REVIEW

BIOTECHNOLOGY and BIOENGINEERING

Vaccine Process Technology

Jessica O. Josefsberg,[1] Barry Buckland[2]

[1]BioEdge Consulting, LLC, 100 Jefferson Avenue, Miami Beach, Florida

[2]Department of Biochemical Engineering, University College London, Torrington Place, London, UK; telephone: 646-3692034; fax 305-675-2713; e-mail: buckland@biologicb.com

Received 2 December 2011; revision received 24 February 2012; accepted 27 February 2012

Published online 30 March 2012 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.24493

ABSTRACT: The evolution of vaccines (e.g., live attenuated, recombinant) and vaccine production methods (e.g., in ovo, cell culture) are intimately tied to each other. As vaccine technology has advanced, the methods to produce the vaccine have advanced and new vaccine opportunities have been created. These technologies will continue to evolve as we strive for safer and more immunogenic vaccines and as our understanding of biology improves. The evolution of vaccine process technology has occurred in parallel to the remarkable growth in the development of therapeutic proteins as products; therefore, recent vaccine innovations can leverage the progress made in the broader biotechnology industry. Numerous important legacy vaccines are still in use today despite their traditional manufacturing processes, with further development focusing on improving stability (e.g., novel excipients) and updating formulation (e.g., combination vaccines) and delivery methods (e.g., skin patches). Modern vaccine development is currently exploiting a wide array of novel technologies to create safer and more efficacious vaccines including: viral vectors produced in animal cells, virus-like particles produced in yeast or insect cells, polysaccharide conjugation to carrier proteins, DNA plasmids produced in *E. coli*, and therapeutic cancer vaccines created by in vitro activation of patient leukocytes. Purification advances (e.g., membrane adsorption, precipitation) are increasing efficiency, while innovative analytical methods (e.g., microsphere-based multiplex assays, RNA microarrays) are improving process understanding. Novel adjuvants such as monophosphoryl lipid A, which acts on antigen presenting cell toll-like receptors, are expanding the previously conservative list of widely accepted vaccine adjuvants. As in other areas of biotechnology, process characterization by sophisticated analysis is critical not only to improve yields, but also to determine the final product quality. From a regulatory perspective, Quality by Design (QbD) and Process Analytical Technology (PAT) are important initiatives that can be applied effectively to many types of vaccine processes. Universal demand for vaccines requires that a manufacturer plan to supply tens and sometimes hundreds of millions of doses per year at low cost. To enable broader use, there is intense interest in improving temperature stability to allow for excursions from a rigid cold chain supply, especially at the point of vaccination. Finally, there is progress in novel routes of delivery to move away from the traditional intramuscular injection by syringe approach.

Biotechnol. Bioeng. 2012;109: 1443–1460.

KEYWORDS: vaccine process technology; fermentation; cell culture; purification; formulation; manufacturing

Introduction

The earliest vaccines were relatively crude and consisted of partially purified live attenuated virus (e.g., smallpox, rabies) or inactivated bacteria (e.g., pertussis). Over time, more refined methods were introduced such as chemical treatment of a protein toxin to form a toxoid (e.g., tetanus, diphtheria), development of a purified and inactivated virus (e.g., hepatitis A), development of virus-like particles (e.g., hepatitis B, human papillomavirus), and use of purified polysaccharides (e.g., pneumococcal vaccines). Vaccines can generally be classified as whole organism, purified macromolecules, combined antigens, recombinant vectors, synthetic peptides, or DNA, and have been historically introduced in approximately this order. As these vaccine types have evolved, the production processes to make them have had to evolve as well. A summary is given in Table I.

The first vaccines used whole live virus and human-to-human or animal-to-human transfer, such as Edward Jenner's cowpox (vaccinia) pus inoculation in 1796, intended to immunize against the more pathogenic smallpox in humans. In fact, the word vaccination (Latin: vaccinus = cow) originated from this first vaccine since it was derived from a virus affecting cows (Dekleva, 1999). Production methods then advanced to live attenuated virus vaccines produced in vivo or in ovo.

Bacterial vaccines were created by Louis Pasteur in the 1880s, including vaccines for chicken cholera and anthrax using weakened bacterial cultures. The Bacille Calmette-Guerin (BCG) vaccine for tuberculosis (TB) was developed around the same time (Bae et al., 2009). The pertussis whooping cough vaccine, licensed in 1918, was the first

Correspondence to: B. Buckland

Table I. A summary of vaccine product classifications.

Product classification	Licensed vaccines	Adjuvant
Live attenuated virus	Smallpox, polio, measles, mumps, rubella, chicken pox, rotavirus, shingles, influenza, and yellow fever	No
Inactivated purified virus	Inactivated polio, japanese encephalitis, hepatitis A, Influenza (seasonal and pandemic), and rabies	Sometimes
Live attenuated bacterium	Tuberculosis and typhoid	No
Whole inactivated bacterium	Whole cell pertussis	Sometimes
Purified protein	Acellular pertussis	Yes
Purified protein toxoid	Tetanus, anthrax, and diphtheria	Sometimes
Purified virus-like particles (VLPs)	Hepatitis B and human papillomavirus	Yes
Purified polysaccharide	Pneumococcal for adults and typhoid	No
Polysaccharide conjugated to carrier protein	Pneumococcal for infants, haemophilus type B, and bacterial meningitis	Sometimes
Plasmid DNA	In development	Yes
Adenovirus DNA delivery	In development	No

whole-cell inactivated bacterial vaccine. It was subsequently combined with diphtheria and tetanus toxoids to make the combination diphtheria, tetanus and whole-cell pertussis vaccine (DTwP) and is increasingly being replaced with an acellular subunit vaccine (DTaP) consisting of individual bacterial proteins (Rappuoli, 1990). Protein subunit and toxoid vaccines came about in the 1930s with the diphtheria and tetanus vaccines, which consisted of bacterial toxins inactivated with formalin.

The first in vitro cultivation of a viral vaccine was polio virus in non-neural human cells by Enders in 1949, followed by Salk's inactivated polio vaccine in primary monkey kidney cells in 1955. This marked the beginnings of the modern cell culture based industry (Aunins et al., 2000). Whole virus vaccines, such as the attenuated rabies vaccine, require specialized facilities for virus propagation and fill and finish to ensure human safety. Due to the safety risks associated with whole cell and whole virus vaccines, scientists began to create vaccines based on individual pathogen antigens. These subunit vaccines are potentially safer, but are often less immunogenic due to the absence of other viral and cellular components. Polysaccharide vaccines typically contain polysaccharides from the surface of a bacterial capsid, such as in the subunit vaccine for typhoid and early versions of pneumococcal and meningococcal vaccines (14-valent in 1977 and 23-valent in 1983).

Historically, vaccines have been developed using these conventional methods that follow the paradigm of isolating, sometimes inactivating, and injecting the disease-causing pathogen or pathogen component (Table II). Some of these methods are still used today for vaccines given to people throughout the world (Aunins et al., 2011). For example, the process used to make modern diphtheria or tetanus vaccines, based on bacterial toxoids, is a refined version of the one developed in the 1930s and the process used to make today's measles, mumps and rubella (MMR) vaccine was developed more than 30 years ago. For some of these important vaccines, further process development is focused on troubleshooting any manufacturing issues, as well as formulation work to either combine with other vaccines (e.g., ProQuad® = Varicella + MMR) or to improve product stability (e.g., novel excipients) (Lightfoot and Moscariello, 2004). These products are generally not protected by patents, but the complexity of manufacturing and key analytics results in a high barrier to entry for new competitors. For other traditional vaccines, novel process approaches are being applied in order to further drive down cost. For example, a new Sabin-based inactivated polio vaccine is being developed to move away from Salk wild-type strains to the safer attenuated Sabin strains, allowing for process optimization including the application of modern purification methods (Bakker et al., 2011). In another

Table II. Evolution of traditional vaccine production processes and vaccine-related events.

