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Development of Virus-Like Particle Technology from Small Highly Symmetric to Large Complex Virus-Like Particle Structures

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Key Words

Virus-like particles · Three-dimensional structure · Vaccines · Drug delivery · Biotechnology

Abstract

Virus-like particle (VLP) technology is a promising approach for the construction of novel vaccines, diagnostic tools, and gene therapy vectors. Initially, VLPs were primarily derived from non-enveloped icosahedral or helical viruses and proved to be viable vaccine candidates due to their effective presentation of epitopes in a native conformation. VLP technology has also been used to prepare chimeric VLPs decorated with genetically fused or chemically coupled epitope stretches selected from immunologically defined target proteins. However, structural constraints associated with the rigid geometrical architecture of icosahedral or helical VLPs pose challenges for the expression and presentation of large epitopes. Complex VLPs derived from non-symmetric enveloped viruses are increasingly being used to incorporate large epitopes and even full-length foreign proteins. Pleomorphic VLPs derived from influenza or other enveloped viruses can accommodate multiple full-length and/or chimeric proteins that can be rationally designed for multifunctional purposes, including multivalent vaccines. Therefore, a second generation of VLP carriers is represented by complex particles reconstructed from natural or chimeric structural proteins derived from complex enveloped viruses. Further development of safe and efficient VLP nanotechnology may require a rational combination of both approaches.

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VLP Concept and Its Evolution from Simple Icosahedral to Complex Enveloped Particles

Recombinant virus-like particles (VLPs) are nanodimensional structures that (1) are built from one or several viral structural constituents in the form of recombinant proteins synthesized in efficient homologous or primarily heterologous expression systems (bacteria, yeast, or eukaryotic cell culture); (3) are identical or closely related by their three-dimensional architecture and immunochemical characteristics to naturally occurring viral structures, and (3) lack genomes or infectivity [1–5]. VLPs can be formed from nucleocapsid or envelope proteins alone and the term VLP will be used here to describe any multimolecular structure formed by viral capsid and/or envelope proteins or by capsid and envelope combinations. The term *non-chimeric VLPs* is used for particles

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that are constructed from the original native non-modified viral proteins, whereas VLPs are defined as *chimeric* when the original structural proteins are covalently modified by the addition or substitution of foreign polypeptide stretches with functional properties, such as immunological epitopes or cell-targeting or encapsidation signals [5]. Covalent integration into chimeric VLPs can be achieved either by the expression of VLP monomer genes containing the appropriate insertions encoding the desired protein stretches [1–3, 5] or by the chemical coupling of peptides, proteins, or other molecules to VLPs [6]. Complex VLPs carrying full-length foreign proteins are defined here as *hybrids.*

Chimeric and/or hybrid VLPs target three main functional applications: (1) presentation of foreign epitopes leading to novel immunological content and subsequently to the creation of novel vaccines; (2) encapsidation of various therapeutic or diagnostic agents, such as nucleic acids as adjuvants for vaccines or gene therapy, proteins or mRNAs for diagnostic or therapeutic purposes, or low-molecular-weight drugs to be delivered to specific cells, and (3) specific targeting of desired organs, tissues, or cells.

Historically, the first two VLPs were derived from hepatitis B virus (HBV) and expressed in *Escherichia coli* and *Saccharomyces cerevisiae* during the mid-1980s. These VLPs were hepatitis B core (HBc) [7–9] and surface (HBs) [10] particles, which possessed icosahedral and octahedral symmetry, respectively [for a detailed review, see 11]. Later, the first representatives of VLPs derived from small RNA bacteriophages, non-enveloped bacterial viruses, were described [12–16]. The first rod-shaped chimeric VLPs with helical symmetry were derived at the same time from tobacco mosaic virus [17]. Enveloped VLPs from complex enveloped viruses, such as influenza viruses or the Ebola virus, were only introduced after more than 20 years of research [18–20].

In this review, we illustrate the evolution of the VLP concept from highly ordered symmetric VLPs to large complex VLPs derived from enveloped viruses. In most cases, symmetric VLPs are structurally well characterized by high-resolution techniques (protein X-ray crystallography and cryo-electron microscopy), whereas complex enveloped VLPs are often less structurally characterized. Figure 1 presents images of crystal structures of non-enveloped icosahedral viruses and experimental photomicrographs of complex enveloped viruses that have been used for the preparation of VLPs. Table 1 summarizes data on the various VLPs and demonstrates that almost all viral families have contributed to the development of VLP technology.

Non-Enveloped Icosahedral and Helical VLPs

Native Non-Chimeric VLPs

Non-enveloped native VLPs composed of unmodified viral proteins have been used as vaccines and configured as epitope display carriers (table 1). Recombinant icosahedral and, to lesser extent, helical VLP structures provide exceptionally effective environments for a high-density symmetric display of foreign oligopeptides, whereas three-dimensional maps of VLPs allow for the rational design of sites for epitope insertion. The regular repetitive pattern and correct conformation of the initial and inserted epitopes of symmetric VLPs have been recognized as critical features for the induction of a strong immunological response and for their potential use as highly efficient vaccines [for reviews, see 5, 6, 11, 21–28].

The interest in rationally designed manipulations of chimeric VLPs has been reinforced by the determination of the structures of many viral capsids by protein X-ray crystallography. The upper part of figure 1 shows crystal structures of representative icosahedral VLPs. As is evident, VLPs have been derived from all classes of viral genomes, i.e., double- and single-stranded DNA and RNA. Thus far, most VLPs have been derived from viruses with single-stranded RNA genomes of positive polarity lacking a DNA intermediate. Complex VLPs were recently constructed from large enveloped negative-strand RNA viruses such as influenza viruses and Ebola virus (see the section 'Complex VLPs derived from enveloped viruses' below).

As indicated in table 1, some icosahedral and helical VLPs have been used as vaccines in their native, non-chimeric form without the introduction of foreign sequences (for references, see table 1). For example, the HBs antigen that represents 22-nm particles produced in yeast has been a successful HBV vaccine since 1986. Another structural HBV protein, a nucleocapsid or HBc particle, is being tested as a component of a therapeutic HBV vaccine (table 1). Yet another example of a native, non-chimeric VLP vaccine are human papillomavirus (HPV) VLPs produced in yeast or baculovirus expression systems, which went onto the market in 2006 and 2007, respectively, as vaccines against cervical cancer. Norwalk virus and rotavirus VLPs against the major etiologic agents of epidemic gastroenteritis in humans are currently in clinical trials.

