



Published in final edited form as:

Expert Rev Vaccines. 2012 August ; 11(8): 995–1007. doi:10.1586/erv.12.70.

Virus-like particles as universal influenza vaccines

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Abstract

Current influenza vaccines are primarily targeted to induce immunity to the influenza virus strain-specific hemagglutinin antigen and are not effective in controlling outbreaks of new pandemic viruses. An approach for developing universal vaccines is to present highly conserved antigenic epitopes in an immunogenic conformation such as virus-like particles (VLPs) together with an adjuvant to enhance the vaccine immunogenicity. In this review, the authors focus on conserved antigenic targets and molecular adjuvants that were presented in VLPs. Conserved antigenic targets that include the hemagglutinin stalk domain, the external domain of influenza M2 and neuraminidase are discussed in addition to molecular adjuvants that are engineered to be incorporated into VLPs in a membrane-anchored form.

Keywords

adjuvant; HA stalk domain; influenza; M2; NA; universal vaccine; VLPs

Influenza virus causes 17,000–51,000 deaths in the USA and 250,000–500,000 deaths worldwide annually; a global pandemic could kill millions [1,2]. Vaccination is the most cost-effective public health measure to prevent disease and mortality caused by influenza virus infection [2]. The envelope of influenza virus contains two major surface glycoproteins: the receptor-binding hemagglutinin (HA) and the sialic acid receptor-cleaving neuraminidase (NA) proteins [3,4]. Human influenza viruses are continually evolving, resulting in numerous variants with distinct antigenic surface glycoprotein properties. Current influenza vaccines consist of virus chemically inactivated with formalin or β -propiolactone and further detergent-treated to produce soluble forms of the viral surface antigens. In addition, a live attenuated influenza vaccine FluMist™ (MedImmune) is licensed for intranasal delivery in humans [5]. A limitation of current vaccines based on immunity to HA is that the antigenic regions of HA are highly susceptible to continual changes by mutations in circulating epidemic virus strains [6,7]. The HA proteins of influenza A viruses are divided into 16 subtypes and are further categorized into two major phylogenetic groups based on the distinct structures in the HA2 stalk domain: Group 1 (subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16) and Group 2 (subtypes H3, H4, H7, H10, H14 and H15 [8]). Influenza vaccines are updated annually to match the

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Financial and competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

antigenicity of the virus strains predicted to circulate in the coming winter season, and current vaccines are not effective in preventing emerging new pandemic strains with distinct HA antigenicity. Therefore, developing broadly cross-protective vaccines is a high priority.

Several new approaches are being applied to develop universal influenza A vaccines inducing broadly cross-protective immunity against conserved antigenic targets such as the HA stalk domain, the extracellular domain of M2 (M2e) and NA. These conserved antigenic targets are designed to be presented in a carrier system or conjugated to adjuvant molecules. In particular, virus-like particles (VLPs) are similar to the virus in structure and morphology, and thus can be a promising platform for presenting surface proteins in a highly immunogenic form. VLPs are produced without viral genomes (Figure 1), representing a high safety feature. The viral glycoproteins are unmodified by fixatives and presented in a membrane-anchored form mimicking their native conformation. VLPs are effective in stimulating APCs such as dendritic cells (DCs) as well as in inducing both B- and T-cell responses [9–14]. VLPs, similar to the virus in structure, are able to induce CD4 T-cell proliferation and cytotoxic T-cell immune responses [13,15]. In particular, influenza VLP vaccine was demonstrated to stimulate CD8 T cells via DC-mediated antigen cross-presentation, probably through the MHC class I pathway [13].

The HA antigen is incorporated into influenza virions at higher levels than NA or M2 and is known to be immune-dominant; thus, immune responses to other antigens such as NA are relatively low [16,17]. In this regard, VLP technology has an advantage by expressing fewer immunogenic components separately (Figure 1). Thus, NA and M2 antigens presented separately on VLPs can be more immunogenic by avoiding an HA immune-dominant component in the vaccines [16,17]. In addition, adjuvant molecules can be directly incorporated into VLP vaccines [18–21]. Here, the current progress in developing universal influenza vaccines using different platforms for presenting conserved target antigens is reviewed and discussed. These conserved target antigens include the HA stalk domain, M2e or NA (Figures 1 & 2). Influenza VLP vaccines presenting conserved influenza antigenic targets as well as adjuvant molecules in a membrane-anchored form have been receiving more attention and are discussed in comparison with other vaccine platforms.

VLPs expressing the conserved HA stalk domain

Influenza virus HA is a homotrimeric protein molecule, and each monomer consists of two disulfide-linked subunit glycoproteins, a globular head of HA1 and a stem domain composed of the N- and C-terminal parts of HA1 and all of HA2 (Figure 2) [22]. Current influenza vaccination primarily induces immunity to the globular head domain of HA. The globular head domain of the receptor-binding pocket is surrounded by variable antigenic sites contributing to the generation of numerous escape mutants, identified by monoclonal antibodies and natural variants [23,24]. The locations of four antigenic sites (A, B, C and D) are consistent with antigenic mapping studies of the HA molecule-based reactivity of distinct monoclonal antibodies [25,26]. The failure or reduced efficacy of influenza vaccination primarily results from mutations occurring in the HA1 globular head domains [25,26].

In an effort to identify conserved epitopes, recent studies have indicated the HA stalk domain as a potential target for developing universal vaccines. The sequence homology of the HA2 subunit among different subtypes is in a range of 51–80%, relatively lower compared with that of the HA1 subunit (34–59% [27]). The sequence homology of the HA2 subunit within the same subtype is even higher [27]. In particular, specific regions in the HA stalk domains were identified to be highly conserved among different subtypes of influenza viruses. A long α -helix domain in the HA2 subunit is one such part that shows a high degree

of conservation in amino acid sequence and amino acid type (Figure 2). Alignment of the 76–130 amino acid region of the HA2 subunit shows this conservation among different HA subtypes including H1, H2, H3, H5 and H7 [28].

Another conserved region is the HA cleavage site that forms an extended, highly exposed loop structure on the surface that is highly conserved in most influenza A viruses (Figure 2) [29]. Particularly, the N-terminal sequence of 11 amino acids in the HA cleavage site is invariant among most influenza A virus strains and differs only by one or two amino acid replacements in influenza B virus. The maintenance of this invariant domain is likely to be required because of functional constraints for being a suitable substrate for host-encoded proteases. This cleavage domain is exposed in a loop structure in the HA precursor and is accessible to antibody on the uncleaved HA precursor expressed on the plasma membrane of infected host cells [29].

