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REVIEW

Exploiting virus-like particles as innovative vaccines against emerging viral infections

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Emerging viruses pose a major threat to humans and livestock with global public health and economic burdens. Vaccination remains an effective tool to reduce this threat, and yet, the conventional cell culture often fails to produce sufficient vaccine dose. As an alternative to cell-culture based vaccine, virus-like particles (VLPs) are considered as a high-priority vaccine strategy against emerging viruses. VLPs represent highly ordered repetitive structures via macromolecular assemblies of viral proteins. The particulate nature allows efficient uptake into antigen presenting cells stimulating both innate and adaptive immune responses towards enhanced vaccine efficacy. Increasing research activity and translation opportunity necessitate the advances in the design of VLPs and new bioprocessing modalities for efficient and cost-effective production. Herein, we describe major achievements and challenges in this endeavor, with respect to designing strategies to harnessing the immunogenic potential, production platforms, downstream processes, and some exemplary cases in developing VLP-based vaccines.

Keywords: virus-like particles, emerging viruses, expression systems, vaccine design, downstream processes

Introduction

Infectious diseases are the leading cause of death among children and adolescents globally, and one of the primary causes of mortality in adults (Andre *et al*., 2008). Most of these deaths disproportionately burden low- and middle-income countries. For these countries, high-impact, low-cost public health interventions remain a key strategy for mitigating health and economic burden by infectious diseases. Immunization remains among the most cost-effective measures, second only to clean water. However, some vaccines are unavailable, inaccessible, and/or unaffordable for the populations most in need (Giersing *et al*., 2016a). Therefore, investments into the research, development, and deployment of vaccines and delivery technologies against emerging and re-emerging pathogens are likely to yield considerable dividends in global health.

Vaccination against infectious diseases is among the most effective of all global public health missions and saves the lives of 2.5 million people among children younger than age 5 every year worldwide (Giersing *et al*., 2016a, 2016b). However, vaccination has some challenges to solve. Most of all, the World Health Organization warned in its 2007 report that infectious diseases are emerging at an unprecedented rate (Andre *et al*., 2008). Since the 1970s, about 40 infectious diseases have been newly discovered, including SARS, MERS, Ebola, chikungunya, avian flu, swine flu, and, most recently, Zika. In the modern world, people travel much more than in the past, live in more densely populated areas, and come into closer contact with wild animals. As a consequence, the potential for emerging infectious diseases to spread rapidly and cause global epidemics becomes a major concern. The 2009 pandemic H1N1 influenza of swine origin is a prime example of such an emerging disease. Frequent changes in the influenza virus genome can cause epidemics and pandemics by successful immune evasion from prior infection or vaccination. Moreover, there has been potential zoonotic transmission from domestic or wild animals, some of which serves as mixing vessels for the generation of novel influenza. In fact, the 2009 pandemic H1N1 influenza turned out to be a genetic reassortment among four different viral species of avian, swine, and human origin (Shapshak *et al*., 2011; York and Donis, 2013). Although direct human-to-human transmission is not officially announced, the highly pathogenic avian influenza (HPAI) H5N1 virus remains on the watch list, especially when considering extremely high mortality among humans. To help meet this challenge, therapeutic and prophylactic interventions are dearly needed. This work encompasses both basic research and more directed research in developing and evaluating vaccines to prevent infection by these agents.

Challenges in the development of vaccines against an emerging virus

There are several challenges in developing a vaccine against

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an emerging virus (Søborg *et al*., 2009; Bowick and McAuley, 2011). First, our understanding about the pathogenicity is limited and protective antigens are poorly characterized, especially with newly discovered viruses. In addition, for highly variable viruses, it is virtually impossible to predict the genetic variants that would cause the next outbreak or pandemic.

Second, it is hard to find appropriate animal challenge models for the evaluation of vaccine efficacy. Vaccine candidates need to be pre-clinically tested in animal models for safety, immunogenicity, and protective efficacy. Ideally, an animal model should have a well-known immune system and similar susceptibility, and immune responses to the pathogen as the natural host. Other important factors in choosing an ideal animal model are price, size, possibility to use in large numbers, and ethical considerations. However, due to the lack of knowledges on pathogenicity (e.g., route of infection and cellular receptors for viral entry, etc), an ideal animal model closely mimicking human infection is hard to establish.

Third, some emerging viruses have high mortality rates, with no treatment or prophylaxis available, and must be manipulated under high bio-safety conditions, sometimes requiring BSL-4 facilities. Vaccine manufacturing in most cases requires the production of target viruses in large quantities by cell culture. Similar concerns apply to veterinary vaccines against highly contagious viruses, e.g., foot and mouth disease virus (FMDV), where BSL-3 level facilities is required during production.

Fourth, the time required to develop, validate, produce in a large scale, and deliver a new vaccine should ideally be as short as possible. This could be facilitated by the use of well-known vaccine platforms that have already been tested and validated against similar pathogens. Vaccines to be used to control an ongoing outbreak should be stockpiled in advance or produced in a timely manner, and provide protection preferably by a single administration. And yet, due to the lack of adaptation in cell substrates approved by regulatory authorities, newly isolated viruses are often difficult or impossible to produce in high titers enough to deliver the vaccine in a timely manner.

Various types of vaccines with wide spectrum of efficacy and safety have been developed in the last century (see Fig. 1). The conventional vaccines, inactivated or live attenuated vaccines require the culture of infectious viruses in cell culture. For newly discovered emerging viruses, the conventional cell culture often fails to produce sufficient vaccine dose for testing of immunogenicity, safety, antigenic variability, and cross-protection across the pathogens. Therefore, other strategies that would circumvent the cell culture method must be considered. First, a viral antigen can be produced by recombinant or synthetic peptides/proteins (Wang *et al*., 2010; Pallister *et al*, 2011; Turley *et al*., 2011). This strategy is safe in production processes and involves no viral replication. The prospective antigens could be easily modified for enhanced protection. However, recombinant soluble antigens are themselves poorly immunogenic and would require potent adjuvants or boosts to enhance immunogenicity. Second strategy involves the use of nucleic acids (DNAs or recently RNAs) as immunogen, where inoculation of cDNAs encoding viral antigens leads to uptake and expression of the cDNA by antigen-presenting cells to trigger protective immune responses (Powell, 2004; Liu *et al*., 2006) (Fig. 1). DNA vaccines have many advantages. Primarily, production from bacterial system in high purity is possible without high-level biosafety requirements. In addition, they can lead to humoral and T cell responses. Introduction of replicons in plasmids could increase immunogenicity by allowing limited level of replication after cellular uptake. And yet, the difficulties in delivery and consequently relatively low level of immunogenicity remains to be improved, preferably by combining with adjuvants or heterologous prime-boost strategies. Third strategy is viral vectored vaccines (Gunther, 2011; Sabchareon *et al*., 2012). For several decades, recombinant viruses have been used as vectors for protein expression and vaccination. For this purpose, viruses are manipulated to enhance their safety and immunogenicity by eliminating virulence factors; to increase coding capacity by eliminating non-essential genes; and to change tropism by changing envelope proteins. For this purpose, various type of vectored vaccine platforms are now available. A major concern is the pre-existing immunity against the viral vector that often compromises the vaccine efficacy. Fourth, recombinant bacteria can serve as vectors for the in vivo delivery of antigens or DNA, as well as production host for subunit vaccines (Pei *et al*., 2005). They elicit both humoral and T cell responses, and inherent replicon activity increase immunogenicity. For this purpose, several non-toxic or even probiotic bacteria are available.

