as DSM ACC 2219). Similarly, WO2001/064846 discloses a MDCK-derived cell line that grows in suspension in serum-free culture ('B-702', deposited as FERM BP-7449). WO2006/071563 discloses non-tumorigenic MDCK cells, including 'MDCK-S' (ATCC PTA-6500), 'MDCK-SF101' (ATCC PTA-6501), 'MDCK-SF102' (ATCC PTA-6502) and 'MDCK- SF103' (PTA-6503). WO2005/113758 discloses MDCK cell lines with high susceptibility to infection, including 'MDCK.5F1' cells (ATCC CRL-12042). Any MDCK cell line, including those provided herein, can be used in the methods of the present disclosure.

For viral growth or propagation on a cell line, such as on MDCK cells, the influenza virus may be grown on cells in suspension or in adherent culture.

The cells according to the present disclosure can be cultured in the course of the process in various serum-free media or media that is substantially free of serum (e.g., contains less than 0.5, 0.1, 0.05 or 0.01 wt% of serum), as are known to the person skilled in the art (e.g., Iscove's medium, ultra CHO medium (Bio Whittaker), EX-CELL (JRH Biosciences)). Otherwise, the cells for replication can alternatively be cultured in the serum-containing media (e.g., MEM or DMEM medium with about 0.5% to about 10%, more particularly about 1.5% to about 5%, of foetal calf serum) or protein-free media (e.g., PF-CHO (JRH Biosciences)). Suitable culture vessels which can be employed in the course of the process according to the present disclosure are all vessels known to the person skilled in the art, such as, for example, spinner bottles, roller bottles or fermenters.

In particular examples, the cells are suitably grown in serum-free culture media and/or protein free media, such as for cell proliferation and/or supporting influenza virus replication. A medium is referred to as a serum-free medium in the context of the present disclosure if it contains no additives or substantially no additives (e.g., less than 0.5%, 0.25% or 0.1% by weight thereof) from serum of human or animal origin. Protein-free refers to a culture media in which multiplication of the cells occurs with the exclusion of proteins, growth factors, other protein additives and non-serum proteins, but can optionally include proteins such as trypsin or other proteases that may be necessary for viral growth. The cells growing in such cultures naturally contain proteins themselves.

Cell lines supporting influenza virus replication are suitably cultured at a temperature below 37°C (e.g., about 30°C to about 36°C, or at about 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or any range therein) during viral replication. Where virus is grown on a cell line then the culture media, and also the viral inoculum used to start the culture, is suitably free from (e.g., will have been tested for and given a negative result for contamination by) contaminating viruses, such as herpes simplex virus, respiratory syncytial virus, parainfluenza virus 3, SARS coronavirus, adenovirus, rhinovirus, reoviruses, polyomaviruses, birnaviruses, circoviruses, and/or parvoviruses.

Where the influenza virus has been grown on a mammalian cell line then a vaccine composition or similar derived therefrom will advantageously be free from egg proteins (e.g., ovalbumin and ovomucoid) and from chicken DNA, thereby reducing the allergenicity thereof. The avoidance of allergens is useful for minimizing Th2 responses. Where cells are used as a culture host in the methods of the present disclosure, the cell culture conditions described

herein (e.g., temperature, cell density, pH value, trypsin concentration, virus dose/MOI etc.) can be variable over a wide range subject to the cell line and the influenza virus strain employed and can be adapted to the requirements of the present methods, as described in more detail herein.

Influenza viruses and influenza virus proteins

Influenza viruses are enveloped RNA viruses that belong to the family of Orthomyxoviridae (Palese and Shaw (2007) Orthomyxoviridae: The Viruses and Their Replication, 5th ed. Fields' Virology, edited by B. N. Fields, D. M. Knipe and P. M. Howley. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, USA, p1647-1689). Influenza A and B viruses are major human pathogens, causing a respiratory disease that ranges in severity from sub-clinical infection to primary viral pneumonia which can result in death. The clinical effects of infection vary with the virulence of the influenza strain and the exposure, history, age, and immune status of the host. The natural hosts of influenza viruses are predominantly avian, but influenza viruses, particularly influenza A viruses (including those of avian origin) can also infect and cause illness in humans and other animal hosts (bats, canines, pigs, horses, sea mammals, and mustelids).

The influenza virus of the present disclosure can be an influenza A virus or an influenza B virus. According to some examples, the influenza virus is an influenza A virus. In alternative examples, the influenza virus is an influenza B virus. The influenza A or B virus may be any strain of virus. By way of examples, the influenza A virus provided herein may include a HA subtype selected from H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In various examples, the influenza A virus is of a H1 or H3 subtype. Moreover, such viruses may contain the influenza A virus NA subtypes N1, N2, N3, N4, N5, N6, N7, N8 or N9. In particular, the influenza A virus can be a strain selected from the group consisting of H1N1, H1N2, H2N2, H3N1, H3N2, H3N8, H5N1, H7N1, H7N2, H7N3, H7N7, H9N2, and H10N7. According to some examples, the influenza A virus is a H3N2 strain. In alternative examples, the influenza A virus is a H1N1 strain.

In view of the above, the influenza virus referred to herein is suitably a seasonal influenza virus strain. As used herein, the term “seasonal influenza virus strain” refers to a strain of influenza virus to which a subject population is exposed to on a seasonal basis. As such, in certain examples, the term seasonal influenza virus strain refers to a strain of influenza A virus. In other examples, the term seasonal influenza virus strain refers to a strain of influenza A virus that belongs to the H1 or the H3 subtype (i.e., the two subtypes that presently persist

or are endemic in the human population). In some examples, the term seasonal influenza virus strain refers to a strain of influenza B virus.

The influenza viruses referred to herein encompass any virus type, subtype or strain including, but not limited to, naturally occurring strains, variants or mutants, mutagenized viruses, reassortant viruses and/or genetically modified viruses.

