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Review

Engineered Nanoparticle Applications for Recombinant Influenza Vaccines

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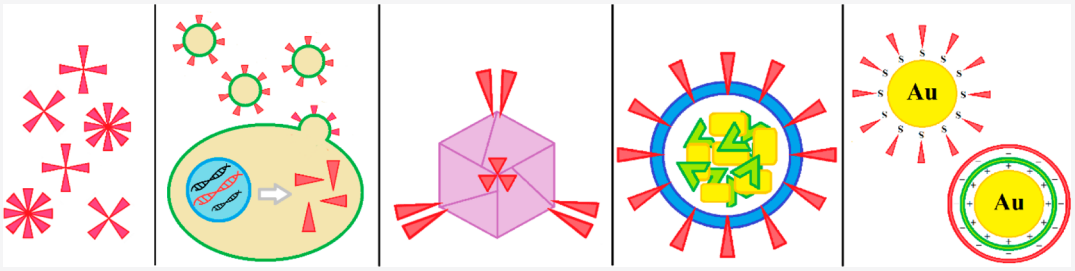

ABSTRACT: Influenza viruses cause seasonal epidemics and represent a pandemic risk. With current vaccine methods struggling to protect populations against emerging strains, there is a demand for a next-generation flu vaccine capable of providing broad protection. Recombinant biotechnology, combined with nanomedicine techniques, could address this demand by increasing immunogenicity and directing immune responses toward conserved antigenic targets on the virus. Various nanoparticle candidates have been tested for use in vaccines, including virus-like particles, protein and carbohydrate nanoconstructs, antigen-carrying lipid particles, and synthetic and inorganic particles modified for antigen presentation. These methods have yielded some promising results, including protection in animal models against antigenically distinct influenza strains, production of antibodies with broad reactivity, and activation of potent T cell responses. Based on the evidence of current research, it is feasible that the next generation of influenza vaccines will combine recombinant antigens with nanoparticle carriers.

KEYWORDS: *influenza, vaccine, nanoparticles, particles, antigens*

1. INTRODUCTION

Influenza vaccination poses an annual challenge in vaccine manufacturing and disease control. Despite the availability of seasonal influenza vaccines, the preliminary burden estimates by the CDC for influenza indicate 410 000−740 000 hospitalizations with 24 000−62 000 deaths resulting from this virus in the United States alone.[1] The greatest challenge to influenza prevention is the high rate of change in influenza epitopes, which allows the virus to escape immune recognition. Influenza virus strains are identified based on the serotyping of two critical surface proteins, hemagglutinin (HA) and neuraminidase (NA), which serve as immunogenic antigens for the host immune response.[2] Currently 18 HA and 11 NA subtypes have been identified, with new HA and NA subtypes discovered as recently as 2014.[3] The rapid evolution of influenza virus strains can be attributed to two mechanisms: antigenic drift and antigenic shift. Antigenic drift involves the gradual accumulation of small mutations to HA and NA, a process that is responsible for the emergence of strains that can infect pre-exposed populations each influenza season, driving the need for reformulation of influenza vaccines each year. Antigenic shift occurs when influenza viruses of different subtypes undergo genetic reassortment with each other, resulting in novel influenza strains for which there is little existing immunity in human populations, resulting in a pandemic risk.[2]

The current approach to producing seasonal influenza vaccines begins with global monitoring of influenza strains to select for virus samples projected to match prediction models for the prevailing strains of that season. Most modern vaccines are quadrivalent, containing influenza A virus strains for the H1N1 and H3N2 subtypes and two influenza B strains.[4] By the traditional method, the genes encoding the HA and NA of selected seed viruses are inserted into the genome of a strain capable of infecting embryonated chicken eggs to perform high-yield production of virus particles.[5,6] Harvested viral particles are used as whole inactivated virus particles (WIV vaccines), split with a detergent into fragments possessing a

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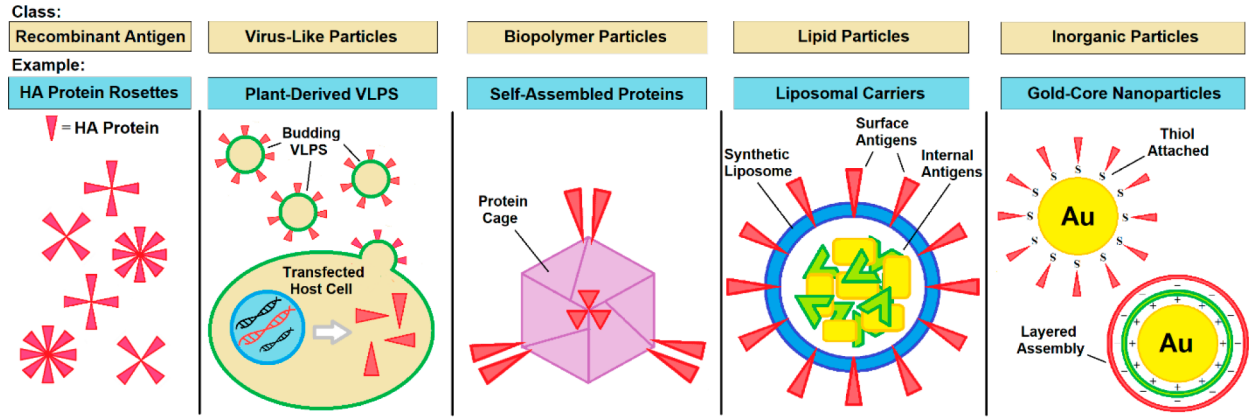

Figure 1. Various methods for producing nanoparticles with recombinant antigens. In solution, recombinant full-sequence hemagglutinin can form radial oligomeric particles termed "rosettes". Cellular hosts, including plant and insect cells, can be induced to produce virus-like particles (VLPs) through transfection with viral surface proteins. Antigen sequences can be formed into protein subunits, which naturally assemble into structures including protein cages, as protein is a type of biopolymer nanoparticle. Synthetic lipids can be used to form liposomes that encapsulate antigens and adjuvants on their surface or interior. Inorganic particles can serve as a core scaffold for the attachment of antigens; in this case, gold can be functionalized with surface antigens using either sulfur association or charge layering methods.

subset of virus proteins (split vaccines), or further purified into subunits of individual surface antigens (subunit vaccines).[6−10]

In the last 10 years, the overall efficacy of seasonal vaccines has ranged between 19 and 60% against circulating strains.[11] A significant reduction in vaccine efficacy results when an emergent strain fails to match the predictive models. Also, because the current methodology for influenza vaccine production relies upon modeling and isolation of existing seed viruses, it cannot reliably prepare for novel pandemic strains that could emerge. The National Institute for Allergy and Infectious Disease (NIAID) has set the criterion for a universal vaccine as one that can produce >75% efficacy against seasonal strains, as well as provide broad protection against influenza A in phylogenetic groups I and II, for at least 1 year.[12] In order to achieve these goals, a new vaccine paradigm must be created. The use of recombinant antigens could be the key to generating broadly protective immune responses by selecting for highly conserved proteins and epitopes, including those not typically targeted by existing vaccines.

Methods for producing influenza antigens using recombinant technology have gained traction based on potential advantages over traditional production methods. A significant benefit of recombinant techniques is bypassing the egg-based proliferation steps required for traditional vaccines. By removing the reliance on a limited supply of vaccine-quality eggs, shortages could be avoided, and vaccine manufacturers could achieve more flexible production to meet changing demands incurred by new virus strains.[13] Recombinant antigens also provide greater control over the antigens produced, avoiding unintended mutations that can occur in the proliferation process.[14] A study of the 2016−2017 seasonal vaccine found that egg-adapted H3N2 virus developed alternative glycosylation in HA, which diminished the efficacy of resulting antibodies against the circulating strain of the virus. When the researchers produced HA through baculovirus expression to exhibit the correct glycosylation motif, antibody recognition of the circulating strain was dramatically improved.[15] These advantages indicate a future for influenza vaccines that are diverging from existing egg-based methods.

In 2013, FluBlok became the first licensed recombinant influenza vaccine in the United States,[16] with a clinical trial during the 2014−2015 influenza season, reporting increased effectiveness over the traditional inactivated vaccine.[17] However, a general disadvantage to the recombinant method is the relative immunogenicity by antigen quantity; a greater concentration of the antigen or the addition of an adjuvant is required to yield protective immunity.[18] Various adjuvants have been tested in the formulation of pandemic influenza vaccines, including aluminum hydroxide (alum),[19] MF59,[20−22] and other squalene adjuvants,[23] some of which have obtained approval for use in certain markets. However, these adjuvants have generally been only modest for enhancing protective immunity against the influenza HA antigen in recombinant subunit vaccines.[23,24] Development of more robust immunopotentiators could overcome the pitfalls of existing vaccine adjuvants.