Animal circovirus and parvovirus VLP vaccines have been designed against infections in pigs and dogs (table 1). Other animal vaccines that are based on non-modified native VLPs have been prepared against calicivirus

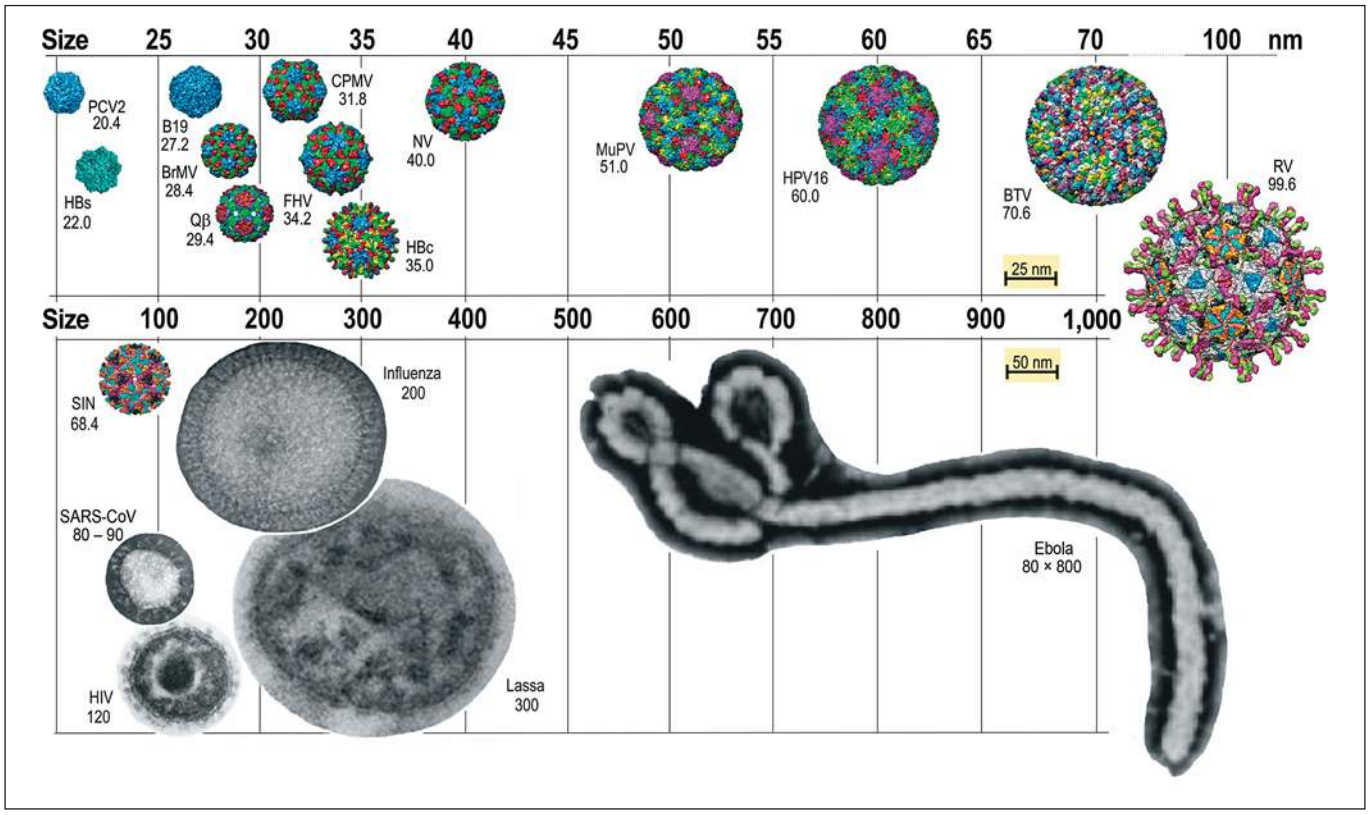

Fig. 1. Size-scaled presentation of the crystal structures of non-enveloped icosahedral viruses and experimental photomicrographs of the complex enveloped viruses that have been used for the preparation of VLPs. Three-dimensional models of structurally resolved particles are from the VIPERdb database [400, 401]. The structure of the HBs particle with octagonal symmetry is from Gilbert et al. [402]. Photomicrographs of enveloped viruses are from publicly available resources. For abbreviations, see table 1.

(RHDV), papillomavirus (BPV and CRPV), reovirus (BTV), and birnavirus (AHSV) infections.

Chimeric VLPs

Table 1 lists the published chimeric VLPs (including hybrid VLPs) that have been prepared by either gene fusion or chemical coupling of epitope peptides to the VLP surface, with an emphasis on vaccine and drug delivery applications.

The earliest chimeric structures, which appeared during the mid-1980s, were either based on tobacco mosaic virus [17] or derived from HBs protein [29, 30]. The development of the HBs protein has recently resulted in the first successful phase III clinical trial of a chimeric VLP vaccine, specifically the RTS,S vaccine against malaria [31, 32]. Among icosahedral VLPs, which appeared at the same time in the 1980s, HBc and VLPs derived from RNA phages have often been used as scaffolds for the construction of both prophylactic and therapeutic vaccine candidates, which are in clinical trials. For example, single-stranded RNA phages have been extensively used for the development of therapeutic vaccines by attaching genetically fused and chemically coupled epitopes (for references, see table 1).

Among other icosahedra that have advanced to chimeric VLPs, capsids from parvoviruses (B19, PPV, and CPV), comoviruses (CPMV), nodaviruses (FHV), polyomaviruses (MuPV and HaPV), and papillomaviruses (HPV and BPV) have proved to be sufficiently flexible to retain the ability to self-assemble after genetic fusion with foreign peptides. CMV, a representative of cucumoviruses, has demonstrated the unique ability to remain replication-competent and to produce progeny of chimeric viruses carrying foreign insertions. Bromoviruses, along with the CPMV [for a review, see 33], have formed a niche of nanotechnological materials involved mostly in a variety of packaging protocols using BrMV and CCMV VLPs [for a review, see 34, 35].

