Review

Virus-like particle-based human vaccines: quality assessment based on structural and functional properties

Qinjian Zhao[1], Shaowei Li[1], Hai Yu[1], Ningshao Xia[1], and Yorgo Modis[2]

[1] National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Public Health, Xiamen University, Xiamen, Fujian 361102, People's Republic of China
[2] Department of Molecular Biophysics and Biochemistry, Yale University, 266 Whitney Avenue, New Haven, CT 06520, USA

Human vaccines against three viruses use recombinant virus-like particles (VLPs) as the antigen: hepatitis B virus, human papillomavirus, and hepatitis E virus. VLPs are excellent prophylactic vaccine antigens because they are self-assembling bionanoparticles (20 to 60 nm in diameter) that expose multiple epitopes on their surface and faithfully mimic the native virions. Here we summarize the long journey of these vaccines from bench to patients. The physical properties and structural features of each recombinant VLP vaccine are described. With the recent licensure of Hecolin against hepatitis E virus adding a third disease indication to prophylactic VLP-based vaccines, we review how the crucial quality attributes of VLP-based human vaccines against all three disease indications were assessed, controlled, and improved during bioprocessing through an array of structural and functional analyses.

Introduction

Ever since the discovery of the non-infectious hepatitis B virus (HBV) particles, or so-called 'Australia antigen', in the blood of infected individuals [1], the idea of using non-infectious virus particles to develop prophylactic human vaccines has been attractive. Virus particles are excellent vaccine antigens because they present on their surface an array of antigenic epitopes that mimics the surface of native virions more faithfully than specific isolated subunits or subcomponents of the virus. Approaches based on virus-like particles (VLPs; see Glossary) also offer a safer method for prophylactic vaccination because recombinant VLPs do not contain viral genetic materials. Spherical particles 22 nm in diameter consisting of HBV surface antigen (HBsAg) and derived from human plasma were licensed as a vaccine in the United States in 1981 [2]. With the dawn of recombinant DNA technology, the 22 nm HBsAg particles were produced in yeast (*Saccharomyces cerevisiae*) and licensed in 1986 to Merck as the first recombinant human vaccine, RECOMBIVAX HB [3–5]. The yeast-derived HBsAg particles were essentially identical to plasma-derived particles in their morphology, surface features, and immunological properties [6]. The first recombinant vaccine based on VLPs was a great success, and more than ten similar HBsAg VLP-based hepatitis B vaccines have since been licensed in various countries (reviewed in [7]). However, it took another 20 years for the next recombinant VLP-based vaccine to be licensed for human use – Gardasil (Merck) against human papillomavirus (HPV)-associated diseases. The success of HPV vaccination was shown by a reduction of over 50% in HPV infection among teenagers by mid-2013 according to a study by the US Centers for Disease Control and Prevention [8].

It was a long journey to turn laboratory research into a licensed vaccine for human use in the clinic. Using HPV16, which causes about half of cervical cancers worldwide,

Glossary

Comparability exercise: an analytical process in which a database of physicochemical and functional properties of the vaccine is generated and compared for every stage of the product life cycle. This database is used to manage the process upgrade and scale up for vaccine or biologic production and to predict the consistency of the vaccine over time of manufacturing and across different lots.
HBsAg: hepatitis B virus (HBV) surface antigen. HBsAg assembles into 22 nm lipid-containing particles. It is the sole protein component of the RECOMBIVAX HB vaccine.
HEV p239: a 239-amino-acid fragment of the 606-amino-acid plasmid open reading frame 2 (pORF2) capsid protein from hepatitis E virus (HEV). p239 assembles into protein-only particles 20–30 nm in diameter. It contains neutralizing and immunodominant epitopes and is the sole protein component of the prophylactic Hecolin vaccine.
HPV L1: is the major capsid protein of human papillomavirus (HPV). L1 forms pentameric capsomeres, which self-assemble into icosahedrally symmetric protein-only particles. L1 is the sole protein component of the Gardasil vaccine.
***In vitro* relative potency assay (IVRP):** IVRPs measure the binding affinity and avidity of neutralizing or functional antibodies to the vaccine antigen. IVRPs offer reliable and quantitative measures of vaccine antigenicity, which correlates to the vaccine potency in humans. IVRPs complement other kinds of potency assay, including mouse (or other animal-based) potency assays, cell-based potency assays, and clinical serological assays.
Virus-like particles (VLPs): are excellent recombinant vaccine antigens because they present multiple epitopes on their surface and are good mimics of native virions.

Corresponding authors: Zhao, Q. (qinjian_zhao@xmu.edu.cn); Modis, Y. (yorgo.modis@yale.edu).
Keywords: subunit vaccine; comparability exercise; potency assay; bionanoparticle; epitope mapping; neutralizing antibody.

0167-7799/$ – see front matter
 http://dx.doi.org/10.1016/j.tibtech.2013.09.002

Frazer *et al.* developed methods for production of VLPs in 1991 [9]. However these methods were costly and complex owing to the requirement to express both of the capsid proteins, L1 and L2, using a recombinant vaccinia virus expression system in mammalian cells [9]. One year later, John Schiller, Douglas Lowy, and others, using a bovine model, demonstrated that the major capsid protein L1 could self-assemble into VLPs without the need for L2 and produced very high level of neutralizing antibodies in animals [10]. In effect, the 'L1 only' VLPs mimicked the HPV virions to a great degree, with the L1 pentamer building blocks, or 'capsomeres', being distinctly visible on the VLP surface by transmission electron microscopy (TEM). Subsequently, Schiller's group showed that, when expressed in baculovirus, HPV16 L1 was able to efficiently self-assemble into VLPs with or without the coexpression of L2 [11]. 'L1-only' VLPs offered a more straightforward path for bioprocessing and bioanalytics of VLP production at commercial scale. Approximately 3 years after Gardasil was licensed in the United States, a second VLP-based HPV vaccine was licensed, Cervarix (GlaxoSmithKline), which is produced in a baculovirus-based insect cell expression system [12].

The recombinant VLP-based vaccine Hecolin (Xiamen Innovax Biotech) was licensed in China in 2011 for prophylactic use against hepatitis E virus (HEV) infection, the third disease indication for a VLP-based vaccine [13,14]. Novel aspects of VLP-based vaccines are currently the subject of intense study, including the emerging work on newly studied pathogens such as BK polyomavirus in patients who have undergone kidney transplant [15], and new approaches such as grafting a new epitope onto an existing VLP platform on well-studied pathogens like influenza virus [16]. This review focuses on the licensed human vaccines, which continue to have important roles in human health. Here we present a summary of the structural and functional analysis of the first and most thoroughly studied commercial VLP-based human vaccines against HBV, HPV and HEV, namely RECOMBIVAX HB, Gardasil, and Hecolin, respectively (Table 1).

Expressing VLPs and enhancing their antigenicity by post-purification reassembly

Each of the human vaccines in Table 1 use aluminum-containing adjuvant to stabilize the antigen and enhance the immune response in vaccines. Although adjuvant is an important factor in stabilizing vaccine antigens in final formulations and in eliciting a high level of protective immunity in animals and in humans, this review focuses on describing the active components of the vaccine, namely the VLP antigens. Multifaceted characterization of the physicochemical, structural, and immunochemical properties of antigens is critically important in delivering consistent and stable lots for preclinical and clinical stages, as well as post-licensure life cycle management, where process upgrade or scale up may be necessary to support the market demand.