By vaccination of mice with bromelain-treated or low pH-exposed influenza virus, previous studies identified monoclonal antibodies that are reactive to the stalk region of the HA molecule [8,30–32]. Some of these monoclonal antibodies recognizing epitopes in the stalk domain were found to show weak but broadly neutralizing activity [33–35]. Specific monoclonal antibodies recognizing epitopes in the fusion peptide region were shown to inhibit the fusion step of virus entry [8,34], to neutralize different influenza viruses [36], and in some cases, to provide survival protection [35]. HA2-specific monoclonal antibodies were also used to develop a simple slot test for detecting all subtypes of influenza HA proteins after a denaturation step [37].

In contrast to many studies on monoclonal antibodies recognizing the conserved stalk domain, fewer studies were reported regarding the development of universal influenza vaccines based on conserved antigenic targets of the HA2 subunit. Vaccines utilizing HA2 subunits include the cleavage region, fusion peptide, long α -helical polypeptides and recombinant HA2 subunits vaccines (Figure 2). Synthetic polypeptides covering the HA1/HA2 cleavage region conjugated to the carrier proteins were shown to induce antibodies recognizing cleavage region peptide epitopes and weak protection of immunized mice, as shown by reduced illness and fewer deaths upon lethal challenge with influenza A virus [38]. Similarly, a peptide conjugate vaccine based on the highly conserved maturational cleavage site of the HA precursor of the influenza B virus elicited a protective immune response against lethal challenge with an antigenically different influenza B virus [39].

Recent studies demonstrate the efficacy of stalk domain-based vaccines. An HA2-based immunogen destabilizing the low-pH conformation of HA2 was expressed in *Escherichia coli* and used for the immunization of mice [40]. This HA2 vaccine was highly immunogenic, protecting mice against lethal challenge with a homologous virus [40]. In addition, the chemically synthesized fusion peptide (amino acids 1–38 of HA2) (Figure 2) was conjugated to the keyhole limpet hemocyanin and tested in mice as a vaccine [40,41]. Similarly, this vaccine provided survival protection against a low-dose challenge with homologous and heterologous virus in immunized mice [40,41].

The Palese laboratory described an HA2-specific monoclonal antibody, 12D1, cloned from hybridoma fusion of lymphocytes from mice repeatedly immunized with H3 subtype HA DNA vaccines and followed by H3 virus boost [33]. This 12D1 monoclonal antibody was shown to recognize a region within amino acids 76–106 of the HA2 subunit stalk domain and to broadly neutralize heterologous viruses within the H3 subtype [33]. A further study by the same group revealed that 12D1 shows maximal binding to the extended structural element of a peptide representing the whole long α -helix domain amino acids 76–130 of the HA2 subunit from the subtype H3 HA (A/Hong Kong/1/1968 [28]). Based on the stalk

domain epitope identified to be recognized by 12D1 (Figure 2), a long α -helix HA2 vaccine consisting of the amino acid 76–130 polypeptide was designed and coupled to the carrier protein keyhole limpet hemocyanin [28]. Sera from mice immunized with α -helix HA2 vaccine showed substantial binding antibodies reactive to heterosubtypic virus [28]. This α -helix HA2 vaccine could provide partial protection against heterosubtypic challenge viruses (10–15 mouse LD50, A/PR8 H1N1, A/Vietnam/04 H5N1 virus) and survival protection against the homologous virus H3 subtype [28]. Although the HA2 vaccine immune sera showed significant breadth in cross-reactivity with different HA molecules, the breadth was limited to Group 2 HAs (subtypes H3, H4, H7, H10, H14 and H15 [28]).

Using recombinant genetic engineering techniques, Steel *et al.* expressed a modified HA molecule lacking the globular head domain [42]. This headless HA is composed of membrane-proximal portions of both the HA1 signal peptide region and HA2 subunits so that headless HA molecules are stably expressed on cell surfaces. Furthermore, this construct was intended to remove the highly immunogenic head domain. A novel approach was to incorporate the headless HA into VLPs (Figure 2). Co-expression of the HIV Gag core protein and headless HA protein by transient DNA co-transfections resulted in the production of chimeric Gag VLPs containing headless HA molecules [42]. A three-dose vaccine regimen was applied to immunize mice. Two vaccinations with DNA constructs (Gag and HA) were followed by boost with chimeric headless HA VLP vaccines (150 ng HA content) in the presence of Freund's complete adjuvant [42]. The headless HA VLP vaccines provided protection against homologous challenge in mice with moderate body weight loss. The neutralizing activity against the homologous virus in the immune sera of mice with headless HA VLP vaccines was marginally higher than the background, but was not conclusively confirmed [42]. Nonetheless, it is important to note that headless HA (A/PR8) VLP immune sera were likely to exhibit greater reactivity to heterologous strains than the full-length HA vaccine [42]. A/Hong Kong/68 (H3N2) headless HA VLP vaccines did not induce antibodies cross-reactive to different Group 1 HAs (subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16). In other studies, vaccines of influenza virions stripped of HA1 by treatment with acid or dithiothreitol were not effective in inducing cross-reactive antibodies and cross-protection [43,44]. Acid or chemical treatment to strip the HA1 part would change the remaining HA2 portion to the extended, low-pH conformation, possibly explaining the low efficacy of protection. By contrast, headless HA VLPs remain in the pre-fusion conformation, which is more likely to be in a native-like structure. Some limitations of these approaches are the low yield of headless HA VLPs produced by transient co-transfection with DNA expression constructs, use of Freund's adjuvant and breadth due to the subtype or HA group.

VLPs expressing M2e

The M2 protein is produced by translation from a spliced mRNA derived from influenza gene segment 7, also coding for the matrix protein M1 [45]. The first nine codons of M2 are shared with those of M1, and this overlapping RNA gene seems to further contribute to the conservation of M2 amino acid sequence. M2 is a type III integral membrane protein, forming a pH-dependent proton-selective ion channel [46–48]. M2 is a specific target of anti-influenza drugs such as amantadine and rimantadine, and these drugs are known to inhibit virus uncoating and maturation [49,50]. Influenza virus enters the target cells via receptor-mediated endocytosis, and M2 lowers the pH of the virus interior by opening the H⁺ ion channel across the virus membrane. The low pH facilitates viral membrane fusion and protein–protein dissociation between the matrix protein and the nucleoprotein (NP) complex. The M2 protein is also known to play a role in virus morphogenesis and assembly [51–55].