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Table 1. Summary of VLPs used for the development of vaccines, vectors/nanocontainers, and cell-targeting tools

Taxonomic description of the VLP sources: family, genus, species	VLPs as vaccines and/or vaccine candidates		VLPs as nanocages for the transportation of the following molecules	VLPs as delivery tools to the specified cell targets
	native non-chimeric VLPs	chimeric and/or hybrid VLPs carrying fused or chemically conjugated epitopes/fragments from the proteins indicated below		
Non-enveloped viruses: icosahedral symmetry				
Circoviridae Circovirus Porcine circovirus 2 (PCV2)	**PCV2** [92] (yeast), [93] *(E. coli)*			
Parvoviridae Erythrovirus Human parvovirus B19	**B19** [94, 95] (insect cells)	HSV-1, murine hepatitis virus (MHV) [96], dengue [97], anthrax [98] (all insect cells)		
Parvoviridae Brevidensovirus Infectious hypodermal and hematopoietic necrosis virus (IHHNV)	IHHNV [99] *(E. coli)*			shrimp hemocytes [99]
Parvoviridae Dependovirus Goose parvovirus (GPV)	**GPV** [100] (insect cells)			
Parvoviridae Parvovirus Porcine parvovirus (PPV)	**PPV** [101] (insect cells)	poliovirus [102], **PCV2** [103] (all insect cells)		
Canine parvovirus (CPV)	**CPV** [104] (insect cells)	poliovirus [105], rabies [106]		transferrin receptors [107]
Hepeviridae Hepevirus Hepatitis E virus (HEV)	HEV [108] (plants)	herpes simplex virus [109] (insect cells)		
Bromoviridae Alfamovirus Alfalfa mosaic virus (AlMV)		rabies [110], respiratory syncytial virus (RSV) [111], anthrax [112] (plants)		
Bromoviridae Bromovirus Brome mosaic virus (BrMV)			nanogold [113]	
Cowpea chlorotic mottle virus (CCMV)			polystyrene [114], nanometal [115], RNA [116], phthalocyanine [117]	
Bromoviridae Cucumovirus Cucumber mosaic virus (CMV)		HCV [118], Newcastle disease virus (NDV) [119], Alzheimer's disease [120] (plants)	DNA, a protein and a fluorophore [121]	
Leviviridae Levivirus Enterobacteria phage MS2		HIV-1 [122, 123], malaria [124] (all *E. coli*)	RNA [125–130], ricin A [131], proteins and drugs [129]	leukaemia cells [126], HCC [129]
Enterobacteria phage PP7		HIV-1 [132], HPV [132, 133] *(E. coli)*		
Enterobacteria phage fr		HBV (Pushko P, unpubl.), HaPV [134] *(E. coli)*	CpG (Renhofa R, unpubl.)	
Enterobacteria phage GA			RNA [135], CpG, LMW drugs (Renhofa R, unpubl.)	CXCR4-overproducing cells (Renhofa R, unpubl.)
Acinetobacter phage AP205		hypertension, GnRH, HIV-1, influenza A [136], WNV [137] *(E. coli)*	RNA, CpG, magnetic iron nanoparticles (Renhofa R, unpubl.)	HBV receptors (Renhofa R, unpubl.)
Leviviridae Allolevivirus Enterobacteria phage Qβ	**Allergy**, therapeutic vaccine [80, 138] *(E. coli)*	HBV [139], influenza [140], therapeutic vaccines: **smoking** [141], **hypertension** [142], melanoma [143], arthritis [144, 145], Alzheimer's disease [146], obesity [147], osteoporosis [148], eosinophilia [149], HIV-1 [150], cat allergy [151] (all *E. coli*)	RNA [78], enzymes [152], proteins [153], drugs [154, 155]	targeting by ZZ domain [156], EGF targeting [157], heparin reversion [158]

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Taxonomic description of the VLP sources: family, genus, species	VLPs as vaccines and/or vaccine candidates		VLPs as nanocages for the transportation of the following molecules	VLPs as delivery tools to the specified cell targets
	native non-chimeric VLPs	chimeric and/or hybrid VLPs carrying fused or chemically conjugated epitopes/fragments from the proteins indicated below		
Leviviridae unclassified Caulobacter phage phiCb5			metal ion-dependent assembly [159] *(E. coli)*	
Secoviridae Comovirus Cowpea mosaic virus (CPMV)		*Pseudomonas aeruginosa* [160], **mink enteritis (MEV)** [161], **CPV** [162] (plants)	nanobuilding blocks (plants) [163–166]	tumour cells [167, 168], intravital imaging [169, 170]
Picornaviridae Cardiovirus Porcine encephalomyocarditis virus (EMCV)	EMCV [171] (insect cells)			
Picornaviridae Enterovirus Enterovirus type 71 (EV71)	EV71 [172] (insect cells)			
Coxsackievirus B (CVB3)	CVB3 [173] (insect cells)			
Picornaviridae Aphthovirus Foot-and-mouth disease virus (FDMV)	FDMV [174] *(E. coli)*, [175] (insect cells)			
Nodaviridae Alphanodavirus Flock house virus (FHV)		anthrax [176] (insect cells)		
Tombusviridae Tombusvirus Tomato bushy stunt virus		ricin toxin [177] (insect cells)		
Nodaviridae Betanodavirus Malabaricus grouper nervous necrosis virus (MGNNV)	**viral nervous necrosis** [178] (insect cells)			
Dragon grouper nervous necrosis virus (DGNNV)	**viral nervous necrosis** [179] *(E. coli)*			
Caliciviridae Norovirus Norwalk virus (NV)	NV [180–182] (plants), [183] (insect cells)			
Caliciviridae Lagovirus Rabbit hemorrhagic disease virus (RHDV)	**RHDV** [184] (insect cells), [185] *(S. cerevisiae)*, [186] *(Pichia pastoris)*	FMDV [187, 188], feline calicivirus (FCV) [188], ovalbumin [189] (insect cells)		dendritic cells [190]
Polyomaviridae Polyomavirus Murine polyomavirus (MuPV)		HBV[191] (yeast), antitumour [192–194] (insect cells), influenza [195] *(E. coli)*		tumour cells [196]
Hamster polyomavirus (HaPV)		HBV [197], hanta [198], antitumour [199, 200], *Gardnerella vaginalis* [201] (all *S. cerevisiae*)	DNA [202]	
Simian virus 40 (SV40)			proteins [203], quantum dots [204]	RGD targeting [205], EGF receptor [206]
JC polyomavirus			RNAi [207], DNA [208]	
Papillomaviridae Alphapapillomavirus Human papillomavirus (HPV)	**HPV**: **Gardasil** [209] (yeast), **Cervarix** [210] (insect cells)	SIV/HIV [211], **HPV**: **L1+E7** [212, 213] (insect cells), influenza [214], HPV E6, E7 [215] (plants)	DNA, packaging in GFP-decorated VLPs [216], HEV ORF [217]	DNA delivery to dendritic cells [218], B cells [219]

