Chapter 3
Recombinant Virus-like Particle Protein Vaccines

Robert D. Sitrin, Qinjian Zhao, Clinton S. Potter, Bridget Carragher and Michael W. Washabaugh

3.1 Introduction

Viral diseases offer a major challenge to vaccine development because of the complex nature of virus structures and the large size of the virus particle needed to generate an effective immune response. Viral diseases frequently stimulate both Th2 (antibody-mediated) and Th1 (cell-mediated) immune pathways. Thus, a viral subunit vaccine candidate should stimulate both immune response pathways and might require a novel adjuvant that favors the Th1 cell-mediated pathway. In general, subunit vaccines with smaller molecular weights have failed to elicit a protective immune response for viral targets. Thus, classical approaches to develop antiviral vaccines have required the use of attenuated (or inactivated) live viruses produced in cell culture. Although this approach has been widely successful (measles, mumps, rubella, rotavirus, varicella, polio, and hepatitis A) and is still in practice, not all viruses are amenable to replication in cell culture, particularly at commercial scale.

R.D. Sitrin (✉)
SitrinSolutions, LLC, 237 Emerson Drive, Lafayette Hill, PA 19444, USA
e-mail: Sitrinsolutions@gmail.com

Q. Zhao
State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics,
School of Public Health, Xiamen University, Xiamen 361102, Fujian, China

C.S. Potter · B. Carragher
NanoImaging Services, Inc., 11099 North Torrey Pines Road,
San Diego, CA 92037, USA

C.S. Potter · B. Carragher
Department of Integrative Structural and Computational Biology,
The Scripps Research Institute, La Jolla, CA 92037, USA

M.W. Washabaugh
MedImmune, Inc., Gaithersburg, MD 20878, USA

With the advent of modern molecular biology, it has become reasonable to consider the use recombinant DNA approaches to produce modern prophylactic vaccines. One interesting problem presents itself here, as the viruses themselves are fairly large (multimillion Dalton molecular weight), and thus pose a special challenge to cloning such a large entity. In fact, the viruses themselves face the same problem, in that if they needed to package the genome coding for a multimillion Dalton entity, they would not have sufficient room inside their viral capsid structure for such a large genome. They have solved this problem by evolving over thousands of years the ability to produce proteins of modest size (24 kDa and higher), which self-assemble into icosahedral virus particles after viral replication inside a host cell (Harrison 1990). In appropriate expression systems, these proteins retain that ability to self-assemble into virus-like particle (VLP) structures whose surface is immunochemically comparable to that of the actual virus. This has been a major success story in vaccines and has enabled the development and commercialization of vaccines against Hepatitis B (HBV) (Sitrin et al. 1993; Stephenne 1990), Human Papillomavirus (HPV) (Shi et al. 2007), and recently flu (FDA 2013; Cox 2011) and Hepatitis E (HEV) (Zhu et al. 2010).

3.2 Common Issues for Recombinant-Derived VLP Vaccines

3.2.1 Comparison with Other Recombinant Protein Products

In general, production and licensure of recombinant vaccines share many of the issues important in licensure of recombinant therapeutic proteins and monoclonal antibodies. Current licensed VLP vaccines use either yeast or Baculovirus expression systems—both amenable to fermentation or cell culture at relatively large scale (Cox 2012). The expressed proteins self-assemble during production or purification to produce VLP entities, often of 20–80 nm in diameter. The EP monographs (EP 2008, 2010a) describe the expected EU testing and release criteria for HBV and HPV vaccines, which are presumably similar to the corresponding FDA release expectations. As is the common practice for recombinant products, master cell banks and banked viral seed lots are tested and released for freedom from adventitious agents (viral or bacterial as appropriate). At the completion of purification, usually to a >95 % value, products are sterile filtered and formulated onto aluminum salts and other adjuvants. In general, release and characterization testing procedures are analogous to those used in the Biotech industry for recombinant proteins such as purity, and host cell and DNA residuals (EP 2008, 2010a). Final dosage forms are routinely tested for sterility, adjuvant content and potency. (EP 2008, 2010a; WHO 2006; CHMP 2006).

3.2.2 Comprehensive Characterization

Recombinant VLP vaccines, particularly pivotal clinical and process validation lots, are typically subjected to comprehensive testing analogous to typical testing done on therapeutic protein products (purity by SDS-PAGE, primary and secondary structure) as well as further tests to measure tertiary and quaternary structure and properties on aluminum adjuvant. This additional analytical characterization is above and beyond the release testing carried out on commercial lots. It has become increasingly clear that structural and functional methods for analysis of recombinant VLP-based vaccines are beneficial during development and postlicensure (Zhao et al. 2012a; Zhao et al. 2013b). In addition, characterizing in vitro antigenicity, in an epitope-specific manner, is emerging as an excellent surrogate marker for in vivo immunogenicity or vaccine efficacy (Shank-Retzlaff et al. 2005; Zhao et al. 2012b). Figure 3.1 summarizes the analytical procedures used to assess primary, secondary, tertiary, and quaternary protein structure of GARDASIL$^{®}$ and demonstrates the array of testing technology that can be applied to any recombinant VLP-based vaccine (Sitrin 2010).

A major issue for vaccines is the ability to manage process or facility changes at commercial scale without the need for repeated clinical trials, which is often the case for "traditional" vaccines. It is important to establish the link or correlation between critical product attributes and critical process conditions. A well-defined process can result in a well-characterized product. This is particularly important for clinically relevant structural features such as neutralizing and immune-dominant epitopes for a prophylactic vaccine. Thus, the ability to characterize a recombinant VLP vaccine offers an excellent tool box to demonstrate "comparability" to license process or facility changes or upgrades.
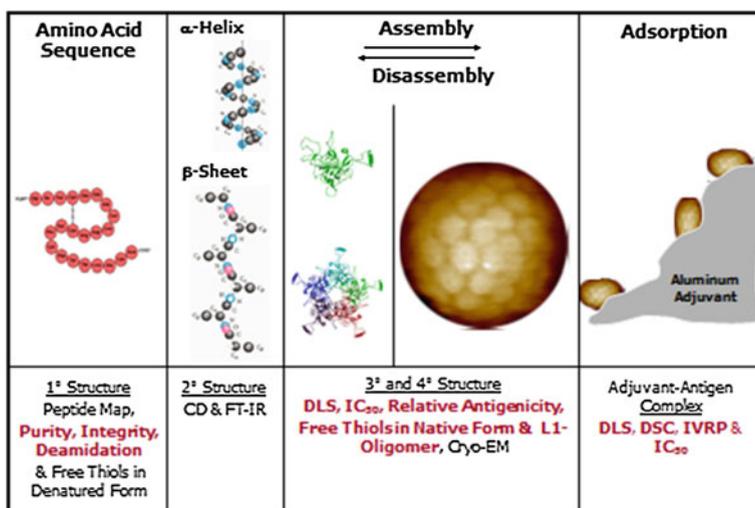

Fig. 3.1 Typical analytical techniques applied to VLP vaccines (Sitrin 2010)

3.2.3 Complexity of Protein Vaccines Versus Small Molecule Drugs

Complexity is the central theme when dealing with the properties of biologics or vaccines. Unlike traditional small molecular weight drugs, where high performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) can fully define structure and function, high molecular weight biological identities display an array of slightly different variations in structures or conformations which cannot fully define the biological aspects of the structure. They have unique signatures, or fingerprints, made up of naturally occurring variations or a complex mixture. These structural variations include heterogeneity in conformation, partial or full modification of N-terminus, disulfide bond pairing, degree or variations in glycosylation or phosphorylation, and different levels of proteolytic clipping of peptide chains occurring during bioprocessing and/or during storage. The complexity reflected by these arrayed variations in structure or conformation may not translate into differences in function. This is why the terms "comparable" or "equivalent" (vs. "same" or "identical") have been used when describing the properties of different batches of products or products made with slightly different processes or at different scales.

3.2.4 What Is Comparability?

For a given product attribute, there are three facets to consider when it comes to defining comparability criteria: process comparability, analytical comparability, and the link of a change to the clinical outcome ("does a change matter?"). Thus, the premise of a comparability exercise is to gauge the future product attributes, when a scale change or process improvement is implemented, using the database (or the fingerprints defined from the past experience) based on the past clinical and commercial manufacturing batches. A weighted approach needs to be adopted, so more meaningful assays, such as the potency assays or in vitro functional binding assays with close links to clinical outcome, can be treated with higher significance. For HBV and HPV vaccines, mouse potency and mAb based in vitro relative potency assays have been extremely important in defining the product efficacy during product development. Orthogonal methods, preferably gauging the bulk property or the "whole sample" (as opposed to a subset of product) properties, are often employed to better assess the products. The recently published A-Vax case study, which explores the use of Quality by Design (QbD) for a VLP vaccine, discusses weighting in terms of a risk assessment and a control strategy that supports comparability studies (PDA 2012).

Better understanding of the structural basis for the functions of VLPs would lead to improved assays to define the specific "fingerprints" and a link to clinical performance. More quantitative analysis and more complete understanding of the

process should lead to more precise control and management of the chemical or biophysical complexity in a vaccine product. This increased knowledge would in turn help to define the design space during process development and process validation to deliver safe and efficacious products.

3.2.5 Special Technology Applied to VLPs

Potency assay: There are unique attributes and tests applied to VLP vaccines that are not generally used to analyze or test recombinant proteins. One of the most critical attributes of any vaccine is that of the potency determination. Classically, in vivo testing (usually mouse or perhaps rat) has been used for release of vaccine products. The Th1 (cell-mediated) response is becoming increasingly important in evaluating vaccine clinical performance, and the mouse potency assay (in vivo immunogenicity) is usually a serology test that only evaluates the Th2 (total polyclonal antibody-mediated) response in the mouse. Consequently, it has been difficult to establish an in vitro antigenicity—in vivo immunogenicity relationship that is also well-correlated with protective immune response in humans. However, because of their unique icosahedral repeat structures with multiple immunological surface epitopes, properly calibrated enzyme linked immunoassay (ELISA) techniques can be used for in vitro potency evaluation and release. These require extensive discussions with regulatory agencies to assure that they are accepted as clinical surrogates of vaccine efficacy.

Sizing analysis: Another unique testing technology not usually applied to recombinant protein products is nanometer scale size determination, either by dynamic light scattering (DLS) or multiangle laser light scattering (MALLS) often in concert with HPLC size exclusion analysis (HPSEC-MALLS) or field flow fractionation (See references in Table 3.1). Size determination of products, using DLS carried out in a routine manner, provides a facile method to track process consistency over time and provides an early warning of potential shifts in performance manifested by changes in size such as aggregation or oligomerization.

Imaging: Finally, because of their large and symmetrical structure, these viral vaccine products are amenable to imaging using Transmission Electron Microscopy (TEM), both in negative stain as well as in a frozen hydrated state (cryoTEM), and by Atomic Force Microscopy (AFM). TEM methods, in combination with modern computational techniques, can also provide three-dimensional visualizations of these vaccines, their interaction with relevant antibodies, and their appearance when adsorbed to adjuvants. In addition, these orthogonal direct visualization methods aid the interpretation of the data generated by the more indirect light scattering methods.