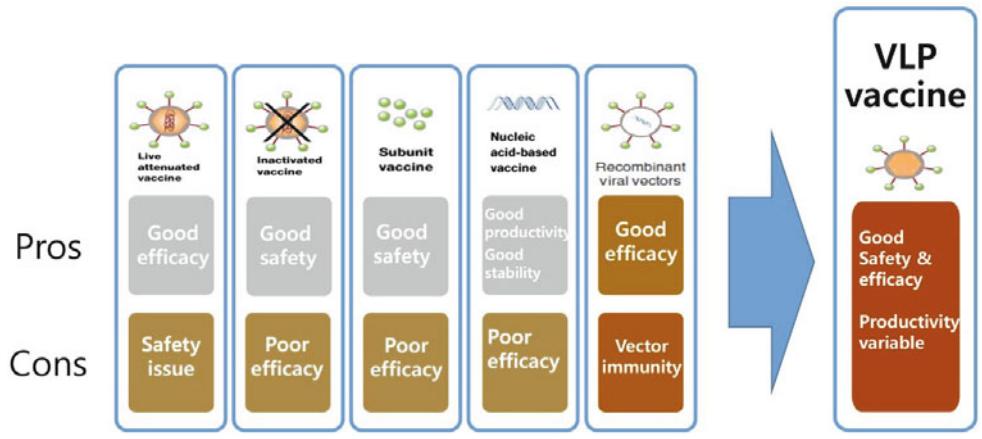

Fig. 1. Contrasting features among various types of vaccines. Pros and cons in terms of safety, efficacy, productivity and stability are compared

Table 1. A brief history of development of VLP-based vaccines for human use

VLP-based vaccines	Year		Method of production
	Development	Approval	
HBV (Hepatitis B virus)	1982	1986	Yeast
HPV (Human papilloma virus)	1991	2006	Yeast, Insect cells
HEV (Hepatitis E virus)	1993	2011	*E. coli*

Fifth, virus-like particles (VLPs), nanoparticles, or peptides in multimeric assembly provides an alternative vaccine platform (Akahata *et al*., 2010; Steel *et al*., 2010). Recombinant surface antigens constituting natural virions are assembled in a highly ordered conformation as an empty particle devoid of viral genetic materials. This feature can be explored as a way to increase the immunogenicity of viral antigens by delivering them in multimeric conformation, preferably as VLPs. Mimicking the morphology of natural infectious virions, but without virally derived genetic materials, the VLPs are highly immunogenic with proven protective immune response and safety profile. As the next wave of technical development as vaccine platform, we discuss VLPs in more details.

Vaccine-like particles as a high-priority vaccine strategy against emerging viruses

VLPs, structurally resembling infectious virions, are non-infectious due to the lack the viral genome required for replication (Kushnir *et al*., 2012; Chen and Lai, 2013; Zeltins, 2013). They still possess immune-stimulatory and self-adjuvanting properties of natural viruses, as they are comprised of capsid proteins that can initiate an immune response. VLPs have distinctive features of size and geometry, which are critical for their ability to potently activate B cells and elicit robust and long-lasting antibody responses. In turn, these two features of VLPs can be exploited as safe and effective vaccines (Fig. 2). First, VLPs are mostly in the range of 20 to 100 nm in diameter, a size that allows for free entry into the lymphatic vessels, passive drainage to the sub-capsular area within lymph nodes, and recognition and uptake by various antigen-presenting cells (Manolova *et al*., 2008). VLPs can traffic into the B cell follicles by the aid of specific interactions with complement components or natural IgM antibodies (Link *et al*., 2012). Second, VLPs also show a special geometry that enables them to highly activate B cells. Epitopes are presented on the multivalent and highly repetitive outer structures of VLPs and thus lead to cross-link B cell receptors. These interactions strongly stimulate B cells and induce potent and long-lasting antibody responses (Jennings and Bachmann, 2008; Bachmann and Jennings, 2010; Zabel *et al*., 2013). Furthermore, these responses can overcome B cell tolerance and allow robust antibody responses to be induced against self-antigens, providing technical platform for therapeutic vaccines against metabolic diseases (Bachmann *et al*., 1993; Bachmann and Zinkernagel, 1997; Chackerian *et al*., 2008). VLPs can stimulate both innate and adaptive immune responses, based on their particulate structure which can be easily recognized and absorbed by antigen-presenting cells (Keller *et al*., 2010). Since the development of the first approved recombinant vaccine against hepatitis B virus in 1986, VLPs have demonstrated success as a reliable vaccine platform (Table 1).

VLPs are diverse in structure and classified as capsid-based (non-enveloped) or enveloped (Fig. 2). Both capsid-based and enveloped VLPs can be single or multilayered and composed of single or multiple proteins. Among the simplest is a non-enveloped single-layered VLP, such as the human papillomavirus (HPV) VLP vaccines. These simple VLPs can be produced in both prokaryotic and eukaryotic expression systems. Sometimes, the capsid proteins of simple VLPs are recombinantly produced and assembled in a cell-free environment to form homogenous VLPs (Salunke *et al*., 1986; Chen *et al*., 2001; Bundy *et al*., 2008; Bundy and Swartz, 2011). Non-enveloped multiple-capsid protein VLPs are more complex and usually produced in higher eukaryotic hosts such as yeast (Rodriguez-Limas *et al*., 2011; Li *et al*., 2013), insect cells (Palomares *et al*., 2012; Fernandes *et al*., 2013) and plants (Scotti and Rybicki, 2013). These expression systems allow

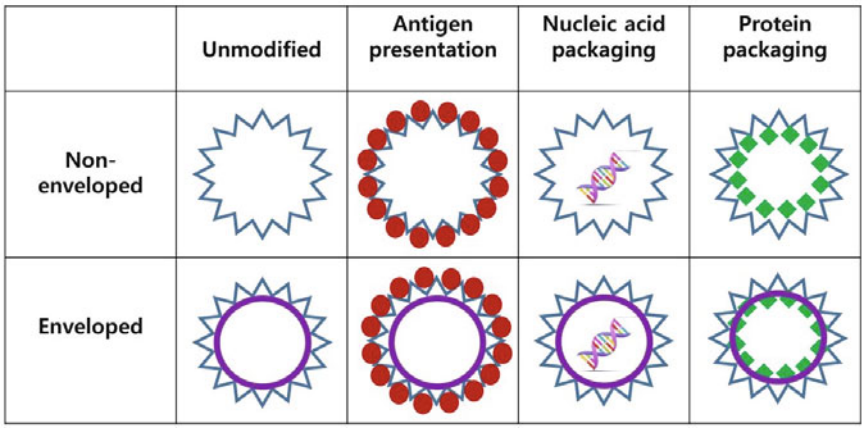

Fig. 2. Different types of VLPs. VLPs share common features of self-assembly into particulate form, which is necessary for immune stimulation. The lack of genetic materials is common requirement for VLP based vaccines. Alternatively, VLPs could also be used as delivery vehicles for nucleic acids or proteins.

for co-expression of the different capsid proteins and complex assembly of the VLPs within a cell.

In enveloped VLPs, the assembly of matrix proteins provides a molecular scaffold, where viral antigens are embedded into lipid membrane. Different types of glycoproteins can be embedded in the lipid bilayer and then become the target immunological antigen for generating neutralizing antibodies. This enveloped VLPs are not structurally uniform and challenging to characterize. Influenza VLPs are a well-studied example of enveloped VLPs (Kang *et al*., 2012), in which matrix M1 proteins and glycoprotein hemagglutinin (and/or neuraminidase) are embedded in the lipid bilayer.