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	native non-chimeric VLPs	chimeric and/or hybrid VLPs carrying fused or chemically conjugated epitopes/fragments from the proteins indicated below		
Papillomaviridae Deltapapillomavirus Bovine papillomavirus (BPV)	**BPV** [220] (insect cells)	HPV [221], Alzheimer's disease [222], antitumour [223] (insect cells)	DNA [224]	
Papillomaviridae Kappapapillomavirus Cottontail rabbit papillomavirus (CRPV)	**CRPV** [225] *(S. cerevisiae)*, [226, 227] (insect cells), [228] (plants)			
Reoviridae Orbivirus Bluetongue virus (BTV)	BTV [229–232] (insect cells)	HBV [233], rabies virus [234], influenza [235]		
African horse sickness virus (AHSV)	AHSV [236] (mammalian cells)	FMDV, AHSV 2 [237]		
Reoviridae Rotavirus Rotavirus (RT)	RT [238–240], RT + noro [241] (insect cells)	chimeric RT, VP8 fusion to VP2 [242] (insect cells)	GFP [243] (insect cells)	intestinal cells [243]
Yeast retrotransposon Ty1		malaria [244–246], **HIV** [247–249], influenza, Sendai virus, VSV [250] (all yeast)		
Podoviridae T7-like viruses Enterobacteria phage T7				HepG2 cells [251]
Podoviridae P22-like viruses Enterobacteria phage P22			small molecules, polymers, metals [252] *(E. coli)*	
Siphoviridae Lambda-like viruses Enterobacteria phage lambda		HIV-1 [253] (*E. coli*, conjugation)		
Adenoviridae Mastadenovirus Human adenovirus B (type 3) (Ad3)		cancer [254] (insect cells)	proteins [255], bleomycin [256]	
Non-enveloped viruses: helical symmetry				
Alphaflexiviridae Potexvirus Papaya mosaic virus (PapMV)		influenza A [257, 258] (plants)		
Potato virus X (PVX)		HIV-1 [259], beet necrotic yellow vein virus (BNYVV) [260], tuberculosis [261], influenza [262], HCV [263], HPV [264, 265] (all plants)		targeting model [266]
Bamboo mosaic virus (BaMV)		FMDV [267], IBDV [268] (all plants)		
Potyviridae Potyvirus Johnsongrass mosaic virus (JGMV)		*Plasmodium falciparum* [269], Japanese encephalitis virus (JEV) [270], gamete epitopes for immunocontraception [271] (all *E. coli*)		
Plum pox virus (PPV)	**Sharka disease** [272] (plants)	CPV [273] (plants)		
Potato virus Y (PVY)		HBV [274] *(E. coli)*		
Virgaviridae Tobamovirus Tobacco mosaic virus (TMV)		poliovirus: [17] *(E. coli)*, [275] (plants); malaria [276], influenza, HIV-1 [277], rabies virus (RV) [278], MHV [278, 279], *P. aeruginosa* [280], FMDV [281], CRPV, rabbit oral papillomavirus (ROPV) [282] (plants)	RNA [283]	

Table 1 (continued)

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	native non-chimeric VLPs	chimeric and/or hybrid VLPs carrying fused or chemically conjugated epitopes/fragments from the proteins indicated below		
Enveloped viruses: icosahedral symmetry				
Hepadnaviridae Orthohepadnavirus Hepatitis B virus (HBV) core (HBc) particles	therapeutic HBV [11, 284], combined with HBs [285] *(E. coli)*	FMDV: eukaryotic cells [286], *E. coli* [287], plants [288]; HPV: *E. coli* [289, 290], *Salmonella typhimurium* [291], HIV-1, HBV [11, 191], hepatocellular carcinoma [292], hanta [293, 294], HCV [295, 296], anthrax [297], Crohn's disease [298], tuberculosis [299], **influenza** [300–303] *(E. coli)*, [304] (plants), **malaria** [305–307], ***Borrelia burgdorferi*** [308–310], **cattle theileriosis** [311], **IBDV** [312] *(E. coli)*	CpG [78, 79]; RNA, CpG, proteins, magnetic iron nanoparticles (Renhofa R, unpubl.)	fibronectin targeting by *B. burgdorferi* BBK32 protein fragments [313]
Hepatitis B virus (HBV) surface (HBs) particles	**HBV: Engerix-B** [314], **H-B-Vax II**, or **Recombivax HB** [315] *(S. cerevisiae)*, **Heplisav** [316] *(Hansenula polymorpha)*, combined with HBc [285] (yeast), edible vaccine [317–320] (plants)	HSV-1 [29], **HBV: preS-HBs therapeutic GenHevac B** [321, 322], **Bio-Hep-B** [323], **Hepacare** (mammalian cells) [324] (mammalian cells), **malaria RTS,S** [31, 32] *(S. cerevisiae)*, poliovirus 1 and 2 [30], SIV [325] (both mammalian cells), HIV-1: mammalian cells [326], yeast [327], plants [328], HPV [290] *(S. cerevisiae)*, HEV [329], dengue [330] (both *P. pastoris*), HCV [331, 332], influenza [333], *Helicobacter pylori* [334] (all mammalian cells)		
Woodchuck hepatitis virus (WHV) core (WHc) particles		influenza [335] *(S. typhimurium)*		
Flaviviridae Flavivirus West Nile virus (WNV)	WNV [336] (mammalian cells)			
Dengue virus	Dengue [337] (mammalian cells), [338] *(P. pastoris)*, [339, 340] *(E. coli)*			
Hepatitis C virus (HCV)	HCV [341–343] (insect cells), [344, 345] *(P. pastoris)*			
Japanese encephalitis virus (JEV)	JEV [346–348] (mammalian cells)			
Tick-borne encephalitis virus (TEV)	TEV [345] (mammalian cells)			
Togaviridae Alphavirus Sindbis virus (SIN)	SIN: core-like particles [349, 350] (*E. coli*)			
Ross river virus (RRV)	RRV: core-like particles [349, 350] *(E. coli)*, VLPs [351] (*E. coli*; mammalian cells)		gold nanoparticles [352]	
Chikungunya virus (CHIKV)	CHIKV [353] (mammalian cells)			
Salmonid alphavirus (SAV)	SAV [354] (insect cells)			
Coronaviridae Betacoronavirus Severe acute respiratory syndrome-related coronavirus (SARS-CoV)	SARS [355] (insect cells)	SARS influenza [48] (insect cells)		

