Review

Biotechnology and Bioengineering

Bioengineering Virus-Like Particles as Vaccines

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ABSTRACT: Virus-like particle (VLP) technology seeks to harness the optimally tuned immunostimulatory properties of natural viruses while omitting the infectious trait. VLPs that assemble from a single protein have been shown to be safe and highly efficacious in humans, and highly profitable. VLPs emerging from basic research possess varying levels of complexity and comprise single or multiple proteins, with or without a lipid membrane. Complex VLP assembly is traditionally orchestrated within cells using black-box approaches, which are appropriate when knowledge and control over assembly are limited. Recovery challenges including those of adherent and intracellular contaminants must then be addressed. Recent commercial VLPs variously incorporate steps that include VLP in vitro assembly to address these problems robustly, but at the expense of process complexity. Increasing research activity and translation opportunity necessitate bioengineering advances and new bioprocessing modalities for efficient and cost-effective production of VLPs. Emerging approaches are necessarily multi-scale and multi-disciplinary, encompassing diverse fields from computational design of molecules to new macro-scale purification materials. In this review, we highlight historical and emerging VLP vaccine approaches. We overview approaches that seek to specifically engineer a desirable immune response through modular VLP design, and those that seek to improve bioprocess efficiency through inhibition of intracellular assembly to allow optimal use of existing purification technologies prior to cell-free VLP assembly. Greater understanding of VLP assembly and increased interdisciplinary activity will see enormous progress in VLP technology over the coming decade, driven by clear translational opportunity.

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KEYWORDS: capsomere; computational; epitope; modular; synthetic biology; vaccine

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Introduction

Virus-like particles (VLPs) are highly ordered repetitive structures, macromolecular assemblies of viral proteins, which effectively crosslink B-cell receptors (Bachmann et al., 1993). VLPs can stimulate both innate and adaptive immune responses, aided by their particulate structure which favors uptake by antigen presenting cells (Keller et al., 2010). These intrinsic immunological characteristics of VLPs, augmented by their self-adjuvanting properties and demonstrated success as products to date (Fig. 1), suggest potential for broad impact in the years ahead. Future success will increasingly be predicated on enhanced bioengineering ability for the predictable carriage and display of antigenic and immunostimulatory modules and encapsulated cargos including biomolecules and nucleic acids. Translation to products at reasonable cost will require improved and new bioprocessing regimes that overcome current limitations pertaining to cost and quality.

Structurally Diverse VLPs

The diversity of VLPs makes them structurally fascinating and functionally versatile. VLPs are classified as non-enveloped or enveloped (Fig. 2). Non-enveloped VLPs are further categorized as single- or multiple-capsid protein VLPs. In general, an enveloped VLP consists of matrix proteins enveloped in a lipid membrane derived from the expression host, with glycoproteins embedded in the lipid.

A structurally simple VLP is a non-enveloped single capsid VLP, such as the licensed human papillomavirus (HPV) VLP vaccines. These simple VLPs, composed of a single capsid protein, can be produced in both prokaryotic and eukaryotic expression systems. In some cases, the capsid proteins of simple VLPs are assembled in a cell-free environment to form homogenous VLPs (Chen et al., 2001; Salunke et al., 1986) or are synthesized and assembled in an entirely cell-free environment (Bundy and Swartz, 2011; Bundy et al., 2008). On the other hand, non-enveloped multiple-capsid protein VLPs are more complex and challenging to produce. These complex VLPs are usually produced in higher eukaryotic hosts such as yeast (Li et al., 2013; Rodriguez-

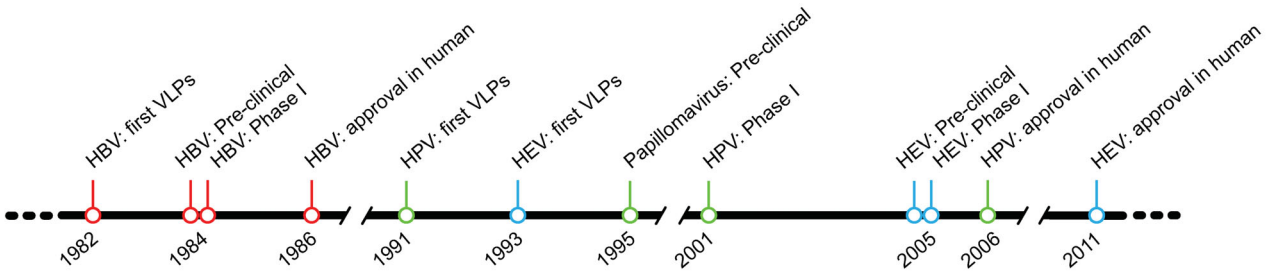

Figure 1. Developmental milestones of currently approved VLP-based vaccines. The development of the three VLP-based vaccines approved for human use followed the path from initial recombinant production (first VLPs), to demonstration of protection in animals (pre-clinical), to human clinical trials (Phase I) and approval (approval in human). Hepatitis B virus (HBV) surface antigen VLPs (red) was the first approved VLP-based vaccine, followed by human papillomavirus (HPV) VLPs (green) and then hepatitis E virus (HEV) VLPs (blue).

Limas et al., 2011), insect cells (Fernandes et al., 2013; Palomares and Ramirez, 2009) and plants (Scotti and Rybicki, 2013), which allow for co-expression of the different capsid proteins and complex assembly of the VLPs within a cell. VLPs from bluetongue virus (BTV; French and Roy, 1990), Enterovirus 71 (Chung et al., 2006, 2008; Lin et al., 2012), infectious bursal disease virus (Kibenge et al., 1999), poliovirus (Bräutigam et al., 1993), and rotavirus (Rodriguez-Limas et al., 2011; Vieira et al., 2005) are examples of multiple interacting capsid proteins successfully assembled into multilayered VLPs when produced in a heterologous host.

Enveloped VLPs acquire their lipid membranes from the expression cell line during the assembly and budding of the VLPs from the cells. One or more types of glycoprotein spikes can be embedded in the lipid bilayer and these glycoproteins are the target immunological antigen for generating neutralizing antibodies. This category of VLPs is poorly characterized biophysically as they are structurally less uniform than to their non-enveloped VLP counterparts. Influenza VLPs are possibly the most studied enveloped VLPs (Kang et al., 2012), commonly consisting of matrix M1 proteins and with glycoprotein hemagglutinin (HA) and/or neuraminidase (NA) embedded in the lipid bilayer. Human immunodeficiency virus (HIV) VLPs (Buonaguro et al., 2013) and Ebola virus VLPs (Warfield and Aman, 2011) have also been well reported, with recent attention on VLPs of Chikungunya virus (Metz et al., 2013; Velez et al., 2012) and Nipah virus (Walpita et al., 2011).

Functionally Versatile VLPs

The versatility of VLPs allows tailorable functionality, thus leading to a wide variety of applications as illustrated in Figure 3. VLPs are best known as immunogens (Noad and Roy, 2003), such as the HPV vaccines Gardasil® and Cervarix®, which are produced using yeast and insect cells, respectively. Hepatitis B virus (HBV) and HPV VLP-based vaccines are structurally similar to their parental virions but devoid of the infectious genetic material. Such recombinantly produced VLPs are assemblies of unmodified capsid proteins derived from the parental virus, such as surface antigen for HBsAg VLPs (Valenzuela et al., 1982) and L1 for HPV VLPs (Kirnbauer et al., 1992). A subset of prophylactic VLP immunogens comprises enveloped VLPs with viral proteins from unrelated viruses [e.g., Newcastle disease virus (NDV) VLPs carrying respiratory syncytial virus (RSV) F and G proteins (McGinnes et al., 2011)] or multiple serotypes of the same virus [e.g., influenza VLPs with 3 distinct HA subtypes (Pushko et al., 2011)]. These hybrid VLPs represent an emerging approach to vaccine design.

A class of novel vaccine candidates has developed as a result of presentation of novel immunological antigens on VLPs. Novel antigens are placed on the surface of VLPs either by molecular fusion or chemical conjugation (Jennings and Bachmann, 2008; Peacey et al., 2007). The highly arrayed structure of VLPs allows ordered repetitive presentation of the heterologous epitopes on the particle surface. Therefore, VLPs offer a favorable platform that is extensively exploited for generation of epitope-based VLPs to target various diseases (Plummer and Manchester, 2011). The desire to generate a specifically understood antibody response against a defined epitope has led to numerous studies of small peptide or protein modules displayed on VLPs, as reviewed elsewhere (Chackerian, 2007; Jennings and Bachmann, 2008; Roose et al., 2013). Whole heterologous antigen presentation is increasingly being explored because of complexity around maintaining the structural authenticity of defined epitopes when removed from a large protein onto a presenting VLP (Anggraeni et al., 2013). Large antigen display also raises a polyvalent antibody response and does not require understanding of neutralizing epitopes which may be limiting. This approach has been explored using HBV (Bisht et al., 2002), flock house virus (FHV; Manayani et al., 2007), and bacteriophage T4 virus (Shivachandra et al., 2006) display.

The properties of some viral capsid proteins allow engineered VLPs to encapsulate proteins, molecules, or nucleic acids. These packaged VLPs act as delivery vessels, designed to deliver its cargo to specific cells, tissues or organs,

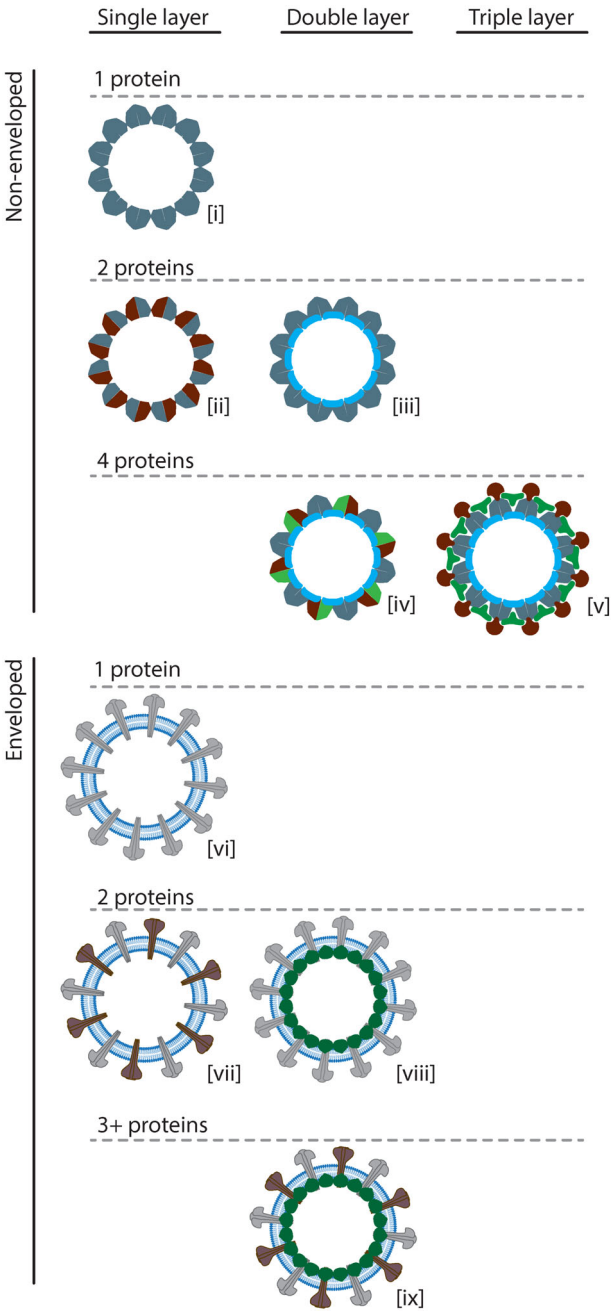

Figure 2. Structural diversity of VLPs. Both capsid-based and enveloped VLPs can be single or multilayered and composed of single or multiple proteins. Single layered non-enveloped VLPs can be assembled from (i) a single protein [e.g., hepatitis B core antigen (Clarke et al., 1987; Whitacre et al., 2009)]; or (ii) two proteins [e.g., cowpea mosaic virus VLPs (Saunders et al., 2009)]. Double layered non-enveloped VLPs can be assembled from (iii) two proteins [e.g., papillomavirus L1 and L2 VLPs (Kirnbauer et al., 1993)]; or (iv) four proteins [e.g., Foot-and-mouth disease virus (Porta et al., 2013)]. Triple layered VLPs (v) have been assembled from four coat proteins of bluetongue virus (Hewat et al., 1994) and rotavirus (Conner et al., 1996). For enveloped VLPs, expression of one (vi) or two glycoproteins (vii) will form a single layer, as demonstrated by the expression of influenza virus hemagglutinin (D'Aoust et al., 2008) and the co-expression of both hemagglutinin and neuraminidase (Chen et al., 2007), respectively. Double layered enveloped VLPs can be formed by the (viii) inclusion of structural proteins [e.g., influenza VLPs (Latham and Galarza, 2001)] and may possess (ix) multiple glycoproteins on their surface (Latham and Galarza, 2001; Pushko et al., 2011).

