

TITLE

VLP BASED VACCINE DELIVERY SYSTEM

FIELD OF THE INVENTION

This invention relates to virus-like particles. More particularly, this invention
5 relates to engineered protein molecules for the production of chimeric polyoma virus-like particles.

BACKGROUND TO THE INVENTION

Not more so than in the modern era of global travel has the potential
catastrophic consequences of a pandemic arising from respiratory-borne pathogens
10 been so acute. As such there is a striking need for quick and efficient large-scale vaccine production. Influenza virus poses a particular threat because of its capacity to evade the adaptive immune response by mutation. Furthermore, the existence of a sizeable animal reservoir in birds of influenza increases the chance of rapid emergence.

15 Influenza viruses continuously undergo antigenic variation (antigenic drift and antigenic shift) to evade the immune system of the host. The antigenic variation of influenza viruses forms the primary basis for the occurrence of annual influenza epidemics and occasional pandemics and necessitates constant evolution of vaccine composition. Existing vaccine production methods for influenza rely upon either use
20 of embryonated eggs or cell culture, which are complex and time-consuming processes. Manufacture using these methods also requires specialized and costly infrastructure not widely available. In particular, the current lead time for initial delivery of even small amounts of influenza vaccine is several months following identification of a new pandemic strain. Clearly this amount of time is unacceptable

in view of the estimated 1.2 billion high-risk people that will need rapid vaccination with the new and possibly as-yet-unknown vaccine in the event of an influenza pandemic. Further, the ideal method of pandemic prevention is initial containment of outbreaks by rapid vaccination of the entire population in the geographical risk region, not only high-risk young and old. This must occur rapidly and certainly faster than is possible using current vaccine biomanufacturing technologies. The issues of speed and scale are daunting considering the complexity and slowness of existing influenza vaccine technologies, and the need for specialized infrastructure.

Virus-like particles (VLPs) provide a potentially powerful tool in a number of applications including as vaccines, as vehicles for delivery of small molecules and in gene therapy. The potential efficacy of VLP-based vaccines has been postulated for some time and has been demonstrated for cervical cancer vaccines. It is thought that the particulate nature of VLPs induces a more effective immune response than denatured or soluble proteins as immunogens.

VLPs have the added advantage that at no stage during biomanufacture is an infectious virus created. This is distinct to existing embryonated egg technologies and some cell-culture technologies where (i) the starting point of manufacture is the creation of an infectious virus, necessitating high biocontainment during manufacture, and (ii) the virus may be disassembled during processing to remove infectivity (i.e. to reduce the possibility that the vaccine itself might cause disease). This disassembly process has the disadvantage that the virus structure is destroyed and consequently that less-effective denatured or soluble immunogens are administered.

SUMMARY OF THE INVENTION

Due to increasing concern over the imminent threat of pandemic arising from respiratory-borne pathogens, there exists a need for broad-spectrum vaccines which can be rapidly and easily manufactured at large-scale and in swift response to disease
5 outbreak

In one broad form, the present invention is directed to a rapid response vaccine manufacturing technology against epidemic virus such as influenza but is not limited thereto. A preferred advantage of the present invention is an automated method using direct PCR ligation to create a multiplicity of antigenic VLPs
10 presenting different parts of a protein of interest.

In another broad form, the invention is directed to generation of a VP1-based VLP which has been engineered and/or modified to include a foreign protein, or a fragment thereof on the surface of said VLP.

In a first aspect, the invention provides an isolated protein comprising a
15 murine polyomavirus VP1 amino acid sequence wherein one or more exposed loops of said murine polyomavirus VP1 amino acid sequence has an insertion of an amino acid sequence of a virus protein other than murine polyomavirus VP1, or a fragment of said virus protein other than murine polyomavirus VP1.

It is contemplated that in particular embodiments that said one or more
20 exposed loops comprise an insertion site selected from the group consisting of site 1, site 3 and site 4.

Preferably, the one or more exposed loops comprise an insertion site selected from the group consisting of site 1 and site 4.

Preferably, the virus protein other than murine polyomavirus VP1 is derived

from influenza virus.

Suitably, although not limited thereto, the influenza virus is a H5N1 strain inclusive of variants and newly arising strains such as those resulting from antigenic drift and antigenic shift.

- 5 It is envisaged that the virus protein other than murine polyomavirus VP1 can be any class of viral protein which is capable of eliciting an immune response such as, but not limited to, transmembrane proteins, structural proteins and non-structural proteins, or a fragment thereof.

- In one embodiment, the virus protein other than murine polyomavirus VP1 is
10 an influenza virus protein selected from the group consisting of hemagglutinin (HA), neuraminidase (NA), nuclear protein (NP), matrix protein M1 and matrix protein M2.

 Preferably, the virus protein other than murine polyomavirus VP1 is selected from the group consisting of HA and M2.

- Advantageously, the virus protein other than VP1 corresponds to a
15 hypervariable region of HA.

 Preferably, the virus protein other than murine polyomavirus VP1 corresponds to an exposed loop of HA selected from the group consisting of loop A, loop B, loop C, loop D and loop E.

- More preferably, the exposed loop of HA is selected from the group
20 consisting of loop A and loop B.

 The invention further contemplates that the virus protein other than murine polyomavirus VP1 may be either the same protein in each insertion site or a different protein in each insertion site.

 In a second aspect, the invention provides for an isolated nucleic acid

encoding the isolated protein of the first aspect.

In one embodiment, the invention provides an isolated nucleic acid encoding a murine polyomavirus VP1 amino acid sequence which has been adapted to receive within one or more exposed loops of said murine polyomavirus VP1 amino acid
5 sequence, an isolated nucleic acid encoding a virus protein other than murine polyomavirus VP1.

In a third aspect, the invention provides an expression construct comprising an isolated nucleic acid according to the second aspect operably-linked to one or more regulatory sequences in an expression vector.

10 In a fourth aspect, the invention provides a host cell comprising the expression construct of the third aspect.

Preferably, the host cell is selected from the group consisting of a prokaryotic cell and an insect cell.

More preferably, the host cell is a bacterium.

15 In a fifth aspect, the invention provides a virus-like particle (VLP) comprising one or more isolated proteins of the first aspect.

In a sixth aspect, the invention provides a method of producing an isolated nucleic acid including the step of inserting into each of one or more nucleotide sequences that encode one or more exposed loops of a murine polyomavirus VP1
20 protein, a nucleotide sequence encoding a virus protein other than murine polyomavirus VP1, or a fragment of said nucleotide sequence encoding a virus protein other than murine polyomavirus VP1.

In a seventh aspect, the invention provides a method of producing a VLP including the steps of:

- (a) introducing the isolated nucleic acid of the second aspect or the expression construct of the third aspect into a host cell;
- (b) culturing said host cell under conditions which facilitate production of the isolated protein of the first aspect;
- 5 (c) optionally purifying the isolated protein of the first aspect; and
- (d) assembling the isolated protein purified according to step (c) to produce the VLP.

In an eighth aspect, the invention provides a pharmaceutical composition comprising an isolated protein, isolated nucleic acid or VLP according to any of the
10 aforementioned aspects and a pharmaceutically acceptable carrier, diluent or excipient.

Preferably, the pharmaceutical composition of the invention is an immunotherapeutic composition.

More preferably, the pharmaceutical composition is a vaccine.

15 Compositions according to this aspect may be used either prophylactically or therapeutically.

In a ninth aspect, the invention provides a method of treating an animal including the step of administering the pharmaceutical composition of the eighth aspect to prophylactically or therapeutically treat a disease, disorder or condition. It
20 will be appreciated that the disease, disorder or condition can be caused by any infectious organism for which a disease-specific or highly-disease associated antigen is, or will be known.

Preferably, the disease, disorder or condition is caused by a virus such as influenza virus, but is not limited thereto. More preferably, the disease, disorder or

condition is caused by an avian influenza virus.

In a tenth aspect, the invention provides a method of immunising an animal including the step of administering the pharmaceutical composition of the eighth aspect to said animal to thereby induce immunity in said animal.

- 5 An animal can be selected from the group consisting of humans, domestic livestock, laboratory animals, performance animals, companion animals, poultry and other animals of commercial importance, although without limitation thereto.

Preferably, the animal is a mammal.

More preferably, the animal is a human.

- 10 Throughout this specification, unless the context requires otherwise, the words “comprise”, “comprises” and “comprising” will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES

- 15 In order that the invention may be readily understood and put into practical effect, preferred embodiments will now be described by way of example with reference to the accompanying figures wherein like reference numerals refer to like parts and wherein:

Figure 1 Amino acid sequences of generic vectors. The sequences can be
20 identified as follows: VP1 (SEQ ID NO:87); VP1-S1 (SEQ ID NO: 88); VP1-S4 (SEQ ID NO:89); VP1-S3 (SEQ ID NO:90); VP1-S1-S3-S4 (SEQ I NO:91).

Figure 2 Amino acid sequences of VP1-S1 vectors with inserted peptides. The sequences can be identified as follows: VP1-S1 (SEQ ID NO: 88); VP1-S1B (SEQ ID NO:92); VP1-S1A (SEQ ID NO:93); VP1-S1M2e (SEQ ID NO:94); VP1-

S1hM2e (SEQ ID NO:95).

Figure 3 Purification of GST-VP1-S1A using GSTrap HP column. X-axis is volume in ml; Y1 is absorbance in mAU; Y2 is conductivity in mS/cm. Line A represents the UV trace at 280nm; Line B represents conductivity trace; the vertical dashed line is the injection point of sample onto the column.

