Different Applications of Virus-Like Particles in Biology and Medicine: Vaccination and Delivery Systems

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ABSTRACT:

Virus-like particles (VLPs) mimic the whole construct of virus particles devoid of viral genome as used in subunit vaccine design. VLPs can elicit efficient protective immunity as direct immunogens compared to soluble antigens co-administered with adjuvants in several booster injections. Up to now, several prokaryotic and eukaryotic systems such as insect, yeast, plant, and E. coli were used to express recombinant proteins, especially for VLP production. Recent studies are also generating VLPs in plants using different transient expression vectors for edible vaccines. VLPs and viral particles have been applied for different functions such as gene therapy, vaccination, nanotechnology, and diagnostics. Herein, we describe VLP production in different systems as well as its applications in biology and medicine. © 2015 Wiley Periodicals, Inc. Biopolymers 105: 113–132, 2016.

Keywords: virus-like particles; prokaryotic and eukaryotic systems; vaccination; delivery system; clinical trials

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INTRODUCTION

Virus-like particles (VLPs) known as viral "empty shells" maintain the same structural properties of virions, without genome. These constructs are considered very efficient as vaccine platforms and therapeutic delivery systems.[1] Many antigens can readily be displayed on the surface of VLPs. These antigens can be genetically or chemically fused to the VLP.[2] Regarding to the reports, the immune stimulation by VLPs contains: (a) Stimulation of innate immunity through TLRs and Pattern recognition receptors (PRRs) due to the expression of multivalent structures; (b) Induction of strong humoral response and also IgM in T-cell independent way; and (c) Enhancement of the uptake, processing and presentation by APCs through MHC I and MHC II cross-presentation pathway due to the particulate nature of VLPs.[3] VLPs can be subcutaneously or intramuscularly injected. Their small diameter facilitates entry into lymphatic vessels and direct drainage into local lymph nodes. Once in the lymph node, VLPs are taken up by lymph node resident dendritic cells (DCs). This uptake is enhanced by the size and form of VLPs. VLPs stimulate CD4 T cells via the MHC II pathway, as well as highly efficient cross-presentation on the MHC class I pathway.[3] Generally, viral-like particles, are considered as vaccine candidates because their natural properties such as multimeric antigens and their specific structures are suitable for the stimulation of efficient humoral and cellular immunity. Currently, the development of recombinant *subunit vaccines* (SUVs) has been significantly increased using heterologous expression systems. Antigens derived from many bacterial, viral, fungal and parasitic pathogens were used for safe and effective vaccination. Five VLP-based vaccines have been already approved including three for HBV and two for HPV, while in the veterinary field; a VLP-based vaccine against porcine circovirus type 2 (PCV2) has been approved. Some VLP-based vaccines targeting human and
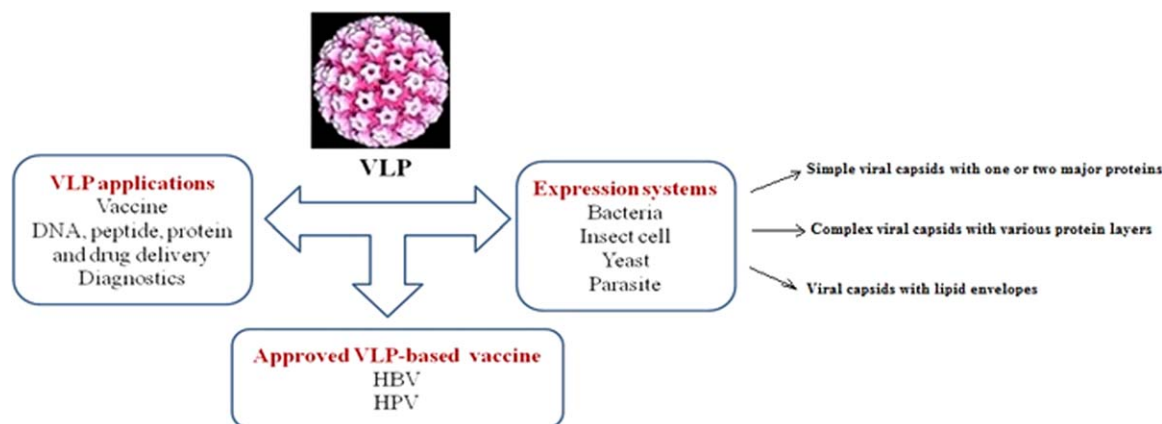
FIGURE 1   General model of VLP along with its applications: The picture shows the recombinant HPV16 L1 pentamers assembled *in vitro* into capsid-like structures. Self-assembly of recombinant viral coat proteins into empty capsids is a promising strategy for production of virus-like particles (VLPs) in vaccine design. The resulting VLPs can induce a protective immune response by mimicking the authentic epitopes of virions.

animal diseases are recently in late stages of clinical trials. VLPs have a positive value as academic, industrial, and commercial systems especially in gene therapy and design of nanomaterials. However, the study of the VLP-based applications (vaccination, gene and drug delivery, and imaging) must be followed to show the reliability, and cost efficiency of this technology. Furthermore, the expression systems would be improved to achieve the best strategy for VLP production from different viral genes. This review will focus on VLP characteristics and its applications especially as vaccines or delivery systems for DNA, SiRNA and drugs. It should be noted that in the vaccination field whenever a viral-like particle carries genetic material is called "vectored vaccines[4]" and in gene therapy, they are called viral vectors. However, for simplicity in this review, we called all particles entitled as viral-like particles (VLPs).

STRUCTURES OF VIRAL-LIKE PARTICLES

Viral-like particles (VLPs) have been generated for over thirty various infectious viruses in animals and humans.[5] VLPs are composed of one or more structural (/capsid) proteins possessing natural properties for self-assembly, and are morphologically similar to authentic viruses.[5,6] Comparing to live viruses, VLPs are non-replicating and non-infective due to the lack of infectious genetic material.[5] Virus-like particles have the potential to be used as safe vaccine candidates without the need for any adjuvant.[7,8]

Different viruses present different structures for generation of viral-like particles such as:

a. Simple viral capsids with one or two major proteins (e.g., Parvoviruses, Papillomaviruses, Circovirses,

Calciviruses, Hepatitis E virus (HEV) and Polyomaviruses).
b. Complex viral capsids with various protein layers, encoded by many distinct mRNAs, or generated from a single polyprotein (e.g., *Picornaviruses*).
c. Viral capsids with lipid envelopes including a lipid bilayer obtained from the host cell, as well as viral glycoprotein spikes (e.g., *Influenza*, HIV and *Hepatitis C*).[5,9] Figure 1 shows the general model of VLP along with its applications.

DIFFERENT EXPRESSION METHODS FOR GENERATION OF VLP

The selection of expression vector is one of the major factors in VLP generation. The reports showed the successful production of 174 VLPs indicating that bacterial systems, yeast and insect systems are used in 28%, 20%, and 28% of the cases. In addition, mammalian cells (15%) and plants (9%) were usually applied to produce VLPs with special properties.[8]

Bacterial Systems

Bacterial systems are often included the commercial *E.coli* strains and expression vectors, to produce non-enveloped VLPs in high levels compared to other systems (Table I).[5] In addition, bacterial cells have been applied for generation of VLPs which need several types of structural proteins, such as the *avibirnavirus* IBDV VP2, VP4, and VP3-polyproteins.[54] The reports indicated that the expression of the *hepatitis B virus* (HBV) capsid protein in *E. coli* leads to the formation of structures similar to the HBV core (HBc) particle.[55] Bacterial