Year	Historical vaccine event
1796	Vaccine using animal-to-human cowpox inoculation (smallpox)
1879	Live-attenuated bacterial vaccine (chicken cholera)
1884	Live-attenuated viral vaccine grown in brain tissue (rabies)
1897	Vaccine prepared from horse serum (bubonic plague)
1918	Whole-cell inactivated bacterial vaccine (pertussis whooping cough)
1923	Toxoid vaccine prepared from inactivated bacterial toxins (diphtheria)
1931	Freeze-dried vaccine approved by FDA (smallpox)
1949	Combination vaccine (diphtheria, tetanus, whole-cell pertussis, and DTwP)
1949	Viral vaccine produced in vitro with non-neural human cells by Enders (polio)
1954	Freeze drying process for smallpox vaccine greatly improved by Collier to allow global distribution
1955	Inactivated viral vaccine produced in vitro with primary monkey kidney cells (polio)
1962	Human diploid cell line (WI-38) established by Hayflick
1977	Last case of smallpox outside of the laboratory
1986	Recombinant virus-like particle vaccine produced in yeast (hepatitis B)
1987	Conjugated polysaccharide-protein vaccine (*Haemophilus influenza* b)
1998	Classical vaccine highly purified using biotech purification approach (hepatitis A)

example, the processes used for polio vaccines have evolved toward larger scale production with an interesting early application of microcarriers on an industrial scale (Montagnon et al., 1984).

It is a different story for novel vaccines; there are major incentives for new vaccines to be more defined. Due to the global desire to avoid exposure to disease-causing organisms by both patients and manufacturers, recombinant expression of proteins and viral vectors is the modern vaccine development method of choice to remove pathogens from the system. The advantages of subunit and recombinant vaccines include: virtual elimination of safety risks, vaccine feasibility even with a difficult-to-cultivate virus, defined process components, more controlled bioprocesses, and a shorter production process (e.g., cell culture vs. egg) which is critical for pandemic response (Buckland, 2005; Eisenstein, 2011). An example is Recombivax HB®, a hepatitis B vaccine that was the first licensed recombinant DNA vaccine. The original vaccine was made by purifying virus particles from infected blood; it was revolutionary to be able to replace this with the inherently safe recombinant approach of expressing hepatitis B surface antigen in *Saccharomyces cerevisiae*.

Several new vaccine development approaches have come to the forefront over the last decade. Reverse vaccinology is an approach that uses genome analysis to identify the complete repertoire of antigens that are surface-exposed and highly antigenically conserved across multiple strains. The most immunogenic epitopes, once sequenced, are typically patented and evaluated for suitability in various vaccine formulations. An example is the influenza vaccines under development that are based on recombinant hemaglutinin protein antigens rather than on live attenuated virus. Systems biology is another approach that integrates multiple fields of study to explore the interactions between different biological systems. Using mathematical models to evaluate data collected from various fields, such as transcriptomics, metabolomics, and proteomics, scientists can begin to elucidate the causes and effects in biological networks. This can lead to a greater understanding of how vaccine antigens interact with different components of the immune system. Using the above scientific approaches, rationally designed vaccines are created, often at the amino acid sequence level, to present antigens through specific intracellular pathways.

Using similar techniques, ground-breaking new virus-like particle (VLP) vaccines have been developed, such as Gardasil® and Cervarix®, effective against human papilloma virus (HPV) and associated cervical cancer. Despite these innovations, many other diseases continue to elude vaccine scientists. Pathogens with high-antigenic variability that may require T-cell dependent immunity, such as those for malaria, TB, and HIV, continue to cause human suffering. Developing effective and long-lasting vaccines for diseases such as these will continue to require new approaches to vaccine design and production (Rinaudo et al., 2009). This article focuses on the production, purification, analytical, formulation, and delivery innovations in the field of vaccines that have surfaced over the last decade.

Virus-Based Vaccines and Viral Vectors

Many viral vaccines were originally produced in primary cell lines, which are cells obtained directly from animal organs and tissues. Diploid cells are derived from primary cultures and can be serially passaged, although they retain their anchorage dependence and limited lifespans (40–60 passages) (Sureau, 1987). These characteristics make it difficult to maintain a high cell density for a prolonged period of time, which leads to challenges in bioreactor design, as was the case of the development of the VAQTA hepatitis A vaccine with diploid MRC-5 cells in roller bottles (Armstrong et al., 1993).

Continuous cell lines originate from diploid cells that have undergone a chromosomal transformation to allow them to divide indefinitely. Those that can be used for the production of viral vaccines fall into two categories. The first is conventional cell lines such as African green monkey kidney-derived Vero cells, Madin–Darby Canine Kidney (MDCK) cells, or PBS-1 cells (HepaLife Technologies, Boston, MA) that are grown either adherently in roller bottles, NUNC cell factories (Thermo Scientific, Roskilde, Denmark), or on microcarriers, or in suspension cultures in stirred-tank bioreactors or disposable wave bioreactors. The second category is proprietary human cell lines such as PER.C6® (Johnson & Johnson, Leiden, the Netherlands), AGE1.CR® (ProBiogen, Berlin, Germany), and EB14® (Vivalis, Nantes, France) which are typically grown in suspension cultures in serum-free media (Genzel and Reichl, 2009).

The Vero cell line was the first continuous mammalian cell line, established from African green monkeys in 1962, and is currently the most widely accepted by regulatory authorizes for vaccine development due to the fact that Vero-derived human vaccines have been in use for nearly 30 years. They can be grown and infected on microcarrier beads in large-scale fermenters (6,000 L reported) and in serum-free medium with no loss in productivity (Barrett et al., 2009). Several innovative vaccines produced in Vero cells have been licensed including the smallpox vaccine ACAM2000® (Sanofi Pasteur, Lyon, France) and the pediatric rotavirus vaccines Rotateq® (Merck, Whitehouse Station, NJ) and Rotarix® (GSK, Brentford, Middlesex, UK), all of which are live attenuated virus vaccines. Also produced from Vero cells is the H5N1 pandemic influenza vaccine Preflucel® (Baxter, Deerfield, IL) and the japanese encephalitis vaccine Ixiaro® (Intercell, Vienna, Austria), both produced from inactivated virus.

Viral vectors allow simultaneous expression of multiple antigenic determinants while avoiding the safety risks associated with the use of the whole pathogenic virus (Liniger et al., 2007). DNA viral vectors that have been used in antigenic delivery systems include poxviruses,

herpesvirus, and adenovirus, while RNA viral vectors, such as retrovirus and flavivirus, have been studied as well (Geels and Ye, 2010). Viral vectors have the advantages of being capable of inducing both antibody and T-cell-mediated immunity in the absence of an adjuvant, do not require complex purification development, may be able to generate antigens with native conformation, and may be able to deliver more than one gene (e.g., multiple antigens from different parasite life stages that could induce a broad protective immunity) (Li et al., 2007).

Adenovirus vectors are one of the most promising gene delivery vectors for vaccines because they are efficient at delivering DNA to target cells, have a large capacity for incorporation of cDNA expression cassettes, and have a low potential for oncogenesis because they do not insert their genome into the host DNA (Subramanian et al., 2007). Adenovirus have been produced in PER.C6®, HEK293, and A549 cell lines, although PER.C6® is the most common despite its limited number of generations. PER.C6® cells can be grown adherently or in suspension culture, the latter having similar growth patterns in large bioreactors to Chinese Hamster Ovary (CHO) cells, with the added complication of an infection step during the exponential growth phase. The challenge of growing a virus that was designed to be replication incompetent was solved using a metabolic engineering approach involving the deletion of the required E1 region from the virus and subsequent insertion into the host cell line (Maranga et al., 2005; Singh and Kostarelos, 2009). The result is that the recombinant adenovirus replicates well in the specific host cell, but does not replicate after injection into the patient.

