Published in final edited form as:
Virus Res. 2009 August ; 143(2): 140–146. doi:10.1016/j.virusres.2009.04.005.

Influenza vaccines based on virus-like particles

Sang-Moo Kang[*], **Jae-Min Song**, **Fu-Shi Quan**, and **Richard W. Compans**[*]
Department of Microbiology and Immunology and Emory Vaccine Center, Emory University School of Medicine, Atlanta, Georgia, 30322

Abstract

The simultaneous expression of structural proteins of virus can produce virus-like particles (VLPs) by a self-assembly process in a viral life cycle even in the absence of genomic material. Taking an advantage of structural and morphological similarities of VLPs to native virions, VLPs have been suggested as a promising platform for new viral vaccines. In the light of a pandemic threat, influenza VLPs have been recently developed as a new generation of non-egg based cell culture-derived vaccine candidates against influenza infection. Animals vaccinated with VLPs containing hemagglutinin (HA) or HA and neuraminidase (NA) were protected from morbidity and mortality resulting from lethal influenza infections. Influenza VLPs serve as an excellent model system of an enveloped virus for understanding the properties of VLPs in inducing protective immunity. In this review, we briefly describe the characteristics of influenza VLPs assembled with a lipid bilayer containing glycoproteins, and summarize the current progress on influenza VLPs as an alternative vaccine candidate against seasonal as well as pandemic influenza viruses. In addition, the protective immune correlates induced by vaccination with influenza VLPs are discussed.

1. Introduction

Influenza virus contains a segmented negative sense RNA genome and belongs to the Orthomyxoviridae family. It is a lipid enveloped RNA virus, and is among the most devastating human and animal diseases due to the ease of its spread as an aerosol and ability to cause severe mortality to a susceptible host. Influenza virus causes 17,000–51,000 deaths in United States annually and a global pandemic can cause millions of human fatalities. The virion is surrounded by lipid membrane containing two major glycoproteins the hemagglutinin (HA) and neuraminidase (NA), and a minor but essential ion-channel protein M2. The HA protein is the most abundant viral surface glycoprotein and is responsible for the attachment of virus to terminal sialic acid residues on host cell receptors (Carroll and Paulson, 1985). Wild birds are a source for 16 influenza A HA subtypes based on antigenic properties of the HA protein, representing a large reservoir for novel glycoproteins to which the human immune system is naïve (Fouchier et al., 2005; Rohm et al., 1996; Webster et al., 1992). Genetic reassortment of RNAs between avian and human influenza viruses or mutations affecting host range can enable a virus to acquire the capability to transmit among the human population, which may lead to a global pandemic with high mortality.

*Corresponding authors: Department of Microbiology and Immunology, Emory University School of Medicine, 1510 Clifton Rd, Atlanta, GA 30322. Richard W. Compans: Phone, 404-727-5950; Fax, 404-727-8250; compans@microbio.emory.edu, Sang-Moo Kang: Phone, 404-727-3228; Fax, 404-727-3659; skang2@emory.edu.

Vaccination has been a most cost-effective means to control the disease resulting from influenza infection. Vaccine strains are selected based on epidemiological and antigenic considerations of circulating human strains and their anticipated prevalence during the coming year. Chemically inactivated influenza vaccines consist of detergent-split virion subunits composed of hemagglutinin (HA) and lesser amounts of other virion proteins including NA. This inactivated, split vaccine is not completely effective, particularly in the elderly. The efficacy of the vaccine is significantly compromised when circulating viruses do not have a good match with vaccine strains due to antigenic drift or inaccurate epidemiological predictions. A live, attenuated influenza virus vaccine (FluMist®) is also licensed for seasonal influenza and is intended for intranasal administration to people six months to 49 years of age (Block et al., 2008; Cintra and Rey, 2006). Both types of licensed influenza vaccines are produced using fertilized chicken eggs as substrates (Johansson and Brett, 2007).

Due to limitations in vaccine supply from the conventional egg-based manufacturing system and its susceptibility to a pandemic outbreak that could threaten egg-substrate availability, it is important to develop alternative influenza vaccines not relying on egg substrates. Several recent studies have focused on developing non-replicating virus-like particles (VLPs) as alternative influenza vaccines. VLPs can be produced from both non-enveloped and enveloped viruses. Among non-enveloped VLPs, human papillomavirus (HPV) VLPs are most thoroughly studied, and the expression of the major capsid protein L1 resulted in the production of VLPs (Kirnbauer et al., 1992; Sasagawa et al., 1995). VLPs produced in yeast or insect cells were highly immunogenic resulting in successful clinical trials for preventing HPV infection and FDA approval for human use (Koutsky et al., 2002; Ljubojevic, 2006; Markowitz et al., 2007; Stanley, 2006). VLPs from viruses with lipid envelopes represent more difficult challenges because of the complexity of the particle budding process for incorporating biologically active glycoproteins. The first human vaccine manufactured using recombinant DNA technology was the enveloped hepatitis B vaccine produced in yeast, which has been used for over a decade (Assad and Francis, 1999). Hepatitis small envelope protein antigen self-assembles to form particles of approximately 22 nm (McAleer et al., 1984). Enveloped influenza VLPs have been developed by several laboratories and were demonstrated to induce protective immunity in animal studies (Tables 1 and 2). In this regard, this review summarizes the current progress on seasonal and pandemic influenza VLP vaccines, and discusses the immune responses that are relevant to protection.

2. Characteristics of influenza VLP vaccine candidates

The non-infectious nature of VLPs and their lack of viral genomic material represent a desirable safety feature as a vaccine candidate that can be repeatedly administered to all populations including high-risk groups. In addition, VLPs as particulate antigens can activate antigen presenting cells such as dendritic cells capturing the antigens for presentation to both T and B lymphocytes (Buonaguro et al., 2006; Da Silva et al., 2001; Lenz et al., 2003; Moron et al., 2002; Moron et al., 2003; Sailaja et al., 2007). For influenza viruses, VLPs are assembled in producer cells and released into culture medium mimicking the viral budding process, which incorporates viral glycoproteins on their surfaces. Influenza VLPs resemble intact virions in structure and morphology, and contain functionally active and immunologically relevant structural proteins (Tables 1 and 2). The viral glycoproteins on VLPs are in a native conformation and unmodified by fixatives or chemicals for inactivation. It is likely that the self-assembled macrostructure of VLPs can present native conformational epitopes of surface proteins to the immune system comparable to those of live virions. Therefore, approaches for developing vial vaccines based on VLPs are important and have significant clinical implications.