Numerous studies have been undertaken with the intent of determining a supporting system to enhance recombinant antigens with many promising results arising from applications of nanotechnology. Nanoparticles are an enticing tool for use in vaccines, capable of performing the dual duties of both delivery vehicle and immunostimulant adjuvant to vaccine antigens.[25,26] Recent studies indicate the potential capability of nanoscale particles with incorporated antigens to achieve immunogenicity comparable to virus-derived methods, as well as expand the breadth of protection. In this Review, various particle candidates for influenza vaccines, including those presented in Figure 1, are discussed, with an emphasis on how these methods could improve upon the production, administration, and breadth of protection provided by current vaccines.

2. PARTICLE ADVANTAGES

Vaccine efficacy is dependent upon the response induced in the B and T cells that compose the adaptive immune system. B cell activation determines the repertoire of antibodies produced in the influenza response, and it has been found that certain responses can result in antibodies that are broadly neutralizing. For example, the stem-binding antibody CR6261 has shown *in vivo* efficacy in protecting mice against pandemic H1, H2, and H5 viruses.[27,28] A method that could tailor the B cell response to a specific epitope could facilitate the

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Table 1. Representative Nanoparticle Vaccines with Efficacy in Preclinical Challenge Studies[a]

particle type	antigen target(s)	route	challenge strain	ref
HA-NA-M1 VLP	H3, H5, H9	IM, IN	H3N2, H5N1, H9N2	66
HA-M1 baculovirus expression VLP cocktail	H1, H3, H5, H7	IN	H1N1, H2N2, H5N1, H7N9, H11N9, chimeric H6, H7, H10	75
HA-trivalent VLP	H1−H3−HA B, H2−H5−H7	IM	H1N1, H2N3, H3N2, H5N1, H7N2, influenza type B	76
HA-NA-Gag VLP	H5, N1	IM	H5N1	79
NP-P22 VLP	nucleoprotein	IM	H1N1, H3N2	81
B10-M2e VLP	M2e	IM	H1N1, H3N2	82
plant-derived HA VLP (adj. Alum, GLA-SE)	H5, H7	IM	H5N1, H7N9	86
ferritin−HA stem	HA stem, H1 derived	IM	H1N1, H3N2, H5N1, H7N9	100
ferritin−HA mosaic	H1 (various strains)	IM	H1N1	99
self-adjuvanted flagellin−M2e	M2e	IM	H1N1	104
4MtG-hrHA double-layered particle	HA stem, H1, H3 derived	IM	H1N1, H3N2, H5N1, H7N9	110
liposome-encapsulated peptide	M2e, nucleoprotein, partial peptides	IN	H1N1, H1N2, H3N2, H5N1	121

[a]Administration routes: intramuscular (IM), intranasal (IN). Strain serotypes for which the particle was effective in preclinical animal model challenge studies are indicated.

production of antibodies with similar potential. Furthermore, T cell activation could provide another route by which broad immunity could be achieved. An investigation into pandemic H1N1 found that individuals with preexisting T cells to conserved influenza core protein epitopes exhibited significantly reduced symptoms.[29] Thus, a method that reinforces the delivery of antigens to T cells could provide a crucial component of broad multistrain protection. Nanoparticles offer a vehicle that could be used to achieve both enhanced activation and specific presentation of antigens to induce these favorable B and T cell responses. A diverse range of nanoparticles, with an array of administration routes, antigenic targets, and adjuvant properties, have been examined in preclinical studies for influenza vaccines, as can be seen in Table 1.

To achieve immunity, the antigen must be able to reach the immune cells. Nanoparticles can facilitate the delivery of antigens across biological barriers and into lymph nodes. Particles have been developed with the capacity to transfer material across mucus membranes,[30] which can be administered through the intranasal (IN) route to activate immunity in the respiratory system. Nanoparticles of virus-like size (20−200 nm in diameter) drain effectively into the lymphatic system to reach lymph nodes.[31] Once they reach targeted tissues, particle vaccines also can increase the uptake of antigen into immune cells such as resident macrophages and dendritic cells,[26,32,33] antigen-presenting cells (APCs) that play a crucial role in mediating T cell activation.[34] Thus, nanoparticles serve as vehicles by which recombinant antigens can be more effectively delivered to cellular targets.

The density of antigens that is presented on the surface of nanoparticles also contributes to B cell activation through B-cell receptor cross-linking. When a significant number of B-cell receptors (BCRs) are activated simultaneously in a localized region on the surface of the B cell, the recognition response is enhanced relative to random monovalent binding events between BCRs and individual soluble antigens (Figure 2).[35] For the same quantity of recombinant antigen, BCR cross-linking can result in higher B cell proliferation and antibody production. In addition, binding of multimeric factors such as IgM and complement proteins may be improved with particlized antigens, further aiding in APC uptake and B cell activation.[31] By this method, particulate vaccines can provide a structural advantage to antigen presentation.

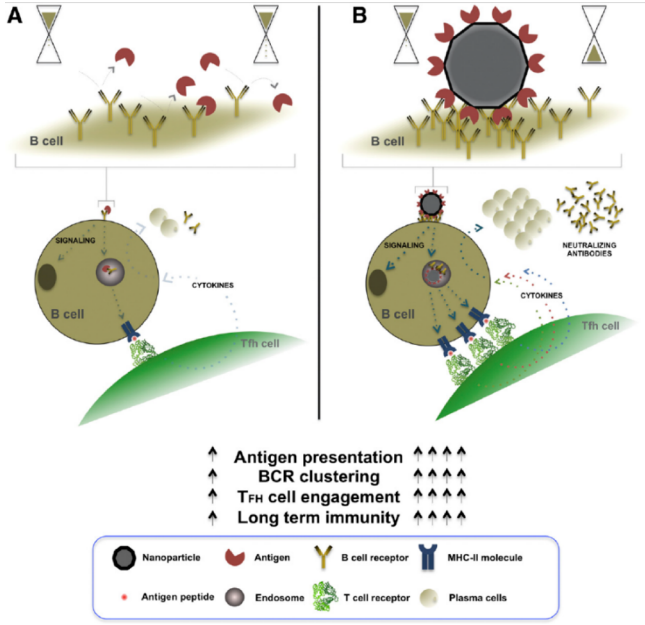

Figure 2. A comparison of soluble antigen binding against nanoparticle-bound antigen binding. The presentation of antigens on a particle results in a high concentration of localized receptor binding on the surface of B cells. Furthermore, while reversible binding limits the exposure time of receptors to soluble antigens, the multivalent binding of particles prolongs the duration of receptor activation to promote a stronger response. This response elicits both intracellular and intercellular signaling, increasing T_{FH} cell engagement, cytokine production, and production of antibodies, resulting in a superior long-term immunity. Reproduced with permission.[35]

Many nanoparticles are also capable of integrating immunostimulant adjuvants in their formulation, acting synergistically with other particle properties to further amplify the immune response. In liposomal vaccines, lipid adjuvants such as MPLA can be incorporated to the membrane and coparticlized with the antigens; this methodology has shown positive results in experimental cancer[25] and malaria vaccines.[33] Biopolymeric particles may act as a self-adjuvant, such as in the case of chitosan[36] or flagellin[37] particles. Even

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synthetic polymers such as PLGA[31] or inorganic materials like gold[38] can be functionalized to present additional immunopotentiators. Between benefits to delivery, uptake, and immune stimulation, nanoparticles may serve as the necessary component to achieve a greater number of viable antigenic targets for influenza vaccines.

3. INFLUENZA ANTIGENS

An ideal next-generation influenza vaccine with a broad application should target components of the virus that are unlikely to change between seasons and that are also present on novel strains. To that end, experimental influenza vaccines have examined various influenza proteins to determine a conserved target for antibodies that would allow binding across many disparate strains. While hemagglutinin is the basis of modern influenza vaccine evaluation as the most effective target for neutralizing antibodies, alternative antigens such as neuraminidase, matrix protein, and nucleoprotein have also been found to elicit some broadly effective immunity, which could contribute to the development of future vaccines.

3.1. Hemagglutinin. Hemagglutinin, abbreviated as HA, is a glycosylated protein that forms trimers on the viral envelope (Figure 3, trimer).[39] It is homo-oligomeric, with each subunit possessing an identical structure (Figure 3, monomer). In a viral infection, the HA protein binds to terminal sialic acids of host cell glycoproteins and glycolipids, facilitating the membrane fusion and entry of the virus into the cell.[2,40] Due to its crucial role in the initial infection stages of the virus, current seasonal vaccines undergo quality control based on the quantity of HA antigens provided by the formulation, and the neutralization of HA activity by host antibodies is considered indicative of a protective response. HA antibody response is commonly evaluated using a hemagglutination inhibition (HAI) assay. Due to the correlation between HAI performance and protection induced by a vaccine, HAI can be used as a surrogate to evaluate vaccine efficacy for initial licensure.[41] However, not all antibodies that can target or neutralize the HA protein yield results that can be measured in an HAI assay. For example, stem-targeted antibodies do not directly inhibit receptor binding, and so cannot be evaluated accurately with HAI, making it more difficult to evaluate correlates of protection.[27] In order to quantify broadly reactive antibody neutralization in future vaccines, new correlates of protection will need to be established.