VLPs can function in various ways and thus have a wide range of applications (see Fig. 2). First, VLPs can function as immunogens: for vaccination against the cognate virus, VLPs can be generated from unmodified components as in the case for non-enveloped HPV vaccines Gardasil1 and Cervarix1 and enveloped influenza virus vaccine candidates (Galarza *et al*., 2005). Second, novel immunological antigens can be displayed on VLPs. Novel antigens are placed on the surface of VLPs either as fusion protein or chemical conjugation (Peacey *et al*., 2007; Jennings and Bachmann, 2008). Therefore, VLPs can be an effective platform that is exploited for generation of epitope-based VLPs to target various diseases (Plummer and Manchester, 2011). Heterologous antigen presentation on non-enveloped VLPs can be achieved through fusion of small epitopes or large antigens [e.g., hepatitis B virus core antigen (Whitacre *et al*., 2009)]. On enveloped VLPs, heterologous antigen presentation can be accomplished by insertion of glycoproteins [e.g., herpes simplex virus glycoprotein inserted into simian immunodeficiency virus VLPs (McGuigan *et al*., 1993)]; or by heterologous head domain fusion onto transmembrane region [e.g., vesicular stomatitis virus glycoprotein ectodomain fused to rabies virus glycoprotein (Kato *et al*., 2011)]. Third, VLPs are recently developed as delivery vectors for nucleic acids or proteins. Here, the properties of some viral capsid proteins allow engineered VLPs to encapsulate proteins, molecules, or nucleic acids. Examples of packaging nucleic acids include the non-enveloped Simian virus 40 (Kawano *et al*., 2013) and enveloped retrovirus VLPs (Keswani *et al*., 2013). Likewise, heterologous proteins can be encapsulated and delivered in both enveloped and non-enveloped VLPs. DNA-loaded VLPs are developed for gene therapy (Ramqvist *et al*., 2007; Seow and Wood, 2009) and therapeutic VLPs encapsulate proteins or drug molecules (Ashley *et al*., 2011; Kaczmarczyk *et al*., 2011).

Design of VLP-based vaccine platforms

Computational structure modeling and bioinformatics techniques have been increasingly applied on design of VLP-based vaccines (Ding *et al*., 2010; Pankavich and Ortoleva, 2012; Roldão *et al*., 2012). Currently, there are 540 completed icosahedral virus capsid structures available from VIPERdb database (http://viperdb.scripps.edu/), from which the designing principle of multi-molecular assembly could be deduced. The data have been generated from structural and computational analyses, as well as high-quality renderings for 3D information. Structural analyses, such as X-ray crystallography and cryo-electron microscopy (Carrillo-Tripp *et al*., 2009), provide a major tool for determining crystal structures of viral capsids (Grimes *et al*., 1998; Prasad *et al*., 1999; Wynne *et al*., 1999). Computational analyses can complement to these empirical structural analyses, and in combination provide a tool to rational design of monomers for expedited assembly into VLPs, followed by experimental verification (Kuroda *et al*., 2012; Zhang *et al*., 2013). Thus, design of VLP-based vaccines will increasingly benefit from protein engineering of monomers especially considering their ability for inter-molecular interactions. Enhanced computer modeling techniques are required for designing engineered VLP with novel epitopes transplanted on its surface.

To maximize the immunogenic potential of particle platform technologies, target antigens must be displayed at high density on the surface of VLPs. Different approaches have been tried to engineer and tailor VLPs as molecular scaffolds for antigen display. In order to maximize this critical feature, VLPs should be able to display foreign antigens of various sizes, structures, and compositions. One way is to generate recombinant fusions in which foreign antigens are inserted into specific sites within the viral structural protein so that the foreign antigen is displayed on the surface of the resulting VLP. However, peptide insertions with a view to introduce novel epitopes, often adversely affect the folding of monomers, which consequently fail to assemble into regular and compact structure. This often results in misfolding into amorphous aggregates, resulting in lowering of immunogenicity and/or vaccine efficacy. Such problems can be circumvented by initially preforming of VLPs as scaffolds to which foreign antigens or epitopes are subsequently conjugated. Post-production modifications of VLPs can take advantage of naturally occurring sites of conjugation, e.g., amino or sulfhydryl groups exposed on the VLP surface.

In HBcAg VLPs, like many other VLPs, foreign antigen insertion sites are identified by a combination of empirical apporach and rational design based on structural categorizations. First, by a comprehensive combinatorial approach with multiple insertion sites, compensatory mutations, and robust screening methods, the Billaud group greatly enhanced the success rate for producing chimeric Woodchuck Hepatitis Virus core antigen VLPs (Billaud *et al*., 2005). Second, transforming the subunits of VLPs into a more thermodynamically stable conformation can dramatically enhance the success rate for engineered VLPs with novel peptide epitope insertions. Third approach is to circumvent potential disruption of folding and assembly by chemically cross-linking antigens to preformed VLPs. These approaches potentially increase the cost of production or decrease scalability along the downstream manufacturing processes, but allow VLPs to display various antigens, including peptides, whole proteins, carbohydrates, and other small molecules.

The VLPs interact with various components of the immune system to provoke protective responses: receptors for the entry into antigen-presenting cells and molecular components in the trafficking pathway. Thus, VLP-based vaccines usually provoke strong immune responses, and by modifying VLP structures the relative degree of immune responses can be further tuned for a balanced induction of humoral and cell-mediated immunity. Overall impact on elicited im-

Table 2. Comparison of different expression platforms for producing VLPs

	Speed	Cost effectiveness	Scalability	Yield	VLP complexity
Bacteria	+++	+++	+++	+++	+
Yeast	+++	+++	+++	++	++
Baculovirus-insect cells	++	+	++	++	+++
Mammalian cells	+	+	++	+	++
Plants	++	++	++	+	++

+, low; ++, medium; +++, high

mune response also depends on the intrinsic half-life of particles and kind of adjuvants (Storni *et al*., 2002; Manolova *et al*., 2008; Link *et al*., 2012). Another critical factor to be considered is the chemical stability of VLPs. Although VLPs are highly stable in most cases, VLP types that can withstand a wide range of temperature and pH conditions will be helpful during manufacturing, storage, and distribution. This feature is particularly important in the developing countries where the cold chain is not readily available. In addition, enhancing the thermal stability of VLPs reduces the production cost by allowing a longer shelf-life and also reduces delivery costs requiring cold storage. Thermo-stability of VLPs can be enhanced by structural modifications, such as the introduction of inter-subunit disulfide bonds (Ashcroft *et al*., 2005; Fiedler *et al*., 2012). Judicious choice of mutations is required to minimized potential structural perturbation, which otherwise would lead to amorphous intermolecular aggregation rather than into regular packing into VLPs, compromising the vaccine efficacy.

Expression platforms for VLPs

In developing VLP-based vaccines, serious considerations should be given to a robust and faithful production platforms that enables the delivery of vaccines in a timely and reproducible manner. As the significance of VLPs has been appreciated for developing next-generation vaccines, several expression platforms for producing VLPs have been developed, including bacteria (*Escherichia coli*), yeast, insect cells, mammalian cells, and plants (Table 2). The *E. coli* expression system is easy to use and the least expensive. A major drawback for bacterial host, however, is that *E. coli* cytoplasm does not provide optimal milieu for the folding of human infecting viral antigens, and thus rarely successful for VLP assembly. Moreover, this system lacks a post-translational modification capacity and thus its use is limited to produce simple VLPs, such as non-enveloped VLPs. So far, commercial success is made for hepatitis E virus (HEV) vaccines based on chemical refolding of inclusion bodies (Hecolin1, Xiamen Innovax Biotech Co. Ltd.). To be widely applicable for VLP vaccines, a robust expression platform is yet to be developed ensuring soluble expression of viral antigens amenable to folding and self-assembly.