Table 3.1 Techniques used in the size analysis of VLPs with or without an in-line separation process

Method	VLP/observation	Reference
Methods with in-line separation		
High-performance size exclusion chromatography (HPSEC)		
HPSEC with UV or FL detection	HPV/30–60 nm	Mach et al. (2006)
HPSEC with MALLS	HPV/30–60 nm	Li et al. (2007), MacNair (2005)
Analytical ultracentrifugation (AUC)	HPV/VLP	Mach et al. (2006)
Disc centrifugation	HPV/VLP	Deschuyteneer et al. (2010)
Asymmetric field flow fractionation (FFFF or AF4)	VLP	Pease et al. (2009), Mohr et al. (2013)
Electrospray differential mobility analysis (ES-DMA)	Virus/VLP	Guha et al. (2012)
Methods without separation		
Dynamic light scattering (DLS)		Mohr et al. (2013, Sitrin (2010)
Negative-stain TEM		Shi et al. (2007)
CryoTEM	3D reconstruction	Zhao et al. (2014), Mulder et al. (2012)
Protein tomography		Deschuyteneer et al. (2010)
Atomic force microscopy (AFM)		Zhao et al. (2012a), (2012b), Mulder et al. (2012), Zhao et al. (2006)

3.2.6 VLPs Are "Well-Characterized Vaccines"

Based on its design, this recombinant VLP approach yields an entity that could be considered well characterized, as are the therapeutic protein products that are manufactured in an analogous manner. Thus, unlike the situation for traditional vaccines, a comprehensive characterization data base provides an excellent and well-accepted means to carry out future process and manufacturing facility changes without repeating clinical studies. It should be noted that this database should contain significant analytical characterization information above and beyond release data.

3.3 HBV Vaccines

3.3.1 Serum-Derived Vaccine

Hepatitis B vaccine (HBV) products bridge several generations of technology and demonstrate a steady progression of the application of modern techniques to the analysis and characterization of vaccines. The original HBV vaccine, Heptavax®, was licensed by Merck in 1981 (Hilleman 1993). The vaccine antigen, Hepatitis B

surface antigen (HBsAg), was isolated from human carriers and purified by a lengthy and extensive process to yield a safe and effective vaccine adsorbed to an aluminum adjuvant. Most of the analytical testing was designed to assure safety from potential human viruses likely present in the source serum. In fact, the World Health Organization (WHO) document on requirements for release of the plasma-derived HBV vaccine, focuses mostly on safety tests, with expectations for residual HBV viral DNA, purity by SDS-PAGE (>95 %), and a relative mouse potency requirement (WHO 1988; Grachev and Magrath 1993). The dose was set from clinical trials as 20 μg protein determined by UV, micro-Kjedahl, or Lowry. Potency was measured by an in vivo assay in mice assessed by a total polyclonal antibody response to the antigen. Given the available technology at the time, this represented an appropriate panel of tests to characterize this vaccine product.

The Hepatitis B surface antigen, comprised of an approximately 24 kDa molecular weight protein along with smaller amounts of extended sequences ("Pre S1" and "Pre S2") contains very significant hydrophobic sequences, which cause it to take on lipids from the producing (liver) cells and assemble into a 22 nm VLP. Early characterization of the product used SDS-PAGE, amino acid analysis, Edman degradation, HPLC, TEM (22 nm particles), and analytical ultracentrifugation. (Peterson 1981). Figure 3.2 is a negative-stain TEM micrograph of a clinical lot of Heptavax® (Hilleman 1993). Note the regular spherical particles of the antigen in this figure. Lipids, presumably from the host producing cells (phospholipids, cholesterol, and triglycerides), were critical to structure and immunogenicity as the protein was insoluble in the absence of lipids (Gavilanes et al. 1982; Peterson et al. 1982; Gavilanes et al. 1990).
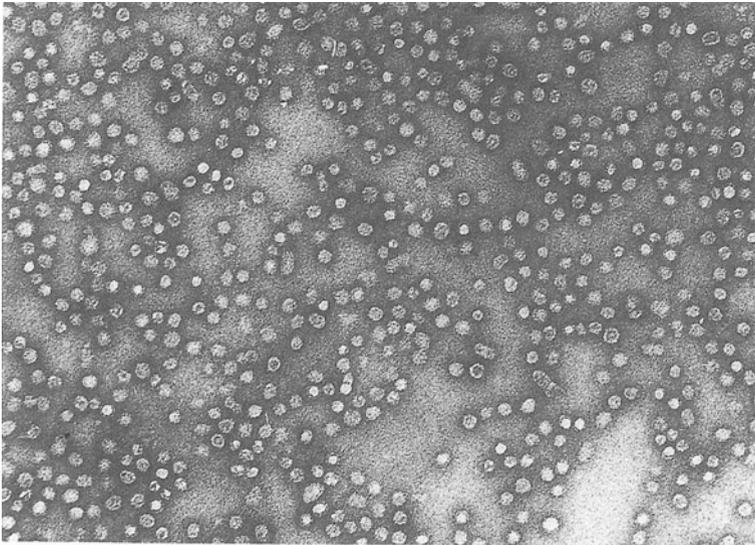

Fig. 3.2 Negative-stain TEM micrograph of Heptavax® (Hilleman 1993). Reprinted with permission

3.3.2 Yeast-Derived Vaccines

Limited supply of source serum and perceived safety issues led to the development of the yeast-derived HBV vaccines developed both by Merck (Recombivax HB[®]) and GSK (Engerix B[®]) (Sitrin et al. 1993). These vaccines, expressed in baker's yeast, *Saccharomyces cerevisiae*, were the first application of modern recombinant DNA technology to commercial products. Although the historical safety tests for Heptavax[®] were no longer required, these new vaccines had to meet all of the newly evolving testing and quality procedures for rDNA products (WHO 1989) with particular focus on purity (SDS-PAGE), acceptable levels of yeast proteins and yeast DNA, as determined by hybridization techniques (<10 ng/dose) (EP 2008; Grachev and Magrath 1993). Although the lipid content changed from human to yeast lipids, the expressed 24 kDa protein was able to assimilate the yeast lipids and make VLPs as effective as the VLP antigen isolated from human carriers. At the time of original licensure, potency was measured by the same technique used for the serum-derived vaccine, a classical ED_{50} determination in mice, using the same specifications (EP 2010b). The product was also tested for sterility, process residuals (formaldehyde or Cesium Chloride), and aluminum (EP 2008) either at the purified bulk or final container stage as appropriate.

3.3.3 Characterization of the Recombinant HBV Vaccines

Both manufacturers applied available technology to characterize their products. Merck reported the use of nonreducing gels to demonstrate the extent of cross-linking of the disulfide containing product into several discrete forms, which were ascribed different levels of antigenicity (Wampler et al. 1985). In GSK publications, the recombinant product was compared to the serum-derived product by EM, amino acid composition, N- and C-terminal data, SDS-PAGE, HPLC, and comparative immunochemistry (Petre et al. 1987; Stephenne 1990). Original data published by GSK (Stephenne 1990) indicated consistent levels of lipid (11–15 mcg per 20 mcg protein), sugars (0.2–0.35 mcg per 20 mcg protein), antigen using a radioimmunoassay called AUSRIA (Abbott_Diagnostics 2012), and protein purity (>98 %) in the bulk product. Further structural studies of the vaccine (Hemling et al. 1988) confirmed 85 % of the sequence by FAB mass spectrometry of proteolytic and CNBr digests. Complete structure confirmation was thwarted by the fact that the protein was 70 % blocked at its N-terminus and contained very hydrophobic sequences making handling of the protein difficult. The lipid-free protein and many of its fragments showed propensity to form water-insoluble aggregates.

3.3.4 Release Strategy and Potency

The doses for the two licensed HBV vaccines are fixed and expressed in terms of protein mass (µg protein as measured by Lowry or the equivalent) (EP 2008; Grachev and Magrath 1993). The protein concentration is measured at a bulk purified stage and this value is used to dilute into the final formulation (adsorption onto aluminum adjuvant). Potency is then measured on the final formulated drug product as a separate measurement from the protein dose. Originally, this potency was determined by an in vivo assay in mice and expressed as an ED_{50} or relative ED_{50} value compared to a standard (Descamps et al. 2011; EP 2010b). An in vitro potency assay based on the Abbott Auszyme Sandwich ELISA kit was later developed to replace the in vivo assay (Descamps et al. 2011). When this kit was discontinued, an in house competitive inhibition ELISA needed to be developed (Descamps et al. 2011). At Merck, an in vitro sandwich ELISA potency test was also developed and validated based on the Abbott Auszyme Sandwich ELISA kit (Schofield 2002). The specifications for Recombivax HB® were based on historical data with Heptavax®. Using extensive concordance data between the two assays (Fig. 3.3), new specifications could be established at the crossover points. This required extensive statistical analysis, given the high variability of the mouse potency assay. Both assays are described in the EP Monograph (EP 2010b).

Both vaccines are formulated on aluminum adjuvant. GSK uses aluminum hydroxide and Merck uses amorphous aluminum hydroxyphosphate sulfate. Final vaccine containers are tested for aluminum, sterility, pyrogens, and potency (EP 2008; Grachev and Magrath 1993).
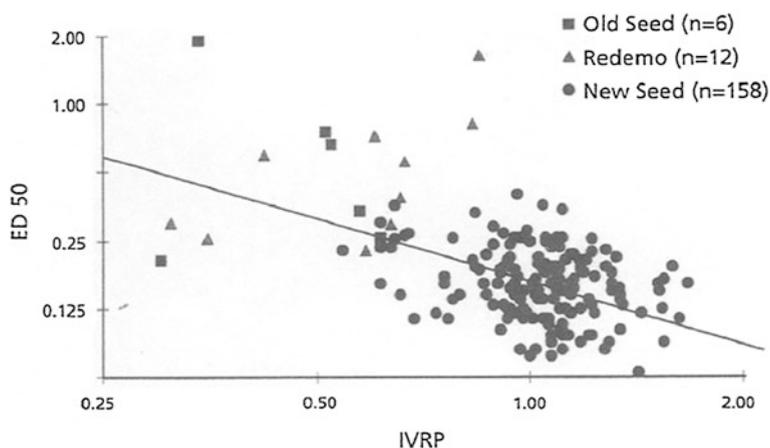

Fig. 3.3 Correlation between mouse potency and in vitro relative potency (IVRP) assays for the recombinant HBV vaccine Recombivax HB®. ED_{50} values are in units of mcg antigen (Schofield 2002). Reprinted with permission

3.3.5 Modern Characterization Methods

Further characterization of the Hepatitis B antigen by more modern techniques has been reported as these have evolved. Using a reversed-phase microisolation procedure and a new matrix 4hcca (4-hydroxy-alpha-cyanocinnamic acid), the sample could be delipidated and the parent molecular ion (25,438 Da) as well as the two major tryptic fragments (13,466 and 11,989 Da could be obtained by MALDI MS (Cohen et al. 2000).