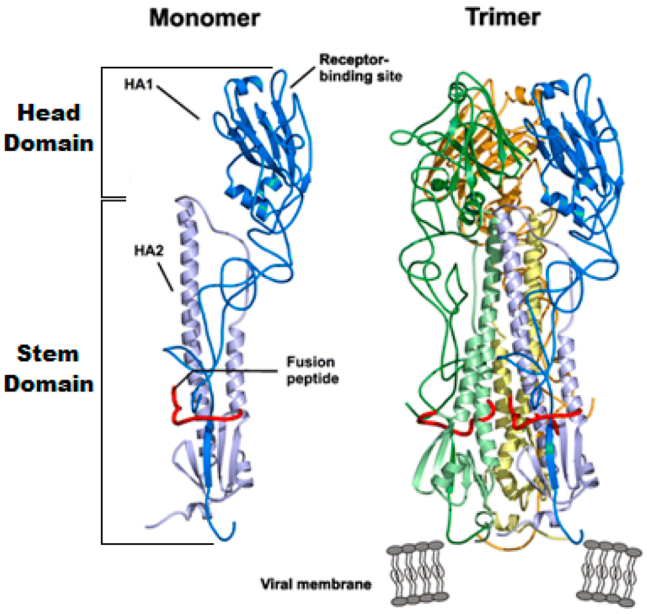

Figure 3. Ribbon diagram representing the three-dimensional structure of a hemagglutinin monomer and trimer. HA1 indicates the head domain, bearing the sialic acid receptor-binding site. HA2 indicates the stalk domain, possessing the fusion peptide, which facilitates cellular entry. Figure modified with permission.[42]

Recombinant production of HA poses a challenge for HA trimerization. Producing only a partial HA protein favoring the most antigenic epitopes is likely to exclude the sequence for the trimerization of the native protein, resulting in a non-native, monomeric form. However, there is research to suggest that the trimerization of HA plays a role in eliciting an effective immune response. Studies comparing recombinant HA produced with or without trimerizing domains found a significant increase in antibody and HAI titers against antigens that trimerize,[43] and the binding efficiency of stalk-targeting antibodies was also improved.[44] On the other hand, other studies have shown that HA from a variety of recombinant expression systems with varying oligomerization statuses is able to induce neutralizing antibodies; thus, trimerization may not be as critical for head domain antibodies as stalk ones.[45] Nevertheless, the polymerization of HA antigens is likely to be a contributing factor to the efficacy of the Flublok vaccine. When recombinant HA is produced as a full-sequence protein (including transmembrane and trimerization domain) as in the Flublok, nanoscale oligomers described as "rosettes" are observed. These oligomers have a characteristically uniform size and structure, containing 4−8 HA units arranged in a radial pattern.[45−47] Their formation can likely be attributed to the transmembrane sequence and trimerization property of HA, although, in the absence of a membrane, their binding tends to exceed 3 units. These particles may confer some of the benefits that are observed in nanoparticle vaccines.

3.2. Neuraminidase. Neuraminidase (NA) is a tetrameric surface protein, which facilitates the release of virus from infected cells by cleaving sialic acids and also assists in viral entry by cleaving through mucins.[48,49] Broadly protective antibodies for NA have been reported,[50−52] and it has been proposed that the combination of HA and NA in vaccines could contribute to a greater breadth of response. A study performed with coadministration of both recombinant H3 and N2 found that this vaccine could suppress viral replication in a distantly related strain of H3N2 to a greater degree than an inactivated virus vaccine. This was attributed to equal immunogenicity observed in dissociated HA and NA, whereas antigenic competition favors an HA-dominant response with WIVs.[53] Neuraminidase inhibition (NAI) assays have also been developed, which can serve a similar role to HAI,[53,54] although they are not currently a standard metric for evaluating influenza vaccines.

3.3. Matrix Proteins. Matrix proteins on the surface of influenza virions have been found to be highly conserved between strains yet are poorly immunogenic. It has been theorized that if the immunogenicity of the matrix proteins, particularly matrix protein 2 (M2) can be increased, the response could be broadly effective across many strains. M2 showed promise in animal models, where vaccination with recombinant M2 afforded protection in lethal challenge,[55] and antibodies against M2 could confer viral inhibition through passive transfer.[56] A recombinant ectodomain of M2, termed

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M2e, has been used as an antigen target in numerous nanoparticle vaccines. However, the practical application of M2-based vaccines faces certain challenges. The mechanism of protection underlying anti-M2e antibodies remains poorly understood, as the infection is not directly affected by these antibodies. As such, there does not exist an assay like HAI or NAI that can be used to evaluate M2 vaccines.[57] Another issue facing M2e applications is the observation of escape mutant strains emerging in mice treated with anti-M2e antibodies. Although the diversity of these mutants was limited, this reveals the potential for M2-based vaccines to lose effectiveness against future strains.[58] Because of the drawbacks, the future of M2e vaccines remains uncertain although research is still ongoing, and it is possible that M2e may contribute to future vaccines as a supplemental antigen to increase cross-protection.

3.4. Nucleoprotein. The highly conserved nucleoprotein of influenza is normally located in the interior of the virus particle, serving a critical role in viral RNA replication.[59] The inclusion of nucleoprotein in influenza vaccines poses certain notable risks. It was found that nucleoprotein artifacts in the influenza vaccine Pandemrix resulted in antibodies that cross-reacted to human HCRT receptor 2, resulting in the development of autoimmune narcolepsy in some patients who received the vaccine.[60] Despite this, nucleoprotein remains a candidate of interest for vaccines due to its potential to induce heterosubtypic immunity through the activation of cross-reactive T cells.[41] One of the most advanced nucleoprotein-targeting approaches in recent years was a viral-vectored vaccine produced by Vaccitech; MVA-NP+M1 was used to deliver sequences encoding for nucleoprotein, as well as matrix protein 1, in a modified Vaccinia Ankara virus to host cells to induce T cell responses to the antigens. This method completed Phase I clinical trials,[61] although it did not reach its Phase II clinical end point.[62] Further study into nucleoprotein vaccines could involve nanoparticles encapsulating a recombinant nucleoprotein, which may serve to facilitate cellular uptake while preventing undesired antibody production.

4. VIRUS-LIKE PARTICLES

Virus-like particles (VLPs) are self-assembling, but non-replicating structures composed of viral capsid proteins. By mimicking the structure of naturally occurring viruses (Figure 4), VLPs can achieve the enhanced antigenicity of attenuated virus vaccines while retaining the advantages of recombinant subunit vaccines. Furthermore, VLPs lack the genetic material required for replication competency, eliminating the risk of reversion into an infectious state that may occur in live-attenuated virus vaccines.[63] To produce influenza VLPs, a plasmid encoding influenza protein is introduced into a host cell, which produces the antigen, induces particle formation and budding in a manner similar to natural viral replication.

4.1. Influenza VLP Particles. Using recombinant baculoviral delivery to transfect Sf9 insect host cells, it is possible to generate synthetic influenza virus capsids capable of self-assembly and budding. Early influenza VLPs were produced through transfection with four structural proteins: HA, NA, and matrix proteins M1 and M2.[65] Further refinement of the VLP design removed M2,[66] then later M1 as well. While M1 was once considered a crucial factor for viral budding, motivating its inclusion in some experimental VLPs, further investigation revealed that budding can be induced by