A large-scale cell culture and infection process was developed at Merck for a recombinant adenovirus type 5 (rAd5) vector expressing the HIV-1 *gag* gene in suspension PER.C6® cells, as shown in Figure 1 (Xie et al., 2003). This process was scaled-up to 250 L under the worst-case sparging conditions projected for 10,000 L scale, which was the scale projected for implementation if this proposed HIV vaccine (p55 *gag* transgene) was commercialized. The process includes a step for removal of polysorbate-80 surfactant and the addition of Pluronic F-68, a nonionic polymer that reduces cell damage incurred during sparging. In 2007, the same team developed a different process for production of rAd5 vectors in PER.C6® cells that bypasses the time-consuming virus cloning and passaging steps. This process involves transfection of adherent PER.C6 cells with bacterially cloned adenovirus plasmid using calcium phosphate coprecipitation (Subramanian et al., 2007). The cells were grown and transfected in NUNC cell factories with a production level of $>5 \times 10^{10}$ VP per NUNC tray, obtained 1 month from the time of plasmid construction.

Few HIV preventative vaccine candidates have made it to Phase III trials and all have failed to prove satisfactory efficacy. The first was called AIDSVAX. VaxGen began trials in Thailand of AIDSVAX B/E in combination with the Aventis Pasteur vaccine, ALVAC-HIV, which used genetic elements of several different HIV strains encapsulated in a harmless canarypox virus vector. Efficacy ranged from 26.1% to 31.4%, too low to be considered successful, but the results are considered an important milestone. The second Phase III vaccine candidate was the rAd5-based vaccine developed by Merck. In the STEP and Phambili HIV trials, the vaccine failed to protect Ad5-seronegative individuals against infection and both trials were halted in September 2007 (Iaccino et al., 2008).

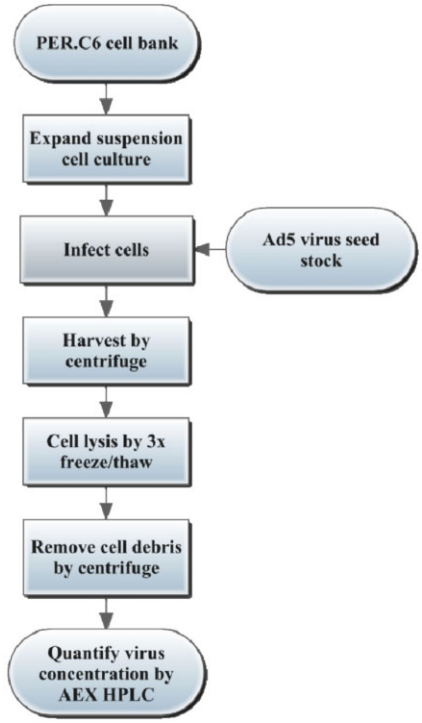

Figure 1. Adenovirus production process developed by Merck involving the infection of PER.C6® mammalian cells with rAd5 viral vectors containing the HIV-1 p55 *gag* transgene. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

Polyethylene glycol (PEG) precipitation and solvent extraction have both been around for some time and are still key components in the purification of many viral vaccines. For example, they are both used in the purification of the highly purified formalin-inactivated hepatitis A vaccine VAQTA (Merck), where chromatography and PEG precipitation concentrate the virus, with subsequent extraction with chloroform causing irreversible denaturation of contaminant proteins at the interface, retaining viable hepatitis A virus in the aqueous phase (Hagen et al., 1997). During development of VAQTA, the PEG precipitation step was found to be sensitive to small changes in growth and harvest conditions. Additional development work indicated that nucleic acids were aggregating during the membrane concentration step and were coprecipitating with the virus. These variable amounts of nucleic acids led to inconsistent virus recovery and product purity. The use of nuclease on the crude lysate decreased the molecular size of

the nucleic acids and reduced their coprecipitation with the virus. Following the nuclease addition step with anion exchange chromatography (AEX) provided further optimization in yield and purity, as well as enhanced process reproducibility (Hagen et al., 1996).

Influenza Vaccines—Evolution of Vaccine Production Processes

The first influenza vaccine was developed in 1937 as an inactivated whole virus vaccine. It has since progressed to live attenuated influenza vaccines (LAIV) and purified split vaccines, followed by a purified surface antigen vaccine, all produced in fertilized eggs. The influenza vaccine has since evolved from this in ovo process that involves a long production time and limited capacity, to cell culture processes that are low-yielding and capital intensive, to newer technologies such as recombinant antigens, VLPs, and the use of advanced adjuvants such as Toll-Like Receptor (TLR) agonists (Price, 2011). This evolution continues to be driven by the risk of an influenza pandemic, especially with types H5N1 and H1N1, and the overall vaccine demand has roughly doubled since 2009 (Gregersen et al., 2011). Current estimates indicate that global spread could occur in 6 months, which is approximately the period required for in ovo vaccine production and the brief clinical studies required in Europe (Hoare et al., 2005). Many countries without virus-based vaccine capacity could not afford to put production facilities in place just for a possible pandemic. The newer recombinant vaccine technologies with shorter production timelines and/or based on platform technologies could be used to solve worldwide capacity issues.

The influenza surface proteins and peptides of greatest current interest, shown in Figure 2A, include the hemaglutinin (HA), neuraminidase (NA), core matrix 1 (M1), and core matrix 2 (M2) domains (Amorij et al., 2008). Neutralizing and receptor-blocking antibodies against HA are the primary means of protection, while antibodies against NA seem to play a secondary role. The seasonal human influenza vaccines currently licensed contain an inactivated split of H1N1, H3N2, and influenza B viral antigens to promote HA and NA subtype and strain specific antibody protection (Barrett et al., 2009). There is little or no known cross-protection against drifted strains within subtypes or to other subtypes (Bright, 2011).

Cell culture based production of LAIV offers the advantages of shorter production cycles, greater process control, well-characterized production substrates, and providing larger quantities of vaccine in a shorter time period (Aggarwal et al., 2011; George et al., 2010; Liu et al., 2009). The disadvantage is relatively low productivity and a long virus adaptation period, which would be problematic during a pandemic. The use of live virus also requires a higher level of biosafety containment.

The most common host cells used for influenza vaccines are MDCK cells that are grown and infected either on microcarrier beads or in suspension culture. MDCK cells demonstrate a high degree of robustness, are easily adapted to different medium formulations, can tolerate a wide pH range, and are less sensitive to aeration than other cell lines (Genzel and Reichl, 2009). There are currently two licensed seasonal influenza vaccines manufactured in MDCK cells. Abbott's (acquired Solvay Pharmaceuticals in 2009) Influvac TC® was licensed in the Netherlands in 2001, while Novartis' Optaflu® was approved in the EU in 2007. Novartis also produces Celtura® based on the H1N1 strain for use in pandemics. Influvac TC® is produced in adherent culture, while Optaflu® and Celtura® are produced in suspension culture.

Recombinant technologies will likely become the way of the future for influenza vaccines since the upstream processes are fast compared to in ovo and cell culture production and due to the fact that they avoid the handling of live virus and the associated costly biosafety containment. A recombinant process would also allow for quick cloning of a new strain and the use of non-specialized production facilities for surge capacity during a pandemic.