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	native non-chimeric VLPs	chimeric and/or hybrid VLPs carrying fused or chemically conjugated epitopes/fragments from the proteins indicated below		
Bunyaviridae Nairovirus Crimean-Congo hemorrhagic fever (CCHF) virus	CCHF [356] (insect cells)			
Bunyaviridae Phlebovirus Rift Valley fever virus (RVFV)	RVFV [357] (mammalian cells)			
Bunyaviridae Hantavirus Hantaan virus (HTNV)	HTNV [358] (mammalian cells)			
Retroviridae Lentivirus Human immunodeficiency virus 1 (HIV-1)	HIV-1 [41, 49, 65, 359] (mammalian cells), [360–362] (insect cells)	HIV-1 [363] (mammalian cells), [46, 364] (insect cells), equine herpesvirus type 1 (EHV-1) [365], pseudorabies virus [366] (both insect cells), HPV [367] (mammalian cells)	proteins [368–370]	
Human immunodeficiency virus 2 (HIV-2)	HIV-2 [371] (insect cells)	HIV-1 and HIV-2 [372, 373] (insect cells)	proteins [368]	
Simian immunodeficiency virus (SIV)	SIV [374] (mammalian cells), [375] (insect cells)	SHIV [376, 377] (insect cells)		
Retroviridae Alpharetrovirus Rous sarcoma virus (Rous SV)	Rous SV [378] (insect cells)	human prorenin receptor (hPRR) [379] (insect cells)		
Retroviridae Gammaretrovirus Murine leukemia virus (MLV)		Alzheimer's disease [380], prion disease [381], VSV [382], cytokines [383], influenza [384] (all insect cells)		
Herpesviridae Lymphocryptovirus Human herpesvirus 4 (Epstein-Barr virus, EBV)	EBV [385] (mammalian cells)			
Enveloped complex viruses				
Orthomyxoviridae Influenzavirus A Influenza A virus	Influenza A [42–44, 54, 58, 386–388] (mammalian, insect cells)	multi-subtype influenza [70], influenza-pseudotyped Gag [52]	CAT RNA [389]	TLR [59, 390]
Arenaviridae Arenavirus Lassa virus	Lassa virus [45] (mammalian cells)			
Tacaribe and Junin viruses		Tacaribe and Junin arenaviruses [391]		
Paramyxoviridae Avulavirus Newcastle disease virus		RSV-NDV (avian) [47, 76]		
Respiratory syncytial virus (RSV)				
Paramyxoviridae Henipavirus Nipah virus (NiV)	NiV [392] (mammalian cells)			
Paramixoviridae Respirovirus Sendai virus (SeV)	SeV [393] (mammalian cells), [87] *(S. cerevisiae)*			
Human parainfluenza virus 1 and 3	[89] *(S. cerevisiae)*			
Paramyxoviridae Rubulavirus Mumps virus (MuV)	MuV [394] (mammalian cells), [85] *(S. cerevisiae)*			

Table 1 (continued)

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	native non-chimeric VLPs	chimeric and/or hybrid VLPs carrying fused or chemically conjugated epitopes/fragments from the proteins indicated below		
Menangle virus (MeV)	[88] (*S. cerevisiae*)			
Tioman virus (TioV)	[90] (*S. cerevisiae*)			
Paramyxoviridae Metapneumovirus Human metapneumovirus (hMPV)	[91] (*S. cerevisiae*)			
Paramyxoviridae Morbillivirus Measles virus (MV)	[86] (*S. cerevisiae*)			
Filoviridae Marburgvirus Marburg marburgvirus (MARV)	Marburg marburgvirus [395] (mammalian cells)	hybrid MARV and EBOV [396]		
Filoviridae Ebolavirus Zaire ebolavirus (EBOV)	Zaire ebolavirus [18, 397–399] (mammalian, insect cells)	hybrid MARV and EBOV [396]		

VLPs are listed in the order of size according to figure 1. Clinically and veterinary-approved vaccines are depicted in bold. Expression systems used for the production of VLPs are shown in parentheses.

The taxonomic orders of VLP sources are provided in accordance with the official ICTV 2011 virus taxonomy available at http://ictvonline.org/virusTaxonomy.asp?version=2011. LMW = Low-molecular-weight.

Rod-shaped plant viruses of helical symmetry provide a number of replication-competent examples allowing the production of chimeric viruses, with the exception of potexviruses (papaya and bamboo mosaic viruses and PVX) and some potyviruses (JGMV, PVA, and PVY), which accept foreign insertions in the VLPs after propagation in *E. coli* and baculovirus expression systems (for references, see table 1).

Complex VLPs Derived from Enveloped Viruses

Construction of Complex Enveloped VLPs

Enveloped viruses are widely used for the development of VLPs. The viral envelope represents a lipoprotein membrane that surrounds a nucleocapsid [36, 37]. The lipid bilayer is derived from host cell membranes and contains embedded viral proteins responsible for receptor recognition or other functions. It can also include carbohydrates and proteins derived from the host cell. Envelope proteins usually contain hydrophobic domains that allow their localization within the lipid bilayer. Similar to non-enveloped VLPs, the repeated regular patterns of epitopes in the native conformation make complex enveloped VLPs exceptionally effective immunogens [24, 38].

From the point of view of their spatial structure, the majority of viral envelopes are asymmetrical. However, for some enveloped viruses, viral proteins within the envelope reflect the symmetry of the inner nucleocapsid. For example, alphaviruses contain 240 heterodimers of E2-E1 proteins arranged in an icosahedral lattice according to the 240 subunits of the capsid protein within the inner icosahedral nucleocapsid [39, 40]. Other viruses, such as the orthomyxovirus influenza virus, contain envelopes that do not possess an apparent regular geometric symmetry, although they may contain elements of symmetry such as the trimers of hemagglutinin (HA) or tetramers of neuraminidase (NA) in influenza viruses.