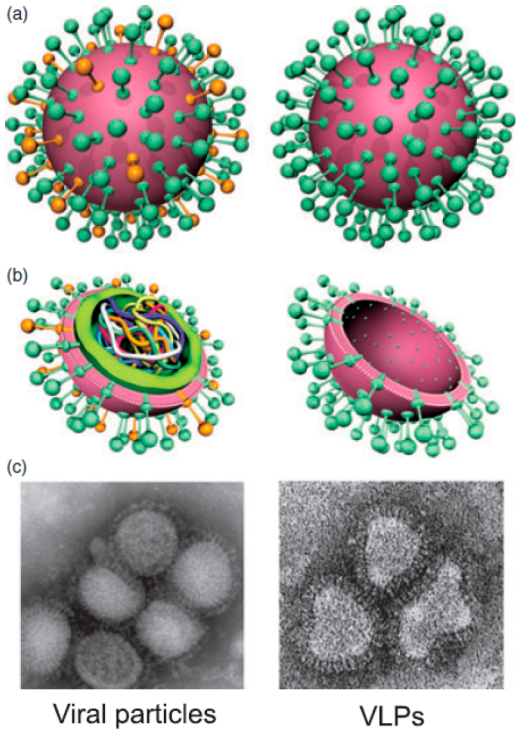

Figure 4. A structural comparison of influenza virus (left) and VLPs (right). (a) Native virus presenting both HA (green) and NA (orange) surface proteins; VLPs may be produced with both proteins or may be produced with HA presented only, as shown. (b) Unlike the virus, VLPs contain no internal proteins or nucleic acids. (c) When viewed with electron microscopy, VLPs bear a physical resemblance to the native virus. Reproduced with permission.[64]

the cytoplasmic tail domains of the HA and NA proteins,[67] possibly involving host cell proteins in assembly.[68] Currently, VLPs are one of the most advanced nanoparticles for influenza vaccines, with a recent phase III clinical performed by Novavax with the sf9-produced quadrivalent vaccine candidate NanoFlu achieving all primary end points.[69,70]

While influenza VLPs are a promising direction for seasonal vaccines, there is also interest in their application for pandemic strains for which there is little preexisting immunity in human populations. Avian influenza A/Hong Kong/1073/99 (H9N2) was used as a basis for a VLP vaccine, with coexpressed HA, NA, and M1 in Sf9 insect cells forming the VLPs for *in vivo* testing. Immunization of BALB/c mice with the VLPs prior to viral challenge reduced the weight loss associated with infection and reduced viral titer in lung and nasal tissues.[66] Further studies based on HA from A/Fujitan/411/2002 (H3N2) and H5N1 from multiple viral clades revealed that VLPs may provide additional benefits over existing recombinant or WIV methods. Mouse and ferret sera were tested against a panel of H3N2 strains isolated within 5 years of the Fujitan/2002 strain used, with the experimental vaccine inducing HAI inhibition titers above the predicted protective threshold (>1:40) for multiple strains, while equal doses of subunit HA or even WIV were relatively ineffective at inducing inhibition across the same breadth of strains. This advantage of VLPs over WIV may be due to the process of viral inactivation, which may alter the conformation of antigens away from their native forms, whereas VLPs generate proteins in their native states.[71] Further evidence of broad multistrain protection was observed when isolates from pandemic candidate H5N1 avian

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influenza from different evolutionary clades were incorporated into VLPs. Production of H5N1 vaccines is a critical consideration, as both clades of H5N1 have caused cases of lethal human infection and isolates have been identified with resistance to antiviral drugs.[72,73] When VLPs bearing HA and NA antigens from either clade 1 or clade 2 were used to vaccinate mice prior to lethal challenge, it was found that even mice vaccinated with VLPs bearing antigens of the heterologous clade were protected. Furthermore, the protective efficacy of the VLPs was enhanced by intranasal administration.[74] The broad protective range elicited by even single-antigen-presenting VLPs provides a basis for an approach toward "universal" influenza vaccines.

One method for generating a broadly protective vaccine is a VLP "cocktail", a single dose that includes multiple VLPs, each bearing a single distinct antigen. A cocktail of HA-M1 VLPs produced by baculovirus expression containing isolates from H1, H3, H5, and H7 strains has been tested in mice, where it was found to elicit protective antibodies against homologous, heterologous, and even heterosubtypic strains of influenza. Despite the lack of a representative HA in the vaccine formulation, the cocktail was also successful at protecting mice in a challenge against H2N1, H6N1, H10N1, and H11N1. These VLPs did not contain NA antigens, strongly indicating that the antibodies induced by the VLP cocktail recognized conserved regions on the HA protein.[75]

However, the antigenic expression of VLPs is not strictly limited to one subtype at a time. Various researchers have also produced multivalent VLPs, which express multiple influenza antigens on the surface of a single particle. Trivalent VLPs supporting HA antigens from seasonal H1, H3, and B strains have been formulated,[76,77] as well as VLPs trivalent for pandemic candidates H2, H5, and H7.[77] Both formulations resulted in a significant reduction of viral titer in ferret challenges against relevant influenza strains. The coexpression of multiple antigen subtypes also invites new challenges, however, because the expression of multiple genes on the same vector can decrease the stability of the plasmid and lead to faults in gene expression. While these trivalent formulations could be successfully produced for research purposes, techniques would need to be developed to allow greater valence capacity and ensure consistent quality at high scales. To address this, a method was developed by which a stable recombinant cell culture could be further transfected to increase valence in a modular fashion, resulting in pentavalent particles. In addition, the negative effects of high cell density on HA production could be mitigated with a novel culturing method, resulting in a scalable strategy for large-scale production of multivalent influenza VLPs.[78] A remaining challenge for the development of multivalent VLPs is accurately quantifying the quantify of each antigen present in the vaccine. When coexpressing HAs on a single VLP, it is a substantial challenge to measure the individual HA quantities.

4.2. Alternative Capsid Proteins. Based on the complexity of native influenza viral assembly and budding, there has been an increased interest in developing a more structurally controlled platform for VLP production, one which could serve as a robust basis for a range of viral antigens. To this end, influenza HA and NA antigens have been combined with structural proteins from noninfluenza virions to generate VLPs with a heterologous core structure and antigen presentation, known as a "pseudotype" VLP. An early influenza-pseudotype VLP was constructed based on the murine leukemia virus Gag protein, leveraging the role of Gag as a budding engine and its formation of lipid raft domains on infected cell membranes, which serves as a crucial step in the process of recruiting viral HA and NA proteins to the forming particles. While the particles physically resembled gamma retroviruses rather than influenza, they were demonstrated to yield protection in ferrets challenged with a highly pathogenic H5N1 strain, regardless of whether the VLPs presented antigens from the homologous strain or a heterologous H5N1.[79]

The combination of influenza and nonflu proteins also opens the path for novel vaccine approaches with alternative antigens. Attenuated vaccines can elicit strong $CD8^+$ T cell responses,[80] and so it has been proposed that the delivery of a nucleoprotein achieved with cargo-carrying VLPs could likewise yield a similar response. To investigate this, a bacteriophage P22 protein cage VLP was produced with an interior-localized nucleoprotein segment attached through genetic fusion to scaffold proteins. Once the particles were uptaken by APCs and processed, the nucleoprotein could then be expressed on MHC I on the cell surface, prompting the activation of $CD8^+$ T cells. When mice were treated with this VLP, a significant increase in nucleoprotein MHC I was observed in lung fluid obtained by bronchoalveolar lavage (BAL). Furthermore, while the nucleoprotein VLPs did not reduce the severity of weight loss due to illness, the VLPs did dramatically increase the survival rate in mice subjected to two consecutive challenges, first with 100 times the lethal dose of H1N1 influenza (PR8) and then with 50 times the lethal dose of an H3N2 strain (X-31). Thus, it was concluded that the nucleoprotein-targeted response contributed to protection against both seasonal influenza serotypes.[81]

A pseudotype VLP may also be effective in producing responses against conserved matrix M2e. The bacteriophage T7 capsid was modified to display M2e through the production of a chimeric B10-M2e recombinant structural protein. The resultant VLP was able to elicit both IgG antibody and IFN-γ-secreting cells specific to M2e, which corresponded with protective immunity against both H1N1 PR8 and H3N2 X47 virus, although a 100% survival rate was only achieved with the inclusion of Freund's adjuvant.[82]

4.3. Plant-Derived Particles. The discovery that HA alone can drive VLP budding has additionally opened a new pathway for influenza VLP generation through the transfection of plant cells. Plant-derived VLPs have been produced for numerous viruses, including Hepatitis B, HPV, and HIV.[83] In the case of influenza, plant cell VLP production is particularly appealing because plant-derived VLPs do not require the involvement of other capsid structural proteins to form. Plant cells do not synthesize sialic acids, which eliminates the requirement of NA to facilitate VLP budding, effectively allowing influenza VLPs to be generated through transfection with only the HA sequence.[64] Quadrivalent formulations developed by Medicago for seasonal influenza have reached phase III clinical trials investigating the efficacy and safety of plant-derived VLPs in adults[84] and elderly subjects.[85]

Plant cell production of influenza VLPs is especially appealing for pandemic vaccines due to the ability to produce a high yield of particles, allowing a rapid response to outbreaks. Plant-derived VLPs based on H5 from avian H5N1 influenza have been tested in preclinical and clinical trials.[86] In ferrets, H5 VLPs provided protection against a lethal viral challenge as well as detectable, cross-clade reactive antibodies that could exceed the protective threshold in HAI assay at sufficient