A novel protein folding vehicle was recently developed that could be implemented for VLP assemblies in *E. coli* (Fig. 3). It has been shown that RNA binding could affect the overall kinetic network of protein folding pathway in favor of enhanced folding over off-pathway aggregation (Choi *et al*., 2008, 2009, 2013). In addition, the RNA binding-mediated solubility is greatly enhanced for increasing soluble yield of passenger proteins and this feature could be usefully implemented for high-throughput protein expression. The RNA-interaction mediated folding vehicle provides novel expre-

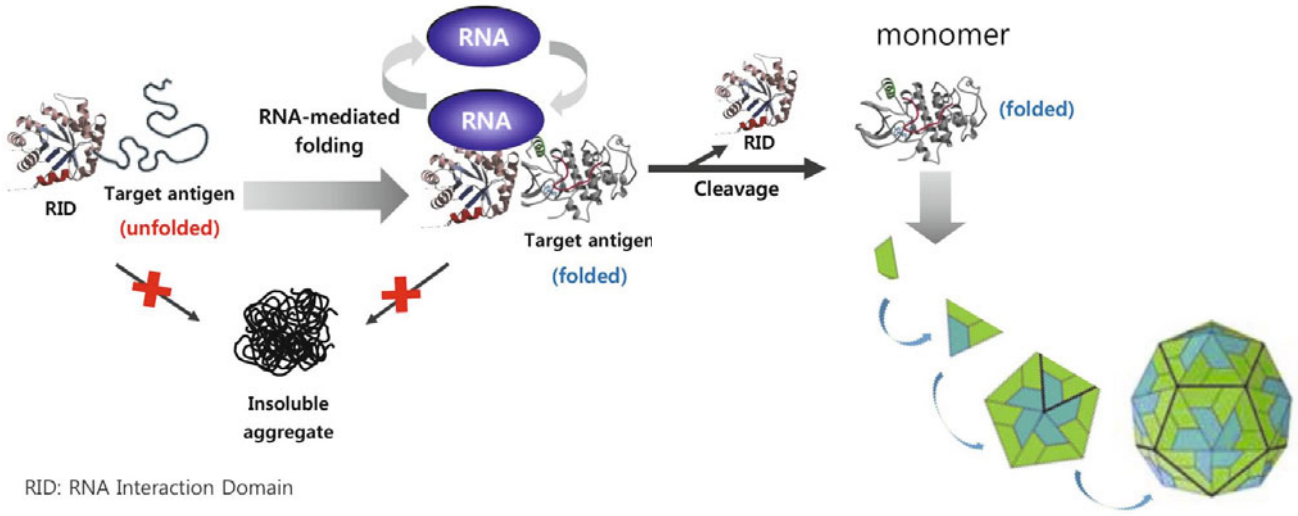

Fig. 3. Novel VLP production platform from bacterial host. Target antigen is expressed as fusion to RNA-interaction domain (RID). Using RNAs as molecular chaperones, the monomers are expressed as soluble, properly folded form, which, upon cleavage, assembles into VLPs.

ssion platform for VLP vaccine, e.g., HPV as well as norovirus (unpublished results). Fascinating possibility remains if the RNA-based molecular chaperones for protein folding could be extended to the production multi-component VLPs.

Yeast systems are popularly used in producing VLP-based vaccines. For example, the hepatitis B virus (HBV) vaccine Engerix-B® (GlaxoSmithKline) and the human papillomavirus (HPV) vaccine Gardasil® (Merck & Co., Inc) (Kim and Kim, 2017) were produced from recombinant yeasts. Yeast systems elicit high expression and easy scale-up and, unlike the *E. coli* system, provide post-translational modification of the expressed proteins. In addition, yeast has low risk of contamination by adventitious agents during scale-up of production. Yeast has been mostly applied to produce VLPs with simple structures, but currently its application has been extended to VLPs for enveloped viruses. Furthermore, it has been recently found that yeast systems enable VLPs to be produced either intracellularly or using secretion pathways. To enhance the quality and quantity of yeast-based VLPs, various factors, such as the type of plasmid and promoter and, especially the secretability and processing on exit out of the yeast cells, need to be carefully taken into account (Bae *et al*., 1998; Lee *et al*., 1999; Han *et al*., 2005).

The baculovirus-insect cell and mammalian-cell systems have an advantage of eliciting more complete post-translational modification including glycosylation and expressing multiple component VLPs (Rodriguez-Limas *et al*., 2011). VLP-base vaccines produced using these systems include the HBV vaccine GenHevac B® (Pasteur-Mérieux Aventis) and the HPV vaccine Cervarix® (GlaxoSmithKline). These systems are more expensive than bacterial and systems, and downstream processes are usually more complex. The bacterial system, if optimized in the downstream recovery process (Fig. 3), would afford VLP vaccines (HPV vaccine, for instance) in developing countries.

Recently, plants become newly appreciated as bioreactors, as it has been realized that plants, as molecular pharming, can work as a factory for recombinant expression of proteins of interest (Ma *et al*., 2015; Marsian and Lomonossoff, 2016). Innovative methods for rapid production of useful proteins in plants rely on transient expression to bypass the requirement for tedious transformation and regeneration steps. This strategy can be applied to produce viral coat proteins to be assembled into VLPs. As a protein production system, plants are cost-effective and highly scalable in manufacturing requiring only water, carbon dioxide, inorganic nutrients, and sunlight for growth (Twyman *et al*., 2003; Lico *et al*., 2008; Paul *et al*., 2011; Marsian and Lomonossoff, 2016). Plant expression systems provide complex but distinctive protein assembly and post-translational modifications as in mammalian-cell systems, and show high-level immunogenicity. Glycosylation in yeast and insect cells is limited to very simple and inconsistent glycoforms, whereas plants can generate a wide variety of glycosylation types (Chen and Lai, 2013). Recent success on genetically engineered plants that support humanized glycosylation plant lines holds a great potential for their applications (Marsian and Lomonossoff, 2016). Furthermore, unlike mammalian cell cultures, they bring a low risk of contamination derived from endotoxins and mammalian pathogens.

Downstream processes of VLP-based vaccines

VLP production in recombinant host is invariably associated with numerous impurities from cell substrates and the manufacturing process. US the Food and Drug Administration (FDA) summarized these impurities into two subgroups, process-related and product-related contaminants (US FDA, 1999). Process-related contaminants are predominantly attributed to host cell impurities, such as cells, cell debris, host cell-derived proteins, DNAs, proteases, endotoxins, polysaccharides, and lipids. Impurities intrinsic to the production process are media components, anti-foam reagents, and other potential hazards supplemented during upstream or downstream processing, such as stabilizers, excipients, proteases, and nucleases. A list of inactive ingredients allowed in final drug formulations is provided by FDA (http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm). The World Health Organization (WHO) states that quality monitoring of final VLP-based vaccine bulks should include, among others (WHO, 2006): testing for sterility (bacteria, fungi); testing for virus clearance; summary of vaccine composition (protein, lipid, polysaccharides); control of residual DNA, protein and endotoxin content; determination of protein purity (by SDS-PAGE or similar); and description of residual chemicals.

Several factors - expression host, the cellular environment, the type of target viral proteins, and the virus subtype - exert influence on the quality of *in vivo*-assembled VLPs in recombinant systems (Mach *et al*., 2006). It is practically very difficult to sufficiently control all of these factors during VLP assembly *in vivo*. As an alternative, therefore, disassembly and reassembly *in vitro* were developed and optimized for several VLPs (McCarthy *et al*., 1998; Ren *et al*., 2006; Xing *et al*., 2010; Link *et al*., 2012; Roldão *et al*., 2012). The disassembly and reassembly *in vitro* allows easy control over encapsulated host cell impurities and lead to increase the homogeneity, stability, and reactivity of VLPs (Zhao *et al*., 2012a, 2012b). Molecular dynamic simulations enable a better understanding of the assembly mechanism and prediction of the quality of newly designed VLP constructs (Zhang *et al*., 2014). Other product-related contaminants include VLP aggregates formed during both upstream and downstream processing. Modeling the competition between aggregation and self-assembly into ordered sturucture has been developed (Shi *et al*., 2005; Ding *et al*., 2010; Sanchez-Rodriguez *et al*., 2012), which, if properly implemented, may help enhance the quality and the purification yields.

The final bulk products need to meet the desired purity criteria for recombinant VLPs (Effio and Hubbuch, 2015). The downstream purification process ranges from the initial product isolation out of cells to concentration (clarification and capturing), intermediate purification, and polishing of the molecule of interest (Effio and Hubbuch, 2016). The methods of choice for these processes include chromatography, filtration, and integrating purification techniques. Process-related contaminants need be separated particularly at the beginning of the process, whereas product-related contaminants and residual process-related impurities are removed preferably in the final polishing step.