3. Production of influenza VLPs

Influenza virus is an enveloped RNA virus and is assembled at the plasma membrane of infected cells. Release of newly assembled virions into the outside environment occurs at the plasma membrane by a budding process (Ali et al., 2000; Gomez-Puertas et al., 2000; Latham and Galarza, 2001; Nayak et al., 2004; Roberts et al., 1998). Influenza virus structural components including the matrix protein (M1) and the viral ribonucleoprotein complex as well as the three transmembrane proteins of viral envelope (hemagglutinin (HA), neuraminidase (NA), and M2) should be produced and transported to the plasma membrane prior to budding. These viral proteins interact with each other at the plasma membrane to initiate the budding processes leading to morphogenesis of virus particles and release of virions containing a host cell-derived lipid bilayer.

A predominant model of influenza virion assembly is that the matrix M1 protein associates with the lipid raft domain of the plasma membrane and interacts with the cytoplasmic tails of HA and NA driving the virus assembly and budding processes (Ali et al., 2000; Gomez-Puertas et al., 2000; Latham and Galarza, 2001). Supporting the role of M1 in assembly and release of particles, VLPs lacking either HA or NA were found to be formed and released from cells expressing M1 from recombinant DNA or virus infection (Gomez-Puertas et al., 2000; Latham and Galarza, 2001; Liu et al., 1995; Pattnaik et al., 1986). Influenza VLP production was demonstrated with different expression systems including recombinant vaccinia viruses (Ali et al., 2000), DNA plasmid transfection with T7 RNA polymerase-expressing vaccinia virus (Gomez-Puertas et al., 2000), recombinant DNA expression vectors (Chen et al., 2007; Szecsi et al., 2006), or recombinant baculoviruses (Table 1). However, the major viral components required for controlling influenza VLP formation are still uncertain. Interactions between M1 and the cytoplasmic tails of HA, NA, and M2 seemed to be important for the efficient virus assembly, morphology, and budding, as suggested by studies that virus budding was impaired when the cytoplasmic tails of the viral glycoproteins were mutated, indicating a role of these proteins (Jin et al., 1997; Zhang et al., 2000). A recent study reported that influenza virus HA and NA, but not M1, were required for assembly and budding of DNA vector-expressed influenza VLPs and that low levels of VLPs were found in the culture supernatants when HeLa cells, but not 293T cells, were transfected with DNA expressing M1 alone (Chen et al., 2007). These differences among studies may result from different expression systems (DNA tranfection versus recombinant viruses; mammalian versus insect cells) and further studies are needed to better understand the contribution of each influenza component to the budding process of VLPs.

For the production of VLPs containing HA in mammalian cells, co-expression of NA or exogenously added NA was required for the effective release of influenza VLPs into culture media, implying an important role of the NA activity in cleaving sialic acids bound to HA of budding particles (Ali et al., 2000; Chen et al., 2007; Gomez-Puertas et al., 2000). In contrast, VLPs containing HA can be produced in insect cells in the absence of NA expression (Galarza et al., 2005; Guo et al., 2003; Latham and Galarza, 2001; Quan et al., 2007). Insect cells do not add sialic acids to the N-glycans during the posttranslational modification [Lanford, 1989 #295], which explains how VLPs containing HA but not NA are effectively released from insect cell surfaces (Galarza et al., 2005; Quan et al., 2007).

The recombinant baculovirus (rBV)/insect cell expression system yields high expression levels of recombinant proteins and allows subsequent large scale manufacturing of a vaccine. Many VLPs have been produced by rBV-infected insect cells, and VLP antigens were highly immunogenic inducing both neutralizing antibodies and cellular immune responses. Examples include simian and human immunodeficiency virus (SIV/HIV) (Buonaguro et al., 2007; Deml et al., 1997; Guo et al., 2003; Kang and Compans, 2003a; Wang et al., 2007; Yao et al.,

2000), human papillomavirus (Harro et al., 2001), rotavirus (O'Neal et al., 1998), hepatitis type C virus (Murata et al., 2003; Qiao et al., 2003; Triyatni et al., 2002), and Ebola virus (Sun et al., 2009; Ye et al., 2006). There may be fewer safety concerns about VLPs production in insect cells as compared with the use of alternative expression systems in mammalian cells because baculoviruses are found in green vegetables and are not able to replicate in mammalian cells.

Recombinant influenza VLPs have been generated in insect cells infected with rBVs expressing either four structural influenza genes HA, NA, M1, and M2 (Latham and Galarza, 2001) or three HA, NA, and M1 genes cloned into a single baculovirus construct (Bright et al., 2007; Pushko et al., 2005; Pushko et al., 2007). Also, the production of influenza VLPs was demonstrated in insect cells using individual rBVs expressing genes of HA and M1 (Galarza et al., 2005; Quan et al., 2007). Electron micrographs of these influenza VLPs displayed 80 to 120 nm diameter particles with apparent HA spikes which are reminiscent of influenza virus. Co-expression of murine leukemia virus (MLV) Gag and influenza HA and NA resulted in retrovirus-like particles containing HA, in that these pseudotyped particles appeared as consistent 100 nm spheres (Haynes et al., 2009). A Gag-to-HA ratio was noted to be approximately 3–4:1 and to exhibit hemagglutination specific activity, which are similar to those found in influenza virus. Therefore, while there may exist specific interactions between cytoplasmic domains of viral envelope proteins and matrix or core components, such virus specific interactions are not required for efficient release of a self sufficient budding protein Gag (Haynes et al., 2009).