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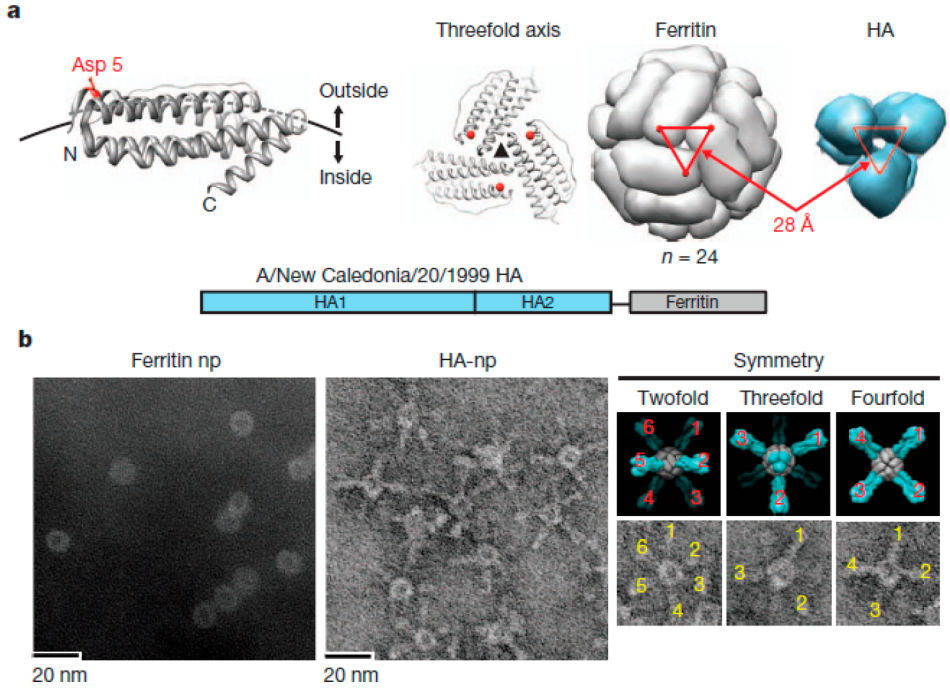

Figure 5. Design of ferritin nanoparticles for influenza antigen presentation. Subunits of ferritin form 3-fold axial symmetry, which allows HA trimers to form at the axis. Assembled ferritin particles are octahedral and have an HA trimer valence of 6, which is valuable for the characterization and quantification of HA for influenza vaccines. Reproduced with permission.[97]

doses. In a randomized, double-blind Phase I clinical trial, H5 VLPs showed no significant reactogenicity, and antibody titers were detectable by HI, MN, and SRH assays. By the EU Committee for Medicinal Products for Human Use (CHMP) guidelines, the criteria for seroconversion and GMI were met by HAI, and additionally, the criteria for seroconversion was met by single-radial hemolysis (SRH). A Phase II clinical trial was performed with attention to both HAI and the humoral and cell-mediated response. When administered with the adjuvant GLA-SE, low human-relevant doses (3.75−7.5 μg) were not only able to meet the licensure criteria for HAI but also induced polyfunctional and cross-reactive $CD4^+$ T cell response that was sustained in subjects up to 6 months following the vaccination course.[87] In an additional Phase II clinical trial, a moderate (30 μg) dose without adjuvant was found to be safely tolerated and immunogenic in subjects age 18−49 as well as over 50.[88]

A benefit of VLPs is the rapid turnaround time from antigen identification to a functional vaccine, presenting a viable solution to emergent pandemics. After the first reported human infection from avian H7N9 influenza in 2013,[89] plant-derived VLPs expressing H7 were successfully generated within 5 months and showed positive results in preclinical challenge studies in both mice and ferrets.[90] On the other hand, while appealing as a method for rapid and scalable vaccine production, plant-based VLPs have also been demonstrated to possess certain limitations that must be addressed to create a practical vaccine. These particles do not have strong self-adjuvant properties, and experimentally tested VLPs with H5 and H7 have needed an additional adjuvant such as alhydrogel (alum) or GLA-SE to achieve ideal efficacy results. Another uncertainty facing VLPs is the question of purity. Surface proteins from the producing host have been observed in the VLP membranes, and there is evidence that this protein impurity may result in reduced vaccine efficacy.[91] Encapsulation of internal components from host cells have also been found in the lumen.[68] Methods have been developed to further purify VLPs;[91,92] however, current methods for quality testing these particles are limiting to practical manufacturing, requiring study into the development of new testing techniques.[93]

5. BIOPOLYMER PARTICLES

A virus-like particle is defined by its mimicry of naturally occurring viral capsids; however, it is also possible to induce immune activation with a protein that is not of viral origin. Among naturally occurring proteins are ferritin, heat shock protein, and enzyme complexes capable of spontaneously forming protein cage structures.[94] The self-assembling protein nanoparticles that result from these interactions can serve as antigen carriers that present immunogenic material to cell receptors in a manner similar to viruses, yet with properties that can allow greater control or immunogenicity. It is also possible to modify immunostimulatory proteins through recombinant methods to produce polymeric structures. Some proteins, while not capable of spontaneous assembly, can be used to form capsulates bearing target antigens. While a broad range of polymers has been explored for influenza vaccines, in this section, we outline protein and chitosan particles as examples of applications with recombinant antigens.

5.1. Self-Assembling Protein Nanoparticles. While early ferritin-based particles were designed for presenting HIV antigens,[95] ferritin has been of interest for influenza applications for a number of reasons. Ferritin is naturally produced in many organisms and, in humans, serves the function of regulating ferrous ion availability,[96] indicating a likelihood for tolerance in the body with low reactogenicity. Furthermore, recombinant ferritins produced in prokaryotic cells are not subject to post-translational modification, allowing greater control over production.[95,97] Most crucially, however, is the fact that, when HA is attached at the N-terminus of the

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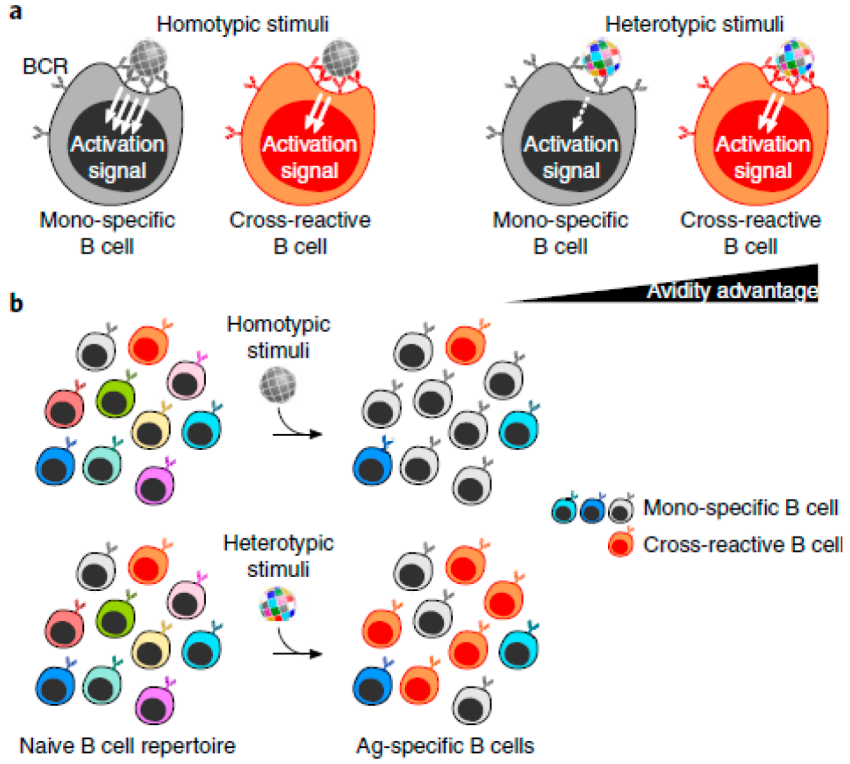

Figure 6. Induction of cross-reactive immunity with multivalent nanoparticles. Utilizing the principles discussed in Figure 3, it is predicted that multivalent "mosaic" particles will preferentially activate B cells with cross-reactive receptors, thus initiating an adaptive immune response favoring cross-reactive B cell proliferation and antibody production. Reproduced with permission.[99]

ferritin, the geometry of the formed particle gives rise to an HA trimer (Figure 5a), replicating the presentation of the antigen on the native virus. This structural similarity has been confirmed through the study of the reactivity of the HA−ferritin particles with stem-directed monoclonal antibodies.[97] The specific geometry of ferritin particles (Figure 5b) allows for optimized antigen spacing; based on an optimal antigen spacing of 50−100 Å for B-cell activation,[98] a 24-meric HA−ferritin particle achieves the optimal spacing between any two neighboring HA trimers.[99] Given these advantageous properties, ferritin NPs have served as a candidate for HA-targeted vaccines.

