



Viral nanoparticles and virus-like particles: platforms for contemporary vaccine design

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Current vaccines that provide protection against infectious diseases have primarily relied on attenuated or inactivated pathogens. Virus-like particles (VLPs), comprised of capsid proteins that can initiate an immune response but do not include the genetic material required for replication, promote immunogenicity and have been developed and approved as vaccines in some cases. In addition, many of these VLPs can be used as molecular platforms for genetic fusion or chemical attachment of heterologous antigenic epitopes. This approach has been shown to provide protective immunity against the foreign epitopes in many cases. A variety of VLPs and virus-based nanoparticles are being developed for use as vaccines and epitope platforms. These particles have the potential to increase efficacy of current vaccines as well as treat diseases for which no effective vaccines are available. © 2010 John Wiley & Sons, Inc. *WIREs Nanomed Nanobiotechnol* 2011 3 174–196 DOI: 10.1002/wnan.119

INTRODUCTION

The goal of vaccination is to initiate a strong immune response that leads to the development of lasting and protective immunity. Vaccines against pathogens are the most common, but approaches to develop vaccines against cancer cells, host proteins, or small molecule drugs have been developed as well.^{1,2} For the purposes of this article, we focus primarily on the use of virus-based platforms in the development of vaccines for infectious disease.

The immune system is composed of the innate (nonspecific) and adaptive (specific) branches. After a pathogen breaches the host's physical barriers such as mucosal surfaces or skin, cells of the innate immune system can recognize general characteristics of the pathogen and initiate a response.³ Pathogen-associated molecular patterns (PAMPs) are molecules that are common to many pathogens, such as lipopolysaccharide (LPS), which can be found on the cell wall of many bacterial species, double-stranded RNA or unmethylated CpG motifs, which

are normally associated with virus infection. PAMPs can be recognized by Toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs) which are present on the surface of host cells.⁴ The intrinsic properties of multivalent display and highly ordered structure present in many pathogens also facilitate recognition by PAMPs, resulting in increased immunogenicity. PAMPs stimulate antigen uptake by antigen presenting cells and the subsequent presentation of antigens to cells of the adaptive immune response. Furthermore the initial response by polymorphonuclear (PMNs) leukocytes, granulocytes, and natural killer (NK) cells induces the release of pro-inflammatory cytokines that promote elimination of pathogens.

The adaptive immune response takes longer to develop, on the order of days. Adaptive responses require the recognition of the pathogen by host lymphocytes through interaction of cell-surface receptors that are unique to the lymphocyte. An enormous repertoire of possible receptor combinations allows for the recognition of almost any antigen presented.⁵ Once the antigen is delivered to the adaptive immune system and stimulates the proliferation of antigen-specific effector cells, these cells begin eliminating the pathogen. The adaptive response is also the basis for immunological memory, which is important for ensuring a fast, strong response when an infectious pathogen is encountered

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again.⁶ Memory lymphocytes can form subsequent initiation for a rapid and immediate immune response upon reintroduction of the pathogen. Vaccines capitalize on immune memory by stimulating the formation of memory B- and T- cells and specific antibodies to a pathogen, which are then available to rapidly recognize the natural pathogen during future exposures. The ideal vaccine utilizes this process by introducing the immune system to a specific pathogen and allowing the formation of memory cells for future recognition, yet avoiding symptoms, illness, or transmission of the pathogen to other individuals.

VACCINE STRATEGIES FOR INITIATING IMMUNE RESPONSES

Viruses are continually evolving attributes that allow them to survive in a hostile environment, and hosts evolve strategies of recognizing and relieving themselves of the pathogen. We can take advantage of these natural strategies of recognition as we develop vaccines. Classical approaches to vaccine development involve chemical inactivation of virus particles or infected tissues (termed 'killed' vaccines), attenuation of virulence during passage in tissue culture or animal hosts (termed 'live-attenuated' vaccines), or immunization with portions of the pathogen that can induce specific immune responses (termed 'subunit' vaccines). To generate vaccines against viruses, the delicate balance of protection and safety was first reached by using whole virus that was inactivated or killed. More recently, as understanding of the immune system activation has improved, it has become apparent that even small immunogenic peptides derived from a pathogen have the potential for inducing protective immunity.⁷

Currently, a large number of successful vaccines are based on whole viruses, whether live-attenuated or inactivated. The first vaccine developed against smallpox by Edward Jenner in 1796, also involved the administration of less pathogenic cowpox (vaccinia) virus.⁸ The method for immunization with vaccinia was crude and imprecise, the mechanism of immune protection was not well understood, and the potential for vaccine-induced illness was high. Over the next century, various vaccines for a range of infectious (primarily bacterial) agents, such as cholera, tetanus, and the bubonic plague were developed and used in humans.

A major advance toward the development of modern vaccines was the ability to grow and produce viruses in laboratory cell cultures, allowing better characterization and standardization. The first licensed vaccine produced from cell culture was

an injected dose of killed poliovirus developed by Jonas Salk and announced to the American public in 1955.^{9,10} Other whole-virus vaccines quickly followed suit and have been tremendously successful at reducing morbidity and mortality worldwide, including measles, mumps, rubella, influenza, and the inactivated hepatitis A vaccine.^{11–13}

In spite of the success of the poliovirus and other vaccines, there are intrinsic disadvantages involved with the use of whole virus particles for immunization.⁷ Live-attenuated viral vaccines are relatively unstable and difficult to deliver. The potential for genetic reversion of the attenuated strain to a more virulent form is always a concern. While administration of killed whole-virus vaccines negates this particular problem, the immune response to these is weaker and a regimen of doses is generally required, increasing the expense and reducing vaccine coverage in a population. In addition, complete inactivation of the vaccine virus must be assured to prevent infection.

Subunit vaccines prime immune responses by delivery of a subset of immunogenic viral proteins, often portions of the virus capsid (Figure 1). Because there is no potential for replication, subunit vaccines are potentially much safer, but are often less immunogenic when expressed and purified in the absence of other viral components. An advance in this area involves the natural ability of many types of viral capsid subunits to self-assemble into virus-like particles (VLPs), which allows for a better mimic of the whole virus particle, and a resulting improvement in effectiveness as vaccines (Figure 2).^{18–22} Further advances utilize the VLP as a platform for the attachment or display of foreign epitopes such as nonstructural viral proteins or nonviral proteins. This strategy couples delivery of the chosen antigen with the ability of the VLP to induce strong immune responses. The ease with which VLPs are often made and various antigens are attached to them makes every potential platform promising from onset. However, application of the platform in a complex system involves unique challenges and problems that may limit their progression in clinical use. Each of these approaches is discussed below, as well as summarized in Table 1.