4. Protective immune responses to seasonal influenza VLPs

Galarza et al (Galarza et al., 2005) first reported the protective effects of immunization with influenza VLPs containing M1 and HA derived from A/Udorn 72 (H3N2). Mice intramuscularly or intranasally immunized twice with influenza VLPs containing 1 μg of HA in the presence or absence of IL-12 cytokine adjuvant were similarly protected against lethal challenges with 5 LD_{50} of heterologous mouse-adapted A/Hong Kong/68 (H3N2) virus. It is important to note that influenza VLPs were able to elicit binding antibody responses to HA at similar or higher levels compared to those induced by two sublethal vaccinations with a live virus.

Influenza M1-HA VLPs derived from the A/PR/8/34 virus provided equivalent protection against homologous and heterologous strains (A/PR/8/34, A/WSN/33) (Quan et al., 2007). However, lung viral titers were over a hundred fold lower in the homologous challenge group than those in the heterologous challenge group, which was consistent with higher neutralizing titers found against to homologous virus. Less than 20% survival was observed after immunization with M1 VLPs without HA, and surviving mice experienced severe loss in body weight. No antibodies specific to influenza were detected in this group indicating the critical role of influenza HA specific antibodies in providing protection (Quan et al., 2007). When heat-treated VLPs with the complete loss of hemagglutination activity were used for immunization, no protective immunity was observed (Quan et al., 2007). Therefore, the integrity of influenza VLPs maintaining hemagglutination activity seems to be a critical factor in inducing functional antibodies that are protective.

Intramuscular immunization of mice with influenza VLPs with HA and NA from A/Fujian/411/2002 (H3N2) induced broader cross-reactive responses, probably due to higher levels of serum antibodies than inactivated influenza whole virions or recombinant HA (rHA) as determined by hemagglutination inhibition (HAI) assay using a panel of H3N2 human isolates (Bright et al., 2007). When equal amounts of HA in soluble rHA form and in VLPs were compared, VLPs induced 10 to 15 fold higher serum titers than rHA, and more than a 20 fold

HA antigen sparing effect was observed with VLPs (Bright et al., 2007). Also, VLP immunization showed 2 fold higher HAI titers than inactivated whole virus. Enhanced titers against drifted strains for the VLP vaccine were induced in ferrets compared to the rHA vaccine (Bright et al., 2007), suggesting that influenza VLPs are superior antigens to rHA in inducing protective immune responses. Regarding types of immune responses, mice (BALB/c) intramuscularly immunized with influenza VLPs with HA and NA from A/Fujian/411/2002 (H3N2) showed IgG2a and IgG2b as dominant serum antibody isotypes with IgG1 as the third major antibody (Bright et al., 2007). In contrast to VLP vaccination, mice immunized with inactivated whole virus elicited both IgG2a and IgG1 as dominant isotypes, but little IgG2b antibodies, and rHA induced primarily an IgG1 response (Bright et al., 2007).

To test the potential for a multivalent influenza vaccine, bivalent influenza VLPs, a mixture of H1 and H3 VLPs, were investigated for their immunogenicity (Quan et al., 2008b). It was found that the breadth of protection was strain-specific as expected from the components of the two VLP vaccine strains, consistent with the current concept of influenza vaccination.

Splenocytes from mice immunized with influenza VLPs were stimulated with HA-specific MHC I- or MHC II- restricted peptides to quantify HA-specific $CD4^+$ and $CD8^+$ cells secreting Th1 type (IFN-γ, IL-2) and Th2 type (IL-4, IL-5) cytokines. Significant levels of IFN-γ and IL-2 in responses to MHC I or MHC II peptide stimulation were detected in mice immunized with VLPs but not in rHA-immunized or naïve mice (Bright et al., 2008; Quan et al., 2008b). $CD4^+$ cells were found to secrete higher levels of the cytokines IL-4 and IL-5 than $CD8^+$ cells. Upon virus infection, mice immunized with VLPs rapidly induced significantly higher levels of lymphocytes secreting IFN-γ, and IL-2 secreting $CD4^+$ and $CD8^+$ cells as compared to those observed prior to challenge. These results suggest that influenza VLPs induce both Th1 and Th2 type cellular immune responses, which can expand rapidly in response to influenza virus infection.

5. Strategies to enhance the immunogenicity of influenza VLPs

Overcoming the strain-specificity of protection and developing a broadly cross-protective influenza vaccine have been a challenge for influenza vaccination. A few previous studies demonstrated that the addition of potent mucosal adjuvants such as cholera toxin or mutant *E. coli* heat-labile enterotoxin significantly enhanced the immunogenicity of inactivated viral vaccines resulting in heterosubtypic immunity (Quan et al., 2008; Tumpey et al., 2001). However, these potent adjuvants would not be appropriate for human use due to safety concerns. The addition of a Novasome adjuvant (non-phospholipid liposome nanoparticles) to influenza VLPs did not enhance the immunogenicity of influenza VLPs, in contrast to its significant effects on enhancing the immunogenicity of rHA soluble protein antigen, probably due to the highly immunogenic intrinsic property of VLPs (Pushko et al., 2007). Strategies to incorporate immunostimulatory molecules into VLPs have also been explored. Dendritic cell stimulating cytokines (GM-CSF, Flt3 ligand) can be genetically engineered to be incorporated into budding VLPs, which resulted in increasing the VLP immunogenicity (Sailaja et al., 2007; Skountzou et al., 2007). In a recent study, the Toll-like receptor 5 ligand flagellin was engineered to be expressed in a membrane-anchored form which was subsequently incorporated into influenza VLPs (Wang et al., 2008). Incorporation of flagellin into influenza A/PR8 HA VLPs (H1N1) was found to significantly increase the immunogenicity of VLPs as well as to enhance the protective efficacy against heterosubtypic challenge, improving the breadth of immune responses.