Unlike VLPs, ferritin particles do not rely on a budding process for release, which may allow greater flexibility in modifying recombinant HA antigens. The conserved stalk is a sought-after vaccine target; however, stalk antibodies generally do not inhibit hemagglutination or viral entry, and it has proven difficult to induce potent stalk antibodies. One proposed method for achieving a broadly reactive immune response to influenza is to target the stalk region of the HA by removing the globular head domain from recombinant designs. This method has met with some success in protecting against both phylogenetic group 1 including H1[100] and group 2 influenza strains including H3 and H7 subtypes.[28,101] Subsequently, an H1 stabilized stem (HA-SS) antigen was applied to ferritin nanoparticles (H1-SS-np) and evaluated for protection against a group 1 heterosubtypic strain of H5N1. In mice and ferrets, the H1-SS-np was capable of providing protection against a lethal challenge, both in cases of direct vaccination and passive serum transfer.[102]

Recently, ferritin particles have been investigated as a multivalent platform for the induction of cross-reactive immune responses. The method takes advantage of the theory that colocalized receptor activation on the B cell surface strengthens the response to the particlized antigen;[35] by presenting a "mosaic" of a heterologous antigen across the particle, the activation of cross-reactive B cells may be promoted over monospecific B cells (Figure 6). When introduced to mice, the antigen mosaic particles generated mean HAI titers that were higher than admixtures of the same array of antigens in monovalent particles, and titers diminished less as valence increased, indicating that multisubtypic antibodies could achieve significant neutralization despite lower specificity. Furthermore, the researchers were able to isolate an antibody, 441D6, which was found to be broadly neutralizing against H1N1 subtypes, validating the method as a means to generate cross-reactive antibodies against a diverse subtype of influenza.[99]

Flagellin-based self-assembling particles have also been under investigation as a method for facilitating a reaction to the M2e influenza matrix protein. Bacterial flagellin derived from pathogenic *Salmonella typhimurium* is a TLR5 agonist, making it an effective immunopotentiator.[103] Furthermore, this TLR5 agonist activates a specific pathogen-associated molecular pattern (PAMP), which has been associated with an increased response to M2e. In the challenge, flagellin with attached M2e achieved markedly better survival rates and clinical scores than coadministration of equimolar flagellin and M2e, indicating an advantage afforded by coparticlization.[104] Flagellin−M2e complexes were determined to be well-tolerated in a double-blind clinical study.[105] Flagellin was also tested as an incorporated adjuvant to VLPs, which increased overall IgG and HI titers by approximately 2-fold over the VLPs compared, and allowed mice to survive a

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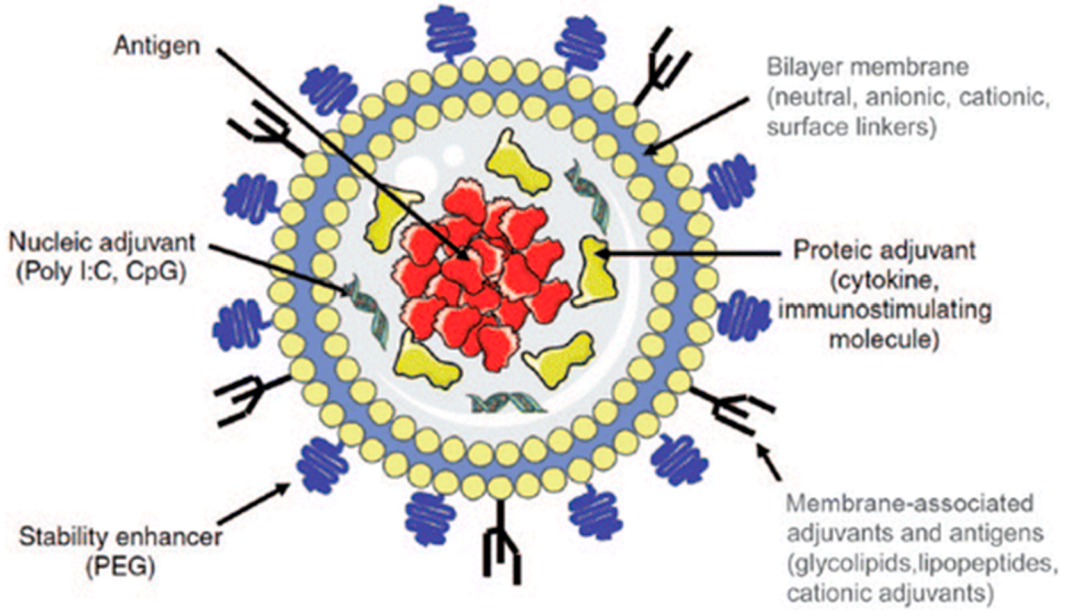

Figure 7. Design schematic for liposomal vaccine nanoparticles. Both the lipid membrane and interior lumen can act as vehicles for antigen and adjuvant transport. This allows B cell activation with membrane-associated surface antigens such as HA, while also facilitating cellular response to conserved internal antigens such as nucleoprotein. Lipid adjuvants, such as monophosphoryl lipid A, can be presented in the liposomal membrane, while cytokines can be delivered to the intracellular compartment. Reproduced with permission.[120]

heterosubtypic lethal challenge.[106] In another study, the N-terminus of the flagellin sequence was joined with a tetramerizing sequence presenting M2e, and researchers constructed a two-subunit nanoparticle that presented both the conserved antigen and the TLR5 agonist. A broad cross-neutralizing response was observed, with antibodies reacting with 7 strains of influenza which between them presented 5 different subtypes of HA and 7 different subtypes of NA.[37]

5.2. Protein Capsulates. While self-assembly is a useful trait in particle design, proteins that cannot undergo self-assembly are not necessarily discounted from use in nanoparticles. Protamine, a protein family with clinical applications that have been approved by the FDA, has been investigated as a carrier vehicle for drug delivery and vaccine antigens.[107] It may act as a TLR7 agonist as well as provide synergistic adjuvanticity when combined with oligonucleotides or polysaccharides.[108,109] While still in early stages of the investigation, protamine nanocapsules containing viral antigens were found to effectively deliver the antigen to macrophages and promote immune response without significant toxicity. Furthermore, capsulates could be converted into a freeze-dried powder form, which could provide a valuable method for transporting and storing future vaccines for mass distribution.[107]

Recently, another protein nanoparticle candidate was investigated for application in a broadly cross-reactive vaccine. This double-layered particle was constructed from substantially modified influenza components; 4MtG, a stabilized tetramer composed of M2e tandem copies, and head-removed HA (hrHA) trimers. The 4MtG tetramers formed the core of the particle, with cross-linking of hrHA forming the surface layer. Particles were formulated bearing hrHA derived from H1, H3, or with both types. In a lethal challenge, it was found that complete protection could be conferred when a particle bore a clade-matched hrHA to the challenge strain, while even cross-clade vaccination yielded a significant increase to survival rates. Particles bearing both hrHA types were effective against both clades. Furthermore, challenge studies demonstrated vaccine effectiveness against both seasonal H1N1 and H3N2 as well as pandemic candidate H5N1 and H7N9 strains. These results indicate that a protein construct derived entirely from recombinant influenza peptides may serve as an effective multistrain vaccine.[110]

5.3. Chitosan Particles. In addition to proteins, carbohydrates can also serve as the basis for the creation of immunostimulatory, antigen-carrying nanoparticles. Chitosan, a naturally occurring polysaccharide, has been considered a promising material for antigen delivery due to its ability to stimulate uptake by mucosal tissue, such as the lining of the nasal passages.[111,112] Where existing vaccines are delivered by an intramuscular injection route (IM), mucosal uptake would allow for vaccines designed to be administered through the nose or mouth, the intranasal (IN) and sublingual (SL) routes. Chitosan has been applied in conjunction with traditional virion-derived influenza vaccines, where it was found to enhance both local and humoral response to the virus as measured by IgA, IgG, and IgM antibody quantification as well as HAI titers.[111,113−115] Chitosan also possesses immunostimulatory effects. Studies have reported formulation-dependent promotion of cytokine stimulation,[112] and trimethyl chitosan (TMC) was found to increase the immunogenicity of the WIV influenza vaccine when coadministered intranasally.[36] With chitosan potentially enhancing current influenza vaccine techniques, its potential for application with recombinant vaccines should also be considered.