The first licensed human vaccine made using recombinant DNA technology was for hepatitis B. Most licensed recombinant DNA hepatitis B vaccines consist of the 226-amino-acid S gene product, HBsAg protein [17]. HBsAg particles consist of approximately 30% lipid by mass. It is therefore essential to express the particles in host cells that provide an appropriate environment and lipid composition for the HBsAg molecules to self-assemble into the 22 nm spherical particles (Figure 1). *Escherichia coli* expression did not yield useful HBsAg particles, presumably owing to the unfavorable environmental conditions (e.g., pH and redox potential) or lipid compositions within the bacteria. By contrast, yeast (*S. cerevisiae, Pichia pastoris*, and *Hansenula polymorpha*) and mammalian cells (Chinese hamster ovary cells) have been used successfully to express the morphologically uniform, antigenically and immunogenically active lipid-containing HBsAg particles [3–6,18,19] (Table 1).

In contrast to HBsAg particles, the HPV and HEV VLPs used in vaccines are nanoparticles (between 30 and 60 nm in diameter) consisting only of the viral capsid protein (or proteins) and do not contain lipids. Successful expression of HPV and HEV VLPs was also achieved with a baculovirus-based expression system in insect cells and with yeast cells. Without the need for lipids, these VLPs self assemble into

Table 1. Crucial structural and functional properties of representative recombinant VLP-based human vaccines[a,b]

Trade name (company)	Year of licensure	VLP diameter (nm)	Source or expression host	Structure	Key activity marker
HEPTAVAX-B (Merck)	1981	22	Human plasma	Octahedral[c]	Auzyme kit [28]
RECOMBIVAX HB (Merck)	1986	22	*Saccharomyces cerevisiae* (yeast)[d]	Octahedral [27]	Auzyme kit [18,28]; RF1 and A1.2 [27,37]
Gardasil (Merck)	2006	40–60	*S. cerevisiae* (yeast)[d]	Icosahedral [21]	mAb-based ELISA[e]; H16.V5; H18.R5; H6.B2; H11.B2 [29]
Cervarix (GlaxoSmithKline)	2009	40–60	*Trichoplusiani*/baculovirus	Icosahedral [12]	H16.V5 [12]
Hecolin (Xiamen Innovax Biotech)	2011	20–30	*Escherichia coli*	NA	mAb 8C11 [23]

[a]These vaccines emulate the exterior of the virions but lack viral genomic material. After RECOMBIVAX HB was licensed in 1986, more than ten other recombinant hepatitis B virus surface antigen (HBsAg)-based hepatitis B vaccines were licensed in various countries (reviewed in [7]).

[b]Abbreviations: NA, not available; mAb, monoclonal antibody; VLP, virus-like particle.

[c]The high-resolution structure of plasma-derived HBsAg particles has not been determined because there are three different forms of the surface protein (large, medium, and small forms) with different post-translational proteolytic processing. Therefore, the reconstruction was performed on HBsAg particles assembled *in vivo* prepared from transgenic mice expressing only the small form of the surface protein (226 amino acids) [69].

[d]In China and India, other expression hosts, such as *Pichia pastoris*, *Hansenula polymorpha*, and Chinese hamster ovary cells were also used to produce recombinant HBsAg in licensed vaccines.

[e]The detection mAb against each of the four human papillomavirus L1 VLPs was listed in the sandwich ELISA-based *in vitro* relative potency tests [29].

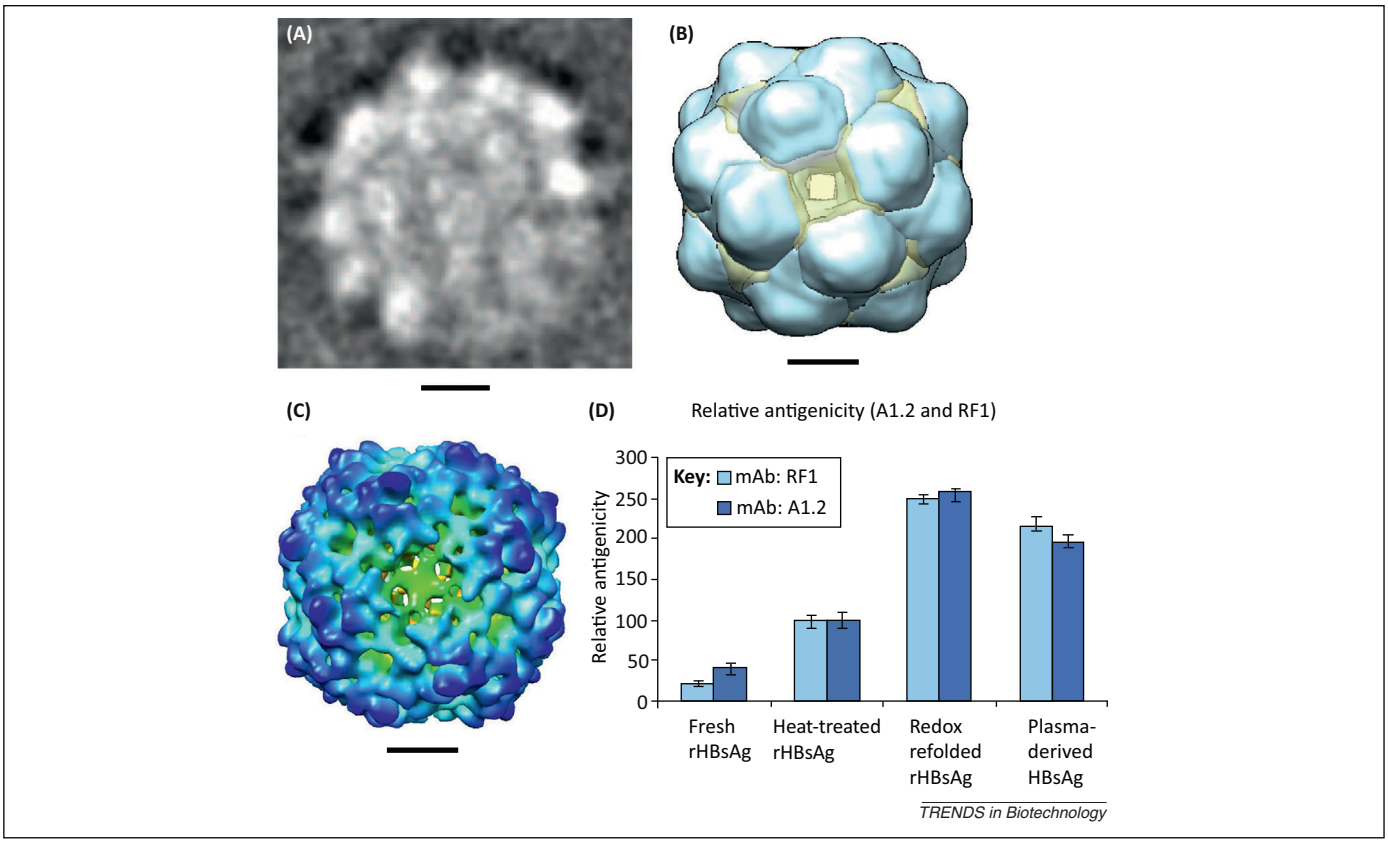

Figure 1. Hepatitis B virus surface antigen (HBsAg)-based hepatitis B vaccines. From 1981 to 1986 these vaccines were plasma derived, but from 1986 to the present they have been derived from yeast and based on recombinant virus-like particles (VLPs). Scale bar = 5 nm. **(A)** 2D cryo-electron microscopy (cryoEM) class average of yeast-derived recombinant HBsAg VLPs (from [27]). **(B)** 3D image reconstruction at 15 Å resolution from cryoEM of the yeast-derived VLPs (from [27]). Segmentation of the map revealed regions of high density, presumed to be protein (gray), surrounded by regions of lesser density, presumed to be lipid (yellow). **(C)** CryoEM image reconstruction at 12 Å resolution of the plasma-derived HBsAg particles (from [69]). The particles are 20–23 nm in diameter and have octahedral symmetry. **(D)** Measurement of recombinant HBsAg (rHBsAg) VLP binding to the neutralizing monoclonal antibodies (mAbs) RF1 and A1.2 by surface plasmon resonance. Binding affinities were interpreted as 'relative antigenicity', with plasma-derived HBsAg particles as the reference [27]. Scale bars represent 5 nm.

particles on expression in *E. coli*, which is the most widely used and most efficient expression system in the biotechnology industry. However, the VLPs derived from *E. coli* have a high degree of heterogeneity in their physical properties, including the shape and diameter of the particles: for example, many ill-formed or incomplete particles are present in HPV L1 VLPs [20]. Post-purification disassembly and reassembly of HPV L1 VLPs was shown to greatly enhance the homogeneity and morphology of the VLPs, enabling high-resolution structure determination by cryo-electron microscopy (cryoEM) or atomic force microscopy (AFM) and development of vaccine formulations with good stability [20,21] (Figure 2). Importantly, the reassembled HPV VLPs had more virion-like epitopes compared to the VLPs prior to reassembly [21,22] (Table 2).