The M2 protein of 96 amino acids has three structural domains: an amino-terminal extracellular domain (23 residues), a transmembrane domain (19 residues) and a cytoplasmic tail domain (54 residues). The native conformation of M2 is a tetrameric membrane protein with disulfide bonds [56,57]. M2 is expressed on infected cell surfaces at high levels [58], in a ratio of approximately two M2 per HA trimer [59]. However, M2 molecules are estimated to be present at low levels (~23–60) on each virion [60]. The amino acid sequence in M2e is highly conserved among human influenza A viruses. For example, the N-terminal epitope SLLTEVET (residues two to nine) in M2e was found to be conserved at a level of 100% among human influenza A virus isolates and approximately over 99% among all influenza A subtypes [61,62]. In other residues in M2e, there are few amino acid changes depending on the host species (human, avian, swine, equine and other hosts) where influenza viruses were isolated [61,62]. The five amino acids within the residues 10–20 of M2e (underlined residues) were observed to be host restricted: PIRNEWGCRCN (amino acids 10–20, human isolates), PTRNGWECKCS (amino acids 10–20, avian isolates) and PIRNGWECRCNCN (amino acids 10–20, swine isolates [62]). Owing to the low degree of variation in the M2 extracellular domain compared with the HA and NA protein, M2 is considered to provide an attractive antigenic target for developing a universal influenza vaccine.

The extracellular 23-amino acid residue of the M2 protein is a small peptide and a very weak immunogen in its native form. Anti-M2e antibodies are detected only in a fraction of infected people [63]. Even mice infected with influenza virus do not induce high levels of antibodies recognizing M2 [64]. Nonetheless, there is evidence that anti-M2 immunity can confer protection against different influenza viruses. M2e-specific monoclonal antibodies were shown to reduce the plaque size or the growth of some influenza A virus strains *in vitro* in a strain-specific manner [53,55,60]. The reduction in plaque size might be due to blocking a late stage of replication by M2 antibodies [53,55] as similarly observed with treatment of antibodies specific to NA [65]. Passive transfer of M2 monoclonal antibodies protected mice by lowering lung virus titers upon subsequent infection with influenza A virus [20,66]. Therefore, induction of adaptive anti-M2 immunity would be a cost-effective and practical strategy for controlling influenza epidemics or pandemics.

One concern about M2e-based influenza A vaccine is its limited efficacy. Hence, different approaches to link M2e peptide to carriers and/or use of potent adjuvants were explored. The first study on cross-protection was reported using an M2 vaccine composed of a partially purified M2-containing membrane fraction derived from the recombinant baculovirus insect-cell expression system in combination with incomplete Freund's adjuvant [67]. This recombinant M2 vaccine provided a survival advantage to immunized mice after lethal challenge [67]. Since then, many studies have focused on developing recombinant M2e fusion constructs using a variety of carrier molecules or systems: HBV core particles [68–70], human papillomavirus L protein VLPs [71], phage Q β -derived VLPs [72], keyhole limpet hemocyanin [73], bacterial outer membrane complex [69,74], liposomes [75] and flagellin [76]. With the inclusion of diverse adjuvants, recombinant M2e-carrier vaccines were demonstrated to provide protection against lethal challenge with H1N1, H3N2 and H5N1 influenza A viruses. In particular, different forms of M2e vaccines fused to the HBV core VLPs were shown to induce high levels of anti-M2e antibody responses [61,68,70,77,78]. This might be due to the particulate nature of the carrier molecules. VLPs as M2e antigen carriers are likely to present M2e epitopes in an ordered array and in a particulate form, which enables a strong immune response as well as increasing their stability and immunogenicity [77,79]. However, M2e-mediated protection was relatively weak or partial compared with HA-mediated protection [64]. In addition, M2e-specific antibodies were not very effective in binding to the virus [64]. It is probable that chemical or

genetic fusion of M2e would not be likely to form the tetrameric structure of M2 in its native conformation.

A novel approach was pursued in an attempt to facilitate the formation and maintenance of tetrameric structure for improving the immunogenicity of M2e. Genetically linking M2e to the tetramer-forming leucine zipper domain of the yeast transcription factor *GCN4* was demonstrated to form recombinant tetrameric M2e vaccines [80]. The recombinant M2e–GCN4 vaccine induced potent M2e-specific antibody responses and 100% survival protection to the vaccinated mice [80].

An alternative approach is to express M2 proteins in a membrane-anchored form mimicking the native conformation on influenza virions. It is possible that the presence of the larger and more abundant HA proteins probably suppresses M2 from interacting efficiently with immune effector cells to induce antibodies (Figures 1 & 3). Thus, a full-length M2 was presented on influenza M1 VLPs (M2 VLPs) without HA and NA using the baculovirus insect-cell expression system [81]. Mice vaccinated with M2 VLP vaccines induced higher levels of M2e-specific antibodies compared with whole inactivated influenza virus vaccination [82]. In addition, M2e-specific antibodies induced by M2 VLP vaccination were highly cross-reactive to subtypes H1N1, H3N2 and H5N1 influenza viruses [81,82]. Mice immunized with M2 VLPs survived lethal infection with different subtypes of influenza A viruses [81]. Therefore, M2 proteins presented in a membrane-anchored form on VLPs can be a promising approach to avoid the immune-dominant HA proteins (Figure 3) and are effective in inducing M2 antibodies reactive to antigenically different influenza virions.

M2e-based immunity alone is infection-permissive and could not eliminate disease symptoms, as also shown by most other candidate universal vaccines studied so far in animal models. A desirable universal influenza vaccine should be able to diminish morbidity and mortality. A suggestion is to use such vaccines as adjunct to current vaccination. In this regard, it was shown that the use of M2e-based vaccines as a supplement could significantly improve the efficacy of cross-protection. The addition of the M2e peptide to an aluminum-adjunct split H3N2 virus vaccines significantly enhanced the cross-protection in mice by intraperitoneal vaccination [83]. Despite enhanced survival protection, significant morbidity as evidenced by weight loss was observed in mice that received M2e-peptide supplemented vaccines after heterosubtypic challenge infection [83]. By contrast, M2 expressed on VLPs in a membrane-anchored form was found to be highly effective in improving cross-protection when used in combination with inactivated whole viral vaccine in mice [82]. Mice that were intranasally immunized with a mixture of an inactivated virus and M2 VLP were protected from both mortality and morbidity. M2 VLP-supplemented inactivated influenza virus vaccine (A/PR/8/34, H1N1) conferred broad cross-protection to the immunized mice against lethal challenge with 2009 H1N1 pandemic virus, heterosubtypic H3N2 or H5N1 influenza viruses [82]. As a new approach to improve cross-protection, M2 VLP supplementation was extended to the seasonal influenza split vaccines as well as to the more commonly used route of intramuscular immunization (data not shown). Ultimately, clinical trials are required to validate this supplementation method as a potential universal vaccine for human use.