VLP AS VACCINES

VLPs are considered a subunit vaccine because they consist of a noninfectious subset of viral components, but mimic killed vaccines since they typically present a whole, but inactive virus particle to the host. Tailoring the viral proteins expressed can enable production of particles that are structurally and morphologically similar to infectious viruses, and retain the ability

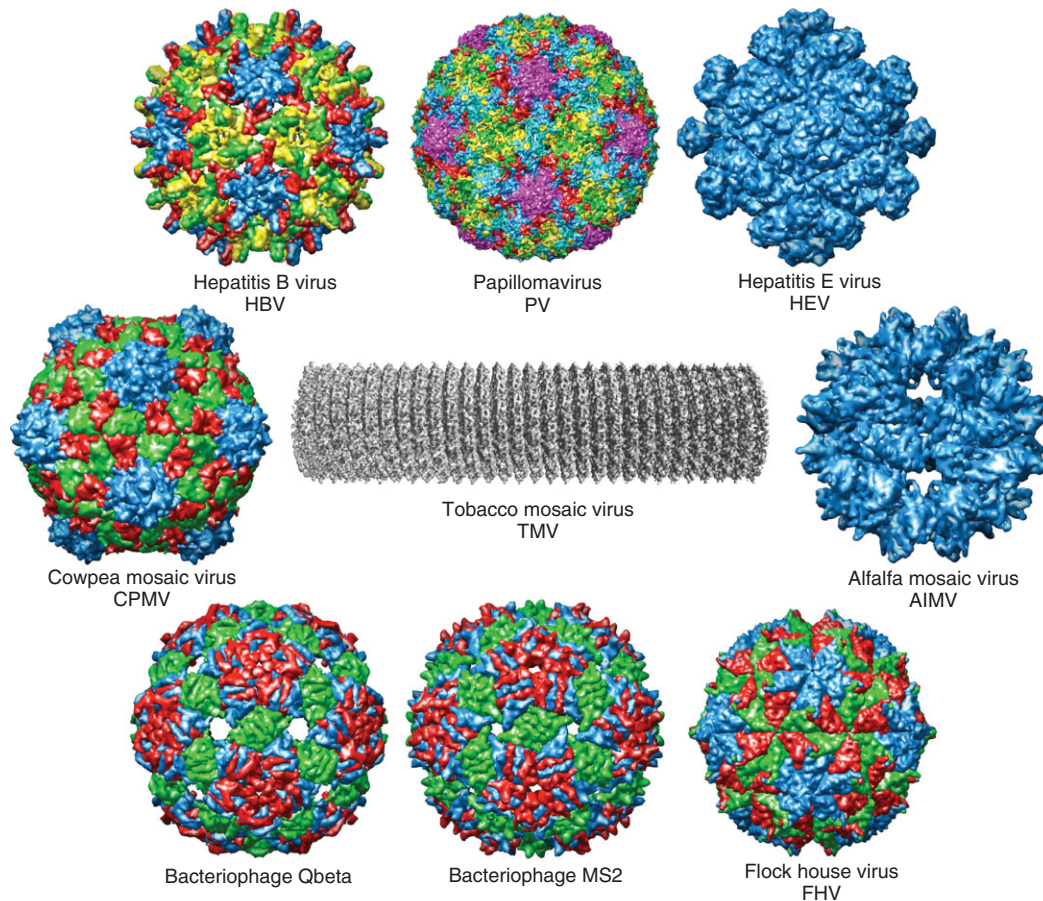


FIGURE 1 | Examples of various virus capsids that have been developed as virus-like particle (VLP) vaccines and platforms for heterologous antigen. PDB IDs: hepatitis B capsid, 2QUJ; Papillomavirus (PV), 3IYJ;^{14,15} Hepatitis E virus (HEV), 2ZTN; Cowpea mosaic virus (CPMV), 1NY7; Alfalfa mosaic virus (AIMV), AMV; bacteriophage Q β capsid, 1QBE; bacteriophage MS2–2MS2; Flock house virus (FHV) VLP, 2Q26. (Tobacco mosaic virus (TMV) image has been reprinted with permission from Ref 16. Copyright 2007 Academic Press). (Other images have been reprinted with permission from Ref 17. Copyright 2009 Oxford University Press).

to bind and penetrate host cells. Noninfectious VLPs are inherently safer than killed or attenuated virus vaccines. In addition, due to their particulate nature, VLPs can be much more stable than soluble antigens, which have failed in many vaccine approaches. The multivalent and highly ordered structure of the VLP is not typical of host proteins, and thus constitutes a PAMP, which triggers innate immune sensing mechanisms. In addition, most VLPs encapsidate nucleic acids during their production, and these nucleic acids may stimulate particular TLRs as well. These qualities contribute to the effectiveness of VLPs as vaccines because they facilitate uptake by antigen presenting cells and long lasting CTL responses and antibody responses are possible.^{23,24}

Nonenveloped VLPs

The two most successful VLP-based vaccines have been licensed and approved for use in humans. The

first to be commercialized was the hepatitis B virus (HBV) vaccine. HBV infection is transmitted by blood or sexual contact, and is a major cause of hepatitis and hepatocellular carcinoma, affecting approximately 300 million people worldwide. In the initial vaccine formulation, empty particles of small HBV-derived surface antigen (HBsAg) that are naturally made during infection with hepatitis B and are present in the blood, were obtained from infected individuals and purified. The limited quantity of antigen produced by this method led to investigation of the potential for making the particles by recombinant protein expression. Expression systems in yeast, baculovirus, and mammalian cells are often utilized because these cultures can produce large amounts of proteins *in vitro*. In the case of HBV, vectors were prepared carrying the cDNA sequence for HBsAg, and the surface antigen proteins were expressed in recombinant yeast cell culture and VLPs formed by self-assembly.²⁵ Further

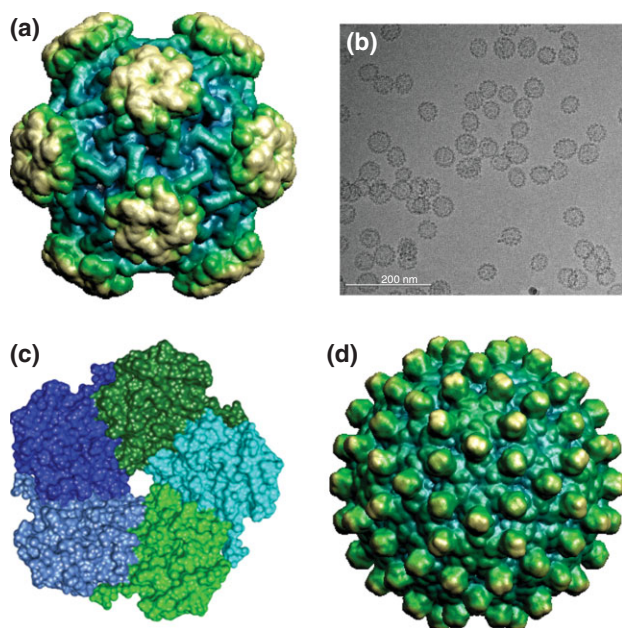


FIGURE 2 | (a) Icosahedral capsid of human papilloma virus (HPV)16 L1 capsid, PDB ID: 1DZL. (b) Electron micrograph of HPV virus-like particles (VLPs) (Image courtesy of Merck&Co and The Scripps Research Institute). (c) L1 pentamer of HPV rendered in chimera, PDB ID: 2R5H. (d) Human hepatitis B viral capsid (HBcAg), PDB ID: 1QGT. ((a) and (d) have been reprinted with permission from Ref 17. Copyright 2009 Oxford University Press).