Insights into the maturation of the HBsAg particle and formation during the manufacturing process were observed using atomic force microscopy (AFM) and nonreduced SDS gels (Zhao et al. 2006). Progressive formation of the appropriate disulfide bonds after thiocyanate treatment could be observed as well as changes in particle flexibility. These are demonstrated in the AFM images in Fig. 3.4 where Samples A and B are the antigen before and after thiocyanate treatment. The AFM technique consists of a cantilever with a sharp tip (probe) at its end that is used to scan the specimen surface. When the tip is brought into proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever. These images were obtained in aqueous environments to minimize the deformation of the lipoprotein particles. The lower figures show the expanded region from the
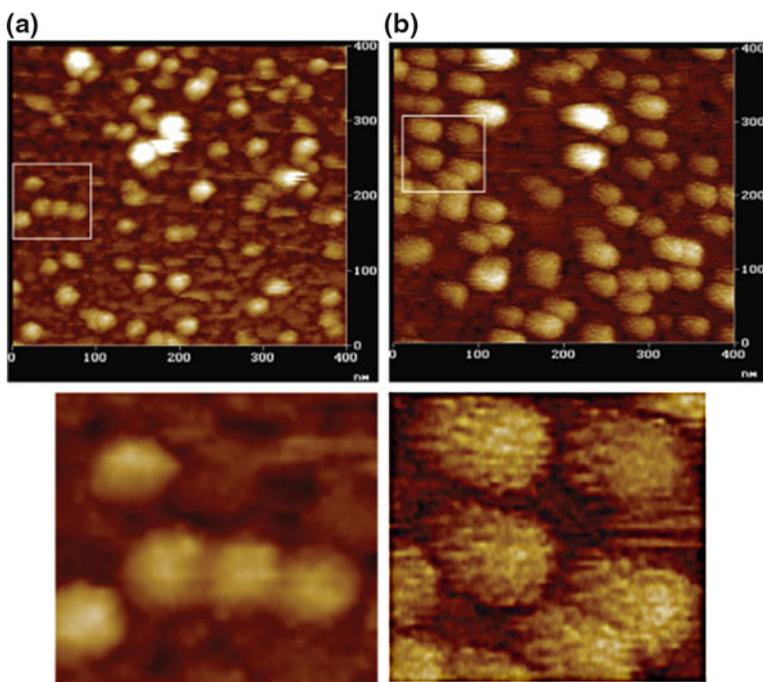

Fig. 3.4 Atomic force microscopy (AFM) images of HBsAg before (**a**) and after (**b**) thiocyanate treatment (Zhao et al. 2006). Reprinted with permission

specified portion of the top figures. Note that the protruded surface features of the HBsAg particles are much better defined after KSCN-induced oxidative maturation with more interchain cross-linking via disulfide bonds (Wampler et al. 1985). Size characterization and monitoring of aggregation during processing steps could be monitored by CD and HPSEC-MALLS (Li et al. 2007).

Further studies on the maturation of the HBsAg particle during manufacture were carried out using immunochemical techniques by surface plasmon resonance (SPR) with monoclonal antibodies known to bind clinically relevant sites on the VLP surface (Zhao et al. 2011a). These tools could also be used to optimize the vaccine antigenicity and immunogenicity using heat treatment or treatment with redox mixtures and to enable rapid process monitoring of manufacturing process intermediates (Zhao et al. 2011b). These modern techniques were summarized as a "Toolbox" for structural and functional characterization of vaccines that includes AFM and CryoTEM studies (Mulder et al. 2012) and so would they fulfill the purpose to demonstrate comparability as mentioned earlier.

3.3.6 CryoTEM Studies

The advantage of CryoTEM is that it provides a very direct means of observing individual particles in a hydrated sample, simultaneously providing information on size, shape, morphology, degree of preservation, and aggregation state. It can also be used to determine the three-dimensional structure of the particles and their interaction with antibodies, and reveal the mode of interaction between the particles and their adjuvants (Fox 2012). In this method, a small drop (3 μL of the sample is placed onto an EM grid covered by a holey carbon support film. The bulk of the sample is removed to leave a thin film, which is then rapidly plunged into a cryogen (e.g., liquid ethane) capable of vitrifying the sample (i.e., freezing it without creating ice crystals). The sample preserved in this vitrified medium is transferred into the electron microscope using a cryogenic stage that maintains the sample temperature below −170 °C. In order to limit damage to the specimen by the electron beam, images are acquired using a very low dose of electrons resulting in images with a low signal-to-noise ratio and limited contrast. The particles are preserved in their natural hydrated state, and thus images provide an accurate description of the morphology of the structures and can be used to extract quantitative metrics such as size distributions and particle fraction counts.

One of the unique advantages of the CryoTEM method is that a three-dimensional map of the structure can be reconstructed by combining particles of the same morphology and conformation but in different relative orientations. The method is generally referred to as "single particle analysis" (described in a recent review (Orlova and Saibil 2011)) and relies on acquiring a large number of images of the randomly oriented particles, which are two-dimensional projections of the three-dimensional object. The individual particles are boxed out of the images and iterative computational methods are used to determine the relative orientation of each

of the two-dimensional projections, which are then backprojected to provide the three-dimensional object. An alternative method, known as electron tomography can also provide three-dimensional reconstructions, although usually at a lower resolution. Analogous to common medical imaging technologies (such as computed tomography), electron tomography acquires a series of images as the sample is rotated in known angular increments about an axis perpendicular to the electron beam. Computational methods are then used to align the images and backproject them to reconstruct the three-dimensional object (Milne et al. 2013).

Yeast-derived HBsAg particles observed by CryoTEM show fairly regular, mostly spherical particles with slightly amorphous boundaries (Fig. 3.5) (Mulder et al. 2012). Further analysis using two-dimensional classification reveals that the VLP surface shows slight protrusions arranged in a regular pattern (Fig. 3.5 inset). Particle-size distribution analysis provides a maximum particle Feret diameter ranging from 18.5 to 22.7 nm (266 particles) and a circularity measure of 0.89 ± 0.03, indicating that the VLP likely deviates from a perfect sphere. Three-dimensional reconstructions show that particles are structured as an empty spherical shell with 24 "knuckle"-like protrusions projecting from a smooth surface and separated by regions of lesser density (Fig. 3.6a, b) (Mulder et al. 2012). The body of each protrusion (presumed to be S-protein) is partially submerged in what is presumed to be lipid monolayer. A region of density corresponding to ~ 18 kDa of protein, protruded into the extracellular space (Fig. 3.6c, d); notably this region of density would be able to accommodate a tetramer of a stretch of amino acid residues (aa 105–156) in the antigenic loop most implicated in infectivity and antigenicity (Le Duff et al. 2009; Salisse and Sureau 2009). Together with previously published results, the analysis of the three-dimensional cryoTEM map

Fig. 3.5 CryoTEM image of HBsAg VLPs. Inset is a two-dimensional class average (Mulder et al. 2012). Reprinted with permission
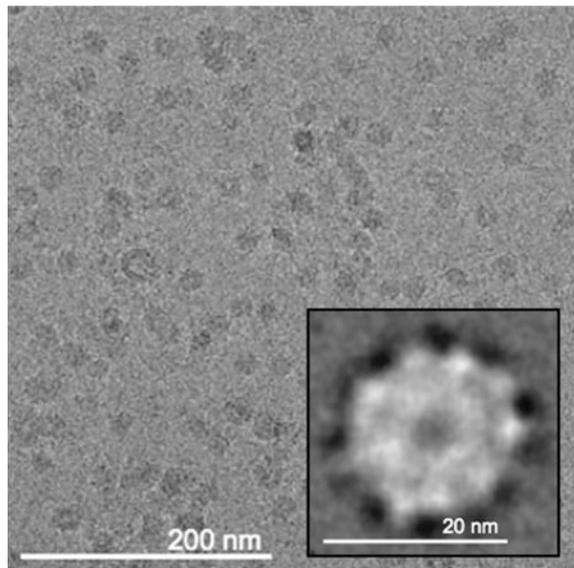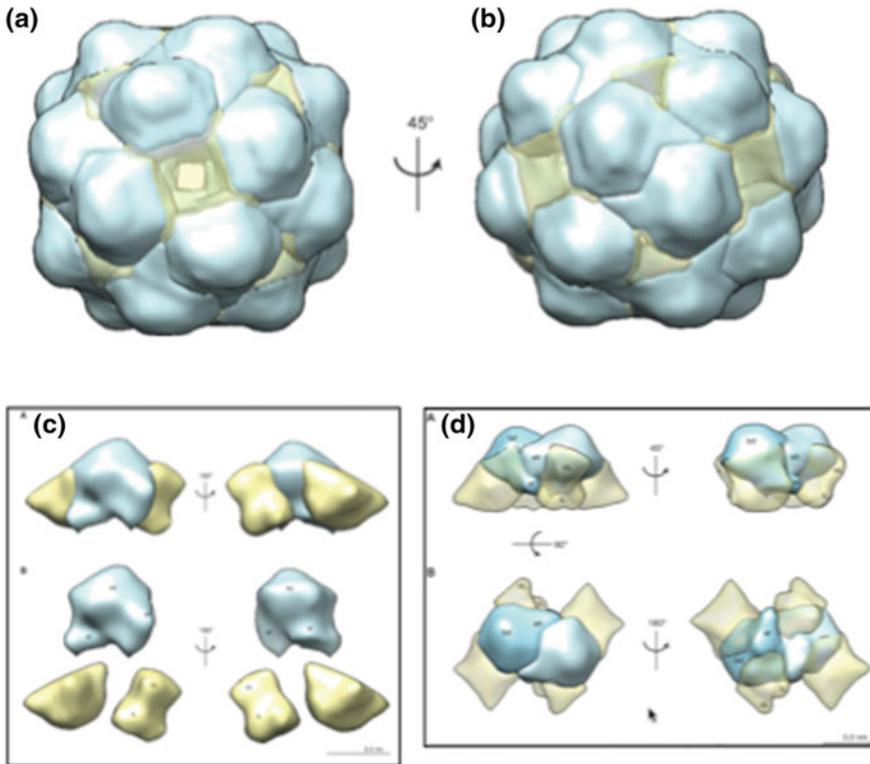
Fig. 3.6 **a/b** CryoTEM map of HBsAg. **c** Structural features of protein containing protrusion and surrounding lipid layer. **d** Arrangement of protein and lipid in the HBsAg particle (Mulder et al. 2012). Reprinted with permission

provides a structural model for the HBsAg vaccine particle that is consistent with previous results regarding the antigenic features, lipid–protein arrangement, and overall particle architecture (Mangold et al. 1995; Stirk et al. 1992; Le Duff et al. 2009; Salisse and Sureau 2009; Gilbert et al. 2005; Greiner et al. 2010; Short et al. 2009).

3.3.7 Closing Comments of HBV

The three decades of HBV vaccine history offer an excellent case study for progressive improvements in technology applied to the same product. Continuous improvement in analytical methods during life cycle management is essential as technologies in the field evolve. The use of these newer analytical techniques provides scientists with new tools to improve processes and production steps and providing additional assurance that a reliable supply of vaccines is maintained.

3.4 HPV Vaccines

3.4.1 History

After over 13 years of development, the U.S. Food and Drug Administration (FDA) approved in 2006 the first preventive HPV vaccine, GARDASIL®, marketed by Merck & Co. (Shi et al. 2007). Glaxo SmithKline gained approval of its HPV vaccine, known as CERVARIX®, in 2007 in Australia, and the European Union, followed by approval in the US in 2009 (Deschuyteneer et al. 2010). HPV vaccines are now licensed in over 100 countries. Since their introduction, the yeast-derived recombinant quadrivalent HPV L1 (Types 6, 11, 16, and 18) vaccine GARDASIL® and the Baculovirus-derived bivalent vaccine (Types 16 and 18) CERVARIX® have played an important role in lowering HPV infection rates in teenage girls (Markowitz et al. 2013) to ultimately reduce incidence of cervical cancer (Dunne and Datta 2008). It is notable that CryoTEM images were used to visualize what the vaccines look like to the general public as evidenced by Fig. 3.7 which was included in a US News and World Report cover story article on the breakthrough launch of anticancer HPV vaccines (Fischman 2006).