Figure 4 Buffer exchange on a desalting column. X-axis is volume in ml; Y1 is absorbance in mAU; Y2 is conductivity in mS/cm. Line A represents the UV trace at 280nm; Line B represents the conductivity trace; the vertical dashed line is the injection point of sample onto the column.

Figure 5 Second purification of GST-VP1-S1A on GSTrap HP column. X-axis is volume in ml; Y1 is absorbance in mAU; Y2 is conductivity in mS/cm. Line A represents the UV trace at 280nm; Line B represents the conductivity trace; the vertical dashed line is the injection point of sample onto the column; the horizontal dashed line is the P960 flow.

Figure 6 Lane 1: eluate after second GST affinity purification; Lane 2: cleavage of GST tag from VP1-S1A.

Figure 7 Size exclusion separation on Superdex 200 to purify VP1-S1A after thrombin treatment. X-axis is volume in ml; Y1 is absorbance in mAU; Y2 is conductivity in mS/cm. Line A represents UV trace at 280nm; Line C represents UV trace at 260nm; line B represents the conductivity trace; the vertical dashed line is the injection point of sample onto the column.

Figure 8 Purified VP1-S1A sample after separation by size exclusion chromatography on a Superdex 200. Lower and upper bands are calibration markers introduced into the purified sample during analysis preparation (i.e. are system

peaks).

Figure 9 AFFF plot showing assembled VLPs. X-axis is time (minutes) and Y-axis is rms radius (nm).

Figure 10 Electron micrograph images showing assembled VLPs.

5 Figure 11 Slot blot showing hM2e antibody reacting with VP1-S1hM2e capsomeres.

Figure 12 Amino acid sequence alignment of HA across various strains of H5N1 subtype of influenza A virus. The boxed regions marked "A" and "B" represent loops A and B respectively. The sequences of full length HA, loop A and loop B
 10 respectively may be identified as follows: DQ343152 (SEQ ID NO: 96; SEQ ID NO: 1 and SEQ ID NO:24); DQ343150 (SEQ ID NO:97; SEQ ID NO: 2 and SEQ ID NO:25); DQ343151 (SEQ ID NO: 98; SEQ ID NO: 3 and SEQ ID NO:26); DQ323672 (SEQ ID NO:99; SEQ ID NO: 4 and SEQ ID NO:27); DQ236085 (SEQ ID NO:100; SEQ ID NO: 5 and SEQ ID NO:28);DQ236077 (SEQ ID NO:101;SEQ
 15 ID NO: 6 and SEQ ID NO:29); DQ211925 (SEQ ID NO:102; SEQ ID NO: 7 and SEQ ID NO:30);DQ182483 (SEQ ID NO: 103; SEQ ID NO: 8 and SEQ ID NO:31); DQ023145 (SEQ ID NO:104; SEQ ID NO: 9 and SEQ ID NO:32); AY741221 (SEQ ID NO:105; SEQ ID NO: 10 and SEQ ID NO:33); AY741219 (SEQ ID NO: 106; SEQ ID NO: 11 and SEQ ID NO:34); AY741217 (SEQ ID NO:108; SEQ ID NO:12
 20 and SEQ ID NO:35); AY741215 (SEQ ID NO:109; SEQ ID NO:13 and SEQ ID NO:36); AY741213 (SEQ ID NO:110; SEQ ID NO:14 and SEQ ID NO:37); AY646175 (SEQ ID NO:111; SEQ ID NO:15 and SEQ ID NO:38); AY646167 (SEQ ID NO:112; SEQ ID NO:16 and SEQ ID NO:39); AY555153 (SEQ ID NO:113; SEQ ID NO:17 and SEQ ID NO:40); AB239125 (SEQ ID NO:114; SEQ ID NO:18 and

SEQ ID NO:41); AB233322 (SEQ ID NO:115; SEQ ID NO:19 and SEQ ID NO:42);
 AB233321 (SEQ ID NO:116; SEQ ID NO:20 and SEQ ID NO:43); AB233320 (SEQ
 ID NO:117; SEQ ID NO:21 and SEQ ID NO:44); AB233319 (SEQ ID NO: 118;
 SEQ ID NO:22 and SEQ ID NO:45); AB212649 (SEQ ID NO:119; SEQ ID NO:23
 5 and SEQ ID NO:46).

Figure 13 Amino acid sequence alignment of HA across various strains of H3N2
 subtype of influenza A virus. The boxed regions marked "A" and "B" represent loops
 A and B respectively. The sequences of full length HA, loop A and loop B
 respectively may be identified as follows:U97740 (SEQ ID NO:120; SEQ ID NO: 47
 10 and SEQ ID NO:61);P03437 (SEQ ID NO:121; SEQ ID NO: 48 and SEQ ID
 NO:62);J02132 (SEQ ID NO: 122; SEQ ID NO:49 and SEQ ID NO:63);J02090
 (SEQ ID NO:123; SEQ ID NO:50 and SEQ ID NO:64);DQ508929 (SEQ ID NO:124;
 SEQ ID NO:51 and SEQ ID NO:65);DQ508865 (SEQ ID NO:125; SEQ ID NO:52
 and SEQ ID NO:66);DQ508849 (SEQ ID NO:126; SEQ ID NO:53 and SEQ ID
 15 NO:67);DQ508833 (SEQ ID NO: 127; SEQ ID NO: 54 and SEQ ID
 NO:68);DQ508825 (SEQ ID NO: 128; SEQ ID NO:55 and SEQ ID
 NO:69);AY779254 (SEQ ID NO:129; SEQ ID NO:56 and SEQ ID
 NO:70);AY779253 (SEQ ID NO:130; SEQ ID NO:57 and SEQ ID
 NO:71);AB019356 (SEQ ID NO:131; SEQ ID NO:58 and SEQ ID
 20 NO:72);AB019354 (SEQ ID NO:132; SEQ ID NO:59 and SEQ ID
 NO:73);AAA43099 (SEQ ID NO: 133; SEQ ID NO:60 and SEQ ID NO:74).

Figure 14 Amino acid sequence alignment of VP1 vectors carrying HA loop A
 and loop B of influenza strain H3N2 inserted in S1 of VP1. The sequences can be
 identified as follows: VP1-S1 (SEQ ID NO:88); VP1-S1H3A (SEQ ID NO:134);

VP1-S1H3B (SEQ ID NO:135).

Figure 15 Amino acid sequence alignment of VP1 vectors carrying HA loop A and loop B of influenza strain H3N2 inserted into S4 of VP1. The sequences can be identified as follows: VP1-S4 (SEQ ID NO:89), VP1-S4-H3A (SEQ ID NO:136),

5 VP1-S4H3B (SEQ ID NO:137).

Figure 16 EM images of VP1-H3-S1A VLPs. Magnification is 200,000x (Scale-bar 100nm).

Figure 17 EM images of VP1-H3-S1B VLPs. Magnification is 200,000x. (Scale-bar 100nm).

10 Figure 18 Amino acid sequence alignment of VP1 vectors carrying HA loop A and loop B of influenza strain H5N1 inserted into S4 of VP1. The sequences can be identified as follows: VP1-S4 (SEQ ID NO:89), VP1-S4A (SEQ ID NO:138), VP1-S4B (SEQ ID NO:139), VP1-S4hM2e (SEQ ID NO: 140).

Figure 19 Purification of GST-VP1-S4A (Figure 19A) and GST-VP1-S4B
15 (Figure 19B) using GSTrap HP column. X-axis is volume in ml; Y1 is absorbance in mAU; Y2 is conductivity in mS/cm. Line A represents the UV trace at 280nm; Line C represents UV trace at 260nm; Line B represents the conductivity trace; the vertical dashed line is the injection point of sample onto the column.

Figure 20 Separation of thrombin digestion and capsomere purification of GST-
20 VP1-S4A (Figure 20A) and GST-VP1-S4B (Figure 20B) on a Superdex S200 10/300 GL size exclusion column (GE Healthcare). X-axis is volume in ml; Y1 is absorbance in mAU; Y2 is conductivity in mS/cm. Line A represents the UV trace at 280nm; ; Line C represents UV trace at 260nm; Line B represents conductivity trace; the vertical dashed line is the injection point of sample onto the column.

Figure 21 Comparison of GST-VP1 with GST-VP1-S4A and GST-VP1-S4B by bioanalyzer analysis.

Figure 22 AFFF plot showing assembled GST-VP1-S4A and GST-VP1-S4B VLPs. X-axis is time (minutes) and Y-axis is rms radius (nm).

5 Figure 23 EM images of VP1-S4A (Figure 23 A), VP1-S4B (Figure 23 B) and wild-type VP1 (Figure 23 C). Magnification of all images is 200,000x (scale-bar 100nm).

DETAILED DESCRIPTION OF THE INVENTION

The isolated proteins of the present invention and VLPs derived therefrom
10 provide a new and advantageous broad spectrum vaccine for treatment of emerging diseases and, in particular, influenza. Surprisingly, the inventors have utilised the natural mechanism which viruses have evolved to evade adaptive immunity to produce a broad-spectrum vaccine which can be manufactured rapidly in response to a new viral threat, has low manufacturing costs and low requirements for specialised
15 infrastructure during manufacture, and complies with regulatory standards.