The baculovirus expression vector system (BEVS) is an excellent option for production of recombinant influenza vaccines, since it involves the infection of insect cells with baculovirus, which are inherently safe because they do not replicate in mammalian cells (Wang et al., 2006). In addition, virtually no known adventitious agents can replicate in both insect cells and mammalian cells. Protein Sciences Flublok® is the first recombinant HA influenza vaccine (trivalent) produced in BEVS and is currently under review for approval in the USA. If approved, it would be the first licensed recombinant influenza vaccine. Figure 2B shows the production process for Flublok®, which involves standard cloning, cell culture, and purification procedures (Cox, 2010; Cox and Hollister, 2009). The HA gene from a new influenza virus is cloned directly into a baculovirus expression vector. The recombinant baculovirus is then used to infect insect cells in large-scale stirred bioreactors. Following protein expression, infected cells are harvested by centrifugation and the antigen is collected using detergent extraction. The HA is purified by two column chromatography steps and two filtration steps. The time from DNA sequence acquisition for a new influenza strain to production of a rosette-shaped protein is rapid using this technology. There are many similarities, both upstream and downstream, between the Protein Science's *expres*SF (derived from Sf9 cells) insect cell process and a CHO-based antibody manufacturing process. This creates a plausible scenario in which an existing CHO-based facility could be used to make influenza vaccine, since the process is readily scalable to large stirred tank bioreactors.

Several other companies are developing recombinant influenza vaccines in standard expression systems as well. Novavax exploits several influenza surface antigens in its VLP vaccine that is also produced in BEVS; it consists of a protein shell decorated with HA, NA, and M1 antigens (Extance, 2011; Singhvi, 2010). The company VaxInnate

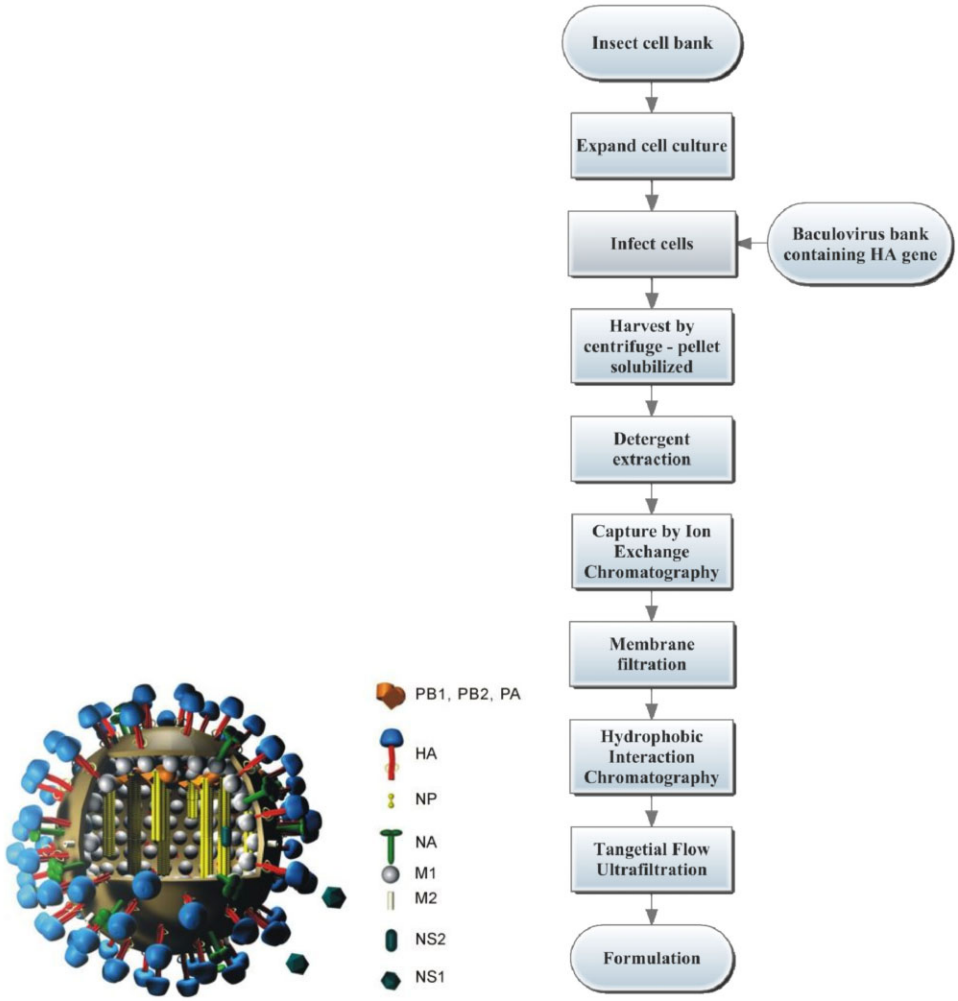

Figure 2. **Panel A**: Influenza virus particle showing the surface peptides of greatest interest in current influenza vaccine research: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), non-structural protein 2 (NS2), and non-structural protein 1 (NS1). **Panel B**: Production and purification process schematic for FluBlok®, an HA influenza vaccine produced in baculovirus expression vector system (BEVS) by Protein Sciences. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

uses recombinant *E. coli* to express HA in the form of a TLR agonist–antigen fusion protein, which has demonstrated strong potency at a tenth of a standard human dose. TLR agonist-antigen fusion proteins mimic natural infection by simultaneously presenting a TLR agonist and an antigen at the same antigen-presenting cell (APC). The bacterial fermentation process requires approximately 3 weeks and can use existing prokaryotic manufacturing facilities globally, allowing for fast surge capacity in response to a pandemic (Shaw, 2006). Both Novavax and VaxInnate have received US government contracts from BARDA in February 2011 totaling $215 million, which they will use to push their respective vaccines toward Phase III trials (Young, 2011).

Other recombinant influenza vaccine approaches include HA delivered by a modified recombinant viral vector (Vaxin, Birmingham, AL) and a plasmid DNA vector (Pfizer, New York, NY). Several universal vaccines based on the M2e peptide (constant across all influenza strains) are also under development, including two by Acambis/Sanofi Pasteur and VaxInnate (Price, 2011).

Conjugate Vaccines

Conjugate vaccines combine a pathogen antigen with a carrier protein that lends additional immunogenicity to the vaccine. For example, the efficacy of three vaccines against encapsulated bacterial pathogens, *Neisseria meningitides*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* type b (Hib), was significantly enhanced by covalently attaching (conjugating) the polysaccharides (PS) in the original

vaccines with carrier proteins (Frasch, 2009). Typhoid vaccines based on the Vi polysaccharide of *Salmonella typhi* were also improved by conjugation to several carrier proteins (Knuf et al., 2011; Thiem et al., 2011), including tetanus or diphtheria toxoids, cholera toxin B subunit, or recombinant mutant *Pseudomonas aeruginosa* exoprotein A (rEPA). Though these particular conjugate vaccines are non-recombinant and require fermentation of the original pathogens in a higher biosafety level facility, they represent an advance in vaccine technology. The PS vaccines stimulate bactericidal anticapsular antibodies, which give good short-term immunogenicity, but lack the ability to mount an adequate immune response in children under 2 years of age. Conjugated PS vaccines trigger a T-cell-dependent antigen response (T-helper cells promote B-cell activation) that induces long-term immunity.

There are five main carrier proteins used in vaccines today: tetanus toxoid (TT), diphtheria toxoid (DT), cross-reactive material 197 (CRM197), *N. meningitides* outer membrane protein (OMP), and non-typeable *H. influenza* derived protein D (PD). Hib, meningococcal, and pneumococcal conjugate vaccines have shown good safety and immunogenicity regardless of the carrier protein used, although data is conflicting as to which protein is most immunogenic and the implications of protein size are unclear. In the future, genetically detoxified vaccine production will likely become more widely used as the mechanism of action becomes better understood (Bae et al., 2009).

In the conjugation process as shown in Figure 3, purified PS must first be chemically modified to generate reactive groups that can link to the protein, for example with either periodate or 1-cyano-4-dimethylaminopyridinium (CDAP). Multiple factors, including low molecular weight impurities in the protein and suboptimal PS to protein ratio, may result in inefficient conjugation with less than 20% of the activated PS becoming conjugated (Lee et al., 2009). Newer conjugation methods have recently been developed (addition of hydrozones to carboxyl groups or oximes to amino groups) that involve generating highly reactive groups on both the PS and carrier protein and have yields approaching 50% (Lees et al., 2006).