The protective immune mechanisms of the immune responses induced by M2 vaccination are not fully elucidated and further studies are needed. Protection by vaccination with M2e-hepatitis B core vaccine was found to be mediated by antibody-dependent, natural killer cell-mediated cytotoxicity [64]. Another M2e-carrier vaccine, C-terminal 28-kDa domain of *Mycobacterium tuberculosis* heat shock protein 70 (M2e-HSP70359–610), was shown to provide protection via alveolar macrophages and Fc receptor-dependent elimination of influenza A virus-infected cells [84]. It was demonstrated that protection by immune sera

after M2 VLP vaccination was dependent on the presence of dendritic and macrophage cells *in vivo* in a mouse model as shown by depletion experiments using clodronate liposomes [81,82]. Other studies suggest that M2 antibodies can restrict the growth of influenza viruses as shown by inhibiting the plaque size or replication of *in vitro* cultured viruses [53,55,60,85,86]. Therefore, multiple mechanisms are likely to be involved in conferring protection by M2 vaccination.

Some recombinant M2e vaccines have been tested in clinical trials. Sanofi Pasteur Biologics Co. has tested the safety and immunogenicity of a recombinant vaccine candidate, M2e-HBc fusion protein (ACAM-FLU-A), in a Phase I trial study [201]. The ACAM-FLU-A vaccine was reported to be immunogenic and well tolerated with no significant side effects [201]. M2e-HBc carrier protein vaccine also conferred partial protection to ferrets with 70% survival from infection by the highly lethal avian H5N1 influenza strain [201]. VaxInnate reported the first Phase I clinical trial study of an M2e-flagellin fusion vaccine (STF2.4xM2e) in healthy young volunteers aged 18–49 years [87,202]. Low doses of flagellin-M2 vaccines (0.3 and 1.0 μg doses) were safe and tolerated in subjects tested. Also, two doses of these vaccines were immunogenic in 75% after the first dose and 96% after the second dose [87,202]. However, two high doses (3 and 10 μg doses) of flagellin-M2 vaccines were associated with the appearance of influenza-like symptoms in some of the subjects. Therefore, toxicity seems to be an issue at higher doses of vaccines. Development of a safer vaccine based on M2 may be advantageous and VLP-based vaccines can be an attractive approach. Also, the flagellin-HA fusion protein vaccine was shown to be immunogenic in elderly vaccinees [88]. These clinical trials encourage further studies for developing safe and effective universal influenza vaccines.

NA-containing VLPs

The NA content in current vaccines is variable and the antibody response to NA in vaccinees is low (18% seroconversion) compared with the HA response (84% [89]). The host immune response to NA is poor in primed individuals and limited by current vaccination because of two factors: a lack of sufficient immunogenic NA in the vaccine and the dominant immunogenic response to HA [16,90,91]. Antigenic drift of NA was found to be slower than the drift in HA [92,93]. The independent and slower rate of NA evolution as well as less host immunity to NA might favor the more rapid emergence of HA escape mutants [16,92]. These considerations suggest that the current influenza vaccines are not effective in inducing antibodies against NA [91]. Inhibition of NA activity either by NA host immunity or by drugs can significantly reduce the morbidity and mortality caused by influenza. Thus, the NA protein has become an important target for antiviral drugs (oseltamivir, zanamivir). For preventive efficacy and lower treatment cost, a vaccine inducing protective immunity to the less variable antigenic target NA would be desirable in addition to the immunity to HA.

Previous studies of the role of NA in vaccination against influenza include use of purified NA proteins [91,94–97], DNA plasmid [98–102] and a variety of live virus-vectored vaccines expressing NA [103–107]. The use of live vectored vaccines may have some concerns about antivector immunity and vaccine safety, particularly in young infants, elderly and immune-compromised individuals. The efficacy of DNA vaccines is relatively low, and thus multiple immunizations are required. The preparation of soluble recombinant protein vaccines is laborious and may increase the vaccine cost. Thus, the development of more affordable and effective NA vaccines should be continued.

Like other enveloped viruses, influenza virus HA and NA are major viral glycoproteins incorporated into virions in a membrane-anchored form (Figure 3). Therefore, expressing NA on VLPs in a membrane-anchored form would mimic the native conformation of NA on

virions. Protective immunity of influenza VLPs containing both HA and NA was also reported [108–110]. However, NA-mediated protective immune responses by vaccination with influenza VLPs have not been well studied. Probably due to the immunodominant effects of HA in the same particles, the host immune response to NA was relatively weak, which made it difficult to investigate the protective roles of NA [16,109,110]. Thus, generation of VLPs containing NA without HA will provide a valuable vaccine modality in understanding the roles of NA in inducing protective immune responses (Figure 3).

Intranasal immunization of mice with influenza M1-derived VLPs containing NA from A/PR8 (H1N1) without HA effectively induced NA-specific immune responses [111]. The mice immunized with NA VLPs were found to have significant NA inhibition activity and to be reactive to homologous virus as well as heterosubtypic whole viral antigen (A/Philippines/82, H3N2 [111]). Importantly, NA VLPs conferred 100% protection after challenge with homologous A/PR/8/34 (H1N1) as well as heterosubtypic A/Philippines/82 (H3N2) virus, although body weight loss was observed after lethal challenge with the heterosubtypic H3N2 virus [111]. Thus, influenza VLP vaccines containing NA in a membrane-anchored conformation appear to be able to induce immunity to homologous as well as antigenically distinct influenza A virus strains.

NA-specific antibodies confer protection by a different mechanism from that of HA-specific antibodies induced by conventional vaccination, although the mechanisms of NA-immune mediated protection are not well understood. Antibodies reactive to the head domain of HA are able to neutralize and block the infection by influenza virus in an antigenically homologous or in a closely related strain-specific manner, capable of inducing sterilizing immunity, whereas NA antibodies can block the release of infectious virus from the surface of infected cells, causing virus aggregation, limiting viral spread and thus allowing the host to develop adaptive immunity [112,113]. Despite the fact that anti-NA sera do not prevent infection of cells *in vitro*, passive immunization reduces morbidity and mortality in mice after infection [113–115]. Immune responses to NA VLP vaccination were found to effectively control lung viral replication resulting in several thousand-fold lower titers, although they were not able to prevent the infection [111]. Less protection was observed when mice were treated with clodronate, indicating that protection by NA VLP immune sera was partially dependent on lung dendritic and macrophage cells [111]. By contrast, immune sera with high neutralizing antibodies from infected mice were completely protective independent of lung dendritic and macrophage cells [111].

Current influenza vaccines contain both HA and NA as major antigens. However, when both HA and NA are presented together in a viral particle, HA immune responses become dominant over NA in both B- and T-cell priming as a result of antigen competition (Figure 3) [16]. Therefore, a better vaccine strategy may include HA and NA as separate components to broaden the immune response against influenza. Immunity to the more slowly evolving NA should reduce the effect of antigenic changes in a potential emerging influenza virus strain. To avoid the immune-dominant effects of HA, it was shown that NA is sufficiently immunogenic when added as a separate component in a vaccine [17,97,116,117].