Suitably, the influenza virus is a reassortant virus. The term "reassortant virus" denotes a virus which contains genetic material that results from the combination of genetic material of at least two donor viruses. When the reassortant virus is used for preparing a vaccine composition, its genetic material usually contains at least the HA and NA genes from a seasonal or pandemic influenza virus, whereas the other genes (i.e., backbone genes) are from one or several other donor viruses which have been selected for their ability to grow easily on the substrate of production used for manufacturing the flu vaccine (e.g., the allantoic cavity of embryonated hen's eggs or a permissive cell line) and/or to be less or non-pathogenic to humans. Examples of donor or seed viruses that contribute as donors of backbone genes include A/Puerto Rico/8/1934 (PR8), A/Texas/1/1977, A/New York/55/2004, AJ Ann Arbor/6/60, A/Leningrad/134/17/57, B/Ann Arbor/1/66, B/Florida/4/2006, B/Panama/45/1990 and B/Lee/1940. Additional exemplary donor or seed virus strains are described in International Patent Application PCT/IB2024/055963, which is incorporated by reference herein. The reassortant virus may be produced by any method known in the art, inclusive of reverse genetics and classical reassortment.

The influenza virus protein referred to herein may include any such protein known in the art. Typically, influenza virus proteins include: the PB1, PB2, PA, HA, NP, NA, M1, M2, NS1 and NS2/NEP proteins for influenza A viruses; or the PB1, PB2, PA, HA, NP, NA, NB, M1, BM2, NS1 and NS2/NEP proteins for influenza B viruses.

For certain examples of the present disclosure, the influenza virus protein is a haemagglutinin protein. The terms "Haemagglutinin" and "HA" refer to any hemagglutinin protein known to those of skill in the art (e.g., influenza A HA subtypes of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16). In certain examples, the haemagglutinin protein is an influenza hemagglutinin protein, such as an influenza A haemagglutinin protein or an influenza B haemagglutinin protein. A typical haemagglutinin protein comprises a signal peptide (optional herein), a stem domain, a globular head domain, a luminal domain, a transmembrane domain and a cytoplasmic domain. In certain examples, a hemagglutinin protein comprises a single polypeptide chain, such as HA0. In other examples, a hemagglutinin protein comprises more than one polypeptide chain in quaternary association

(e.g., HA1 and HA2). In certain examples, a hemagglutinin protein lacks a signal peptide (i.e., the hemagglutinin protein is a mature hemagglutinin). The haemagglutinin proteins provided herein may also be modified by post-translational processing, such as signal peptide cleavage, disulfide bond formation, glycosylation (e.g., N-linked glycosylation), protease cleavage and lipid modification (e.g. S-palmitoylation).

Vaccines

The present disclosure envisages that the influenza virus and/or the influenza virus protein derived from the influenza virus produced according to the methods described may be utilised in vaccine compositions.

Influenza vaccines are generally based either on a live attenuated virus or on an inactivated virus. Inactivated vaccines may be based on whole virions, "split" virions, or on purified surface antigens. Antigens can also be presented in the form of virosomes. The present methods can be used for manufacturing any of these types of vaccine. Where an inactivated influenza virus is used, the vaccine may comprise whole virion, split virion, or purified surface antigens (e.g., hemagglutinin and optionally neuraminidase). Chemical means for inactivating a virus include treatment with an effective amount of one or more of the following inactivating agents: detergents, formaldehyde, b-propiolactone, methylene blue, psoralen, carboxyfullerene (C60), binary ethylamine, acetyl ethyleneimine, or combinations thereof. Non-chemical methods of viral inactivation are also known in the art, such as UV light or gamma irradiation.

Virions can be harvested from virus-containing fluids, such as cell culture supernatant, by various methods. For example, a purification process may involve zonal centrifugation using a linear sucrose gradient solution (that optionally includes detergent to disrupt the virions) or affinity chromatography methods. Antigens may then be purified, after optional dilution, by diafiltration.

The vaccine composition may contain a pharmaceutically-acceptable carrier, diluent or excipient. By "pharmaceutically-acceptable carrier, diluent or excipient" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, diluent and excipients well known in the art may be used. These may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and

sulfates, organic acids such as acetates, propionates and malonates, water and pyrogen-free water.

A useful reference describing acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991) which is incorporated herein by reference.

Suitably, for the purposes of eliciting an immune response, certain immunological or immunogenic agents may be used in combination with the immunogenic protein described herein. The term "immunogenic agent" includes within its scope carriers, delivery agents, immunostimulants and/or adjuvants as are well known in the art. As will be understood in the art, immunostimulants and adjuvants refer to or include one or more substances that enhance the immunogenicity and/or efficacy of a composition. Non-limiting examples of suitable immunostimulants and adjuvants include squalane and squalene (or other oils of plant or animal origin), inclusive of squalene oil-in-water emulsions (e.g., MF59, AS03 and AF03); block copolymers; TLR agonists, such as pathogen-derived compounds, including lipopeptides, glycolipids, nucleotides, small-molecule inhibitors and bacterial-derived components, such as flagellin; detergents such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; *Corynebacterium*-derived adjuvants such as *Corynebacterium parvum*; *Propionibacterium*-derived adjuvants such as *Propionibacterium acne*; *Mycobacterium bovis* (Bacille Calmette and Guerin or BCG); *Bordetella pertussis* antigens; tetanus toxoid; diphtheria toxoid; surface active substances such as hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin, dimethyldioctadecylammonium bromide, *N,N*-dicoctadecyl-N′, N′bis(2-hydroxyethyl-propanediamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines such as pyran, dextransulfate, poly IC carbopol; peptides such as muramyl dipeptide and derivatives, dimethylglycine, tuftsin; oil emulsions; and mineral gels such as aluminium phosphate, aluminium hydroxide or alum; interleukins such as interleukin 2 and interleukin 12; monokines such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon; immunostimulatory DNA such as CpG DNA, combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide; saponins, such as Matrix-M; liposomes; ISCOM® and ISCOMATRIX® adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as muramyl dipeptides or other derivatives; Avridine; Lipid A derivatives; dextran sulfate; DEAE-Dextran alone or with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer

emulsions such as Neocryl A640 (*e.g.* U.S. Pat. No. 5,047,238); water in oil emulsifiers such as Montanide ISA 720; poliovirus, vaccinia or animal poxvirus proteins; or mixtures thereof.