For both products, the development strategy was to clone the L1 protein (∼55 kDa) specific for each serotype in a recombinant expression system, either full length L1s in yeast (Merck) or as C-terminal truncated proteins in a Baculovirus expression system (GSK). For all of the HPV serotypes, the L1 protein contains in its structure the ability to assemble to capsomers of five monomers (MW ∼ 300 kDa),
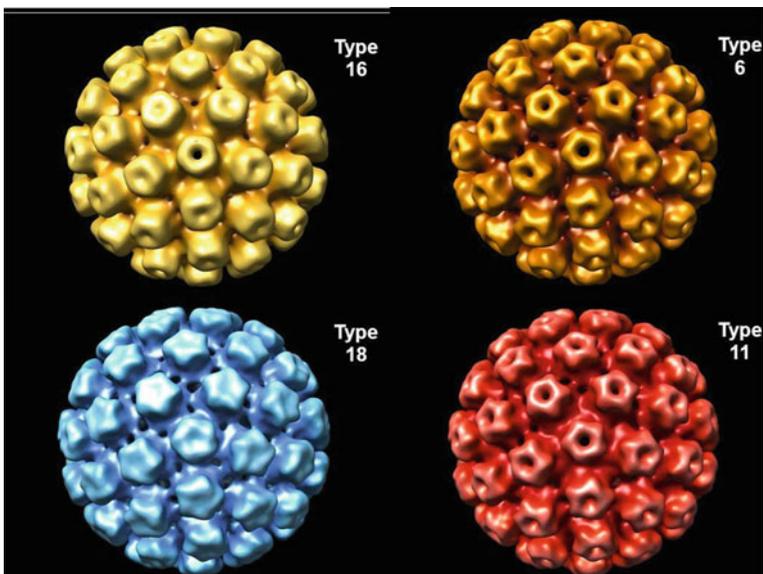

Fig. 3.7 CryoTEM reconstructions of GARDASIL® (Fischman 2006). Reprinted with permission

which themselves assemble into icosahedral structures. The final vaccines contain these VLPs of about 60 nm in size, which mimic the natural viruses in antigenicity and immunogenicity. For both products, the vaccines are purified either as capsomers or VLPs by a variety of chromatographic techniques, with a disassembly/reassembly (D/R) step added at the end for the Merck vaccine process for some types to further enhance the particle morphology, homogeneity, and stability. Both products are formulated on aluminum adjuvant with the GSK product also containing an additional novel adjuvant (ASO4) to enhance its immunogenicity (Deschuyteneer et al. 2010).

3.4.2 Biochemical Analysis

Using established techniques (Fig. 3.1) for recombinant protein characterization, the primary structure of the constituent L1 proteins could be confirmed for both vaccines. Reduced SDS-PAGE was used to confirm the molecular weights and purities (Deschuyteneer et al. 2010). Purified L1 VLPs were analyzed by MALDI-TOF MS on peptide fragments, ISOQUANT® (deamidation), free thiol groups, circular dichroism (CD), and Fourier Transform Infrared (FT-IR) spectroscopy (WHO 2006; CHMP 2006). No evidence has been found of N-linked glycosylation since the observed mass values in SDS-PAGE and in MALDI-TOF MS closely matched the theoretical values (Cohen et al. 1999). GSK published data on peptide mapping of the L1 proteins by tandem LC-MS/MS, N- and C-terminal determinations, Differential Scanning Calorimetry (DSC), TEM (including samples on aluminum adjuvant), CryoTEM, amino acid analysis, disc centrifugation, and relative antigenicity (Deschuyteneer et al. 2010).

3.4.3 Sizing Analysis of VLP-Based Vaccines

Particle size is a key attribute unique to VLP vaccines. A variety of techniques have been applied to determine size and size distribution as shown in Table 3.1. These techniques are particularly helpful to monitor consistency of full-scale manufacturing lots either by DLS for GARDASIL® as shown in Fig. 3.8 (Sitrin 2010) or disc centrifugation for CERVARIX®, as shown in Fig. 3.9 (Deschuyteneer et al. 2010).

3.4.4 Analytical Tools Applied to the Disassembly–Reassembly Process Products

In the GARDASIL® manufacturing process for some of the types, the purified VLP is subjected to a disassembly–reassembly (D/R) step in order to create a more stable and properly assembled particle (Mach et al. 2006). Since this step imparts significant
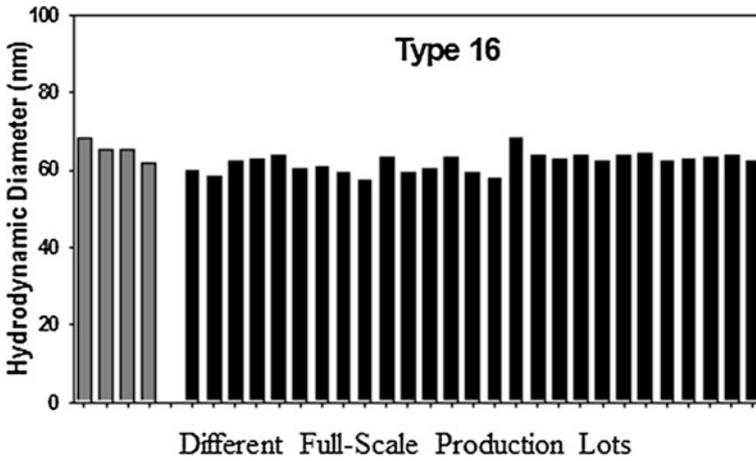
Fig. 3.8 Use of dynamic light scattering (DLS) to monitor consistency of manufacture of VLP vaccine (Sitrin 2010)
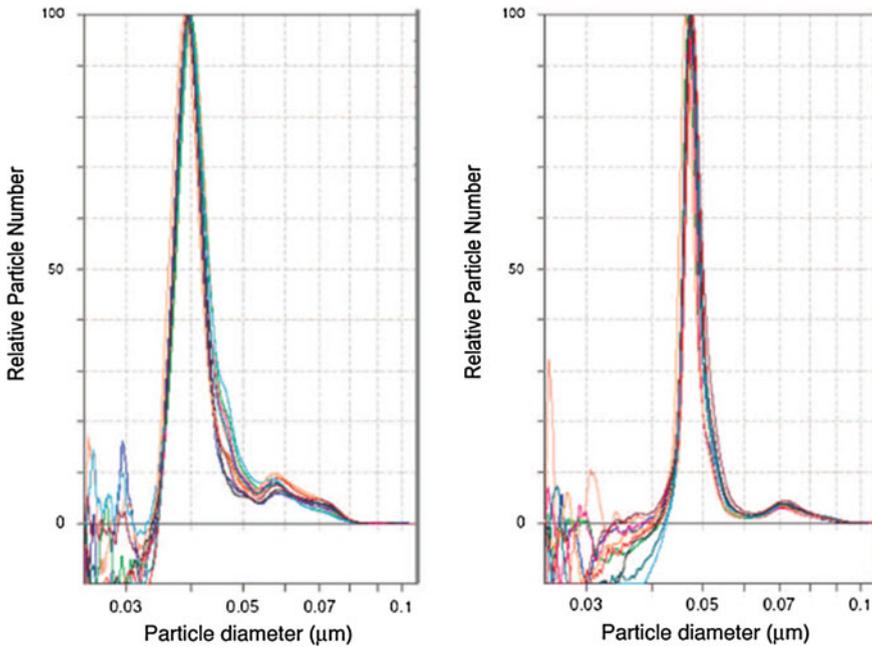

Fig. 3.9 Use of disc centrifugation to monitor consistency of manufacturing of CERVARIX® types 16 (*top*) and 18 (*bottom*) involving ten batches (one color/batch) (Deschuyteneer et al. 2010). Reprinted with permission
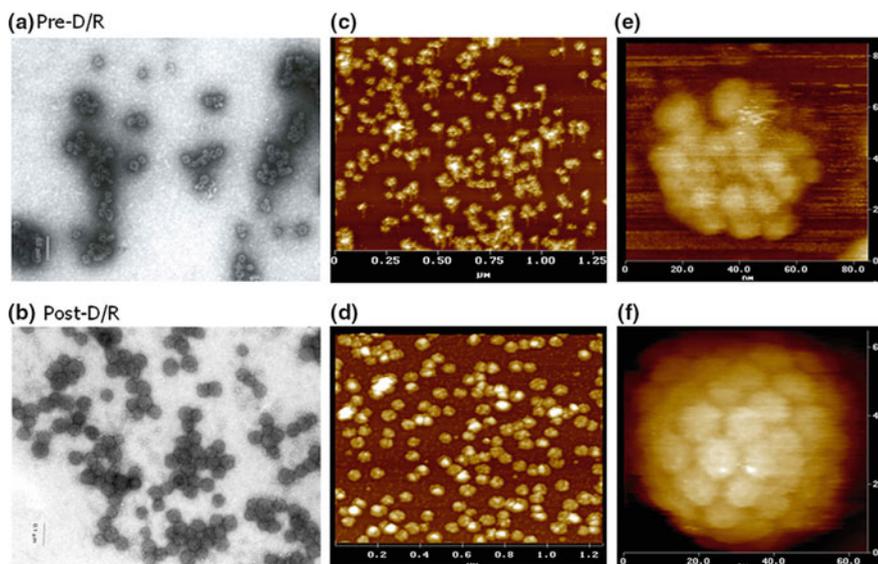
Fig. 3.10 Changes in morphology of VLPs before and after disassembly/reassembly (D/R) as measured by negative-stain TEM (panels **a** & **b**), and atomic force microscopy (AFM) (panels **c**–**f**) (Zhao et al. 2012b). Reprinted with permission

improvements in stability and potency, it is important to determine which characterization tools can discern between VLPs before and after D/R as they would have verified utility for meaningful parameters. A variety of analytical techniques were applied to observe the VLPs before and after reassembly. For example, Fig. 3.10 (Zhao et al. 2012a) shows the differences by negative-stain TEM as well as by AFM. The changes from irregular to well-defined icosahedral structures are particularly evident in the AFM images. These techniques are especially useful to characterize pivotal samples during process development and manufacturing process demonstrations.