Prevnar 13® is a pneumococcal vaccine by Pfizer, licensed in 2010, that contains cell membrane PS of 13 bacterial serotypes conjugated to diphtheria CRM197 carrier protein, a non-toxic variant of diphtheria toxin (Cooper et al., 2011). Each PS is manufactured independently with subsequent combination during the formulation process, requiring almost a year from manufacture to release. The number of serotypes makes the analytical aspects and manufacturing of this vaccine particularly complex.

Ancora is currently exploring a synthetic approach to carbohydrate production, which offers the advantages of easier purification (vs. natural carbohydrate antigens), improved manufacturing options, and the addition of an anchor for convenient conjugation to a carrier protein. An example was recently described for making a synthetic oligosaccharide for Group A Streptococcus (GAS) that was subsequently conjugated to CRM197 and showed promising immunogenicity in mice (Kabanova et al., 2010).

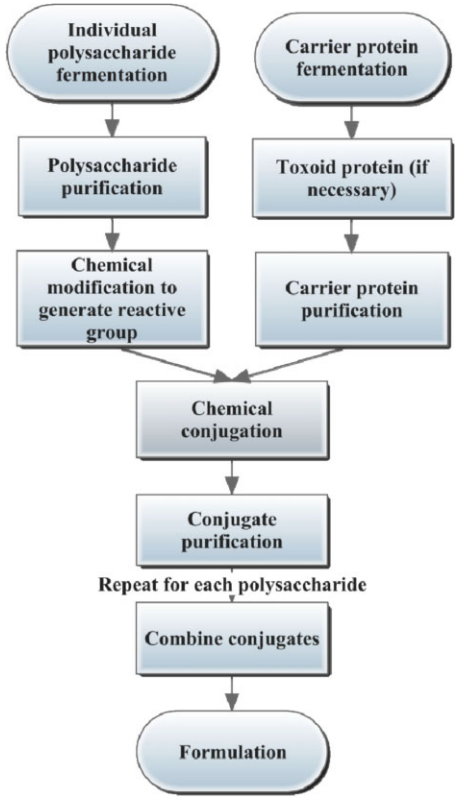

Figure 3. A typical process for production of a multivalent conjugate vaccine in which the polysaccharides are individually produced by fermentation, individually conjugated to a carrier protein, individually purified, and then combined into the final formulation. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

DNA Vaccines

DNA vaccines can lead to a strong and long-lasting immune response through the inoculation of a plasmid containing a gene for a particular protein antigen, which is subsequently expressed by the cellular machinery of the person receiving the vaccine. DNA vaccines offer the potential for immunotherapy of diseases like tumors because they can induce a cytotoxic T-effector lymphocyte response for antigen-specific apoptosis of infected cells (Polakova et al., 2009). Although only veterinary DNA vaccines have been approved to-date (e.g., equine vaccine for protection against west nile virus), there are numerous DNA vaccines in various stages of development for targets such as HIV, cancer, and multiple sclerosis (Stuve et al., 2007).

Production of DNA plasmids by bacterial fermentation at large scale is relatively straightforward. The main process challenge lies with developing effective and economical

cellular purification protocols, made difficult because the supercoiled plasmid DNA is quite similar in size and structure to the contaminating RNA, genomic DNA, and open circular plasmid DNA that are released upon cellular lysis along with the supercoiled DNA product.

To satisfy regulatory guidelines for DNA vaccines, the plasmid DNA (pDNA) must be a highly purified, homogenous preparation of supercoiled circular covalently closed (ccc) plasmids. Purification to this level of quality is expensive and could account for a majority of total operational costs.

Figure 4 depicts several of the most frequently used pDNA purification strategies, all of which start with centrifugation

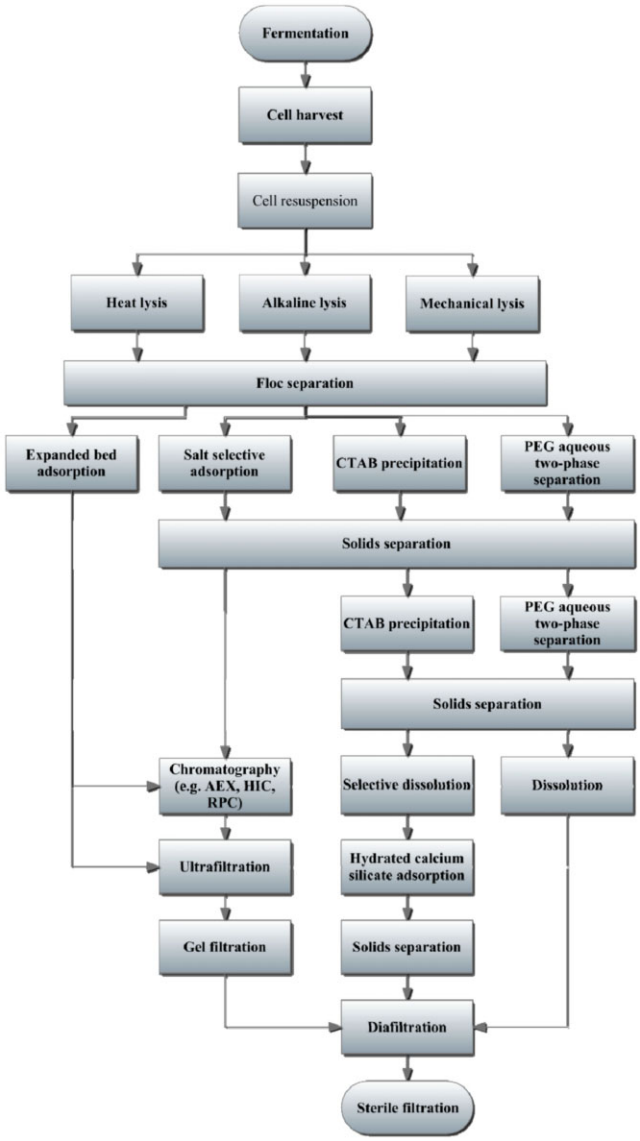

Figure 4. Relevant plasmid DNA purification processes for the large-scale preparation of DNA vaccines. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

or, more commonly, filtration of the fermentation cell mass to reduce the overall volume (Freitas et al., 2009; Hoare et al., 2005; Lis and Schleif, 1975; Murphy et al., 2005; Stadler et al., 2004; Williams et al., 2009). If centrifugation is used, a solid-bowl centrifuge was found to be preferable to a disc-stack centrifuge, which has an additional high-stress stage of nozzle discharge into the lower bowl (Kong et al., 2008). The additional degraded DNA resulting from nozzle discharge was not effectively removed during flocculation and led to increased fouling of the chromatographic columns. It was also found that a freeze/thaw step or a lengthy hold of fresh cells in resuspension buffer following harvest resulted in higher levels of contaminating DNA. The use of a genetically engineered *E. coli* with an *endA* deletion mutation may overcome some of this DNA degradation.

This is followed by a heat-based, alkaline, or mechanical lysis of the bacterial cell walls to release the plasmids into solution (Cheetham et al., 1982). Heat lysis is a relatively simple method that can make use of standard industrial heat exchangers, thereby reducing capital costs. It has the disadvantage of requiring a significant amount of costly lysozyme for many protocols, although a heat lysis method without lysozyme has been reported that gives a comparable yield to an alkaline method (Wang et al., 2002). There are also reports of the use of autolytic *E. coli* strains that express lysozyme (endolysin) in the cytoplasm, which is released to the periplasm upon induction (Carnes and Williams, 2007).