studies confirmed the immunogenicity of these particles in human vaccination.²⁶ Inclusion of surface proteins (preS1 and preS2 proteins), which are present in the native HBV envelope at lower levels than in HBsAg, allows for immune responses in the small percentage of patients who do not respond to VLPs of HBsAg alone.^{27–29} In this case, the better mimic of the authentic virion surface results in broadened effectiveness. The commercially successful vaccine, licensed in 1986, is a safe and effective example to which other vaccines are compared. The effectiveness of this vaccine has facilitated exploration into more complex issues such as administration in poorer countries where HBV is an epidemic and application of the current vaccine is limited due to cost. The most recent advances have tested the possibility of oral delivery of HBV particles that are produced in transgenic plants.³⁰

The second commercially available VLP-based vaccine is to human papillomavirus (HPV). An effective HPV vaccine is of particular interest because of the link of HPV infection, particularly genotypes HPV-16 and HPV-18, to cervical intraepithelial neoplasia (cervical cancer).^{31–33} The HPV capsid consists of two proteins, L1 and L2. Expression of the L1 major capsid protein of HPV16, either

alone or in combination with L2 resulted in the production of 40 nm VLPs that induced high-titer neutralizing antisera in rabbits.^{34,35} Immunizations in human females demonstrated that the HPV vaccine protected them from infection with HPV, and also reduced or prevented entirely the development of intraepithelial neoplasia. Of the control patients who received placebo, a subset was infected with HPV and some developed cervical intraepithelial neoplasia. The current HPV vaccine shows great promise, as it is almost completely protective against HPV types from which it is derived even in the absence of an adjuvant, and with adjuvant achieves partial protection against other phylogenetically related types.³⁶ This VLP vaccine is considered a breakthrough for preventing cervical cancer, and it will be interesting to observe the impact of the vaccine on cervical cancer rates in the future. Since HPV-16 has also recently been associated with oropharyngeal cancer,³⁷ vaccination may impact the future rates of this disease as well. As with the HBV vaccine, production and characterization of VLPs is expensive and results in higher vaccine costs. Approaches to reduce costs by expressing the L1 protein as pentameric capsomeres, or in combinations with other viral proteins, are being explored.³⁶

Other VLP-based vaccines are in various stages of development.³⁸ VLPs of hepatitis E virus (HEV), a virus thought to cause an acute severe liver disease in some individuals, were shown to be immunogenic and protective in macaques, and have now passed Phase I of clinical trials.^{39,40} Experimentation with the protein requirements for HEV VLP formation has facilitated further understanding of the formation of VLPs.^{41,42} However, circumstances specific to the disease hamper the testing of protective efficacy. The clinical attack rate of HEV is low and sporadic, requiring a large number of vaccinated and control individuals to achieve any interpretable results. VLPs of Norwalk virus, a highly infectious virus that is a major cause of gastrointestinal illness, have been tested in adults and are immunogenic.⁴³ Strides toward an oral Norwalk virus vaccine produced in tobacco leaves and potato tubers have been successful, and oral immunization of mice resulted in production of serum and mucosal antibodies.^{44,45} In addition, a betanodavirus-based VLP vaccine has found much success in preventing viral nervous necrosis disease in fish.⁴⁶

Enveloped VLPs

Enveloped VLPs of viruses such as influenza, HIV, and HCV are similarly replication-incompetent, and the immunogen consists of assembled particles containing

TABLE 1 | VLP Vaccines and VLPs Developed for Display of Heterologous Epitopes

Platform	Composition	Vaccine Target	Stage of Development	References
Poliovirus	VP0, VP3, VP1	Poliovirus	Particles in development	120
HBV	Energix-B	HBV	Testing in humans	25,26,30
HBV	HBsAg, envelope (major S, minor pre-S2, pre-S1)	HBV	Testing in humans	27–29,121
HPV	L1 and L2	HPV	Approved	31–34,122,123
Rotavirus	Variety	Rotavirus	Animal studies	124–126
HEV	Structural protein with truncated N terminus	HEV	Testing in humans	39–42
Flu	HA, NA, and matrix proteins	Flu	Animal studies	47–49
HCV	Core, E1, E2	HCV	Testing in nonhuman primates	54–57
HIV/SIV	HIV or SIV capsid, and HIV immune epitopes (gag, pol)	HIV	Animal studies	20,50,127,128
Ebola virus and Marburg virus	Glycoprotein and matrix protein VP40	EBOV and MARV	Testing in nonhuman primates	58–60
Norwalk virus	Major capsid protein	Norwalk virus	Testing in humans; potential as oral	43–45
SARS	S, E, M, and N structural proteins	SARS	Particles in development	61
FHV	FHV capsid with E1 of HCV	HCV	Particles in development	112,113
FHV	Core and E1 protein epitopes and HBsAg epitope on FHV capsid	HBV/HCV	Animal studies	114,115
FHV	FHV capsid with neutralizing domain from V3 loop of HIV-1	HIV	Animal studies	116
FHV	FHV capsid with ANTXR 2 PA-binding domain	Anthrax—both protective and as a treatment	Animal studies	65
FHV	FHV capsid with CCR5 (HIV receptor)	HIV	Testing in humans	129
HBV	HBV core antigen and HIV env protein, conserved region of gp41, or p34 peptide	HIV	Animal studies	69–71
HBV	HBc with CS protein of malaria	Plasmodium falciparum	Testing in humans	76,77
HBV	HBsAg with env protein of DENV	Dengue virus	Animal studies	118,119
HBV	Epitope of FMDV on HBcAg	Foot and mouth disease virus	Animal studies	73
HBV	HBcAg with immunogenic HCMV epitope	Human cytomegalo virus	Animal studies	130
HBV	PUU nucleocapsid protein on HBc	Puumala hantavirus	Animal studies	75

TABLE 1 | Continued

Platform	Composition	Vaccine Target	Stage of Development	References
HBV	HBc with M2 protein of influenza A	Influenza	Animal studies	72
HBV	HBcAg with CTL epitopes of HPV	HPV	Animal studies	131
HEV	HEV VLPs with B cell epitope tag	Stimulate mucosal immunity	Animal studies	132
HPV	L1 of HPV with SIV gag, HIV tat, and HIV rev	HIV	Testing in nonhuman primates	79
M13	M13 version of S3Pvac	Taenia solium	Animal studies	106
M13	Parasitic worm protein on M13	Schistosoma japonicum	Animal studies	107
NDV	NP and M proteins of NDV with RSV G protein	RSV	Animal studies	133
Q β	Q β with allergen Der p1	Allergen	Testing in humans	134
Q β	Q β with nicotine	Nicotine	Testing in humans	2
Q β	CCR5 on Q β	HIV	Animal studies	135
AP205	Various heterologous epitopes on AP205	<i>Salmonella typhi</i> , HIV, Influenza A	Animal studies	108
CPMV	CPMV with VP2 capsid protein of CPV	Canine parvovirus	Animal studies	84,85
CPMV	CPMV with outer membrane protein F of <i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i>	Animal studies	89,90,101
CPMV	Mink enteritis virus protein on CPMV	Mink enteritis virus	Animal studies	86
CPMV	Staphylococcus aureus protein on CPMV	Staphylococcus aureus	Animal studies	91
CPMV	Gp41 peptide inserted onto CPMV	HIV	Animal studies	87,88
CPMV	Outer membrane protein F of <i>Pseudomonas aeruginosa</i> on CPMV	<i>Pseudomonas aeruginosa</i>	Animal studies	90
Tobacco mosaic virus	Peptides from VP1 of FMDV on TMV	Foot and mouth disease virus	Animal studies	95,96
Tobacco mosaic virus	Peptide from spike protein of murine hepatitis virus on TMV	Murine hepatitis virus	Animal studies	94
Tobacco mosaic virus	Outer membrane protein F from <i>Pseudomonas aeruginosa</i> on TMV	<i>Pseudomonas aeruginosa</i>	Animal studies	97
Cucumber mosaic virus	R10 epitope of HCV on CMV	Hepatitis C virus	Animal studies	98
Alfalfa mosaic virus	G protein peptide from RSV on AIMV coat protein	RSV	Testing in nonhuman primates	100
PapMV	LCMV peptide p33 on PapMV	LCMV	Animal studies	64