6. VLPs as pandemic vaccine candidates

Although there is a licensed influenza H5N1 vaccine, limitations exist particularly for potential pandemic influenza viruses. There are known problems with growing highly pathogenic avian

influenza viruses in embryonated eggs because they can kill the embryos which would hamper virus production, and there are associated human safety concerns in working with live pathogenic viruses. Because of the high pathogenicity of pandemic strains, recombinant genetic modifications were used to generate reassortant and attenuated seed viruses for developing pandemic vaccines. To increase the safety of H5N1 vaccine strains, polybasic amino acids in the HA cleavage site were mutated (Li et al., 1999; Lipatov et al., 2005). Also, using reverse genetics engineering technologies, reassortant pandemic vaccine strains were generated which contain the gene segments encoding the internal proteins derived from A/PR/8/34 or other attenuated strains but with high growth properties in eggs (Li et al., 1999; Lipatov et al., 2005; Subbarao et al., 2003; Suguitan et al., 2006; Webby et al., 2004). All these vaccines are dependent on the egg-substrates for their production. Diseases that affect chicken flocks due to an avian influenza virus outbreak could easily disrupt the supply line of eggs available for vaccine manufacturing. These factors as well as the requirement for biosafety level 3 or higher containment facilities for safe handling of pathogenic avian influenza viruses support the urgent need to develop a new influenza vaccine modality. Therefore, development of pandemic vaccines based on influenza VLPs has received high emphasis during recent years as summarized in Table 2.

Since 1997 when the first human infection of H5N1 highly pathogenic avian influenza occurred in Hong Kong, highly pathogenic avian influenza viruses (HPAI) have diverged and been classified into 10 clades (0–9). Thus, induction of cross-clade protective immunity is one of the most important factors in vaccine strain selection. Influenza VLPs containing HA and NA from the clade 2 influenza isolate, A/Indonesia/05/2005 (Indo_05), were tested for a cross-clade protective immune response (Bright et al., 2008). In this study, two doses of VLPs containing 3 μg or 600 ng HA provided broadened protective immune responses including humoral and cell-mediated immunity, which was found to be protective against challenge infections with reassortant homologous (A/Indonesia/05/2005) or a heterologous virus, A/Vietnam/1203/2004 (Viet_04) clade 1. However, all mice vaccinated with a rHA vaccine died or suffered severe body weight loss (Bright et al., 2008; Crevar and Ross, 2008). In order to elicit protective immunity against various clades of H5N1 HPAI, a bivalent pandemic influenza VLP vaccine has been investigated (Crevar and Ross, 2008). Mice immunized twice with a mixture of two H5N1 VLPs containing clades 1 and 2 of H5 HA (Viet-04 and Indo_05) induced HAI activity against clade 1, 2 and 2.3 (A/Anhui/1/2005) but not clade 2.2 (A/Bar headed goose/Qinghai/1A/2005). Even mice vaccinated only with clade 2 VLPs (Indo-05) exhibited considerable cross-protective immunity and were protected from clade 1 or 2 lethal challenge, indicating that influenza H5N1 VLPs derived from clade 2 A/Indonesia/05/2005 virus induce cross-clade protective immune responses. In contrast to this, mice vaccinated with clade 1 VLPs (Viet_04) showed a significant weight loss against clade 2 challenge. These results demonstrate that a bivalent H5N1 VLP vaccine is effective in eliciting protective immunity and also revealed that clade 2 VLPs induced somewhat greater cross-clade immunity than clade 1 VLP. In addition, influenza H5N1 VLPs (A/Viet Nam/1203/04) were able to induce long-term protective immunity as well as memory B and T cell responses in mice (Kang et al., 2009).

Considering animal models for influenza experiments, ferrets which can develop fever and respiratory symptoms are thought to be a more suitable system for their similarity to human influenza disease. A recent report has shown that H5N1 Indo_05 VLPs vaccine with two immunizations protected against homologous and heterologous clade 1 Viet_04 reassortant virus even at a low dose (0.6 μg HA) (Mahmood et al., 2008). Interestingly, vaccinated ferrets survived challenge infection with 10 FLD_{50} of heterologous virus even in the absence of detectable HAI titers. Thus, there seemed to be less correlation between levels of HAI titers and protection against HPAI than that observed with seasonal vaccines (Mahmood et al., 2008).

The use of plant-based expression system for producing VLPs can be another option for influenza vaccine production because of safety and mass production capacity. Indo-05 H5 VLPs were produced by agroinfiltration of Nicotiana benthamiana plants (D'Aoust et al., 2008). Mice immunized and boosted intramuscularly with plant-derived H5 VLPs VLPs formulated in alum adjuvant were protected against lethal infection with a low dose challenge (one LD50 of A/Vietnam/1194/04 heterologous strain). Whether the H5 HA containing VLPs produced in plant cells maintain biological activities has not been reported.

Similar to the M1 protein, MLV Gag protein can be used as a driving force for VLP budding. Gag H5N1 VLPs (A/Vietnam/1203/04 or A/Indonesia/5/05) produced by a baculovirus insect cell expression system were shown to induce protection against highly pathogenic A/Vietnam/1203/04 challenge in mice and ferrets after prime-boost immunization (Haynes et al., 2009). These results suggest that Gag VLPs containing influenza HA are as immunogenic as influenza M1-derived VLPs.

There are other subtypes of avian influenza viruses with pandemic potential. The H7 subtype of avian influenza is also considered as a potential pandemic threat because of a case report of direct transmission to human (Kurtz et al., 1996). Retrovirus Gag derived VLPs containing HA, NA, and M2 (A/Chicken/FPV/Rostock/1934 (H7N1) or A/Thailand/KAN-1/04 (H5N1)) were produced in 293T mammalian cells by plasmids transfection (Judit Szécsi, 2006). These pseudotyped influenza VLPs were immunogenic and found to induce strain-specific neutralizing activities after immunization of mice two times although no cross-reactive antibodies were found between two subtypes.

Some studies reported VLP vaccine strategy for the control of low pathogenic avian influenza virus (LPAI) (Prel et al., 2008). H5N3 LPAI VLPs containing HA and NA (A/Duck/France/02166/2002) were produced using the rBV/insect cell expression system and their immunogenicities were tested in ducks for developing animal vaccines. Positive HAI titers detected in immune sera suggest the feasibility for developing VLP-based subunit vaccines in this species. Another LPAI VLP was generated with HA and NA derived from A/HongKong/1073/99 (H9N2) virus (Pushko et al., 2005; Pushko et al., 2007). Mice were inoculated with H9N2 VLPs (10 μg) on days 0 and 28 subcutaneously for immunological studies and intramuscularly for challenge experiment. The HAI activity and virus specific antibodies were detected after a single immunization and the titer increased after boosting. Following 100 MID_{50} (50% mouse infectious dose) homologous challenge, vaccinated mice showed less weight loss whereas control group lost around 17% of body weight.