In the case of the p239-based HEV Hecolin vaccine, a robust process was developed using *E. coli* as the expression system. High refolding efficiency and overall production yields were achieved [23–25]. Particle assembly of p239 was performed post-purification [23,26]. Biophysical analysis of the p239 VLPs by high-performance size-exclusion chromatography (HPSEC), analytical centrifugation, and electron microscopy clearly demonstrated that p239 assembles into particles with diameters of 20–30 nm (Figure 3). However, the shape of the particles is too heterogeneous for structure determination by electron microscopy (EM) image reconstruction (heterogeneity precludes the symmetry averaging used in EM structure determination), possibly because p239, with 239 amino acids, only contains approximately 40% of the full-length capsid open reading frame 2 protein (pORF2; 606 amino acids). Most importantly, the neutralizing antibodies elicited by the p239 vaccine showed high neutralization titers in *in vitro* cell-based models and *in vivo* challenge studies in chimpanzees, showing that the neutralizing and immunodominant epitopes are preserved on the p239 VLPs [23].

Functional properties of VLPs used in human vaccines

The functional properties of VLPs, including those in the RECOMBIVAX HB, Gardasil and Hecolin vaccines, have been assessed with biochemical, biophysical, immunochemical, and immunological assays. The application of these assays constitutes a 'comparability exercise' that can be used to generate a database for gauging the product characteristics over a broad timeframe and different manufacturing scales. After the introduction of the concept of 'well-characterized biologics' in the mid-to-late 1990s, the yeast-derived HBV vaccine RECOMBIVAX HB was the first vaccine for which a such a comparability exercise was used to improve the production process and scale up approach to achieve the VLP quality required for licensing [27].

Data from the quantitative analytical assays used in the comparability exercise were used to generate a

Table 2. Improved biophysical and immunochemical properties of recombinant HPV16 VLPs on post-purification reassembly during bioprocessing[a]

Property	Observations in post-reassembly VLPs	Method	Refs
Physical and structural attributes			
Morphology	More closed VLPs	TEM, AFM	[21,22]
	Larger VLPs	TEM, AFM	[21]
	Pronounced surface protrusions	TEM, AFM	[21]
Monodispersity	More monodisperse	TEM	[22]
	Single peak (versus two in pre-)	SEC–HPLC	[22]
Propensity to aggregate	Fewer aggregates	DLS	[22]
	Less prone to aggregation	Cloud point	[21]
	Less non-specific binding to dextran surface	SPR	[21,22]
Thermal unfolding	Higher thermal stability	DSC	[21]
Resistance to proteolysis	More resistant to proteolysis	SDS-PAGE	[21]
Activity-based attributes			
Epitope-specific antigenicity (label-free analysis)	Improved antigenicity with multiple mAbs	SPR	[27]
Solution antigenicity (competition ELISA)	Higher antigenicity when probed with neutralizing mAbs	IC_{50}	[21]
Epitope mapping (2×2 or 8×8)	More focused map	SPR	[43]
Solution dissociation constant (K_D)	Higher affinity to neutralizing mAbs	ELISA	[75]
IVRP (for release and stability testing)	30–50% increase in potency		[21,22]
In vivo potency (mouse ED_{50})	>Fivefold decrease in ED_{50} (enhanced immunogenicity)		[22]

[a]Abbreviations: AFM, atomic force microscopy; DSC, differential scanning calorimetry; HPV, human papillomavirus; IVRP, *in vitro* relative potency; mAb, monoclonal antibody; SEC–HPLC, size-exclusion chromatography–high-performance liquid chromatography; SPR, surface plasmon resonance; TEM, transmission electron microscopy; VLP, virus-like particle.

database for gauging the product characteristics over a broad timeframe and different manufacturing scales. This database, along with data generated from product that was batch-produced with the optimized process, validated the process improvement and scale up, and facilitated regulatory approval [27]. This approach also provided guidance in predicting the quality of future in-line lots of the vaccine or lots from scale-down model process [27]. As a result of the recent initiatives of quality by design (QbD) for product development and life-cycle management, the use of a reference database of product characteristics is particularly important in defining the critical quality attributes (CQAs) for newly developed human vaccines. Work space can then be defined on the basis of these CQAs and critical process parameters (CPPs), thus enabling smooth and successful scale up, which is essential to meet the increasing market demands after initial licensure and product launch.

Both HBV and HPV vaccines have gone through rigorous comparability tests of their structure and antigenicity after scale up. In the following sections, we review the critical functional analyses and potency assays. We then describe the physical properties and structural features of these recombinant vaccines, as determined using an array of biophysical approaches.

Potency assay of VLP-based vaccines

Animal-based potency assay

One way to measure the potency of a vaccine is to directly measure how effective the vaccine is in an animal disease model. It is crucial to have one or more animal-based assays to model the human response to a vaccine during various developmental phases of the vaccine. An animal potency assay, usually in mice, is also needed to study the relative dose required to elicit biologically significant (in some cases functional) titers. A more quantitative measure, such as ED_{50} or geometric mean titer (GMT) can be used as a release assay for potency and to track the stability of the product. Although animal-based potency assays are critically important during preclinical and clinical testing stages, they are less useful during post-marketing support because they are generally not sufficiently precise for product characterization and stability tracking. More precise *in vitro* relative potency assays using polyclonal antibodies, or more preferably monoclonal antibodies, for probing distinct epitopes are widely used in the lead-up to release and in evaluating stability of a licensed vaccine [28,29].

Potency assays in animals, the closest mimics of a human response to a vaccine, are essential during the preclinical and clinical development stages of a vaccine to provide a good preview of how the vaccine would work inside a human body. However, animal potency assays have slow turn-around times, consume large numbers of animals, and have poor precision and large intrinsic variations when it comes to assessing lot-to-lot variations and product stability over time. For all of these reasons, animal potency assays are not ideal for long-term product stability studies in support of the product after commercial release.