Immunogenic agents may include carriers such as thyroglobulin; albumins such as human serum albumin; toxins, toxoids or any mutant cross-reactive material (CRM) of the toxin from tetanus, diphtheria, pertussis, *Pseudomonas*, *E. coli*, *Staphylococcus*, and *Streptococcus*; polyamino acids such as poly(lysine:glutamic acid); influenza; Rotavirus VP6, Parvovirus VP1 and VP2; hepatitis B virus core protein; hepatitis B virus recombinant vaccine and the like. Alternatively, a fragment or epitope of a carrier protein or other immunogenic protein may be used. For example, a T cell epitope of a bacterial toxin, toxoid or CRM may be used. In this regard, reference may be made to U.S. Patent No 5,785,973 which is incorporated herein by reference.

Oil-in-water emulsions have been found to be particularly suitable for use in adjuvanting influenza virus vaccines. Various such emulsions are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolisable) and biocompatible. The oil droplets in the emulsion are generally less than 5 μm in diameter, and may even have a sub-micron diameter, with these small sizes being achieved with a microfluidiser to provide stable emulsions. Droplets with an average size less than 220 nm are preferred as they can be subjected to filter sterilization.

In various examples, the oil-in-water emulsion is uniform. A uniform emulsion is characterized in that a majority of droplets (particles) dispersed therein is within a specified size range (e.g., in diameter). A suitable specified size range can be, for example, between 50-220 nm (e.g., 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220 nm or any range therein), between 50-180 nm, between 80-180 nm, between 100-175 nm, between 120-185 nm, between 130-190 nm, between 135-175 nm, between 150-175 nm. In some examples, the uniform emulsion contains <10% of the number of droplets (particles) that are outside of the specified range of diameters. In certain examples, the mean particle size of oil droplets in the oil-in-water emulsion preparation is between 135-175 nm, e.g., 155 nm ± 20 nm as measured by dynamic light scattering, and such a preparation contains not more than 1 x 10^7 large particles per mL of the preparation, as measured by optical particle sensing. "Large particles" as used herein mean those having diameters ≥1.2 μm, typically between 1.2-400 μm. In particular examples, the uniform emulsion contains less than 10%, less than 5%, or less than 3% of the droplets that fall outside of the preferred size range. In some examples, the mean droplet size of particles in an oil-in-water emulsion preparation is between 125-185 nm, e.g., about 130 nm, about 140 nm, about 150 nm, about 155 nm, about 160 nm, about 170 nm, or

about 180 nm, and the oil-in-water emulsion is uniform in that less than 5% of the number of droplets in the preparation fall outside the 125-185 nm range.

The vaccine composition described herein can be used with oils, such as those from an animal (such as fish) or vegetable source. Sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Jojoba oil obtained from the jojoba bean can also be used. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like may also be used. 6-10 carbon fatty acid esters of glycerol and 1,2- propanediol, while not occurring naturally in seed oils, may be prepared by hydrolysis, separation and esterification of the appropriate materials starting from the nut and seed oils. Fats and oils from mammalian milk are metabolizable and may therefore be used in the vaccine composition described herein. The procedures for separation, purification, saponification and other means necessary for obtaining pure oils from animal sources are well known in the art. Most fish contain metabolizable oils which may be readily recovered. For example, cod liver oil, shark liver oils, and whale oil, such as spermaceti, exemplify several of the fish oils which may be used herein.

A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as terpenoids. Shark liver oil contains a branched, unsaturated terpenoid known as squalene, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene. Squalane, the saturated analogue to squalene, may also be utilised in the present vaccine composition. Fish oils, including squalene and squalane, are readily available from commercial sources or may be obtained by methods known in the art. Other suitable oils are the tocopherols. Mixtures of oils are also envisaged.

Surfactants can be classified by their 'HLB' (hydrophile/lipophile balance). Suitably, surfactants described herein have a HLB of at least 10, more particularly at least 15, and even more particularly at least 16. The vaccine composition may include one or more surfactants including, but not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially polysorbate 20 and polysorbate 80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-l,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol); (octylphenoxy)polyethoxy ethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); polyoxyethylene fatty

ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Non-ionic surfactants are preferred. Exemplary surfactants for including in the emulsion are Tween 80 (polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100.

Mixtures of surfactants can also be used (e.g., Tween 80/Span 85 mixtures). A combination of a polyoxyethylene sorbitan ester, such as polyoxyethylene sorbitan monooleate (Tween 80) and an octoxynol, such as t-octylphenoxypolyethoxyethanol (Triton X-100) is also suitable. Another envisaged combination comprises laureth 9 plus a polyoxyethylene sorbitan ester and/or an octoxynol.

Exemplary amounts of surfactants (% by weight) are: polyoxyethylene sorbitan esters (such as Tween 80) 0.01% to 1%, in particular about 0.1%; octyl- or nonylphenoxy polyoxyethanols (such as Triton X-100, or other detergents in the Triton series) 0.001% to 0.1 %, in particular 0.005% to 0.02%; polyoxyethylene ethers (such as laureth 9) 0.1% to 20 %, more particularly 0.1% to 10 % and even more particularly 0.1% to 1 % or about 0.5%.

In particular examples, the oil-in-water emulsions are squalene-in-water emulsions, and more particularly, submicron squalene-in-water emulsions.

Any suitable procedure is contemplated for producing vaccine compositions. Exemplary procedures include, for example, those described in New Generation Vaccines (1997, Levine *et al.*, Marcel Dekker, Inc. New York, Basel, Hong Kong), which is incorporated herein by reference.

Any safe route of administration may be employed, including oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intranasal, intraocular, intraperitoneal, intracerebroventricular, topical, mucosal and transdermal administration, although without limitation thereto.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, nasal sprays, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release may be effected by coating with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Compositions may be presented as discrete units such as capsules, sachets, functional foods/feeds or tablets each containing a pre-determined amount of one or more therapeutic agents of the disclosure, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the agents of the disclosure with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as effective. The dose administered to a subject, in the context of the present disclosure, should be sufficient to effect a beneficial response in a subject over an appropriate period of time (e.g., generate a protective immune response). The quantity of agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

Also disclosed herein is a container comprising the immunogenic or vaccine compositions disclosed herein. Any suitable container known in the art may be used. For example, the container may be selected from the group consisting of a vial, a syringe, an ampoule, a flask, a fermenter, a bioreactor, a bag, a jar, an ampoule, a cartridge and a disposable pen. In one example, the container is a vial, ampoule or a syringe.

The container may be made of glass, metals (*e.g.*, steel, stainless steel, aluminium, etc.) and/or polymers (*e.g.*, thermoplastics, elastomers, thermoplastic-elastomers). The container may be at least partially siliconized.