Table I   Preclinical and Clinical Studies of VLPs in Vaccine Development

Virus Type	Recombinant Protein	Expression System	VLP Type	Vaccine Name	Animal Model	Clinical Trial/ Approved	Reference
Hepatitis B virus (HBV)	HBsAg	*S. cerevisiae*	Non-enveloped	Engerix-B®	—	Licensed/Engerix-B®	5,10
		P. pastoris	Non-enveloped	Enivac HB®	—	Licensed/Enivac HB®	5,11
		S. cerevisiae	Non-enveloped	Euvax B®	—	Licensed/Euvax B®	5,12
		H. polymorpha	Non-enveloped	Gene Vac-B®	—	Licensed/Gene Vac-B®	5,13
		P. pastoris	Non-enveloped	Heberbiovac HB	—	Licensed/Heberbiovac HB	5,14
		H. polymorpha	Non-enveloped	Hepavax-Gene®	—	Licensed/Hepavax-Gene®	5,15
		S. cerevisiae	Non-enveloped	Recombivax HB®	—	Licensed/Recombivax HB®	5,16
	HBsAg/HBcAg	*P. pastoris*	Non-enveloped	Revac-B®	—	Licensed/Revac-B®	5,17
	HBsAg/HBcAg	*P. pastoris*	Non-enveloped	Shanvac-B®	—	Licensed/Shanvac-B®	5,18
		Plant	Non-enveloped	Shanvac-B®	—	Clinical trial: Phase I	5,19
	HBsAg/HBcAg	*E. coli*	Non-enveloped	Nasal vaccine	—	Clinical trial: Phase III	20
	HBsAg/HBcAg	Plant	Non-enveloped	Edible vaccine	—	Clinical trial: Phase I	20
	HBsAg	*H. polymorpha*	Non-enveloped	Heplisav	—	Clinical trial: Phase III	20
Hepatitis C virus (HCV)	Core, E1, and E2	Insect cells/ *P. pastoris*	Non-enveloped	—	Mice, chimpanzee	—	20–22
Hepatitis E virus (HEV)	Capsid protein	Plant	Non-enveloped	—	Mice, Monkey	—	20
Birnaviridae infectious bursal disease virus (IBDV)	VP2, VPX, PP	Insect cells	Non-enveloped	—	Bird	—	23
Human immunodeficiency virus 1 (HIV-1)	HIV-1 Gag p17/ p24	*S. cerevisiae*	Non-enveloped	—	—	Clinical trial: Phase II	5,24
		Mammalian cells	Non-enveloped	—	Mice	—	25
		Insect cells	Non-enveloped	—	Mice	—	20
Papillomavirus	HPV6/11/16/ 18 L1	*S. cerevisiae*	Non-enveloped	Gardasil®	—	Licensed/Gardasil®	5,26
	HPV16/18 L1	Insect cells	Non-enveloped	Cervarix®	—	Licensed/Cervarix®	5,27
	HPV6/11/16/18/ 31/33/45/52/ 58 L1	*S. cerevisiae*	Non-enveloped	V503	—	Clinical trial: Phase III	5,28
	HPV-16 L1	*Lactobacillus casei*	Non-enveloped	—	Mice	—	29
	HPV-16 L1	*Lactococcus lactis*	Non-enveloped	—	Mice	—	30
Human parvovirus	B19 VP1, VP2	Insect cells	Non-enveloped	VAI-VP705	Mice	Clinical trial: Phase I/II	5,31
Porcine encephalomyocarditis virus (EMCV)	VP1 protein	Insect cells	Non-enveloped	EMCV	Mice	—	20,32
Enterovirus type 71 (EV71)	EV71 P1 and 3CD proteins	insect cells	Non-enveloped	EV71	Mice	—	20,33
Influenza virus A		Insect cells	Enveloped	Influenza	—	Clinical trial: Phase I/II	5,34

Table I   *Continued*

Virus Type	Recombinant Protein	Expression System	VLP Type	Vaccine Name	Animal Model	Clinical Trial/ Approved	Reference
	A/California/04/09, (H1N1) HA, NA	Plant	Enveloped	Influenza	–	Clinical trial: Phase I	5,35
	A/California/04/09, (H1N1) HA	Insect cells	Enveloped	Influenza	–	Clinical trial: Phase I/IIa	5,36
	A/Indonesia/05/05, (H5N1) HA, NA	Plant	Enveloped	Influenza	–	Clinical trial: Phase II	5,37
	A/Indonesia/05/05, (H5N1) HA	Insect cells	Enveloped	Influenza	–	Clinical trial: Phase IIa	5,38
	A/Brisbane/59/07 (H1N1), A/Brisbane/10/07, B/Florida/04/06 (H3N2), HA, NA	Cell-free	Enveloped	Inflexal® V	–	Licensed/Inflexal® V	5,39
	A (H1N1), A (H3N2), B, HA, NA						
Norwalk virus (NV)	NV CP	Insect cells	Non-enveloped	NV	–	Clinical trial: Phase I	5,20
	NV CP	Plant	Non-enveloped	NV	–	Clinical trial: Phase I	5,40
Severe acute respiratory syndrome related coronavirus (SARS-CoV)	RSV F protein	Insect cells	Non-enveloped	RSV	–	Clinical trial: Phase I	5
Porcine parvovirus (PPV)	VP2	Insect cell	Non-enveloped	PPV	guinea pigs	–	41
Human parvovirus B19	VP1 and VP2	Insect cells	Non-enveloped	HPVB19	–	Clinical trial: Phase I/II	42,43
Goose parvovirus (GPV)	VP1 and VP2 and VP3	Insect cells	Non-enveloped	GPV	Goose/Duck embryo	–	20,44
Coxsackievirus (CVB3)	B Virus proteins 1–4 (VP1–4)	Insect cells	Non-enveloped	CVB3	Mice	–	20
Malabaricus grouper nervous necrosis virus (MGNNV)	MGNNV coat protein	Insect cells	Non-enveloped	Malabaricus grouper nervous necrosis virus (MGNNV)	Sea bass Dicentrarchus labrax	–	20
	VP60 protein	Insect cells, *S.cerevisiae,*	Non-enveloped	Rabbit hemorrhagic disease virus (RHDV)	Rabbit	–	20

Table I *Continued*

Virus Type	Recombinant Protein	Expression System	VLP Type	Vaccine Name	Animal Model	Clinical Trial/ Approved	Reference
Rabbit hemorrhagic disease virus (RHDV)		*P. pastoris*					
Rotavirus (RT)	VP2/VP6 CP	Insect cells	Non-enveloped	RT	Pig/Mice	–	20,45
West Nile virus (WNV)	M proteins, E proteins, immature prM proteins	Mammalian cells	Enveloped	West Nile virus (WNV)	Mice	–	20,46
Flavivirus Dengue virus	premembrane (prM) and E proteins, PD24,recNLP virus	Mammalian cells, *P. pastoris, E. coli*	Enveloped	Dengue virus	Mice	–	20
Flavivirus encephalitis virus (JEV)	(E) proteins	Mammalian cells	Enveloped	Encephalitis virus (JEV)	Mice	–	20
Chikungunya virus (CHIKV)	C and E1/E2 proteins	Mammalian cells	Enveloped	Chikungunya virus (CHIKV)	Mice	–	20
Hantaan virus (HTNV)	Hantaan virus nucleocapsid (N) protein, Glycoproteins (Gn and Gc)	Mammalian cells	Enveloped	Hantaan virus (HTNV)	Mice	–	20,47
Human herpesvirus 4 (Epstein–Barr virus, EBV)	LMP2a???	Mammalian cells	Enveloped	EBV	Mice	–	20,48
Lassa virus	GP1, GP2, NP, and Z proteins	Mammalian cells	Enveloped	Lassa virus	Mice	–	20,49
Mumps virus (MuV)	Nucleocapsid (NP), and fusion (F) proteins; M protein	Mammalian cells, *S. cerevisiae*	Enveloped	MuV	Mice	–	20,50
Menangle virus (MeV)	MenV nucleocapsid protein	*S. cerevisiae*	Enveloped	MeV	Pig	–	20,51
Tioman virus (TioV)	TioV nucleocapsid (N) protein	*S. cerevisiae*	Enveloped	TioV	Rabbit	–	20,52
Marburg marburgvirus (MARV)	MARV or EBOV VP40	Mammalian cells	Enveloped	Marburg marburgvirus	Guinea pig	–	20,53

systems are not always a desired plan for VLP production due to several factors, such as (a) lack of ability to generate recombinant proteins with mammalian-like post-translational modifications (PTM), (b) failure to produce the correct disulfide bonds, (c) drawbacks of protein solubility, and d) the existence of lipopolysaccharides (LPS)/or endotoxins in production of recombinant proteins (rp).[5] Viral coat proteins (CPs) can be efficiently produced as insoluble inclusion bodies, purified under denaturing conditions, refolded, and self-assembled, as indicated in the *parvovirus* B19 and the CCMV and CMV plant viruses.[56] A simple change in the cultivation conditions such as low-temperature can solve the problem of inclusion bodies and induce the formation of soluble VLPs, as performed for two viral systems, the *densovirus* IHHNV,[57] and the *potyvirus* PVY.[58] Some factors including the resistance markers of the expression plasmids and the composition of the cultivation medium can also change the VLP assembly (*e.g.*, *bacteriophage* Qβ).[59] Another strategy applied to increase expression levels and solubility involves the use of different fusion protein systems, *e.g.*, glutathione-S-transferase (GST) fusion proteins such as the *papillomavirus* L1, the *polyomavirus* MuPyV, and the *picornavirus* FMDV.[60–63] Other prokaryotic hosts have been recently used to generate VLP, *e.g.*, *Lactobacillus*.[8] The intracellular assembly of HPV16 L1 VLP was reported in *Lactobacillus casei*, a lactose-inducible expression strain.[5] Furthermore, the production of L1 VLPs using *Lactobacillus* developed new live mucosal prophylactic vaccines (Table I).[29,30]