TABLE 1 | Continued

Platform	Composition	Vaccine Target	Stage of Development	References
MS2	Peptide from V3 of HIV gp20 or CCR5 on MS2 coat protein	HIV	Animal studies	105
HBV	Peptide from Bordatella pertussis on HBV core protein	Bordatella pertussis	Animal studies	136
HBV	Peptide from SPAG-1 of Theileria annulata on HBcAg	Theileria annulata	Animal studies	137,138
ISCOMs	H1N1 peptide	H1N1	Animal studies	139,140
ISCOMs	Core protein IMX	Hepatitis C virus	Testing in nonhuman primates	141,142
ISCOMs	Gp120, env, or gag peptides	HIV	Testing in nonhuman primates	143–146
ISCOMs	Peptide F and G from RSV	RSV	Animal studies	147,148
ISCOMs	HSV-2 antigen	Herpes simplex virus	Animal studies	149
ISCOMs	Rotavirus antigen	Rotavirus	Animal studies	150,151
Liposomes	Hepatitis A peptide	Hepatitis A virus	Testing in humans	152–154
Liposomes	Peptide from circumsporozoite protein of Plasmodium falciparum	Plasmodium falciparum	Testing in humans	155
Liposomes	Ha and Na from influenza	Influenza	Testing in humans	156
Liposomes	Gp120 from HIV	HIV	Animal studies	157,158
Liposomes	Toxins	Diphtheria or tetanus	Animal studies	159,160
Liposomes	HA from influenza	Influenza	Animal studies	161
Virosomes	HA and NA from influenza	Influenza	Approved	162
Virosomes	Inactivated Hepatitis A virus	Hepatitis A virus	Animal studies	153,163
PLG microspheres	Whole cell lysate of Helicobacter pylori	Helicobacter pylori	Animal studies	164
PLG microspheres	Tetanus toxoid	Tetanus	Animal studies	165
PLG microspheres	HBsAg	Hepatitis B virus	Animal studies	166,167
PLG microspheres	Plasmodium falciparum antigen	Plasmodium falciparum	Animal studies	168
PLG microspheres	Yersinia pestis F1 antigen	Yersinia pestis	Animal studies	169
PLG microspheres	Phosphorylcholine antigen of Salmonella typhimurium	Salmonella typhimurium	Animal studies	170
Nanoemulsion	Influenza A	Influenza	Animal studies	171
Nanoemulsion	Protective antigen from Bacillus anthracis	Anthrax	Animal studies	172
Nanoemulsion	Gp120 from HIV	HIV	Animal studies	173
Nanoemulsion	Vaccinia virus	Smallpox	Animal studies	174
Nanoemulsion	HBsAg	Hepatitis B virus	Animal studies	175

some or all of the surface components of the virus embedded in plasma membrane. VLPs expressed in baculovirus and composed of influenza hemagglutinin (HA), neuraminidase (NA), and matrix structural proteins can induce production of specific antibodies in mice.^{47,48} Route of entry and presence of the cytokine interleukin-12 (IL-12) as an adjuvant alters the immune response and affords different levels of protection.⁴⁹ In addition, although individual influenza proteins were capable of inducing protective immune responses, the use of baculovirus-expressed recombinant revealed a problem that may be an underlying problem in many VLP-based vaccines using recombinant proteins. The recombinant HA proteins were poorly immunogenic in people, perhaps due to differences in post-translational modification such as glycosylation.^{47,48}

The ability of HIV to consistently and effectively evade the immune system over time makes the development of an effective vaccine to HIV particularly difficult. On the surface, a VLP vaccine may be at a disadvantage compared to current replication-based vaccine strategies because HIV-1 VLPs are similar to the wild-type viral structure, which does not elicit a protective response in infected individuals. However, modifications in the HIV VLP could advance this strategy. In addition, mucosal administration of the particles elicits strong neutralizing antibody and CTL responses.⁵⁰ Efficient induction of broadly neutralizing antibodies is thought to be an essential component for inducing protection. Nevertheless, while such antibodies have been identified in a small subset of HIV-infected patients, it is not yet possible to induce such antibodies experimentally. One significant challenge is the difficulty of producing antigens that are structurally similar to those found on the intact HIV particles. The production of an HIV VLP that confronts this problem has been attempted by mimicking the intact HIV particle containing the envelope proteins that associate with the viral gag protein core.^{50–52} The focus of recent research has been on the more conserved epitopes of the HIV-1 gp-41 envelope protein.⁵³ Nevertheless the challenge of an efficient HIV vaccine will require further manipulation of the different VLPs and expression systems available.

Other VLP-based vaccines for enveloped viruses have the potential to ultimately be used in humans. Progress has been made in developing VLPs for hepatitis C virus (HCV),^{54–57} filoviruses Ebola virus (EBOV), Marburg virus (MARV),^{58–60} SARS coronavirus,⁶¹ and Chikungunya virus.⁶²

USING VLPS AS PLATFORMS FOR DISPLAYING AND INDUCING IMMUNE RESPONSES AGAINST HETEROLOGOUS ANTIGENS

VLPs can also serve as a platform for multivalent heterologous epitope display, for the purpose of mounting an immune response to the protein or peptide that is attached through fusion or through genetic insertion into the capsid (Figure 3). VLPs thus serve as a multivalent particle platform; the capsid serves both as a presentation scaffold for epitopes from another viral, bacterial, or parasitic pathogen, and as an adjuvant to boost the immune response.^{2,63–66} Because of the particulate nature of the VLPs, they are readily taken up into antigen-presenting cells (APCs) and stimulate MHC Class I and Class II responses and therefore prime lasting T-cell and antibody responses.

VLP-based systems for epitope presentation are limited by certain constraints, including the difficulty of adding larger epitopes and proteins that might hinder VLP assembly. The viral capsid surface is a varied landscape, with local regions differing in charge, immunogenicity, and accessibility. Placement of antigen on this varied surface is the key to the immunogenicity and success of the vaccine. The first and most easily adaptable strategy is to introduce antigens into VLPs such as HBV and VLP that already have FDA approval as vaccines, such as HBV and HPV, and these are described below. In addition, novel VLP platforms displaying heterologous antigens are discussed.