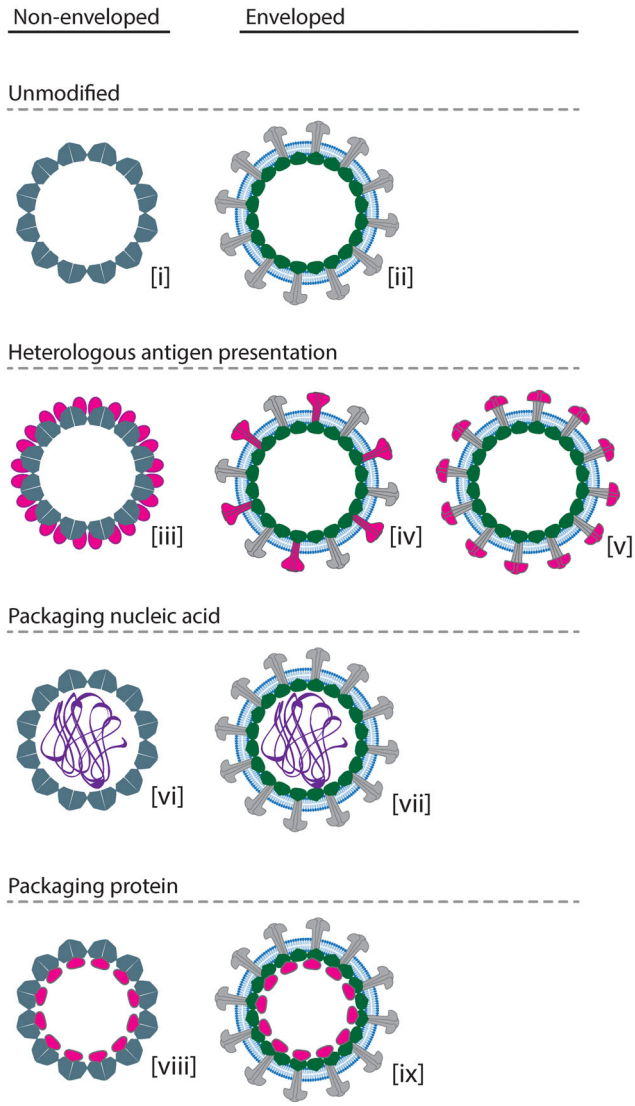

Figure 3. Functional diversity offered by VLPs. For vaccination against the cognate virus, VLPs may be generated from unmodified components as in the case for (i) non-enveloped human papillomavirus vaccines Gardasil® and Cervarix® and (ii) enveloped influenza virus vaccine candidates (Galarza et al., 2005). Heterologous antigen presentation on (iii) 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 (iv) insertion of glycoproteins [e.g., herpes simplex virus glycoprotein inserted into Simian immunodeficiency virus VLPs (McGuigan et al., 1993)]; or by (v) heterologous head domain fusion onto transmembrane region [e.g., vesicular stomatitis virus glycoprotein ectodomain fused to rabies virus glycoprotein (Kato et al., 2011)]. A number of VLPs are being developed as delivery vehicles for nucleic acids including the (vi) non-enveloped Simian virus 40 (Kawano et al., 2013); and (vii) enveloped retrovirus VLPs (Keswani et al., 2013). Lastly, both enveloped (viii) and non-enveloped (ix) VLPs are also being developed for the encapsulation and delivery of heterologous proteins.

via targeting antibodies or cell-specific ligands on the surface of the 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).

Computational VLP-Based Vaccine Design (and Redesign)

A structural understanding of VLPs will allow their exploitation as immunological particles and accelerate VLP vaccine development. There are already more than 350 completed icosahedral virus capsid structures within the VIPERdb database (Carrillo-Tripp et al., 2009). These structures were determined by either X-ray crystallography or cryo-electron microscopy (cryo-EM; Carrillo-Tripp et al., 2009), and in combination with icosahedral symmetry averaging, these complementary methods become a common procedure for solving crystal structures of viral capsids (Grimes et al., 1998; Prasad et al., 1999; Wynne et al., 1999). While structure determination approaches have enabled further understanding of HPV VLPs (Stanley et al., 2006) and characterization of peptide presentation on viral surfaces (Lin et al., 1996; Taylor et al., 2000), crystallized structures may not represent the proteins in their native solution conditions. Furthermore, the information provided is limited to a static conformation and gives no detail on the dynamics of the biological system (Joshi et al., 2011b).

Computational methods are almost universally accepted as complementary to empirical structural analysis methods, and in combination provide a means to generate hypotheses, guide experiments, and aid data interpretation (Kuroda et al., 2012). With the advancement of computers and informatics, new approaches have been devised to facilitate vaccine research (He et al., 2010), including bioinformatics and 3D structure prediction. Computational techniques have been extensively applied to biological research and drug discovery (Galeazzi, 2009; Jorgensen, 2004); although their application in VLP research is in the early stages.

Computer modeling of VLPs has largely focused on the self-assembly kinetics of VLPs (Ding et al., 2010; Pankavich and Ortoleva, 2012; Roldao et al., 2012), directed at minimizing protein aggregation during processing. A limiting factor for computational modeling of VLPs is their large size, as it requires extensive computational resources and long time periods for simulation. Multiscale models have been used to reduce these required computing resources for cowpea chlorotic mottle virus (CCMV; Miao and Ortoleva, 2006, 2010) and dengue virus capsids (Shreif et al., 2008). Nevertheless, computational power available now from supercomputers enables molecular dynamics (MD) simulations of multi-million atom systems, thus allowing in silico investigation of macromolecular structures including viruses, such as the whole poliovirus (Roberts et al., 2012). The energetics of polyomavirus VLP subunits stability in different buffers was probed using MD simulations and further validated with experimental analyses (Zhang et al., 2013). Joshi et al. (2011a) used MD simulations to investigate the conformational changes of surface exposed loops between subunits and capsids of HPV VLPs, and the effects on immunogenicity. All capsid simulations to date have focused on unmodified VLP vaccine development, as opposed to the exploitation of the VLP surface for heterologous epitope display.

Bioinformatics analyses have been used to optimize epitopes for presentation on VLPs, though this was phylogeny based rather than via structural analyses (Giles and Ross, 2011; Giles et al., 2012a,b). Three-dimensional structure homology modeling of influenza epitopes for presentation on FHV VLPs was employed to predict and understand experimental immunological results (Schneemann et al., 2012). Antibodies produced against these modular VLPs were not protective in an animal challenge study, emphasizing the need for better structural epitope prediction. MD simulations on HIV epitopes presented on rhinovirus capsid subunits were performed to predict epitope native-like conformation for antibody binding (Lapelosa et al., 2009). Although this study was on viruses and not VLPs, it highlights that the length and hydrophobicity of the epitope and the site of epitope insertion are crucial for achieving the greatest structural similarity to the native HIV epitope (Lapelosa et al., 2010). The importance of native structure to the desired immunological result was recently evidenced in a study on modular polyomavirus VLPs presenting a hypervariable helix epitope (Anggraeni et al., 2013). MD simulations on variant epitope peptides supported the immunological animal results, where the peptide design with less structural deviation from the native helical epitope achieved a higher quality of immune response. This further validated the use of computational analyses for both the design of modular VLPs and as a complementary approach to experimentation.

The prediction of functional epitopes and the ability to present the native 3D structure of the epitope on the carrier molecule are current challenges in epitope-based VLP vaccine design (He et al., 2013). Computer modeling enables informed prediction of the unknown structure of a peptide or protein sequence using an array of computational tools (Fig. 4). This information applies to design, redesign, or experimental data interpretation, particularly for application on newly emerging modular VLPs, as illustrated in Figure 4. Through acceleration of computing technology, we can expect simulations of whole modular subunits and their VLPs, for vaccine design, in the not too distant future.

Current and Emerging Paradigms for VLP Production

Commercial VLP production has been achieved with *Escherichia coli*, yeast, baculovirus-insect cell and CHO mammalian cell expression systems. HBsAg VLPs produced in yeast (*Saccharomyces cerevisiae* and *Pichia pastoris*) or CHO mammalian cells, HPV VLPs expressed in *S. cerevisiae* (Gardasil®, Merck & Co) or in insect cells (Cervarix®, GlaxoSmithKline), and hepatitis E virus (HEV) VLPs expressed in *E. coli* and assembled in vitro (Hecolin®, Xiamen Innovax Biotech Co. Ltd.), are the only licensed VLP-based human vaccines (Fig. 1). Numerous VLP-based vaccine candidates are currently in clinical trials and are produced using various expression systems (Roldao et al., 2010).

The structural complexity and immunogenicity of the desired VLPs are key determinants for the choice of

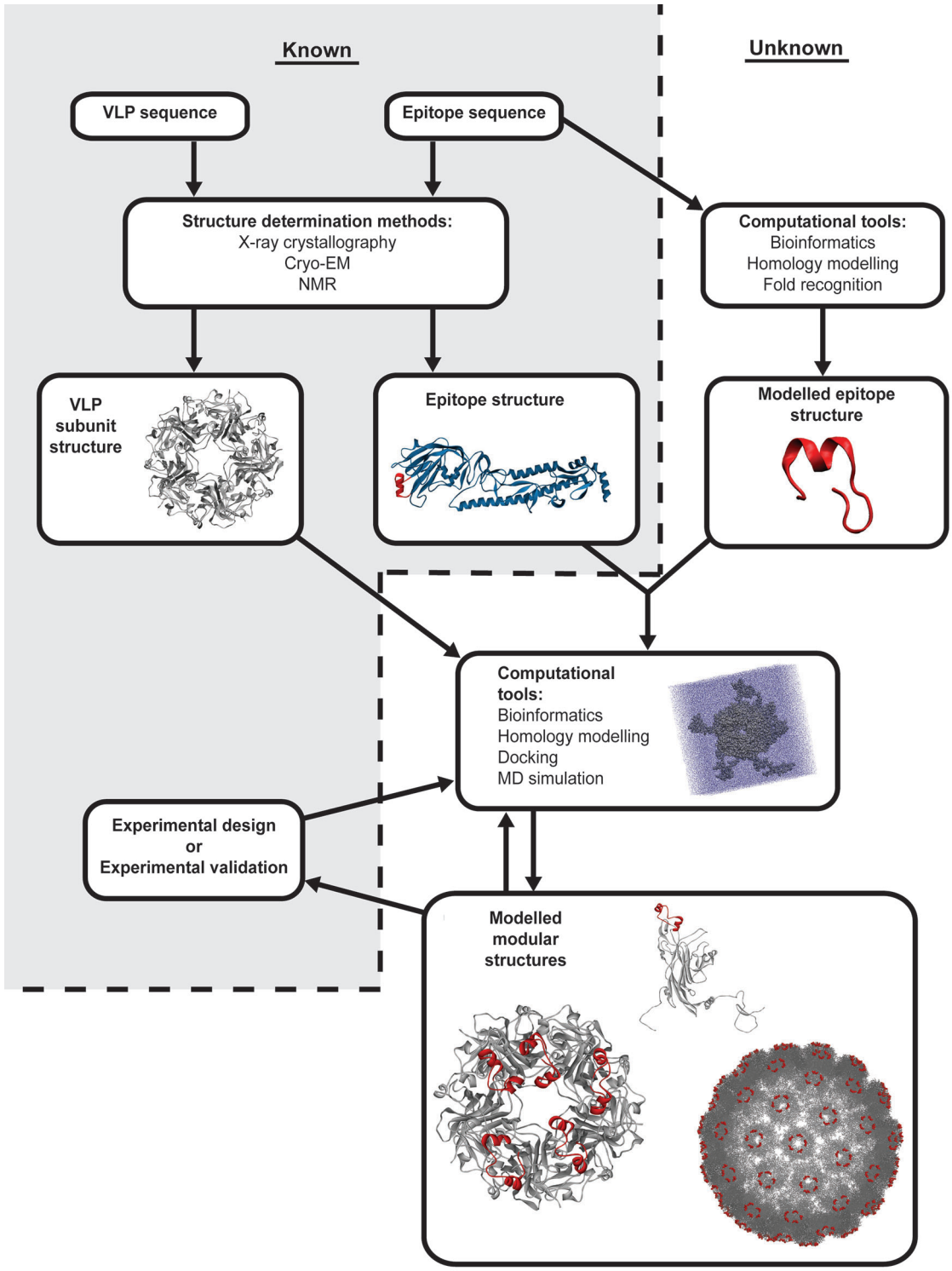

Figure 4. Application of computational tools for modular VLP design. Computational tools are used to predict and model unknown structures, such as antigenic epitopes, modular subunits and VLPs, for either experimental design or validation. With a known sequence, an unknown structural model can be derived using a variety of computational tools such as bioinformatics, homology modeling and fold recognition. The unknown structure of modular proteins, where two or more proteins/peptides are genetically fused together, can be derived using the same tools. The molecular interactions and dynamics of the derived modular proteins can be probed using molecular dynamics simulation, while immunological inferences can also be made by drug or antibody docking simulations. Here, influenza hemagglutinin epitope (3MLH.pdb), colored red, is modeled on murine polyomavirus subunits (1SID.pdb)-colored gray, and presented on the surface of modular VLPs. Homology models were created and visualized using Accelrys Discovery Studio 3.0 and capsid was visualized using VMD.