Another consideration is the development of VLP vaccines against influenza virus such as H2N2 which also possess pandemic potential. Historically, the most widespread influenza pandemic was the 1918 "Spanish flu". To determine the vaccine potency of VLP against Spanish flu, VLPs bearing HA, NA (from 1918 influenza A virus), M2, and M1 (from A/Udorn, H3N2) were prepared in insect cells and formulated with or without CpG oligodeoxynucleotide adjuvant (Matassov et al., 2007). Mice immunized intranasally two times were challenged with A/Swine/Iowa/15/30 (H1N1) which is antigenically related with the 1918 influenza virus. Immunization with VLPs alone could provide protection against lethal infection, and the addition of CpG adjuvant improved the protective efficacy as evidenced by lower viral titers in the upper respiratory tract.

7. Conclusions

As presented in recent studies, VLP platform vaccines have received significant attention for their potential promise in developing effective and safe vaccines against viral pathogens. Licensed hepatitis B and HPV vaccines are non-replicating particles based on the concept of

VLPs, which are capsid-based, non-enveloped particles. Influenza VLPs represents a new vaccine format because of their enveloped structure containing membrane-anchored glycoproteins. As reviewed here, many recent studies have provided pre-clinical evidence that influenza VLPs can be developed as promising vaccines against both seasonal influenza and pandemic influenza viruses. In addition to the desirable safety features of non-replicating, non-infectious VLPs, influenza VLPs may provide a broader range of protection against antigenic variants of the virus. Also, they are likely to induce cellular immune responses known to play a contributing role in broadening protection, which will be especially beneficial in high-risk groups such as the elderly population.

As mentioned above, overcoming the antigenic changes of influenza viruses is a difficult challenge in developing effective vaccines. Antigenic drift occurs throughout the waves of a pandemic and throughout annual influenza epidemics. Recombinant VLP vaccines have demonstrated the ability to protect against homologous and closely related heterologous H5N1 viruses in animal models without an adjuvant (Bright et al., 2008; Haynes et al., 2009). Immunogenicity and lethal challenge studies in ferrets have demonstrated that influenza VLP vaccines induce cross-protection and cross-reactivity against drifted strains of H5 and H3 influenza strains (Bright et al., 2007; Haynes et al., 2009). However, we do not know the underlying mechanisms by which immune responses can induce protection against drifted pandemic strains. In contrast to seasonal influenza viruses, there is less correlation between levels of neutralizing antibodies (or HAI titers) and homologous or heterologous protective efficacies although the observed cross protection is still limited to closely related strains. A better understanding of protective immune correlates among pandemic strains will provide insightful information for developing more effective pandemic vaccines. Enhancing the mucosal immunogenicity and/or targeting to more conserved antigenic targets will provide an approach for broadening the protective efficacy, which might be possible by modification of VLPs using genetic engineering technologies.

The route of vaccination is also an important issue for developing effective vaccines. Influenza VLPs were demonstrated to provide protective immunity via either the intranasal or intramuscular route in the absence of adjuvants. Intranasal immunization is known to induce immune responses at mucosal sites where respiratory pathogens are entering the body. Particularly, influenza is a respiratory disease and thus a vaccination route inducing mucosal immunity should be effective. Live attenuated influenza vaccines are approved for nasal delivery in humans. However, safety and efficacy after intranasal delivery of non-replicating vaccines remain to be determined in humans. Most vaccines are traditionally administered to humans via intramuscular immunization. As a systemic vaccination route, intramuscular immunization does not induce significant levels of mucosal immunity, and intranasal and intramuscular routes of influenza VLP vaccinations should both be clinically tested in humans.

Despite the theoretical advantages and promising pre-clinical protective efficacies, issues relating to host cell and viral components incorporated into VLPs during production need to be addressed. Determining their effects on affecting the immunogenicity of vaccine antigens and/or effects on repeated administration of VLP vaccines will be important for clinical advancement of such vaccines. Since adult populations have a certain level of pre-existing immunity against seasonal influenza virus, it will be informative to determine the immunogenicity of pandemic H5 VLPs in the presence of immunity to seasonal influenza virus. Also, immune responses to VLPs in aged mice will provide helpful insight into vaccinating the elderly populations which are more susceptible to influenza infection. Finally, the safety and predictive protective immunity by influenza VLP vaccines should be tested in human clinical trials.

Acknowledgments

This work was supported by NIH/NIAID grant AI0680003 (R.W.C.).

References

Ali A, Avalos RT, Ponimaskin E, Nayak DP. Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein. J Virol 2000;74(18):8709–19. [PubMed: 10954572]

Assad S, Francis A. Over a decade of experience with a yeast recombinant hepatitis B vaccine. Vaccine 1999;18(1–2):57–67. [PubMed: 10501235]

Block SL, Yogev R, Hayden FG, Ambrose CS, Zeng W, Walker RE. Shedding and immunogenicity of live attenuated influenza vaccine virus in subjects 5–49 years of age. Vaccine. 2008

Bright RA, Carter DM, Crevar CJ, Toapanta FR, Steckbeck JD, Cole KS, Kumar NM, Pushko P, Smith G, Tumpey TM, Ross TM. Cross-Clade Protective Immune Responses to Influenza Viruses with H5N1 HA and NA Elicited by an Influenza Virus-Like Particle. PLoS ONE 2008;3(1):e1501. [PubMed: 18231588]

Bright RA, Carter DM, Daniluk S, Toapanta FR, Ahmad A, Gavrilov V, Massare M, Pushko P, Mytle N, Rowe T, Smith G, Ross TM. Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. Vaccine 2007;25(19):3871–8. [PubMed: 17337102]

Buonaguro L, Devito C, Tornesello ML, Schroder U, Wahren B, Hinkula J, Buonaguro FM. DNA-VLP prime-boost intra-nasal immunization induces cellular and humoral anti-HIV-1 systemic and mucosal immunity with cross-clade neutralizing activity. Vaccine 2007;25(32):5968–77. [PubMed: 17629365]