An alternative promising approach is to use purified NA as a supplement to standard influenza A vaccine. Chromatographically purified NA from influenza virus was used to supplement conventional monovalent-inactivated influenza A vaccine [94]. Supplementing the inactivated viral vaccine with purified NA protein induced high titers of antibodies to both HA and NA at equivalent levels for each antigen to those in animals immunized with either antigen alone, and resulted in greater reduction in viral replication following heterosubtypic challenge than the non-supplemented vaccine [94]. Alternative production

methods such as recombinant baculovirus or yeast expression systems could be explored to reduce current egg substrate-based production time and cost. Influenza HA VLPs were superior to soluble HA protein vaccine in the induction of binding and neutralizing antibodies, in levels of long-lived memory B cells and antibody-secreting plasma cells, in levels of IFN- γ secreting T cells and in clearing lung viral loads [118]. Therefore, presenting NA on VLPs in a membrane-anchored form for a supplemental vaccine to the HA-based current influenza vaccine is expected to be more effective in broadening and enhancing cross-protection compared with the soluble subunit NA vaccines.

Adjuvant-containing VLPs

Adjuvants are being used as a means of improving the immunogenicity of weak immunogens such as M2 vaccines, which is important for developing vaccines against potential pandemic influenza virus strains. Stimulating the innate immune system is important for inducing adaptive immune responses. Viruses and other pathogens can interact with innate immune cells through pathogen-specific molecular patterns that are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors, and thus are highly effective in inducing potent adaptive immunity. Binding of a PRR by its ligand or a pathogen triggers a signaling cascade inducing cytokines and chemokines. The resulting cytokine and chemokine milieu plays a significant role in developing adaptive immune responses [119–121].

APCs such as DCs or macrophage cells initially capture and process pathogen or vaccine-specific antigens, and present these antigens to T and B cells. The interaction of pathogen-associated molecular patterns (pathogen or vaccine antigens) with PRRs on the APCs stimulates their activation, resulting in the expression of costimulatory molecules (CD40, CD80/86) and cytokines. B cells have multiple roles in recognizing antigens and producing antigen-specific antibodies. B cells also express PRRs, and capture, process and present antigens to T cells. Some adjuvant molecules activate APCs and have a significant impact on enhancing T- and B-cell adaptive immune responses [122–126].

Replicating viruses are highly effective in inducing potent B- and T-cell responses, whereas many isolated recombinant proteins or T-cell epitopes are very poor in inducing immune responses unless given with strong adjuvants [127,128]. By contrast, the highly repetitive and particulate properties of VLPs are considered an attractive feature contributing to strong and long-lasting IgG responses in the absence of exogenous adjuvants [79,118, 129–131]. The highly organized form of antigens on VLPs induces strong B-cell responses [118,131]. In addition, VLPs are more effective for MHC class I and II pathway antigen presentation compared with a soluble antigen [13,118,132]. However, VLPs are less effective in inducing cytotoxic CD8 T-cell and helper CD4 T-cell responses as compared with replicating viral antigens because of their nonreplicating property [128]. In an attempt to enhance the T-cell immunogenicity, viral epitope-containing hepatitis B core VLPs that were conjugated to an adjuvant molecule (anti-CD40 antibody or CpG oligonucleotide) were found to effectively induce cytotoxic CD8 T-cell responses conferring protection *in vivo* in a mouse model [133,134]. Incorporating immunostimulatory molecules into VLPs would be an approach to enhance the immunogenicity of influenza VLPs, thus resulting in broader cross-protection.

VLPs with lipid envelopes need special attention in choosing an adjuvant owing to the nature of their membranes. Utilizing recombinant genetic engineering techniques, new approaches have been applied to express novel adjuvant molecules on VLPs in a membrane-anchored form (Figure 1). Chimeric HIV VLPs were generated to incorporate influenza HA into VLPs containing HIV envelope proteins, aiming to enhance mucosal immunity by using HA for binding to sialic acid present on epithelial cells on the mucosa [135,136]. Chimeric

simian human immunodeficiency virus (SHIV) VLPs containing HA were shown to have significantly increased their immunogenicity even in a mouse lacking CD4 T cells [135,136]. HIV VLPs containing membrane-anchored Flt3 ligand, a DC growth factor, were also generated to target VLPs to DCs and were shown to increase DC and monocyte/macrophage populations in the spleen when administered to mice [14]. GM-CSF is clinically used to increase the immunogenicity of various antigens, probably by stimulating APCs. It was found that the chimeric simian immunodeficiency virus (SIV) VLPs containing GM-CSF were more effective in inducing SIV-specific antibody and neutralizing activities compared with standard SIV VLPs with or without equivalent amounts of soluble GM-CSF [18]. CD40 ligand is expressed on the surfaces of activated T cells and has multiple immune functions, such as enhancing DC maturation and CD8 T-cell activity and stimulating antibody isotype switching and maturation. HIV or SIV VLPs containing CD40 ligand were demonstrated to enhance DC activation, cytokine-expressing T-cell responses and antibody titers at a moderate level [18,137]. These approaches have desirable advantages because the low level of immune-enhancing molecules incorporated into VLPs are effective in enhancing immune responses and thus side effects are unlikely [14,18,137].

Flagellin is the major proinflammatory protein component of Gram-negative bacterial organisms and is a potent immune stimulator by triggering Toll-like receptor 5 on host cells [138,139]. It was found that enhancing the immunogenicity of inactivated influenza vaccines by cholera toxin adjuvant could be an approach to broaden cross-protection [140]. Thus, to develop a more effective VLP vaccine inducing broad cross-protection, flagellin derived from *Salmonella* bacteria was engineered to be expressed on the cell surfaces of insect cells in a membrane-anchored form by linking the ectodomain of flagellin to the transmembrane-cytoplasmic tail domain of HA [20,21,141]. This membrane-anchored flagellin was incorporated into influenza HA VLPs (derived from A/PR8/34, H1N1 subtype), resulting in chimeric HA VLPs [20,141]. Intramuscular immunization of mice with the flagellin-containing VLPs elicited significantly enhanced levels of IgG2a/2b antibody responses, cytokine secreting T-cell responses in response to both MHC I and II peptides, and higher survival protection (67%) against heterosubtypic challenge (A/Philippines/82, H3N2 subtype [20]). In addition, mice intranasally immunized with HA (H1) VLPs incorporating flagellin were fully protected (100% survival) against H3N2 heterosubtypic challenge [141]. By contrast, use of soluble flagellin as an adjuvant was not as effective at improving the protective immunity compared with the chimeric HA VLPs with membrane-anchored flagellin, as determined by both systemic and mucosal immunization studies [20,141]. In a clinical study, a recombinant HA influenza-flagellin fusion vaccine (VAX125, STF2.HA1 SI) was demonstrated to be highly immunogenic, overcoming poor immune responses in the elderly (older than 65 years [88]). In addition, a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) was shown to be safe and immunogenic in healthy adults [87]. Recombinant flagellin-adjuvanted influenza vaccines could be a promising new candidate for the prevention of influenza A disease in both young adults and the elderly. The novel approach of anchoring an immunostimulatory molecule in VLPs can target low levels of a molecular adjuvant specifically to the VLP vaccines, and enhance the vaccine immunogenicity and cross-protection.