In particular, present conventional approaches in eukaryotes to VLP vaccine production principally rely upon *in vivo* assembly of the VLP from overexpression of the viral proteins and extensive *ex vivo* post-expression processing to separate the VLP from structurally related contaminants, DNA and harmful unrelated viruses that
20 may be present as a result of the cell culture process or else through contamination during the extended times required for cell culture. Separation of the VLP from viruses having closely related physical properties presents a significant processing challenge. VLP vaccines to influenza based on eukaryotic expression of full-length HA and M protein, with or without co-expression of NA, also incorporate an ill-

defined membrane which is labile during processing, giving complex and heterogeneous VLP structures following purification. These problems with existing VLP vaccines remain largely unresolved for influenza vaccines, particularly for complex structures such as those comprising HA, M and/or NA protein that must be
5 assembled *in vivo*.

The present invention overcomes the purification-separation problems and avoids the creation of a VLP having a complex structure including a labile membrane. By creating a simple minimalist VLP structure, this invention enables disassembly-reassembly processing of the VLP structure.

10 For culture methods that create a relatively simple VLP structure *in vivo*, that is for example a VLP comprising one foreign protein, such VLPs can be disassembled and the VLP protein purified with high efficiency using conventional technologies widely available at large scale. Purified protein can be sterile filtered to ensure removal of adventitious virus, as routinely practised for conventional protein
15 therapeutics. Following the preparation of purified sterile-filtered protein, the VLP is recreated *in vitro* through appropriate chemical control of the reactor environment.

For culture methods that do not create a VLP structure *in vivo* (e.g. using fast-growing bacterium such as *E. coli*), the process of manufacture is further speeded and simplified. In such cases the current invention is not predicated on the availability of
20 specialised infrastructure nor extended cultivation times, allowing for rapid production in diverse manufacturing sites using technologies widely available for the production of protein therapeutics. The use of a simple and minimalist VLP structure allows for the creation of a VLP structure through *in vitro* assembly after protein expression and purification.

Regardless of the culture method employed, the approach of present invention of *in vitro* assembly following protein purification and sterile filtering provides for precise control of the VLP assembly step. The assembly of the VLP structure is under control of the process operator thus removing limitations inherent for *in vivo* assembly approaches. This improved control overcomes problems with conventional VLP technologies, and in particular, for influenza VLP vaccines, and produces a superior VLP which has higher morphological homogeneity. Precise control of VLP assembly also allows for the incorporation of precisely defined antigenic DNA sequences into the growing VLP structure, under the control of the process operator, giving opportunity for rapid evolution of vaccine design.

Isolated proteins

It is appreciated by a person of skill in the art that VP1 from polyomavirus is particularly well suited to the present invention. Preferably, the VP1 is derived from murine polyomavirus.

Although not wishing to be bound by any particular theory, the presence of loops on the surface of a virus particle provide ideal candidates for recognition by the immune system. Such loops are particularly suitable if tolerant to insertion of foreign sequences without disruption to the protein structure.

Although not limited thereto, VP1 comprises four surface exposed loops which span around about residues 82-89, 221-224, 247-249 and 292-297 and are referred to as site 1, site 2, site 3 and site 4 respectively. In the context of the present invention, typically, although not exclusively, at least one exposed loop site has an insertion. It can be appreciated that to facilitate generation of a more potent or, alternatively a broad-spectrum VLP, one, two, three or four sites may comprise an

insertion.

Preferably, one, two or three exposed loops comprise an insertion. More preferably, the one or more exposed loops comprising an insertion are selected from the group consisting of site 1, site 3 and site 4.

- 5 In more preferred embodiments, the one or more exposed loops comprising an insertion are selected from the group consisting of site 1 and site 4.

The isolated protein of the invention may be referred to hereinafter as a “*VPI chimera*”. By “*chimera*” is meant a fusion between at least two proteins or a fusion between fragments of said at least two proteins. Typically, although not exclusively,
10 the proteins are unrelated however it is readily contemplated that the proteins may be homologues.

By “*protein*” is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids, D- or L- amino acids as are well understood in the art.

- 15 The term “*protein*” includes and encompasses “*peptide*”, which is typically used to describe a protein having no more than fifty (50) amino acids and “*polypeptide*”, which is typically used to describe a protein having more than fifty (50) amino acids.

For the purposes of this invention, by “*isolated*” is meant material that has
20 been removed from its natural state or otherwise been subjected to human manipulation. Isolated material 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 or recombinant form.

The invention also contemplates use of a fragment of the VP1 chimera or alternatively, a fragment of the virus protein other than VP1.

In one embodiment, a “*fragment*” includes a protein comprising an amino acid sequence that constitutes less than 100% of an amino acid sequence of an entire
5 VP1 or a virus protein other than VP1.

In the general embodiments which contemplate a fragment of the VP1 chimera, preferably the fragment comprises less than 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 85%, 80%, 75% or 70%, but greater than 50%, of the entire protein.

10 In the other general embodiments which contemplate a fragment of the virus protein other than VP1, preferably the fragment comprises less than 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55% or 50% but can also relate to a peptide as hereinbefore described.

Preferably, the fragment is a “*biologically active*” fragment, which retains
15 biological, structural and/or physical activity of a given protein, or an encoding nucleic acid. In the context of VP1, the biologically active fragment preferably has the ability to self-assemble and form VLPs.

The choice of which virus protein other than VP1 to insert into the exposed loops can be made taking into account the particular desired end result. The invention
20 is well suited to proteins which are involved in eliciting an immune response to a pathogen such as, but not limited to, influenza virus, human immunodeficiency virus, hepatitis C virus, Ebola virus, measles virus, parainfluenza virus and respiratory syncytial virus. The invention is particularly suited to proteins that are subject to continuous alteration in circulating epidemic strains. It is envisaged that fragments

which correspond to antigenic epitopes of the virus protein other than VP1 can be used for insertion.

By "*corresponds to*" in the context of the present invention, is meant an amino acid sequence which shares primary sequence characteristics of another amino acid sequence but is not necessarily derived or obtained from the same source as said
5 another amino acid sequence.

In one embodiment, the virus protein other than VP1 is a protein from influenza virus such as HA, NP, NA, M1 or M2.

In a preferred embodiment, the virus protein other than VP1 is selected from
10 the group consisting of HA and M2.

In another preferred embodiment, the virus protein other than VP1 is a domain of M2. Typically, although not exclusively, the domain of M2 is an ectodomain.

In particular preferred embodiments, the virus protein other than VP1 corresponds to an exposed loop of HA selected from the group consisting of loop A,
15 loop B, loop C, loop D and loop E, or a fragment thereof. Preferably, the exposed loop of HA is selected from the group consisting of loop A, loop B, loop C and loop E. More preferably, the exposed loop of HA is selected from the group consisting of loop A and loop B.

In other particular preferred embodiments, the fragment of a virus protein other
20 than VP1 is an antigenic epitope. In more preferred embodiments, the fragment is an antigenic epitope of HA. In even more preferred embodiments, the fragment is an antigenic epitope of an exposed loop of HA selected from the group consisting of loop A, loop B, loop C and loop E.

A person skilled in the art will appreciate that viruses have evolved a number

of mechanisms to evade the host cell immune response and, as a consequence, lead to generation of escape mutants. One such mechanism is the presence of a region within a virus protein/s with a high degree of variability, the so named hypervariable region.

It will further be appreciated that the variability within an antigenic epitope
5 between virus subtypes, and in particular influenza virus subtypes, can be substantial. Advantageously, although not exclusively, loop A and loop B comprise a minimal region which display a high degree of variability across virus subtypes.

Therefore in general embodiments where the virus protein other than VP1 is HA derived from a H5 subtype of influenza virus, at least one consensus amino acid
10 sequence for an antigenic epitope of loop A is PYqGKSS (SEQ ID NO:75) (there is common q<-->N and K<-->R variability) whereas at least one consensus sequence for an antigenic epitope of loop B is PNDAAEQTKLYQNPTTY (SEQ ID NO:76) (there is common K<-->R variability), although without limitation thereto.

In other general embodiments where the virus protein other than VP1 is HA
15 derived from a H3 subtype of influenza virus, at least one consensus amino acid sequence for an antigenic epitope of loop A is KRGPgSG (SEQ ID NO:77) (there is common PgS<-->PaS variability) whereas at least one consensus amino acid sequence for an antigenic epitope of loop B is PSTNQEQTsLYVQASGR (SEQ ID NO:78) (there is common TsL<-->TNL variability), although without limitation
20 thereto.

In yet other general embodiments where the virus protein other than VP1 is HA derived from a H1 subtype of influenza virus, at least one consensus amino acid sequence for an antigenic epitope of loop A is SHKGKSS (SEQ ID NO:79), whereas at least one consensus amino acid sequence of loop B is PSNIEDQKTIYRKENAY

(SEQ ID NO:80), although without limitation thereto.

The present invention also contemplates variants of the virus protein other than VP1.

In light of the foregoing, it will be appreciated that a variant includes within its
5 scope a natural variant such as, although not limited to, a variant arising from natural
antigenic variation of a virus wherein one or more residues are different to at least
one consensus sequence. It will be appreciated that an absence of primary amino acid
consensus sequence between antigenic epitopes from different virus subtypes is not
unexpected considering the propensity for a number of viruses, including influenza,
10 to undergo antigenic shift and antigenic drift in order to generate escape mutants.
Hence a variant includes variation within the subtypes of a virus and/or variation
between subtypes of a virus, but is not limited thereto. By way of example only, at
least one consensus amino acid sequence for loop A derived from a H5 variant of
influenza A virus is PYNGKSS (SEQ ID NO: 81) whereas the corresponding region
15 in HA from H3 subtype is KRGP GSG (SEQ ID NO: 82).

According to the aforementioned embodiments, variants are contemplated
where the substitutions in amino acid sequences are conservative in nature.

Exemplary variant antigenic epitopes of loop A and loop from HA are listed in
Figure 12 and Figure 13.