In the past, process development and optimization in downstream processes mostly depended on empirical approaches

by trial and error. By virtue of recently developed emerging technologies, the FDA has implemented 'quality by design' (QbD) and 'process analytical technology' (PAT) for biopharmaceutical products. The idea of these approaches is to develop a better knowledge and control of products and manufacturing processes by using PAT methods and other rational development strategies. PAT methods include online high-performance liquid chromatography (HPLC) systems or light-scattering techniques which enable process surveillance and control of unit operations in real-time (Rathore and Winkle, 2009; Yu *et al*., 2013). Monitoring the size of VLPs during the manufacture is especially important for the quality control of VLP-based vaccines. New rational development strategies for biopharmaceutical products involve the approaches of design of experiments (DoE), scale-down, high- throughput experimentation, and process modeling.

New strategies and technologies for VLP purification and downstream processes have recently arisen (Effio and Hubbuch, 2015). Ultracentrifugation steps are progressively replaced by scalable chromatography and filtration unit operations. New stationary phases and alternative methods such as aqueous two-phase extraction, precipitation, and expanded bed adsorption chromatography are increasingly used.

An exemplary case: norovirus VLP-based vaccines

Noroviruses are the most common cause of nonbacterial acute gastroenteritis in humans worldwide (Patel *et al*., 2009; Hall *et al*., 2011). These highly infectious viruses have recently been referred to as 'The Perfect Human Pathogen' since the virus is highly stable in the environment, infectious at low doses, and moderately virulent yet rapidly shed in large quantities by the host following natural infection. In addition, the virus is antigenically diverse and evolving, and induces limited immunity after natural infection (Hall, 2012). The lack of proper cell culture methods and animal infection models pose challenges to the development of the vaccine. As an alternative to cell culture method, the VLPs has enabled significant progress toward effective vaccine candidates designed to protect against multiple circulating norovirus strains. Vaccination with norovirus VLP vaccines has been shown to both induce antibodies that block virus-derived VLP carbohydrate binding and protect against homologous viral challenge in a clinical study. The first finding that recombinant norovirus VP1 capsid sequences can be expressed and self-assembled into VLPs that are morphologically similar to the infective virion (Jiang *et al*., 1992) led to extensive use of VLPs for understanding norovirus structure, immunogenicity, and receptor-binding activity.

In case of noroviruses, the inability to propagate viruses in cell culture precludes the development of live-attenuated or inactivated vaccine candidates. Thus, several expression systems have been developed for the production of norovirus VLPs including yeast, baculovirus-infected insect cells and transgenic plants. Initial methods described for norovirus VLP production typically involved small-volume cell culture, with VLP purification subsequently carried out by centrifugation and/or sucrose density gradient fractionation (Jiang *et al*., 1992; Ausar *et al*., 2006; Huhti *et al*., 2010). Although such production methods generate sufficient levels of material for general characterization and animal immunogenicity studies, robust and scalable production methods are required for human clinical trials. More recent production and purification development has focused on commercially scalable unit processes including bioreactor production, column chromatography and ultrafiltration/diafiltration (UF/DF) (Kissmann *et al*., 2008; Taylor, 2009; Koho *et al*., 2012).

Large-scale bioprocessing of plant-derived Norwalk GI.1 VLPs has been accomplished using pH-dependent precipitation of process contaminants followed by UF/DF of the clarified harvest fraction and chromatographic purification by anion-exchange chromatography (Lai and Chen, 2012). The purified proteins are properly assembled and stable VLPs, and contain low levels of residual host cell protein and nucleic acid impurities. Stabilization of Norwalk GI.1 VLP structure in vaccine formulations can be further enhanced with carbohydrate excipients (Kissmann *et al*., 2008). The future development of low-cost, large-scale manufacturing processes preferably using the bacterial host (Fig. 3) is required for the delivery of this unmet need vaccines to low- or middle-income countries.

Other cases: HBV and papillomavirus VLP-based vaccines

After the successful launch in 1986 of the first recombinant vaccine produced from yeast, the production platform for the VLP-based hepatitis B virus vaccines has been extended to plants (Mechtcheriakova *et al*., 2006). It is demonstrated that a monomeric form of the core antigen HBcAg can be expressed and spontaneously form core-like particles in different heterologous expression systems including bacteria and plants. HBc particles with a spiky surface and repetitive presentation of antigenic determinants show a strong inherent immunogenicity and can be used for the display of foreign peptides (Peyret *et al*., 2015). Using HBcAg dimers produced by the tandem fusion of two HBcAg open reading frames, a carrier system for antigens is developed. These 'tandem core' system can still self-assemble into core-like particles and can be used to display correctly folded GFP or nanobodies on their surface. These, in turn, can be used to bind their cognate antigens. Therefore, it is possible to display whole proteins on the particle surface.

Papillomaviruses are non-enveloped tumor-inducing DNA viruses, with over 100 serotypes mostly infecting humans, accounting for over 5% of all human cancers (Münger *et al*., 2004). Two types of HPV VLP vaccines, based on the self-assembly of L1 (the major capsid protein) expressed in yeast or insect cells, are currently on the market (Rybicki, 2014). As an alternative to yeast- or insect cell-based production, a low-cost plant-based vaccines are being developed, where HPV-8 L1 is successfully expressed as VLPs (Matić *et al*., 2012).

Challenges and perspectives

Over the last few decades, significant advances are made in

the design and manufacture of VLP vaccines, as vital alternative to traditional cell-culture vaccines. Capitalizing on strong immune stimulation intrinsic to particulate form of immunogen, continued research has focused on engineering the VLP assembly as to enhance the particle stability, and the diversity of antigens to be displayed on the surface of VLPs. Although only a few VLP-based vaccine have reached the market and no VLP-based vaccine against emerging viruses is currently available yet, there are great expectations for several VLP vaccines based on the positive results in clinical trials. Technical challenges still remains, however. As compared with VLPs made of single component, e.g., norovirus or HPV, the assemblies of viral capsids requiring multi-components, e.g., FMDV, HFMV, is more complex. Further understanding on the folding of individual monomers and the complexities involved in multimeric macromolecular assemblies, as well as the enabling protein folding technologies, are required. On the downstream process especially for enveloped VLPs, the purification of VLPs from baculo-viral particles of similar size and biophysical characters still poses daunting challenge. To meet the regulatory requirements on the safety issue, proper inactivation of contaminating baculo-virions without sacrificing the immunogenicity of VLPs should also be combined. Despite such huddles, the recombinant VLP vaccine platform, as alternative to cell-culture methods, would emerge in the foreseeable future as a major manufacturing platform for vaccines combating emerging and re-emerging infectious diseases.

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References

Akahata, W., Yang, Z.Y., Andersen, H., Sun, S., Holdaway, H.A., Kong, W.P., Lewis, M.G., Higgs, S., Rossmann, M.G., Rao, S., *et al.* 2010. A virus-like particle vaccine for epidemic Chikungunya virus protects nonhuman primates against infection. *Nat. Med.* **16**, 334–338.

Andre, F.E., Booy, R., Bock, H.L., Clemens, J., Datta, S.K., John, T.J., Lee, B.W., Lolekha, S., Peltola, H., Ruff, T.A., *et al.* 2008. Vaccination greatly reduces disease, disability, death and inequity worldwide. *Bull. World Health Organ.* **86**, 140–146.

Ashcroft, A.E., Lago, H., Macedo, J.M., Horn, W.T., Stonehouse, N.J., and Stockley, P.G. 2005. Engineering thermal stability in RNA phage capsids via disulphide bonds. *J. Nanosci. Nanotechnol.* **5**, 2034–2041.

Ashley, C.E., Carnes, E.C., Phillips, G.K., Durfee, P.N., Buley, M.D., Lino, C.A., Padilla, D.P., Phillips, B., Carter, M.B., Willman, C.L., *et al.* 2011. Cell-specific delivery of diverse cargos by bacteriophage MS2 virus-like particles. *ACS Nano.* **5**, 5729–5745.

Ausar, S.F., Foubert, T.R., Hudson, M.H., Vedvick, T.S., and Middaugh, C.R. 2006. Conformational stability and disassembly of Norwalk virus-like particles. Effect of pH and temperature. *J. Biol. Chem.* **281**, 19478–19488.

Bachmann, M.F. and Jennings, G.T. 2010. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat. Rev. Immunol.* **10**, 787–796.