A *Pseudomonas fluorescens (P. fluorescens)* expression system is an efficient choice against *E. coli*, because of simple manipulation, high yields of active and soluble proteins, and large-scale cultivation. Some differences between *P. fluorescens* and *E. coli* including the various sizes of genome, and diverse metabolic approaches can influence the generation of recombinant proteins.[64] The capsid protein of a plant *bromovirus*, the *cowpea chlorotic mottle virus* (CCMV), has been recently expressed as a soluble form in *P. fluorescens*, and assembled into VLPs *in vivo*. This construct was structurally similar to the natural viral particles provided from plants.[64]

Yeast Systems

Eukaryotic expression systems are a striking alternative to bacteria, especially for solving the problem of bacterial endotoxins in vaccine development. Some structural genes of mammalian viruses expressed in yeast are able to form the VLP. This expression host has been efficiently applied to generate the first licensed HBV vaccine.[65] HBsAg is one of the antigens commonly utilized for production of VLP-based HBV vaccine. HBsAg has been expressed in *Pichia pastoris* (*P. pastoris*), *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Hansenula polymorpha* (*H. polymorpha*) (Table I).[5,16] It is critical to consider that the viral-like particles are not always formed during the cultivation procedure of the yeast cells. These studies showed that the self-assembly of the VLPs in *Pichia* system should be completed during the protein purification.[16,66–68] The expression and self-assembly of recombinant bacteriophage Q coat protein (Q-CP) was indicated in *Saccharomyces cerevisiae* and *Pichia pastoris*. The yeast-derived Q-VLPs were greatly immunogenic in mouse similar to that in *E.coli*-derived Q-VLPs.[69] MS2 VLPs produced in *Saccharomyces cerevisiae* could package functional heterologous mRNAs. For example, the linkage of the MS2 packaging sequence to the human growth hormone mRNA allowed the packaging of the mRNA in MS2 VLPs. Indeed, the high stability of MS2 VLPs suggests them as an efficient delivery system for RNA-based vaccines.[70]

The *P. pastoris* system was also utilized as a potent alternative for expression of CCMV coat protein VLPs due to easy manipulation and high expression levels.[71] In addition, this system has been utilized to express efficiently the pre-membrane and envelope glycoproteins of *dengue virus type 2* (DENV-2),[72] HBsAg,[73,74] HCcAg[75] resulting in the generation of VLPs.[72] The major advantage of yeast systems is the PTM including phosphorylation or glycosylation, as indicated in HBV VLPs.[8] The studies indicated that HBc phosphorylation plays a major role in viral replication and capsid formation. Such yeast-derived HBc VLPs are valuable for vaccination and diagnostics.[76] Furthermore, the potent multigene expression systems have been constructed in yeasts. For example, the expression of three *rotavirus* structural genes from a single plasmid vector led to the generation of triple layered VLPs in *Saccharomyces cerevisiae*.[8,77] However, the multimerization of protein into VLPs is not supported for the enveloped viruses (*e.g.*, Gag VLPs of HIV-2), suggesting that yeast does not have the essential factors of host.[8] Thus, the generation of enveloped HIV-1 Pr55Gag VLPs has been performed using *S. cerevisiae* spheroplasts, morphologically similar to immature viral particles.[5,78] In general, the construction of yeast expression systems, especially *Hansenula* and *Pichia* strains, are more difficult than bacterial vectors. In addition, the yield of VLP production is less than that in *E.coli*.[8] Other limitation of yeast system is its dissimilarity with mammalian cells in the PTM of proteins, especially glycosylation.[79,80] Therefore, this system is more suitable for the generation of non-enveloped viral-like particles.

Insect Systems

Another attractive system utilized broadly for production of VLP is the *baculovirus*-insect cell expression system, due to

some advantages, such as the rapid growth ratios, the culture preparation in large-scale, and the PTM of the target proteins similar to mammalian cells.[81–83] The results showed that both yeast and insect cells were previously used for the VP1 expression of several polyomaviruses, and its assembly into viral-like particle.[84] In addition, insect cells were used to provide VLP-based vaccines, e.g., the approved HPV vaccine, *Cervarix*. Indeed, insect cells are able to generate both VLP types (*i.e.*, enveloped and non-enveloped). There are enveloped VLPs in clinical trials.[9] The main limitation of insect cell system is protein contamination with the enveloped *baculovirus* particles, suggesting the development of efficient plans for purification of VLPs.[85] Recently, co-expression of four genes of human *influenza* H3N2 virus (i.e., HA, NA, M1, and M2) in insect cells led to generate *influenza* VLPs which protected mice against H3N2 virus challenge.[86] These data suggested that viral-like particles are a hopeful vaccine candidate for H9N2 *influenza* and probably other subtypes of virulent avian *influenza* viruses.[87] The non-infectious viral-like particles of the *alphavirus* SAV was also generated using the recombinant *baculoviruses* expressing SAV capsid protein and two major immunodominant viral glycoproteins (E1 and E2) in insect cells.[88] Moreover, *baculovirus* expression system was utilized to generate VLPs from *cowpea mosaic virus* (CPMV), *tomato bushy stunt virus*, and *entorovirus*−71 (EV71).[8,89,90] Recently, non-replicative *baculovirus* have been developed to cope with the problem of *baculovirus* contamination.[91] Stable systems using insect cells have been also tested.[92] Moreover, silkworm expression systems were efficiently applied to generate VLPs and the surface of VLPs could be changed by some strategies, irrespective if their constructs are enveloped or not. Silkworms show a high capability for production of recombinant proteins, in comparison with insect cells, and also easy and inexpensive protein preparation similar to *E.coli* expression system.[81]

Mammalian Cells

For over two decades, different mammalian cell lines have been developed as a source of commercial therapeutic proteins for clinical applications,[93] because of their ability for proper protein folding, assembly and PTM (*e.g.,* the correct glycosylation pattern).[8,93] However, high costs of production and potential safety concerns remained a challenge for these systems. The mammalian cells were progressively utilized to produce VLP-based vaccines[5,94], *e.g.,* for *influenza* viruses. For instance, the generation of a stable mammalian cell line (*e.g.,* Vero cells) expressing four *influenza* structural proteins (HA, NA, M1, and matrix 2 (M2)) led to form hybrid VLPs containing matrix proteins, and surface glycoproteins of H3N2 and H5N1 *influenza* types, respectively.[8,95] Another examples are the produc-

tion of *Newcastle disease virus* (NDV) VLP in ELL cells (East Lansing line), and *Porcine circovirus* (PCV), *Porcine parvovirus* (PPV), *Lassa virus* (LASV), *Marburg virus* (MARV) and *Ebola virus* (EBOV) VLPs in HEK293 cells,[7,96] and bacteriophage T7 VLP in HepG2 cells,[97] and HIV-1 VLP in COS-7/Vero cells,[98] and HBV VLP in CHO cells.[99–101]