Buonaguro L, Tornesello ML, Tagliamonte M, Gallo RC, Wang LX, Kamin-Lewis R, Abdelwahab S, Lewis GK, Buonaguro FM. Baculovirus-derived human immunodeficiency virus type 1 virus-like particles activate dendritic cells and induce ex vivo T-cell responses. J Virol 2006;80(18):9134–43. [PubMed: 16940524]

Carroll SM, Paulson JC. Differential infection of receptor-modified host cells by receptor-specific influenza viruses. Virus Res 1985;3(2):165–79. [PubMed: 4060886]

Chen BJ, Leser GP, Morita E, Lamb RA. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. J Virol 2007;81(13):7111–23. [PubMed: 17475660]

Cintra OA, Rey LC. Safety, immunogenicity and efficacy of influenza vaccine in children. J Pediatr (Rio J) 2006;82(3 Suppl):S83–90. [PubMed: 16794746]

Crevar CJ, Ross TM. Elicitation of protective immune responses using a bivalent H5N1 VLP vaccine. Virol J 2008;5:131. [PubMed: 18957098]

D'Aoust MA, Lavoie PO, Couture MM, Trepanier S, Guay JM, Dargis M, Mongrand S, Landry N, Ward BJ, Vezina LP. Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. Plant Biotechnol J 2008;6(9):930–40. [PubMed: 19076615]

Da Silva DM, Velders MP, Nieland JD, Schiller JT, Nickoloff BJ, Kast WM. Physical interaction of human papillomavirus virus-like particles with immune cells. Int Immunol 2001;13(5):633–641. [PubMed: 11312251]

Deml L, Kratochwil G, Osterrieder N, Knuchel R, Wolf H, Wagner R. Increased incorporation of chimeric human immunodeficiency virus type 1 gp120 proteins into Pr55gag virus-like particles by an Epstein-Barr virus gp220/350-derived transmembrane domain. Virology 1997;235(1):10–25. [PubMed: 9300033]

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005;79(5):2814–22. [PubMed: 15709000]

Galarza JM, Latham T, Cupo A. Virus-like particle (VLP) vaccine conferred complete protection against a lethal influenza virus challenge. Viral Immunol 2005;18(1):244–51. [PubMed: 15802970]

Gomez-Puertas P, Albo C, Perez-Pastrana E, Vivo A, Portela A. Influenza virus matrix protein is the major driving force in virus budding. J Virol 2000;74(24):11538–47. [PubMed: 11090151]

Guo L, Lu X, Kang SM, Chen C, Compans RW, Yao Q. Enhancement of mucosal immune responses by chimeric influenza HA/SHIV virus-like particles. Virology 2003;313(2):502–13. [PubMed: 12954217]

Harro CD, Pang YY, Roden RB, Hildesheim A, Wang Z, Reynolds MJ, Mast TC, Robinson R, Murphy BR, Karron RA, Dillner J, Schiller JT, Lowy DR. Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. J Natl Cancer Inst 2001;93(4):284–92. [PubMed: 11181775]

Haynes JR, Dokken L, Wiley JA, Cawthon AG, Bigger J, Harmsen AG, Richardson C. Influenza-pseudotyped Gag virus-like particle vaccines provide broad protection against highly pathogenic avian influenza challenge. Vaccine 2009;27(4):530–41. [PubMed: 19026705]

Jin H, Leser GP, Zhang J, Lamb RA. Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. Embo J 1997;16(6):1236–47. [PubMed: 9135140]

Johansson BE, Brett IC. Changing perspective on immunization against influnza. Vaccine 2007;25(16): 3062–5. [PubMed: 17276554]

Kang SM, Compans RW. Enhancement of mucosal immunization with virus-like particles of simian immunodeficiency virus. J Virol 2003a;77(6):3615–23. [PubMed: 12610137]

Kang SM, Yoo DG, Lipatov AS, Song JM, Davis CT, Quan FS, Chen LM, Donis RO, Compans RW. Induction of long-term protective immune responses by influenza H5N1 virus-like particles. PLoS ONE 2009;4(3):e4667. [PubMed: 19252744]

Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci U S A 1992;89 (24):12180–4. [PubMed: 1334560]

Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, Chiacchierini LM, Jansen KU. A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med 2002;347(21):1645–51. [PubMed: 12444178]

Kurtz J, Manvell RJ, Banks J. Avian influenza virus isolated from a woman with conjunctivitis. Lancet 1996;348(9031):901–2. [PubMed: 8826845]

Latham T, Galarza JM. Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. J Virol 2001;75(13):6154–65. [PubMed: 11390617]

Lenz P, Thompson CD, Day PM, Bacot SM, Lowy DR, Schiller JT. Interaction of papillomavirus virus-like particles with human myeloid antigen-presenting cells. Clin Immunol 2003;106(3):231–7. [PubMed: 12706410]

Li S, Liu C, Klimov A, Subbarao K, Perdue ML, Mo D, Ji Y, Woods L, Hietala S, Bryant M. Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. J Infect Dis 1999;179(5):1132–8. [PubMed: 10191214]

Lipatov AS, Webby RJ, Govorkova EA, Krauss S, Webster RG. Efficacy of H5 influenza vaccines produced by reverse genetics in a lethal mouse model. J Infect Dis 2005;191(8):1216–20. [PubMed: 15776365]

Liu C, Eichelberger MC, Compans RW, Air GM. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. J Virol 1995;69(2):1099–106. [PubMed: 7815489]

Ljubojevic S. The human papillomavirus vaccines. Acta Dermatovenerol Croat 2006;14(3):208. [PubMed: 17010274]

Mahmood K, Bright RA, Mytle N, Carter DM, Crevar CJ, Achenbach JE, Heaton PM, Tumpey TM, Ross TM. H5N1 VLP vaccine induced protection in ferrets against lethal challenge with highly pathogenic H5N1 influenza viruses. Vaccine 2008;26(42):5393–9. [PubMed: 18706956]