Bachmann, M.F., Rohrer, U.H., Kündig, T.M., Bürki, K., Hengartner, H., and Zinkernagel, R.M. 1993. The influence of antigen organization on B cell responsiveness. *Science* **262**, 1448–1451.

Bachmann, M.F. and Zinkernagel, R.M. 1997. Neutralizing antiviral B cell responses. *Annu. Rev. Immunol.* **15**, 235–270.

Bae, C.S., Yang, D.S., Chang, K.R., Seong, B.L., and Lee, J. 1998. Enhanced secretion of human granulocyte colony-stimulating factor directed by a novel hybrid fusion peptide from recombinant Saccharomyces cerevisiae at high cell concentration. *Biotechnol Bioeng.* **57**, 600–609.

Billaud, J.N., Peterson, D., Barr, M., Chen, A., Sallberg, M., Garduno, F., Goldstein, P., McDowell, W., Hughes, J., Jones, J., *et al.* 2005. Combinatorial approach to hepadnavirus-like particle vaccine design. *J. Virol.* **79**, 13656–13666.

Bowick, G.C. and McAuley, A.J. 2011. Vaccine and adjuvant design for emerging viruses: mutations, deletions, segments and signaling. *Bioeng. Bugs* **2**, 129–135.

Bundy, B.C., Franciszkowicz, M.J., and Swartz, J.R. 2008. *Escherichia coli*-based cell-free synthesis of virus-like particles. *Biotechnol. Bioeng.* **100**, 28–37.

Bundy, B.C. and Swartz, J.R. 2011. Efficient disulfide bond formation in virus-like particles. *J. Biotechnol.* **154**, 230–239.

Carrillo-Tripp, M., Shepherd, C.M., Borelli, I.A., Venkataraman, S., Lander, G., Natarajan, P., Johnson, J.E., Brooks, C.L. 3rd, and Reddy, V.S. 2009. VIPERdb2: an enhanced and web API enabled relational database for structural virology. *Nucleic Acids Res.* **37**(Database issue), D436–442.

Chackerian, B., Durfee, M.R., and Schiller, J.T. 2008. Virus-like display of a neo-self antigen reverses B cell anergy in a B cell receptor transgenic mouse model. *J. Immunol.* **180**, 5816–5825.

Chen, X.S., Casini, G., Harrison, S.C., and Garcea, R.L. 2001. Papillomavirus capsid protein expression in *Escherichia coli*: purification and assembly of HPV11 and HPV16 L1. *J. Mol. Biol.* **307**, 173–182.

Chen, Q. and Lai, H. 2013. Plant-derived virus-like particles as vaccines. *Hum. Vaccin Immunother.* **9**, 26–49.

Choi, S.I., Han, K.S., Kim, C.W., Ryu, K.S., Kim, B.H., Kim, K.H., Kim, S.I., Kang, T.H., Shin, H.C., Lim, K.H., *et al.* 2008. Protein solubility and folding enhancement by interaction with RNA. *PLoS One* **3**, e2677.

Choi, S.I., Kwon, S., Son, A., Jeong, H., Kim, K.H., and Seong, B.L. 2013. Protein folding *in vivo* revisited. *Curr. Protein Pept. Sci.* **14**, 721–733.

Choi, S.I., Ryu, K., and Seong, B.L. 2009. RNA-mediated chaperone type for *de novo* protein folding. *RNA Biol.* **6**, 21–24.

Ding, Y., Chuan, Y.P., He, L., and Middelberg, A.P.J. 2010. Modeling the competition between aggregation and self-assembly during virus-like particle processing. *Biotechnol. Bioeng.* **107**, 550–560.

Effio, C.L. and Hubbuch, J. 2015. Next generation vaccines and vectors: Designing downstream processes for recombinant protein-based virus-like particles. *Biotechnol. J.* **10**, 715–727.

Fernandes, F., Teixeira, A.P., Carinhas, N., Carrondo, M.J., and Alves, P.M. 2013. Insect cells as a production platform of complex virus-like particles. *Expert Rev. Vaccines* **12**, 225–236.

Fiedler, J.D., Higginson, C., Hovlid, M.L., Kislukhin, A.A., Castillejos, A., Manzenrieder, F., Campbell, M.G., Voss, N.R., Potter, C.S., Carragher, B., *et al.* 2012. Engineered mutations change the structure and stability of a virus-like particle. *Biomacromolecules* **13**, 2339–2348.

Galarza, J.M., Latham, T., and Cupo, A. 2005. Virus-like particle vaccine conferred complete protection against a lethal influenza virus challenge. *Viral Immunol.* **18**, 365–372.

Giersing, B.K., Modjarrad, K., Kaslow, D.C., and Moorthy, V.S. 2016a. WHO Product Development for Vaccines Advisory Committee;

WHO Product Development for Vaccines Product Development Advisory Committee. Report from the World Health Organization's Product Development for Vaccines Advisory Committee (PDVAC) meeting, Geneva, 7-9th Sep 2015. *Vaccine* **34**, 2865–2869.

Giersing, B.K., Modjarrad, K., Kaslow, D.C., Okwo-Bele, J.M., and Moorthy, V.S. 2016b. The 2016 Vaccine Development Pipeline: A special issue from the World Health Organization Product Development for Vaccine Advisory Committee (PDVAC). *Vaccine* **34**, 2863–2864.

Grimes, J.M., Burroughs, J.N., Gouet, P., Diprose, J.M., Malby, R., Ziéntara, S., Mertens, P.P., and Stuart, D.I. 1998. The atomic structure of the bluetongue virus core. *Nature* **395**, 470–478.

Gunther, S., Feldmann, H., Geisbert, T.W., Hensley, L.E., Rollin, P.E., Nichol, S.T., Stroher, U., Artsob, H., Peters, C.J., Ksiazek, T.G., *et al.* 2011. Management of accidental exposure to Ebola virus in the bios afety level 4 laboratory, Hamburg, Germany. *J. Infect. Dis.* **204**, S785–S790.

Hall, A.J. 2012. Noroviruses: the perfect human pathogens? *J. Infect. Dis.* **205**, 1622–1624.

Hall, A.J., Rosenthal, M., Gregoricus, N., Greene, S.A., Ferguson, J., Henao, O.L., Vinjé, J., Lopman, B.A., Parashar, U.D., and Widdowson, M.A. 2011. Incidence of acute gastroenteritis and role of norovirus, Georgia, USA, 2004–2005. *Emerg. Infect. Dis.* **17**, 1381–1388.

Han, K.S., Kim, S.I., Choi, S.I., and Seong, B.L. 2005. N-Glycosylation of secretion enhancer peptide as influencing factor for the secretion of target proteins from *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **337**, 557–562.

Huhti, L., Blazevic, V., Nurminen, K., Koho, T., Hytönen, V.P., and Vesikari, T. 2010. A comparison of methods for purification and concentration of norovirus GII-4 capsid virus-like particles. *Arch. Virol.* **155**, 1855–1858.

Jennings, G.T. and Bachmann, M.F. 2008. The coming of age of virus-like particle vaccines. *Biol. Chem.* **389**, 521–536.

Jiang, X., Wang, M., Graham, D.Y., and Estes, M.K. 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. *J. Virol.* **66**, 6527–6532.

Kaczmarczyk, S.J., Sitaraman, K., Young, H.A., Hughes, S.H., and Chatterjee, D.K. 2011. Protein delivery using engineered virus-like particles. *Proc. Natl. Acad. Sci. USA* **108**, 16998–17003.

Kang, S.M., Kim, M.C., and Compans, R.W. 2012. Virus-like particles as universal influenza vaccines. *Expert Rev. Vaccines* **11**, 995–1007.

Kato, S., Kobayashi, K., Inoue, K., Kuramochi, M., Okada, T., Yaginuma, H., Morimoto, K., Shimada, T., Takada, M., and Kobayashi, K. 2011. A lentiviral strategy for highly efficient retrograde gene transfer by pseudotyping with fusion envelope glycoprotein. *Hum. Gene Ther.* **22**, 197–206.