Plant Systems

Plants were successfully used to express specific gene products. The feasibility of recombinant plants for generation of vaccine antigens were shown in tobacco plants, potato tubers, and others.[102] This approach develops vaccine strategies which can stimulate mucosal as well as systemic immune responses. In addition, it can be delivered orally as part of a normal biologic function in human.[102] The antigen expressed in plant systems shows extensive disulphide cross-linking and oligomerization for formation of virus-like particles. For example, the hepatitis B major surface antigen has been expressed in several plant systems.[103] Plants are able to express and assemble both types of VLPs (*i.e.*, enveloped and non-enveloped) as multimeric and chimeric proteins. The high expression of VLPs in plant is easy and rapid (e.g., 1–2 weeks) using a *tobacco mosaic virus* (TMV) RNA replicon system and/or a *bean yellow dwarf virus* (BeYDV) DNA replicon system.[104] Another advantage of plants is the use of plant virus particles as a delivery system to present foreign epitopes. Furthermore, the problem of plant-specific glycans has been partially solved using the development of transgenic plants with "humanized" glycosylation pathways.[104] Plant-derived VLPs can be used for oral delivery of vaccines. Viral-like particles are more resistant to digestive enzymes than soluble proteins in body, because of their highly ordered and packed structures. For example, the gastrointestinal viruses-derived VLPs including *noroviruses* and *rotaviruses* were utilized orally as potent candidates for mucosal immunization.[105] Plant-derived VLPs showed the same structures with VLPs generated in other expression systems accompanied by a comparable or higher immunogenicity. Some plant-derived VLPs could induce protective humoral and cellular immunity and also safety in clinics.[105] The studies showed that the level of protein expressed in the recombinant plants is variable and often low. Therefore, further increase in expression will be necessary for practical and efficient products.[102] Recent progress in the glyco-engineering of plants allows human-like glycol-modification and optimization of desired glycan structures for increasing safety and functionality of recombinant pharmaceutical glycoproteins.[1] Some plant-based systems can stabilize antigen and thus reduce storage and distribution costs.[103]

Parasite Systems

Toxoplasma gondii (*T. gondii*) is an obligate intracellular parasite infecting the nucleated cells of warm-blood vertebrates. This parasite is able to stimulate strong humoral, cellular and mucosal immunity, and thus it can be used as an efficient delivery system for heterologous antigens. *T. gondii* was applied as a vector for live vaccination against infectious pathogens.[104–109] Recently, a non-pathogenic kinetoplastida, *Leishmania tarentolae*, was utilized to express heterologous proteins. The studies showed that expression of mammalian glycoproteins in this parasite leads to their modification with mammalian-like oligosaccharides.[110–113] Recently, our group has focused on its use as a live vector or killed vaccine,[114–116] and also generation of viral coat proteins and their assembly as VLP in this system.[117]

IMMUNOSTIMULATION BY VLPS

Virus-like particles show an efficient strategy to deliver antigens to the immune system, inducing both arms of the adaptive immunity.[118] Indeed, VLPs present antigenic epitopes in the proper conformation, leading to induce humoral responses.[5] For example, preclinical trials with influenza VLPs indicated their capacity to induce both humoral and cellular immune responses at low antigen doses. Several authors have reported antibody response to parenterally or orally administered plant-derived antigens.[119,120] As exogenous antigens, VLPs are taken up by professional antigen presenting cells (APCs), especially DCs, followed by antigen processing and presentation via MHC class II molecules, DC activation and maturation through up-regulation of co-stimulatory molecules and cytokine production, and stimulation of CD4+ T helper cells. All these events can efficiently induce both humoral and cellular immunity.[5] In addition, the exogenous VLPs can enter the cytosol of DCs, be processed and presented by MHC class I molecules to cytotoxic T lymphocytes (CTLs) using cross-presentation.[5,121,122] Furthermore, the B-cell activation using VLPs is robust enough to induce T cell-independent IgM antibodies.[7,8] DCs loaded with yeast-derived HIV VLPs can alter Gag-specific memory CD8+ T cells into effector cells through cross-presentation in chronically HIV-infected individuals, although some Gag-specific T cells in these patients did not show any response.[123] The reports showed that the expression system used for generation of VLP might significantly affect direction, type and outcome of immune responses.[121] For example, potent and specific immunomodulatory effects were assigned to yeast-derived HIV VLPs in comparison with other expression systems.[123] On the other hand, plant- or insect-derived VLPs, consisting of the L1 capsid protein of HPV, were both immuno genic to an equal degree. Half of mice fed transgenic potatoes expressing HPV VLPs developed L1-specific antibodies.[124] The studies indicated that the VLPs are taken up by clathrin-dependent macropinocytosis and phagocytosis before being degraded in acidic lysosomal compartments. VLP-derived peptides are loaded onto MHC I that have been recycled from the cell surface.[125] A study showed that uptake and activation of DC by VLP involves proteoglycan receptors, TLR4 and NF-kB, and can be inhibited by heparin.[126] Several data suggest different routes of VLP uptake by DC and Langerhans cells (LC).[127] For example, LCs and DCs internalize similar amounts of HPV-VLPs in vaccine design, albeit through different uptake mechanisms.[128,129] VLP uptake by DCs results in activation and cross-presentation of MHC I-restricted peptides with co-stimulation to T-cells. On the other hand, VLP uptake by LC leads to cross-presentation in the absence of co-stimulation. Efficient VLP cross-presentation by LCs with co-stimulation can be achieved by addition of CD40 ligand.[128] The lack of a protective immune response after viral contact with LCs may explain why some women fail to induce an immune response against the virus. LCs endocytose HPV VLPs via a non-clathrin, non-caveolae, actin-independent pathway, whereas DCs take up HPV VLPs both by a clathrin-mediated mechanism and via macropinocytosis in an actin-dependent manner. This difference in endocytosis resulted in processing and presenting HPV VLP peptides by LCs similar to that by DCs on their surface, but in the absence of co-stimulation. With the addition of CD40L, LCs incubated with HPV VLPs generated the efficient amounts of the pro-inflammatory cytokine (IL-12) and could stimulate a HPV-specific immune response after incubation with T cells.[128] Despite these differences, VLPs taken up by DC and LC were able to prime naive CD8+ T cells and induce cytolytic effector T cells *in vitro*.[127] Furthermore, HIV-1 Pr55^{Gag} virus-like particles could stimulate strong humoral and cellular immune responses. VLP expressed by recombinant *baculoviruses* activated human PBMC to release pro-inflammatory (IL-6, TNF-α), anti-inflammatory (IL-10) and Th1-polarizing (IFN-γ) cytokines as well as GM-CSF and MIP-1α in a dose-and time-dependent manner. Furthermore, VLP-induced monocyte activation was shown by up-regulation of molecules involved in antigen presentation (MHC II, CD80, and CD86) and cell adhesion (CD54). Exposure of VLP to serum inactivated its capacity to stimulate cytokine production.[130] The linking of VLPs to adjuvant molecules was also shown to improve the immunogenicity of the nano-bioparticles.[131] Adjuvanted VLPs [*e.g.,* CpG ODN1826 or poly (I: C) adjuvants] elicited a higher titer of total specific IgG compared to VLPs alone. Furthermore, while VLPs alone induced a balanced Th2 pattern, VLPs formulated with adjuvant elicited a Th1-biased IgG subclasses (IgG2a and IgG3), with poly (I: C) more potent than CpG ODN1826 in

animal model.[118] In addition, mice immunization with chimeric *simian immunodeficiency virus* (SIV) VLPs containing GM-CSF significantly induced SIV Env-specific antibodies as well as neutralizing activity at higher levels than those elicited by standard SIV VLPs, SIV VLPs containing CD40L, or standard VLPs mixed with soluble GM-CSF. On the other hand, the incorporation of immunostimulatory molecules showed significantly increased CD4+ and CD8+ T-cell responses to SIV Env, compared to standard SIV VLPs.[132] Formulation of VLPs with rough LPS (R-LPS) adjuvant as well as DNA primed-VLP boosted regimen were led to increase specific immune responses as compared to VLPs alone, but among them the VLP/R-LPS highly enhanced immune response.[133] Recent studies demonstrated the potential of the HBc VLPs as an oral immunogen. Intraperitoneal immunization with the HBc VLP induced a strong, mixed Th1/Th2 response. In contrast, oral administration of the HBc VLP generated a high humoral response with mainly IgG2a antibodies, directing toward a Th1 response which is essential in the control of intracellular pathogens.[134] In addition, the intranasal monovalent adjuvanted Norwalk VLP vaccine was well tolerated and highly immunogenic.[135]