The improved stability of the VLPs after D/R was evident on differential scanning calorimetry (DSC) analysis, which measures thermal transitions as a sample is warmed. A significant upward shift in the lower transition temperature from 53° to 57° as well as a significant change in the relative observed peak heights could be observed with four different batches before and after D/R (see Fig. 3.11) (Zhao et al. 2012a). (Shank-Retzlaff et al. 2006). The results of a cloud point study also supported the decreased propensity for post-D/R VLPs to aggregate, as compared to pre-D/R VLPs, during thermal treatment (Mach et al. 2006).
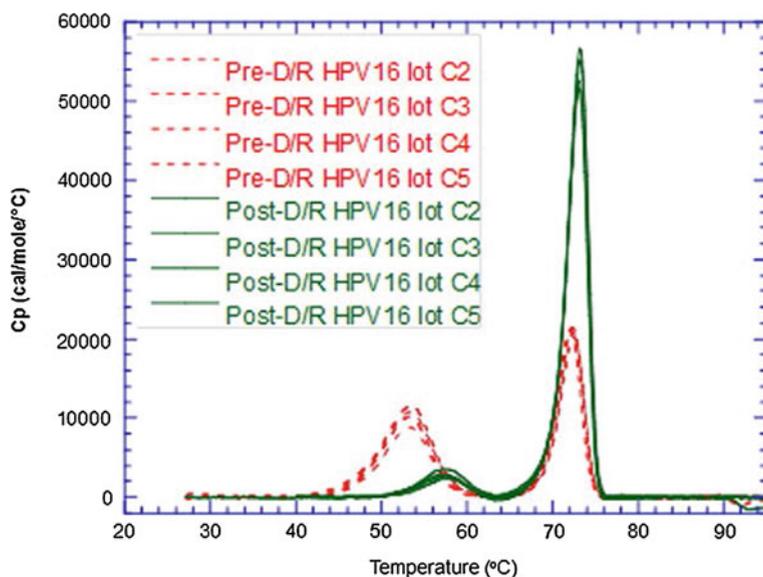
Fig. 3.11 Differential scanning calorimetry (DSC) thermograms of four HPV-16 VLP lots of pre-D/R (traces in *dash lines—red*) and post-D/R treatment (traces in *solid lines—green*). D/R refers to disassembly/reassembly (Zhao et al. 2012a). Reprinted with permission

3.4.5 *Immunochemical Characterization and Antigen Quantitation*

For GARDASIL®, a panel of highly specific monoclonal antibodies (mAbs) against L1 protein were used to characterize the purified recombinant VLPs in a set of various assays, namely, epitope mapping using label-free SPR technology for the relative footprint size/degree of overlap of different mAbs, inhibitory concentration at 50 % response (IC_{50}) by competitive ELISA (Zhao et al. 2012b) and affinity of mAbs to the different epitopes (solution K_D determination) (Towne et al. 2013). The GSK group demonstrated by ELISA that the same protective epitopes were present in their truncated L1 VLPs as in the full-length L1 pseudovirions (Fig. 3.12) (Deschuyteneer et al. 2010) and demonstrated binding of protective antibodies to their truncated VLPs using SPR.

3.4.6 *Immunochemical Analysis of D/R Products*

Epitope mapping was performed on the different HPV types prior to and after D/R using an SPR technique with a panel of mAbs that recognizes conformational or linear epitopes. Along with significant improvements in morphology and stability
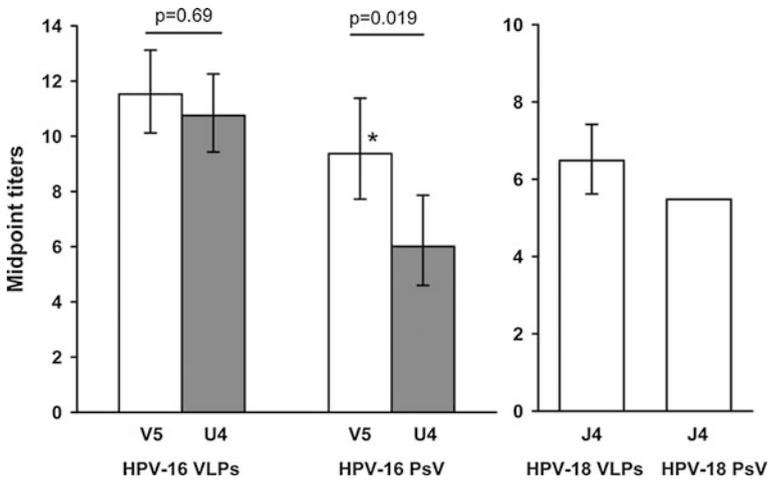
Fig. 3.12 Binding capacity of monoclonal antibodies to truncated L1-derived HPV-16/18 VLPs and HPV-16/18 pseudovirions (PsV), as determined by ELISA (Deschuyteneer et al. 2010). Reprinted with permission

profiles, post-D/R VLPs showed markedly higher antigenicity ($\sim$2 to 3.5 fold) to conformational and neutralizing mAbs, whereas the binding to mAbs recognizing linear epitopes was greatly reduced and for one mAb below the detection threshold (Fig. 3.13a) (Zhao et al. 2012b; Towne et al. 2013). A much more focused epitope map was observed for the postreassembly VLPs indicating the decreased heterogeneity in different VLP forms (VLP to VLP) and within VLP conformational heterogeneities (Fig. 3.13b).

3.4.7 Potency Assay

For any new vaccine product, critical attention is required to developing a suitable potency assay and assuring it correlates with clinical performance. This can be particularly challenging for a new vaccine product where a clinical correlate does not yet exist.

3.4.7.1 Dose Versus Potency

As with the case of the Hepatitis B vaccines described earlier, both commercial HPV vaccines express their doses in terms of protein mass (µg protein as measured by Lowry or the equivalent). The protein concentration is measured at a bulk purified stage and this value is used to dilute into the final formulation (adsorption onto aluminum adjuvant). Relative potency is then measured on the final formulated drug

Fig. 3.13 a Relative immunochemical changes of HPV-16 before and after disassembly/reassembly (D/R). **b** Relative footprint before and after D/R (Zhao et al. 2012b). Reprinted with permission
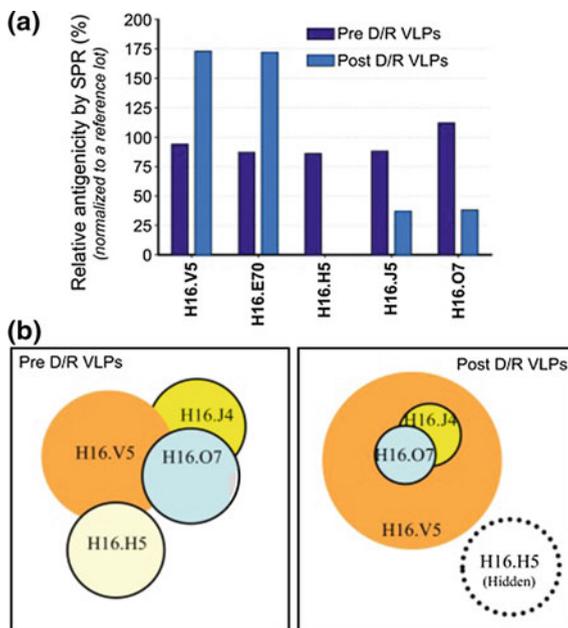
product as a separate measurement from the protein dose (EP 2010a) by comparison to a reference (clinical) lot. Originally, this potency was determined by an in vivo assay in mice and expressed as an ED_{50} or relative ED_{50} value compared to a standard. For GARDASIL®, a unique pair of mAbs for each type was selected to develop a type-specific sandwich ELISA assay for measuring the specific antigenicity of a given VLP preparation in aqueous solution. This ELISA was then reformatted to become the in vitro Relative Potency (IVRP) assay on the (aluminum adjuvant-adsorbed) final vaccine product after dissolution treatment and comparison to a reference standard lot (Shank-Retzlaff et al. 2005). Because four different pairs of mAbs were used, each antigen could be tested for release and stability in the presence of the others in the final container. For CERVARIX®, the original in vivo assay used during program development was also replaced by an in vitro ELISA assay which was very effective for demonstrating long-term stability of the vaccine (Le Tallec et al. 2009).

3.4.7.2 Analyzing Adjuvanted Vaccines

Analyzing antigens on adjuvanted vaccines is not as straightforward as analyzing antigens in solution. The quadrivalent vaccine, GARDASIL®, uses amorphous aluminum hydroxyphosphate sulfate adjuvant, while the bivalent CERVARIX® vaccine uses AS04 (500 μg aluminum hydroxide 50 μg 3-O-deacyl-4'-monophosphoryl lipid A) (Deschuyteneer et al. 2010). Adsorbed VLPs have been analyzed by
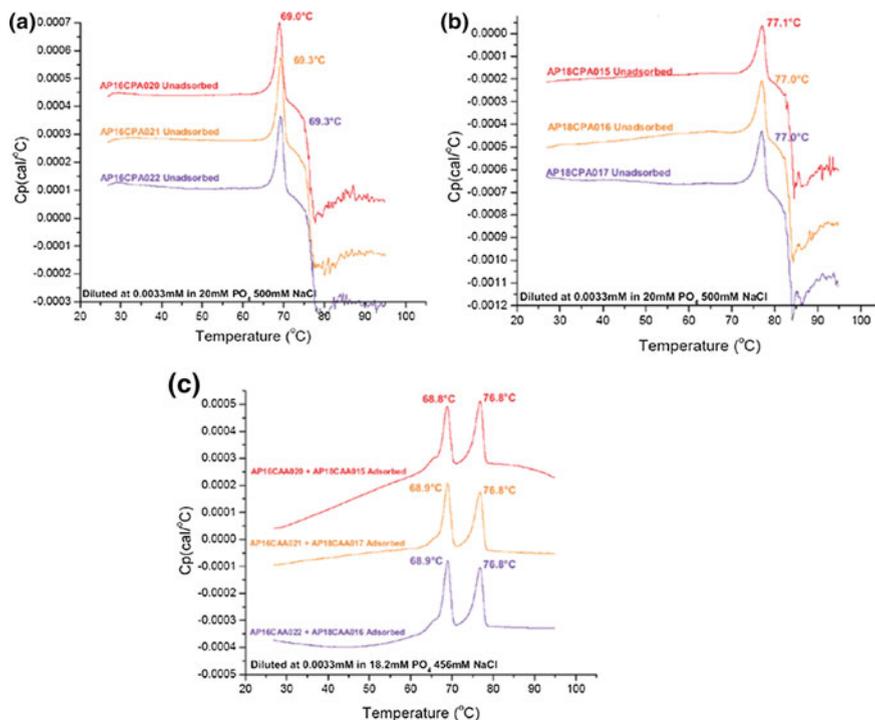
Fig. 3.14 Differential scanning calorimetry (DSC) thermograms of VLPs in solution (**a** and **b**) and of VLPs adsorbed on aluminum hydroxide (**c**). **a** DSC profiles of three different batches of HPV-16 L1 VLPs. **b** DSC profiles of three different batches of HPV-18 L1 VLPs. **c** DSC profiles of three different batches of mixed aluminum-adsorbed HPV-16 and HPV-18 L1 VLPs (Deschuyteneer et al. 2010). Reprinted with permission

mouse potency, IVRP as well as DSC (Deschuyteneer et al. 2010; Shank-Retzlaff et al. 2005). The GSK team used DSC analysis to demonstrate consistency of manufacturing and stability of VLPs when adsorbed to aluminum hydroxide (Fig. 3.14) (Deschuyteneer et al. 2010; Le Tallec et al. 2009). It was concluded that adsorption to the aluminum adjuvant did not result in significant changes in VLPs structure as evidenced by similar morphology and antigenicity pre- and post-adjuvant adsorption. For further discussion on this, see later section for TEM and CryoTEM of VLPs on aluminum adjuvants without pretreatment.