Precipitation is a method of solid–liquid separation that can deal with very large volumes. Fractional precipitation with the cationic detergent cetyltrimethylammonium bromide (CTAB) isolates ccc pDNA from not only proteins, RNA, and endotoxin, but also host genomic DNA and relaxed and denatured forms of the plasmid (Lander et al., 2002). The ccc pDNA can then be selectively dissolved into a controlled salt concentration as an initial recovery step. Using the minimum required salt concentration to dissolve the pDNA allows any remaining impurities not removed by the prior precipitations to be separated in an insoluble, filterable form. Since precipitation and dissolution are conventional unit operations, manufacturing scale-up is relatively straightforward. Figure 5 is a graph from Lander et al. (2002) that shows the standardized concentration (% w/v) of CTAB required for precipitation of various forms of plasmid and genomic DNA. This graph illustrates that there is a specific concentration of CTAB in which the contaminating forms of plasmid DNA precipitate but the supercoiled plasmid product remains soluble.

Hydrated calcium silicate (gyrolite) selectively adsorbs DNA impurities, including genomic and open-circular DNA, leaving the ccc pDNA in solution (Winters et al., 2003). This selective adsorption step often follows precipitation methods as the gyrolite also adsorbs surfactants from upstream processing steps, such as CTAB and Triton X-100, as well as endotoxin. Since gyrolite is far less expensive than conventional wide-pore chromatographic resins, its use in plasmid purification is both scalable and economical.

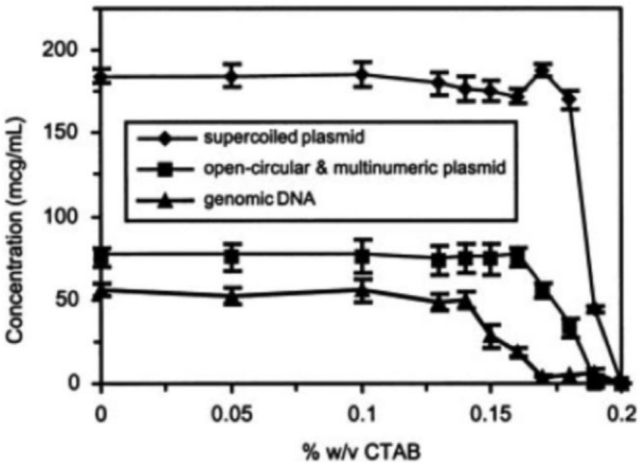

Figure 5. Graph from Lander et al. (2002) displaying the solubility of supercoiled, open-circular, and multimeric forms of plasmid DNA and genomic DNA as a function of CTAB concentration in clarified lysate. The concentration of total DNA (plasmid forms plus genomic DNA) was measured by HPLC assay. Genomic DNA concentration was measured using qPCR assay. The percentage of supercoiled plasmid DNA from agarose gel assays illustrates that open-circular and multimeric plasmid forms and genomic DNA precipitate before supercoiled plasmid DNA. The error bars are based on duplicate measurements from qPCR and agarose gel assays.

Virus-Like Particles

VLPs are viral structural proteins that are expressed in cells and possess native conformational epitopes. However, VLPs are subunit vaccines that do not contain a genome and are incapable of a spreading infection. VLPs have a repetitive antigenic structure that is capable of efficiently stimulating both cellular and humoral immune responses (Bolhassani et al., 2011; Roy and Noad, 2008). Potentially, VLPs could also serve as excellent carrier molecules for the delivery of epitopes in vaccines. Notable work in this area includes hepatitis B core particles, HPV VLPs, and parvovirus VLPs displaying T-cell specific epitopes from another protein on their capsid (Cook et al., 1999).

Recombivax HB® for prevention of hepatitis B infection was the first recombinant protein vaccine for human use, licensed in the US in 1986 by Merck. Engerix-B by GlaxoSmithKline (GSK) is a similar vaccine that was licensed soon after. These were considered revolutionary vaccines at the time, both because they were the first to use virus-like particles (VLPs) and because they were arguably the first anti-cancer vaccines (hepatitis B infection increases the incidence of liver cancer). They replaced a previous hepatitis B vaccine that was produced by the labor-intensive process of purifying antigen from the blood of infected donors. There was an intense urgency to develop these new recombinant vaccines due to the advent of HIV infection in the general population, which raised additional questions about the safety of blood-derived products.

Both vaccines were made by inserting the gene for a hepatitis B surface antigen (HBsAg) into genetically engineered strains of *S. cerevisiae*. Figure 6 shows a process for producing HBsAg starting with fermentation of recombinant *S. cerevisiae* in a complex fermentation medium (Dekleva, 1999; Geels and Ye, 2010; Kee et al., 2008). The HBsAg protein is released into solution after cell disruption and detergent extraction, and is then purified by a series of physical and chemical methods, including fumed silica adsorption, hydrophobic interaction chromatography, and several ultrafiltration steps (not shown). The purified protein is treated in a potassium thiocynate solution with formaldehyde and then co-precipitated with potassium aluminum sulfate (alum).

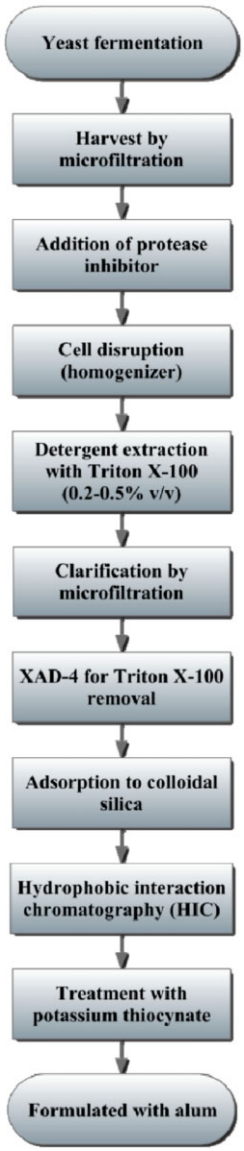

Figure 6. Recombinant hepatitis B vaccine manufacturing process that involves fermentation of genetically engineered strains of *Saccharomyces cerevisiae* containing the genetic sequence encoding for hepatitis B surface antigen (HBsAg). Recombivax HB® was the first recombinant vaccine approved by the FDA in 1986. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

Two VLP-based vaccines for HPV have since been FDA approved, Gardasil® (Merck) and Cervarix® (GSK), both for the prevention of HPV and cervical cancer. Numerous other particle vaccines are in various stages of clinical trials, including influenza and respiratory syncytial virus (RSV) vaccines produced using BEVS with insect cells (Novavax, Gaithersburg, MD) and an influenza vaccine produced in plant cells (Medicago, Quebec City, Canada). Vaccines for melanoma, malaria, and hepatitis B and C vaccines that use chimeric hepatitis B core VLPs are also being explored (Kazaks et al., 2008; Plummer and Manchester, 2010).

The Gardasil® quadrivalent vaccine (HPV types 6, 11, 16, and 18) is produced by expressing the type-specific papillomavirus major capsid protein, L1, in *S. cerevisiae* (Shi et al., 2007). Previous work has shown that recombinant HPV16 L1 pentamers assemble in vitro into capsid-like structures, and truncation of 10 N-terminal residues leads to a homogeneous preparation of 12-pentamer, icosahedral particles (Chen et al., 2000). The fermentation process uses a recombinant yeast expression system with a galactose-inducible promoter (GAL1) expressing HPV capsid proteins, which permits high cell densities to be achieved before the L1 protein is expressed (premature expression of high levels of foreign proteins may be detrimental to the host cells). Harvest is initiated when galactose reaches a designated level. The L1 protein makes up approximately 15% of the total soluble protein. The main technical challenges occur downstream and include releasing the intracellular VLPs, typically by homogenization, and preserving the intact VLP particles during cell lysis and purification.

A major VLP vaccine development challenge is that fermentation monitoring requires frequent removal of samples, followed by centrifugation, cell disruption, and chromatography to partially purify the VLPs. A miniaturized version of this was recently described, in which the key steps of cell disruption and chromatography are run at very small scale (Wenger et al., 2007). The multi-step chromatography process was reduced 1,000-fold using microliter volumes of resin in a pipette tip and automated on a liquid-handling robotic workstation. This high-throughput platform was found to be predictive of laboratory-scale purification in terms of yield and purity, allowing optimization of a fermentation process while reducing overall time and materials.