Table I. Comparison of production host systems [reported yields obtained from (Liew et al., 2010; Roldao et al., 2010; Scotti and Rybicki, 2013)].

	E. coli	Yeast	Baculovirus-insect cells	Mammalian cells	Plants
Speed	++++	+++	++	++	++
Scalability	++++	+++	++	++	++
Yield	++++	+++	++	+	+
Reported yields	4.38 g/L Polyomavirus VP1	400 mg/L Hepatitis B surface antigen	662 mg/L Rotavirus VP2, VP6 and VP7	500 mg/L Adeno-associated virus	3 g/kg Papillomavirus L1
VLP complexity	+	++	++++	++	+++
Regulatory	++++	++++	++	++++	+

expression system for VLP production, however, a system amenable to cost-effective industrial production is important (Table I). Non-enveloped single protein VLPs are generally expressed in *E. coli* or in yeast. Both *E. coli* and yeast offer high expression yield and ease of scale-up (Table I), with the yeast expression system providing for post-translational modification. Baculovirus-insect cell expression is recognized as the workhorse system for production of complex non-enveloped and enveloped VLPs (Palomares and Ramirez, 2009), as insect cells can readily co-express multiple proteins and assemble them into structurally complex VLPs. Nevertheless, there is increasing research activity using alternative systems such as yeasts and plants for large-scale cost-effective VLP production. Enveloped VLPs acquire lipid envelopes, typically with embedded immunogenic glycoprotein spikes, as they bud from the host cells. Hence, they are produced exclusively by eukaryotic hosts. The choice of expression system for VLP production has been extensively reviewed elsewhere (Kushnir et al., 2012; Pushko et al., 2013; Roldao et al., 2010; Zeltins, 2013).

Most VLPs produced in these expression systems will assemble in vivo and the assembled VLPs are purified from the host contaminants after assembly. Evidently, the choice of production system, thereof the contaminant spectrum, greatly impact on the downstream processes and thus overall production cost. The purification in itself is often non-trivial as VLPs may need to be separated from similarly sized particles deriving from the host cell; the issue of adventitious virus removal must also be addressed during validation. Indeed, particle separation processes are themselves an area of active research and the full complexity is not well understood. However, it is clear that cell-assembled VLPs may contain contaminating host proteins and/or DNA (Lipin et al., 2008a). Depending on the contaminant levels, these encapsulated contaminants may have undesirable biological responses, thus require further processing to meet the stringent requirements of biopharmaceutical products (Pattenden et al., 2005). Furthermore, VLPs may themselves associate with proteins from the host cells, contaminating to some extent the exterior of the VLP. These adherent contaminants will themselves increase processing complexity, as it will be necessary to break the physical connection between the VLP and contaminant prior to further processing. Such treatments may themselves drive structural modification or disassembly of the VLP, which is held together by the same weak forces that associate the adherent contaminants.

The cell-orchestrated in vivo VLP assembly process is not well understood. This cell-aided approach is extensively exploited for VLP production, particularly for structurally complex VLPs. Density gradient ultracentrifugation is commonly utilized in most laboratories to purify cell-assembled VLPs. This method does allow the separation of empty VLPs from those packaged with host proteins and DNA (Lipin et al., 2008a), however, it is not economically scalable. In the early development of Gardasil® HPV VLP vaccine, ion exchange (IEX) chromatography was introduced to purify VLPs from *S. cerevisiae* (Cook et al., 1999), with an overall purification yield of 10%. A purification process was later developed using ceramic hydroxyapatite chromatography post IEX to improve both the recovery and purity of HPV VLPs (Baek et al., 2011; Cook, 2003). With increasing demand for purification of macromolecular structures, a new generation of chromatographic media such as CIM® Monoliths (BIA Separations) and Capto™ Core 700 (GE Healthcare, Little Chalfont, Buckinghamshire, UQ) have been developed, and optimized for purification of new VLP modalities. Despite these developments in VLP recovery via chromatography, the challenges associated with ensuring complete removal of packaged and adherent contaminants from cell-assembled VLPs, and issues related to heterogeneity, still remain for cell-orchestrated VLP assembly approaches.

It is well reported that yeast-derived HPV VLPs are highly heterogeneous, consisting of irregularly shaped VLPs with broad size distribution (Mach et al., 2006). Disassembly of purified HPV VLPs and reassembly in vitro produced monodispersed VLPs with enhanced thermal stability and immunogenicity. Recent reports from the same group further support the importance of disassembly and reassembly of HPV VLPs to produce particles with more consistent morphology, improved thermal stability, reduced propensity to aggregate and higher antigenicity (Zhao et al., 2012a,b). On the other hand, GSK's insect cell-derived HPV Cervarix® vaccine consists of C-terminally truncated L1 proteins, to prevent VLP assembly in the cells, thus avoiding DNA encapsulation and improving both purification processes and product purity. These modified capsomeres were purified through a series of chromatographic columns and assembled in vitro to obtain homogeneous VLPs (Deschuyteneer et al., 2010). With intracellular assembly, it can be difficult

to direct the cellular localization and stoichiometric ratio of capsid proteins for the assembly of multilayered VLPs such as rotavirus and BTV VLPs. For example, high production of rotavirus structural proteins inside insect cells did not result in high yields of correct architectural VLPs (Palomares and Ramirez, 2009), and introduced bioprocess complexity associated with the need to separate highly immunogenic triple layered VLPs from the double layered VLPs (Castro-Acosta et al., 2010). Improved control can potentially be obtained through better understanding of the in vitro disassembly and reassembly of rotavirus VLPs (Mellado et al., 2009). Other VLPs orchestrated by diverse hosts have also undergone disassembly and reassembly in vitro, including CCMV (Lavelle et al., 2009; Zhao et al., 1995) and bacteriophage MS2 (Wu et al., 1995), to eliminate host nucleic acid in the VLPs.

An alternative to intracellular assembly processes which eliminates the embedded disassembly–reassembly processing along with purification from adherent and encapsulated contaminants would enable simpler VLP processing. For non-complex VLPs the problem is easily inverted. The biopharmaceutical industry has evolved over an extended period to make purified protein at scale prior to formulation. It is then attractive to view the formation of a non-enveloped VLP simply as an additional "formulation" step, using previously purified protein. This approach eliminates the problem of adherent contaminants as the weak forces that drive VLP formation (Chuan et al., 2010) are suppressed until purified protein product is obtained. Encapsulation of contaminants also cannot occur if contaminants have been removed prior to VLP formation. This emerging paradigm of cell-free VLP assembly is evident in the literature for papillomavirus (Chen et al., 2001) and polyomavirus (Pattenden et al., 2005; Salunke et al., 1986). These examples also fully exploit the cheap, fast, easily scalable *E. coli* system for the preparation of highly purified capsid protein, sometimes at gram-per-litre levels (Liew et al., 2010).

A bacterial-based production platform enables low cost vaccine manufacture, deploying the well established *E. coli* industrial bioprocesses in biopharmaceutical production. The ease and rapid molecular manipulation of this expression system supports the building of modular VLPs and capsomeres to target highly mutable and variable pathogens, providing for rapid response during epidemics and pandemics and at low cost for neglected diseases. Middelberg et al. (2011) presents a robust and flexible modular platform that is tailorable to different diseases, by using polyomavirus capsid protein VP1 to present antigenic modules of pathogens. The versatility of the platform was demonstrated with modular VLPs and capsomeres against Group A Streptococcus (GAS; Chuan et al., 2013; Middelberg et al., 2011; Rivera-Hernandez et al., 2013) and influenza (Anggraeni et al., 2013; Wibowo et al., 2013). All modular capsomeres synthesized in *E. coli* were purified to high purity with similar chromatographic techniques (Lipin et al., 2008b, 2009) and then either assembled in vitro into monodispersed VLPs (Chuan et al., 2010; Liew et al., 2012) or formulated directly as capsomere vaccine candidates (Wibowo et al., 2013). This VLP technology combines the benefits of *E. coli* high productivities and controllable cell-free assembly of contaminant-free capsomeres into modular VLPs, presenting an innovative manufacturing bioprocess for cost-effective production of VLP-based vaccines.

VLP Characterization

Characterization of purified VLP preparations is essential during VLP vaccine development. Misinterpretation of immunological data due to poorly characterized VLP vaccine candidates can impact both VLP design (and redesign) and bioprocess development. Moreover, the propensity for aggregation of VLP subunits and VLPs stipulates analytical characterization as an important quality check on the manufacturing bioprocesses and vaccine batch-to-batch consistency. The functionality of epitope-based VLPs and the physical properties of VLPs, such as size and polydispersity, contribute to the potency and safety of VLP vaccines. Comprehensive VLP characterization, including biochemical, biophysical and biological, for vaccine development or vaccine manufacturing, necessitates multiple complementary analytical tools (Fig. 5), particularly to encompass the structural and functional diversity of engineered VLPs.

Characterization of VLPs uses the standard array of techniques embedded in the biopharmaceutical industry, as a starting point. Analysis includes the determination of amino acid composition, molecular weight, and VLP purity. Mass spectrometry (MS) is an indespendible tool for analysis of protein sequence and it informs the molecular mass of protein analyte as well as its amino acid composition. This technique is useful in proteolytic and post-translational modification studies as shown for potato virus Y-like particles and HBV core protein VLPs (Freivalds et al., 2011; Kalnciema et al., 2012). Protein purity analysis is often conducted on denatured and/or reduced VLPs using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and reversed-phase high performance liquid chromatography (RP-HPLC). SDS–PAGE coupled with Western Blot analysis is useful for analyzing multiple capsid protein VLPs (Bright et al., 2007; Crawford et al., 1994; Latham and Galarza, 2001). Native agarose gel electrophoresis is used to analyze nucleic acid encapsulation within VLPs (Birnbaum and Nassal, 1990; Ibañez et al., 2013; Phelps et al., 2007), to monitor VLPs assembly in the presence of RNA (Caldeira and Peabody, 2007), and to analyze modifications to the external surface of VLPs in comparison to unmodified VLPs (Sainsbury et al., 2011; Steinmetz et al., 2007).