Markowitz LE, Dunne EF, Saraiya M, Lawson HW, Chesson H, Unger ER. Quadrivalent Human Papillomavirus Vaccine: Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2007;56(RR2):1–24. [PubMed: 17380109]

Matassov D, Cupo A, Galarza JM. A novel intranasal virus-like particle (VLP) vaccine designed to protect against the pandemic 1918 influenza A virus (H1N1). Viral Immunol 2007;20(3):441–52. [PubMed: 17931114]

McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR. Human hepatitis B vaccine from recombinant yeast. Nature 1984;307(5947):178–80. [PubMed: 6318124]

Moron G, Rueda P, Casal I, Leclerc C. CD8alpha- CD11b+ dendritic cells present exogenous virus-like particles to CD8+ T cells and subsequently express CD8alpha and CD205 molecules. J Exp Med 2002;195(10):1233–45. [PubMed: 12021304]

Moron VG, Rueda P, Sedlik C, Leclerc C. In vivo, dendritic cells can cross-present virus-like particles using an endosome-to-cytosol pathway. J Immunol 2003;171(5):2242–50. [PubMed: 12928368]

Murata K, Lechmann M, Qiao M, Gunji T, Alter HJ, Liang TJ. Immunization with hepatitis C virus-like particles protects mice from recombinant hepatitis C virus-vaccinia infection. Proc Natl Acad Sci U S A 2003;100(11):6753–8. [PubMed: 12748380]

Nayak DP, Hui EK, Barman S. Assembly and budding of influenza virus. Virus Res 2004;106(2):147–65. [PubMed: 15567494]

O'Neal CM, Clements JD, Estes MK, Conner ME. Rotavirus 2/6 viruslike particles administered intranasally with cholera toxin, Escherichia coli heat-labile toxin (LT), and LT-R192G induce protection from rotavirus challenge. J Virol 1998;72(4):3390–3. [PubMed: 9525668]

Pattnaik AK, Brown DJ, Nayak DP. Formation of influenza virus particles lacking hemagglutinin on the viral envelope. J Virol 1986;60(3):994–1001. [PubMed: 3783822]

Prel A, Le Gall-Recule G, Jestin V. Achievement of avian influenza virus-like particles that could be used as a subunit vaccine against low-pathogenic avian influenza strains in ducks. Avian Pathol 2008;37(5):513–20. [PubMed: 18798027]

Pushko P, Tumpey TM, Bu F, Knell J, Robinson R, Smith G. Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. Vaccine 2005;23(50):5751–9. [PubMed: 16143432]

Pushko P, Tumpey TM, Van Hoeven N, Belser JA, Robinson R, Nathan M, Smith G, Wright DC, Bright RA. Evaluation of influenza virus-like particles and Novasome adjuvant as candidate vaccine for avian influenza. Vaccine 2007;25(21):4283–90. [PubMed: 17403562]

Qiao CL, Yu KZ, Jiang YP, Jia YQ, Tian GB, Liu M, Deng GH, Wang XR, Meng QW, Tang XY. Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 haemagglutinin and N1 neuraminidase genes. Avian Pathol 2003;32(1):25–32. [PubMed: 12745375]

Quan FS, Compans RW, Nguyen HH, Kang SM. Induction of heterosubtypic immunity to influenza virus by intranasal immunization. J Virol 2008;82(3):1350–9. [PubMed: 18032492]

Quan FS, Huang C, Compans RW, Kang SM. Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. J Virol 2007;81(7):3514–24. [PubMed: 17251294]

Quan FS, Steinhauer D, Huang C, Ross TM, Compans RW, Kang SM. A bivalent influenza VLP vaccine confers complete inhibition of virus replication in lungs. Vaccine 2008b;26(26):3352–61. [PubMed: 18468740]

Roberts PC, Lamb RA, Compans RW. The M1 and M2 proteins of influenza A virus are important determinants in filamentous particle formation. Virology 1998;240(1):127–37. [PubMed: 9448697]

Rohm C, Zhou N, Suss J, Mackenzie J, Webster RG. Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. Virology 1996;217(2):508–16. [PubMed: 8610442]

Sailaja G, Skountzou I, Quan FS, Compans RW, Kang SM. Human immunodeficiency virus-like particles activate multiple types of immune cells. Virology 2007;362(2):331–41. [PubMed: 17276476]

Sasagawa T, Pushko P, Steers G, Gschmeissner SE, Hajibagheri MA, Finch J, Crawford L, Tommasino M. Synthesis and assembly of virus-like particles of human papillomaviruses type 6 and type 16 in fission yeast Schizosaccharomyces pombe. Virology 1995;206(1):126–35. [PubMed: 7831768]

Skountzou I, Quan FS, Gangadhara S, Ye L, Vzorov A, Selvaraj P, Jacob J, Compans RW, Kang SM. Incorporation of Glycosylphosphatidylinositol-Anchored Granulocyte- Macrophage Colony-

Stimulating Factor or CD40 Ligand Enhances Immunogenicity of Chimeric Simian Immunodeficiency Virus-Like Particles. J Virol 2007;81(3):1083–94. [PubMed: 17108046]

Stanley MA. Human papillomavirus vaccines. Rev Med Virol 2006;16(3):139–49. [PubMed: 16710836]

Subbarao K, Chen H, Swayne D, Mingay L, Fodor E, Brownlee G, Xu X, Lu X, Katz J, Cox N, Matsuoka Y. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. Virology 2003;305(1):192–200. [PubMed: 12504552]

Suguitan AL Jr, McAuliffe J, Mills KL, Jin H, Duke G, Lu B, Luke CJ, Murphy B, Swayne DE, Kemble G, Subbarao K. Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. PLoS Med 2006;3(9):e360. [PubMed: 16968127]

Sun Y, Carrion R Jr, Ye L, Wen Z, Ro YT, Brasky K, Ticer AE, Schwegler EE, Patterson JL, Compans RW, Yang C. Protection against lethal challenge by Ebola virus-like particles produced in insect cells. Virology 2009;383(1):12–21. [PubMed: 18986663]