Binding-based potency assay

Measuring the binding affinity or antigenicity of the VLPs in the vaccine for neutralizing antibodies provides a more reliable and quantitative measure of vaccine potency. Antigenicity is determined using neutralizing antibodies and correlates with the effectiveness of the vaccine in eliciting neutralizing antibodies after being injected into humans. This type of binding-based assay is usually referred to as an *in vitro* relative potency (IVRP) assay. For the HBV vaccine, binding analyses measure the binding affinity and avidity of neutralizing antibodies such as RF1 and A1.2 to the VLPs [30–32] (Figure 1D). In the case of HPV L1-derived VLPs, certain monoclonal antibodies had high neutralization efficiencies [33,34] and recognized

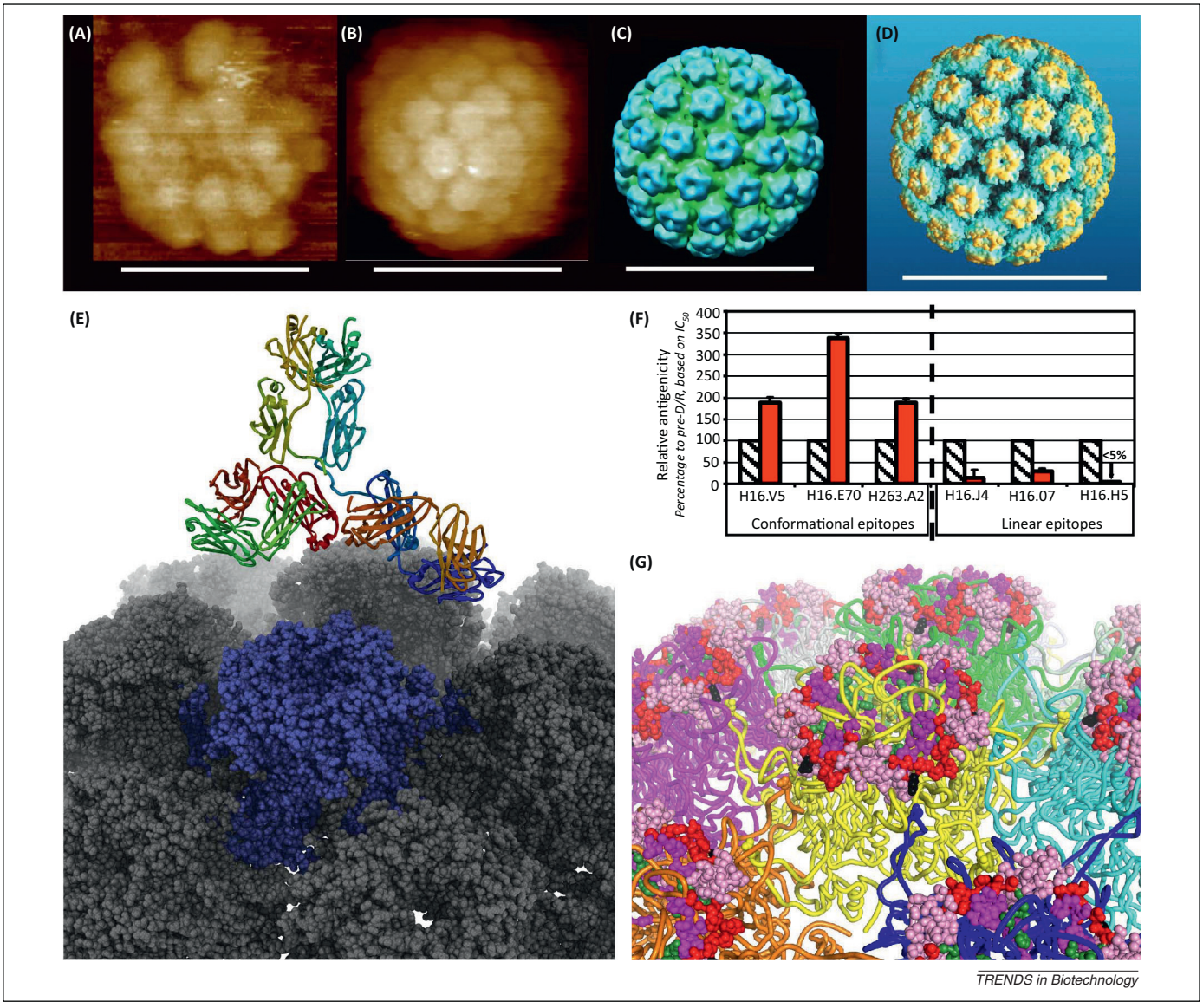

Figure 2. Structure, assembly, and antigenic determinants of human papillomavirus (HPV) virus-like particles (VLPs). Scale bar = 50 nm. Atomic force microscopy (AFM) images of HPV16, **(A)** before and **(B)** after disassembly and reassembly treatment (from [20,53]). **(C)** Cryo-electron microscopy (CryoEM) structure of HPV16 [21]. **(D)** Atomic model of HPV16 generated from crystal structure of HPV16 L1 [66] and cryoEM structures of bovine papillomavirus [61,64]. **(E)** The surface of the atomic model of HPV16 shown in panel **(D)** with the pentavalent capsomere colored in blue, the hexavalent capsomeres in gray, and a full-length IgG crystal structure in cartoon representation drawn to scale, with the antigen-binding fragment (Fab) moieties pointing in the general direction of epitopes. **(F)** Binding activities of various neutralizing antibodies to VLPs before (black stripes) and after (red) disassembly and reassembly treatment (from [21]). Percentage to pre-D/R: the binding activities for the post-reassembly samples were normalized to those of the control samples which were the samples without disassembly/reassembly treatment. **(G)** Close-up of a capsomere from the atomic model of HPV16 shown in panel **(D)**, with each key antibody epitope shown in a different color [21]. Scale bars represent 5 nm.

immunodominant epitopes when analyzed with human sera from naturally infected individuals [35]. Such monoclonal antibodies with neutralizing activity and immunodominant epitopes – for example, HPV16.V5 – are preferred for use in IVRPs [18,36–39], but an antibody recognizing a non-immunodominant epitope and a polyclonal antibody were also used for lot release of the vaccine [40,41]. However, the correlation between IVRP and mouse potency, measured as the ED_{50}, needs to be established prior to claiming an IVRP assay to be a release assay [28,29]. IVRP assays were also used to set the product specifications for product lot release and stability [42]. In addition to the lot release assays, it is necessary to further characterize the recombinant antigen using monoclonal or polyclonal antibodies in order to gain a more complete picture of the VLP conformation or antigen quality [43].

Cell-based potency assay

Vaccine antigens such as the recombinant HPV L1 protein might elicit robust titers of binding antibodies in animals or humans, but the binding activity of an antibody to an antigen does not necessarily correspond to its functional antibody levels. This is because a recombinant protein may adopt many different conformations with different levels of denaturation or aggregation, particularly when antigens are coated onto a solid surface (as in enzyme immunoassays or radioimmunoassays). To solve this issue, it may be necessary to develop assays in which the binding activity can better mimic the binding to the native virions, such as an *in vitro* virus neutralization assay. In the case of HPV, owing to the difficulty in culturing the viruses, a pseudovirion-neutralizing assay was developed using alkaline phosphatase as a reporter gene [44]. Later, different