3.4.7.3   Correlation Between in Vivo and in Vitro Assays

For GARDASIL®, the mouse potency (ED_{50}) assay was performed on BALB/c mice in which anti-HPV antibodies were measured using an ELISA-based assay 4 weeks postimmunization. This in vivo potency assay was used to establish the

Fig. 3.15 Comparison of
in vitro (IVRP) and in vivo
(mouse potency) assays for
HPV-16 in GARDASIL®.
ED$_{50}$ values are in units of
mcg antigen **a** plotted by
sample type (non-reassembled
vs reassembled) and **b** by
sample age (months)
(Shank-Retzlaff et al. 2005).
Reprinted with permission
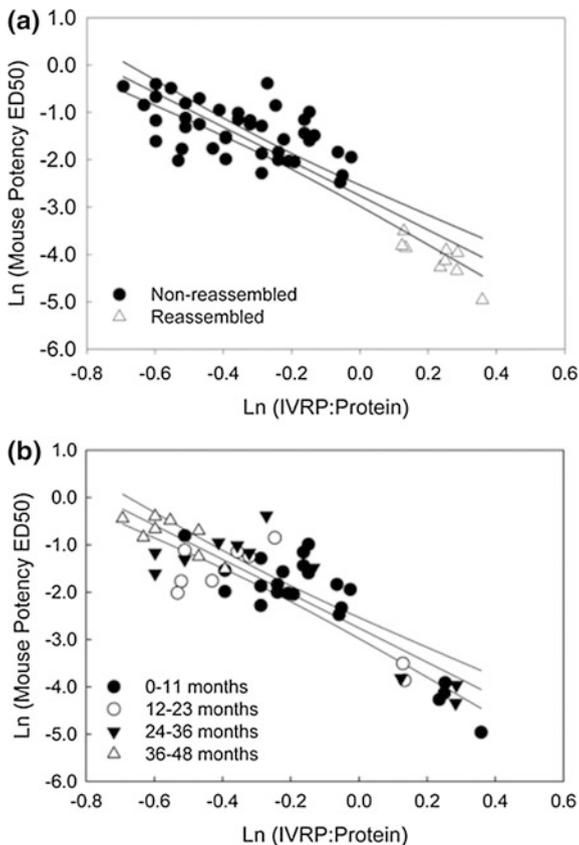
immunogenicity of formulated products in mice. Both IVRP and in vivo mouse
potency assay (ED$_{50}$) were used to release clinical lots and to characterize the
product stability. By the time of licensure, the correlation between the in vivo and
in vitro assays had been demonstrated and the IVRP assay (see Fig. 3.15) was
approved as the release potency assay (Shank-Retzlaff et al. 2005).

3.4.7.4 Setting Specifications

With the correlation between mouse potency and the IVRP assays established, a
novel "quality-by-design" approach was used to set specifications for GARDA-
SIL®. In this study, a statistical evaluation of variability of the ELISA assay and the
formulation/filling process along with stability variability was used to propose
specifications for the ELISA (see Fig. 3.16) that were confirmed with a specially
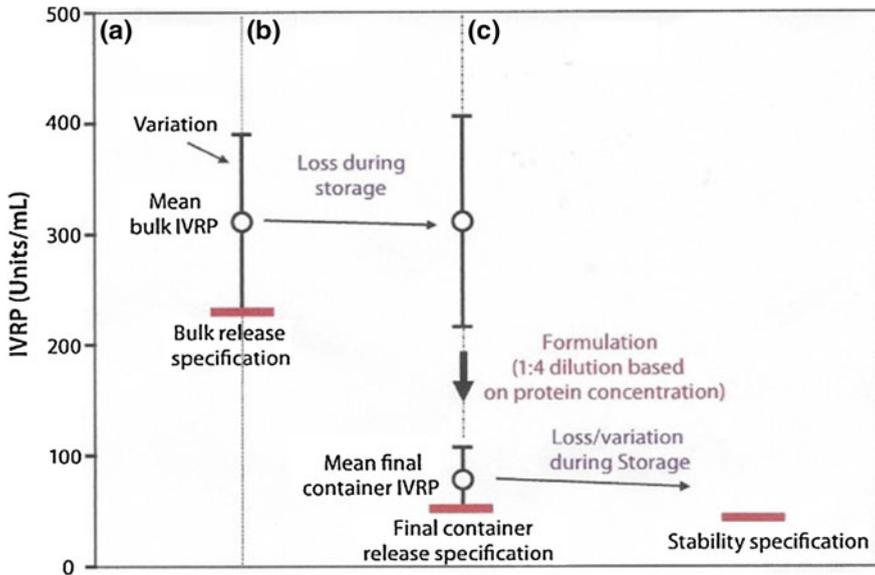designed dose-ranging clinical trial (Capen et al. 2007).

Fig. 3.16 Model used for setting specifications for GARDASIL®. IVRP refers to the in vitro relative potency assay used to measure vaccine potency (Capen et al. 2007). Reprinted with permission

3.4.8 CryoTEM Studies

3.4.8.1 CryoTEM Imaging and Analysis

CryoTEM (see earlier description in Sect. 3.3.6 above) was used to image each of the four GARDASIL® HPV types as shown in Fig. 3.17 (Zhao et al. 2014). The majority of the VLPs appear to be fully assembled, are observed to have a range of sizes, are predominantly spherical or ellipsoidal in shape, with no evidence of filaments or other large aggregates. These observations are similar to those reported using negative-staining EM for the C-terminal truncated VLPs in CERVARIX® (Deschuyteneer et al. 2010).

A set of particles of similar diameter (54 ± 3 nm) were selected from images of VLPs of type 11 and type 16 and single particle analysis methods were used to reconstruct three-dimensional maps of these serotypes as shown in Fig. 3.18a, c (Zhao et al. 2014).

3.4.8.2 VLPs Interacting with Fabs

CryoTEM and single-particle 3D reconstruction was also applied to VLPs interacting with monoclonal antibodies (mAbs), which were known to be protective and
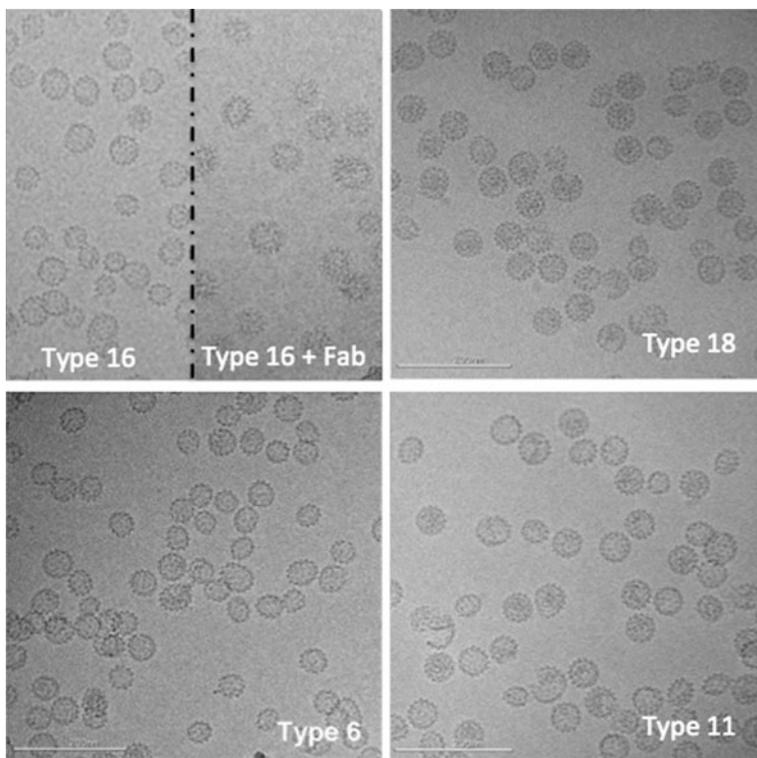
Fig. 3.17 CryoTEM images of VLPs in GARDASIL® (Zhao et al. 2014). Reprinted with permission

which had been used in the potency assay. To avoid cross particle binding, the mAbs were digested and then purified into their respective Fabs. After three-dimensional reconstruction and difference mapping, VLPs can be observed directly interacting with antibody fragments (see Fig. 3.17 inset). Three-dimensional volumes of decorated particles for two different serotypes were reconstructed using methods as described above in Sect. 3.3.6. The antigenic site of interaction is quite different for the two serotypes and their two individual antibodies; for Type 11, the antigenic site is located at the center of the capsomer (Fig. 3.18b), whereas for the Type 16 serotype, the antigenic site for this antibody is located off to the side at the outer edge of the capsomers (Fig. 3.18d) (Zhao et al. 2014).

3.4.8.3 VLPs Interacting with Alum Adjuvant

Images of the VLPs adsorbed to aluminum adjuvant were obtained using CryoTEM (Fig. 3.19) for GARDASIL® and using negative-staining TEM (Fig. 3.20) for CERVARIX®. In both cases, intact VLPs can be observed attached to the adjuvant
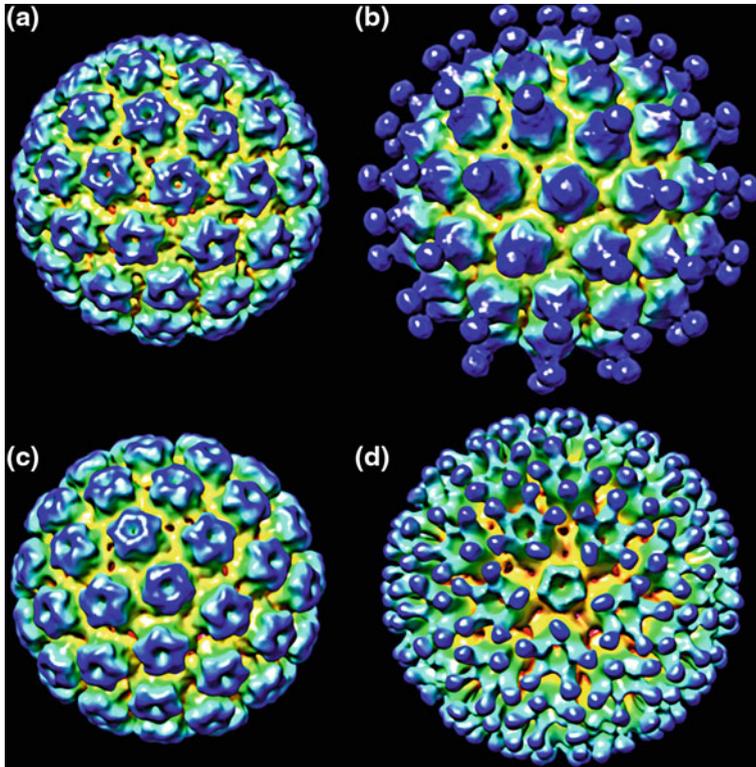
Fig. 3.18 Three-dimensional reconstructions of **a** HPV-11; **b** HPV-11 decorated with antibody fragment H11.B2; **c** HPV-16; and **d** HPV-16 decorated with antibody fragment H16.V5 (Zhao et al. 2014). Reprinted with permission

particles. For GARDASIL®, tomographic methods were used to obtain three-dimensional reconstructions of the structures to confirm that the morphology of the individual is unchanged on binding to the adjuvant (Murphy and Jensen 2007).