The preparation of complex enveloped VLPs is challenging. Unlike simple icosahedral particles, enveloped VLPs often contain multiple species of lipid-associated structural proteins. In most cases, the protein-protein interactions and the roles of the proteins, lipids, and host cell factors in the assembly of VLPs are not fully understood. To achieve correct assembly of enveloped VLPs, proper interactions must occur between the nucleocapsid proteins, between the envelope proteins within the lipid bilayer, and between the envelope and the nucleocapsid proteins. The production must include an appropriate cell line and expression system, which should express suf-

ficient quantities of enveloped VLPs that are immunologically similar to their cognate virus, including the arrangement of the proteins within the envelope. Furthermore, purification of enveloped VLPs can be a difficult task. During production and purification of VLPs, various cellular lipids, carbohydrates, proteins, and nucleic acids co-purify with VLPs, which may cause difficulties in purification as well as safety and regulatory concerns if VLPs are intended for clinical applications.

Despite the challenges, enveloped VLPs of several viruses have been constructed, including VLPs for HIV, influenza viruses, Ebola virus, Lassa virus, and other viruses (table 1). VLPs have been shown to induce advantageous immune responses compared with soluble recombinant proteins. For example, membrane-embedded HIV-1 envelope proteins on the surface of VLPs elicited broader immune responses than did soluble envelope proteins [41]. Similarly, influenza VLPs containing HA protein within the envelope demonstrated greater immunogenicity than subunit HA [19, 42, 43]. In some studies, native enveloped VLPs could be made with unmodified viral proteins [19, 38, 44–46]. In other cases, extensive optimization and engineering of component proteins were required to generate VLPs [46–48].

Rational Design of Enveloped VLPs

Rational design of VLPs has been used extensively to improve the properties of enveloped VLPs, including expression levels, stability, immunogenicity, and other characteristics. First, attempts have been made to improve the expression of component proteins to improve the production of the assembled VLPs. For example, codon optimization of the expressed viral genes has improved the expression of HIV and HTLV-1 VLPs [49, 50]. Second, efforts have been made to optimize the composition of VLPs and the interactions between the individual VLP components. For example, in many cases, influenza VLPs were prepared using HA, NA, and M1 proteins derived from the same strain [19, 42–44]. Other configurations of VLPs have included a heterologous M1 derived from an unrelated influenza virus strain [51] or even a retrovirus Gag protein in place of M1 [52]. Furthermore, influenza VLPs have been made from HA and NA only [53], from HA and M1 proteins only [54], and from NA and M1 only [55]. VLPs have also been generated in the presence of additional influenza virus proteins, such as M2 [56–58], or in the presence of immunostimulatory antigens such as flagellin, a ligand for Toll-like receptor 5 [59]. These studies have helped elucidate the contributions of individual components to influenza VLP formation. For example, M1 protein is the major component of the virus and an important antigen due to the presence of subtype cross-reactive epitopes [60]. However, the role of M1 in the formation of VLPs or viral particles is not completely understood. According to one view, M1 is the driving force for influenza virus particle formation, penetrating through the cell membrane from inside the cell and interacting with the cytoplasmic tails (CT) of the structural glycoproteins HA and NA during budding [61–63]. An alternative model suggests that HA and NA partition into lipid raft microdomains and recruit the internal viral components, including M1, into the budding viral particle [53]. VLP studies have shown that VLPs can be formed either with or without M1, suggesting both M1-dependent and M1-independent mechanisms of VLP formation. This hypothesis is further supported by the fact that both M1-deficient and M1-containing types of the mature enveloped virions have been observed by cryo-electron microscopy [64].

Rational design has also been applied to HIV VLPs to improve immunogenicity and efficacy. One approach utilized consensus Env protein sequences [65]. To address the sequence diversity in Env sequences, consensus sequences were made for clade B and clade C envelope proteins. The rationale for using a consensus sequence is that the genetic difference between the vaccine strain and any given viral isolate is decreased, and, therefore, a consensus sequence will provide broader vaccine coverage. The consensus Env sequence elicited broader cell-mediated peripheral and mucosal immune responses than did polyvalent and monovalent Env protein vaccines [65]. In another study, the consensus approach was combined with protein engineering methods to increase the density of Env glycoprotein within the VLP envelopes [46]. Chimeric Env gene constructs have been engineered, in which the coding sequences for the signal peptide (SP), transmembrane (TM), and CT domains of HIV-1 Env were replaced with those of other viral or cellular proteins. Substitution of the SP from the honeybee mellitin protein resulted in 3-fold higher expression of recombinant HIV-1 Env on insect cell surfaces and increased Env incorporation into VLPs. Substitution of the HIV TM-CT with sequences derived from mouse mammary tumour virus (MMTV) envelope glycoprotein, influenza virus HA, or baculovirus (BV) gp64, but not Lassa fever virus glycoprotein, was shown to enhance Env incorporation into VLPs. The highest level of Env incorporation into VLPs was observed in constructs containing the MMTV and BV gp64 TM-CT domains in which the Gag/

Env molar ratios were estimated to be 4:1 and 5:1, respectively, compared with a 56:1 ratio for full-length consensus gp160 [46].

Additional examples of rational design include engineering of simian human immunodeficiency virus (SHIV) VLPs containing mutant HIV Env with reduced glycosylation, variable loop-deleted mutations, or combinations of both types of mutations [66].

Presentation of Foreign Proteins within Enveloped VLPs

Unlike VLPs from icosahedral viruses such as HPV, which are composed of a specific number of subunits symmetrically arranged in a geometric structure [67, 68], enveloped VLPs allow considerable structural flexibility. The lipid bilayer is relatively fluid, allowing greater flexibility than the rigid geometric lattice of icosahedral VLPs. This flexibility may provide considerable structural capacity and allow the expression of large epitopes or even entire foreign proteins as an integral part of enveloped VLPs.

In addition to accommodating larger foreign epitopes, the structural flexibility of enveloped VLPs makes possible the preparation of chimeric and hybrid VLPs that colocalize proteins from distinct strains or even from distinct viruses.