The studies showed that chimeric HPV-VLPs are able to elicit potent CTL responses in mice against HPV16-transformed tumors; however, the mechanism of T cell priming has remained obscure. HPV VLP could bind to human MHC class II-positive APCs through interaction with FcγRIII, and immature DCs were activated after incubation with HPV VLP.[136] It was shown that binding and uptake of VLP by DC from FcγRII, FcγRIII, and FcγRII/III deficient mice are reduced by up to 50% compared with wild-type mice. In addition, maturation of murine DC from FcγRII/III-deficient mice by VLP is also reduced, indicating that DC maturation, and thus Ag presentation, is diminished in the absence of expression of FcγR.[136]

Poor immunogenicity of mucosally administered proteins has been a major barrier for development of efficient oral vaccines. One way to overcome this obstacle is the use of appropriate adjuvants. Also, delivery of antigen to mucosal surfaces as VLP provides an efficient way of inducing mucosal immunity. After oral or intranasal immunization with *Norwalk* VLP, or *Rotavirus* VLP without adjuvant, intestinal IgA was detected in immunized mice, which were protected from virus challenge.[137] In addition, the plasma cell precursors that migrate to the genital tract are derived primarily from mucosal lymphoid tissues and often secrete IgA.[138] The studies indicated that immune responses generated by mucosal administration of VLP were generally weaker than systemic administration. VLP specific IgA was higher in intestine washes following intrarectally (*i.r.*) than intravaginally (*i.va.*) immunization, and higher in vaginal washes following intramuscularly (*i.m.*) than *i.r.* or *i.va* immunization. Some studies suggested that the immunogenicity of virus particles at mucosal surfaces is probably a property of particulate antigens assembled as multimers of subunits. Indeed, VLP might be actively taken up by mucosal APC through the integrin receptors.[137]

LIPOPARTICLE TECHNOLOGY

Lipoparticles are stable, highly purified, homogeneous, and specialized VLPs containing high concentrations of an integral membrane protein. Integral membrane proteins are involved in different biological functions and are targeted by ~ 50% of existing therapeutic drugs. However, because of their hydrophobic domains, membrane proteins are difficult to manipulate outside of living cells. Lipoparticles can incorporate a wide variety of the membrane proteins, including G protein-coupled receptors, ion channels, and viral envelopes. Lipoparticles provide a platform for different applications such as antibody screening, production of immunogens, and ligand binding assays.[139–141] During the assembly of enveloped viruses, lipid ordered domains of the host cell plasma membrane, known as lipid rafts, frequently function as a natural target for viral proteins. The role of lipid rafts in the organization of complex combinations of immune receptors during antigen presentation and T cell signaling is extensively recognized.[142] On the other hand, in order to improve the immunogenicity of HIV-1 envelope glycoproteins, the fusion of gp120 was performed to a carrier protein, hepatitis B surface antigen (HBsAg) which is capable of spontaneous assembly into virus-like particles. The HBsAg-gp120 hybrid proteins assembled efficiently into 20-30 nm particles. The particles resembled native HBsAg particles in size and density, consistent with a lipid composition of about 25% and a gp120 content of about 100 per particle. Particulate gp120 folded in its native conformation and was biologically active, as shown by high affinity binding of CD4. Because the particles are lipoprotein micelles, an array of gp120 on their surface closely mimics gp120 on the surface of HIV-1 virions. These gp120-rich particles can enhance the quality, and also quantity of antibodies elicited by a gp120 vaccine.[143]

THE APPLICATIONS OF VLPS IN BIOLOGY AND MEDICINE

Virus-like particles show an expanding spectrum of applications such as gene therapy, nanotechnology, vaccination, and diagnostics.[55,77] Recently, the studies showed a pattern of direct conjugation of some ligands, including nucleic acids and proteins attached to VLP surface.[144,145] In addition, because of the

superior accessibility of cysteine and lysine residues on VLPs, bio-conjugation has been performed by commercial homo- or hetero-bifunctional linkers.[146–149] For example, three foreign proteins were chemically conjugated to the VLP surface of CPMV by proper bifunctional cross-linkers.[147] On the other hand, the researchers could produce an *alphavirus* VLP surrounding a functional gold nanoparticle.[150] VLPs have been also used to stimulate immune responses and generate antitumor responses, *e.g.*, *alphavirus*-based virus-like replicon particles (VRP) expressing various melanoma antigens.[151–153] It is interesting that the first viral-associated cancer vaccines were founded on HBV VLP and HPV VLP to prevent HBV-associated hepatocellular carcinoma (HCC) and HPV-associated cervical carcinoma, respectively.[153,154] It should be noted that these VLP formulations are viral vaccines that prevent a viral infection that may progress to carcinoma after a long time. We indicate some applications of VLPs against viral diseases as following:

VLPs in Recombinant Viral Vaccines

In several studies, specific vaccine antigens were generated by various expression systems to induce protective immune responses and apply in licensed recombinant viral vaccines.[100,155] Some examples of preventive VLP-based vaccines are recently commercialized worldwide including GlaxoSmithKline's Engerix® (HBV) and Cervarix® (HPV), and Merck and Co., Inc.'s Recombivax HB® (HBV) and Gardasil® (HPV). Other VLP-based vaccines undergo preclinical evaluation or clinical trials, including *parvovirus*-, *influenza*-, *Norwalk*-derived VLPs and also different chimeric VLPs.[156]

For generation of immunogenic VLPs, eukaryotic expression hosts including yeast (*S. cerevisiae*, *P. pastoris* and *H. polymorpha*) and mammalian cells (Chinese hamster ovary cell line [CHO]) were used. The studies indicated that the recombinant HBsAg generated in CHO and *H. polymorpha* have significant differences in size, molecular weight (MW), and monomer number. Furthermore, the CHO-derived viral-like particles include a combination of glycosylated and non-glycosylated HBsAg proteins, similar to those in patients' sera, while yeast-derived antigens were reported to be non-glycosylated. CHO-based vaccines were provided by Pasteur-Mérieux Aventis in France (GenHevac B®) and SciGen in Israel (Sci-B-Vac™). Both vaccines contained not only the HBsAg S protein but also the M protein (GenHevac B) or the M and L protein (Sci-B-Vac).[156] On the other hand, Gardasil approved by the FDA in 2006 is a quadrivalent HPV types 6/11/16/18 L1 VLP vaccine produced in *S. cerevisiae*. Cervarix is the other licensed HPV vaccine approved by the FDA in 2009.[156] Cervarix is a

bivalent HPV types 16/18 L1 VLP vaccine expressed via a recombinant *baculovirus* vector.[25,157]

Different experiments have been concentrated on HPV VLP vaccination in mouse and human models including: (a) activation of immature human DCs by chimeric HPV16 VLPs, (b) determination of systemic cytokine pattern elicited by HPV L1 VLPs, (c) identification of gene expression signatures in HPV16 L1 VLP-induced human PBMCs, (d) generation of potent and prolonged neutralizing L1 antibodies using a single intramuscular (IM) mice injection with recombinant adeno-associated virus encoding HPV16 L1 protein (rAAV-16L1), (e) augmentation of immunogenicity of HPV L1 DNA vaccines using genetic linkage to a chemokine and secretory signal peptide sequences, (f) potent stimulation of systemic and mucosal immune responses to VLP vaccines using the encapsulation of a genetic cytokine adjuvant (e.g., IL-2), (g) improvement of HPV16 VLP immunogenicity by linkage to the modified adjuvant, and m) nasal immunization of mice with HPV16 VLPs.[158] HPV16 L1-E7 chimeric virus-like particles (cVLP) could induce E7- and L1- specific CTLs. The therapeutic potential of the cVLP also indicated a considerable safety in high grade cervical intraepithelial neoplasia patients (CIN 2/3).[159]

Several improvements in vaccine design by VLP are still in preclinical trials. Some main examples are referred as following:

A. Co-injection of the HPV16 L1 VLP with *E. coli* heat-labile enterotoxin (LT) as an adjuvant significantly increased the levels of serum IgG and vaginal IgA after nasal or bronchial mice immunization.[160,161]

B. Enveloped *influenza* VLPs could stimulate protective immunity during preclinical and clinical studies. The immunogenicity of *influenza* VLPs by the recombinant *baculoviruses* expressing HA and M1 genes, has been detected in several animal studies (mice, rats and ferrets) even after a single dose vaccination. For example, Novavax (MD, USA) was applied in three clinical trials to assay immunogenicity and safety of seasonal H5N1 and H1N1 VLPs (Table I).[5,156]