20 Therefore it is further contemplated that either a hypervariable region or a
conserved region of a virus protein other than VP1 may be inserted into the VP1
chimera, or a combination thereof.

The invention therefore also contemplates variants of epitopes, isolated
proteins and encoding nucleic acids which share an appropriate level of sequence

identity with isolated proteins and encoding nucleic acids as set forth herein.

The term "*sequence identity*" is used herein in its broadest sense to include the number of exact nucleotide or amino acid matches having regard to an appropriate alignment using a standard algorithm, having regard to the extent that sequences are
5 identical over a window of comparison. Thus, a "*percentage of sequence identity*" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U) or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total
10 number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For example, "*sequence identity*" may be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA).

15 In particular embodiments, variants will share at least 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or 90% and more preferably at least 95%, 96%, 97%, 98% or 99% sequence identity with the isolated proteins and/or isolated nucleic acids of the invention. It will be appreciated that a variant comprises all integer values less than 100%, for example the percent value as set forth above and others.

20 The VP1 chimera may also comprise one or more additional amino acid sequences. "*Additions*" of amino acids may include fusion of a VP1 protein of the invention or a fragment thereof with other proteins or peptides. The other protein may, by way of example, assist in the purification of the protein. For instance, these include a polyhistidine tag, maltose binding protein (MBP), green fluorescent protein

(GFP), Protein A or glutathione S-transferase (GST). Other additions include "epitope tags" such as FLAG and c-myc epitope tags. It is further contemplated that more than one fusion partner may be included into the isolated protein.

Well known examples of fusion partners include, but are not limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP), NusA and hexahistidine (HIS₆), which are particularly useful for isolation of the fusion protein by affinity chromatography. For the purposes of fusion protein purification by affinity chromatography, relevant matrices for affinity chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpressTM system (Qiagen) useful with (HIS₆) fusion partners and the Pharmacia GST purification system.

In some cases, the fusion partners also have protease cleavage sites, such as for Factor X_a, Thrombin or human rhinovirus 3C protease, which allow the relevant protease to partially digest the fusion protein of the invention and thereby liberate the recombinant protein of the invention therefrom. The liberated protein can then be isolated from the fusion partner by subsequent chromatographic separation.

The invention also contemplates chemical derivatives of the VP1 chimera, such as produced using techniques described in CURRENT PROTOCOLS IN PROTEIN SCIENCE Chapter 15, for example.

In particularly preferred embodiments, the isolated protein or VP1 chimera of the present invention comprises an amino acid sequence selected from the group consisting of SEQ ID NO:92; SEQ ID NO:93; SEQ ID NO:94; SEQ ID NO:95; SEQ ID NO:134; SEQ ID NO:135; SEQ ID NO:136; SEQ ID NO:137; SEQ ID NO:138;

SEQ ID NO:139; and SEQ ID NO: 140.

Isolated nucleic acids and expression constructs

It will be appreciated from the foregoing and also from pharmaceutical compositions described in more detail hereinafter, that the invention also provides
5 use of an isolated nucleic acid encoding the VP1 chimeric protein of the invention. Non-limiting examples of isolated nucleic acids are provided in Figures 1 and 2.

The term “*nucleic acid*” as used herein designates single-or double-stranded mRNA, RNA, cRNA and DNA inclusive of cDNA, genomic DNA and DNA-RNA hybrids. Nucleic acids may also be conjugated with fluorochromes, enzymes and
10 peptides as are well known in the art.

A “*probe*” may be a single or double-stranded oligonucleotide or polynucleotide, suitably labeled for the purpose of detecting complementary sequences in Northern or Southern blotting, for example.

A “*primer*” is usually a single-stranded oligonucleotide, preferably having 15-
15 50 contiguous nucleotides, which is capable of annealing to a complementary nucleic acid “template” and being extended in a template-dependent fashion by the action of a DNA polymerase such as *Taq* polymerase, RNA-dependent DNA polymerase or SequenaseTM.

It can be readily appreciated by a person of skill in the art that the isolated
20 nucleic acids of the present invention may be produced and manipulated using an assortment of recombinant techniques. Examples of preparation of such isolated nucleic acids is given in Examples 1 and 2.

In a preferred embodiment, the nucleotide sequences encoding the one or more exposed loops of VP1 are adapted to receive an isolated nucleic acid encoding a

virus protein other than VP1. By "*adapted to receive*" is meant the manipulation, by recombinant means or other, of nucleotide sequences in order to facilitate insertion, excision or mutagenesis of native or foreign nucleotide sequences. By way of example only, a nucleotide sequence may be engineered to incorporate new
5 restriction enzyme sites in order to improve the speed and efficiency of cloning procedures. Although not limited thereto, restriction enzymes that do not create an overhang are the most amenable to high speed cloning of amplification products generated by PCR with super high-fidelity polymerases such as *Pfx*. A nucleotide sequence adapted in such a manner is also amenable to high-throughput screening of
10 vaccine candidates.

A person of skill in the art would readily appreciate that mutagenesis can be performed using an assortment of recombinant techniques. Non-limiting examples of suitable techniques include random mutagenesis (*e.g.*, transposon mutagenesis), oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis and
15 cassette mutagenesis.

In a preferred embodiment, the isolated nucleic acid comprises a nucleotide sequence encoding the VP1 chimera wherein the nucleotide sequence of one or more exposed loops has been mutated to include a recognition site for a restriction enzyme which generates blunt-ends.

20 The invention also contemplates variant VP1 nucleic acids having one or more codon sequences altered by taking advantage of codon sequence redundancy.

A particular example of a variant VP1 nucleic acid is optimization of a nucleic acid sequence according to codon usage, as is well known in the art. This can effectively "tailor" a nucleic acid for optimal expression in a particular organism, or

cells thereof, where preferential codon usage has been established.

The invention further contemplates an expression construct comprising an isolated nucleic acid encoding the VP1 chimera operably-linked to one or more regulatory sequences in an expression vector.

- 5 An “*expression vector*” may be either a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into a host genome, inclusive of vectors of viral origin such as adenovirus, lentivirus, poxvirus and flavivirus vectors as are well known in the art.

- By “*operably linked*” is meant that said regulatory nucleotide sequence(s)
10 is/are positioned relative to the recombinant nucleic acid of the invention to initiate, control, regulate or otherwise direct transcription and/or other processes associated with expression of said nucleic acid.

- Regulatory nucleotide sequences will generally be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and
15 suitable regulatory sequences are known in the art for a variety of host cells.

- Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, splice donor/acceptor sequences, enhancer or activator
20 sequences and nucleic acid packaging signals.

 Preferably, said promoter is operable in a bacterial cell. Non-limiting examples include T7 promoter, *tac* promoter and T5 promoter.

 Inducible/repressible promoters (such as *tet*-repressible promoters and IPTG-, alcohol-, metallothionine- or ecdysone-inducible promoters) are well known in the art

and are contemplated by the invention, as are tissue-specific promoters such as α -crystallin promoters. It will also be appreciated that promoters may be hybrid promoters that combine elements of more than one promoter (such as SR α promoter).

- 5 The expression construct may also include a fusion partner (typically provided by the expression vector) so that the VP1 chimera is expressed as a fusion protein with said fusion partner, as hereinbefore described. Expression constructs may also include a selection marker nucleic acid that confers transformed host cell resistance to a selection agent. Selection markers useful for the purposes of selection
- 10 of transformed bacteria include *bla*, *kanR* and *tetR* while transformed eukaryotic cells may be selected by markers such as hygromycin, G418 and puromycin, although without limitation thereto.

- Expression constructs may be introduced into cells or tissues, inclusive of cells capable of VLP production, by any of a number of well known methods
- 15 typically referred to as “*transfection*” “*transduction*”, “*transformation*” and the like. Non-limiting examples of such methods include transformation by heat shock, electroporation, DEAE-Dextran transfection, microinjection, liposome-mediated transfection (e.g. lipofectamine, lipofectin), calcium phosphate precipitated transfection, viral transformation, protoplast fusion, microparticle bombardment and
- 20 the like.

It is readily contemplated that any recombinant protein expression system may be used for the present invention such as bacterial, yeast, plant, mammalian and insect-based expression systems but is not limited thereto.

In one preferred embodiment, recombinant protein expression occurs in cells

of prokaryotic origin. Suitable host cells for recombinant protein expression are bacterial cells such as *Escherichia coli* (BL21 and various derivative strains thereof which have been optimised for certain applications, such as Rosetta, for example).

Methodology for production of VLPs

5 One broad application of the isolated proteins, isolated nucleic acids and expression constructs of the present invention is a platform technology for production of a vaccine. In particular, the present invention facilitates rapid assembly and production of VLPs *in vitro*.

Many methods are well known in the art for VLP assembly. Pattenden and co-
10 workers (2005, Trends in Biotechnology 10: 523-529) provides a non-limiting example of such methodology. The present invention readily contemplates both *in vivo* and *in vitro* approaches to VLP assembly however the invention is particularly suited to *in vitro* processes for assembly of VLPs. Advantages conferred by *in vitro* methods include high compositional consistency and a process which is readily
15 controlled and amenable to scale-up.