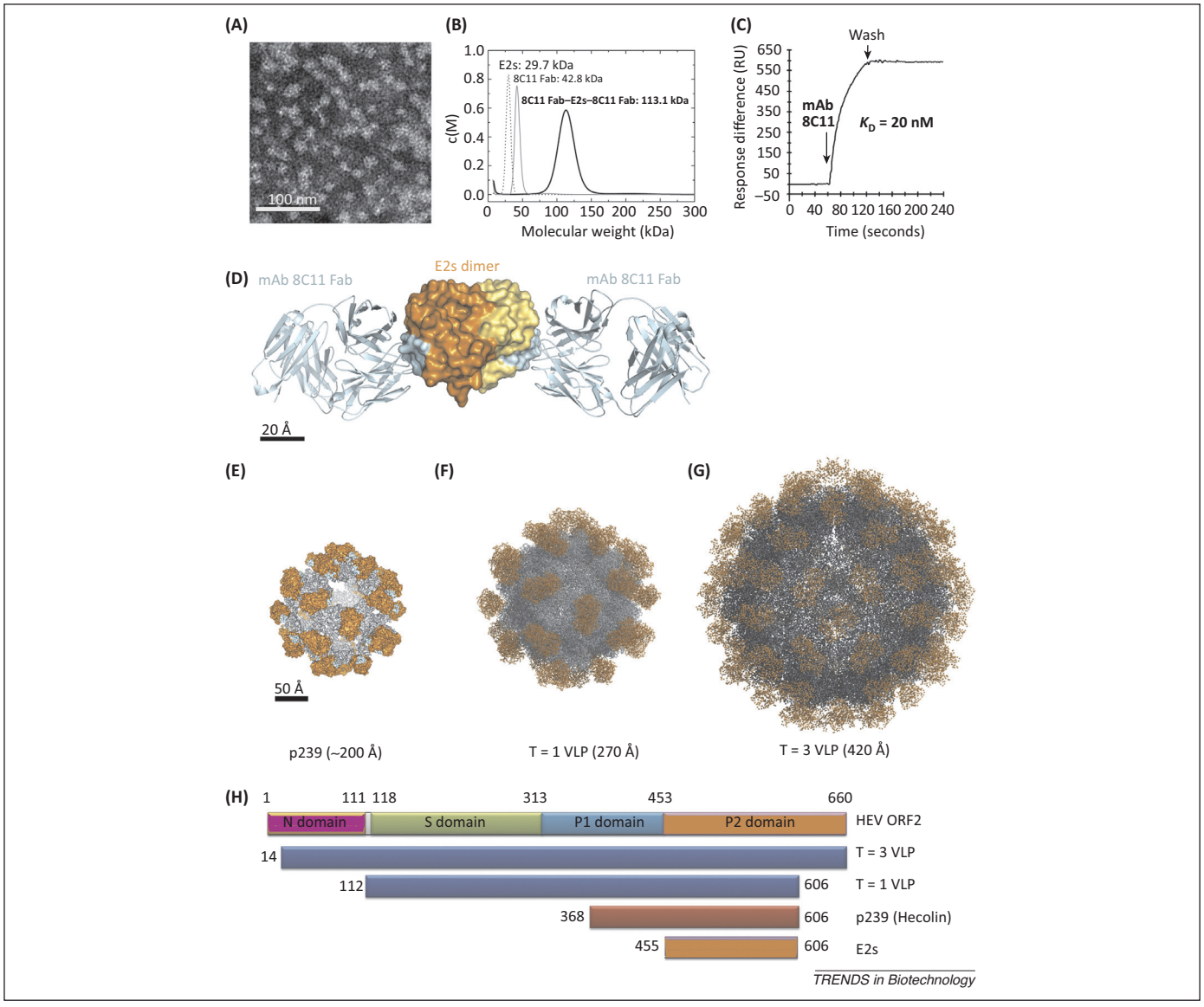

Figure 3. Structural and functional analysis of the hepatitis E vaccine. **(A)** Negatively stained transmission electron micrograph of p239 virus-like particles (VLPs) (in bulk from Hecolin vaccine). **(B)** Sedimentation velocity analytical ultracentrifugation (SV-AUC) profiles of the protrusion domain of E2s, the neutralizing monoclonal antibody (mAb) 8C11 Fab (antibody-binding fragment), and the E2s–8C11 Fab complex. The sedimentation profiles show that E2s binds 8C11 Fab with a 1:1 stoichiometry, or one E2s dimer to two Fabs. **(C)** The association–dissociation curve of E2 protein binding with the hepatitis E virus (HEV) neutralizing antibody 8C11 and the binding constant were calculated by surface plasmon resonance (Biacore). **(D)** Crystal structure of the E2s–Fab 8C11 immune complex [Protein Data Bank (PDB) code 3RKD], revealing an immunodominant neutralizing epitope of the HEV capsid. **(E)** A p239 'shrunken VLP' model constructed by *in silico* truncation of the icosahedral T (triangulation number) = 1 E2 VLP (PDB code 2ZTN) [26]. **(F)** Crystal structure of T = 1 VLPs (PDB code 2ZTN). **(G)** Cryo-electron microscopy structure of T = 3 VLPs (PDB code 3IYO). **(H)** Schematic profile of HEV open reading frame 2 (ORF2) functional domains and some historic constructs.

versions of this pseudovirion-neutralizing assay were adopted in different laboratories to assess the titers and quality of the antibodies elicited by the recombinant vaccine in animals and humans [45–47]. Similarly, owing to the difficulty of *in vitro* HEV replication, the neutralization capacity of antibodies blocking cell adsorption entry into Huh7 cells was assessed [48–50]. Infectious cDNA clones of HEV also showed potential to be used in assessing the neutralizing efficacy of a polyclonal or monoclonal antibody [51,52].

Biophysical and structural analysis of recombinant VLPs

Electron microscopy with negative staining

TEM with uranyl acetate as a 'negative staining' contrast-enhancing agent is the most commonly used technique to assess the morphology of a VLP preparation. TEM, with its quick turn-around, ease of handling, and low cost, is a useful tool for quickly checking the size distribution and particle morphology of VLPs in a preparation [53]. However, the staining and drying of samples can potentially introduce artifacts owing to particle dehydration because VLPs are empty capsid shells without packed nucleic acids to increase the integrity of the protein shell [54].

Field-flow fractionation and electrospray differential mobility analysis

Electrospray–differential mobility analysis (ES–DMA) provides a quantitative measurement of VLP size distributions [55,56]. ES–DMA is similar to electrospray–mass spectrometry (ES–MS) but measures the effective particle size, rather than mass [57]. VLPs are aerosolized and their electrical mobility is measured at ambient temperature.

Another very distinct approach for accurately measuring VLP particle size distributions is asymmetric flow field-flow fractionation with multi-angle light scattering detection (AFFFF–MALS or AF4–MALS) [58]. AF4–MALS analysis provides accurate size and distribution information even for heterogeneous samples without causing aggregation [58–60]. ES–DMA and AF4–MALS thus provide orthogonal, quantitative approaches to monitor batch consistency for new vaccine products [56]. Moreover, ES–DMA and AF4–MALS are both faster and more cost effective than TEM, and provide greater statistical significance than TEM or dynamic light scattering [56].

3D image reconstructions of VLPs from electron cryomicroscopy

If the VLP preparation is homogeneous enough, the electron cryoEM method can be used to obtain 3D structures of the VLPs. The VLPs are flash frozen in a vitrified (glass-like) and fully hydrated form without stain, promoting capsid stability and reducing the number of artifacts that limit the usefulness of TEM. CryoEM can reveal the detailed surface structural features on the VLPs more faithfully than negative-staining TEM because the VLP structure is preserved in the vitrified form, and artifacts resulting from surface adsorption, staining, and drying procedures are minimized. By averaging data from thousands of particles and using internal icosahedral symmetry averaging within each particle, it is possible to obtain highly detailed structures of virus particles (Figure 2C). This approach has been applied to bovine papillomavirus to determine its structure at pseudo-atomic (3.6 Å) resolution [61,62]. In the case of the HPV vaccine, the arrangement of the T (triangulation number) = 7 icosahedral structure of the full-sized VLP of HPV16 was clearly recognizable even from data collected from a single VLP [21]. Moreover, the binding of a functional monoclonal antibody fragment, Fab H16.V5, could be clearly visualized on the surface of VLPs [21].