The vaccine compositions disclosed herein may further comprise a buffer. The buffer may be any suitable buffer known in the art. For example, the buffer may be a TRIS, acetate, glutamate, lactate, maleate, tartrate, phosphate, citrate, carbonate, glycinate, histidine, glycine, succinate or triethanolamine buffer. In one example, the buffer is a phosphate buffer. In another example, the buffer is a succinate buffer. In another example, the buffer is a histidine buffer. In another example, the buffer is a citrate buffer.

The buffer may be selected from USP compatible buffers for parenteral use, in particular, when the pharmaceutical formulation is for parenteral use. For example, the buffer may be selected from the group consisting of monobasic acids such as acetic, benzoic, gluconic,

glyceric and lactic; dibasic acids such as aconitic, adipic, ascorbic, carbonic, glutamic, malic, succinic and tartaric, polybasic acids such as citric and phosphoric; and bases such as ammonia, diethanolamine, glycine, triethanolamine, and TRIS.

Methods for eliciting an immune response and treatment

The influenza virus proteins and the vaccine compositions described herein may be suitable for administration to human or non-human animal subjects, such that the present disclosure provides methods of raising an immune response and/or preventing and/or treating an influenza-associated disease, disorder or condition in a subject. The present disclosure also provides a composition as described herein for use as a medicament, and provides the use of such a composition for the manufacture of a medicament for raising an immune response and/or preventing and/or treating an influenza-associated disease, disorder or condition in a subject.

Accordingly, in one form, the present disclosure provides a method of eliciting an immune response in a subject, said method including the step of administering a therapeutically effective amount of the influenza virus, the influenza virus protein or the vaccine composition provided herein to the subject to thereby elicit the immune response in the subject.

In a related form, the present disclosure provides a method of preventing and/or treating an influenza-associated disease, disorder or condition in a subject, said method including the step of administering a therapeutically effective amount of the influenza virus, the influenza virus protein or the vaccine composition described herein to the subject to thereby prevent and/or treat the influenza-associated disease, disorder or condition.

With respect to the aspects described herein, the term "subject", "patient" and "individual" includes, but is not limited to, mammals, inclusive of humans, performance animals (such as horses, camels, greyhounds), livestock (such as cows, sheep, horses) and companion animals (such as cats and dogs). Suitably, the subject is a human.

By "elicit an immune response" is meant generate or stimulate the production or activity of one or more elements of the immune system inclusive of the cellular immune system, humoral immune system (i.e., antibodies) and/or the native immune system. Suitably, the immune response described herein includes one or more elements of the immune system, such as T lymphocytes, B lymphocytes, antibodies, neutrophils, dendritic cells inclusive of plasmacytoid dendritic cells, cytokines and/or chemokines. Non-limiting examples of cytokines include pro-inflammatory cytokines such as TNF-α, IL-2, IL-6, IL-8, IL-17A and IL-1 *(e.g.*, IL-1β). A non-limiting example of a chemokine is the neutrophil chemo-attractant

IL-8. In certain examples, the immune response that is elicited by the vaccine compositions described herein is protective.

As generally used herein, the terms "immunize", "vaccinate" and "vaccine" refer to methods and/or compositions that elicit a protective immune response against an influenza virus, whereby subsequent infection by the influenza virus, or a related serotype, strain or variant, is at least partly prevented or minimized.

As used herein, "treating", "treat" or "treatment" refers to a therapeutic intervention that at least partly ameliorates, eliminates or reduces a symptom or pathological sign of an influenza-associated disease, disorder or condition, such as an influenza infection, after it has begun to develop. Treatment need not be absolute to be beneficial to the subject. The beneficial effect can be determined using any methods or standards known to the ordinarily skilled artisan.

As used herein, "preventing", "prevent" or "prevention" refers to a course of action initiated prior to infection by, or exposure to, an influenza virus or molecular components thereof and/or before the onset of a symptom or pathological sign of the disease, disorder or condition, so as to prevent infection and/or reduce the symptom or pathological sign. It is to be understood that such preventing need not be absolute to be beneficial to a subject. A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of the disease, disorder or condition, or exhibits only early signs for the purpose of decreasing the risk of developing a symptom or pathological sign of the disease, disorder or condition.

The term "therapeutically effective amount" describes a quantity of a specified agent, such as the influenza virus, the influenza virus protein or the vaccine composition described herein, sufficient to achieve a desired effect in a subject being treated with that agent or composition. For example, this can be the amount of the agent and optionally one or more further therapeutic agents (e.g., one or more further anti-viral agents), necessary to reduce, alleviate and/or prevent an influenza-associated disease, disorder or condition, such as an influenza infection. Suitably, a "therapeutically effective amount" is sufficient to reduce or eliminate a symptom of an influenza-associated disease, disorder or condition. More particularly, a "therapeutically effective amount" may be an amount sufficient to achieve a desired biological effect, for example an amount that is effective to decrease or prevent disease progression.

Ideally, a therapeutically effective amount of an agent is an amount sufficient to induce the desired result without causing a substantial cytotoxic effect in the subject. The therapeutically effective amount of an agent useful for reducing, alleviating and/or preventing the diseases, disorders and conditions described herein will be dependent on the subject being

treated, the type and severity of any associated disease, disorder and/or condition (*e.g.*, disease progression), and the manner of administration of the therapeutic composition. Suitably, a therapeutically effective amount of the agent is administered parenterally to the subject.

The vaccines described herein may be used to treat both children and adults. Influenza vaccines are currently recommended for use in paediatric and adult immunisation, from the age of 6 months. Thus, a human subject may be less than 1 year old, 1-5 years old, 5-15 years old, 15-55 years old, or at least 55 years old. Preferred subjects for receiving the vaccines are the elderly (e.g. ≥50 years old, ≥60 years old, and preferably ≥65 years), the young (e.g. ≤5 years old), hospitalised subjects, healthcare workers, armed service and military personnel, pregnant women, the chronically ill, immunodeficient subjects, subjects who have taken an antiviral compound in the 7 days prior to receiving the vaccine, people with egg allergies and people travelling abroad. The vaccines are not suitable solely for these groups, however, and may be used more generally in a population. For pandemic strains, administration to all age groups is preferred.