Expert commentary & five-year view

HA-based current influenza vaccination is not fully protective and needs to be improved. Several additional antigenic targets and new approaches are being developed. The HA stalk domain contains conserved regions that can be a target for developing universal influenza vaccines. The cleavage region of the N-terminal HA2 domain contains 11 highly conserved amino acids. Also, the long α -helix region of HA2 involved in viral fusion is highly conserved among different viral subtypes. Monoclonal antibodies were identified that

recognize an epitope in the conserved HA2 stalk domain and exhibit broadly neutralizing activity against different strains. However, the reactivity is HA-subtype group specific, and there is a limitation in its breadth. As an approach to develop an HA2 stalk domain-based vaccine, VLPs expressing the whole HA2 domain without the HA1 globular head are likely to be more promising than alternatives such as fusion peptide conjugate vaccines. It is desirable to develop a more immunogenic epitope design as well as scalable approaches to produce VLP vaccines expressing headless HA in a membrane-anchored form, likely to represent the native conformation. As a future direction, it is also suggested to develop insect-cell or other expression systems producing headless HA VLPs. Also, incorporating a molecular adjuvant into these headless HA VLPs will be an attractive method to enhance their immunogenicity.

M2e is a target extensively used to develop universal influenza vaccines using different platforms for presentation. Some candidates (M2e-HBc fusion protein, an M2e-flagellin fusion vaccine) are being tested in Phase I/II human clinical trials. M2 immunity is broad and highly cross-reactive among influenza A viruses when the M2e epitopes are in an immunogenic form such as presented in VLPs or conjugated to potent adjuvant molecules. However, as demonstrated in numerous efficacy studies in preclinical animal models, the strength of protection is relatively weak compared with HA-based immunity. This is evident since most animals immunized with M2 vaccine experience a certain level of morbidity as shown by significant weight loss and viral loads in the lungs. Therefore, studies to improve the efficacy of M2 vaccines should be continued. Designing multiple copies of M2e epitopes and presenting them on VLPs will be an attractive approach to present the membrane proteins in a native-like conformation, and incorporating adjuvant molecules in a membrane-anchored form as well as M2 into VLPs can enhance immunogenicity. An M2e vaccine can be used singly or can be added as a supplement to seasonal vaccines. In either approach, M2e vaccination is expected to reduce the risk of mortality when new pandemic outbreaks occur.

Current influenza vaccines contain some NA, although regulation of its content is not required and its efficacy in clinical studies remains unknown. Preclinical and clinical studies demonstrated that supplementary NA enhanced the immune responses to NA. Also, NA is under less immune pressure to mutate and thus has a slower rate of evolutionary change. New production methods for NA vaccines should be developed to reduce the cost. NA vaccines based on VLPs can provide a feasible method because the NA is presented in a membrane-anchored and immunogenic conformation, mimicking the native structure. Insect cell-derived NA vaccines were proven to be safe and immunogenic in clinical studies, and can be an attractive option for NA VLP production [111].

Most universal antigenic targets (HA2 stalk domain, M2, NA) are less immunogenic and induce relatively weak protection despite their greater breadth of cross-reactivity. Therefore, approaches to enhance their immunogenicity should be a priority for future studies. In this respect, new recombinant techniques are being utilized to develop new designs of vaccine epitopes and novel VLP vaccines. Universal antigenic targets can be engineered to present them on VLPs in a membrane-anchored form, making them highly immunogenic. In addition, molecular adjuvants such as flagellin conjugated to a conserved target (M2e) can be designed and produced in a VLP format [20,141,142]. Thus, VLPs have enormous potential for developing universal influenza vaccines, which can include several conserved targets in a particulate and immunogenic form as well as molecular adjuvants. In particular, such adjuvanted VLPs will be highly desirable since low concentrations of adjuvant molecules minimize the potential side reactions, but specifically target VLP vaccines to immune cells [14,18,20].

Approaches to combine multiple conserved antigenic targets into a vaccine will be important since these multiple targets are likely to reduce the chances of escape mutants once universal influenza vaccines are used in humans. Including T-cell epitopes in vaccines would be an advantage in broadening the cross-protection. Influenza NP is a relatively well-conserved target for inducing T-cell immunity but T-cell immunity alone would induce weak protection, whereas previous studies demonstrated that combining the NP component with M2 vaccine could provide enhanced crossprotection [73,143–146]. In addition, more attention will be needed to cover both human influenza A and B strains as well as potential avian-origin pandemic influenza viruses. M2e-like approaches including the conserved ectodomain of NB or BM2 could provide protection against multiple influenza B strains and should be further explored. Some divergence and deviation in the M2e sequence are present in the strains from swine- and avian-origin influenza viruses, and these need to be considered in designing M2e vaccines to cover potential pandemic strains. These conserved target epitopes could be delivered as separate vaccine antigens. Preclinical studies also need to be expanded to more relevant animal models. Ferrets are an important animal model for developing human influenza vaccines, and chickens and pigs as potential natural reservoirs need to be included in the efficacy tests of universal influenza vaccines. Such studies should be expanded because this is an essential part of developing universal influenza vaccines.

Acknowledgments

The authors would like to thank Erin-Joi Collins for her valuable assistance in preparing this manuscript.

This work was supported in part by NIH/NIAID grant AI0680003 (RW Compans), and NIH/NIAID grants AI081385 (SM Kang) and AI093772 (SM Kang).

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Key issues

- Continuous emergence of new strains cause influenza epidemics and pandemics, and require the development of effective universal influenza vaccines.
- Multiple conserved epitopes for inducing broadly cross-reactive antibodies have been identified and are being developed, which include the hemagglutinin stalk domain, M2 external domain and neuraminidase proteins.
- Virus-like particles (VLPs) presenting conserved epitopes in a membrane-anchored form are likely to be more immunogenic and display conserved epitopes in a virus-like conformation.
- Incorporating molecular adjuvants into VLPs is desirable to specifically target adjuvants to VLP vaccines at low concentrations.
- Use of influenza universal vaccines as stand-alone vaccines or as supplements needs to be considered and tested using preclinical and clinical studies.
- For the development of effective universal vaccines, their evaluation in animal models is essential.