Typically, although not exclusively, *in vitro* assembly method of VLPs comprises overexpression of a pentameric subunit of a self-assembly protein, such as VP1 or the VP1 chimera of the present invention, in a recombinant expression system as described above. Although not limited thereto, advantageously the recombinant
20 protein is expressed in the soluble fraction of the cell. The recombinant protein may be subsequently purified using a combination of tag removal, filtration and/or chromatography techniques, followed by sterile filtration, which are well known in the art. This lack of a requirement for specialised infrastructure and the use of well-known readily scaleable operations makes the current invention distinctly

advantageous. Subsequent self-assembly of these subunits into a VLP is achieved by means of controlled changes in the physicochemical environment containing these subunits. By way of example only, assembly of VLPs may be more favourable under high salt or alternatively, low salt conditions. Inclusion and/or removal of low-
5 molecular weight compounds such as glycerol and carbohydrates may also facilitate the assembly reaction. Methods typically known in the art such as buffer exchange by dialysis, dilution or size exclusion chromatography may be utilised to effect the changes in the physicochemical conditions of the subunits, but is not limited thereto.

A recombinant protein may be conveniently prepared by a person skilled in
10 the art using standard protocols as for example described in Sambrook *et al.*, MOLECULAR CLONING. A Laboratory Manual (Cold Spring Harbor Press, 1989), incorporated herein by reference, in particular Sections 16 and 17; CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.*, (John Wiley & Sons, Inc. 1995-1999), incorporated herein by reference, in particular Chapters 10
15 and 16; and CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, Inc. 1995-1999) which is incorporated by reference herein, in particular Chapters 1, 5 and 6.

By “purify”, “purified” and “purification”, particularly in the context of recombinant protein purification, is meant enrichment of a recombinant protein so
20 that the relative abundance and/or specific activity of said recombinant protein is increased compared to that before enrichment.

By “chromatography” such as in the context of chromatographic steps of the invention, is meant any technique used for the separation of biomolecules (eg protein and/or nucleic acids) from complex mixtures that employs two phases: a stationary

bed phase and a mobile phase that moves through the stationary bed. Molecules may be separated on the basis of a particular physicochemical property such as charge, size, affinity and hydrophobicity.

Chromatography may be performed by a person skilled in the art using
5 standard protocols as for example described in CURRENT PROTOCOLS IN
PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, Inc. 1995-1999)
which is incorporated by reference herein, in particular Chapters 8 and 9.

The invention also readily contemplates overexpression of the isolated
proteins and isolated nucleic acids of the present invention to generate an intact VLP,
10 which obviates the need for an *in vitro* assembly process. A person skilled in the art
will appreciate that there are a variety of expression systems which are suited for this
application such as, but not limited to, mammalian-based and insect-based expression
systems. In general embodiments, the baculovirus expression system is particularly
amenable to expression of an intact VLP of the invention.

15 It will be appreciated that because of the flexibility of the present invention, a
VLP assembled according to the method of the present invention may comprise a
VP1 backbone with the following combinations inserted into the exposed loops: (a) a
single protein from a single virus; (b) a plurality of different proteins from the same
virus; or (c) a mixture of proteins from various viruses. The present invention is also
20 readily amenable to production of a mixed population of VLPs wherein one or more
VP1 chimeras according to (a), (b) or (c) are assembled into VLPs to thereby produce
a multivalent vaccine delivery system. The present invention is also contemplates
production of a mixed population of VLPs by means of introduction of one or more
expression constructs into a cell.

Pharmaceutical and immunotherapeutic compositions, vaccines and methods of treatment

The present invention provides a VP1 chimera which exploits the self-assembly capacity of VP1 and the potential immunogenicity of exposed loops as a vehicle for delivery for VLP production and vaccine delivery. However, the invention more broadly provides a pharmaceutical composition not limited to use in vaccine delivery, but inclusive of pharmaceutical compositions in several forms such as vessels for the delivery of a number of palliatives such as, but not limited to, small molecules, catalytic nucleic acids such as ribozymes and nucleic acids for gene therapy.

The pharmaceutical composition may further comprise a pharmaceutically-acceptable carrier, diluent or excipient.

In a preferred form, the pharmaceutical composition of the invention is an immunogenic composition comprising an isolated protein, isolated nucleic acid or VLP and an immunologically acceptable carrier, diluent or excipient.

By “*immunogenic*” is meant capable of eliciting an immune response, preferably a protective immune response, upon administration to a host.

Thus in a particular form, the immunogenic composition of the invention may be a vaccine which induces a protective immune response when administered to a host.

It will be appreciated that compositions, vaccines and methods of treatment may be used therapeutically or prophylactically.

Suitably, the immunogenic composition and/or vaccine of the invention is administrable to an animal host, inclusive of domestic animals, livestock,

performance animals and humans.

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.

As hereinbefore described, the immunogenic composition and/or vaccine of the invention may include an “*immunologically-acceptable carrier, diluent or excipient*”.

Useful carriers are well known in the art and include for example: thyroglobulin; albumins such as human serum albumin; toxins, toxoids or any mutant crossreactive 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.

The “*immunologically-acceptable carrier, diluent or excipient*” includes within its scope water, bicarbonate buffer, phosphate buffered saline or saline and/or an adjuvant as is well known in the art.

As will be understood in the art, an “*adjuvant*” means one or more substances that enhances the immunogenicity and/or efficacy of a vaccine composition. Non-limiting examples of suitable adjuvants include squalane and squalene (or other

- oils of animal origin); block copolymers; 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*;
- 5 *Mycobacterium bovis* (Bacille Calmette and Guerin or BCG); interleukins such as interleukin 2 and interleukin 12; monokines such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon; surface active substances such as hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin, dimethyldioctadecylammonium bromide, *N,N*-dioctadecyl-*N'*, *N'*bis(2-
- 10 hydroxyethyl-propanediamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines such as pyran, dextran sulfate, poly IC carbopol; peptides such as muramyl dipeptide and derivatives, dimethylglycine, tuftsin; oil emulsions; and mineral gels such as aluminum phosphate, aluminum hydroxide or alum; combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide;
- 15 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 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); vaccinia or animal poxvirus proteins;
- 20 sub-viral particle adjuvants such as cholera toxin, or mixtures thereof.

Any safe route of administration may be employed for providing a patient with the immunotherapeutic composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, intranasal, inhalational, intraocular, intraperitoneal,

intracerebroventricular, transdermal and the like may be employed. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions and vaccines.

Dosage forms include tablets, dispersions, powders, suspensions, injections, solutions, syrups, troches, capsules, 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 of the therapeutic agent may be effected by coating the same, for example, 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.

Pharmaceutical compositions of the present invention suitable for oral or parenteral administration may be presented as discrete units such as capsules, sachets or tablets each containing a pre-determined amount of one or more therapeutic agents of the invention, 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 invention 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 is pharmaceutically-effective. The dose administered to a patient, in the context of the present invention, should be
5 sufficient to effect a beneficial response in a patient over an appropriate period of time. 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.

Immunotherapeutic compositions of the invention may be used to
10 prophylactically or therapeutically immunize animals such as humans.

However, other animals are contemplated, preferably vertebrate animals including domestic animals such as livestock and companion animals.

Immune responses may be induced against viruses by expressing appropriately immunogenic proteins and peptide epitopes inclusive of polyepitopes
15 using the vaccine of the invention. A non-limiting list of potential viruses include influenza virus, human immunodeficiency virus, hepatitis C virus, Ebola virus, measles virus, parainfluenza virus and respiratory syncytial virus.

It is envisaged that an immune response involves induction of antibodies and/or T-cells.

20 So that the invention may be readily understood and put into practical effect, the following non-limiting Examples are provided.

EXAMPLES

Example 1

Construction of generic vectors carrying VP1 sequences able to accept antigenic

peptide sequences

Murine polyomavirus VP1 sequence (GenBank accession number: M34958) was cloned between the *Bam*HI and *Xho*I sites within the multiple cloning site of the commercial vector pGEX-4T-1 (GE healthcare). Surface-exposed loops of polyomavirus VP1 are known as Site 1, Site 2, Site 3 and Site 4. These sites can tolerate insertion of peptide sequences without disrupting the structure of VP1.

Sites 1-4 (S1-4) were identified on murine polyomavirus VP1 (GenBank accession number: M34958). Three unique blunt-end restriction sites were created in the S1, S3 and S4 of VP1. No restriction site was created in S2 as it is not a favourable site for insertion of foreign peptides. Blunt-end restriction sites were chosen, as they allowed rapid cloning of any peptide sequences into VP1 without the need to create sticky ends on vectors/inserts. This strategy also removes the possibility of incompatible restriction sites that may be present on target peptide sequence.

The amino acid sequence of VP1 present in the generic vectors is depicted in Figure 1. S1 of VP1 was mutated to include *Nae*I restriction site (LATSDTED mutated to LATSAGTED). S3 of VP1 was mutated to include *Pml*I restriction site (GTTT mutated to GTHV). S4 of VP1 was mutated to include *Afe*I restriction site (TRNYDV mutated to TRSAYDV).

Resulting generic vectors with modified VP1 sequences to enable the insertion of one or multiple foreign peptides are: pGEX4T1-VP1 S1, pGEX4T1-VP1 S3, pGEX4T1-VP1 S4, pGEX4T1-VP1 S1/S4 and pGEX4T1-VP1 S1/S3/S4.

Example 2**Construction and sequencing of vectors expressing a foreign antigen**

Construction and sequencing of vectors carrying HA epitopes from loop A and loop B of H5N1:

E. coli codon optimised oligonucleotides were designed for epitopes A (7 amino acids, PYNGKSS) and B (17 amino acids, PNDAAEQTKLYQNPTTY) for insertion
 5 into generic vectors. The sequences of epitopes A and B are of H5N1 (A/Vietnam/3028/2004).