Treatment can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. In a multiple dose schedule the various doses may be given by the same or different routes (e.g., a parenteral prime and mucosal boost, a mucosal prime and parenteral boost). Administration of more than one dose (typically two doses) is particularly useful in immunologically naive patients (e.g., for subjects who have never received an influenza vaccine before), or for vaccinating against a new HA subtype (e.g., in a pandemic outbreak). Multiple doses will typically be administered at least 1 week apart (e.g., about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, etc.).

So that preferred embodiments of the present disclosure may be fully understood and put into practical effect, reference is made to the following non-limiting examples.

Examples

Example 1.

The aim of the present Example was to optimise end of infection purity by investigating how pH, viral dose, and trypsin concentration can affect the end of infection purity, as well as

investigate how the optimal conditions for maximizing GUC purity compare to the optimal conditions for maximizing HA yield across a range of influenza virus strains.

Materials and Methods

All cell expansion was executed according to the following work instructions:

Suspension MDCK cells were grown at 37°C in DM134 media in increasing volumes for 4-6 passages. Following growth phase, MDCK cells were seeded at $2.5x10^6$ cells/mL in suitable benchtop bioreactor vessels and infected at different pH, virus dilution, and trypsin levels as determined by Design of Experiments (DOE; Table 1). A central composite design was created using three continuous factors pH (6.8 – 7.2), trypsin (4000-8000 U/L), and virus dilution (-4 to -9). Influenza infected cultures were grown for 60-72 hours and harvested by centrifugation. Following removal of cells, ultracentrifugation (UC) is performed to pellet the virus and quantify HA yield by HA-HPLC. In addition to virus, the UC pellet will also contain host cell contaminates. BCA analysis is performed to determine total protein of each sample and calculate relative purity by using the HA/BCA ratio. This ratio has been shown to correlate well with purity following downstream purification.

Experimental Design

The experimental design for each of the strains studied used response surface methodology.
A 3 factor (pH, virus dilution, and trypsin), with 3 levels was used in the design of experiment (Table 1). An I-optimal design or central composite designed experiments were created to optimize the factors towards HA yield and % purity. The continuous factors evaluated were pH (6.8 to 7.2), trypsin (4000 to 8000) and virus dilution (10^{-4} to 10^{-9}) with HA yield and % purity as response.

Table 1: Experimental Factors and their Coded Levels of Independent Variables in the experimental Design.

Factors	Code	Coded variable levels

		-1	0	1
pH	X_1	6.8	7.0	7.2
Trypsin (U/L)	X_2	4000	6000	8000
Virus Dilution	X_3	10^{-4}	$10^{-6.5}$	10^{-9}

Results

A/Florida/50/2022 (H3N2)

Figure 1 demonstrates the model prediction that resulted in the highest HA yield and % purity for this virus strain. This showed that at a pH of 6.8 and a trypsin concentration of 8000 U/L should have an end of infection purity of around 37.9% and an HA titer of around 30.1mg/kg. The pH trend for HA yield and purity was similar, with an optimal value of 6.8. The trypsin data demonstrates similar profiles for HA yield and purity with increases in trypsin concentration producing increases in both read outs and maximum yields for both HA yield and purity being achieved at a trypsin concentration of 8000 U/L. Suitable viral dilutions were observed for this strain across the range of dilutions tested (i.e., between dilutions of -6 and -9).

A/Michigan/41/2022 (H3N2)

Figure 2 shows the model prediction for the maximized HA yield and %Purity for this strain. Based on prediction profiler maximum HA yield and % purity was obtained at pH 6.9 and trypsin concentration of 8000 U/L. This model predicts that at optimized infection parameters should yield around 34 mg/kg HA with a purity of around 34%. Suitable viral dilutions were observed for this strain across the range of dilutions tested (i.e., between dilutions of -7 and -9).

A/Darwin/11/2021 CVR-88 (H3N2)

Figure 3 demonstrates the model prediction for the maximized HA yield for this strain. As can be seen from this figure, for pH there is an inverse relationship between HA yield and purity with the optimal range for HA yield being pH 7.1 – 7.2 and the optimal range for purity being pH 6.8 – 6.9. The trypsin data demonstrates similar profiles for HA yield and purity with increases in trypsin concentration producing increases in both read outs and maximum yields for both HA yield and purity being achieved at a trypsin concentration of 7500 U/L. Suitable

viral dilutions were observed for this strain across the entire range of dilutions tested (i.e., between dilutions of -6 and -8).

A/Canberra/407/2019 (H3N2)

Figure 4 demonstrates the model prediction for the maximized HA yield for this strain. As can be seen from this figure, for pH there is an inverse relationship between HA yield and purity with the optimal range for HA yield being pH 7.0 – 7.2 and the optimal range for purity being pH 6.8 – 6.9. The trypsin data demonstrates similar profiles for HA yield and purity with increases in trypsin concentration producing increases in both read outs and maximum yields for both HA yield and purity being achieved at a trypsin concentration of 8000 U/L. Suitable viral dilutions were observed for this strain at dilutions of -7 to -9.

A/NorthCarolina/01/2021 (H1N1)

Figure 5 demonstrates the model prediction for the maximized HA yield for this strain. As can be seen from this figure, for pH there is an inverse relationship between HA yield and purity with the optimal range for HA yield being pH 7.1 – 7.4 and the optimal range for purity being pH 6.8 – 7.0. The trypsin data demonstrates similar profiles for HA yield and purity with increases in trypsin concentration producing increases in both read outs up until a trypsin concentration of 6000 U/L above which HA purity and yield decrease slightly. Suitable viral dilutions were observed for this strain at dilutions of -6.5 to -8.

A/Washington/19/2020 (H1N1)

Figure 6 demonstrates the model prediction for the optimised HA yield and purity for this strain. As can be seen from this figure, pH has little effect on purity, but HA yield increases as pH increases with the optimal range for HA yield being pH 7.1 – 7.2 and the optimal range for purity being pH 6.9 – 7.2. The trypsin data demonstrates similar profiles for HA yield and purity with increases in trypsin concentration producing increases in both read outs up until a trypsin concentration of 7000 - 8000 U/L above which HA purity and yield plateau or slightly decrease. Suitable viral dilutions were observed for this strain at dilutions of -7 to -9.