Beyond compositional analyses as detailed above, basic information on VLP morphology and state is obtained by standard laboratory techniques including analytical ultracentrifugation (AUC), density gradient ultracentrifugation, and transmission electron microscopy (TEM; Akinobu et al., 1996; Crawford et al., 1994; Deschuyteneer et al., 2010; Freivalds et al., 2006; Gleiter and Lilie, 2001; Li et al., 2003; Mulder et al., 2012; Pease et al., 2009; Rasmussen

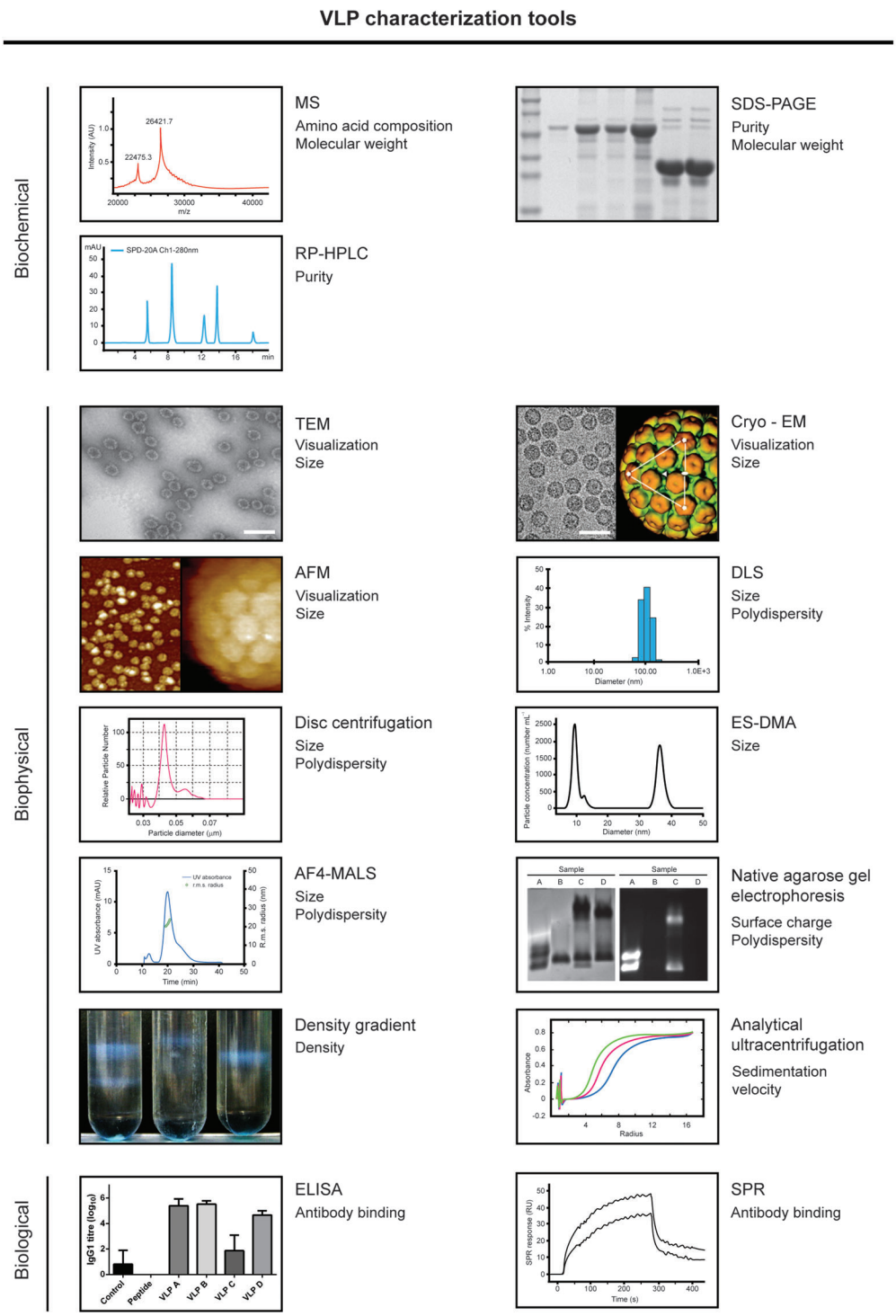

Figure 5. Analytical tools for VLP characterization. A range of analytical tools is available for VLP characterization based on their biochemical, biophysical, and biological properties. MS: mass spectrometry; SDS–PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; TEM: transmission electron microscopy; Cryo-EM: cryo electron microscopy; AFM: atomic force microscopy; DLS: dynamic light scattering; ES-DMA: electrospray differential mobility analysis; AF4-MALS: asymmetric flow field-flow fractionation coupled with multiple-angle light scattering; ELISA: enzyme-linked immunosorbent assay; SPR: surface plasmon resonance. Cryo-EM image is reprinted from Virology, 311 (1), Tian-Cheng Li, Naokazu Takeda, Kenzo Kato, Josefina Nilsson, Li Xing, Lars Haag, R. Holland Cheng, Tatsuo Miyamura, Characterization of self-assembled virus-like particles of human polyomavirus BK generated by recombinant baculoviruses, page no. 115–124, Copyright (2003), with permission from Elsevier (Li et al., 2003). AFM image is reprinted from Nanomedicine: Nanotechnology, Biology and Medicine, 8 (7), Qinjian Zhao, Michael J. Allen, Yang Wang, Bei Wang, Ning Wang, Li Shi, Robert D. Sitrin, Disassembly and reassembly improves morphology and thermal stability of human papillomavirus type 16 virus-like particles, page no. 1182–1189, Copyright (2012), with permission from Elsevier (Zhao et al., 2012b). Native agarose gel image is reprinted from ChemBioChem, 12 (16), Frank Sainsbury, Keith Saunders, Alaa A. A. Aljabali, David J. Evans, George P. Lomonossoff, Peptide-Controlled Access to the Interior Surface of Empty Virus Nanoparticles, Copyright (2011), with permission from John Wiley and Sons (Sainsbury et al., 2011).

et al., 1990; Salunke et al., 1986). AUC informs the molecular weight, conformation and heterogeneity of a sample, while density gradient ultracentrifugation provides density of a VLP sample, and both techniques are complemented with TEM for visual confirmation of VLP-like structure. Although relatively simple, TEM sample preparation can cause VLP deformation and artifacts, and imaging can provide for non-quantitative user-bias leading to preferential visualization of VLP structures. Alternative visualization techniques by Cryo-EM and atomic force microscopy (AFM) are less likely to cause structure deformation due to rapid freezing during sample processing (Adrian et al., 1984) and in-solution analysis (Kuznetsov and McPherson, 2011), respectively. These techniques have been extensively used for characterization of human polyomavirus BK, HPV, and HBsAg VLPs (Li et al., 2003; Milhiet et al., 2011; Mulder et al., 2012; Zhao et al., 2012a).

VLP biophysical characterization with quantitative outputs is accomplished through dynamic light scattering (DLS; Hu et al., 2011; Kissmann et al., 2011), disc centrifugation particle size analysis (Deschuyteneer et al., 2010), electrospray differential mobility analysis (ES-DMA; Pease et al., 2009), and asymmetric flow field-flow fractionation coupled with multiple-angle light scattering (AF4-MALS; Chuan et al., 2008; Citkowicz et al., 2008; Lang et al., 2009, 2010; Mohr et al., 2013; Pease et al., 2009). DLS is the simplest to perform and can accurately and quickly determine the particle size of a monodispersed sample. However, DLS is highly sensitive to large scattering particles and cannot resolve a polydispersed sample well. On the other hand, disc centrifugation particle size analysis, ES-DMA and AF4-MALS provide size separation prior to size measurement, thus allowing particle size determination with high resolution and accuracy. An orthogonal study of ES-DMA and AF4, validated with TEM, has demonstrated the robustness and superiority of these new methods (Pease et al., 2009).

Biological characterization of VLPs is often achieved through analysis of VLP binding to a known antibody. Methods such as enzyme-linked immunosorbent assay (ELISA) (Deschuyteneer et al., 2010; Freivalds et al., 2006; Li et al., 2003), dot blot (Li et al., 2003), immune precipitation or immune diffusion assay (Freivalds et al., 2006; White et al., 1997) are often used to analyze antibody binding to VLPs.

Formulation of VLP Vaccines

Formulation development has traditionally focused on ensuring that the marketed therapeutic products are efficacious, safe to administer and remain stable during shipping and storage (Maddux et al., 2011). For vaccine products, adjuvants and excipients are commonly used to achieve such goals. All the current licensed VLP-based vaccines are formulated with aluminum salts (Table II), the most widely used adjuvant in humans, to enhance the immunogenicity of the antigens despite the self-adjuvanting ability of VLPs (Guillen et al., 2010; Liu et al., 2000; Zhang et al., 2000). The AS04 adjuvant system used in Cervarix®, which contains the toll-like receptor (TLR) 4 agonist 3-*O*-desacyl-4′ monophosphoryl lipid A (MPL) in addition to the alum salt used, has been shown to enhance antigen-specific activation of T-cells (Didierlaurent et al., 2009). Excipients are used in licensed vaccines (Table II) for various purposes: polysorbate 80 as a surfactant stabilizer to reduce surface-enhanced VLP aggregation (Shi et al., 2005), l-histidine, sodium borate, and sodium phosphate as buffering agents, and 2-phenoxyethanol as a preservative agent. The use of adjuvants and excipients in pharmaceutical formulations has been reviewed extensively elsewhere (Aguilar and Rodriguez, 2007; Nema et al., 1997; Petrovsky and Aguilar, 2004).

Innovative effort in formulation has been made to investigate novel VLP formulations that may (i) reduce dosage, (ii) improve immunogenicity, (iii) eliminate the cold chain, and (iv) improve VLP stability over prolonged storage. Numerous formulations to stabilize VLPs against freeze-drying have been reported. By pre-treating the bacteriophage

Table II. Compositions of VLP-based vaccines in the market.

	Composition per dose			
Vaccine	Protein	Adjuvant	Others	Reference
Engerix®-B hepatitis B vaccine (1 mL/dose)	HBsAg (20 μg)	Aluminum hydroxide (500 μg)	2-phenoxyethanol (5.0 mg)	(GlaxoSmithKline, 2013b)
Recombivax HB® hepatitis B vaccine (1 mL/dose)	HBsAg (10 μg)	Aluminum hydroxyphosphate (500 μg)	Sodium chloride (9 mg) Sodium borate (70 μg)	(Merck, 2013b)
Gardasil® human papillomavirus vaccine (0.5 mL/dose)	HPV 6 L1 (20 μg) HPV 11 L1 (40 μg) HPV 16 L1 (40 μg) HPV 18 L1 (20 μg)	Aluminum hydroxyphosphate sulfate (225 μg)	Sodium chloride (9.56 mg) L-histidine (0.78 mg) Polysorbate 80 (50 μg) Sodium borate (35 μg)	(Merck, 2013a)
Cervarix® human papillomavirus vaccine (0.5 mL/dose)	HPV 16 L1 (20 μg) HPV 18 L1 (20 μg)	AS04 adjuvant system (50 μg of 3-*O*-desacyl-4′ monophosphoryl lipid A, 500 μg aluminum hydroxyphosphate)	Sodium chloride (4.4 mg) Sodium dihydrogen phosphate dehydrate (0.624 mg)	(GlaxoSmithKline, 2013a)
Hecolin® hepatitis E vaccine (0.5 mL/dose)	HE antigen (30 μg)	Aluminum hydroxide (800 μg)	Buffered saline	(Bin Park, 2012; Zhu et al., 2010)

Qβ VLPs with polysorbate 20 and disaccharide trehalose, VLPs were stabilized in a lyophilized form for up to 6 months; full biological activity was retained when the VLPs were reconstituted to isotonic formulations (Lang et al., 2009). In addition, lyophilized Norwalk virus (NV) VLPs formulated with GelSite polymer, when delivered intranasally, has been shown to induce superior NV-specific serum and mucosal antibody titers compared to liquid formulations (Velasquez et al., 2011). Enveloped HIV-1 Pr55gag VLPs formulated with 15% trehalose were stable over a period of 12 months and could withstand freeze-thawing (Lynch et al., 2012). Other excipients, such as glycerol, chitosan glutamate, and glycine, have also been reported to confer a significant stabilizing effect on VLPs (Fernandez et al., 2013; Kissmann et al., 2008, 2011). However, VLP formulation remains very much an empirical activity, requiring screening of a large number of sample conditions. A miniaturized high-throughput screening method using DLS microplate reader to screen a large number of formulations, using as little as 2 μg sample per formulation, has been recently reported (Mohr et al., 2013). Screening was followed by analysis of a smaller set of promising formulation conditions using the high-resolution AF4-MALS method (Mohr et al., 2013). Enhanced VLP stability at elevated temperatures up to 58°C was achieved by addition of polysorbate 20 with either sucrose or sorbitol. The addition of polysorbate 20 and sorbitol effectively protected polyomavirus VLPs during freeze-thawing and freeze-drying, yielding $98 \pm 5\%$ and $83 \pm 4\%$ recovery, respectively. There remains need for deeper understanding, at a fundamental level, of how excipient mixtures enhance VLP stability.

Innovative VLP-Inspired Vaccines

The diversity found among VLPs allows for great variety in approaches to exploiting the properties of VLPs, thus leading to remarkable versatility in VLP technologies. A recurrent theme in VLP-based vaccine design concerns the engineering of viral structural proteins to create novel immunogenic structures to tailor immunogenicity of VLPs towards heterologous pathogens. Aided by molecular modeling and computational design, recent innovations are expanding the available toolbox provided by viral structural proteins for the generation of novel immunogens against a range of pathogens.