C. IM immunization with VLPs comprising of the 58 kDa the *Norwalk virus* (NV) coat protein (CP) stimulated high levels of NV-specific serum antibodies in mice, rabbits and guinea pigs.[162] In addition, oral immunization with NV CP VLPs stimulated both mucosal and systemic antibody responses in mouse model,[163,164] as well as safety and immunogenicity in humans.[165,166]

D. A recombinant VLP vaccine including VP1 and VP2 proteins was improved to inhibit human *parvovirus* B19 infection in clinical settings[71,167] (Table I). In

Phase I clinical trial, viral-like particles formulated with aluminum hydroxide stimulated low levels of neutralizing antibody, But VLPs formulated with the *Novartis* squalene-based MF59c.1 adju vant stimulated high titers of antibody responses.[156]

E. The immunogenicity and safety of the chimeric M2-HBcAg VLP vaccine containing HBV core VLPs (generated in bacteria), displaying *influenza* A M2 peptides on their surface, was confirmed in Phase I clinical trials.[168]

F. Yeast transposon Ty VLPs presenting HIV p17/p24 antigens (HIV-1 p17/p24: Ty VLP) was also immunogenic and well-tolerated in Phase I clinical trials.[24,169]

G. Several groups have focused on improving bacteriophage-based VLP vaccines, *e.g.*, RNA bacteriophage Qβ. These chimeric VLP vaccines were targeted against non-infectious diorders including hypertension, allergy, neurodegenerative and autoimmune diseases (*e.g.,* diabetes mellitus type II and Alzheimer), cancer (*e.g.,* melanoma). The vaccine candidate against Alzheimer (CAD-106) was constructed to display chemically coupled amyloid beta (Aβ1-6) peptide derived from the N-terminal B cell epitope of Aβ protein, on the surface of Qβ-CP VLPs. This vaccine could stimulate Aβ-specific IgG and decrease amyloid accumulation in animal models expressing Aβ precursor protein, without eliciting T-cells or inflammatory reactions in brain tissue.[170,171] In addition, the angiotensin II vaccine was synthesized by covalently conjugation of a peptide derived from angiotensin II to the RNA bacteriophage Qβ VLP capsid. This modified VLP could decrease blood pressure in spontaneously hypertensive rats.[172] Table I shows preclinical and clinical studies of VLPs in vaccine development.

VLPs as Delivery Systems

Generally, a major application of VLPs is the stimulation of immunity against foreign protein epitopes by genetically fusing or chemically conjugating them to VLPs entitled as chimeric VLP (cVLPs).[173] Antigens can be fused to VLPs through either covalent or non-covalent bonds. The most common covalent bond is generated by the hetero-bifunctional chemical cross-linkers with amine and sulfhydryl-reactive arms.[104] For instance, cysteine-containing antigens can be conjugated to lysine residues of VLPs surface at a high density (*e.g.*, three peptides per coat protein molecules). The non-covalent conjugation strategy contains the use of streptavidin as linkers to attach biotinylated antigens and VLPs through their efficient and specific interactions.[104] SV40 VLPs can also encapsulate various materials such as DNA (∼5 kb) and proteins as antigens. Insertion of a special

exogenous peptide into the surface loops of VP1 produced SV40 VLPs with the ability of cell targeting. Moreover, SV40 VLPs stimulated innate immunity as a natural adjuvant. Indeed, SV40 VLPs may be a promising vaccine candidate to deliver heterologous antigens followed by the induction of CTLs without synthetic adjuvants.[174] Several chimeric VLP vaccines have entered clinical trials, such as the *anti-influenza A* M2–HBcAg VLP vaccine (HBcAg VLPs displaying M2 epitope of *influenza A*), the *anti-HIV* p17/p24: Ty VLP, two *anti-malaria* vaccines (HBcAg VLPs displaying *malaria* epitopes), the nicotine-Qβ VLP and the anti-Ang II Qβ VLP.[175]

Generation of cVLPs by Genetic Linkage

Genetic linkage contains a stable bond between VLP and antigen. The studies showed that only peptides shorter than 30 amino acids (small peptides) can be presented without interfering with the correct assembly of VLPs. Other limitations contain the improper folding of displayed antigens and the formation of cVLPs with heterogeneous size. To prevent these issues (*e.g.*, assembly problems), structural studies have identified domains for different VLPs such as HBcAg, HBsAg and HIV Gag that were not necessary for VLP assembly as well as allowed insertion of foreign antigens.[104] The simplest way for generation of single component cVLPs, is the insertion of peptides at the N- or C-terminal regions of chimeric VLPs. Multiple fusion positions should be identified to produce multi-component cVLPs inducing broad immune responses.[104] The direction and intensity of the immune responses are significantly influenced by the VLP type, the foreign antigen density, and its accessibility on or within the VLP. Furthermore, pre-existing immunity against the epitopes of the VLP as a delivery system may importantly change the response against the heterogenous antigen. For example, HBcAg was also utilized to display a neutralizing epitope of HPV16 L2 protein. The nasal delivery of HBcAg-HPV16 L2 epitope cVLPs expressed in tobacco induced antigen-specific antibody responses in mouse model. On the other hand, an HPV16 L1-based chimeric VLP was generated in transgenic tomato to present several T-cell epitopes from HPV16 E7 and E6 proteins. The HPV L1-E7/E6 VLPs elicited a neutralizing antibody response similar to that from an equal amount of the commercial vaccine (Gardasil) in preclinical study. Moreover, the chimeric VLPs induced CTL responses against the E7 and E6 epitopes. Chimeric HPV L1 VLPs were also designed using genetic fusion to display epitopes of *influenza* M2 protein.[104]

Generation of cVLPs by Chemical Attachment

To overcome the problems associated with genetic fusion including the antigen size, conformation and VLP assembly,

chemical conjugation approaches were applied to construct cVLPs. In this strategy, target antigens and native VLPs were generated individually and coupled together by attachment of the antigen to the surface of the pre-assembled VLPs. Two main advantages of this strategy include: (a) Various sizes and types of antigens can be exposed, and (b) The antigen-VLP binding site can be manipulated for further presentation of the conjugated antigen. For example, VLPs were used to display full-length and correctly folded proteins, such as interleukin-17 (IL-17).[104]

VARIOUS COMPOUNDS DELIVERED BY VLPS

Generally, VLPs were used for delivery of protein/peptide, DNA, siRNA and drugs as a brief description in following:

Protein/Peptide Delivery

Viral-like particles were used as a peptide/protein carrier, *in vitro* and *in vivo*. There are several examples for delivery of protein/peptide using VLPs as following:

a. Chimeric VLP vaccines have been improved based on RNA bacteriophage AP205, presenting peptides of self-antigens or pathogens fused to either the N- or C-terminal regions of *AP205 coat protein*. AP205-derived VLPs were highly immunogenic in mice. Furthermore, *Influenza* M2 VLPs stimulated an efficient M2-specific antibody response and full protection against lethal *influenza virus* challenge.[176]

b. VLPs containing Flt3 ligand (FL-VLPs), a DC growth factor, could effectively increase immunogenicity in mice. DCs exposed to VLPs also produced high levels of IL-6.[177]

c. A plant VLP-based approach was used to develop *Respiratory syncytial virus* (RSV) vaccine. A target peptide displaying amino acids 170-190 of the RSV G protein was delivered on the surface of *recombinant alfalfa mosaic virus* (AlMV) particles. This construct induced high pathogen-specific immune responses in immunized animals.[178,179]

d. In a recent study, a peptide from an external loop of mouse CCR5 protein was inserted into a neutralizing epitope of HPV L1. The particles generated by this chimeric L1 could elicit high levels of CCR5 antibodies that specifically recognized the surface of CCR5-transfected cells and blocked *in vitro* infection of an M-tropic HIV strain in mice.[161] In addition, chimeric VLPs containing the full length HPV16 E7 oncoprotein linked to L2, or the N-terminal region of E7 fused to L1, could induce antigen-specific protection of mice from lethal challenge with E7-expressing tumor cells.[180–182]