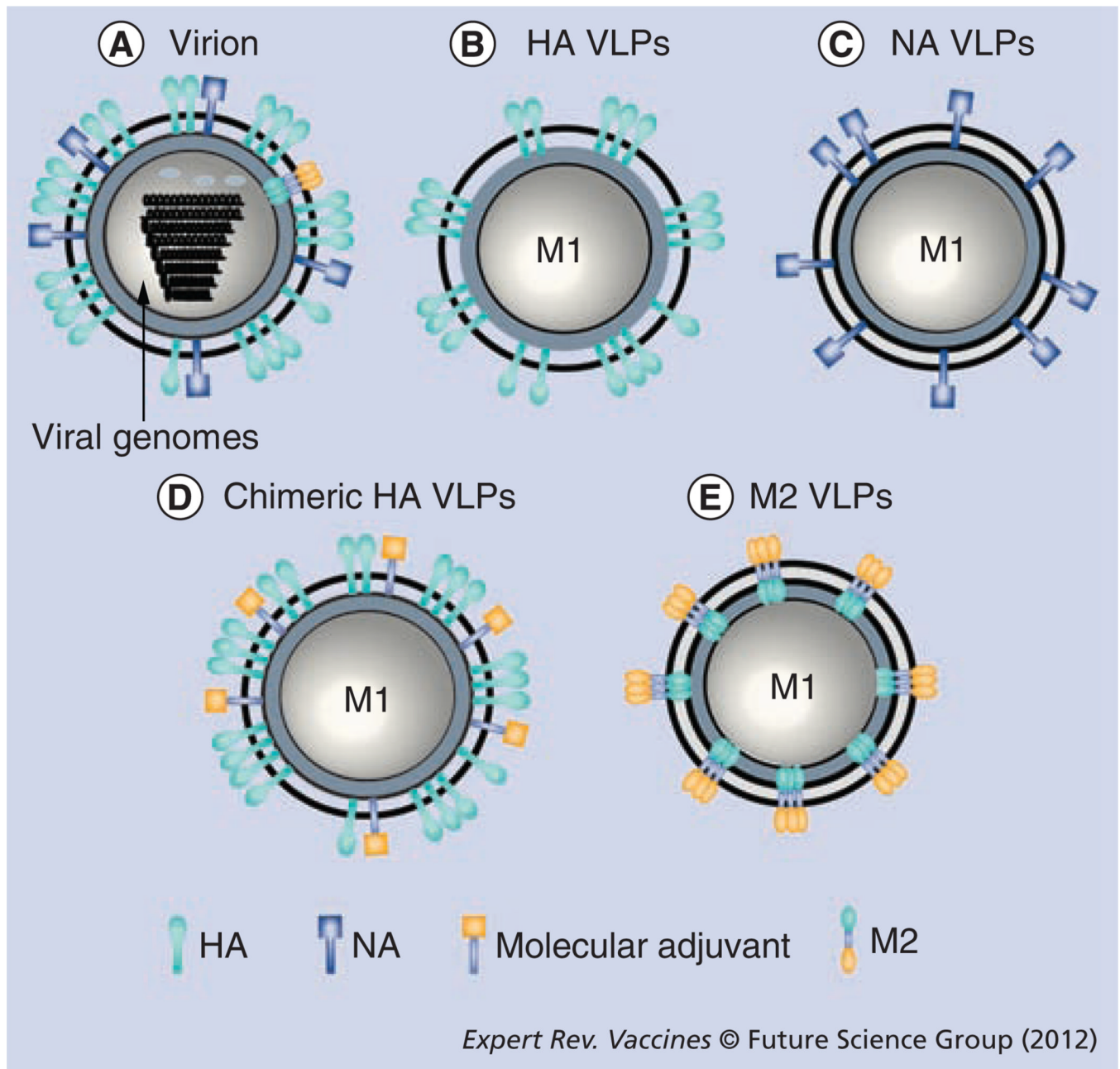


Figure 1. Schematic diagrams of influenza virions and virus-like particles

(A) Influenza virion showing surface proteins, HA, NA, ion channel protein M2 and viral nucleoproteins. (B) HA VLPs: influenza VLPs containing matrix protein M1 and HA. (C) NA VLPs: influenza VLPs containing matrix protein M1 and NA. (D) Chimeric HA VLPs: influenza VLPs containing matrix protein M1, HA and a molecular adjuvant such as GM-CSF, CD40 ligand or flagellin as immune-stimulating molecules. (E) M2 VLPs: influenza VLPs containing matrix proteins M1 and M2. The matrix protein (M1) is thought to be positioned along the inner surface of the membrane and stabilizes the particle [147]. HA: Hemagglutinin; NA: Neuraminidase; VLP: Virus-like particle.