Influenza VLPs have been used as carriers for foreign antigenic epitopes or proteins. For example, it has been shown that influenza VLPs can incorporate vesicular stomatitis virus (VSV) G protein [56]. As mentioned previously, influenza VLPs can also incorporate flagellin [59]. In another study, *Bacillus anthracis* protective antigen (BPA) fragments 90 or 140 aa in length were inserted at the C-terminal flank of the HA SP and expressed as the HA1 subunit [69]. The chimeric proteins could be cleaved into the HA1 and HA2 subunits by trypsin and incorporated into influenza viruses, suggesting that the viral envelope can tolerate foreign insertions without precluding assembly. The inserted BPA domains were maintained in the HA gene segments following several passages in MDCK cells or embryonated chicken eggs. Immunization of mice with viruses that expressed the chimeric BPA/HA proteins induced antibody responses against both the HA and BPA components of the protein [69]. Although VLPs were not generated in this study, these experiments suggest that similar modifications of HA with foreign epitopes may be compatible with the formation of chimeric influenza VLPs.

A novel influenza VLP platform containing HA subtypes derived from three distinct strains has also been reported [70]. This recombinant VLP design resulted in the expression of three HA subtypes co-localized within a VLP (fig. 2). Experimental triple-HA VLPs containing HA proteins derived from seasonal H1N1, H3N2, and type B influenza viruses were immunogenic and protected ferrets from challenge with all three seasonal viruses. Similarly, VLPs containing HA subtypes derived from H5N1, H7N2, and H2N2 viruses protected ferrets from three potentially pandemic influenza viruses [70]. This technology may represent a novel strategy for the rapid development of trivalent seasonal and pandemic influenza vaccines.

The possibility of co-localizing proteins from distinct viruses within the VLP is somewhat similar to the phenotypic mixing that has been observed with live viruses. Phenotypic mixing of proteins from different enveloped viruses has been described between SV5, a paramyxovirus, and VSV, a rhabdovirus, as well as between Sendai and VSV [71, 72]. Reassortant viruses have also been generated by co-infection of embryonated chicken eggs with influenza type A and type B viruses [73]. Recently, live-attenuated chimeric viruses containing HA from A/PR8 (H1), A/HK68 (H3), or A/VN (H5) strains in the backbone of the B/Yamagata/88 virus were reported [74]. Reassortment is routinely used in licensed influenza vaccines. In the reassortant vaccine virus, vaccine-relevant HA and NA glycoproteins are embedded into the envelope, whereas other viral proteins are derived from type-specific donor strains, such as the A/PR/8/34 (H1N1) or B/Ann Arbor/1/66 viruses [75].

In some cases, foreign antigens can only be expressed as VLPs by displaying them within chimeric/hybrid VLPs. For example, VLPs composed entirely of RSV proteins were produced at levels that were inadequate for their use as vaccines [47]. However, VLPs composed of the Newcastle disease virus (NDV) nucleocapsid and membrane proteins and chimeric proteins containing the ectodomains of RSV F and G proteins fused to the TM and cytoplasmic domains of NDV F and HN proteins, respectively, were quantitatively prepared from cultured avian cells. Immunization with a single dose of VLPs resulted in the complete protection of mice against RSV replication in lungs [47]. Another chimeric VLP was composed of the NP and M proteins of NDV and a chimeric protein containing the cytoplasmic and TM domains of the NDV HN protein and the ectodomain of the human RSV G protein (H/G) [76]. The VLP-H/G candidate vaccine was immunogenic in BALB/c mice and prevented replication of RSV in murine lungs with no evidence of immunopathology [76]. Chimeric VLPs consist-

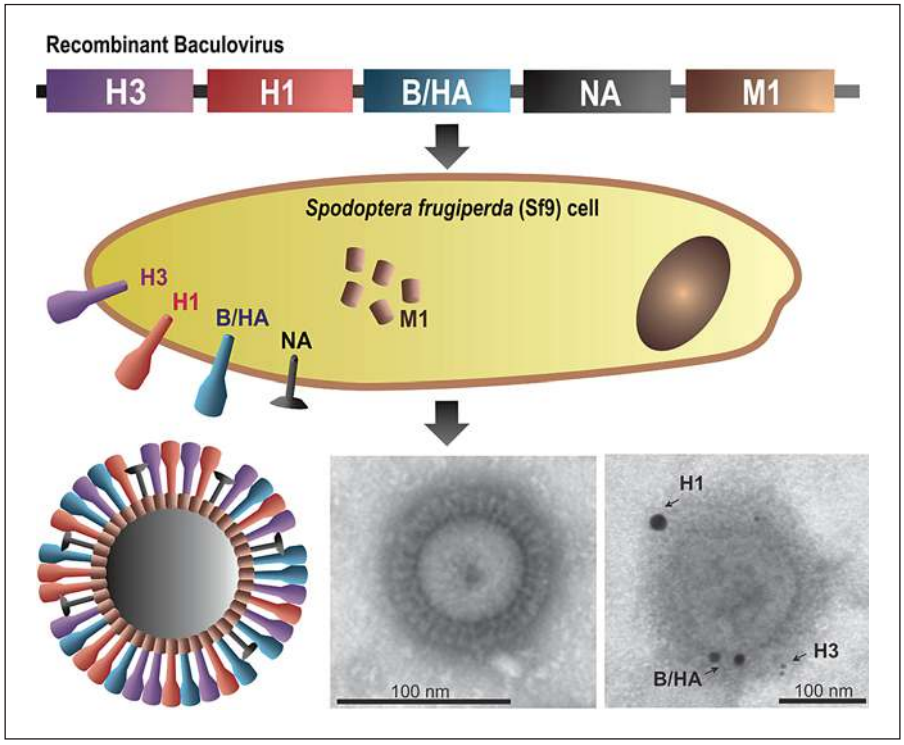

Fig. 2. Preparation and electron microscopy of a triple-subtype VLP vaccine containing seasonal influenza A H1 and H3 subtypes and influenza B virus HA. Recombinant baculovirus (rBV) for the expression of the triple-HA VLPs in Sf9 cells contained influenza HA gene sequences derived from A/New Caledonia/20/1999 (H1N1), A/New York/55/2004 (H3N2) and B/Shanghai/361/2002. NA and M1 gene sequences were from the A/Indonesia/05/2005 (H5N1) virus [70]. Negative stain transmission electron microscopy (bottom middle) was performed by staining with 1% phosphotungstic acid. Immunoelectron microscopy (bottom right) was performed using sucrose gradient-purified VLPs. VLPs were probed with a mixture of primary antibodies specific for H1, H3, and type B influenza from rabbit, mouse, and guinea pigs, respectively. Secondary antibodies were donkey anti-rabbit labelled with 18-nm gold particles, anti-mouse labelled with 6-nm gold particles, and anti-guinea pig labelled with 12-nm gold particles. Bars: 100 nm.