Atomic force microscopy in solution

AFM studies are typically carried out on a solid surface or on nanoparticles adsorbed onto a solid surface. In order to better preserve the morphology and surface features of VLPs, a method for AFM analysis of VLPs in solution has been developed in which the mica surface is immersed in a chamber with flowing buffer. This AFM-in-solution approach reduces artifacts resulting from drying, allowing the particle size distribution to be analyzed more accurately [20,63]. More importantly, in high-quality VLP preparations, high-resolution surface probing could be achieved on a single-particle level with well-formed and fully assembled VLPs. The surface features on individual capsomeres as well as the interacting portions of the neighboring capsomeres were clearly visible [20]. Images from single-particle solution AFM bear a high degree of resemblance to those obtained with cryoEM [20] (Figure 2B,C).

X-ray crystallographic studies

The VLPs in the HPV and HEV vaccines consist of a rigid protein shell, which in the case of HPV VLPs is crosslinked and stabilized by cysteine disulfides [61,64]. Although the VLPs used in vaccines have not been crystallized owing to some heterogeneity in their shapes and sizes, the crystal structures of the capsomeres (the building blocks of the icosahedral VLP assemblies) have been obtained. The capsomeres harbor the key neutralizing epitopes. Crystal structures of the pentameric capsomeres formed by the HPV L1 protein have been determined for a total of four HPV types (types 11, 16, 18, and 35) by Chen and colleagues [65,66]. For HPV, a crystal structure is available for small icosahedral particles [66], but these T = 1 particles are smaller than those used in the HPV vaccines and have different inter-capsomeric contacts from native virions [64]. A combination of crystallographic and cryoEM data has allowed reliable atomic models to be generated for HPV [61,64]. The atomic models reveal the precise location and extent of the principal antibody epitopes. The epitopes are formed by various loops on the capsomere surface, and certain key epitopes involve loops from L1 subunits in different capsomeres [66]. This type of inter-capsomeric epitope would not be present in a subunit vaccine consisting of L1 monomers or even pentameric L1 capsomeres and is a good example of why VLPs are superior antigens. For the HEV VLPs, the key building block is the pORF2 E2 dimer (or E2s, as shown in Figure 3) [24,50]. The structures of two genotypes of HEV VLP were determined at pseudo-atomic resolution (~3.5 Å) by X-ray crystallography [67,68], and the structure of the dimer was determined at high resolution (2.0 Å). In addition, the structure of the complex of E2s with an Fab fragment of a functional antibody (8C11) was also determined at high resolution [50] (Figure 3). 8C11 binds E2 tightly on the surface of native HEV particles and effectively captures the virions, making the antibody an ideal tool for IVRP assay development (M.X. Wei *et al.*, unpublished). The 8C11-based IVRP may potentially replace mouse potency for lot release and stability testing.

The HBsAg particles in the HBV vaccine have not been crystallized, perhaps owing to their high lipid content, which may lead to structural heterogeneity. The HBsAg subunits have not been crystallized either, possibly owing to their unusually high cysteine content, with eight cysteines in the major hydrophilic region, a key antigenic region of ~70 amino acids. The cryoEM structure of the HBsAg particles shows that the major hydrophilic regions of four subunits are clustered in protrusions on the particle surface [69], so that each protrusion contains a total of 32 cysteine residues. These protrusions have been postulated to be the main epitopes for neutralizing antibodies [27]. The large cysteine cluster suggests that the HBsAg particles contain extensive disulfide crosslinking, possibly with different possible combinations of intra- and inter-subunit crosslinks [70]. Variability in disulfide crosslinking may be limiting the resolution at which HBsAg particle structures can be determined. Thus, although the first recombinant HBV vaccine was licensed in 1986, there are still no high-resolution structures of the key epitopes or the major hydrophilic regions of the HBsAg particles.

Structure–activity relationship in VLP vaccines: integrity of key epitopes

Presence of native virion-like epitopes

VLPs that mimic the native virions more faithfully in their assembly are more antigenic than VLPs with non-native

assemblies. Neutralizing antibodies from animals or humans can be used to assess how faithfully natural epitopes are exposed in VLPs in binding assays such as surface plasmon resonance assays [37], solution competition ELISA [21], sandwich ELISA, or IVRP assays. These assays are highly useful for analyzing the antigenicity of VLPs. More importantly, a neutralization assay must be in place to evaluate the quality of the immune response in terms of the neutralization titer, rather than just the binding titer. Thus, the neutralizing epitopes on the antigen surface are the surrogate markers for the vaccine potency *in vivo*. The neutralizing antibodies, by recognizing important epitopes, have a key role in defining the product attributes of a vaccine antigen during production for preclinical and clinical development, as well as during post-marketing life-cycle management. Therefore, the process goal is to maximize the quantity of virion-like epitopes (Table 2), and to keep them intact and stable under favorable conditions during bioprocessing and at the downstream formulation, filling, and storage stages.

VLPs as bionanoparticles with multiple epitopes on their surface

VLPs provide an important enhancement of the immune response owing to the multiplicity of arrayed virion-like epitopes on the VLP surface. In addition to the above-mentioned IVRP and other potency assays, immunochemical assays, such as binding affinity determination (e.g., by surface plasmon resonance), solution competition ELISA, and epitope mapping are needed to fully define the quality and quantity of epitopes on the recombinant antigen. This is critical when implementing a process improvement or a scale up. It is important to have neutralizing or functional monoclonal antibodies as tools to quantitatively analyze the antigen properties from different processes prior to claiming that products or lots to be comparable. A panel of monoclonal antibodies against the protein (or proteins) in the VLP can be used to effectively probe the structural features on the VLP surface in solution (as in a competitive ELISA assay [21,27]) or when adsorbed onto a surface (as in a sandwich ELISA or pairwise epitope mapping). In pairwise epitope mapping, the VLP surface is saturated with a first monoclonal antibody before exposure to a second antibody. In 2×2 pairwise epitope mapping, the relative relationship of the epitopes of the two antibodies is deduced for a given VLP preparation by performing the binding analyses using two different approaches. Consistency in the mapping data with the two approaches reports on the reproducibility of the process and the quality of the VLP preparation [43]. The numbers of antibodies and types of binding analyses can be multiplied further, for example in an 8×8 epitope mapping, to yield a more complete picture of the epitope composition on the VLP surface [43]. From this kind of multifaceted analysis, a complete composite picture can be established from the information gained from each individual approach. Orthogonal approaches must be used when choosing the antibodies for defining the key product attributes, such as the nature of the epitopes recognized (linear or conformational) or the ability to neutralize the virus *in vitro*.

Life-cycle management of vaccine manufacturing

Vaccines must be monitored at different product stages – the early, clinical development, and post-licensure stages – using a database from past manufacturing experience to gauge the quality of future lots. Process changes are implemented based on the comparability exercise, which relies on the database of physical and functional properties of the vaccine to manage and predict the consistency of the vaccine over time and across different lots. Multifaceted characterization is essential to ensure the safety and efficacy of the vaccine. Structural and functional analyses presented in this review (such as the methods listed in Table 2) are an important part of this characterization package for recombinant VLPs to ensure process robustness and product consistency.

Formulation and stability of VLP-based vaccines

It is possible to produce highly immunogenic VLP preparations, but the antigen might not be viable as a VLP-based vaccine candidate until stable formulations can be developed. Therefore, preserving critical epitopes on VLPs is equally important to generating them in the first place. Multiple-year stability is required for a marketed vaccine, and all three vaccines highlighted here (Table 1) have aluminum-based adjuvants for antigen stabilization and for enhanced immunogenicity [53]. The introduction of non-ionic surfactants into HPV VLP aqueous solutions provides substantially enhanced stabilization of HPV VLPs against aggregation on exposure to low salt and protein concentration, as well as protection against surface adsorption and aggregation as a result of heat stress and physical agitation [71]. After adsorption onto adjuvant, accessible epitopes can be probed using antibodies in a competitive format such as a competitive ELISA IC_{50} assay [21] (Table 2); however, for total antigen analysis, proper dissolution conditions need to be developed to remove antigen from adjuvants for morphological and antigenicity analysis [29]. No change in morphology or antigenicity was observed in the case of HBsAg VLPs after recovery from adjuvants using proper dissolution procedures [72]. IVRP or mouse potency with proper dissolution conditions to fully recover the antigen was used to show the stability of the vaccines over many months [29,36]. The tools for process monitoring and for demonstrating product comparability are also crucial for demonstrating the stability of the vaccine over time. However, AF4–MALS and ES–DMA are perhaps the two most promising and powerful techniques that have emerged for quantitative monitoring of VLP size and distribution during product development and process analytics [55–60].