While free antigen solutions may be able to induce protective immunity when delivered IM, mucosal and epithelial barriers within the nasal passages render IN delivery of recombinant antigen impossible without a vehicle to facilitate absorption. To that end, recombinant HA was encapsulated in biodegradable poly-(ε-caprolactone) (PCL) and then coated with chitosan. The theory behind this design

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stated that the chitosan would provide the function of transmission across the mucus membrane, after which the PCL would provide sustained release of HA over numerous days. As anticipated, vaccination in mice showed a significant increase in systemic HAI and IgG titers and a local increase in secretory IgA. However, the particles also had a substantial effect on the cell-mediated response, resulting in highly significant frequencies of IFN-γ and IL-4 producing spleen cells. This effect was more pronounced when chitosan was delivered IN, which could suggest that IN vaccination with chitosan could increase the cross-protective immunity of the vaccine.[116]

6. LIPID PARTICLES

Amphipathic phospholipids are the primary component of cell membranes due to their ability to self-assemble into bilayer structures. The influenza virus uses cell-derived lipid membranes as an outer envelope, so it is natural to assume that nanoparticles based on amphipathic lipids could be used as artificial transport vehicles for influenza antigens. Whereas lipid emulsions act independently from soluble antigens as an immunostimulant, these lipid particles can incorporate antigens as surface targets for a humoral response or as internal cargo for delivery into antigen-presenting cells. In addition, lipid particles can be designed to incorporate lipid adjuvants, allowing the immunostimulatory properties of the particles to be enhanced and modified. Current particles of this type include liposomes and liposome-derived virosomes.

6.1. Liposomes. Liposomes are composed of a phospholipid bilayer, and their capacity to carry bioactive molecules in the interior lumen and on the exterior surface layer has made them a candidate for the delivery of vaccine antigens. For over four decades, liposomes have been studied as immunogenic carriers, with properties such as size, surface charge, and membrane fluidity being characterized and optimized for immunogenicity. Cationic liposome designs such as VaxiSome, Vaxfectin, and CAF01 have been tested in clinical trials for influenza vaccines.[117] Liposomes can be designed to incorporate a wide array of biomolecules including peptides, lipids, and nucleic acids, which can serve as either antigens or adjuvants (Figure 7). For example, liposomes can encapsulate DNA for the M1 gene of influenza, resulting in expression and a strong immune response in mice, while complexation with the plasmid DNA adjuvant CLDC with liposomes resulted in increased protection against a split H5N1 vaccine.[118] The functional range of liposomes can also be modified. When chitosan was incorporated into the membrane, researchers were able to access the sublingual delivery route with a coadministered split vaccine.[119] While liposomal vaccines are highly versatile in their applications, studies involving recombinant influenza antigens are especially important for the design of future vaccines.

One proposed liposomal vaccine for influenza tested the encapsulation of recombinant M2 and nucleoprotein from H5N1 Such a vaccine was capable of significantly reducing the lung viral load and improving lung histopathology in mice, in addition to increasing survival rates.[121] Another encapsulation method applied 10 highly conserved peptide sequences, including M2e, in an IN-administration to a swine model with a challenge against swine H1N1. Results suggested increased HAI titers not only against the challenge strain but also heterologous H1N2 and heterosubtypic H3N2.[122] It has also been shown that, when adsorbed to the surface of cationic liposomes, the immunogenicity of purified subunit HA can be increased.[123] Future liposomes may develop new techniques for the surface presentation of antigens, including the use of histidine tags to associate peptides to liposomal surfaces.[33,124,125] Tandem delivery of surface and encapsulated influenza antigens in a single particle may also be possible.

6.2. Virosomes. Virosomes bear similarities to liposomal and VLP vaccines, although their distinct method of production sets them apart from the particles previously discussed. Virosomes are produced when the lipid membranes of whole influenza virus capsids are disrupted, then reconstituted such that they maintain their original surface antigens but exclude the internal components required for replication. This process is flexible in that it allows for the integration of synthetic lipids and adjuvants to the membrane, and potential for the encapsulation of molecular cargo to the virosome lumen, generating a virosomal delivery (Figure 8).[126,127] Subunit antigens have been proposed as a cargo for

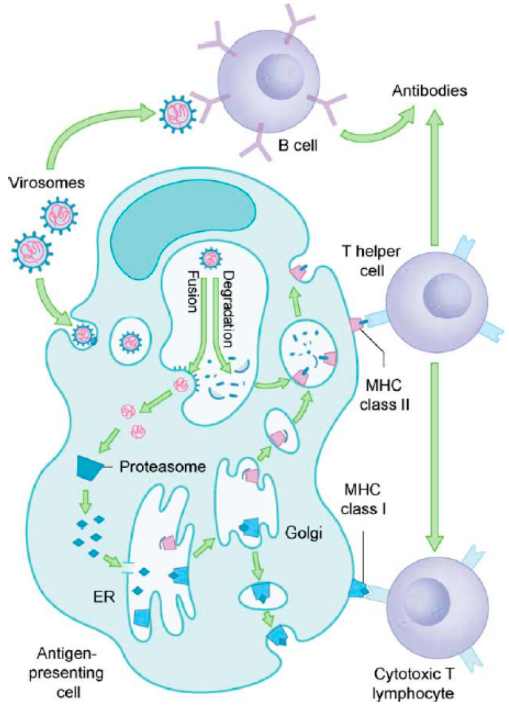

Figure 8. Routes of immune stimulation with virosomes. Virosomes can activate B cells directly through the binding of HA to receptors. Virosome HA can also facilitate the entry of the particle into antigen-presenting cells. Surface antigens are degraded in lysosomes and enter the MHC II pathway for presentation to T helper cells. Internal antigens enter the cytosol through virosomal fusion escape, where they undergo proteasomal degradation and are presented on MHC I to cytotoxic T cells. Reproduced with permission.[126]

virosomes, which would promote APC uptake and major histocompatibility complex (MHC) presentation for stimulating T cell activation.[128]

The Inflexal V virosomes vaccine has already been approved and was introduced to the European Union beginning in 1997. It has undergone numerous clinical studies investigating its safety and immunogenicity. These virosomes are composed of purified HA and NA proteins generated in monovalent virus pools matched to WHO recommended strains, which are integrated into a synthetic liposome. Inflexal V is approved for use in all age groups but is especially advantageous for use in

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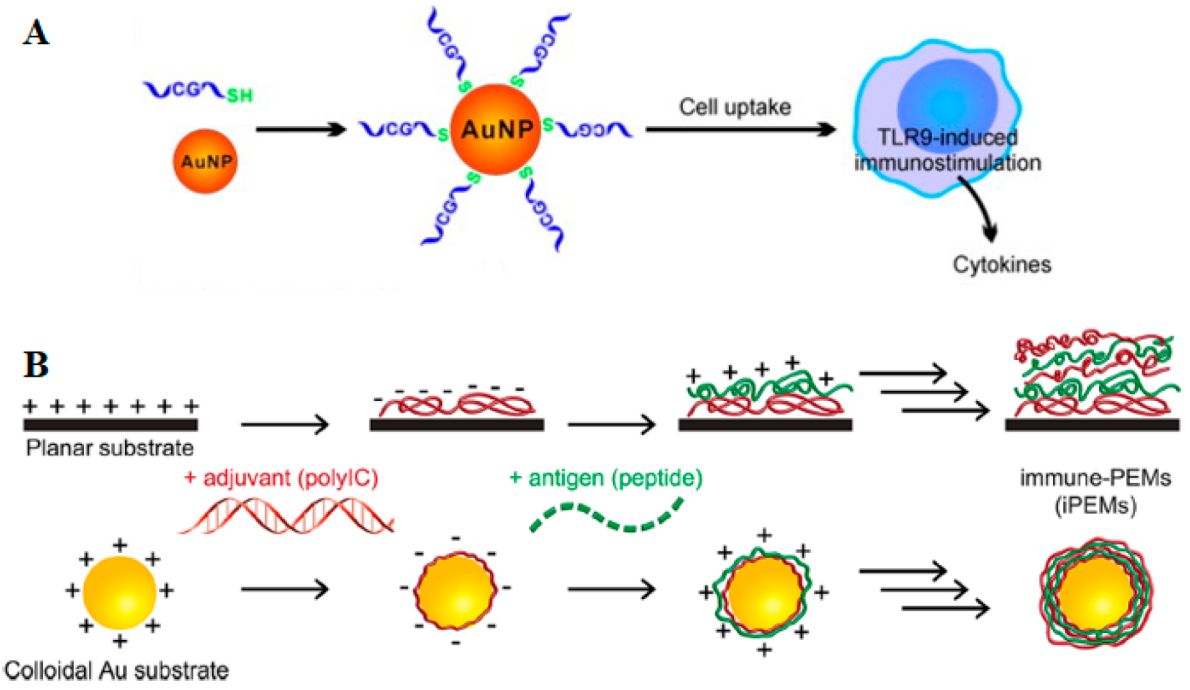

Figure 9. Methods for associating biomolecules to gold-core nanoparticles. (A) Thiol groups associate with gold and can be used to bind molecules, such as the oligonucleotide adjuvant CpG (shown) to the surface of the nanoparticle. (B) Layers of adjuvant and antigen can also be deposited to the surface of the particle through electrostatic interactions. Schematic panel A reproduced with permission.[137] Schematic panel B reproduced with permission.[140]

the elderly, young children, and immunocompromised patients, with results comparable to adjuvanted Fluad vaccine.[129,130] The scalability of Inflexal V exemplifies another promising benefit of virosomes, as a standard lot size produces enough virosomes for half a million doses.[131] The methodology underlying virosomes could be made to integrate recombinant antigens. Virosomes generated from recombinantly produced VLPs, rather than native viruses, could be feasible, as could be the encapsulation of recombinant cellular response targets, such as nucleoprotein, to further enhance the breadth of protection.