B/Philippines/28/2019 (BVIC)

Figure 7 demonstrates the model prediction for the optimised HA yield and purity for this strain. As can be seen from this figure, HA yield and purity both increase with an increase in pH to about a pH of 7.2 above which they decline slightly, with the optimal range for HA yield

being pH 7.0 – 7.2 and the optimal range for purity being pH 7.0 – 7.2. The trypsin data demonstrates similar profiles for HA yield and purity with increases in trypsin concentration producing increases in both read outs and maximum yields for both HA yield and purity being achieved at a trypsin concentration of 7000 U/L. Suitable viral dilutions were observed for this strain at dilutions of -4 to -8.

B/Connecticut/01/2021 (BVIC)

Figure 8 demonstrates the model prediction for the optimised HA yield and purity for this strain. As can be seen from this figure, HA yield increases with an increase in pH to about a pH of 7.2, whilst purity increases with increases in pH to about 7.1 above which this parameter declines slightly, with the optimal range for HA yield being pH 7.1 – 7.2 and the optimal range for purity being pH 7.0 – 7.1. The trypsin data demonstrates that HA yield decreases as the trypsin concentration increases and purity increases as the trypsin concentration increases, with optimal yield being achieved at a trypsin concentration of 5000 U/L and optimal purity being achieved at a trypsin concentration of 8000 U/L. Suitable viral dilutions were observed for this strain at dilutions of -7 to -9.

A/Sydney/5/2021 CVR-112 (H1N1)

Figure 9 demonstrates the model prediction for the optimised HA yield and purity for this strain. As can be seen from this figure, for pH there is an inverse relationship between HA yield and purity, similar to that observed for A/NorthCarolina/01/2021. The optimal range for HA yield being pH 7.0 – 7.2 and the optimal range for purity being pH 6.8 – 7.0. The trypsin data demonstrates similar profiles for HA yield and purity with increases in trypsin concentration producing increases in both read outs up until a trypsin concentration of 7000 U/L above which HA purity and yield plateau.

A/Victoria/10/2020 (H3N2)

Figure 10 demonstrates the model prediction for the optimised HA yield and purity for this strain. As can be seen from this figure, for pH there is an inverse relationship between HA yield and purity similar to that observed for A/Canberra/407/2019 (H3N2). The optimal range for HA yield being pH 7.0 – 7.1 and the optimal range for purity being pH 6.8 – 6.9. The trypsin data demonstrates similar profiles for HA yield and purity with increases in trypsin concentration producing increases in both read outs and maximum yields for both HA yield

and purity being achieved at a trypsin concentration of 7500 U/L above which HA purity and yield plateau.

B/South Australia/06/2019 (B/Yam)

Figure 11 demonstrates the model prediction for the optimised HA yield and purity for this strain. As can be seen from this figure, HA yield and purity both increase with an increase in pH to about a pH of about 7.0 or 7.1 above which they decline slightly, with the optimal range for HA yield being pH 7.0 – 7.2 and the optimal range for purity being pH 6.8 – 7.1. The trypsin data demonstrates similar profiles for HA yield and purity with increases in trypsin concentration producing increases in both read outs and maximum yields for both HA yield and purity being achieved at a trypsin concentration of 6000 to 7000 U/L above which HA purity and yield plateau or decrease slightly.

Conclusions

This Example showed that end of infection purity and yield can be affected by changing the infection conditions of pH and trypsin concentration. Generally, for the influenza A strains, decreasing pH and increasing trypsin activity tended to result in increases in HA purity at end of infection. Conversely, increasing pH (and also increasing trypsin) tended to result in increases in HA yield at end of infection for influenza A strains. Increases in trypsin concentration also tended to increase purity levels for influenza B strains at end of infection.

Itemized Listing of Embodiments

1. A method of determining culture conditions for cells infected with an influenza virus, said method including the steps of:

(a) incubating the influenza virus-infected cells in a plurality of test culture media in which one or more culture conditions thereof are varied between the respective plurality of test culture media, wherein the one or more culture conditions are selected from a pH, a trypsin concentration and a combination thereof; and

(b) selecting the one or more culture conditions that optimise yield and/or purity of the influenza virus.

2. The method of Embodiment 1, wherein varying the one or more culture conditions between the respective plurality of the test culture media comprises:

(i) increasing the trypsin concentration from a baseline level to a maximum level thereof; and/or

(ii) decreasing the pH from a baseline level to a minimum level thereof;

in the plurality of the test culture media.

3. The method of Embodiment 2, wherein the trypsin concentration is increased to the maximum level of 10000 U/L and/or the pH is decreased to the minimum level of 6.0 in the plurality of the test culture media.

4. The method of any one of the preceding embodiments, wherein the pH is varied within a pH range of between about 6.0 to about 8.0.

5. The method of Embodiment 4, wherein the pH is varied within a pH range of about 6.5 to about 7.5.

6. The method of Embodiment 4 or Embodiment 5, wherein the pH is varied within a pH range of about 6.7 to about 7.3.

7. The method of any one of the preceding embodiments, wherein the trypsin concentration is varied within a concentration range of between about 2000 U/L to about 10000 U/L.

8. The method of Embodiment 7, wherein the trypsin concentration is varied within a concentration range of between about 4000 U/L to about 8000 U/L.

9. The method of any one of the preceding embodiments, wherein the cells are incubated with a virus dose of between about 10^{-8} and about 10.

10. The method of any one of the preceding embodiments, wherein step (b) comprises selecting the one or more culture conditions that optimise yield and/or purity of a protein produced by the influenza virus.

11. The method of Embodiment 10, wherein the protein is a haemagglutinin protein.

12. A method of culturing cells infected with an influenza virus, said method including the step of incubating the influenza virus-infected cells at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of at least about 5000 U/L.

13. The method of Embodiment 10, further including the step of harvesting the influenza virus and/or an influenza virus protein from the culture media.

14. A method of producing an influenza virus and/or an influenza virus protein, said method of including the steps of:

(a) incubating cells infected with an influenza virus at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of at least about 5000 U/L; and

(b) harvesting the influenza virus and/or the influenza virus protein produced thereby from the culture media.

15. The method of any one of Embodiments 12 to 14, wherein the influenza virus-infected cells are cultured at a pH of about 6.8 to about 7.1.

16. The method of any one of Embodiments 12 to 15, wherein the culture media contains trypsin at a concentration of between about 5000 U/L to about 10000 U/L.