Antigen Display on HBV VLPs

In many cases, the assembly and stability of VLPs is limited by the size of potential insertions, and small peptides (<50 aa) are often sufficient for the initiation of pathogen-specific antibody production. For example, the addition of small peptides to the immunogenic *c/e1* region of the VLP results in relatively strong responses. However, although the HBV core antigen (HBcAg) was shown to be strongly immunogenic after green fluorescent protein (GFP) was fused to the core protein and introduction resulted in robust humoral immune responses against native GFP in rabbits, the maintenance of the favorable properties of HBcAg with the addition of such a large protein remains to be determined.⁶⁸

For HIV-1, antigenic regions of the HIV envelope protein were linked to HBsAg, producing particles morphologically similar to HBcAg and which exhibited antigenic and immunogenic characteristics

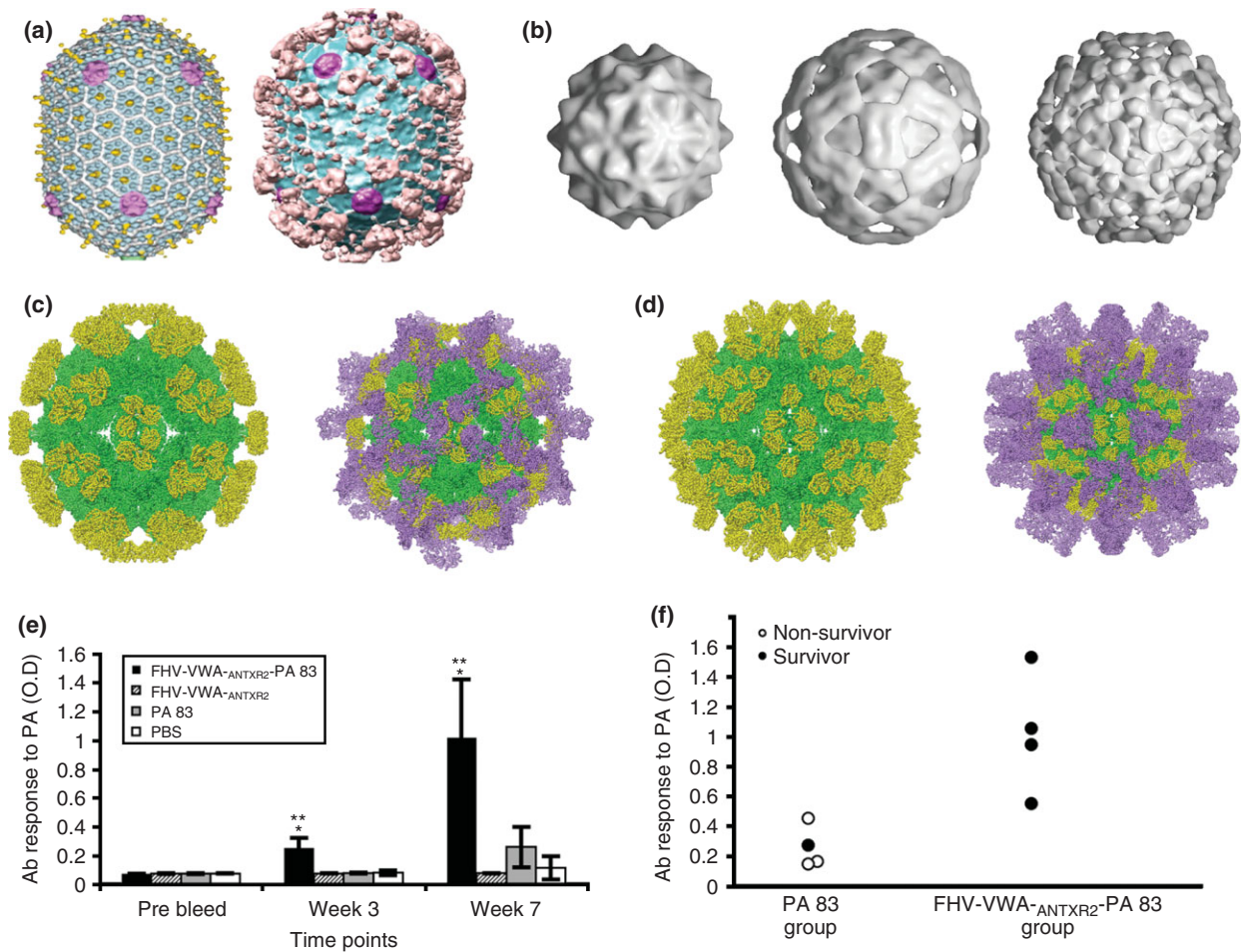


FIGURE 3 | (a) Cryo-EM reconstructions for wild-type T4 capsid (left) and capsid with attached protective antigen fragment and N-terminal domain of lethal factor of anthrax (right). (b) Three dimensional (*) surface shaded reconstructions of wild-type flock house virus (FHV) (left), FHV-ANTXR2-protective antigen (PA) chimera 206 (center), and FHV-ANTXR2-PA chimera 264 (right). (c) Pseudoatomic models of FHV chimera 206 capsid protein (green) and anthrax VWA domain (yellow, left) and model including the protective antigen fragment bound to the surface (purple, right). (d) Pseudoatomic models of FHV chimera 264 capsid protein (green) and anthrax VWA domain (yellow, left) and model including the protective antigen fragment bound to the surface (purple, right). (e) Rats were immunized with FHV-ANTXR2-PA complex or controls and serum samples were collected at indicated time-points and tested for IgG-specific antibody responses to protective antigen. (f) Relationship between anti-PA antibody level and survival of individual rats following challenge. ((a) has been reprinted with permission from Ref 67. Copyright 2007 Academic Press). ((b)–(f) have been reproduced with permission from Annette Schneemann Ref 65. Copyright 2007 Public Library of Science).

of both HBV and HIV.⁶⁹ The short (6 aa) yet highly conserved region of HIV-1 gp41 displayed on these particles elicited high titers of gp41-reactive antibodies in mice, however they failed to neutralize HIV *in vitro*.⁷⁰ This same epitope was more successful in neutralizing antibody production when presented on the influenza A vaccine platform, thus the molecular context seems to be important when considering the ability to elicit protective responses. However, correct processing of particles or T-cell recognition of HIV epitopes on particles is implied on HBV because both peptides and particles with gp41 epitope elicited similar responses.⁷¹

Other epitopes have found varying levels of success when displayed on the HBV platform. The invariant extracellular domain M2 of influenza A fused to HBc and administered intraperitoneally or intranasally gave >90% antibody-mediated protection in mice.⁷² The attachment of an epitope of foot and mouth disease virus (FMDV) to HBcAg VLPs provides immunogenicity stronger than the peptide alone, approaching that of the FMDV particles themselves.^{73,74} The nucleocapsid of Puumala hantavirus on HBc provided protective immunity in voles.⁷⁵

Parasitic infection can also be stemmed using HBV-based vaccines. The circumsporozoite (CS)

protein from malaria was expressed on Hbc proteins, and proved to be highly immunogenic in both mice and monkeys and the first Phase I of trial of these particles was successful.^{76,77} Thus, HBV VLPs have proven to be a productive platform for attachment of epitopes for potential use as vaccines.