7. SYNTHETIC POLYMERS AND INORGANIC PARTICLES

While much of the research into immunostimulatory molecules in recent years has focused on organic substances, the success of biodegradable, synthetic, drug delivery particles indicates the potential for particles of inorganic or synthetic origin to meet the necessary criteria for a new-generation influenza vaccine. Immune responses can be enhanced not only by the strength of the initial immune response but also by prolonging the circulation of antigens, effectively increasing the exposure of the adaptive immune system to the antigen.

Synthetic polymer and inorganic particles may provide a nonspecific substrate for the presentation of antigens, allowing them to act as a flexible platform for multiple vaccines. When studying synthetic particles for vaccines, influenza may be only a single candidate for which the method is being considered. Still, this flexibility in application especially benefits the field of influenza vaccines, as designing separate seasonal and epidemic vaccines may remain the most achievable approach for the near future, until an ideal universal formulation can be rigorously tested and proven effective.

7.1. PLGA Polymers. Based on the principle that prolonged antigen release results in a sustained immune response with more effective antigen adaptation, PLGA polymer particles have been investigated as a biodegradable vehicle for recombinant antigens. Split and inactivated virus-derived influenza particles have been encapsulated, with results indicating advantages in the production of neutralizing antibodies[132] and heterologous T cellular response.[133] To determine if such advantages were conferred to recombinant vaccines, investigators loaded PGLA-NPs with a cocktail of 5 antigens: chimeric M2e-norovirus (M2e-P), two peptides derived from pandemic H1N1 epitopes, and two peptides from seasonal H1N1 epitopes. These PGLA-NPs were administered through the intranasal route in pigs and resulted in the absence of observable influenza symptoms, as well as an increased response in CD4 and CD8 T cells, albeit without a significant increase to antibody titers.[134]

PLGA nanoparticles have also been tested as a vehicle for the codelivery of antigen and immunostimulant TLR agonists. PLGA particles were loaded with antigen and either the TLR4 ligand MPL or the TLR7 ligand R837, or dual-loaded with both ligands. Interestingly, it was found that administering an admixture of antigen and dual-loaded ligands in separate nanoparticles resulted in a stronger antibody response than combining the antigen and ligands in a single particle formulation. Antibody IgG titers were nearly 10-fold higher with the dual-ligand formulation than either ligand applied individually, and analysis of excised germinal centers from mouse lymph nodes indicated the stimulation of long-lived antibody-secreting cells with a predicted persistence of over 1 year. The antigen-specific CD4 and CD8 T cell response was also significantly increased. When the dual-ligand method was used in rhesus macaques with WIV from the H1N1 pandemic strain, significant increases in IgG and neutralization titers were

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observed, indicating the dual-ligand particles could likewise be effective in humans.[135]

7.2. Gold Particles. Gold nanoparticles (AuNPs) have been explored in the study of vaccines against cancer, HIV, encephalitis, hepatitis, and influenza.[136] Through the affinity of thiol groups to gold nanospheres, it is possible to functionalize the particle with immunostimulants such as oligonucleotide cytosine-phosphate-guanosine (CpG) or synthetic carbohydrates (Figure 9A). The application of nucleotides or polysaccharides provides new opportunities not accessed by the peptide or lipid methods previously discussed. For example, CpG was found to act as a novel TLR9 agonist, which could be used to activate intracellular responses for immune signaling that are distinct from the TLR4 and TLR5 responses.[137] Experiments with carbohydrate antigens from bacteria revealed that conjugation to AuNPs could successfully elicit significant IgG responses to the saccharide epitopes,[138] suggesting a potential role as an antigen carrier. Such novel properties of AuNPs could contribute to novel solutions in the field of influenza vaccines.

An investigation was done into the application of AuNPs as a carrier for the M2e antigen, for use in the intranasal vaccination route, and with CpG as an adjuvant to further enhance M2e recognition. The complete formulation of Au−M2e with CpG yielded serum IgG compared to the controls, including unadjuvanted Au−M2e particles, and the IgG produced were cross-reactive with seasonal H1N1 and H3N2 as well as pandemic H1N1. Lethal challenge confirmed the efficacy of the particle-based immunization, with 100% survival observed among mice treated with Au−M2e with CpG.[38] Interestingly, the CpG in this study was included in a soluble state; it is possible that, when using the currently known methods for conjugating CpG with AuNPs, it would be possible to produce self-adjuvanted particles by colocalizing CpG and M2e to the surface of the AuNPs.

Further development of AuNP vaccines could also be applied to future influenza vaccines. For example, polyelectrolyte multilayering of AuNPs could be used to produce modular vaccines with antigens integrated to the surface layer via electrostatic interactions, a type of particle known as iPEMs (Figure 9B). PEM capsules have been produced to include M2e from influenza,[139] and iPEMs with gold cores could allow influenza vaccines to be produced with tunable TLR signaling.[140]

7.3. Silicon Nanoparticles. Silicon dioxide, also called silica, may also serve as an inorganic nanoparticle platform for influenza vaccines. There is evidence to suggest that virus-sized (approximately 50 nm diameter) silica nanoparticles can increase the immunogenicity of a vaccine when coadministered without binding. When tested with M2e, the increase in response was similar to the alum adjuvant.[141] However, a greater range of particle types that can be generated with silica could allow this class of nanoparticles to serve functions not available to the alum adjuvant. One novel vaccine combined silica with plant-produced recombinant HA as well as an additional adjuvant, guanosine monophosphate (GMP), to create particles, which could be introduced to the respiratory tract via an intratracheal (IT) route as an inhalant. While HAI from serum antibodies was lower than the injection of the recombinant antigen with alum, splenocyte stimulation and cytokine secretion were significantly increased, indicating that this method could be used to generate an enhanced cellular response to the target antigen.[142] Silica might also provide a method by which influenza vaccines could be introduced earlier in life than traditional vaccines allow. Using nanoscale silica ($NanoSiO_2$), the researchers were able to successfully protect neonatal mice with an immunization at 1 week of age.[143] While safety considerations make this application difficult to investigate clinically, this case serves to show that silica particles may have applications in future influenza vaccines that warrant further study.

8. OUTLOOK

Due to the advantages in production and control provided by recombinant antigens, it is likely that next generations of influenza vaccines will make use of recombinant and particle-based technology. There is ample research to suggest that recombinant methods can be used to produce antigens for broadly neutralizing vaccines. However, to make the immunogenicity of such conserved-epitope vaccines feasible, nanotechnology will need to develop in tandem to produce a combined antigen-particle approach. In order to achieve this, it may first be necessary to establish the safety and efficacy of nanoparticles in marketable influenza vaccines. Virosomes set a positive precedent for virus-like particle methods, as does the development of VLPs to the stage of phase III clinical studies. However, while current VLPs may benefit from the administration with an additional adjuvant, the self-adjuvanting properties of certain biopolymer or functionalized inorganic particles may prove to be useful for universal vaccines, especially if less immunodominant antigen targets with conserved epitopes are necessitated. If mosaic designs prove sufficient for broad immunity, then self-assembled proteins such as ferritin exhibit properties that make their use favorable, while considerations have also gone into producing multivalent VLPs. Ultimately, the direction of nanoparticle vaccine development may be guided by the first antigen methodology that can achieve broad protective efficacy in humans.

The ideal particle will need to reflect the advantages provided by recombinant antigens, providing a means that is safe and realistically producible on a large scale with properties that can be characterized and controlled for quality. Whether the basis for future mainstream influenza vaccines will include VLPs, biopolymers, liposomes, or synthetic particles, the various research thrusts within this field have yielded numerous promising approaches, working toward both short-term improvements upon existing methods as well as the ultimate goal of universal influenza vaccines. Even once the first broadly protective influenza vaccine reaches the market, it is likely that this field will continue to expand with the ever-improving development of future vaccines.

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Notes

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