Kawano, M., Matsui, M., and Handa, H. 2013. SV40 virus-like particles as an effective delivery system and its application to a vaccine carrier. *Expert Rev. Vaccines* **12**, 199–210.

Keller, S.A., Schwarz, K., Manolova, V., von Allmen, C.E., Kinzler, M.G., Bauer, M., Muntwiler, S., Saudan, P., and Bachmann, M.F. 2010. Innate signaling regulates cross-priming at the level of DC licensing and not antigen presentation. *Eur. J. Immunol.* **40**, 103–112.

Keswani, R.K., Pozdol, I.M., and Pack, D.W. 2013. Design of hybrid lipid/retroviral-like particle gene delivery vectors. *Mol. Pharm.* **10**, 1725–1735.

Kim, H.J. and Kim, H.J. 2017. Yeast as an expression system for producing virus-like particles: what factors do we need to consider? *Lett. Appl. Microbiol.* **64**, 111–123.

Kissmann, J., Ausar, S.F., Foubert, T.R., Brock, J., Switzer, M.H., Detzi, E.J., Vedvick, T.S., and Middaugh, C.R. 2008. Physical stabilization of Norwalk virus-like particles. *J. Pharm. Sci.* **97**, 4208–4218.

Koho, T., Mäntylä, T., Laurinmäki, P., Huhti, L., Butcher, S.J., Vesikari, T., Kulomaa, M.S., and Hytönen, V.P. 2012. Purification of norovirus-like particles (VLPs) by ion exchange chromatography. *J. Virol. Methods* **181**, 6–11.

Kuroda, D., Shirai, H., Jacobson, M.P., and Nakamura, H. 2012. Computer-aided antibody design. *Protein Eng. Des. Sel.* **25**, 507–521.

Kushnir, N., Streatfield, S.J., and Yusibov, V. 2012. Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. *Vaccine* **31**, 58–83.

Lai, H. and Chen, Q. 2012. Bioprocessing of plant derived virus-like particles of Norwalk virus capsid protein under current Good Manufacture Practice regulations. *Plant Cell. Rep.* **31**, 573–584.

Lee, J., Choi, S.I., Jang, J.S., Jang, K., Moon, J.W., Bae, C.S., Yang, D.S., and Seong, B.L. 1999. Novel secretion system of recombinant *Saccharomyces cerevisiae* using an N-terminus residue of human IL-1 beta as secretion enhancer. *Biotechnol. Prog.* **15**, 884–890.

Li, H.Y., Han, J.F., Qin, C.F., and Chen, R. 2013. Virus-like particles for enterovirus 71 produced from *Saccharomyces cerevisiae* potently elicits protective immune responses in mice. *Vaccine* **31**, 3281–3287.

Lico, C., Chen, Q., and Santi, L. 2008. Viral vectors for production of recombinant proteins in plants. *J. Cell. Physiol.* **216**, 366–377.

Link, A., Zabel, F., Schnetzler, Y., Titz, A., Brombacher, F., and Bachmann, M.F. 2012. Innate immunity mediates follicular transport of particulate but not soluble protein antigen. *J. Immunol.* **188**, 3724–3733.

Liu, M.A., Wahren, B., and Karlsson Hedestam, G.B. 2006. DNA vaccines: recent developments and future possibilities. *Hum. Gene Ther.* **17**, 1051–1061.

Ma, J.K.C., Barros, E., Bock, R., Christou, P., Dale, P.J., Dix, P.J., Fischer, R., Irwin, J., Mahoney, R., Pezzotti, M., *et al.* 2005. Molecular pharming for new drugs. *EMBO Rep.* **6**, 593–599.

Mach, H., Volkin, D.B., Troutman, R.D., and Wang, B.E.I. 2006. Disassembly and reassembly of yeast-derived recombinant human papillomavirus virus-like particles (HPV VLPs). *J. Pharm. Sci.* **95**, 2195–2206.

Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P., and Bachmann, M.F. 2008. Nanoparticles target distinct dendritic cell populations according to their size. *Eur. J. Immunol.* **38**, 1404–1413.

Marsian, J. and Lomonossoff, G.P. 2016. Molecular pharming - VLPs made in plants. *Curr. Opin. Biotechnol.* **37**, 201–206.

Matić, S., Masenga, V., Poli, A., Rinaldi, R., Milne, R.G., Vecchiati, M., and Noris, E. 2012. Comparative analysis of recombinant Human Papillomavirus 8 L1 production in plants by a variety of expression systems and purification methods. *Plant Biotechnol. J.* **10**, 410–421.

McCarthy, M.P., White, W.I., Palmer-Hill, F., Koenig, S., and Suzich, J.A. 1998. Quantitative disassembly and reassembly of human papillomavirus type 11 virus-like particles *in vitro*. *J. Virol.* **72**, 32–41.

McGuigan, L.C., Stallard, V., Roos, J.M., and Payne, L.G. 1993. Recombinant-expressed virus-like particle pseudotypes as an approach to vaccine development. *Vaccine* **11**, 675–678.

Mechtcheriakova, I.A., Eldarov, M.A., Nicholson, L., Shanks, M., Skryabin, K.G., and Lomonossoff, G.P. 2006. The use of viral vectors to produce hepatitis B virus core particles in plants. *J. Virol. Methods* **131**, 10–15.

Münger, K., Baldwin, A., Edwards, K.M., Hayakawa, H., Nguyen, C.L., Owens, M., Grace, M., and Huh, K. 2004. Mechanisms of human papillomavirus-induced oncogenesis. *J. Virol.* **78**, 11451–11460.

Pallister, J., Middleton, D., Wang, L.F., Klein, R., Haining, J., Robinson, R., Yamada, M., White, J., Payne, J., Feng, Y.R., *et al.* 2011. A recombinant Hendra virus G glycoprotein-based subunit vac-

cine protects ferrets from lethal Hendra virus challenge. *Vaccine* **29**, 5623–5630.

Palomares, L.A., Mena, J.A., and Ramírez, O.T. 2012. Simultaneous expression of recombinant proteins in the insect cell-baculovirus system: production of virus-like particles. *Methods* **56**, 389–395.

Pankavich, S. and Ortoleva, P. 2012. Multiscaling for systems with a broad continuum of characteristic lengths and times: Structural transitions in nanocomposites. *J. Math. Phys.* **51**, 63303.

Patel, M.M., Hall, A.J., Vinjé, J., and Parashar, U.D. 2009. Noroviruses: a comprehensive review. *J. Clin. Virol.* **44**, 1–8.

Paul, M., van Dolleweerd, C., Drake, P.M., Reljic, R., Thangaraj, H., Barbi, T., Stylianou, E., Pepponi, I., Both, L., Hehle, V., *et al.* 2011. Molecular Pharming: future targets and aspirations. *Hum. Vaccin.* **7**, 375–382.

Peacey, M., Wilson, S., Baird, M.A., and Ward, V.K. 2007. Versatile RHDV virus-like particles: incorporation of antigens by genetic modification and chemical conjugation. *Biotechnol. Bioeng.* **98**, 968–977.

Pei, H., Liu, J., Cheng, Y., Sun, C., Wang, C., Lu, Y., Ding, J., Zhou, J., and Xiang, H. 2005. Expression of SARS-coronavirus nucleocapsid protein in *Escherichia coli* and *Lactococcus lactis* for serodiagnosis and mucosal vaccination. *Appl. Microbiol. Biotechnol.* **68**, 220–227.

Peyret, H., Gehin, A., Thuenemann, E.C., Blond, D., El Turabi, A., Beales, L., Clarke, D., Gilbert, R.J., Fry, E.E., Stuart, D.I., *et al.* 2015. Tandem fusion of hepatitis B core antigen allows assembly of virus-like particles in bacteria and plants with enhanced capacity to accommodate foreign proteins. *PLoS One* **10**, e0120751.

Plummer, E.M. and Manchester, M. 2011. Viral nanoparticles and virus-like particles: platforms for contemporary vaccine design. *Wiley Interdiscip. Rev. Nanomed Nanobiotechnol.* **3**, 174–196.