Accumulating evidence demonstrates that effective immunogens can be generated through engineering of the capsid proteins such that assembly into VLPs is precluded. Reducing the complexity of VLP production by eliminating the need for complex expression systems, tightly controlled assembly conditions or uncontrollable assembly of multiple capsids and/or multilayered VLPs, could reduce cost while increasing the speed of vaccine manufacture. Figure 6 illustrates the bioprocess simplicity for capsomere-based vaccines. Assembly-incompetent capsomeres of papillomavirus and polyomavirus, produced in bacterial expression system, are suitable for heterologous epitope presentation as well as immunization against their cognate virus (Middelberg et al., 2011; Rose et al., 1998). As the structural building blocks of VLPs, capsomeres are often more stable than VLPs (McCarthy et al., 1998; Schmidt et al., 2000), and have high absolute stability as evidenced by recent MD simulations (Zhang et al., 2013). Since the demonstration that immunization with HPV capsomeres induced similar virus neutralization titers as immunization with HPV virions or recombinant VLPs (Rose et al., 1998), a number of capsomere-based vaccine candidates to HPV serotypes have been developed (Dell et al., 2006; Fligge et al., 2001; Wu et al., 2011; Yuan et al., 2001). Used as an antigen carrier system, HPV L1 capsomeres have been used to present RSV epitopes (Murata et al., 2009) and the self-antigen peptide human mucin-1 as a cancer vaccine candidate (Pejawar-

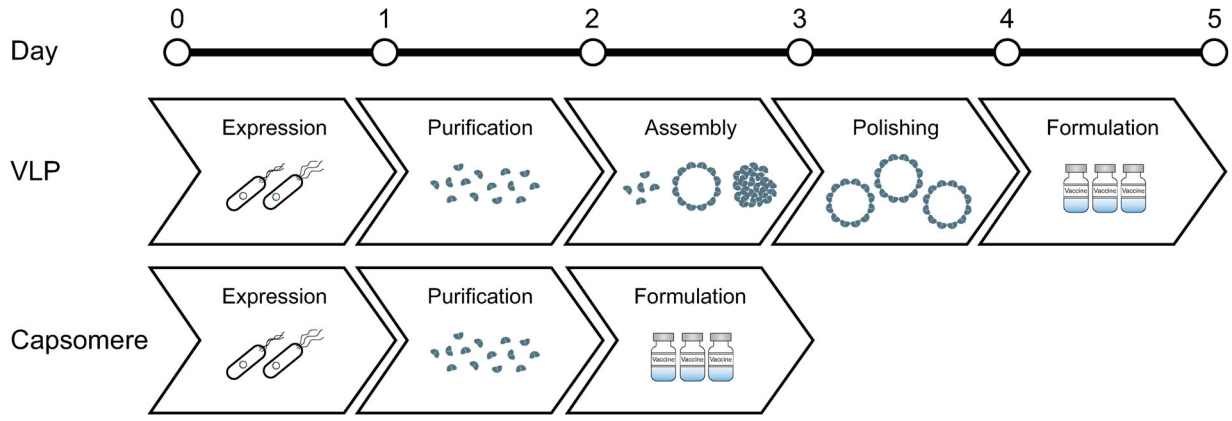

Figure 6. Microbial platform for VLP- and capsomere-based vaccines. A microbial platform for the production of VLP- and capsomere-based vaccines involves the expression of VLP precursors or capsomeres in *E. coli* and the purification of capsomeres from host contaminants. For VLP-based vaccines, purified capsomeres are assembled into VLPs in a cell-free in vitro environment before additional polishing purification to remove unassembled capsomeres and misformed particles. Capsomere-based vaccine platform simplifies bioprocessing requirements and further increases the speed of vaccine manufacturing.

Gaddy et al., 2010). Similarly, modularly engineered polyomavirus VP1 capsomere vaccine candidates against GAS (Middelberg et al., 2011) and influenza (Wibowo et al., 2013) were designed to fully exploit the capsomeres, both of which generated strong antigen-specific immune responses.

The number of published studies describing capsomere-based vaccines is currently limited and we are far from understanding their immunogenic and translational potential. A number of studies report the superb immunogenicity of capsomeres, even in the absence of adjuvant (Ohlschlager et al., 2003; Pejawar-Gaddy et al., 2010; Schadlich et al., 2009; Wibowo et al., 2013; Yuan et al., 2001), whereas others report weak immunogenicity compared to assembled VLPs (Fligge et al., 2001; Jagu et al., 2010; Thones et al., 2008). It is important to note that the thermodynamic properties of capsomere favor molecular association (Chuan et al., 2010) and random association of capsomeres would create macromolecular aggregates that may not induce the desirable immunogenicity. Therefore, careful design and comprehensive characterization of capsomere-based vaccine candidates are necessary in understanding their applications.

The minor coat protein of polyomavirus, VP2/3, also provides an interesting opportunity for the development of novel vaccine designs. Using the interaction between VP2/3 and the interior facing surface of VP1 pentamers, antigens may be encapsulated within polyomavirus VLPs by fusion to the internal protein (Abbing et al., 2004; Barouch and Harrison, 1994). This approach has been used in proof of concept studies into cancer immunotherapy in mice. Dendritic cells loaded with VLPs encapsulating prostate specific antigen (Eriksson et al., 2011) or Her2 (Tegerstedt et al., 2007) were able to prevent outgrowth of tumors expressing these antigens through the induction of T-cell mediated immunity. While the aim of VLP vaccines has traditionally been to elicit a strong antibody response through the humoral branch of the immune system, attention has recently turned towards stimulating a cellular response, which is thought to have great potential in the prevention of major diseases such as HIV (Ahlers and Belyakov, 2009), malaria (Good and Engwerda, 2011), and influenza (Brown and Kelso, 2009; Hillaire et al., 2011).

VLP platforms therefore have future potential as biologics designed to stimulate cellular immunity. In addition to various polyomaviruses (Abbing et al., 2004; Inoue et al., 2008; Ohtake et al., 2010), other capsid-based VLPs developed for the encapsulation of foreign proteins include hepatitis B core antigen VLPs (Beterams et al., 2000), CCMV (Minten et al., 2009) and the bacteriophages MS2 (Glasgow et al., 2012), Qβ (Fiedler et al., 2010), and P22 (O'Neil et al., 2011). Various approaches have been engineered to enable efficient protein encapsulation by these capsid VLPs including both native and engineered heterologous protein–protein interactions as well as protein–nucleic acid interactions. A number of enveloped VLPs have also been developed for foreign protein encapsulation using fusion to the main structural protein. Viruses amenable to this approach include a number of retroviruses such as HIV (Weldon et al., 1990) and murine retrovirus (Voelkel et al., 2010). Enveloped VLPs have proved especially useful in that they can easily be targeted to specific cell types through the insertion of targeting domains as fusions to glycoproteins in a manner similar to pseudotyping (Kaczmarczyk et al., 2011). Through this multiplicity of options afforded by assorted VLP formats, we would expect imminent demonstration of the delivery of internal antigens to the immunoproteasome for T-cell-mediated protection against pathogens.

Enveloped VLPs provide a unique flexibility for the engineering of novel immunogens. Influenza VLPs consisting of three distinct HA subtypes have shown protective immunity in ferrets when challenged by the three influenza viruses that the HA subtypes are derived (Pushko et al., 2011). Another group of hybrid VLPs emerging is fusion of dominant immunological proteins of different viruses to overcome poor expression and/or VLP assembly, as demonstrated for SARS-influenza VLPs [SARS spike protein fused to influenza HA (Liu et al., 2011)], and NDV-RSV VLPs [RSV G protein fused to NDV HN protein, RSV F protein fused to NDV F protein, (McGinnes et al., 2011; Murawski et al., 2010)]. Promising animal immunological results were obtained with these hybrid VLPs. This VLP technology may allow generation of novel VLP immunogens to target diseases which was previously not achievable, provided the issues around efficient and cost-effective bioprocessing, including purification, can be resolved.

Concluding Remarks

The structural diversity and functional versatility of VLPs in current development provides us with a prospective preview to the potential of fast-moving VLP research. Robust and flexible production platforms are emerging to produce a range of VLPs targeting different diseases. One significant challenge for heterologous epitope presentation on VLPs is producing antigenic epitopes that are structurally similar to those found on the parental pathogens. In this context, VLP-based vaccine design will increasingly benefit from computational simulation of whole VLP subunits and VLPs. Enhanced computer modeling to predict the structure of inserted epitopes on a VLP surface, to guide the design of efficacious VLPs, is urgently needed. Biomolecular engineering advances enabling the presentation of large antigens on VLPs may also overcome challenges inherent with epitope-based VLPs. Such approaches will greatly reduce vaccine development time and the high vaccine costs associated with current VLP production. With recent developments in VLP technologies, credited to the versatility of VLPs and ongoing biomolecular engineering advances, we can expect new frontier research on the development of VLP-based vaccines that are multivalent and multi-functional, and enabled by new bioprocessing technologies.

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References

Abbing A, Blaschke UK, Grein S, Kretschmar M, Stark CM, Thies MJ, Walter J, Weigand M, Woith DC, Hess J, Resier CO. 2004. Efficient intracellular delivery of a protein and a low molecular weight substance via recombinant polyomavirus-like particles. J Biol Chem 279(26):27410–27421.

Adrian M, Dubochet J, Lepault J, McDowall AW. 1984. Cryo-electron microscopy of viruses. Nature 308(5954):32–36.

Aguilar JC, Rodriguez EG. 2007. Vaccine adjuvants revisited. Vaccine 25(19):3752–3762.

Ahlers JD, Belyakov IM. 2009. Strategies for recruiting and targeting dendritic cells for optimizing HIV vaccines. Trends Mol Med 15(6):263–274.

Akinobu K, Fumio A, Makoto T, Hiroaki Y, Akihiro K, Hiroshi H. 1996. Purification and characterization of virus-like particles and pentamers produced by the expression of SV40 capsid proteins in insect cells. Biochimica et Biophysica Acta 1290(1):37–45.

Anggraeni MR, Connors NK, Wu Y, Chuan YP, Lua LHL, Middelberg APJ. 2013. Sensitivity of immune response quality to influenza helix 190 antigen structure displayed on a modular virus-like particle. Vaccine 31(40):4428–4435.

Ashley CE, Carnes EC, Phillips GK, Durfee PN, Buley MD, Lino CA, Padilla DP, Phillips B, Carter MB, Willman CL, et al. 2011. Cell-specific delivery of diverse cargos by bacteriophage MS2 virus-like particles. Acs Nano 5(7):5729–5745.

Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H, Zinkernagel RM. 1993. The influence of antigen organization on B-cell responsiveness. Science 262(5138):1448–1451.

Baek J-O, Seo J-W, Kim I-H, Kim CH. 2011. Production and purification of human papillomavirus type 33 L1 virus-like particles from Spodoptera frugiperda 9 cells using two-step column chromatography. Protein Expres Purif 75(2):211–217.

Barouch DH, Harrison SC. 1994. Interactions among the major and minor coat proteins of polyomavirus. J Virol 68(6):3982–3989.

Beterams G, Bottcher B, Nassal M. 2000. Packaging of up to 240 subunits of a 17 kDa nuclease into the interior of recombinant hepatitis B virus capsids. FEBS Lett 481(2):169–176.

Bin Park S. 2012. Hepatitis E vaccine debuts. Nature 491(7422):21–22.

Birnbaum F, Nassal M. 1990. Hepatitis B virus nucleocapsid assembly: Primary structure requirements in the core protein. J Virol 64(7):3319–3330.

Bisht H, Chugh DA, Raje M, Swaminathan S, Khanna N. 2002. Recombinant dengue virus type 2 envelope/hepatitis B surface antigen hybrid protein expressed in Pichia pastoris can function as a bivalent immunogen. J Biotechnol 99(2):97–110.

Bräutigam S, Snezhkov E, Bishop DHL. 1993. Formation of poliovirus-like particles by recombinant baculoviruses expressing the individual VP0, VP3, and VP1 proteins by comparison to particles derived from the expressed poliovirus polyprotein. Virology 192(2):512–524.

Bright RA, Carter DM, Daniluk S, Toapanta FR, Ahmad A, Gavrilov V, Massare M, Pushko P, Mytle N, Rowe T, et al. 2007. Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. Vaccine 25(19):3871–3878.

Brown LE, Kelso A. 2009. Prospects for an influenza vaccine that induces cross-protective cytotoxic T lymphocytes. Immunol Cell Biol 87(4):300–308.

Bundy BC, Swartz JR. 2011. Efficient disulfide bond formation in virus-like particles. J Biotechnol 154(4):230–239.

Bundy BC, Franciszkowicz MJ, Swartz JR. 2008. Escherichia coli-based cell-free synthesis of virus-like particles. Biotechnol Bioeng 100(1):28–37.

Buonaguro L, Tagliamonte M, Visciano ML, Tornesello ML, Buonaguro FM. 2013. Developments in virus-like particle-based vaccines for HIV. Expert Rev Vaccines 12(2):119–127.