Szecsi J, Boson B, Johnsson P, Dupeyrot-Lacas P, Matrosovich M, Klenk HD, Klatzmann D, Volchkov V, Cosset FL. Induction of neutralising antibodies by virus-like particles harbouring surface proteins from highly pathogenic H5N1 and H7N1 influenza viruses. Virol J 2006;3:70. [PubMed: 16948862]

Triyatni M, Saunier B, Maruvada P, Davis AR, Ulianich L, Heller T, Patel A, Kohn LD, Liang TJ. Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry. J Virol 2002;76(18):9335–44. [PubMed: 12186916]

Tumpey TM, Renshaw M, Clements JD, Katz JM. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J Virol 2001;75(11):5141–50. [PubMed: 11333895]

Wang BZ, Liu W, Kang SM, Alam M, Huang C, Ye L, Sun Y, Li Y, Kothe DL, Pushko P, Dokland T, Haynes BF, Smith G, Hahn BH, Compans RW. Incorporation of high levels of chimeric human immunodeficiency virus envelope glycoproteins into virus-like particles. J Virol 2007;81(20):10869–78. [PubMed: 17670815]

Wang BZ, Quan FS, Kang SM, Bozja J, Skountzou I, Compans RW. Incorporation of membrane-anchored flagellin into influenza virus-like particles enhances the breadth of immune responses. J Virol 2008;82(23):11813–23. [PubMed: 18786995]

Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, McClain-Moss LR, Peiris JS, Rehg JE, Tuomanen EI, Webster RG. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet 2004;363(9415):1099–103. [PubMed: 15064027]

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56(1):152–79. [PubMed: 1579108]

Yao Q, Kuhlmann FM, Eller R, Compans RW, Chen C. Production and characterization of simian--human immunodeficiency virus- like particles. AIDS Res Hum Retroviruses 2000;16(3):227–236. [PubMed: 10710211]

Ye L, Lin J, Sun Y, Bennouna S, Lo M, Wu Q, Bu Z, Pulendran B, Compans RW, Yang C. Ebola virus-like particles produced in insect cells exhibit dendritic cell stimulating activity and induce neutralizing antibodies. Virology 2006;351(2):260–70. [PubMed: 16678231]

Zhang J, Leser GP, Pekosz A, Lamb RA. The cytoplasmic tails of the influenza virus spike glycoproteins are required for normal genome packaging. Virology 2000;269(2):325–34. [PubMed: 10753711]

Table 1

VLP vaccines for seasonal influenza viruses[1]

Subtype	Components	Dose/route	Challenge, assay	Reference
H3N2 (A/Udorn)	HA, M1	HA (1 μg)/IN, IM, twice	A/HK/68 (H3N2)	(Galarza et al., 2005)
H1N1 (A/PR8/34)	HA, M1	VLP (10 μg)/IN, twice	A/PR8/34 A/WSN/33	(Quan et al., 2007)
H3N2 (A/Fujian/411/2002)	HA, NA, M1	HA (0. 24 –3 μg)/ IM, twice (mouse, ferrets)	HAI titers (in vitro)	(Bright et al., 2007)
H1 + H3 (A/PR8/34+ A/Aichi/ 2/68 (X31))	HA (H1+H3), M1	VLP (10 μg)/IM, twice	A/PR8/34, A/Aichi (X31) A/WSN, A/Philippines	(Quan et al., 2008b)
A/PR8/34 (H1N1)	HA, M1	VLP (10 μg)/IM, twice	A/PR8, A/Philippines	(Wang et al., 2008)

[1] All vaccines were produced in insect cells. Unless specified, mice were used for testing vaccine efficacy. IN, intranasal; IM, intramuscular

Table 2

VLP vaccines for pandemic influenza viruses[1]

Subtype	Components (cell system)	Dose/route	Challenge, assay	Reference
H5N1 clade 1 (VN/04), clade 2 (Indo/05)	HA, NA, M1 (Insect)	HA (0.6 – 3 μg)/ IM, once or twice	H5N1 clade 1 (VN/04), clade 2 (Indo/05)	(Bright et al., 2008)
H5N1 (Indo/05, clade 2.1)	HA, NA, M1 (Insect)	HA (0.6 –15 μg)/IM, twice (ferrets)	H5N1 (Indo/05, VietNam 03/04)	(Mahmood et al., 2008)
Bivalent H5N1 (clades 1 +2)	HA, NA, M1 (Insect)	HA (0.6 μg)/IM, twice	H5N1 clade 1 (VN/04), clade 2 (Indo/05, WS/05, Anh/05)	(Crevar and Ross, 2008)
H5N1 clade 1 (VN/04)	HA, NA, M1 (Insect)	HA (0.1 – 0.3 μg)/IN	H5N1 (wild type, VN/04),	(Kang et al., 2009)
H5N1 (Indo/05), H1N1 (A/ New Caledonia)	HA, M1 (Plant)	0.1ug VLP 0.5ug VLP	H5N1 clade 1 (VN/04)	(D'Aoust et al., 2008)
H5N1 clade 1 (VN/04), clade 2 (Indo/05) (Pseudotyped)	Gag, HA, NA (Insect)	0.7–1 ug HA/ IM, IP, twice (mouse, ferrets)	A/Vietnam/1203/04	(Haynes et al., 2009)
H5N1, H7N1 (Pseudotyped)	Gag, HA, NA, M2 (293T cells)	10^8 particles	Neutralizing titers	(Szecsi et al., 2006)
H5N3 (LPAI)	HA, NA, M1	VLP (2– 20 μg) (ducks)	HAI (H5N3, A/duck/France)	(Prel et al., 2008)
H9N2 (A/HK/1073/99)	HA, NA, M1 (Insect)	VLP (10 μg)/SC	A/HK/1073/99	(Pushko et al., 2005)
H9N2 (A/HK/1073/99)	HA, NA, M1 (Insect)	HA (0.12 – 15 μg/IM, twice (mouse, rat, ferrets)	A/HK/1073/99	(Pushko et al., 2007)
Pandemic 1918 A virus (H1N1)	HA, NA, M1, M2 (Insect)	HA (1 μg)/IN, twice	A/swine/Iowa (H1N1)	(Matassov et al., 2007)

[1] Unless specified, mice were used for testing vaccine efficacy. IN, intranasal; IM, intramuscular