Powell, K. 2004. DNA vaccines — back in the saddle again? *Nat. Biotechnol.* **22**, 799–801.

Prasad, B.V., Hardy, M.E., Dokland, T., Bella, J., Rossmann, M.G., and Estes, M.K. 1999. X-ray crystallographic structure of the Norwalk virus capsid. *Science* **286**, 287–290.

Ramqvist, T., Andreasson, K., and Dalianis, T. 2007. Vaccination, immune and gene therapy based on virus-like particles against viral infections and cancer. *Expert. Opin. Biol. Ther.* **7**, 997–1007.

Rathore, A.S. and Winkle, H. 2009. Quality by design for biopharmaceuticals. *Nat. Biotechnol.* **27**, 26–34.

Ren, Y., Wong, S.M., and Lim, L.Y. 2006. *In vitro*-reassembled plant virus-like particles for loading of polyacids. *J. Gen. Virol.* **87**, 2749–2754.

Rodríguez-Limas, W.A., Flores-Samaniego, B., de la Mora, G., Ramírez, O.T., and Palomares, L.A. 2011. Molecular and process design for rotavirus-like particle production in *Saccharomyces cerevisiae*. *Microb. Cell Fact.* **10**, 33.

Roldão, A., Mellado, M.C., Lima, J.C., Carrondo, M.J., Alves, P.M., and Oliveira, R. 2012. On the effect of thermodynamic equilibrium on the assembly efficiency of complex multi-layered virus-like particles (VLP): The case of rotavirus VLP. *PLoS Comput. Biol.* **8**, e1002367.

Rybicki, E.P. 2014. Plant-based vaccines against viruses. *Virol. J.* **11**, 205.

Sabchareon, A., Wallace, D., Sirivichayakul, C., Limkittikul, K., Chanthavanich, P., Suvannadabba, S., Jiwariyavej, V., Dulyachai, W., Pengsaa, K., Wartel, T.A., *et al.* 2012. Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. *Lancet* **380**, 1559–1567.

Salunke, D.M., Caspar, D.L., and Garcea, R.L. 1986. Self-assembly of purified polyomavirus capsid protein VP1. *Cell* **46**, 895–904.

Sánchez-Rodríguez, S.P., Münch-Anguiano, L., Echeverría, O., Vázquez-Nin, G., Mora-Pale, M., Dordick, J.S., and Bustos-Jaimes, I. 2012. Human parvovirus B19 virus-like particles: *In vitro* assembly and stability. *Biochimie* **94**, 870–888.

Scotti, N. and Rybicki, E.P. 2013. Virus-like particles produced in plants as potential vaccines. *Expert Rev. Vaccines* **12**, 211–224.

Seow, Y. and Wood, M.J. 2009. Biological gene delivery vehicles: beyond viral vectors. *Mol. Ther.* **17**, 767–777.

Shapshak, P., Chiappelli, F., Somboonwit, C., and Sinnott, J. 2011. The influenza pandemic of 2009: lessons and implications. *Mol. Diagn. Ther.* **15**, 63–81.

Shi, L., Sanyal, G., Ni, A., Luo, Z., Doshna, S., Wang, B., Graham, T.L., Wang, N., and Volkin, D.B. 2005. Stabilization of human papillomavirus virus-like particles by non-ionic surfactants. *J. Pharm. Sci.* **94**, 1538–1551.

Søborg, C., Mølbak, K., Doherty, T.M., Ulleryd, P., Brooks, T., Coenen, C., and van der Zeijst, B. 2009. Vaccines in a hurry. *Vaccine* **27**, 3295–3298.

Steel, J., Lowen, A.C., Wang, T.T., Yondola, M., Gao, Q., Haye, K., Garcia-Sastre, A., and Palese, P. 2010. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio* **1**, e00018-10.

Storni, T., Lechner, F., Erdmann, I., Bächi, T., Jegerlehner, A., Dumrese, T., Kündig, T.M., Ruedl, C., and Bachmann, M.F. 2002. Critical role for activation of antigen-presenting cells in priming of cytotoxic T cell responses after vaccination with virus-like particles. *J. Immunol.* **168**, 2880–2886.

Taylor, R.M. 2009. Production and downstream processing of norovirus virus-like particles. Presented at: 12th International Meeting, Baculovirus and Insect Cell Culture. San Antonio, TX, USA.

Turley, C.B., Rupp, R.E., Johnson, C., Taylor, D.N., Wolfson, J., Tussey, L., Kavita, U., Stanberry, L., and Shaw, A. 2011. Safety and immunogenicity of a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) in healthy adults. *Vaccine* **29**, 5145–5152.

Twyman, R.M., Stoger, E., Schillberg, S., Christou, P., and Fischer, R. 2003. Molecular farming in plants: host systems and expression technology. *Trends Biotechnol.* **21**, 570–578.

U.S. Food and Drug Administration, CBER. 1999. Guidance for Industry Information and Establishment Description Information for a *Vaccine*.

Wang, T.T., Tan, G.S., Hai, R., Pica, N., Petersen, E., Moran, T.M., and Palese, P. 2010. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. *PLoS Pathog.* **6**, e1000796.

Whitacre, D.C., Lee, B.O., and Milich, D.R. 2009. Use of hepadnavirus core proteins as vaccine platforms. *Expert Rev. Vaccines* **8**, 1565–1573.

World Health Organization. 2006. Guidelines to assure the quality, safety and efficacy of recombinant human papillomavirus virus-like particle vaccines, pp. 23–27.

Wynne, S.A., Crowther, R.A., and Leslie, A.G. 1999. The crystal structure of the human hepatitis B virus capsid. *Mol. Cell.* **3**, 771–780.

Xing, L., Li, T.C., Mayazaki, N., Simon, M.N., Wall, J.S., Moore, M., Wang, C.Y., Takeda, N., Wakita, T., Miyamura, T., *et al.* 2010. Structure of hepatitis E virion-sized particle reveals an RNA-dependent viral assembly pathway. *J. Biol. Chem.* **285**, 33175–33183.

York, I. and Donis, R.O. 2013. The 2009 pandemic influenza virus: where did it come from, where is it now, and where is it going? *Curr. Top. Microbiol. Immunol.* **370**, 241–257.

Yu, Z., Reid, J.C., and Yang, Y.P. 2013. Utilizing dynamic light scattering as a process analytical technology for protein folding and aggregation monitoring in vaccine manufacturing. *J. Pharm. Sci.* **102**, 4284–4290.

Zabel, F., Kündig, T.M., and Bachmann, M.F. 2013. Virus-induced humoral immunity: on how B cell responses are initiated. *Curr. Opin. Virol.* **3**, 357–362.

Zeltins, A. 2013. Construction and characterization of virus-like particles: a review. *Mol. Biotechnol.* **53**, 92–107.

Zhang, L., Tang, R., Bai, S., Connors, N.K., Lua, L.H., Chuan, Y.P., Middelberg, A.P., and Sun, Y. 2013. Molecular energetics in the capsomere of virus-like particle revealed by molecular dynamics simulations. *J. Phys. Chem. B.* **117**, 5411–5421.

Zhang, L., Tang, R., Bai, S., Connors, N.K., Lua, L.H., Chuan, Y.P., Middelberg, A.P., and Sun, Y. 2014. Energetic changes caused by antigenic module insertion in a virus-like particle revealed by experiment and molecular dynamics simulations. *PLoS One* **9**, e107313.

Zhao, Q., Allen, M.J., Wang, Y., Wang, B., Wang, N., Shi, L., and Sitrin, R.D. 2012a. Disassembly and reassembly improves morphology and thermal stability of human papillomavirus type 16 virus-like particles. *Nanomedicine* **8**, 1182–1189.

Zhao, Q., Modis, Y., High, K., Towne, V., Meng, Y., Wang, Y., Alexandroff, J., Brown, M., Carragher, B., Potter, C.S., *et al.* 2012b. Disassembly and reassembly of human papillomavirus virus-like particles produces more virion-like antibody reactivity. *Virol. J.* **9**, 52.