Caldeira J, Peabody D. 2007. Stability and assembly in vitro of bacteriophage PP7 virus-like particles. J Nanobiotechnol 5(1):10.

Carrillo-Tripp M, Shepherd CM, Borelli IA, Venkataraman S, Lander G, Natarajan P, Johnson JE, Brooks CL, Reddy VS. 2009. VIPERdb(2): An enhanced and web API enabled relational database for structural virology. Nucleic Acids Res 37:D436–D442.

Castro-Acosta RM, Revilla AL, Ramirez OT, Palomares LA. 2010. Separation and quantification of double- and triple-layered rotavirus-like particles by CZE. Electrophoresis 31(8):1376–1381.

Chackerian B. 2007. Virus-like particles-flexible platforms for vaccine development. Expert Rev Vaccines 6(3):381–390.

Chen XS, Casini G, Harrison SC, Garcea RL. 2001. Papillomavirus capsid protein expression in Escherichia coli: Purification and assembly of HPV11 and HPV16 L1. J Mol Biol 307(1):173–182.

Chen BJ, Leser GP, Morita E, Lamb RA. 2007. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. J Virol 81(13):7111–7123.

Chuan YP, Fan YY, Lua L, Middelberg APJ. 2008. Quantitative analysis of virus-like particle size and distribution by field-flow Fractionation. Biotechnol Bioeng 99(6):1425–1433.

Chuan YP, Fan YY, Lua LHL, Middelberg APJ. 2010. Virus assembly occurs following a pH- or Ca_{2+} -triggered switch in the thermodynamic attraction between structural protein capsomeres. J R Soc Interface 7(44):409–421.

Chuan YP, Rivera-Hernandez T, Wibowo N, Connors NK, Wu Y, Hughes FK, Lua LHL, Middelberg APJ. 2013. Effects of pre-existing anti-carrier immunity and antigenic element multiplicity on efficacy of a modular virus-like particle vaccine. Biotechnol Bioeng 110(9):2343–2351.

Chung YC, Huang JH, Lai CW, Sheng HC, Shih SR, Ho MS, Hu YC. 2006. Expression, purification and characterization of enterovirus-71 virus-like particles. World J Gastroenterol 12(6):921–927.

Chung YC, Ho MS, Wu JC, Chen WJ, Huang JH, Chou ST, Hu YC. 2008. Immunization with virus-like particles of enterovirus 71 elicits potent immune responses and protects mice against lethal, challenge. Vaccine 26(15):1855–1862.

Citkowicz A, Petry H, Harkins RN, Ast O, Cashion L, Goldmann C, Bringmann P, Plummer K, Larsen BR. 2008. Characterization of virus-like particle assembly for DNA delivery using asymmetrical flow field-flow fractionation and light scattering. Anal Biochem 376(2):163–172.

Clarke BE, Newton SE, Carroll AR, Francis MJ, Appleyard G, Syred AD, Highfield PE, Rowlands DJ, Brown F. 1987. Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. Nature 330(6146):381–384.

Conner ME, Zarley CD, Hu B, Parsons S, Drabinski D, Greiner S, Smith R, Jiang B, Corsaro B, Barniak V, et al. 1996. Virus-like particles as a rotavirus subunit vaccine. J Infect Dis 174(Suppl 1):S88–S92.

Cook JC. 2003. Process for purifying human papillomavirus virus-like particles.

Cook JC, Joyce JG, George HA, Schultz LD, Hurni WM, Jansen KU, Hepler RW, Ip C, Lowe RS, Keller PM, et al. 1999. Purification of virus-like particles of recombinant human papillomavirus type 11 major capsid protein L1 from Saccharomyces cerevisiae. Protein Expres Purif 17(3):477–484.

Crawford SE, Labbé M, Cohen J, Burroughs MH, Zhou YJ, Estes MK. 1994. Characterization of virus-like particles produced by the expression of rotavirus capsid proteins in insect cells. J Virol 68(9):5945–5952.

D'Aoust MA, Lavoie PO, Couture MM, Trepanier S, Guay JM, Dargis M, Mongrand S, Landry N, Ward BJ, Vezina LP. 2008. Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. Plant Biotechnol J 6(9):930–940.

Dell K, Koesters R, Linnebacher M, Klein C, Gissmann L. 2006. Intranasal immunization with human papillomavirus type 16 capsomeres in the presence of non-toxic cholera toxin-based adjuvants elicits increased vaginal immunoglobulin levels. Vaccine 24(13):2238–2247.

Deschuyteneer M, Elouahabi A, Plainchamp D, Plisnier M, Soete D, Corazza Y, Lockman L, Giannini S, Deschamps M. 2010. Molecular and structural characterization of the L1 virus-like particles that are used as vaccine antigens in Cervarix™, the AS04-adjuvanted HPV-16 and -18 cervical cancer vaccine. Hum Vaccines 6(5):407–419.

Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, Kielland A, Vosters O, Vanderheyde N, Schiavetti F, et al. 2009. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol 183(10):6186–6197.

Ding Y, Chuan YP, He LZ, Middelberg APJ. 2010. Modeling the competition between aggregation and self-assembly during virus-like particle processing. Biotechnol Bioeng 107(3):550–560.

Eriksson M, Andreasson K, Weidmann J, Lundberg K, Tegerstedt K, Dalianis T, Ramqvist T. 2011. Murine polyomavirus virus-like particles carrying full-length human PSA protect BALB/c mice from outgrowth of a PSA expressing tumor. PLoS ONE 6(8):e23828.

Fernandes F, Teixeira AP, Carinhas N, Carrondo MJT, Alves PM. 2013. Insect cells as a production platform of complex virus-like particles. Expert Rev Vaccines 12(2):225–236.

Fernandez E, Toledo JR, Mendez L, Gonzalez N, Parra F, Martin-Alonso JM, Limonta M, Sanchez K, Cabrales A, Estrada MP, et al. 2013. Conformational and thermal stability improvements for the large-scale production of yeast-derived rabbit hemorrhagic disease virus-like particles as multipurpose vaccine. PLos ONE 8(2):e56417.

Fiedler JD, Brown SD, Lau JL, Finn MG. 2010. RNA-directed packaging of enzymes within virus-like particles. Angew Chem Int Ed Engl 49(50):9648–9651.

Fligge C, Giroglou T, Streeck RE, Sapp M. 2001. Induction of type-specific neutralizing antibodies by capsomeres of human papillomavirus type 33. Virology 283(2):353–357.

Freivalds J, Dislers A, Ose V, Skrastina D, Cielens I, Pumpens P, Sasnauskas K, Kazaks A. 2006. Assembly of bacteriophage Qβ virus-like particles in yeast Saccharomyces cerevisiae and Pichia pastoris. J Biotechnol 123(3):297–303.

Freivalds J, Dislers A, Ose V, Pumpens P, Tars K, Kazaks A. 2011. Highly efficient production of phosphorylated hepatitis B core particles in yeast Pichia pastoris. Protein Expres Purif 75(2):218–224.

French TJ, Roy P. 1990. Synthesis of bluetongue virus (BTV) corelike particles by a recombinant baculovirus expressing the two major structural core proteins of BTV. J Virol 64(4):1530–1536.

Galarza JM, Latham T, Cupo A. 2005. Virus-like particle (VLP) vaccine conferred complete protection against a lethal influenza virus challenge. Viral Immunol 18(1):244–251.

Galeazzi R. 2009. Molecular dynamics as a tool in rational drug design: Current status and some major applications. Curr Comput Aided Drug Des 5(4):225–240.

Giles BM, Ross TM. 2011. A computationally optimized broadly reactive antigen (COBRA) based H5N1 VLP vaccine elicits broadly reactive antibodies in mice and ferrets. Vaccine 29(16):3043–3054.

Giles BM, Bissel SJ, DeAlmeida DR, Wiley CA, Ross TM. 2012a. Antibody breadth and protective efficacy are increased by vaccination with computationally optimized hemagglutinin but not with polyvalent hemagglutinin-based H5N1 virus-like particle vaccines. Clin Vaccine Immunol 19(2):128–139.

Giles BM, Crevar CJ, Carter DM, Bissel SJ, Schultz-Cherry S, Wiley CA, Ross TM. 2012b. A computationally optimized hemagglutinin virus-like particle vaccine elicits broadly reactive antibodies that protect non-human primates from H5N1 infection. J Infect Dis 205(10):1562–1570.

Glasgow JE, Capehart SL, Francis MB, Tullman-Ercek D. 2012. Osmolyte-mediated encapsulation of proteins inside MS2 viral capsids. ACS Nano 6(10):8658–8664.

GlaxoSmithKline. 2013a. Cervarix product monograph.

GlaxoSmithKline. 2013b. Energix-B product monograph.

Gleiter S, Lilie H. 2001. Coupling of antibodies via protein Z on modified polyoma virus-like particles. Protein Sci 10(2):434–444.

Good MF, Engwerda C. 2011. Defying malaria: Arming T cells to halt malaria. Nat Med 17(1):49–51.

Grimes JM, Burroughs JN, Gouet P, Diprose JM, Malby R, Zientara S, Mertens PPC, Stuart DI. 1998. The atomic structure of the bluetongue virus core. Nature 395(6701):470–478.

Guillen G, Aguilar JC, Duenas S, Hermida L, Guzman MG, Penton E, Iglesias E, Junco J, Torrens I, Lobaina Y, et al. 2010. Virus-like particles as vaccine antigens and adjuvants: Application to chronic disease, cancer immunotherapy and infectious disease preventive strategies. Global Vaccine Res Forum 2(2):128–133.

He YQ, Rappuoli R, De Groot AS, Chen RT. 2010. Emerging vaccine informatics. J Biomed Biotechnol 2010. (2010):1–26.

He YQ, Cao ZW, De Groot AS, Brusic V, Schonbach C, Petrovsky N. 2013. Computational vaccinology and the ICoVax 2012 workshop. BMC Bioinformatics 14. (suppl4):l1.

Hewat EA, Booth TF, Roy P. 1994. Structure of correctly self-assembled bluetongue virus-like particles. J Struct Biol 112(3):183–191.

Hillaire ML, Osterhaus AD, Rimmelzwaan GF. 2011. Induction of virus-specific cytotoxic T lymphocytes as a basis for the development of broadly protective influenza vaccines. J Biomed Biotechnol 2011: 939860.

Hu L, Trefethen JM, Zeng Y, Yee L, Ohtake S, Lechuga-Ballesteros D, Warfield KL, Aman MJ, Shulenin S, Unfer R, et al. 2011. Biophysical characterization and conformational stability of Ebola and Marburg virus-like particles. J Pharma Sci 100(12):5156–5173.

Ibañez LI, Roose K, De Filette M, Schotsaert M, De Sloovere J, Roels S, Pollard C, Schepens B, Grooten J, Fiers W, et al. 2013. M2e-displaying virus-like particles with associated RNA promote T Helper 1 Type adaptive immunity against influenza A. PLoS ONE 8(3):e59081.

Inoue T, Kawano MA, Takahashi RU, Tsukamoto H, Enomoto T, Imai T, Kataoka K, Handa H. 2008. Engineering of SV40-based nano-capsules for delivery of heterologous proteins as fusions with the minor capsid proteins VP2/3. J Biotechnol 134(1–2):181–192.

Jagu S, Kwak K, Garcea RL, Roden RBS. 2010. Vaccination with multimeric L2 fusion protein and L1 VLP or capsomeres to broaden protection against HPV infection. Vaccine 28(28):4478–4486.

Jennings GT, Bachmann MF. 2008. Coming of age of virus-like particle vaccines. Biol Chem 389(5):521–536.

Jorgensen WL. 2004. The many roles of computation in drug discovery. Science 303(5665):1813–1818.

Joshi H, Cheluvaraja S, Somogyi E, Brown DR, Ortoleva P. 2011a. A molecular dynamics study of loop fluctuation in human papillomavirus type 16 virus-like particles: A possible indicator of immunogenicity. Vaccine 29(51):9423–9430.

Joshi H, Singharoy A, Sereda YV, Cheluvaraja SC, Ortoleva PJ. 2011b. Multiscale simulation of microbe structure and dynamics. Prog Biophy Mol Biol 107(1):200–217.

Kaczmarczyk SJ, Sitaraman K, Young HA, Hughes SH, Chatterjee DK. 2011. Protein delivery using engineered virus-like particles. Proc Natl Acad Sci U S A 108(41):16998–17003.

Kalnciema I, Skrastina D, Ose V, Pumpens P, Zeltins A. 2012. Potato virus Y-like particles as a new carrier for the presentation of foreign protein stretches. Mol Biotechnol 52(2):129–139.