The structural and biophysical approaches described above (AF4–MALS, ES–DMA, HPSEC, dynamic light scattering, analytical centrifugation, TEM, cryoEM, X-ray crystallography, and AFM) provide valuable quantitative data for each lot of a vaccine product, although it has not been possible to assign a unique identifying numerical matrix to each lot. Solution antigenicity, sandwich-based IVRP or label-free surface plasmon resonance-based antigenicity analyses complement the database of the quantitative analysis of critical product attributes. This database can in turn be used for QbD as part of process analytical

technology to better define the work space during bioprocessing of the VLP-based antigens – a critically important part in the life-cycle management of a marketed vaccine [27,36,37,73,74].

Concluding remarks

VLP-based vaccines will continue to play a critical part in improving human health. Better serotype coverage for HPV-associated diseases is being obtained by adding new, high-risk types. Chimeric systems, such as grafting HPV L2 epitopes into L1 VLPs, are also being explored as alternatives for widening the coverage spectrum. New diseases, including influenza, malaria, mosquito-borne chikungunya virus and food-borne norovirus infection, are actively being studied in preclinical and clinical stages using VLP-based approaches to provide protective immunity. VLPs seem to elicit more robust immune responses than do DNA vaccination or the subunit approach. To ensure production robustness, analytics need to be implemented to enable the QbD approach using quantitative methods to assess VLP quality and stability. Monoclonal antibody-based binding assays are attractive replacements of animal-based potency assays owing to greater assay precision, shorter turn-around times, and greatly reduced animal use in product lot release and stability testing. Although activity in potency assays is the most important product quality attribute, a multifaceted and weighted approach is needed for a whole analytical package, and this needs to be in place throughout the life cycle of a successful vaccine, from the early clinical development stage onwards.

Acknowledgments

Financial support for this work was provided by Institute Reconstruction Fund (2011FU125Z04), National science Foundation of China funds (81172885, and 81273327), Xiamen City Municipal platform fund on viral biotherapeutics (3502Z20131001); and by a Burroughs Wellcome Investigator Award to Y.M.

References

1 Millman, I. *et al.* (1969) Australia antigen detected in the nuclei of liver cells of patients with viral hepatitis by the fluorescent antibody technic. *Nature* 222, 181–184
2 Krugman, S. (1982) The newly licensed hepatitis B vaccine. Characteristics and indications for use. *JAMA* 247, 2012–2015
3 Valenzuela, P. *et al.* (1982) Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 298, 347–350
4 McAleer, W.J. *et al.* (1984) Human hepatitis B vaccine from recombinant yeast. *Nature* 307, 178–180
5 Scolnick, E.M. *et al.* (1984) Clinical evaluation in healthy adults of a hepatitis B vaccine made by recombinant DNA. *JAMA* 251, 2812–2815
6 Andre, F.E. (1990) Overview of a 5-year clinical experience with a yeast-derived hepatitis B vaccine. *Vaccine* 8 (Suppl.), S74–S78 discussion S79–S80
7 Kushnir, N. *et al.* (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
8 Markowitz, L.E. *et al.* (2013) Reduction in human papillomavirus (HPV) prevalence among young women following HPV vaccine introduction in the United States, National Health and Nutrition Examination Surveys, 2003–2010. *J. Infect. Dis.* 208, 385–393
9 Zhou, J. *et al.* (1991) Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology* 185, 251–257
10 Kirnbauer, R. *et al.* (1992) Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. U.S.A.* 89, 12180–12184
11 Kirnbauer, R. *et al.* (1993) Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *J. Virol.* 67, 6929–6936
12 Deschuyteneer, M. *et al.* (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. Vaccin.* 6, 407–419
13 Zhu, F.C. *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, 895–902
14 Wu, T. *et al.* (2012) Hepatitis E vaccine development: a 14 year odyssey. *Hum. Vaccin. Immunother.* 8, 823–827
15 Pastrana, D.V. *et al.* (2012) Neutralization serotyping of BK polyomavirus infection in kidney transplant recipients. *PLoS Pathog.* 8, e1002650
16 Anggraeni, M.R. *et al.* (2013) Sensitivity of immune response quality to influenza helix 190 antigen structure displayed on a modular virus-like particle. *Vaccine* 31, 4428–4435
17 Shouval, D. (2003) Hepatitis B vaccines. *J. Hepatol.* 39 (Suppl. 1), S70–S76
18 Zhao, Q. *et al.* (2011) In-depth process understanding of RECOMBIVAX HB® maturation and potential epitope improvements with redox treatment: multifaceted biochemical and immunochemical characterization. *Vaccine* 29, 7936–7941
19 Zajac, B.A. *et al.* (1986) Overview of clinical studies with hepatitis B vaccine made by recombinant DNA. *J. Infect.* 13 (Suppl. A), 39–45
20 Zhao, Q. *et al.* (2012) Disassembly and reassembly improves morphology and thermal stability of human papillomavirus type 16 virus-like particles. *Nanomedicine* 8, 1182–1189
21 Zhao, Q. *et al.* (2012) Disassembly and reassembly of human papillomavirus virus-like particles produces more virion-like antibody reactivity. *Virol. J.* 9, 52
22 Mach, H. *et al.* (2006) Disassembly and reassembly of yeast-derived recombinant human papillomavirus virus-like particles (HPV VLPs). *J. Pharm. Sci.* 95, 2195–2206
23 Li, S.W. *et al.* (2005) A bacterially expressed particulate hepatitis E vaccine: antigenicity, immunogenicity and protectivity on primates. *Vaccine* 23, 2893–2901
24 Li, S. *et al.* (2009) Dimerization of hepatitis E virus capsid protein E2s domain is essential for virus-host interaction. *PLoS Pathog.* 5, e1000537
25 Zhao, Q. *et al.* (2013) Antigenic determinants of hepatitis E virus and vaccine-induced immunogenicity and efficacy. *J. Gastroenterol.* 48, 159–168
26 Yang, C. *et al.* (2013) Hepatitis E virus capsid protein assembles in 4M urea in the presence of salts. *Protein Sci.* 22, 314–326
27 Mulder, A.M. *et al.* (2012) Toolbox for non-intrusive structural and functional analysis of recombinant VLP based vaccines: a case study with hepatitis B vaccine. *PLoS ONE* 7, e33235
28 Schofield, T. (2002) *In vitro* versus in vivo concordance: a case study of the replacement of an animal potency test with an immunochemical assay. *Dev. Biol. (Basel)* 111, 299–304
29 Shank-Retzlaff, M. *et al.* (2005) Correlation between mouse potency and *in vitro* relative potency for human papillomavirus type 16 virus-like particles and Gardasil vaccine samples. *Hum. Vaccin.* 1, 191–197
30 Waters, J.A. *et al.* (1992) Analysis of the antigenic epitopes of hepatitis B surface antigen involved in the induction of a protective antibody response. *Virus Res.* 22, 1–12
31 Shearer, M.H. *et al.* (1998) Structural characterization of viral neutralizing monoclonal antibodies to hepatitis B surface antigen. *Mol. Immunol.* 35, 1149–1160
32 Tajiri, K. *et al.* (2010) Analysis of the epitope and neutralizing capacity of human monoclonal antibodies induced by hepatitis B vaccine. *Antiviral Res.* 87, 40–49
33 Christensen, N.D. *et al.* (1990) Monoclonal antibody-mediated neutralization of infectious human papillomavirus type 11. *J. Virol.* 64, 5678–5681
34 Culp, T.D. *et al.* (2007) Binding and neutralization efficiencies of monoclonal antibodies, Fab fragments, and scFv specific for L1 epitopes on the capsid of infectious HPV particles. *Virology* 361, 435–446
35 Wang, D. *et al.* (1997) A monoclonal antibody against intact human papillomavirus type 16 capsids blocks the serological reactivity of most human sera. *J. Gen. Virol.* 78, 2209–2215