e. A pre-S1 epitope of HBV was also inserted into the EF loop of HPV VLP recognized by HBV-specific antibody.[6] Chimeric VLPs produced in *E.coli* carried a virus-neutralizing HBV pre-S1 epitope in the major immunodominant region (MIR) and a highly conserved N-terminal HCV core epitope (aa 1 to 60) at the C-terminal region of the truncated HBV core VLPs (HBc). The presence of two different foreign epitopes within the HBc molecule did not interfere with its VLP-forming potential, with the HBV pre-S1 epitope exposed on the surface and the HCV core epitope buried within the VLPs. Mice vaccination showed a specific T cell activation by both foreign epitopes and a high-level antibody response against the pre-S1 epitope, whereas an antibody response against the HBc carrier was inhibited.[183]

f. The researchers have shown that the nanosized HBc-VLPs bearing mycobacterial antigen CFP-10 (HBc-VLP: CFP-10 fusion protein) induced an increased immune response in Balb/c mice compared to mixtures of native antigen.[184]

g. Chimeric *papillomavirus* VLPs based on the bovine *papillomavirus* type 1 (BPV-1) L1 protein were designed by replacing the 23-carboxyl-terminal amino acids of the BPV1 major protein L1 with a synthetic "polytope" minigene, containing known CTL epitopes of human PV16 E7 protein, HIV IIIB gp120 P18, Nef, and reverse transcriptase (RT) proteins, and an HPV16 E7 linear B epitope. The chimeric L1 protein assembled into VLPs in insect cells. Polytope VLPs could deliver multiple B and T epitopes as immunogens to the MHC class I and class II pathways. This study has demonstrated that hybrid VLPs can be used as an efficient antigen delivery system to transfer more than one CTL epitope through MHC class I pathways.[185]

h. The chimeric HPV VLPs were generated in which HPV16 L2 neutralization epitopes (L2 residues 69–81 or 108–120) are inserted within an immunodominant surface loop (between residues 133 and 134) of the L1 major capsid protein of BPV1. Immunization of rabbits with assembled particles elicited high L2-specific serum antibody responses.[186,187] The self-assembly of a chimeric HPV16 L1 harboring the M2e *influenza* epitope in plants also represented an efficient expression of chimeric HPV 16 L1 harboring an epitope of a heterologous virus.[188]

i. The chimeric BPV L1 protein with insertion of a polyglutamic-cysteine residue in the BC, DE, HI loops and the H4 helix was constructed. The polyanionic sites

on the surface of VLPs were recognized with a polycationic MUC1 peptide containing a polyarginine-cysteine residue linked to twenty amino acids of the MUC1 tandem repeat through electrostatic interactions and redox-induced disulfide bond formation. MUC1-fully assembled VLPs induced MUC1-specific CTL, delayed the growth of MUC1 transplanted tumors and induced complete tumor rejection in some animals.[189]

j. In a study, six amino acids encoding for the epitope 78-83 (DPASRE) of the HBc antigen were introduced within the different loops of the L1 protein at positions 56/57, 140/141, 179/180, 266/267, 283/284 or 352/353. All these chimeric L1 proteins were able to self-assemble into VLPs. All VLPs could stimulate neutralizing antibodies in different levels.[190]

k. Plant viruses can be genetically modified to generate chimeric VLPs harboring heterologous peptides.[191] The studies demonstrated the potential to produce multigram amounts of chimeric CPMV VLPs in plants for development of peptide-based vaccines.[192] In addition, *potato virus X* (PVX) was used to display the H-2Db-restricted epitope ASNENMETM of *influenza A virus* nucleoprotein (NP) on VLP surface. The results indicated that cVLPs activate ASNENMTEM specific CD8+ T cells without co-administartion of adjuvants.[191] Furthermore, the highly conserved ELDKWA epitope from HIV-1 glycoprotein 41 was expressed as an N-terminal translational fusion with the PVX coat protein. The resulting cVLPs in plant could induce high-titers of HIV-1-specific IgG and IgA antibodies in mice model.[193]

l. The studies showed that the C-terminal region of Gag fused by T cell epitopes from human cytomegalovirus pp65 led to the formation of hybrid VLPs activating antigen-specific CD8+ memory T cells *ex vivo*.[161] Regarding to previous studies, the Gag polyprotein is the only *retroviral* protein required for VLP formation.[194–196] VLPs, derived from an avian *retrovirus*, were applied to deliver proteins to cells, either as part of Gag fusion proteins (intracellular delivery) or on the surface of VLPs. The construct is an effective system because the VLPs are completely made of the Gag fusion protein, and a single VLP will deliver 2000-5000 copies of Gag fusion protein into a transduced cell.[197]

DNA Delivery

Delivery of foreign genes to the digestive tract mucosa by oral administration of non-replicating gene transfer vectors would be a very useful method for vaccination and gene therapy.[198] The studies indicated that plasmid DNA could be packaged *in vitro* into a VLP composed of open reading frame 2 (ORF2) of HEV, which is an orally transmissible virus. These VLPs could deliver this foreign DNA to the intestinal mucosa *in vivo*, eliciting high mucosal and systemic immunity in mice, without the use of adjuvants. An orally administered HIV DNA vaccine encapsulated in HEV-VLPs could induce mucosal and systemic cellular and humoral immune responses.[198] Moreover, the ability of HPV VLPs was examined to mediate delivery and expression of DNA plasmids *in vitro* and *in vivo*.[199] HPV *pseudoviruses* were provided by disrupting HPV-VLP, mixing them with DNA plasmids and reassembling them into the *pseudoviruses* (VLPs with plasmids inside). The *pseudovirus* induced more potent immune responses than DNA vaccines. The *pseudovirus* could be used in gene therapy by transferring the therapeutic genes into lymphoid tissues in human.[5] In addition, the recombinant HPV16 L1 VLPs, produced in insect cells, could efficiently encapsulate a plasmid harboring either a gene for the GFP or β-galactosidase during *in vitro* disassembly-reassembly of VLPs.[200] VLP-mediated delivery of a GFP reporter construct *in vitro* showed to be highly dependent on the presence of full-length L2 protein within the VLPs. Similarly, expression of GFP and luciferase reporter plasmids *in vivo* was efficiently enhanced by co-administration of L1/L2 VLPs. In addition, co-administration of VLPs with a HPV16 E6-expressing plasmid increased significantly E6-specific cellular immune responses.[201] The reports indicated that the recombinant major structural protein of the BK *polyomavirus* (BKV VP1) was shown to self-assemble into VLPs with a diameter of 45-50 nm. The potential of BKV VP1 VLPs was investigated to transfer gene into COS-7 cells using three methods for the formation of *pseudovirions*: disassembly/reassembly, osmotic shock and direct interaction between VLPs and plasmid DNA. The most efficient method is the direct interaction between VLPs and linearized plasmid DNA. The findings generally demonstrated that BKV VLPs have exogenous DNA-binding activity, as a promising vehicle for gene transfer studies.[200]

SiRNA Delivery

There is a major challenge to identify novel approaches for specific and effective delivery of new types of drugs like siRNAs and peptides. Systemic delivery of small interfering RNA (siRNA) was restricted by its poor stability and low cell-penetrating properties. To overcome these limitations, an efficient siRNA delivery system was designed using polyethyleneimine (PEI)-coated VLPs derived from *adeno-associated virus* type 2 (PEI-AAV2-VLPs). Generally, one of the strategies to integrate siRNA into nanoparticles was to coat these particles with positively charged polymers, including PEI, poly β-amino

ester, or poly L-lysine. Electrostatic coating could increase the efficiency of systemic siRNA delivery due to its protective effects and improved cellular uptake. An *insect/baculovirus* expression system was used to generate AAV2-VLPs. PEI-AAV2-VLPs could condense siRNA, protect it from enzymatic degradation, transfer it with high efficiency and induce cell death in MCF-7 breast cancer cells, for breast cancer therapy.[201] Furthermore, microRNAs (miRNAs) play an essential role in immunoregulation and may be involved in the pathogen esis of *systemic lupus erythematosus* (SLE). Among these SLE-related miRNAs, miR-146a, acts as a significant inhibitor of autoimmunity, myeloproliferation, and cancer. A novel miRNA-delivery approach was described via bacteriophage MS2 VLPs for evaluation of the therapeutic effects of miR-146a, in BXSB lupus-prone mice. Treatment with MS2-miR-146a VLP increased the level of mature miR-146a, leading to a significant reduction in the expression of autoantibodies and total IgG. Furthermore, the levels of inflammatory cytokines, including IFN-α, IL-1β and IL-6 were decreased in mice. The stimulation of dysregulated miRNAs by an MS2 VLP-based delivery system may be considered as a novel therapy.[202–204] The use of MS2 VLPs was reported for selective delivery of nanoparticles, chemotherapeutic drugs, siRNA cocktails, and protein toxins to human HCC.[205] In addition, the researchers used *JC virus* (JCV) VLPs as a vector for delivering RNAi in silencing the IL-10 cytokine gene. JCV VLPs were non-toxic, and showed the therapeutic use as a gene therapy approach for autoimmune diseases (AID) including SLE.[206,207]