The HBV infection elicits a strong humoral response directed against the single immunodominant c/e1 epitope in humans. As expected, insertions at this epitope elicited much better responses than those at the C terminus.⁶⁸ A thorough investigation into the effect of position on immunogenicity studied inserted epitopes of HBV envelope proteins at the amino terminus, the amino terminus with linker, truncated carboxy terminus, and an internal site of HbcAg. The most effective was the internal site, which resulted in very high antibody response. The weakest immune response was to the amino terminus because the epitope was not surface exposed.⁷⁸

Antigen Display on HPV VLPs

Since the HPV vaccine has been approved for use in humans only recently, the study of these VLPs as platforms for vaccines is more limited. Chimeric HPV VLPs have made some progress as an HIV vaccine. Although administration of chimeras with epitopes from HIV showed significant protection in those macaques that showed protection, only partial protection was afforded, so there is plenty of opportunity for further improvement.⁷⁹ To test how the location of the insertion affected immunogenicity of foreign peptides, an Hbc epitope was introduced in various positions in the HPV VLP in order to assess and compare different positions. Although insertion sometimes reduced immunogenicity, each chimera formed particles and induced neutralizing antibodies against Hbc.⁸⁰ The development of HPV VLPs as a platform is still in the early stages, and the future success of these particles is uncertain but highly promising. Exploring the limits of increased sizes of inserted epitopes and tethering of proteins to the external portions of L1 or L2, will likely allow for more efficient formation and immunogenicity of chimeric VLPs.⁷⁹

DISPLAY OF HETEROLOGOUS EPITOPES ON PLANT VIRUSES

Plant viruses in particular have shown promise as a platform for vaccine development. Many plant viruses are unable to replicate in mammals, and this characteristic allows the use of whole virus particles (often termed viral nanoparticles or VNPs)

instead of VLPs. The viruses are modified to multivalently display antigens in a multivalent pattern through genetic introduction of foreign epitopes and proteins. These features augment the favorable safety profile and ease and low cost of large-scale production, creating attractive alternatives to the currently available platforms.

Cowpea Mosaic Virus (CPMV)

CPMV has been extensively studied for vaccine applications.^{81,82} CPMV is a comovirus in the Picornavirus superfamily, and forms a 31 nm icosahedral particle composed of two capsid proteins termed large (L) and small (S). CPMV is a promising plant virus for epitope display for multiple reasons. Large amounts of icosahedral CPMV VLPs can be produced in the natural host *Vigna unguiculata* (cowpea), underscoring the potential for cost-effective manufacture. CPMV particles are also taken up by a number of different antigen-presenting cell types both *in vitro*, and *in vivo* following parenteral or oral administration.⁸³ Particles are also found in the Peyer's patch-associated lymphoid tissue after oral administration, underscoring their potential as a possible oral plant-based vaccine.⁸³ Heterologous peptides displayed on CPMV produce strong antibody responses when administered by parenteral and mucosal routes. As a result of mucosal administration, production of IgA antibodies indicates potential to protect against systemic and mucosal infections.^{84,85} Several exposed loops on the capsid surface, particularly one composed of amino acids 20–27 of the S capsid subunit, are of particular interest regarding development of CPMV as a vaccine.

CPMV particles have been genetically modified to include epitopes from Mink enteritis virus (MEV) VP2 and Canine parvovirus (CPV) VP2. Mink immunized with MEV CPMV particles were protected from viral challenge.⁸⁶ Dogs immunized with CPV CPMV particles were protected from lethal challenge with CPV.^{84,85} Epitopes from the gp41 protein of HIV-1 have also been inserted into the CPMV capsid and the resulting production of neutralizing antibodies suggests there is potential for application as an HIV vaccine.^{87,88}

CPMV nanoparticles have been extensively studied for applications in bacterial vaccines as well. Administration of CPMV particles with peptides from *Pseudomonas aeruginosa* outer membrane (OM) protein F provided pathogen specific antibodies and protection from challenge.⁸⁹ Expressing the same OM epitope in tandem with protein F peptide 18 on CPMV particles, it was shown that the site of peptide expression influences immune recognition.⁹⁰

Administration of CPMV particles with a truncated D2 domain of *Staphylococcus aureus* induced neutrophil activity and macrophage phagocytosis *in vitro*, and provided protection from endocarditis *in vivo*.⁹¹

The CPMV platform provides another example where the positioning of the inserted epitope influences the efficiency of protection. The relationship between a three-dimensional structure of an epitope in the inserted site on CPMV and its immunogenicity were investigated with the display of N1m-1A epitope from human rhinovirus 14 in different positions on the CPMV capsid. In this case, it was demonstrated that when the epitope was presented as a closed loop, the resulting polyclonal antisera had much greater capacity to bind intact human rhinovirus particles.⁹² One drawback toward using CPMV VLPs is frequent proteolytic cleavage of the inserted epitope; this can result in loss of the epitope and/or destabilization and heterogeneity of the particles.⁸² A recent advance toward generating noninfectious VLPs using *Agrobacterium tumefaciens*-based heterologous expression in plants may solve these problems while retaining the possibility for large-scale production.⁹³

Tobacco Mosaic Virus (TMV)

Tobacco mosaic virus was the first plant virus capsid to be genetically modified to contain a foreign epitope. TMV is a rod-shaped RNA virus that infects tobacco and other plants in the *Solanaceae* family. The 18 by 300 nm capsid is made up of 2130 monomers surrounding genomic RNA. It provides an ideal platform for vaccines because it cannot infect animals and large quantities can be easily obtained. Particles with epitopes from murine hepatitis virus and FMDV were successful in providing protection against infectious particles in mice and guinea pigs, respectively.^{94–96}

TMV particles have also been used for platforms for bacterial epitopes, including the outer membrane protein F of *Pseudomonas aeruginosa*. This formulation elicited protection from challenge when administered in mice.⁹⁷ TMV may be a more efficient platform for epitope display when a highly ordered antigen array is required, because the number of potential epitopes per particle and the density of spacing is much higher than in an icosahedral arrangement.

Other Plant Viruses: CMV, AIMV, PVX, and PapMV

Cucumber mosaic virus (CMV) has a wide host range. Its genome is a linear positive-sense single-stranded

RNA and its 31 nm icosahedral capsid is made up of 180 copies of a single capsid protein. CMV particles have been used for presentation of epitopes from HCV.⁹⁸ Particles with the R9 epitope were successful in eliciting a pathogen specific response in rabbits, and HCV patients had a significant release of interferon-gamma and interleukin-12 and interleukin-15.⁹⁹ Alfalfa mosaic virus (AIMV) has four particle types (3 bacilliform and 1 spheroidal), all made up of the same coat protein. The virus infects over 600 plant species in 70 families. An antigenic epitope from Respiratory syncytial virus G protein was expressed on the capsid of AIMV, and specific antibodies were produced *in vitro* in human dendritic cells, and *in vivo* in nonhuman primates.¹⁰⁰ Potato virus X (PVX) is a flexuous rod shape and the type member of the Potexvirus group. The capsid is made up of 1270 identical coat protein subunits. PVX particles have been utilized for attachment of an epitope of HIV-1, and even yielded neutralizing antibodies in mice.¹⁰¹

Papaya mosaic virus (PapMV) forms a rod-shaped 14 by 500 nm particle, and its immunogenicity is dependent on multimerization of the PapMV coat protein. Interestingly, PapMV has demonstrated efficacy as a platform when used to induce antigen-specific CD8⁺ T-cell responses, a process that is typically very inefficient when using a nonreplicating vaccine platform. Nevertheless, immunization with PapMV displaying the well-characterized MHC Class I epitope peptide p33 (derived from lymphocytic choriomeningitis virus (LCMV)) that was expressed on the PapMV surface was able to protect vaccinated mice from subsequent challenge with LCMV.⁶⁴ A similar result was observed with parvovirus VLPs displaying the same epitope,¹⁰² however most VLP platforms are not efficient at inducing Class I-restricted CTL responses. It is not clear why some VLPs result in more efficient cross-presentation of epitopes to Class I, but it may be due to differing routes of endocytosis of VLPs into antigen-presenting cells governing their access to the antigen presentation pathway.