36 Shank-Retzlaff, M.L. *et al.* (2006) Evaluation of the thermal stability of Gardasil. *Hum. Vaccin.* 2, 147–154
37 Zhao, Q. *et al.* (2011) Real time monitoring of antigenicity development of HBsAg virus-like particles (VLPs) during heat- and redox-treatment. *Biochem. Biophys. Res. Commun.* 408, 447–453
38 Cuervo, M.L. *et al.* (2008) Validation of a new alternative for determining *in vitro* potency in vaccines containing hepatitis B from two different manufacturers. *Biologicals* 36, 375–382
39 Shanmugham, R. *et al.* (2010) Immunocapture enzyme-linked immunosorbent assay for assessment of *in vitro* potency of recombinant hepatitis B vaccines. *Clin. Vaccine Immunol.* 17, 1252–1260
40 Giffroy, D. *et al.* (2006) Validation of a new ELISA method for *in vitro* potency assay of hepatitis B-containing vaccines. *Pharmeuropa Bio* 2006, 7–14
41 Karimzadeh, H. *et al.* (2010) Validation of an *in-vitro* method for hepatitis B vaccine potency assay: specification setting. *Panminerva Med.* 52, 177–182
42 Capen, R. *et al.* (2007) Establishing potency specifications for antigen vaccines. *Bioprocess Int.* 5, 30–42
43 Towne, V. *et al.* (2013) Pairwise antibody footprinting using surface plasmon resonance technology to characterize human papillomavirus type 16 virus-like particles with direct anti-HPV antibody immobilization. *J. Immunol. Methods* 388, 1–7
44 Pastrana, D.V. *et al.* (2004) Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. *Virology* 321, 205–216
45 Kemp, T.J. *et al.* (2012) Kinetic and HPV infection effects on cross-type neutralizing antibody and avidity responses induced by Cervarix®. *Vaccine* 31, 165–170
46 Roberts, C. *et al.* (2012) Evaluation of the HPV 18 antibody response in Gardasil® vaccinees after 48 mo using a pseudovirion neutralization assay. *Hum. Vaccin. Immunother.* 8, 431–434
47 Einstein, M.H. *et al.* (2009) Comparison of the immunogenicity and safety of Cervarix and Gardasil human papillomavirus (HPV) cervical cancer vaccines in healthy women aged 18–45 years. *Hum. Vaccin.* 5, 705–719
48 Li, S.W. *et al.* (2005) Mutational analysis of essential interactions involved in the assembly of hepatitis E virus capsid. *J. Biol. Chem.* 280, 3400–3406
49 He, S. *et al.* (2008) Putative receptor-binding sites of hepatitis E virus. *J. Gen. Virol.* 89, 245–249
50 Tang, X. *et al.* (2011) Structural basis for the neutralization and genotype specificity of hepatitis E virus. *Proc. Natl. Acad. Sci. U.S.A.* 108, 10266–10271
51 Emerson, S.U. *et al.* (2004) In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. *J. Virol.* 78, 4838–4846
52 Huang, F.F. *et al.* (2005) Construction and characterization of infectious cDNA clones of a chicken strain of hepatitis E virus (HEV), avian HEV. *J. Gen. Virol.* 86, 2585–2593
53 Shi, L. *et al.* (2007) GARDASIL: prophylactic human papillomavirus vaccine development–from bench top to bed-side. *Clin. Pharmacol. Ther.* 81, 259–264
54 Melchior, V. *et al.* (1980) Stacking in lipid vesicle-tubulin mixtures is an artifact of negative staining. *J. Cell Biol.* 86, 881–884
55 Pease, L.F., 3rd (2012) Physical analysis of virus particles using electrospray differential mobility analysis. *Trends Biotechnol.* 30, 216–224
56 Pease, L.F., 3rd *et al.* (2009) Quantitative characterization of virus-like particles by asymmetrical flow field flow fractionation, electrospray differential mobility analysis, and transmission electron microscopy. *Biotechnol. Bioeng.* 102, 845–855
57 Guha, S. *et al.* (2012) Electrospray–differential mobility analysis of bionanoparticles. *Trends Biotechnol.* 30, 291–300
58 Chuan, Y.P. *et al.* (2008) Quantitative analysis of virus-like particle size and distribution by field-flow fractionation. *Biotechnol. Bioeng.* 99, 1425–1433
59 Ding, Y. *et al.* (2010) Modeling the competition between aggregation and self-assembly during virus-like particle processing. *Biotechnol. Bioeng.* 107, 550–560
60 Mohr, J. *et al.* (2013) Virus-like particle formulation optimization by miniaturized high-throughput screening. *Methods* 60, 248–256
61 Wolf, M. *et al.* (2010) Subunit interactions in bovine papillomavirus. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6298–6303
62 Trus, B.L. *et al.* (1997) Novel structural features of bovine papillomavirus capsid revealed by a three-dimensional reconstruction to 9 A resolution. *Nat. Struct. Biol.* 4, 413–420
63 Zhao, Q. *et al.* (2006) Maturation of recombinant hepatitis B virus surface antigen particles. *Hum. Vaccin.* 2, 174–180
64 Modis, Y. *et al.* (2002) Atomic model of the papillomavirus capsid. *EMBO J.* 21, 4754–4762
65 Bishop, B. *et al.* (2007) Crystal structures of four types of human papillomavirus L1 capsid proteins: understanding the specificity of neutralizing monoclonal antibodies. *J. Biol. Chem.* 282, 31803–31811
66 Chen, X.S. *et al.* (2000) Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Mol. Cell* 5, 557–567
67 Yamashita, T. *et al.* (2009) Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12986–12991
68 Guu, T.S. *et al.* (2009) Structure of the hepatitis E virus-like particle suggests mechanisms for virus assembly and receptor binding. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12992–12997
69 Gilbert, R.J. *et al.* (2005) Hepatitis B small surface antigen particles are octahedral. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14783–14788
70 Mangold, C.M. and Streeck, R.E. (1993) Mutational analysis of the cysteine residues in the hepatitis B virus small envelope protein. *J. Virol.* 67, 4588–4597
71 Shi, L. *et al.* (2005) Stabilization of human papillomavirus virus-like particles by non-ionic surfactants. *J. Pharm. Sci.* 94, 1538–1551
72 Greiner, V.J. *et al.* (2012) The structure of HBsAg particles is not modified upon their adsorption on aluminium hydroxide gel. *Vaccine* 30, 5240–5245
73 Li, M. and Qiu, Y.X. (2013) A review on current downstream bio-processing technology of vaccine products. *Vaccine* 31, 1264–1267
74 Josefsberg, J.O. and Buckland, B. (2012) Vaccine process technology. *Biotechnol. Bioeng.* 109, 1443–1460
75 High, K. *et al.* (2005) Determination of picomolar equilibrium dissociation constants in solution by enzyme-linked immunosorbent assay with fluorescence detection. *Anal. Biochem.* 347, 159–161