The purification of VLPs is similar to the purification of virus vaccine, with the advantage of less stringent biosafety requirements due to the VLP's inability to replicate and cause disease. Figure 7 shows the purification process for the Gardasil® vaccine against HPV (Cook et al., 1999). The yeast cells are harvested from the fermenter and frozen. After thawing, benzonase is added to the cells to weaken the cell membrane and the cell slurry is passed twice through a homogenizer, which achieves a cellular disruption of greater than 95%. The mixture is incubated at 4°C for 12–20 h to complete the lysis. The lysate is then clarified by cross-flow microfiltration in diafiltration mode and followed by two

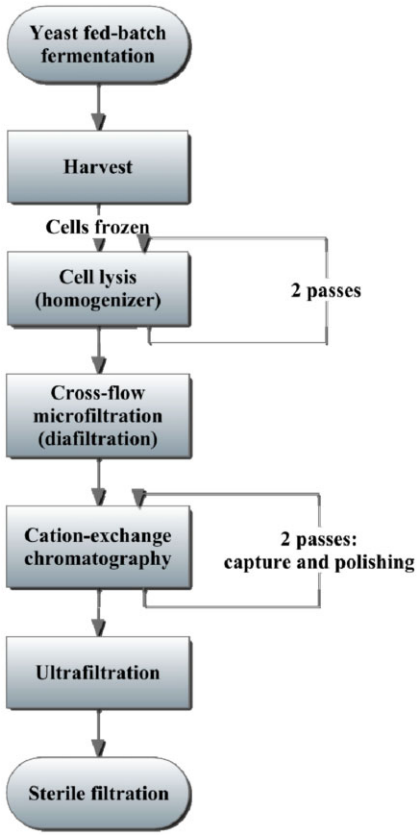

Figure 7. Initial purification process for the Gardasil® (Merck) virus-like particle vaccine, which is produced in *Saccharomyces cerevisiae* and requires cell disruption to release the virus-like particles. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

CEX fractionation steps; the first pass captures the VLPs and the second pass serves as a polishing step. Using this process, the purified VLPs are 98% homogeneous (purity) and overall purification yield is 10% (Ding et al., 2010).

Recently, a novel approach to create VLPs based on the VP1 structural protein from murine polyomavirus (MuPyV) in *E. coli* was described (Middleberg et al., 2011). Two insertion sites on the surface of MuPyV VP1 are exploited for the presentation of the M2e antigen from influenza and the J8 peptide from Group A Streptococcus (GAS).

The most widely used yeast expression system is *S. cerevisiae*, but another yeast strain is becoming increasingly popular. The methylotrophic yeast *Pichia pastoris*, with its efficient and tightly regulated promoter from alcohol oxidase I gene (AOX1), utilizes economical carbon sources to reach the high cell densities needed for large-scale production of vaccines (Geels and Ye, 2010). This has been successfully applied to the manufacture of low cost Hepatitis B vaccine by various Indian manufacturers. The recombinant proteins are often secreted into the media, which facilitates downstream purification and analysis. Proteins expressed in *P. pastoris* have high levels of

glycosylation which may enhance the efficacy of vaccines in some cases. One team developed a large-scale chromatographic purification protocol for production of HBsAg from *P. pastoris* (Hardy et al., 2000), although chromatography-based purification can become prohibitively expensive at large scale (Curling and Gottschalk, 2007). Another team increased production threefold of a recombinant disulfide-rich malaria protein in *P. pastoris* by genetically engineering the strain to overproduce the protein disulfide isomerase (Tsai et al., 2006).

Cancer Vaccines

It was recently estimated that the total of infection-attributable cancer in the year 2002 was 1.9 million cases, or 17.8% of the global cancer burden (Parkin, 2006). The principal agents were the bacterium *Helicobacter pylori*, hepatitis B and C viruses, Epstein–Barr virus, HIV, herpes virus, and HPV. Preventing cancer by protecting the host against infectious pathogens shows a paradigm shift in prophylactic vaccines. A new report predicts that the cancer vaccine market will swell to a $7 billion industry by 2015, which is contingent on success in clinical trials and in the ability to scale-up the technology (Martino, 2011). There are six types of vaccines in development: antigen/adjuvant vaccines, DNA vaccines, vector-based vaccines, tumor cell vaccines, dendritic cell vaccines, and anti-idiotype vaccines (Bolhassani et al., 2011; Schuster et al., 2009).

The goal of therapeutic cancer vaccines is to induce immunity against tumor-associated antigens (TAAs). An important advance is the development of techniques to identify antigens that are recognized by tumor-specific T lymphocytes (Bolhassani et al., 2011). These tumor antigens fall into two broad categories: (1) tumor-specific shared antigens or TAAs, and (2) tumor-specific unique antigens. TAAs are expressed by more than one type of tumor cells, such as human epidermal growth factor receptor 2 (HER2) and carcinoembryonic antigen (CEA), but can also be expressed on normal tissues in differing amounts. Unique tumor antigens result through mutations induced through physical or chemical carcinogens. Both shared and unique tumor antigens can be used in developing cancer vaccines, although an effective vaccine should provide long-term memory to prevent tumor recurrence. Some scientists believe that for total tumor elimination, both the innate and adaptive immune systems should be activated (Pejawar-Gaddy and Finn, 2008). T-cell-based immunotherapy has demonstrated the proof-of-principle that immune control of cancer is possible even with metastatic disease. The challenges, however, are formidable and will likely need to include strategies not only to stimulate tumor-specific immunity but also ways to overcome the immune suppression associated with malignancy (Douek and Nabel, 2011).

Dendreon's patient-specific prostate cancer vaccine Provenge (Sipuleucel-T) was the first therapeutic cancer vaccine to be approved by the FDA. It was approved in April 2010 for metastatic hormone-refractory prostate cancer and is considered a breakthrough in cancer treatment. The active component is antigen-presenting cells (APCs) that present prostatic acid phosphatase (PAP) to T-cells. Figure 8 shows the process where the APCs are patient leukocytes obtained via apheresis and combined with PA2024, which is PAP linked to granulocyte-macrophage colony stimulating factor (GM-CSF), a protein that induces a powerful immune response (Division of Cell and Gene Therapies, 2010). The PA2024 activates the leukocytes, which are infused back into the patient where they present PAP to T-cells, which in turn target and kill cancer cells.

In vitro generation of dendritic cells (DC) loaded with TAAs has also been investigated against human glioblastoma multiforme, an aggressive primary brain tumor (Bolhassani et al., 2011). It has been reported that the injection of exosomes derived from DCs loaded with tumor peptides

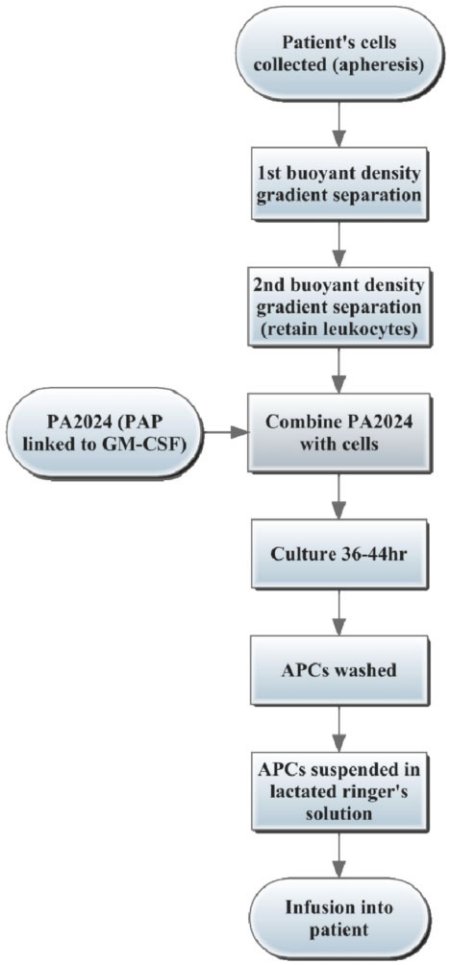

Figure 8. Patient-specific process for production of Dendreon's prostate cancer vaccine Provenge® (Sipuleucel-T) which involves obtaining a patient's leukocytes through apheresis, activating the leukocytes with PA2024, and returning them to the patient. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

induces a potent anti-tumor immune response. Peptide vaccine efficacy is determined by how the peptides are recognized and processed by the immune system. Specifically, peptide concentration, multi-valency, secondary structure, length, and the presence of helper T-cell epitopes can significantly affect the immune response.