17. The method of any one of Embodiments 12 to 16, wherein the cells are incubated with a virus dose of between about 10^{-8} and about 10.

18. The method of any one of Embodiments 13 to 17, further including the step of purifying the influenza virus and/or the influenza virus protein harvested from the culture media.

19. The method of any one of Embodiments 13 to 18, wherein the influenza virus protein is a haemagglutinin protein.

20. The method of Embodiment 19, wherein the haemagglutinin protein harvested from the culture media has a purity level of at least about 10% per total protein concentration thereof.

21. The method of Embodiment 19 or Embodiment 20, wherein the haemagglutinin protein is present in the culture media at a concentration of at least about 10 mg/L.

22. The method of any one of the preceding embodiments, wherein the influenza virus is an influenza A virus.

23. The method of Embodiment 22, wherein the influenza A virus is a H3N2 strain or a H1N1 strain.

24. The method of any one of the preceding embodiments, wherein the cells are or comprise MDCK cells.

25. The method of any one of the preceding embodiments, wherein the incubating step is performed for about 48 hours to about 96 hours.

26. An influenza virus or influenza virus protein produced by the method according to any one of Embodiments 13 to 25.

27. A vaccine composition comprising the influenza virus protein of Embodiment 26 and a pharmaceutically acceptable carrier, diluent or excipient.

28. A method of eliciting an immune response in a subject, said method including the step of administering a therapeutically effective amount of the influenza virus protein of Embodiment 26 or the vaccine composition of Embodiment 27 to the subject to thereby elicit the immune response in the subject.

29. A method of preventing and/or treating an influenza-associated disease, disorder or condition in a subject, said method including the step of administering a therapeutically effective amount of the influenza virus protein of Embodiment 26 or the vaccine composition of Embodiment 27 to the subject to thereby prevent and/or treat the influenza-associated disease, disorder or condition.

30. A method of culturing cells infected with an influenza virus, said method including the step of culturing the influenza virus-infected cells in a culture media containing trypsin at the one or more culture conditions selected in step (b) of the method of any one of Embodiments 1 to 11.

31. A method of improving yield and/or purity in the manufacture of an influenza vaccine, said method including the steps of:

(a) incubating influenza virus-infected cells in a plurality of test culture media in which one or more culture conditions thereof are varied between the respective plurality of test culture media, wherein the one or more culture conditions are selected from a pH, a trypsin concentration and a combination thereof;

(b) selecting the one or more culture conditions that optimise yield and/or purity of the influenza virus;

(c) culturing suspension mammalian cells capable of influenza virus infection in a serum free media; and

(d) infecting the mammalian cells with an influenza virus; and

(e) incubating the influenza virus-infected mammalian cells at the one or more culture conditions selected in (b).

32. The method of Embodiment 31, wherein varying the one or more culture conditions between the respective plurality of the test culture media comprises:

(i) increasing the trypsin concentration from a baseline level to a maximum level thereof; and/or

(ii) decreasing the pH from a baseline level to a minimum level thereof;
in the plurality of the test culture media.

33. The method of Embodiment 32, wherein the trypsin concentration is increased to the maximum level of 10000 U/L and/or the pH is decreased to the minimum level of 6.0 in the plurality of the test culture media.

34. The method of any one of the preceding embodiments, wherein the pH is varied within a pH range of between about 6.0 to about 8.0.

35. The method of Embodiment 4, wherein the pH is varied within a pH range of about 6.5 to about 7.5.

36. The method of Embodiment 4 or Embodiment 5, wherein the pH is varied within a pH range of about 6.7 to about 7.3.

37. The method of any one of the preceding embodiments, wherein the trypsin concentration is varied within a concentration range of between about 2000 U/L to about 10000 U/L.

38. The method of Embodiment 7, wherein the trypsin concentration is varied within a concentration range of between about 4000 U/L to about 8000 U/L.

39. The method of any one of the preceding embodiments, wherein the cells are incubated with a virus dose of between about 10^{-8} and about 10.

40. The method of any one of the preceding embodiments, wherein step (b) comprises selecting the one or more culture conditions that optimise yield and/or purity of a protein produced by the influenza virus.

41. The method of Embodiment 40, wherein the protein is a haemagglutinin protein.

CLAIMS:

1. A method of determining culture conditions for cells infected with an influenza virus, said method including the steps of:

(a) incubating the influenza virus-infected cells in a plurality of test culture media in which one or more culture conditions thereof are varied between the respective plurality of test culture media, wherein the one or more culture conditions are selected from a pH, a trypsin concentration and a combination thereof; and

(b) selecting the one or more culture conditions that optimise yield and/or purity of the influenza virus.

2. The method of Claim 1, wherein varying the one or more culture conditions between the respective plurality of the test culture media comprises:

(i) increasing the trypsin concentration from a baseline level to a maximum level thereof; and/or

(ii) decreasing the pH from a baseline level to a minimum level thereof;

in the plurality of the test culture media.

3. The method of Claim 2, wherein the trypsin concentration is increased to the maximum level of 10000 U/L and/or the pH is decreased to the minimum level of 6.0 in the plurality of the test culture media.

4. The method of Claim 1, wherein the pH is varied within a pH range of between about 6.0 to about 8.0.

5. The method of Claim 4, wherein the pH is varied within a pH range of about 6.5 to about 7.5.

6. The method of Claim 4, wherein the pH is varied within a pH range of about 6.7 to about 7.3.

7. The method of Claim 1, wherein the trypsin concentration is varied within a concentration range of between about 2000 U/L to about 10000 U/L.

8. The method of Claim 7, wherein the trypsin concentration is varied within a concentration range of between about 4000 U/L to about 8000 U/L.

9. The method of Claim 1, wherein the cells are incubated with a virus dose of between about 10^{-8} and about 10.

10. The method of Claim 1, wherein step (b) comprises selecting the one or more culture conditions that optimise yield and/or purity of a protein produced by the influenza virus.

11. The method of Claim 10, wherein the protein is a haemagglutinin protein.

12. The method of Claim 1, wherein the influenza virus is an influenza A virus.

13. The method of Claim 12, wherein the influenza A virus is of a H1 subtype or a H3 subtype.

14. The method of Claim 12, wherein the influenza A virus is a H3N2 strain or a H1N1 strain.

15. The method of Claim 1, wherein the cells are or comprise MDCK cells.

16. The method of Claim 1, wherein the incubating step is performed for about 48 hours to about 96 hours.

17. A method of culturing cells infected with an influenza virus, said method including the step of incubating the influenza virus-infected cells at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of at least about 5000 U/L.