Bacteriophage Platforms for Heterologous Epitope Display: Q β , MS2, AP205, M13, and T4

Bacteriophages present yet another platform for delivery of vaccine to animals. Bacteriophage Q β is an RNA virus that infects *Escherichia coli*. It belongs to the positive-stranded RNA phages family *Leviviridae*. The 24 nm icosahedral capsid is composed of 180 copies of a single coat protein. Displaying antigens on the Q β particles has expanded the technology in other

areas of disease besides infections. Immunization with Q β particles coupled to nicotine succeeded in blocking entry of nicotine into the brain because the antibodies produced can sequester the molecules in the blood of immunized smokers. This is a very exciting advance for the treatment of smoking cessation, and these particles are currently in Phase II clinical trials.^{2,66}

MS2 is another well-characterized bacteriophage which has been utilized for attachment and display of viral epitopes.¹⁰³ Structurally, MS2 is very similar to Q β ; sequence determination for the entire Q β cDNA copy and replicase beta-subunit of MS2 demonstrated regions of homology, and the coat proteins show 23% sequence identity.¹⁰⁴ Peptides from the V3 loop of gp20 of HIV have been inserted into the coat proteins of MS2 and these particles were shown to be immunogenic in mice.¹⁰⁵

Another bacteriophage, M13, has been used primarily for developing vaccines against parasitic infections. M13 is a filamentous bacteriophage made from 2700 copies of the major coat protein P8 and capped with five copies of each minor coat protein P9, P7, P6, and P3. A recent regimen of anticysticercosis tripeptide vaccine in pigs reduced incidence of infection by *Taenia solium*. This is a parasitic infection that primarily infects humans and pigs in Mexico and other Latin American countries and is characterized by headaches and seizures when infection is localized to the nervous system.¹⁰⁶ Potential vaccine candidates have also been identified for *Schistosoma japonicum* infection, which infects humans and other wild mammals with a chronic illness that can damage internal organs.¹⁰⁷ An effective vaccine is important in this case because there is little natural immunity to reinfection.

The RNA bacteriophage AP205 was investigated as a new platform for heterologous display of many antigens. The VLP is formed from 180 copies of the coat protein and allows both N-terminal and C-terminal fusion of epitopes. A fusion of a gonadotropin releasing hormone (GnRH) epitope successfully induced antibodies and a fusion of an extracellular domain of the Influenza A M2 protein elicited protective immunity in mice. Peptides from *Salmonella typhi*, HIV1 Nef, and others were also attached to this platform and resulted in immune responses.¹⁰⁸

Bacteriophage T4 offers an ideal binding platform for antigen on the Hoc and Soc proteins because these accessory structural proteins are on the outer surface of the particle, provide little stabilization, and Hoc in particular is highly immunogenic.^{109–111} The four domains of the Hoc molecule have sites where exposure of epitopes to the

immune system is ideal, in particular the linker regions between Domains 2 and 3, or between Domains 1 and 4. The T4 system is further discussed below.

INSECT VIRUS PLATFORMS FOR ANTIGEN DISPLAY

Flock House Virus (FHV)

Although the ability to act as an adjuvant may be reduced, nonanimal viruses also have potential for use as platform in animal vaccines. The insect virus flock house virus (FHV) is widely used for antigen display attachment and delivery in animals (Figure 3). FHV is a member of the insect virus family nodaviridae, but crosses the kingdom barrier and also replicates in plants and in neonatal mice. The icosahedral capsid is composed of 180 subunits of coat protein, and several surface exposed loops are promising sites for insertion of foreign epitopes. Insertions of HBV and HCV epitopes on various areas of the FHV capsid have been studied and compared. Many highly specific and reactive areas were identified for attachment.^{112–114} Testing in guinea pigs and in hepatitis B and C patients confirmed the potential for VLP-based vaccines against HBV and HCV.¹¹⁵ FHV particles displaying HIV epitopes have also been tested, whereby a neutralizing domain of the V3 loop of HIV-1 was inserted in the FHV capsid, and these particles initiated a strong immune response in guinea pigs.¹¹⁶

DISPLAY OF LARGE ANTIGENS ON VLPS

In some cases, it is extremely difficult to elicit effective antibody responses using epitopes displayed in a loop or on a carrier protein. This environment is very different from native and seldom mimicks the normal conformation of the epitopes. These constraints may require that whole or large protein domains be conjugated to a VLP surface. The large regions generally fold independently, allowing for native conformation of the important immunogenic epitopes. The potential for this has been investigated in the FHV, T4, and HBV systems.

A recent study showed that, in addition to small epitopes, the FHV surface loops at amino acids 206 and 264 could accommodate larger insertions of several hundred amino acids as confirmed by electron cryomicroscopy^{65,117} [Figure 3(B)–(D)]. To capitalize on this feature, FHV particles displaying large protein insertions were developed to potentially function as both, an antitoxin and vaccine for *Bacillus*

anthracis, the causative agent of anthrax. Two enzymatic A subunits of the AB type toxin of *B. anthracis* are responsible for the toxic effects. These are delivered to the cell by binding to the B subunit, termed protective antigen (PA) that binds to cell-surface receptors ANTXR1 and ANTXR2. The adenylate cyclase edema factor (EF) raises intracellular cyclic adenosine monophosphate levels, and the zinc protease lethal factor (LF) cleaves mitogen-activated protein kinase kinases.⁶⁵ Once PA binds to ANTXR1 or 2, it is cleaved to form heptamers that bind EF and LF to form edema toxin and lethal toxin, respectively. The FHV particles were genetically engineered to display the PA-binding domain of anthrax receptor ANTXR2 at position 206 or 264, and the binding domain was deemed functional by its ability to bind PA and inhibit PA-cellular receptor binding and intoxication *in vitro* and *in vivo*. Since they could bind PA, the FHV-ANTXR2 particles were then tested as a multivalent scaffold for displaying the antigenic PA protein. Following immunization with the FHV-ANTXR2-PA complex, strong toxin-neutralizing antibody response along with protection against lethal toxin challenge in rats confirmed the dual-action ability for treatment and protection.⁶⁵ Interestingly, increased antibody responses and protection were observed following immunization multivalent FHV-ANTXR2-PA compared to monomeric PA alone (Figure 3(E) and (F)). In addition, protective responses were observed after a single immunization without additional adjuvants. These results further confirm the idea that multivalency of antigen presentation facilitates effective antibody responses. The use of the FHV platform to display other large antigens is currently under investigation.