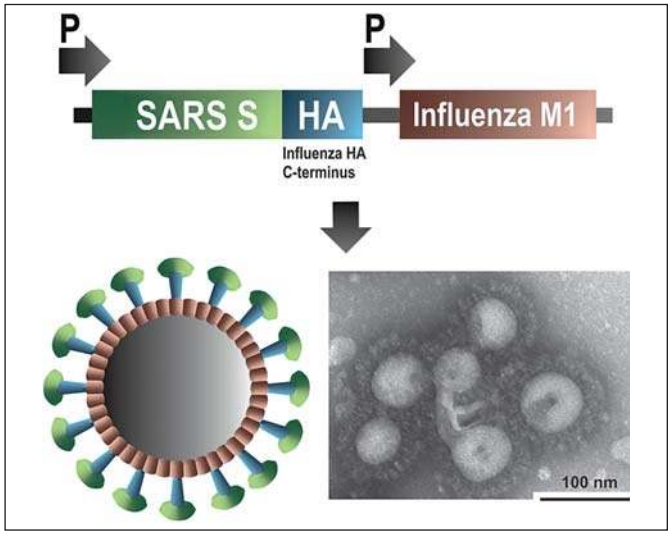

Fig. 3. Preparation of hybrid/chimeric SARS-CoV VLPs using influenza M1 protein and chimeric SARS-CoV S protein (GenBank AAP13441), containing a carboxy-terminal TM sequence derived from influenza HA. TM helices were predicted using TransMem based upon a Hidden Markov Model. The TM domain was derived from influenza A/Indonesia/5/2005 HA (aa 531–568). Chimeric SARS-CoV S and influenza M1 proteins (ABI36004) were co-expressed from recombinant baculovirus in *Spodoptera frugiperda* Sf9 cells [48]. An electron photomicrograph of hybrid/chimeric SARS-CoV VLPs is shown with a schematic model.

ing of an influenza virus matrix (M1) protein core and RSV-F or RSV-G on the surface have also been produced [77]. These data support the further development of VLPs as vaccines for protection against RSV.

Similar to RSV VLPs, VLPs composed entirely of SARS-CoV proteins have been produced at low levels [48]. A method was developed to produce higher levels of SARS VLPs containing the SARS spike (S) protein and the influenza M1 protein (fig. 3). The SARS VLPs protected mice from death when administered via the intramuscular or intranasal routes [48].

Conclusions

It has been more than 25 years since the idea of using highly symmetric self-assembling VLPs for vaccine purposes attracted considerable attention (fig. 1). Applications of VLPs have included their use as carriers to improve the immunogenicity of poorly immunogenic epitopes. With time, over-optimistic expectations for simple and rapid generation of effective VLP-based vaccines have encountered numerous challenges. Nevertheless, as shown in this review, many promising vaccine candidates have been produced (table 1). Renewal of the

previous optimism is due at least in part to the recent development of VLPs of large complex viruses, such as orthomyxoviruses, filoviruses, and paramyxoviruses. Due to their larger size and versatile structure, complex VLPs demonstrate greater capacity and flexibility than highly symmetric icosahedral VLPs for the inclusion of full-length, properly folded foreign proteins. Furthermore, complex VLPs are capable of simultaneously accepting various foreign proteins, enabling construction of multivalent vaccines. Full-length foreign proteins or epitopes delivered by large complex VLPs offer many immunological advantages over short epitopes, which have been inserted onto the surface of icosahedral scaffolds. First, complex VLPs can enhance T helper and cytotoxic T cell responses by inducing highly specific immunological responses directed against conformational epitopes, which is difficult to achieve with chimeric symmetric VLPs. Second, due to their size and organization, complex VLPs may be stable in the blood and thereby obviate the need for adjuvants. Third, if necessary, complex VLPs can be configured to contain specific and efficient adjuvants, including immune stimulatory sequences such as CpGs [78–81]. The presence of a lipid envelope may contribute to the correct folding of certain vaccine antigens and thereby improve immunogenicity. For example, the immunogenicity of purified subunit HA could be improved by adding liposomes [19]. The immunogenicity of split influenza vaccines could also be improved by adding liposomes or lipid-based adjuvants, such as MF59, an oil-in-water emulsion containing the unsaturated aliphatic hydrocarbon squalene [82]. Interestingly, highly immunogenic influenza vaccines, such as whole virus vaccines, VLPs, and virosomes, all have a lipid component in common, suggesting that lipids may play an important role in influenza vaccine immunogenicity.

Growing interest in knowledge-based applications of complex VLPs is strengthened by the development of high-resolution methods [64, 83, 84].

The rapid development of therapeutic candidates based on complex VLPs is propelled by the constant progress of cGMP-compliant production systems, such as baculovirus system-driven production, and the recent approval of a baculovirus-derived HPV VLP vaccine by the US Food and Drug Administration and other regulatory agencies (table 1). However, purification of complex enveloped VLPs to a standard that meets regulatory and safety requirements can be a difficult task. Unlike icosahedral VLPs such as HPV, complex enveloped VLPs are difficult to purify by conventional methods such as refolding in vitro. Therefore, the icosahedral VLP platform remains a good option for many applications due to the advantages of purification as well as a clear regulatory pathway due to the availability of licensed vaccines. The introduction of yeast expression systems into the production of complex VLPs can potentially improve the safety and quality control of biotechnological processes [85–91].

One potential method for the development of VLP-based vaccines may involve the combination of both complex and icosahedral particles, from a simple mixing of potential vaccine candidates (e.g., the therapeutic hepatitis B vaccine DV-601, http://www.dynavax.com/hepatitis_bt.html) to the knowledge-based packaging of small icosahedral VLPs into large complex VLPs in the future.

Finally, both symmetric and complex VLPs, which were initially used primarily for vaccine development, are now evolving in the direction of broader applications, including gene and drug therapies. New applications for VLPs include the targeting of specific cells and encapsidation of new materials, from oligonucleotides, genes, proteins, and peptides to therapeutic drugs and inorganic compounds such as gold and iron nanoparticles. For each application, careful consideration of both non-enveloped and enveloped VLPs may be necessary.

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