3.4.9 Comparability Testing for Life Cycle Management

Extensive characterization provides a database to confirm whether future process changes yield a comparable product. It is critical to determine which tests confirm important similarities or differences. For the GARDASIL® VLP, notable changes could be observed before and after disassembly/reassembly for the TEM, Cryo-TEM, AFM, DLS, HPSEC, DSC, and stability profiles as well as the relative response of various surface epitopes. Since improved antigenicity, stability, mouse potency, and clinical performance were also improved after the D/R step, the utility of these assays for future comparability studies was verified.
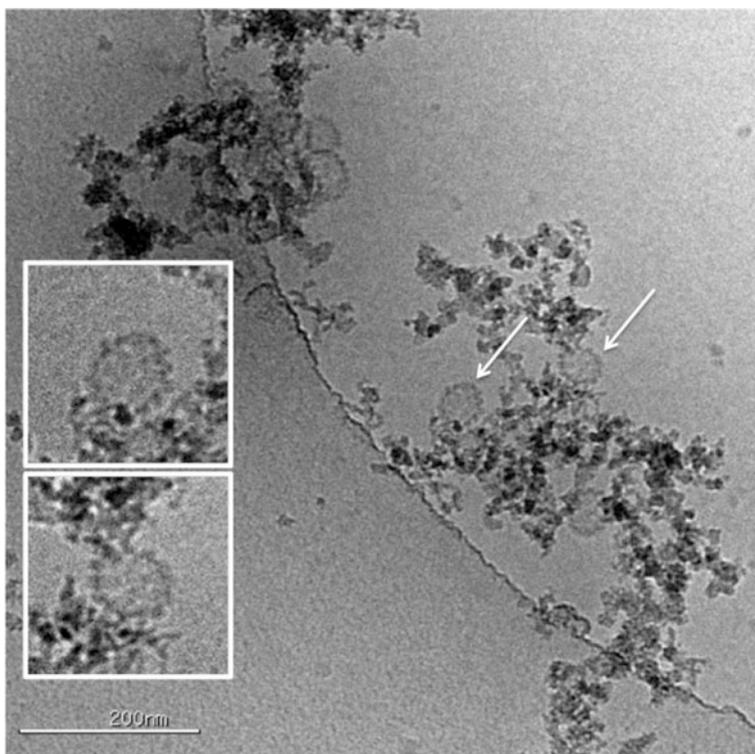
Fig. 3.19 HPV VLPs can be observed directly interacting with the alum adjuvant. The VLPs indicated by the *arrows* are shown at larger scale in the insets indicating that the VLPs are intact and retain their morphology and *spherical shape* upon absorption onto the adjuvant (Zhao et al. 2014). Reprinted with permission
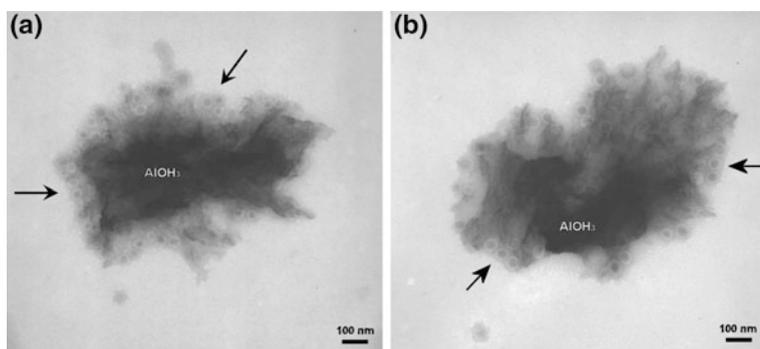

Fig. 3.20 Negative-stain TEM pictures of CERVARIX®, showing that HPV-16 (**a**) and -18 (**b**) L1 VLPs (*arrows*) retain their structure when adsorbed onto aluminum hydroxide (Deschuyteneer et al. 2010). Reprinted with permission

3.5 HEV VLP Vaccine

Recently, a recombinant VLP-based vaccine against Hepatitis E virus infection, Hecolin®, was licensed and launched in China (Zhao et al. 2013a) after demonstration of vaccine safety and efficacy with over 115,000 volunteers enrolled in a large-scale Phase III trial (Zhu et al. 2010). The key antigenic determinants reside on the viral capsid comprised of a single protein encoded by ORF2. Conformation epitopes are essential to mimic the virion surface for desired antigenicity and immunogenicity of the vaccine antigen as evidenced by the antigen-binding activity to a panel of neutralizing mAbs (Zhang et al. 2005; Li et al. 2009). Analytical centrifugation (AUC) was used extensively to assess the particle assembly of the truncated version of the viral capsid protein as a vaccine antigen (Yang et al. 2013).

3.6 Newer VLP-Based Vaccines in Clinical Development

Given the success of the recombinant VLP approach to develop antiviral vaccines for HBV, HPV, HEV and influenza, this approach is likely to be applied to other viral vaccines where direct cell culture techniques are problematic. We will discuss here two such potential products which have advanced into the clinic and meet potential needs which otherwise could not be targeted by classical vaccine methods.

3.6.1 Chikungunya (CHIKV)

Chikungunya (CHIKV) is an arthropod-borne alphavirus (family *Togaviridae*). The alphaviruses are small enveloped positive-strand RNA viruses of 65–70 nm in diameter. This arboviral disease has been epidemic in Africa and parts of Asia with transmission occurring through *multiple* mosquito species. As of 2010 as reported by the Centers for Disease Control and Protection, more than 35 countries have documented cases of CHIKV infection excluding those countries where only imported cases have been documented (Ross 1956).

At present, there are no licensed vaccines or approved antiviral therapy currently available for the prevention or treatment of CHIKV infection. However, the US Department of Defense previously studied a live, attenuated vaccine for the prevention of chikungunya disease (Eckels et al. 1970). In that study, 98 % of vaccinees developed neutralizing antibodies after a single subcutaneous immunization, confirming feasibility for a vaccine for chikungunya disease. The Vaccine Research Center at the National Institute of Allergy and Infectious Diseases has developed a noninfectious VLP technology to induce robust immune responses for the prevention

of chikungunya infection (Akahata and Nabe 2012). The CHIKV VLPs developed by the NIAID contain the E1 and E2 glycoproteins that express the major antigen determinants and the capsid protein. The VLPs are produced by transient transfection of an HEK 293 cell line variant with a DNA plasmid encoding the structural genes of the CHIKV (capsid, E3, E2, 6K and E1, although the 6K and E3 proteins have not been specifically detected in the VLPs). The enveloped VLPs self-assemble and are released into the culture medium as approximately 65 nm particles. This vaccine entered clinical study under IND at the NIH Clinical Center through the VRC Clinical Trials Core.

3.6.2 Norovirus

Norovirus infection, more commonly known as the "winter vomiting disease", is the most common cause of nonbacterial acute gastroenteritis in the U.S., estimated by the CDC to afflict 23 million people per year (Mead et al. 1999). Recent studies from the CDC underscore the potential of norovirus infection to lead to severe complications, especially in children and vulnerable elderly individuals. A systematic review of studies that employed sensitive molecular assays for diagnosis revealed that norovirus causes an estimated, annual 64,000 hospitalizations and 900,000 clinical visits among children in industrialized nations and up to 200,000 deaths of children <5 years of age in developing countries (Patel et al. 2008).

Norovirus infection usually presents as acute-onset vomiting, watery nonbloody diarrhea with abdominal cramps, nausea, and fever. Dehydration is the most common complication, especially among the very young, elderly, and immunocompromised populations and frequently requires medical intervention including hospitalization. Such outbreaks are also a concern for the military where they interfere with combat readiness. Outbreaks are now often seen in special circumstances such as aboard large vessels or cruise ships.

Takeda Vaccines Inc. is developing a vaccine for the prevention of acute gastroenteritis caused by norovirus. The vaccine, Norovirus GI.1/GII.4 VLP (Norovirus Bivalent VLP) vaccine, consists of norovirus virus-like particles (VLP) formulated with aluminum hydroxide and monophosphoryl lipid A (MPL) adjuvants. The two VLPs in the vaccine formulation are a Norwalk virus VLP from genogroup GI.1 and a norovirus VLP consensus sequence from genogroup GII.4. The norovirus consensus VLP is a construct representing a consensus sequence from several norovirus GII.4 strains. The two VLPs in the vaccine are intended to provide broad protection against norovirus infections. The VLPs are produced in a baculovirus/insect cell expression system. A stable recombinant baculovirus encoding the norovirus VP-1 subunit is prepared in adherent Sf9 insect cells.

3.7 VLPs are Well-Characterized Vaccines

Based on the above discussed analytical methods, recombinant HPV VLP-based vaccines can be classified as "well-characterized vaccines," thus making it possible to use analytical characterization data (in addition to routine release data) to implement, after regulatory approval, a process change or scale-up without requiring a new clinical trial. It is important to have a comprehensive plan for comparability testing to support postlicensure scale-up, process improvement, and facility changes. A database is needed to capture the data based on the lots manufactured during process development, and particularly on the lots that had been used in the clinical trials. A weighted approach should be employed when analyzing the data, for instance, the potency assay or in vitro antigenicity analysis should carry more weight than a parameter such as VLP size. Maintaining a dynamic database on the process experience, on pre- and post-licensure lots, is critically important for the life cycle management of a marketed vaccine. Clearly, as new methods for characterizing proteins evolve, they should be applied to VLP vaccines. It would be critical to hold retains of pivotal production lots for this purpose.

References

Abbott_Diagnostics (2012) 40 years of hepatitis leadership. http://international.abbottdiagnostics.com/About_Us/Hepatitis_Leadership/

Akahata W, Nabe LG (2012) A specific domain of the Chikungunya virus E2 protein regulates particle formation in human cells: implications for alphavirus vaccine design. J Virol 86:8879–8883

Capen R, Shank-Retzlaff M, Sings H, Esser M, Sattler C, Washabaugh M et al. (2007) Establishing potency specifications for antigen vaccines. BioProcess Int 5:30–42

CHMP (2006) European medicines agency final report GARDASIL scientific discussion. EMEA

Cohen S, Ward G, Tsai P (1999) MALDI-MS characterization of human papillomavirus protein. Proceedings of the 47th American society for mass spectrometry conference, Dallas, TX, pp 13–17

Cohen S, Ward G, Oswald B, Tsai P (2000) A novel approach to analyze membrane proteins and peptides by mass spectrometry. Proceedings of the 48th American society for mass spectrometry conference, Long Beach, CA

Cox M (2011) A fast track influenza virus vaccine produced in insect cells. J Invertebr Pathol 107:531–541

Cox M (2012) Recombinant protein vaccines produced in insect cells. Vaccine 30:1759–1766

Descamps J, Giffroy D, Remy E, Mortiaux F, Mareschal J-C, Ponsar C et al. (2011) A case study of development, validation and acceptance of a non-animal method for assessing human vaccine potency. Procedia Vaccinol 5:184–191

Deschuyteneer M, Elouahabi A, Plainchamp D, Plisnier M, Soete D, Corazza Y et al (2010) Molecular and structural characterization of the L1 virus-like particles that are used as vaccine

antigens in Cervarix, the ASO4-adjuvanted HPV-16 and -18 cervical cancer vaccine. Human Vaccines 6:407–419

Dunne E, Datta S (2008) A review of prophylactic human papillomavirus vaccines: recommendations and monitoring in the US. Cancer 113:2995–3003

Eckels K, Harrison V, Hetrick F (1970) Chikungunya virus vaccine prepared by tween-ether extraction. Appl Microbiol 19:321–325

EP (2008) Hepatitis B vaccine (rDNA) 01/2008:1056. In: EP, European Pharmacopea, EDQM