Resulting constructs carrying either HA epitopes A or B are pGEX4T1-VP1 S1A, pGEX4T1-VP1 S1/S3/S4 1A, pGEX4T1-VP1 S1/S3/S4 3A, pGEX4T1-VP1 S1/S3/S4 4A, pGEX4T1-VP1 S1B, pGEX4T1-VP1 S1/S3/S4 1B, pGEX4T1-VP1
 10 S1/S3/S4 3B and pGEX4T1-VP1 S1/S3/S4 4B.

Construction of vectors carrying M2e:

E. coli codon optimised oligonucleotides were designed to insert M2e peptide (SLLTEVETPTRNEWECRCSDSSD; SEQ ID NO: 83) into the generic vectors.

Resulting constructs carrying M2e are designated pGEX4T1-VP1 S1M2e,
 15 pGEX4T1-VP1 S1/S3/S4 1M2e, pGEX4T1-VP1 S1/S3/S4 3M2e and pGEX4T1-VP1 S1/S3/S4 4M2e.

Construction of vectors carrying hM2e:

hM2e (23 amino acids) antibody is commercially available and has been shown to target the specific hM2e amino acid sequence
 20 (SLLTEVETPIRNEWGCRCNDSSD; SEQ ID NO: 84).

E. coli codon optimised oligos for hM2e were designed to insert hM2e into the generic vectors. This system is used as a proof-of-concept model since no commercial antibody against other cloned peptides is available.

Resulting constructs carrying hM2e are designated as follows pGEX4T1-VP1 S1hM2e, pGEX4T1-VP1 S1/S3/S4 1hM2e, pGEX4T1-VP1 S1/S3/S4 3hM2e and pGEX4T1-VP1 S1/S3/S4 4hM2e.

The amino acid sequence of VP1-S1 with inserted peptides from
5 representative constructs is exemplified in Figure 2.

Example 3

Expression and purification GST-VP1-S1A and assembly of VP1-S1A into multimerically-defined VLPs

Expression

10 VP1-S1A protein was expressed in Rosetta (DE3) pLysS (Novagen) as a glutathione-s-transferase-VP1-S1A (GST-VP1-S1A) fusion protein using the pGEX-4T-1-VP1-S1A expression vector. For solid agar medium, 15 g/L of agar was added to Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0). Liquid medium used for cell cultures was Terrific Broth (TB) containing 12 g/L
15 peptone, 24 g/L yeast extract, 0.4% (v/v) glycerol, 2.31 g/L KH_2PO_4 and 12.54 g/L K_2HPO_4 . All media used were supplemented with 50 $\mu\text{g/mL}$ of ampicillin and 34 $\mu\text{g/mL}$ of chloramphenicol. Overnight cultures were set up by inoculating single colonies of the transformed cells into 5 mL of TB medium and cultivated at 30 °C on a rotary shaker at 180 rpm. After incubation of 16 hours, the overnight cultures were
20 diluted 1000 times into 400 mL cultures in 2 L baffled flasks. These cultures were cultivated to an OD_{600} of 0.5 at 37 °C and then cooled to 26 °C under running tap water and subsequently induced with IPTG at final concentration of 0.2 mM. The induced cultures were then cultivated at 26 °C for a further 16 hours and harvested by centrifugation at 8000 g, 4°C for 30 mins.

Purification of VP1-S1A capsomeres

Two pellets from 2x400 mL cultures were re-suspended in 150 mL of buffer L (200 mM NaCl, 40 mM Tris, 5mM dithiothreitol, 1 mM EDTA, 5% v/v glycerol, pH 8 with HCl). The cell suspension was passed through a high-pressure
5 homogenizer (Niro-Soavi) once at 1000 bar; dilution gave a final volume of 175 mL. The resulting homogenate was centrifuged at 16000 g, 4°C for 30 mins and the supernatant which contained soluble GST-VP1-S1A was filtered through an 0.45 µm syringe filters (Millipore) giving a recovery of 135 mL.

Protein purification was performed with an Äkta Explorer system from GE
10 Healthcare. GST-VP1-S1A in the clarified homogenate was captured using a GSTrap™ HP 5 mL column from GE Healthcare pre-equilibrated with buffer L. The bound GST-VP1-S1A was eluted with elution buffer (Buffer L with 10 mM reduced glutathione). Eluted samples were stored at -80°C until required.

Purified thawed GST-VP1-S1A was buffer exchanged into buffer L by
15 desalting (Desalting column 15/12, in-house packed) on the Akta Explorer system.

The eluate was pooled and loaded onto a 5 mL GSTrap™ HP column equilibrated in buffer L. Elution was achieved with elution buffer (buffer L with 10 mM reduced glutathione). Eluate (4.2 mg/mL) was stored at -80°C.

The eluate from above was thawed on ice and 8 M urea was added to a final
20 concentration of 0.5 M (to inhibit capsomere aggregation after release from the solubilising GST tag). Thrombin (20 U/mg of GST-VP1-S1A, Sigma) was added followed by 3 hours incubation at room temperature on a roller.

500 µL of thrombin treated capsomere was injected onto a Superdex S200 10/300 GL size exclusion column (GE Healthcare) equilibrated in buffer L

supplemented with 0.5 M urea, to separate capsomeres from aggregates, GST and thrombin. Capsomere fractions eluting at a volume of 10-12 mL were pooled, aliquoted and frozen at -80°C for subsequent VLP assembly.

Assembly of capsomeres into multimerically-defined VLPs

- 5 The starting material after S200 separation is shown in Figure 8. Capsomeres were assembled by dialysis against buffer 1 (0.5 M (NH₄)₂SO₄, 20 mM Tris, pH 7.4, 5 % (v/v) glycerol, 1 mM CaCl₂) for 17.5 hours, and then against buffer 2 (200 mM NaCl, 20 mM Tris, pH 7.4, 5 % (v/v) glycerol, 1 mM CaCl₂) for another 24 hours. Dialysis was at room temperature with a 10,000 MWCO dialysis membrane.

10 Physical Characterisation of multimerically-defined VLPs

- Assembled VLPs were analysed by both asymmetric field-flow fractionation (AFFF, Wyatt Technologies) and electron microscopy (EM). The Eclipse AFFF system purchased from Wyatt Technology Corporation. The separation channel comprised a polyetheretherketone (PEEK) lower block fitted with a stainless steel
15 frit. The upper block was an aluminum frame to which a replaceable polycarbonate inlay was secured. The spacer used was trapezoidal with a thickness of 350 µm and a length of 26.5 cm. The breadths of the spacer near the channel inlet and outlet were 1.5 and 0.5 cm, respectively. A regenerated cellulose membrane with a molecular weight cut-off of 10 kDa was utilized as the accumulation wall. The cross-flow and
20 injection flow rates were each monitored and regulated by a LiquiFlow device (Bronkhorst). For precise control of the focusing point, motor-driven needle valves were also used to regulate the focus and injection flows. The channel pressure was monitored by a pressure sensor, and overpressure of the channel module was prevented by means of a pressure-relief valve. Separation methods were written using

the Eclipse software, which served to control the AFFF system. AFFF analysis of VLP samples were performed in GL buffer 2. The flow rate through the detectors was maintained at 0.75 mL/min during operation. A typical AFFF separation involved an initial increase of flow from zero to 0.75 mL/min in elution mode.

5 The cross-flow rate was then maintained when the focusing mode was started. 100 μ L of sample was then injected into the channel through the injection port at 0.2 mL/min, after which focusing was maintained for another 7 mins. To minimize the perturbations of detector signals at the switching between focusing and elution modes, a stream of bypass flow was maintained through the detectors during focusing

10 mode at 0.75 mL/min. At the end of focusing mode, the analytes were eluted under the same cross-flow rate for 30 mins, after which the cross-flow rate was ramped down to zero to allow elution of highly retained analytes. The results of the AFFF analysis are shown in Figure 9.

For EM, 2 μ L of samples were applied to glow-discharged, Formvar carbon-coated grids. The remaining liquid on the grids were drained off after 2 mins, and the

15 grids were then negatively stained with 2 % uranyl acetate for 20 s. The samples on the grids were viewed under the Philips TECNAI 12 electron microscope and digital images were acquired using the integrated CCD camera and image acquisition software. EM images showing assembled VLPs are in Figure 10. The results show

20 that (i) protein was produced at high expression levels and purified to high homogeneity using conventional chromatographic operations and with efficient removal of the tag (Figure 6), and (ii) this protein could be efficiently assembled into VLP structures (Figure 10) having a size appropriate for VP1 VLPs (Figure 9).

Example 4

Expression of VP1-S1hM2e capsomeres and antibody challenge

GST-VP1-S1hM2e protein was prepared as described in example 3 for GST-VP1-S1A. Protein was purified only once by GST chromatography and stored at -80°C.

5 GST-VP1-S1hM2e protein was thawed and challenged in a slot blot with mouse monoclonal [14C2] antibody to influenza A virus M2 protein from Abcam (Cambridge, UK) recognizing the hM2e sequence inserted into S1 of VP1 (α hM2e), or else α VP1 antibody (Dr Ke An, Kansas State University). Figure 11 shows the result of slot blot analysis. As a negative control, VP1 native VLPs (prepared by
10 expression in baculovirus) were challenged with the same antibodies. VP1 concentrations ranged from 10 μ g/ml (lane 2) to 0.1 pg/ml (lane 10) with 200 μ L loaded per well. Negative control was BSA. The membrane was blocked, challenged with primary antibody, washed 3x in PBS/0.1% Tween, and challenged with secondary antibody followed by chromogenic development.

15 Figure 11 shows conclusively that the antibody to the hM2e epitope recognised the chimeric VP1 containing the hM2e sequence, but did not react with the unmodified VP1 protein, suggesting that this epitope will be well presented to the immune system.