Kang SM, Kim MC, Compans RW. 2012. Virus-like particles as universal influenza vaccines. Expert Rev Vaccines 11(8):995–1007.

Kato S, Kobayashi K, Inoue K, Kuramochi M, Okada T, Yaginuma H, Morimoto K, Shimada T, Takada M, Kobayashi K. 2011. A lentiviral strategy for highly efficient retrograde gene transfer by pseudotyping with fusion envelope glycoprotein. Hum Gene Ther 22(2):197–206.

Kawano M, Matsui M, Handa H. 2013. SV40 virus-like particles as an effective delivery system and its application to a vaccine carrier. Expert Rev Vaccines 12(2):199–210.

Keller SA, Bauer M, Manolova V, Muntwiler S, Saudan P, Bachmann MF. 2010. Cutting edge: Limited specialization of dendritic cell subsets for MHC Class II-associated presentation of viral particles. J Immunol 184(1):26–29.

Keswani RK, Pozdol IM, Pack DW. 2013. Design of hybrid lipid/retroviral-like particle gene delivery vectors. Mol Pharm 10(5):1725–1735.
Kibenge FSB, Qian B, Nagy E, Cleghorn JR, Wadowska D. 1999. Formation of virus-like particles when the polyprotein gene (segment A) of infectious bursal disease virus is expressed in insect cells. Can J Vet Res 63(1):49–55.
Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. 1992. Papillomavirus L1 Major Capsid Protein Self-Assembles into Virus-Like Particles That Are Highly Immunogenic. Proc Natl Acad Sci U S A 89(24):12180–12184.
Kirnbauer R, Taub J, Greenstone H, Roden R, Durst M, Gissmann L, Lowy DR, Schiller JT. 1993. Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. J Virol 67(12):6929–6936.
Kissmann J, Ausar SF, Foubert TR, Brock J, Switzer MH, Detzi EJ, Vedvick TS, Middaugh CR. 2008. Physical stabilization of norwalk virus-like particles. J Pharma Sci 97(10):4208–4218.
Kissmann J, Joshi SB, Haynes JR, Dokken L, Richardson C, Middaugh CR. 2011. H1N1 influenza virus-like particles: Physical degradation pathways and identification of stabilizers. J Pharma Sci 100(2):634–645.
Kuroda D, Shirai H, Jacobson MP, Nakamura H. 2012. Computer-aided antibody design. Protein Eng Des Sel 25(10):507–521.
Kushnir N, Streatfield SJ, 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(1):58–83.
Kuznetsov YG, McPherson A. 2011. Atomic force microscopy in imaging of viruses and virus-infected cells. Microbiol Mol Biol Rev 75(2):268–285.
Lang R, Winter G, Vogt L, Zurcher A, Dorigo B, Schimmele B. 2009. Rational design of a stable, freeze-dried virus-like particle-based vaccine formulation. Drug Dev Ind Pharm 35(1):83–97.
Lang R, Vogt L, Zurcher A, Winter G. 2010. Virus-like particle characterization using AF4 channel technology. Am Lab 42(5):13.
Lapelosa M, Gallicchio E, Arnold GF, Arnold E, Levy RM. 2009. In silico vaccine design based on molecular simulations of rhinovirus chimeras presenting HIV-1 gp41 epitopes. J Mol Biol 385(2):675–691.
Lapelosa M, Arnold GF, Gallicchio E, Arnold E, Levy RM. 2010. Antigenic characteristics of rhinovirus chimeras designed in silico for enhanced presentation of HIV-1 gp41 epitopes. J Mol Biol 397(3):752–766.
Latham T, Galarza JM. 2001. Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. J Virol 75(13):6154–6165.
Lavelle L, Gingery M, Phillips M, Gelbart WM, Knobler CM, Cadena-Nava RD, Vega-Acosta JR, Pinedo-Torres LA, Ruiz-Garcia J. 2009. Phase diagram of self-assembled viral capsid protein polymorphs. J Phys Chem B 113(12):3813–3819.
Li T-C, Takeda N, Kato K, Nilsson J, Xing L, Haag L, Cheng RH, Miyamura T. 2003. Characterization of self-assembled virus-like particles of human polyomavirus BK generated by recombinant baculoviruses. Virology 311(1):115–124.
Li H-Y, Han J-F, Qin C-F, Chen R. 2013. Virus-like particles for enterovirus 71 produced from Saccharomyces cerevisiae potently elicits protective immune responses in mice. Vaccine 31(32):3281–3287.
Liew MWO, Rajendran A, Middelberg APJ. 2010. Microbial production of virus-like particle vaccine protein at gram-per-litre levels. J Biotechnol 150(2):224–231.
Liew MWO, Chuan YP, Middelberg APJ. 2012. High-yield and scalable cell-free assembly of virus-like particles by dilution. Biochem Eng J 67(0): 88–96.
Lin TW, Porta C, Lomonossoff G, Johnson JE. 1996. Structure-based design of peptide presentation on a viral surface: The crystal structure of a plant/animal virus chimera at 2.8 angstrom resolution. Fold Des 1(3):179–187.
Lin YL, Yu CI, Hu YC, Tsai TJ, Kuo YC, Chi WK, Lin AN, Chiang BL. 2012. Enterovirus type 71 neutralizing antibodies in the serum of macaque monkeys immunized with EV71 virus-like particles. Vaccine 30(7): 1305–1312.
Lipin DI, Chuan YP, Lua LHL, Middelberg APJ. 2008a. Encapsulation of DNA and non-viral protein changes the structure of murine polyomavirus virus-like particles. Arch Virol 153(11):2027–2039.
Lipin DI, Lua LHL, Middelberg APJ. 2008b. Quaternary size distribution of soluble aggregates of glutathione-S-transferase-purified viral protein as determined by asymmetrical flow field flow fractionation and dynamic light scattering. J Chromatogr A 1190(1–2):204–214.
Lipin DI, Raj A, Lua LHL, Middelberg APJ. 2009. Affinity purification of viral protein having heterogeneous quaternary structure: Modeling the impact of soluble aggregates on chromatographic performance. J Chromatogr A 1216(30):5696–5708.
Liu WJ, Liu XS, Zhao KN, Leggatt GR, Frazer IH. 2000. Papillomavirus virus-like particles for the delivery of multiple cytotoxic T cell epitopes. Virology 273(2):374–382.
Liu YV, Massare MJ, Barnard DL, Kort T, Nathan M, Wang L, Smith G. 2011. Chimeric severe acute respiratory syndrome coronavirus (SARS-CoV) S glycoprotein and influenza matrix 1 efficiently form virus-like particles (VLPs) that protect mice against challenge with SARS-CoV. Vaccine 29(38):6606–6613.
Lynch A, Meyers A, Williamson A-L, Rybicki E. 2012. Stability studies of HIV-1 Pr55gag virus-like particles made in insect cells after storage in various formulation media. Virol J 9(1):210.
Mach H, Volkin DB, Troutman RD, Wang B, Luo Z, Jansen KU, Shi L. 2006. Disassembly and reassembly of yeast-derived recombinant human papillomavirus virus-like particles (HPV VLPs). J Pharm Sci 95(10):2195–2206.
Maddux NR, Joshi SB, Volkin DB, Ralston JP, Middaugh CR. 2011. Multidimensional methods for the formulation of biopharmaceuticals and vaccines. J Pharm Sci 100(10):4171–4197.
Manayani DJ, Thomas D, Dryden KA, Reddy V, Siladi ME, Marlett JM, Rainey GJA, Pique ME, Scobie HM, Yeager M, et al. 2007. A viral nanoparticle with dual function as an anthrax antitoxin and vaccine. PLoS Pathog 3(10):1422–1431.
McCarthy MP, White WI, Palmer-Hill F, Koenig S, Suzich JA. 1998. Quantitative Disassembly and reassembly of human papillomavirus type 11 viruslike particles in vitro. J Virol 72(1):32–41.
McGinnes LW, Gravel KA, Finberg RW, Kurt-Jones EA, Massare MJ, Smith G, Schmidt MR, Morrison TG. 2011. Assembly and Immunological Properties of Newcastle Disease Virus-Like Particles Containing the Respiratory Syncytial Virus F and G Proteins. J Virol 85(1):366–377.
McGuigan LC, Stallard V, Roos JM, Payne LG. 1993. Recombinant-expressed virus-like particle pseudotypes as an approach to vaccine development. Vaccine 11(6):675–678.
Mellado MCM, Mena JA, Lopes A, Ramirez OT, Carrondo MJT, Palomares LA, Alves PM. 2009. Impact of physicochemical parameters on in vitro assembly and disassembly kinetics of recombinant triple-layered rotavirus-like particles. Biotechnol Bioeng 104(4):674–686.
Merck 2013a. Gardasil product monograph.
Merck 2013b. Recombivax HB product monograph.
Metz SW, Gardner J, Geertsema C, Le TT, Goh L, Vlak JM, Suhrbier A, Pijlman GP. 2013. Effective chikungunya virus-like particle vaccine produced in insect cells. PLoS Negl Trop Dis 7(3):e2124.
Miao YL, Ortoleva PJ. 2006. Viral structural transitions: An all-atom multiscale theory. J Chem Phy 125(214901):1–11.
Miao YL, Ortoleva PJ. 2010. Viral structural transition mechanisms revealed by multiscale molecular dynamics/order parameter eXtrapolation simulation. Biopolymers 93(1):61–73.
Middelberg APJ, Rivera-Hernandez T, Wibowo N, Lua LHL, Fan YY, Magor G, Chang C, Chuan YP, Good MF, Batzloff MR. 2011. A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines. Vaccine 29(41):7154–7162.
Milhiet P-E, Dosset P, Godefroy C, Le Grimellec C, Guigner J-M, Larquet E, Ronzon F, Manin C. 2011. Nanoscale topography of hepatitis B antigen particles by atomic force microscopy. Biochimie 93(2):254–259.
Minten IJ, Hendriks LJ, Nolte RJ, Cornelissen JJ. 2009. Controlled encapsulation of multiple proteins in virus capsids. J Am Chem Soc 131(49):17771–17773.
Mohr J, Chuan YP, Wu Y, Lua LHL, Middelberg APJ. 2013. Virus-like particle formulation optimization by miniaturized high-throughput screening. Methods 60(3):248–256.

Mulder AM, Carragher B, Towne V, Meng Y, Wang Y, Dieter L, Potter CS, Washabaugh MW, Sitrin RD, Zhao Q. 2012. Toolbox for non-intrusive structural and functional analysis of recombinant VLP based vaccines: A case study with hepatitis B vaccine. PLoS ONE 7(4):e33235.

Murata Y, Lightfoote PM, Rose RC, Walsh EE. 2009. Antigenic presentation of heterologous epitopes engineered into the outer surface-exposed helix 4 loop region of human papillomavirus L1 capsomeres. Virol J 6:10.

Murawski MR, McGinnes LW, Finberg RW, Kurt-Jones EA, Massare MJ, Smith G, Heaton PM, Fraire AE, Morrison TG. 2010. Newcastle disease virus-like particles containing respiratory syncytial virus G protein induced protection in BALB/c mice, with no evidence of immunopathology. J Virol 84(2):1110–1123.

Nema S, Washkuhn RJ, Brendel RJ. 1997. Excipients and their use in injectable products. PDA J Pharm Sci Technol 51(4):166–171.

Noad R, Roy P. 2003. Virus-like particles as immunogens. Trends Microbiol 11(9):438–444.

Ohlschlager P, Osen W, Dell K, Faath S, Garcea RL, Jochmus I, Muller M, Pawlita M, Schafer K, Sehr P, et al. 2003. Human papillomavirus type 16 L1 capsomeres induce L1-specific cytotoxic T lymphocytes and tumor regression in C57BL/6 mice. J Virol 77(8):4635–4645.

Ohtake N, Niikura K, Suzuki T, Nagakawa K, Mikuni S, Matsuo Y, Kinjo M, Sawa H, Ijiro K. 2010. Low pH-triggered model drug molecule release from virus-like particles. Chembiochem 11(7):959–962.

O'Neil A, Reichhardt C, Johnson B, Prevelige PE, Douglas T. 2011. Genetically programmed in vivo packaging of protein cargo and its controlled release from bacteriophage P22. Angew Chem Int Ed Engl 50(32):7425–7428.

Palomares LA, Ramirez OT. 2009. Challenges for the production of virus-like particles in insect cells: The case of rotavirus-like particles. Biochem Eng J 45(3):158–167.