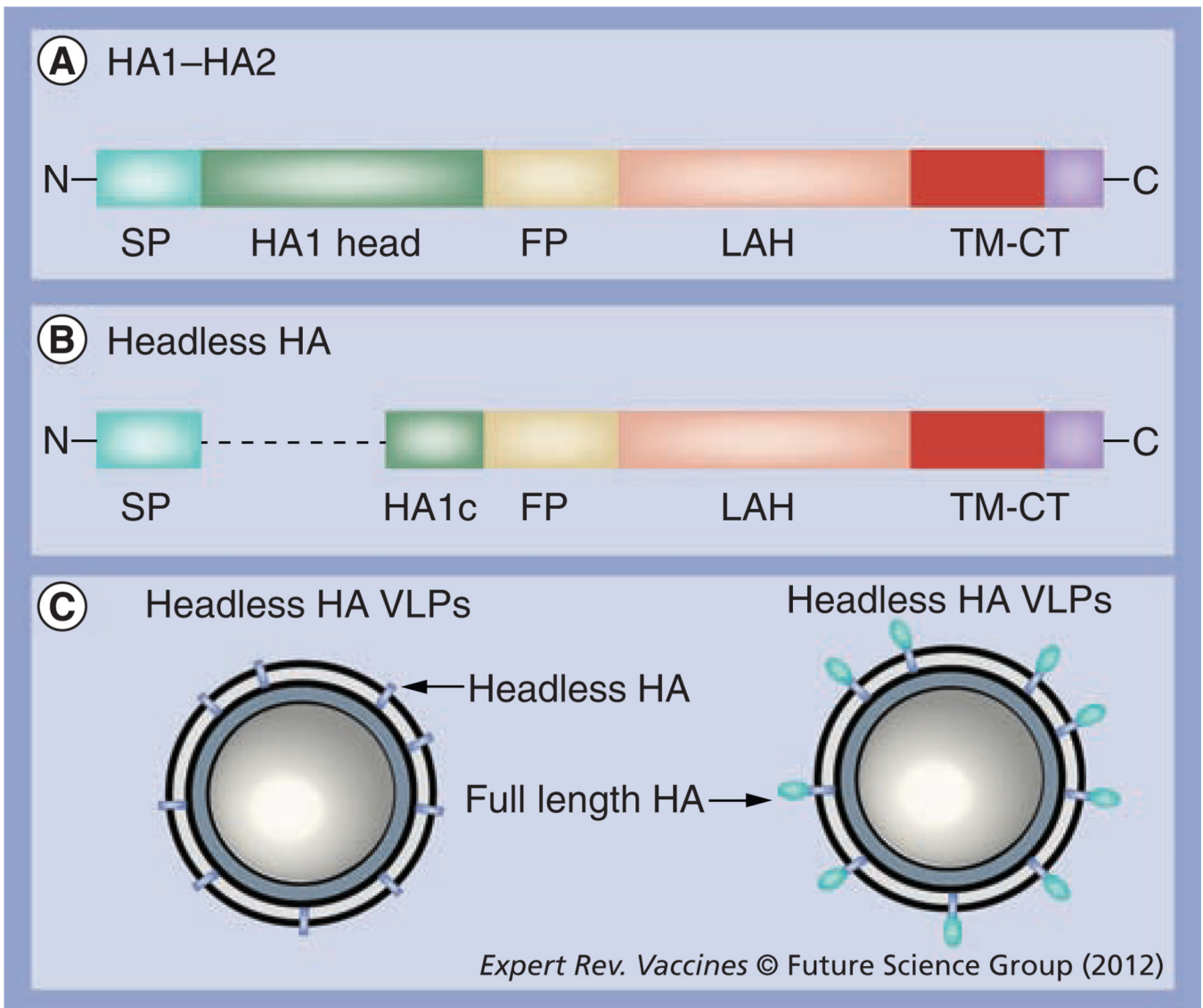


Figure 2. HA2 stalk domain and virus-like particles

(A) HA1–HA2 subunits: the HA1 subunit contains the globular head domain (HA1 head), a major antigenic target of current vaccination. The HA2 subunit contains the FP, the LAH domain and TM-CT. The FP domain includes 11 highly conserved amino acids in the N-terminal cleavage region and the presumed fusion peptide region of amino acids 1–38. The LAH region contains the amino acids 76–130, a region recognized by monoclonal antibody 12D1 [28]. (B) A diagram of a headless HA protein design reported by Steel *et al.* [42]. A SP and HA1 subunit C-terminal adjoining region (HA1c) with the globular head domain deleted are connected to the HA1 subunit for expression on the cell surfaces [42]. (C) A schematic diagram of headless HA-containing VLPs. FP: Fusion peptide; HA: Hemagglutinin; LAH: Long α -helix; SP: Signal peptide; TM-CT: Transmembrane-cytoplasmic tail; VLP: Virus-like particle.

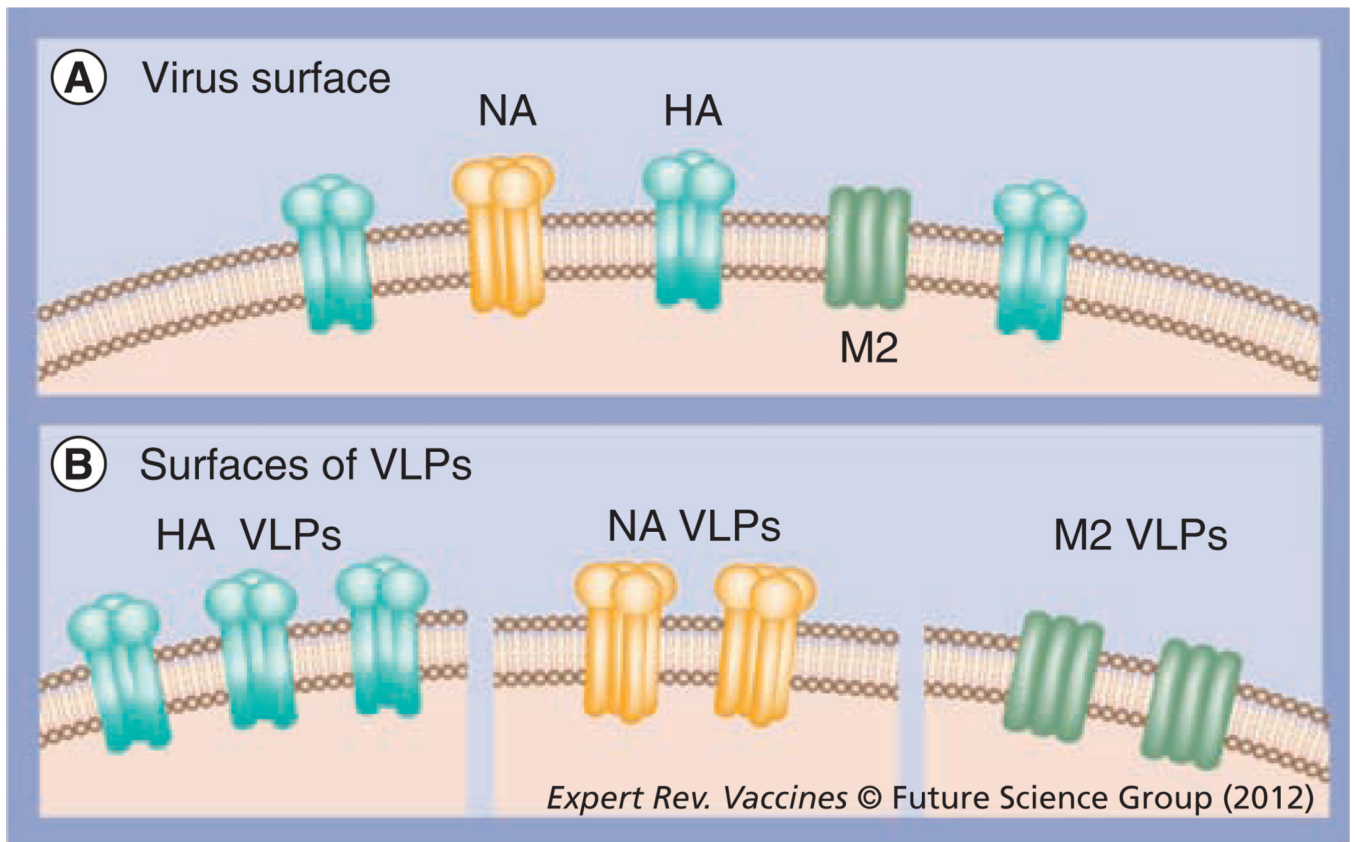


Figure 3. A model of influenza viral surface proteins and virus-like particles expressing influenza surface proteins separately

(A) The viral surface is represented with large trimeric globular HA, tetrameric NA and small tetrameric M2. (B) Influenza VLP surface expressing influenza HA, NA or M2 protein.

HA: Hemagglutinin; NA: Neuraminidase; VLP: Virus-like particle.