Example 5**20 Sequence Alignment of HA from subtypes of Influenza virus**

A sequence alignment of HA from various strains of H5 and H3 subtypes of influenza was performed. The results of this alignment are presented in Figure 12 and Figure 13.

The consensus H5N1 sequence for the epitopes is:

A: PYqGKSS (there is common q<-->N and K<-->R variability)

B: PNDAAEQTKLYQNPTTY (there is common K<-->R variability)

Note the q<-->N variability in A between the consensus. There is also K<-->R variability in both A&B. All of these mutations are conservative (e.g. K<-->R preserves the charge structure). The variability between H5 types is in the A&B regions is small compared with the variability across virus variants.

Mem71 is a H3 variant. The sequence was obtained and aligned; the corresponding peptide loops are:

A: KRGPgSG (there is common PgS<-->PaS variability)

10 B: PSTNQEQTsLYVQASGR (there is common TsL<-->TNL variability)

Again the variability is conservative, although there is a lack of primary sequence consensus with H5.

Although the alignment is not presented, starting with H1N1 accession P18875, a consensus was generated by Blastp against UNIPROT and then ClustalW then CONSP. The resulting loops A and B are:

A: SHKGKSS (SEQ ID NO:85)

B: PSNIEDQKTIYRKENAY (SEQ ID NO:86)

Example 6

Immunization with VLPs and Challenge Experiments in Animal Model

20 Challenge will be intranasally with Mem71 at week 5, after vaccination at week 0 and week 3 with VP1-S1 VLPs containing inserted peptide antigen from loops A (KRGPgSG; SEQ ID NO: 81) and loops B (PSTNQEQTsLYVQASGR; SEQ ID NO: 78) of the H3 Mem71 virus.

Two doses of 3µg of HA will be given in the form of split Mem71 virus

vaccine as a positive control as this will give complete protection after two doses.

Assuming that HA is 30% of the virion, this would be the equivalent of 9µg/dose of virus. Whole, unsplit virus is more immunogenic than split. A volume of 50µL of VLP vaccine will be injected. The carrier VLP with no insertion will also

5 be tested.

The challenge design is:

VLP Testing

Mouse group	# of animals	Immunogen	Challenge (Mem71, 10 ^{4.5} pfu)
1	8	VLP 1 (H3 Peptide A (KRGPGSG) inserted into VP1-S1)	+
2	8	VLP 2 (H3 Peptide B PSTNQEQTSLYVQA SGR inserted into VP1-S1)	+
3	8	wt VLP (VP1 with no insertion)	+
4	8	Split Mem/Bel (3ug/dose)	+
5	8	PBS	+

Week 0

*s.c. – base of tail injection

10 Duration of Study: 7 weeks, continuous monitoring

Vaccination: week 0 (1st shot sc), week 3 (2nd shot sc), intranasal challenge week 5.

Mice: 40 BALB/c mice

Readouts: ELISA, HAI, and virus neutralisation assay of serum

15 post primary and secondary vaccinations and plaque assay on lung homogenates 5

days post challenge

Vaccine samples: 50 μ L injected per sc shot. VLPs were expressed in baculovirus, purified by sucrose and CsCl ultracentrifugation, and dialysed into PBS. See Example 8 for experimental detail of how VLPs used as vaccine samples were prepared. Final composition was 77 μ g of VLP protein and 45 μ g Al(OH)₃ adjuvant per 50 μ L dose. Endotoxin was tested to ensure acceptability.

Example 7

Construction and sequencing of vectors carrying HA loop A and loop B of influenza strain H3N2 inserted in S1 and S4 for expression in *E. coli* and insect cells

Figures 14 and Figure 15 present two alignments. First one shows insertion of loop A and loop B in S1 of VP1 (Figure 14), second one shows insertion of loop A and loop B in S4 of VP1 (Figure 15).

Resulting constructs are pGEX4T1-VP1 S1H3A, pGEX4T1-VP1 S1H3B, pGEX4T1-VP1 S4H3A, and pGEX4T1-VP1 S4H3B for expression in *E. coli* and pENTR-VP1 S1H3A and pENTR-VP1 S1H3B for expression in insect cells.

Example 8

Preparation of VLPs for challenge experiments using baculovirus

The following VLPs were prepared for challenge experiments detailed in example 6:

- VP1-H3-S1A: VP1 containing H3N2 loop A peptide inserted into the S1 site of VP1
- VP1-H3-S1B: VP1 containing H3N2 loop B peptide inserted into the S1 site of VP1

- VP1-wt: wild-type VP1 sequence not containing any insertion nor modification

VP1 proteins for the above constructs were heterologously expressed in *Spodoptera frugiperda* (Sf9) cells infected with recombinant baculovirus encoding the chosen VP1 chimeric protein. Cells were harvested approximately 72h postinfection, pelleted by centrifugation, and frozen at -80°C. For preparation of VLPs (all the steps were performed at 4°C), cell pellets were resuspended in Lysis buffer (50 mM MOPS, 500mM NaCl, 0.002% Tween 80, pH 7.0) and lysed with Branson Ultra-sonics Sonifier. Homogenized lysate was then centrifuged at 16,000xg for 30 min. The supernatant containing soluble VLPs was layered on the top of a 30% sucrose cushion and centrifuged for 1.5 h at 32000 rpm using a Beckman SW 32 Ti Rotor. The pellet was then resuspended in Lysis buffer and subjected to 15 seconds of sonication at low power setting to disperse any aggregates. Homogenate was then centrifuged at 15,000 rpm for 30 min. Clarified supernatant with CsCl added to a final concentration of 35% (wt/vol) was centrifuged at 50,000rpm for 16 hours using a Beckman SW 60 Ti Rotor. Purified VLPs were collected by extracting the top band from the CsCl gradients. Recovered VLPs were dialyzed into PBS overnight and stored at 4°C. Protein concentration was determined by Agilent Bioanalyzer. Samples for electron microscopy were prepared as described in Example 3 and imaged at 200,000x (scale-bar 100nm). After dialysis, VLPs in PBS were formulated with aluminium hydroxide gel (colloidal Al(OH)₃) to give a final dose of 50 µL consisting of 77 µg (protein equivalent) of VLP and 45 µg Al(OH)₃, balance PBS. Figure 16 shows EM images of VP1-H3-S1A VLPs. Figure 17 shows EM images of VP1-H3-S1B VLPs.

Example 9**Construction and sequencing of vectors carrying HA epitopes of loop A and loop B of influenza strain H5N1 inserted in S4**

Figure 18 shows sequence alignment of HA loop A and loop B of influenza strain H5N1 inserted in S4 of VP1. Resulting constructs are pGEX4T1-VP1 S4A, pGEX4T1-VP1 S4B and pGEX4T1-VP1 S4hM2e.

Example 10**Expression and purification of GST-VP1-S4A and GST-VP1-S4B and assembly of VP1-S4A and VP1-S4B into multimerically defined VLPs**

The following VP1 variants were processed:

- GST-VP1 (wild-type protein with no inserted antigen; control)
- GST-VP1-S4A
- GST-VP1-S4B

The purpose of this example is to demonstrate that multimerically defined VLPs can be obtained using the same process used for unmodified VP1 protein, even after very different insertions (A or B) into site S4 of VP1.

In this example, “A” in the above constructs refers to antigenic peptide taken from influenza virus H5N1 corresponding to loop A in the HA protein, as in example 2 (7 amino acids, PYNGKSS). “B” in the above constructs refers to antigenic peptide taken from influenza virus H5N1 corresponding to loop B in the HA protein, as in example 2 (17 amino acids, PNDAAEQTKLYQNPTTY).

For each construct, protein was expressed as described in Example 2.

For purification of each construct, a pellet from 800 mL of culture was re-suspended in 40 mL of buffer L (200 mM NaCl, 40 mM Tris, 1 mM EDTA, 5 mM

dithiothreitol, 5% v/v glycerol, pH 8 with HCl). The cell suspension was passed through a high-pressure homogenizer (Niro-Soavi) once at 1000 bar. The homogenate was diluted with L buffer and centrifuged. Supernatant containing soluble VP1 fusion protein was filtered through two 0.45 µm syringe filters
5 (Millipore), giving a recovery of 100-125 mL per construct examined. Protein purification was performed with an Äkta Explorer system from GE Healthcare. 100 mL of filtered homogenate containing the selected GST-VP1 fusion protein (listed above) was loaded onto a GSTrap™ HP 5 mL column from GE Healthcare pre-equilibrated with buffer L (Figure 19A and B). The bound GST-VP1 fusion protein
10 was eluted with elution buffer (Buffer L with 10 mM reduced glutathione). Eluted fractions of 250 µL were stored at -80°C until required.

The eluate from above was thawed on ice and thrombin (50 U/mL, Sigma) was added followed by 2 h incubation at room temperature on a roller. 200 µL of thrombin-treated capsomere was injected onto a Superdex S200 10/300 GL size
15 exclusion column (GE Healthcare) equilibrated in buffer L, to separate capsomeres from aggregates, GST and thrombin (Figure 20A and B). Capsomere fractions (150 µL) were assembled by dialysis against buffer 1 (0.5 M (NH₄)₂SO₄, 20 mM Tris, pH 7.4, 5 % (v/v) glycerol, 1 mM CaCl₂) for 17 h, and then against buffer 2 (200 mM NaCl, 20 mM Tris, pH 7.4, 5 % (v/v) glycerol, 1 mM CaCl₂) for another 24 hours.
20 Dialysis was at room temperature with a 10,000 MWCO dialysis membrane.