HBV and T4 have also been used as platforms for display of large antigens. HBV was also used to display larger portions of Dengue virus antigens. A 395 amino acid portion of Dengue virus envelope protein type 2 (E2) is effective for priming a protective immune response. This large portion of E2 was expressed as a fusion protein with HBsAg in yeast and the resulting VLPs were purified and characterized.¹¹⁸ These well-structured VLPs were shown to function as bivalent immunogens in mice.¹¹⁹ The Hoc and Soc molecules on bacteriophage T4 are ideal candidates to allow the attachment of large antigens because these proteins are not integral to capsid assembly. T4 can bind to up to 155 Hoc proteins, and fusions between Hoc and either PA, LF, or EF were shown to bind to T4 and induce immune responses against all of the individual antigens, proving the concept that this scaffold could be used for display of multiple antigens simultaneously. Consistent with the FHV

studies, responses against multimeric PA were higher than monomeric PA.¹⁰⁹ After assembly of the Soc-PA proteins on T4 phage, cleaved anthrax protective antigen heptamers were attached and served as further scaffold for binding LF [Figure 3(A)]⁶⁷. Together these results indicate that VLP scaffolds have excellent potential to display antigens with complex structure, and combinations of antigens. Since larger and more complex immunogens are in many cases more similar to natural antigens, it may be that these systems for immunizing with larger antigenic proteins will be particularly fruitful for VLP-based vaccine design.

USING VLPS TO DISPLAY SELF ANTIGENS

Because of the success of attachment of antigen from foreign particles, exploration into attachment of self-peptides for the purposes of treating diseases has been attempted. The antibodies formed following administration of self-antigen often serve as competitive inhibitors to fight diseases. There are often intrinsic problems involved when administering the peptides alone, because in general it is difficult to break immunologic tolerance to self proteins and peptides are typically not very immunogenic. Attachment of self-antigen to a VLP often produces a heightened immune response because of the addition of foreign protein and multivalent display that has the potential to break tolerance. A variety of VLPs have been used to display self-antigens including HBV, FHV, MS2, and HPV.

One potential therapeutic avenue to prevent HIV infection is the production of autoantibodies against the HIV cellular receptor CCR5 as an 'indirect' route to vaccination, avoiding the problem of constant mutation of the viral antigens. A peptide from CCR5 was attached to HPV VLPs and when administered in mice, these particles initiated production of autoantibodies, which inhibited binding of the ligand and blocked infection of an indicator cell line expressing CCR5.¹⁷⁶ Q β has been used in a similar strategy for induction of anti-CCR5 antibodies. Production of IgA antibodies at mucosal sites following administration of an aerosolized pulmonary formulation of the Q β -based vaccine confirmed the potential for focusing the attack on HIV at the first line of contact at the mucosa of the human body.¹³⁵ The indirect route targeting CCR5 has been addressed using the FHV platform as well. Insertion of a CCR5 peptide on FHV, followed by administration results in reduced CCR5 on CD4+ cells in humans and mice.¹²⁹ Furthermore, peptides from the ECL2 loop of the CCR5 coreceptor of HIV have been inserted

into the coat proteins of MS2. The MS2 particles were shown to be potentially immunogenic.¹⁰⁵ While it is potentially risky to induce self-responses, HIV may be one example where further risk is warranted since other strategies vaccine development against HIV infection have not been successful.

Alzheimer's disease is a neurodegenerative disease that results in accumulation of A β peptide, neurofibrillary tangles, and loss of neurons leading to dementia. One therapeutic strategy has been the use of vaccination against A β to eliminate the accumulation of plaques in the brain in the hope of preserving neurons. The attachment of an A β peptide to papillomavirus VLP capsids induced high levels of specific antibodies and inhibited effective assembly of peptides into neurotoxic peptides *in vitro*. A β deposits were also reduced after immunization of a mouse model of Alzheimer's. However, clinical trials of another vaccine formulation against A β peptide resulted in development of encephalitis; therefore, it remains to be determined if vaccination will be effective in this and similar diseases.¹⁷⁷

OTHER NANOPARTICLE PLATFORMS FOR VIRAL VACCINES

Since researchers have come to better understand the requirements to generate an effective vaccine, the goal of a synthetic mimic of VNPs has become more attainable. In principle, synthetic particles might be even safer and easier to produce than VLPs, but their properties of immunogenicity and elimination *in vivo* are not as favorable. Nevertheless, some progress has been made toward synthetic nanoparticle vaccines. A few examples follow.

Biodegradable and biocompatible microparticles have been utilized in oral immunizations for induction of local and systemic immune responses. Poly (D, L-lactide-coglycolide) (PLG) is a biodegradable material, and manipulation of biodegradation is possible through changes to polymer composition and molecular weight. Oral and intragastric administration of these microparticles has resulted in immune responses to antigens trapped inside the particles, including peptide, bacterial toxoids, and bacterial cells.^{178–181} Although PLG microspheres have been studied more extensively as carriers of bacterial vaccines, a few examples of viral vaccines exist.^{166–168} This platform shows particular promise because it has been certified by the US Food and Drug Administration for its preparation as a delivery system.

Liposomes are made up of a phospholipid bilayer, often with cholesterol included to stabilize the artificial membrane.^{152–156,161} The preparation of

empty liposomes has not been challenging; however, successful incorporation of antigen presents some difficulties.¹⁸² In addition, liposomes often do not elicit a strong immune response necessitating the use of adjuvants. Although there has continued to be a slow and steady increase, only formulations which perform very well in experimental animals ever enter expensive clinical trials. These products continue to perform below expectancy in man.¹⁸²

ISCOMs, which are roughly 40 nm spherical micells composed of the saponin mixture Quil A (strong adjuvant), cholesterol, and phospholipids,¹⁸² have been licensed for use in horses and have made progress as an HSV-2 and a rotavirus vaccine.^{149–151,182} In spite of these advances, ISCOMs perform similarly to liposomes, and are therefore limited in progression.

Nanoemulsion (NE)-based vaccines are a promising noninflammatory mucosal adjuvant for use as nasal vaccine platform. The requirement of many current vaccines to be administered by injection has limited broad use of these vaccines worldwide. The use of a nasal-based delivery platform would facilitate vaccination in the developing world.^{171–173,175} These synthetic platforms are an exciting area of development, and as more is learned about how biologically based particles efficiently prime protective immune responses these principles will likely be translated into synthetic systems as well.

CONCLUSION

Virus-based vaccines share some of the same problems encountered with any vaccine. Even with efficient cellular and humoral immune responses, they rely on the longevity of the host response. In addition, use of VLPs as vaccines in immunocompromised patients will encounter the same problems with efficient immune response as other vaccine approaches. VLPs have shown more promise than many other subunit vaccines because they are conformationally authentic and safer due to lack of genetic material.¹⁸³ The success of HBV and HPV-based particles in humans has spurred intense interest in the development of VLPs and VNPs of all kinds, particularly the engineering of more complex structural antigens and multiple antigen delivery systems.

There are several obstacles unique to virus-based vaccines when considering bringing these particles from research tool to use in patient populations. One of these is the concern over potential toxicity of the nanoparticles in the human body. The slow speed of biodegradability of some materials, the ability to span biological membranes, and high

surface area and reactivity may lead to unacceptable toxic effects in humans. Another consideration is the complexity of clinical trials. Because vaccines are usually administered in healthy humans from infant to adult, trials in nonhuman primates are often needed to demonstrate safety and tolerability of the vaccine. In addition, preclinical studies involve immunization immediately followed by challenge to

test for protection. Human trials require waiting for an outbreak to occur before protection can be analyzed. Nevertheless, the benefits to human health have historically outweighed these drawbacks, as vaccines are considered one of the most effective means of improving global health worldwide and thus warrant sustained efforts to continually improve safety and efficacy.

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