EP (2010a) Human papillomavirus vaccine (rDNA) 01/2010:2441. In: European Pharmacopeia, EDQM

EP (2010b) Assay of hepatitis B vaccine 01/2008:20715. In: European Pharmacopea, EDQM

FDA (2013) http://www.flublok.com/pscp2.pdf

Fischman J (2006) Sticking it to cancer. US news and world report

Fox C (2012) Characterization of TLR4 agonist effects on alhydrogel(R) sedimentation: a novel application of laser scattering optical profiling. J Pharm Sci 101:4357–4364

Gavilanes F, Gonzalez-Ros JM, Peterson DL (1982) Structure of hepatitis B surface antigen. J Biol Chem 257:7770–7777

Gavilanes F, Gomez-Gutierrez J, Miguel Gonzalez-Pos J, Ferragut J, Guerrero E et al (1990) Hepatitis B surface antigen role of lipids in maintaining the structural and antigenic properties of protein components. Biochem J 265:857–864

Gilbert R, Beales L, Blond D, Simon M, Lin B, Chisari F et al. (2005) Hepatitis B small surface antigen particles are octahedral. Proc Natl Acad Sci USA 102:14783–14788

Grachev VP, Magrath DI (1993) Quality control of hepatitis B vaccine. In: Ellis RW (ed) Hepatitis B vaccines in clinical practice. Dekker, New York, pp 103–121

Greiner V, Egele C, Oncul S, Ronzon F, Manin C, Klymchenko A et al (2010) Characterization of the lipid and protein organization in HBsAg viral particles by steady-state and time-resolved fluorescence spectroscopy. Biochimie 92:994–1002

Guha S, Li M, Tarlov MJ, Zachariah MR (2012) Electrospray–differential mobility analysis of bionanoparticles. Trends Biotechnol 291–300

Harrison S (1990) Fields virology. In: Fields virology, vol 2. Raven, New York, pp 37–61

Hemling ME, Carr SA, Capiau C, Petre J (1988) Structural characterization of recombinant hepatitis B surface antigen protein by mass spectrometry. Biochemistry 27:699–705

Hilleman M (1993) Plasma-derived hepatitis B vaccine: a breakthrough in preventive medicine. In Ellis RW (ed) Hepatitis B vaccines in clinical practice. Dekker, New York, pp 17–39

Le Duff Y, Blanchet M, Sureau C (2009) The pre-S1 and antigenic loop infectivity determinants of the hepatitis B virus envelope proteins are functionally independent. J Virol 3:12443–12451

Le Tallec D, Doucet D, Ekouahabii P, Deschuyteneer M, Deschamps M (2009) Cervarix, The GSK HPV-16/HPV-18 AS04-adjuvanted cervical cancer vaccine, demonstrates stability upon lon-term storage and under simulated cold chain break conditions. Human Vaccines 5:467–474

Li Y, Bi J, Zhao W, Huang Y, Sun L, Zeng A-P et al (2007) Characterization of the large size aggregation of hepatitis B virus surface antigen (HBsAg) formed in ultrafiltraion process. Process Biochem 42:315–319

Li S, Tang X, Seetharaman J, Yang C, Gu Y et al. (2009) Dimerization of hepatitis E virus capsid protein E2 s domain is essential for virus–host interaction. PLoS Pathog 5(8):e1000537

Mach H, Volkin D, Troutman R, Wang B, Luo Z, Jansen K et al (2006) Disassembly and reassembly of yeast derived recombinant human papillomavirus-like particles (HPV VLPs). J Pharm Sci 95:2195–2206

MacNair JEDT (2005) Alignment of absolute and relative molecular size specifications for a polyvalent pneumococcal polysaccharide vaccine (PNEUMOVAX 23). Biologicals 33:49–58

Mangold C, Unckell F, Wer RM, Streeck R (1995) Secretion and antigenicity of hepatitis B virus small envelope proteins lacking cysteines in the major antigenic region. Virology 211:535–543

Markowitz L, Hariri S, Lin C, Dunne E, Steinau M, McQuillan G 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

Mead P, Slutsker L, Dietz V, McCaig L, Bresee J, Shapiro C et al (1999) Food-related illness and death in the United States. Emerg Infect Dis 607–625

Milne J, Borgnia M, Bartesaghi A, Tran E, Earl L, Schauder D et al (2013) Cryo-electron microscopy–a primer for the non-microscopist. FEBS J 280:28–45

Mohr J, Chuan Y, Wu Y, Lua L, Middelberg A (2013) Virus-like particle formulation optimization by miniaturized high-throughput screening. Methods 60:248–256

Mulder A, Carragher B, Towne V, Meng YW, Dieter L, Potter C 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

Murphy G, Jensen G (2007) Electron cryotomography. Biotechniques 43:413

Orlova E, Saibil H (2011) Structural analysis of macromolecular assemblies by electron microscopy. Chem Rev 111:7710–7748

Patel M, Widdowson M, Glass R, Akazawa K, Vinje J, Parashar U (2008) Systematic literature review of role of noroviruses in sporadic gastroenteritis. Emerg Infect Dis 2008:1224–1231

PDA (2012) A-VAX: applying quality by design to vaccines. http://www.ispe.org/2013-biotechconference/a-vax-applying-qbd-to-vaccines.pdf

Pease LF, Lipin D, Tsai D-H, Zachariah M, Lua L, Tarlov M 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

Peterson DL (1981) Isolation and characterization of the major protein and glycoprotein of hepatitis B surface antigen. J Biol Chem 256:6975–6983

Peterson DL, Nath N, Gavilanes F (1982) Structure of hepatitis B surface antigen correlation of subtype with amno acid sequence and locatio of the carbohydrate moeity. J Biol Chem 257:10414–10420

Petre J, Van Wijnendaele F, De Neys B, Conrath K, Van Opstal O, Hauser P et al (1987) Development of a hepatitis B vaccine from transformed yeast cells. Postgrad Med J 63(Suppl 2):73–81

Ross R (1956) The Newala epidemic. III. The virus: isolation, pathogenic properties and relationship to the epidemic. J Hyg 54:177–191

Salisse J, Sureau C (2009) A function essential to viral entry underlies the hepatitis B virus a determinant. J Virol 83:9321–9328

Schofield T (2002) In vito versus in vivo concordance: a case study of the replacement of an animal potency test with an immunochemical assay. In: Karger BF (ed) Advancing science and elimination of the use of laboratory animals for development and control of vaccines and hormones. Karger, Basel, pp 299–304

Shank-Retzlaff M, Wang E, Morley T, Anderson C, Hamm M, Brown 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. Human Vaccines 1:191–197

Shank-Retzlaff M, Zhao Q, Anderson C, Hamm M, High K, Nguyen M et al (2006) Evaluation of the thermal stability of gardasil. Human Vaccines 2:147–154

Shi L, Sings H, Bryan J, Wang B, Wang Y, Mach H et al (2007) GARDASIL: prophylactic human papillomarvirus vaccine development—from bench top to bed-side. Clin Phamacol Ther 81:259–264

Short J, Chen S, Roseman A, Butler P, Crowther RA (2009) Structure of hepatitis B surface antigen from subviral tubes determined by electron cryomicroscopy. J Mol Biol 390:135–141

Sitrin R (2010) After the license approval: how analytics can keep you in the market. In: Vaccine technology III, Nuevo Vallarta, Mexico: engineering conferences international, http://dc.engconfintl.org/cgi/viewcontent.cgi?article=1028&context=vaccine_iii

Sitrin RD, Wampler DE, Ellis RW (1993) Survey of licensed hepatitis B vaccines and their production processes. In: Ellis RW (ed) Hepatitis B vaccines in clinical practice. Dekker, New York, pp 83–102

Stephenne J (1990) Development and production aspects of a recombinant yeast-derived hepatitis B vaccine. Vaccine 8 suppl:S69-S73

Stirk H, Thornton J, Howard C (1992) A topological model for hepatitis B surface antigen. Intervirology 33:148–158

Towne V, Zhao Q, Brown M, Finnefrock A (2013) Pairwise antibody footprinting using suface plasmon resonance technology to characterize human papilloamavirus type 16 virus like particles with direct anti-HPV antibody immobilization. J Immunol Methods 388:1–7

Wampler DE, Lehman ED, Bodger J, McAleer WL, Scolnick EM (1985) Multiple chemical forms of hepatitis B surface antigen produced in yeast. Proc Nat Acad Sci 82:6830–6834

WHO (1988) Requirements for hepatitis B vaccine prepared from plasma. Requirements for biological substances 31. World health organization technical report series 771; annex 8, pp 181 = -207

WHO (1989) Requirements for hepatitis B vaccines made by recombinant DNA techniques. WHO, Requirements for biological substances no 45. World health organization, technical report series, no 786, pp 38–70

WHO (2006) Guidelines to assure the quality, safety and efficacy of recombinant papillomavirus virus-like particle vaccines. Expert comitttee on biological standardization WHO/BS/06.2050

Yang C, Pan H, Wei M, Zhang X, Wang N, Gu Y et al (2013) Hepatitis E virus capsid protein assembles in 4M urea in the presence of salts. Protein Sci 22:314–326

Zhang J, Gu S, Li S, He Z, Huang G, Zhuang H et al (2005) Analysis of hepatitis E virus neutralization sites using monoclonal antibodies directed against a virus capsid protein. Vaccine 23:2881–2892

Zhao Q, Wang Y, Freed D, Fu T-M, Gimenez J, Sitrin R et al (2006) Maturation of recombinant hepatitis B surface antigen particles. Human Vaccines 2:174–180

Zhao Q, Towne V, Brown M, Wang Y, Abraham D, Oswald CB et al (2011a) In-depth process understanding of RECOMBIVAX HB maturation and potential epitope improvements with redox treatment: multifaceted biochemical and immunochemical characterization. Vaccine 29:7936–7941

Zhao Q, Wang Y, Abraham D, Towne V, Kennedy R, Sitrin R (2011b) Real time monitoring aof antigenicity development of HBsAg virus like particles (VLPs) during heat- and reox-treatment. Biochem Biophys Res Commun 408:447–453

Zhao Q, Allen M, Wang Y, Wang B, Wang N, Shi L et al (2012a) Disassembly and reassembly improves morphology and thermal stability of human papillomavirus type 16 virus like particles. Nanomed Technol Biol Med 8:1182–1189

Zhao Q, Modis Y, High K, Towne V, Meng Y, Alexandrof J et al (2012b) Disassembly and reassembly of human papillomavirus virus like particle produces more virion like antibody activity. Virol J 9:1–13

Zhao Q, Jun Z, Jun Wu T, Li S-W, Ng M-H et al (2013a) Antigenic determinants of hepatitis E virus and vaccine-induced immunogenicity and efficacy. J Gastroenterol 48:159–168

Zhao Q, Li S, Yu H, Xia N, Modis Y (2013b) Virus-like particle-based human vaccines: quality assessment based on structural and functinal properties. Trends Biotechnnol 31:654–663

Zhao Q, Potter C, Carragher B, Alexandroff J, Towne V, Abraham D et al (2014) Use of cryo electron microscopy to visualize the structural features and binding to functional antibodies of virus-like particles in GARDASIL®. Human Vaccines Immunother 10:734–739

Zhu F, Zhang J, Zhang X, Zhou C, Wang Z, Huang S 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