18. The method of Claim 17, further including the step of harvesting the influenza virus and/or an influenza virus protein from the culture media.

19. The method of Claim 17, wherein the influenza virus-infected cells are cultured at a pH of about 6.8 to about 7.1.

20. The method of Claim 17, wherein the culture media contains trypsin at a concentration of between about 5000 U/L to about 10000 U/L.

21. The method of Claim 17, wherein the cells are incubated with a virus dose of between about 10^{-8} and about 10.

22. The method of Claim 18, further including the step of purifying the influenza virus and/or the influenza virus protein harvested from the culture media.

23. The method of Claim 18, wherein the influenza virus protein is a haemagglutinin protein.

24. The method of Claim 23, wherein the haemagglutinin protein harvested from the culture media has a purity level of at least about 10% per total protein concentration thereof.

25. The method of Claim 23, wherein the haemagglutinin protein is present in the culture media at a concentration of at least about 10 mg/L.

26. The method of Claim 17, wherein the influenza virus is an influenza A virus.

27. The method of Claim 26, wherein the influenza A virus is of a H1 subtype or a H3 subtype.

28. The method of Claim 26, wherein the influenza A virus is a H3N2 strain or a H1N1 strain.

29. The method of Claim 17, wherein the cells are or comprise MDCK cells.

30. The method of Claim 17, wherein the incubating step is performed for about 48 hours to about 96 hours.

31. An influenza virus or influenza virus protein produced by the method according to Claim 18.

32. A method of producing an influenza virus and/or an influenza virus protein, said method of including the steps of:

(a) incubating cells infected with an influenza virus at a pH of about 6.7 to about 7.3 and in a culture media containing trypsin at a concentration of at least about 5000 U/L; and

(b) harvesting the influenza virus and/or the influenza virus protein produced thereby from the culture media.

33. The method of Claim 32, wherein the influenza virus-infected cells are cultured at a pH of about 6.8 to about 7.1.

34. The method of Claim 32, wherein the culture media contains trypsin at a concentration of between about 5000 U/L to about 10000 U/L.

35. The method of Claim 32, wherein the cells are incubated with a virus dose of between about 10^{-8} and about 10.

36. The method of Claim 32, further including the step of purifying the influenza virus and/or the influenza virus protein harvested from the culture media.

37. The method of Claim 32, wherein the influenza virus protein is a haemagglutinin protein.

38. The method of Claim 37, wherein the haemagglutinin protein harvested from the culture media has a purity level of at least about 10% per total protein concentration thereof.

39. The method of Claim 37, wherein the haemagglutinin protein is present in the culture media at a concentration of at least about 10 mg/L.

40. The method of Claim 32, wherein the influenza virus is an influenza A virus.

41. The method of Claim 40, wherein the influenza A virus is of a H1 subtype or a H3 subtype.

42. The method of Claim 40, wherein the influenza A virus is a H3N2 strain or a H1N1 strain.

43. The method of Claim 32, wherein the cells are or comprise MDCK cells.

44. The method of Claim 32, wherein the incubating step is performed for about 48 hours to about 96 hours.

45. An influenza virus or influenza virus protein produced by the method according to Claim 32.

46. A vaccine composition comprising the influenza virus protein of Claim 31 and a pharmaceutically acceptable carrier, diluent or excipient.

47. A vaccine composition comprising the influenza virus protein of Claim 45 and a pharmaceutically acceptable carrier, diluent or excipient.

48. A method of eliciting an immune response in a subject, said method including the step of administering a therapeutically effective amount of the influenza virus protein of Claim 31 to the subject to thereby elicit the immune response in the subject.

49. A method of preventing and/or treating an influenza-associated disease, disorder or condition in a subject, said method including the step of administering a therapeutically effective amount of the influenza virus protein of Claim 31 to the subject to thereby prevent and/or treat the influenza-associated disease, disorder or condition.

50. A method of culturing cells infected with an influenza virus, said method including the step of culturing the influenza virus-infected cells in a culture media containing trypsin at the one or more culture conditions selected in step (b) of the method of Claim 1.

51. A method of improving yield and/or purity in the manufacture of an influenza vaccine, said method including the steps of:

(a) incubating influenza virus-infected cells in a plurality of test culture media in which one or more culture conditions thereof are varied between the respective plurality of test culture

media, wherein the one or more culture conditions are selected from a pH, a trypsin concentration and a combination thereof;

(b) selecting the one or more culture conditions that optimise yield and/or purity of the influenza virus;

(c) culturing suspension mammalian cells capable of influenza virus infection in a serum free media; and

(d) infecting the mammalian cells with an influenza virus; and

(e) incubating the influenza virus-infected mammalian cells at the one or more culture conditions selected in (b).

52. The method of Claim 51, wherein varying the one or more culture conditions between the respective plurality of the test culture media comprises:

(i) increasing the trypsin concentration from a baseline level to a maximum level thereof; and/or

(ii) decreasing the pH from a baseline level to a minimum level thereof;

in the plurality of the test culture media.

53. The method of Claim 52, wherein the trypsin concentration is increased to the maximum level of 10000 U/L and/or the pH is decreased to the minimum level of 6.0 in the plurality of the test culture media.

54. The method of Claim 51, wherein the pH is varied within a pH range of between about 6.0 to about 8.0.

55. The method of Claim 51, wherein the pH is varied within a pH range of about 6.5 to about 7.5.

56. The method of Claim 51, wherein the pH is varied within a pH range of about 6.7 to about 7.3.

57. The method of Claim 51, wherein the trypsin concentration is varied within a concentration range of between about 2000 U/L to about 10000 U/L.

58. The method of Claim 51, wherein the trypsin concentration is varied within a concentration range of between about 4000 U/L to about 8000 U/L.

59. The method of Claim 51, wherein the cells are incubated with a virus dose of between about 10^{-8} and about 10.

60. The method of Claim 51, wherein step (b) comprises selecting the one or more culture conditions that optimise yield and/or purity of a protein produced by the influenza virus.

61. The method of Claim 60, wherein the protein is a haemagglutinin protein.