Comparison of GST-VP1 with GST-VP1-S4A and GST-VP1-S4B by Bioanalyzer analysis is shown in Figure 21.

Assembled VLPs were analysed by both asymmetric field-flow fractionation (AFFF, Wyatt Technologies) (Figure 22) and electron microscopy (EM) as described

in Example 3 (see Figure 23 A, B and C).

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

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

CLAIMS

1. An isolated protein comprising a murine polyomavirus VP1 amino acid sequence, or a fragment thereof wherein one or more exposed loops of said murine polyomavirus VP1 amino acid sequence has an insertion of an amino acid sequence
5 of a virus protein other than murine polyomavirus VP1, or a fragment said virus protein other than murine polyomavirus VP1.
2. The isolated protein of claim 1, wherein the one or more exposed loops of said murine polyomavirus VP1 amino acid sequence comprise an insertion site selected from the group consisting of site 1, site 3 and site 4.
- 10 3. The isolated protein of claims 1 or 2, wherein the insertion site is selected from the group consisting of site 1 and site 4.
4. The isolated protein of any one of claims 1, 2 or 3, wherein the virus protein other than murine polyomavirus VP1 or a fragment thereof, is an influenza virus protein.
- 15 5. The isolated protein of claim 4, wherein the influenza virus protein is selected from the group consisting of HA, NP, NA, M2 and M1.
6. The isolated protein of claim 5, wherein the influenza virus protein is selected from the group consisting of HA and M2.
7. The isolated protein of any one of claims 1 or 5, wherein the virus protein
20 other than murine polyomavirus VP1 corresponds to an exposed loop of HA selected from the group consisting of loop A, loop B, loop C, loop D and loop E, or a fragment thereof.
8. The isolated protein of claim 7, wherein the exposed loop of HA is selected from the group consisting of loop A and loop B, or a fragment thereof.

9. The isolated protein of claim 8, wherein the fragment of loop A is an antigenic epitope comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 75, SEQ ID NO: 77 and SEQ ID NO: 79, or a variant thereof.
- 5 10. The isolated protein of claim 9, wherein the variant comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1; SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4; SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:9; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:12; SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:22; SEQ ID NO:23; SEQ ID NO:47; SEQ ID NO:51; SEQ ID NO:52; SEQ ID NO:53; SEQ ID NO:54; SEQ ID NO:55; SEQ ID NO:56; SEQ ID NO:57; SEQ ID NO:58; SEQ ID NO:59 and SEQ ID NO:60.
- 10 11. The isolated protein of claim 9, wherein the variant has at least 30% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NO: 75, SEQ ID NO: 77 and SEQ ID NO: 79.
- 15 12. The isolated protein of claim 8, wherein the fragment of loop B is an antigenic epitope comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 76, SEQ ID NO: 78 and SEQ ID NO: 80, or a variant thereof.
- 20 13. The isolated protein of claim 12, wherein the variant comprises an amino acid sequence selected from the group consisting of SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:30; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:42; SEQ ID NO:43; SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:61; SEQ ID NO:65; SEQ ID NO:66; SEQ ID NO:67; SEQ ID NO:69; SEQ ID NO:70; SEQ ID NO:71; SEQ ID NO:72; SEQ ID NO:73 and SEQ ID

NO:74.

14. The isolated protein of claim 12, wherein the variant has at least 30% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NO: 76, SEQ ID NO: 78 and SEQ ID NO: 80.

5 15. The isolated protein of any one of claims 1 to 14, wherein the said virus protein other than murine polyomavirus VP1, or a fragment thereof, is a single protein or a plurality of proteins.

16. An isolated nucleic acid comprising a nucleotide sequence encoding the isolated protein of any one of the preceding claims.

10 17. The isolated nucleic acid of claim 16, which is DNA.

18. The isolated nucleic acid of claim 16, wherein the nucleotide sequence encoding the murine polyomavirus VP1 amino acid sequence is adapted to receive within one or more exposed loops of said murine polyomavirus VP1 amino acid sequence, an isolated nucleic acid comprising a nucleotide sequence encoding a virus
15 protein other than murine polyomavirus VP1 or a fragment of said nucleotide sequence encoding a virus protein other than murine polyomavirus VP1.

19. An expression construct comprising the isolated nucleic acid of claim 16 operably-linked to one or more regulatory sequences in an expression vector.

20. The expression construct of claim 19, wherein the one or more regulatory
20 sequences is selected from the group consisting of an enhancer of transcription, an enhancer of translation, a nucleotide sequence for autonomous replication, a regulatory element for mRNA processing, a selectable marker and a screenable marker.

21. A host cell comprising the expression construct of claim 19.

22. The host cell of claim 21, which is selected from the group consisting of a prokaryotic cell and an insect cell.
23. The host cell of claim 22, which is a bacterium.
24. A virus-like particle comprising one or more isolated proteins of claim 1.
- 5 25. A method of producing an isolated nucleic acid, said method including the step of inserting into each of one or more nucleotide sequences that encode one or more exposed loops of a murine polyomavirus VP1 protein, a nucleotide sequence encoding a virus protein other than murine polyomavirus VP1, or a fragment of said virus protein other than murine polyomavirus VP1, wherein the one or more
- 10 nucleotide sequences that encode one or more exposed loops of the murine polyomavirus VP1 protein is adapted to receive the nucleotide sequence encoding the virus protein other than murine polyomavirus VP1.
26. The method of claim 25, wherein the virus protein other than murine polyomavirus, or a fragment thereof, is an influenza virus protein selected from the
- 15 group consisting of HA, NP, NA, M2 and M1.
27. The method of claim 26, wherein the influenza virus protein is selected from the group consisting of HA and M2.
28. The method of claim 25 and claim 26, wherein the virus protein other than VP1 corresponds to an exposed loop of HA selected from the group consisting loop
- 20 A, loop B, loop C, loop D and loop E, or a fragment thereof.
29. The method of claim 28, wherein the exposed loop of HA is selected from the group consisting of loop A and loop B, or a fragment thereof.
30. The method of claim 29, wherein the fragment of loop A is an antigenic epitope comprising an amino acid sequence selected from the group consisting of

SEQ ID NO: 75, SEQ ID NO: 77 and SEQ ID NO: 79, or a variant thereof.

31. The method of claim 30, wherein the variant comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1; SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4; SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:9; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:12; SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:22; SEQ ID NO:23; SEQ ID NO:47; SEQ ID NO:51; SEQ ID NO:52; SEQ ID NO:53; SEQ ID NO:54; SEQ ID NO:55; SEQ ID NO:56; SEQ ID NO:57; SEQ ID NO:58; SEQ ID NO:59 and SEQ ID NO:60.

32. The method of claim 30, wherein the variant has at least 30% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NO: 75, SEQ ID NO: 77 and SEQ ID NO: 79.

33. The method of claim 29, wherein the fragment of loop B is an antigenic epitope comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 76, SEQ ID NO: 78 and SEQ ID NO: 80, or a variant thereof.

34. The method of claim 33, wherein the variant comprises an amino acid sequence selected from the group consisting of SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:30; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:42; SEQ ID NO:43; SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:61; SEQ ID NO:65; SEQ ID NO:66; SEQ ID NO:67; SEQ ID NO:69; SEQ ID NO:70; SEQ ID NO:71; SEQ ID NO:72; SEQ ID NO:73 and SEQ ID NO:74.

35. The method of claim 33, wherein the variant has at least 30% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NO: 76, SEQ ID NO: 78 and SEQ ID NO: 80.

36. A method of producing a virus-like particle, said method including the steps

of:

(a) introducing the isolated nucleic acid of claim 16 or an expression construct of claim 19 into a host cell;

(b) culturing said host cell under conditions which facilitate production of
5 the isolated protein of claim 1;

(c) optionally purifying the isolated protein of claim 1; and

(d) assembling the isolated protein purified according to step (c) to produce the virus-like particle.

37. The method of claim 36, wherein the host cell is a bacterium.

10 38. A virus-like particle producing according to the method of claim 36.

39. A pharmaceutical composition comprising: the isolated protein of claim 1, the isolated nucleic acid of claim 16 or the virus-like particle of claim 24 or claim 36 and a pharmaceutically-acceptable carrier, diluent or excipient.

40. The pharmaceutical composition of claim 39, wherein the pharmaceutical
15 composition is an immunotherapeutic composition.

41. The pharmaceutical composition of claim 40, which is a vaccine.

42. The pharmaceutical compositions of any one of claims 39 to 41, wherein the pharmaceutical composition is capable of prophylactically or therapeutically treating a disorder, disorder or condition caused by an influenza virus.

20 43. The pharmaceutical composition of claim 42, wherein the influenza virus is avian influenza virus.

44. A method of treating an animal, said method including the step of administering the pharmaceutical composition of any one of claims 39 to 43 to said animal to prophylactically or therapeutically treat a disease, disorder or condition.

45. The method of claim 44, wherein the disease, disorder or condition is caused by an influenza virus.
46. The method of claim 44, wherein said animal is a mammal.
47. The method of claim 46, wherein said mammal is a human.
- 5 48. A method of immunising an animal, said method including the step of administering the pharmaceutical composition of any one of claims 39 to 43 to said animal to thereby induce immunity in said animal.
49. The method of claim 48, wherein the animal is a mammal.
50. The method of claim 49, wherein the mammal is a human.
- 10 51. The method of claim 48, wherein the animal is prophylactically or therapeutically treated for a disorder, disease or condition caused by an influenza virus.
52. The method of claim 51, wherein the influenza virus is avian influenza virus.