Pankavich SD, Ortoleva PJ. 2012. Nanosystem self-assembly pathways discovered via all-atom multiscale analysis. J Phys Chem B 116(29):8355–8362.

Pattenden LK, Middelberg APJ, Niebert M, Lipin DI. 2005. Towards the preparative and large-scale precision manufacture of virus-like particles. Trends Biotechnol 23(10):523–529.

Peacey M, Wilson S, Baird MA, Ward VK. 2007. Versatile RHDV virus-like particles: Incorporation of antigens by genetic modification and chemical conjugation. Biotechnol Bioeng 98(5):968–977.

Pease LF, Lipin DI, Tsai D-H, Zachariah MR, Lua LHL, Tarlov MJ, Middelberg APJ. 2009. Quantitative characterization of virus-like particles by asymmetrical flow field flow fractionation, electrospray differential mobility analysis, and transmission electron microscopy. Biotechnol Bioeng 102(3):845–855.

Pejawar-Gaddy S, Rajawat Y, Hilioti Z, Xue J, Gaddy D, Finn O, Viscidi R, Bossis I. 2010. Generation of a tumor vaccine candidate based on conjugation of a MUC1 peptide to polyionic papillomavirus virus-like particles. Cancer Immunol Immunother 59(11):1685–1696.

Petrovsky N, Aguilar JC. 2004. Vaccine adjuvants: Current state and future trends. Immunol Cell Biol 82(5):488–496.

Phelps JP, Dao P, Jin H, Rasochova L. 2007. Expression and self-assembly of cowpea chlorotic mottle virus-like particles in Pseudomonas fluorescens. J Biotechnol 128(2):290–296.

Plummer EM, Manchester M. 2011. Viral nanoparticles and virus-like particles: Platforms for contemporary vaccine design. Wiley Interdiscip Rev Nanomed Nanobiotechnol 3(2):174–196.

Porta C, Kotecha A, Burman A, Jackson T, Ren J, Loureiro S, Jones IM, Fry EE, Stuart DI, Charleston B. 2013. Rational engineering of recombinant picornavirus capsids to produce safe, protective vaccine antigen. PLoS Pathog 9(3):e1003255.

Prasad BVV, Hardy ME, Dokland T, Bella J, Rossmann MG, Estes MK. 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science 286(5438):287–290.

Pushko P, Pearce MB, Ahmad A, Tretyakova I, Smith G, Belser JA, Tumpey TM. 2011. Influenza virus-like particle can accommodate multiple subtypes of hemagglutinin and protect from multiple influenza types and subtypes. Vaccine 29(35):5911–5918.

Pushko P, Pumpens P, Grens E. 2013. Development of virus-like particle technology from small highly symmetric to large complex virus-like particle structures. Intervirology 56(3):141–165.

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

Rasmussen L, Battles JK, Ennis WH, Nagashima K, Gonda MA. 1990. Characterization of virus-like particles produced by a recombinant baculovirus containing the gag gene of the bovine immunodeficiency-like virus. Virology 178(2):435–451.

Rivera-Hernandez T, Hartas J, Wu Y, Chuan YP, Lua LHL, Good M, Batzloff MR, Middelberg APJ. 2013. Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS). Vaccine 31(15):1950–1955.

Roberts JA, Kuiper MJ, Thorley BR, Smooker PM, Hung A. 2012. Investigation of a predicted N-terminal amphipathic et-helix using atomistic molecular dynamics simulation of a complete prototype poliovirus virion. J Mol Grap Model 38:165–173.

Rodriguez-Limas WA, Tyo KEJ, Nielsen J, Ramirez OT, Palomares LA. 2011. Molecular and process design for rotavirus-like particle production in Saccharomyces cerevisiae. Microb Cell Fact 10(33): 1–10.

Roldao A, Mellado MCM, Castilho LR, Carrondo MJ, Alves PM. 2010. Virus-like particles in vaccine development. Expert Rev Vaccines 9(10):1149–1176.

Roldao A, Mellado MCM, Lima JC, Carrondo MJT, Alves PM, 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(2):e1002367.

Roose K, De Baets S, Schepens B, Saelens X. 2013. Hepatitis B core-based virus-like particles to present heterologous epitopes. Expert Rev Vaccines 12(2):183–198.

Rose RC, White WI, Li ML, Suzich JA, Lane C, Garcea RL. 1998. Human papillomavirus type 11 recombinant L1 capsomeres induce virus-neutralizing antibodies. J Virol 72(7):6151–6154.

Sainsbury F, Saunders K, Aljabali AAA, Evans DJ, Lomonossoff GP. 2011. Peptide-controlled access to the interior surface of empty virus nanoparticles. ChemBioChem 12(16):2435–2440.

Salunke DM, Caspar DL, Garcea RL. 1986. Self-assembly of purified polyomavirus capsid protein VP1. Cell 46(6):895–904.

Saunders K, Sainsbury F, Lomonossoff GP. 2009. Efficient generation of cowpea mosaic virus empty virus-like particles by the proteolytic processing of precursors in insect cells and plants. Virology 393(2):329–337.

Schadlich L, Senger T, Gerlach B, Mucke N, Klein C, Bravo IG, Muller M, Gissmann L. 2009. Analysis of modified human papillomavirus type 16 L1 capsomeres: The ability to assemble into larger particles correlates with higher immunogenicity. J Virol 83(15):7690–7705.

Schmidt U, Rudolph R, Bohm G. 2000. Mechanism of assembly of recombinant murine polyomavirus-like particles. J Virol 74(4):1658–1662.

Schneemann A, Speir JA, Tan GS, Khayat R, Ekiert DC, Matsuoka Y, Wilsona IA. 2012. A virus-like particle that elicits cross-reactive antibodies to the conserved stem of influenza virus hemagglutinin. J Virol 86(21):11686–11697.

Scotti N, Rybicki EP. 2013. Virus-like particles produced in plants as potential vaccines. Expert Rev Vaccines 12(2):211–224.

Seow Y, Wood MJ. 2009. Biological gene delivery vehicles: Beyond viral vectors. Mol Therapy 17(5):767–777.

Shi L, Sanyal G, Ni A, Luo Z, Doshna S, Wang B, Graham TL, Wang N, Volkin DB. 2005. Stabilization of human papillomavirus virus-like particles by non-ionic surfactants. J Pharm Sci 94(7):1538–1551.

Shivachandra SB, Rao M, Janosi L, Sathaliyawala T, Matyas GR, Alving CR, Leppla SH, Rao VB. 2006. In vitro binding of anthrax protective antigen on bacteriophage T4 capsid surface through Hoc-capsid interactions: A strategy for efficient display of large full-length proteins. Virology 345(1):190–198.

Shreif Z, Adhangale P, Cheluvaraja S, Perera R, Kuhn R, Ortoleva P. 2008. Enveloped viruses understood via multiscale simulation: Computer-aided vaccine design. Sci Model Simul 15(1–3):363–380.

Stanley M, Lowy DR, Frazer I. 2006. Chapter 12: Prophylactic HPV vaccines: Underlying mechanisms. Vaccine 24 Supplement 3 (0):S106–S113.

Steinmetz NF, Evans DJ, Lomonossoff GP. 2007. Chemical introduction of reactive thiols into a viral nanoscaffold: A method that avoids virus aggregation. ChemBioChem 8(10):1131–1136.

Taylor KM, Lin TW, Porta C, Mosser AG, Giesing HA, Lomonossoff GP, Johnson JE. 2000. Influence of three-dimensional structure on the immunogenicity of a peptide expressed on the surface of a plant virus. J Mol Recogn 13(2):71–82.

Tegerstedt K, Franzen A, Ramqvist T, Dalianis T. 2007. Dendritic cells loaded with polyomavirus VP1/VP2Her2 virus-like particles efficiently prevent outgrowth of a Her2/neu expressing tumor. Cancer Immunol Immunother 56(9):1335–1344.

Thones N, Herreiner A, Schadlich L, Piuko K, Muller M. 2008. A direct comparison of human papillomavirus type 16 L1 particles reveals a lower immunogenicity of capsomeres than viruslike particles with respect to the induced antibody response. J Virol 82(11):5472–5485.

Valenzuela P, Medina A, Rutter WJ. 1982. Synthesis and assembly of hepatitis-b virus surface-antigen particles in yeast. Nature 298(5872):347–350.

Velasquez LS, Shira S, Berta AN, Kilbourne J, Medi BM, Tizard I, Ni Y, Arntzen CJ, Herbst-Kralovetz MM. 2011. Intranasal delivery of Norwalk virus-like particles formulated in an in situ gelling, dry powder vaccine. Vaccine 29(32):5221–5231.

Velez RA, de Matos APA, Parreira R, Piedade J, Matos B, Correia C, Esteves A. 2012. Expression of Chikungunya virus-like particles. Microsc Microanal 18:59–60.

Vieira HLA, Estevao C, Roldao A, Peixoto CC, Sousa MFQ, Cruz PE, Carrondo MJT, Alves PM. 2005. Triple layered rotavirus VLP production: Kinetics of vector replication, mRNA stability and recombinant protein production. J Biotechnol 120(1):72–82.

Voelkel C, Galla M, Maetzig T, Warlich E, Kuehle J, Zychlinski D, Bode J, Cantz T, Schambach A, Baum C. 2010. Protein transduction from retroviral Gag precursors. Proc Natl Acad Sci U S A 107(17):7805–7810.

Walpita P, Barr J, Sherman M, Basler CF, Wang LF. 2011. Vaccine potential of Nipah virus-like particles. PLoS ONE 6(4):e18437.

Warfield KL, Aman MJ. 2011. Advances in virus-like particle vaccines for filoviruses. J Infect Dis 204:S1053–S1059.

Weldon RA, Jr., Erdie CR, Oliver MG, Wills JW. 1990. Incorporation of chimeric gag protein into retroviral particles. J Virol 64(9):4169–4179.

Whitacre DC, Lee BO, Milich DR. 2009. Use of hepadnavirus core proteins as vaccine platforms. Expert Rev Vaccines 8(11):1565–1573.

White LJ, Hardy ME, Estes MK. 1997. Biochemical characterization of a smaller form of recombinant Norwalk virus capsids assembled in insect cells. J Virol 71(10):8066–8072.

Wibowo N, Chuan YP, Lua LHL, Middelberg APJ. 2013. Modular engineering of a microbially-produced viral capsomere vaccine for influenza. Chem Eng Sci 103:12–20.

Wu M, Brown WL, Stockley PG. 1995. Cell-specific delivery of bacteriophage-encapsidated ricin A chain. Bioconjug Chem 6(5):587–595.

Wu WH, Gersch E, Kwak K, Jagu S, Karanam B, Huh WK, Garcea RL, Roden RBS. 2011. Capsomer vaccines protect mice from vaginal challenge with human papillomavirus. PLoS ONE 6(11):e27141.

Wynne SA, Crowther RA, Leslie AGW. 1999. The crystal structure of the human hepatitis B virus capsid. Mol Cell 3(6):771–780.

Yuan H, Estes PA, Chen Y, Newsome J, Olcese VA, Garcea RL, Schlegel R. 2001. Immunization with a pentameric L1 fusion protein protects against papillomavirus infection. J Virol 75(17):7848–7853.

Zeltins A. 2013. Construction and characterization of virus-like particles: A review. Mol Biotechnol 53(1):92–107.

Zhang LF, Zhou J, Chen S, Cai LL, Bao QY, Zheng FY, Lu JQ, Padmanabha J, Hengst K, Malcolm K, et al. 2000. HPV6b virus like particles are potent immunogens without adjuvant in man. Vaccine 18(11–12):1051–1058.

Zhang L, Tang RH, Bai S, Connors NK, Lua LHL, Chuan YP, Middelberg APJ, Sun Y. 2013. Molecular energetics in the capsomere of virus-like particle revealed by molecular dynamics simulations. J Phys Chem B 117(18): 5411–5421.

Zhao X, Fox JM, Olson NH, Baker TS, Young MJ. 1995. In vitro assembly of cowpea chlorotic mottle virus from coat protein expressed in Escherichia coli and in vitro-transcribed viral cDNA. Virology 207(2):486–494.

Zhao Q, Allen MJ, Wang Y, Wang B, Wang N, Shi L, Sitrin RD. 2012a. Disassembly and reassembly improves morphology and thermal stability of human papillomavirus type 16 virus-like particles. Nanomedicine 8(7):1182–1189.

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

Zhu F-C, Zhang J, Zhang X-F, Zhou C, Wang Z-Z, Huang S-J, Wang H, Yang C-L, Jiang H-M, Cai J-P, et al. 2010. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: A large-scale, randomised, double-blind placebo-controlled, phase 3 trial. Lancet 376(9744):895–902.