Drug Delivery

A major challenge in pharmacology is to find methods that drugs (especially anti-cancer drugs) can be delivered specifically to target tissues. A potential strategy would be to package or encapsulate the drug molecules inside a particle which is bound to the cancerous tissue. Such encapsulation would protect the drug from degradation in blood. For this purpose, it will be necessary to develop particles which can be modified on their outer surface to carry drug molecules into the target cells. Novel nanocarriers such as dendrimers, liposomes, polymersomes, micelles, and VLPs indicated high potency in improving drug delivery, and targeting strategies. All of these delivery systems make drugs more biocompatible, water-soluble, or colloidal, indicating low toxicity and high uptake in cells.[208] Different virus-based materials were studied for drug delivery such as: the CCMV, the CPMV, the *red clover necrotic mosaic virus* (RCNMV), MS2 RNA-containing *bacteriophage*, the *bacteriophage* Qβ, M13 *bacteriophage*, the TMV.[208] Drug cargo can be loaded through covalent attachment of drugs or their analogs to particular reactive residues on the capsid pro-

teins.[209] Several cancer cell targeting ligands were attached to different types of VLPs, including small molecules, antibodies, peptides and proteins, as well as DNA aptamers. Folic acid (FA) was broadly used in drug delivery targeted to cancer cells. Uptake of FA into cells is mediated by the folate receptor (FR).[210] Recently, lactobionic acid (LA) was applied for the specific targeting of a *rotavirus* capsid VP6 to hepatocytes or hepatoma cells bearing asialoglycoprotein receptors (ASGPRs).[211] Human holo-transferrin (Tfn) is essential for iron homeostasis. Tfn is especially recognized by the Tfn receptor (TfnR), which is over-expressed on the surface of various tumor cells and efficiently taken up by cells in the clathrin-mediated endocytosis.[212,213] Tfn has been conjugated to CPMV[214] and bacteriophage Qβ.[215] The cellular uptake of the Qβ-Tfn particles was relative to the Tfn density; while the internalization was prevented by comparable concentrations of free Tfn. Antibodies contain another group of targeting proteins that could be chemically linked to VLPs. For instance, a single-chain (*scFv*) antibody that recognizes the carcinoembryonic antigen (CEA) over-expressed in a variety of tumor cells, has been attached to CPMV.[216] An important strategy to improve cellular uptake of therapeutic molecules is the use of cell-penetrating peptides (CPPs).[217] The HIV-1 tat peptide is one of the CPPs that were extensively used in the delivery of VLPs.[218,219] In general, virus-like particles represents an attractive system for drug delivery *in vitro*.[220] The efficient delivery of hydrophobic drugs into target cells without the use of organic solvents or chemical linkage to delivery carriers is a critical issue in the biological field. Recently, the intracellular delivery of hydrophobic dyes or drugs encapsulated in VLPs through cyclodextrins (CDs) showed high efficiency. As a model anticancer drug, paclitaxel (PTX)-CD complexes encapsulated inside VLPs exhibited a dose-dependent cytotoxic effect with a 20-fold smaller IC50 than that of free PTX dissolved in DMSO.[221]

CELL TARGETING

Cell targeting is aimed to effective uptake of therapeutic and/or diagnostic reagent in a special location such as a tumor.[222] Targeting can also be achieved using proteins (mainly antibodies), peptides, nucleic acids (aptamers), small molecules, vitamins and carbohydrates. By attachment of targeting ligands, specificity for cell targeting was obtained by receptor-mediated endocytosis. For instance, bacteriophage MS2 VLPs, were chemically conjugated to a targeting peptide (SP94) for the selective delivery of nanoparticles, chemotherapeutic drugs, siRNA cocktails and protein toxins to human HCC.[223–225] Recently, the chemical conjugation of human epidermal growth factor (EGF) to *simian virus* 40 VLPs allowed for cell

selective targeting.[226] *Simian virus*es 40 VLPs have attracted a great attention in gene delivery due to their high stability and low toxicity in blood.[172]

BIOIMAGING

In design of polymeric nanoassemblies, chemical modification is necessary to conjugate the dye or probe for *in vitro* and *in vivo* imaging. However, in the case of nanobioassemblies, chemical or genetic modification can be applied for bioconjugation of fluorescent dyes or other probes. Another advantage of nanobioassemblies such as VLPs for bioimaging is their biological compatibility. Quantum dots (QDs) and GFP were used broadly for *in vitro* and *in vivo* imaging as alternatives to labelling. For example, fluorescent chimeric VLPs of canine *parvovirus* were expressed in insect cells.[227] To create the fluorescent chimeric VLPs of canine *parvovirus,* GFP was genetically engineered onto the N-terminal region of the viral protein VP2, as a visualization tool to understand mechanisms of viral infections. GFP was also used to design chimeric HIV VLPs allowing protein to be followed during assembly and transmission using live-cell imaging.[228,229]

ADVANTAGES AND DISADVANTAGES OF VLPS

Advantages of VLPs include: (a) no need to propagate pathogenic organisms, (b) Repetitive and ordered surface structures, (c) Multivalent as well as particulate in nature, (d) Safer than other vaccines because of non-infectious and non-replicating properties: The studies showed that there is no risk of disease progress in vaccinated groups with VLP-based vaccines as compared to attenuated viral vaccines, because they lack the genomic material needed for the replication and the spread of the viruses, (e) Stable in extreme environmental conditions, depending on VLP structure (*i.e.*, envelope or non-envelope), and (f) As carrier to express foreign antigen.[230] The potential of VLPs to target DCs is a main advantage of VLP vaccines, for activating the innate and adaptive immune responses. They have a special benefit against other delivery systems in size, stability, and capacity to transfer biological molecules across cell barriers. Particles in the 20–200 nm range can stimulate CD4+, CD8+ cells and especially generate Th1 responses. In addition, despite a limited number of VLP vaccines approved for human use, they represent a promising platform for the development of novel mucosal vaccine strategies. Indeed, VLPs are sufficiently small, and the composition of their surface chemistry can be designed to minimize hydrophobic and electrostatic adhesive interactions with mucus. They can also be engineered for recombinant expression of multiple antigenic epitopes and for incorporation of co-stimulatory and immuno-regulatory proteins. However, VLP technology can be limited by difficulties of scale-up and the need for purification from the expression systems.[231] Other limitation in chimeric VLP vaccine is to determine the compatibility of peptide with assembly of VLP and its immunogenicity property. Under the host immune defence, pathogens undergo mutation which render the VLP vaccine ineffective and will be effective for only highly conserved B or T cell epitopes.[230] The major challenge is to develop novel production platforms that can deliver VLP vaccines while significantly reducing production times and costs.[104]

CONCLUSION AND FUTURE PERSPECTIVES

Viral-like particles (VLPs) have shown high ability for the improvement of vaccines against infectious and non-infectious diseases. Several recombinant expression systems were successfully applied for VLP production, with different efficiency. The use of VLPs in vaccine development showed that they are considered safe. In addition, nano-sized VLPs, can act as an adjuvant as well as antigen delivery system through increasing the antigen uptake by APCs. Thus, it is not necessary for the use of adjuvants along with VLPs to stimulate potent immune responses. VLPs have shown a natural affinity to target host cells, and this property has been used for cell-targeting applications. Regarding the advantages of VLPs, it is necessary for further studies in various aspects especially easy and low-cost purification of VLPs as well as their application as a delivery system *in vivo.*

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