Geron's autologous cancer vaccine candidate, GRNVAC1, is also a dendritic cell product (Dimond, 2010). It has been studied in PhI trials and currently is in a PhII study of acute myelogenous leukemia patients in remission. It is distinct from Provenge as Geron transfects immature dendritic cells through electroporation with mRNA encoding the sequences for the human telomerase catalytic subunit (hTERT) linked to a lysosomal targeting sequence (LAMP). The transfected cells express hTERT-LAMP as protein, which can be presented in the context of each patient's specific human leukocyte antigen. The transfected cells are then matured in medium containing a cytokine mix. Cryopreserved aliquots of the final mature DC preparations are ultimately supplied to the clinic for intradermal injections.

In January 2011, Amgen bought Biovex and acquired their lead product candidate OncoVEXGM-CSF, a therapeutic oncolytic vaccine for melanoma and head and neck cancer that is in PhIII clinical trials. OncoVEX GM-CSF is a version of herpes simplex virus which has been engineered to replicate selectively in tumors, leaving surrounding healthy tissues unharmed, and also engineered to express GM-CSF. This enhances the anti-tumor immune response to tumor antigens released following lytic virus replication.

Analytical

Process development for a vaccine occurs in parallel to the development of analytical methods. Techniques need to be developed for measuring the product and any impurities, in addition to assessing the potency of the final product. This topic is beyond the scope of this review but a few key points are included here.

Stringent regulatory requirements are often the basis for establishing a purification paradigm and accurate analytical techniques are essential for validating each step. Except for vaccines prepared in diploid human cells, residual host-cell DNA concentrations must be <10 ng per human dose and the size of residual DNA should be no more than the size of a gene (Board on Life Sciences, 2011). Host-cell protein concentrations also need to be controlled and new vaccines generally contain <1 μg per dose. There exist numerous assays that can reliably quantify protein concentrations including immunoassay, immunoblot, HPLC, enzyme activity, flow cytometry, and optical biosensors, although these assays cannot effectively determine the range of heterogeneity in key surface properties such as size, charge, and aggregation (Rathore et al., 2008, 2010). Changes in fermentation conditions during process development can impact intracellular aggregation, proteolysis, post-translational modifications, and differences in relative impurity levels, all of which can have a significant impact on downstream processing and the quality of the final product (Metz et al., 2009).

Tests for adventitious agents have gradually become more extensive and complex. Where possible, viral clearance filtration is included as a process step and part of risk mitigation for removal of adventitious agents. New approaches based on massively parallel sequencing of the transcriptome have been described as a technique for use in an integrated testing program for release of a cell line that utilizes both specific and broad-based detection methods (Onions et al., 2011).

A key vaccine release assay is the potency assay, which is, ideally, a predictor of clinical performance. Development of a potency assay needs to start as soon as representative vaccine material is available. Historically, many potency assays were developed using animals, but new vaccines typically rely on in vitro testing methods. There are essentially four different approaches to developing a potency assay: (1) immunization-challenge test, (2) serological analyses, (3) cell-based assays, and (4) titration assays. The immunization-challenge test requires labor-intensive and intrinsically variable animal models that are seldom used for newer vaccines. Serological analyses typically involve measuring antibody concentration, antibody avidity (surrogate marker for memory), or cellular immune responses (T-cell proliferation and cytokine quantification), but can also involve functional antibody assays (e.g., pathogen or toxin neutralizing). Antibody responses can be measured with ELISA or in multiplex assays that analyze for several antigens simultaneously in very small volumes (Metz et al., 2009). Cell-based assays detect cellular immune responses in T-cells, such as cytotoxic CD8+ and CD4+. Many T-cell quantification and functional characterization assays are based on flow cytometry, such as fluorescence-activated cell sorting (FACS) analysis of intracellular cytokines and effector molecules. A recent advance is the development of microsphere-based multiplex assays that quantify multiple soluble T-cell factors in small volumes. Titration assays measure potency indirectly, for example, by measuring number of viable particles, determined by colony counting or by virus titration, in the case of live-attenuated virus vaccines. In some cases, it may be feasible to use quantitative PCR, which is faster, less labor-intensive, and likely more accurate.

The development of appropriate potency assays is difficult because the exact epitopes that determine product quality are often not defined. In addition, adjuvants with only partially understood mechanisms of action have to be added to achieve the desired immune response. It is often best to develop "marker" assays for product quality that can be linked to animal responses, and after clinical trials, human responses. This information can be used to develop a better scientific understanding of the production process and its impact on product quality, as promoted by the FDA Process Analytical Technology (PAT) initiative, which is discussed

in the Globalization and Regulatory Influences section of this article.

Formulation and Delivery

Two of the main goals of formulation development are: (1) to improve the immunogenicity of a vaccine and (2) to improve the stability of the vaccine components. These goals are typically accomplished by implementing one or more of the following: adjuvant vehicles, excipients, binders, preservatives, carrier coupling, buffering agents, emulsifying agents, wetting agents, non-viral vectors, and transfection facilitating compounds (Jones, 2008).

One of the main challenges for recombinant protein-based vaccines is that the protein itself may be poorly immunogenic and many require the addition of an adjuvant to boost immune responses. Tested adjuvants include synthetic emulsions (oil-in-water), alum (aluminum hydroxide gel or aluminum phosphate), lipid formulations, and cytokine-based adjuvants like type-one interferon, with alum being the most widely used (Li et al., 2007). Adjuvants typically increase inflammation and activate the compliment cascade, possibly creating particulate multivalency, as well as allowing increased exposure of the antigen by lengthening its release into the injection site. In practice, the regulatory approach to novel adjuvants is conservative with a preference for those with a history of safe use (e.g., alum). Exceptions include MF59 (squalene emulsion), which has been used in more than 20 countries within the last decade as an adjuvant for influenza antigens HA and NA to enhance uptake by APCs, and monophosphoryl lipid A (MPL), which is a bacterial component lipopolysaccharide that acts on the highly expressed TLR-4 on APCs inducing cell-mediated immunity (Tritto et al., 2009). MPL is used in the GSK's HPV vaccine Cervarix® as a component of their proprietary Adjuvant System 04 (AS04).

A major formulation challenge for Gardasil® (Merck) was the preparation of an aqueous solution that was stable under a variety of purification, processing, and storage conditions (Shank-Retzlaff et al., 2006). To achieve this, non-ionic surfactants were introduced and the salt concentration was adjusted, which stabilized the VLPs against surface adsorption, conformational change, inducible antigen aggregation, and loss of antigenicity. The formulation for Gardasil® quadrivalent contains Merck Aluminum Adjuvant (MAA), 0.32 N NaCl, 0.01% polysorbate 80, 10 mM histidine, and pH 6.2, with disassembled and reassembled HPV VLPs (Shi et al., 2007). The MAA significantly enhanced accelerated stability of the vaccine against heat stress-induced degradation, believed to be due to the physical properties of the adjuvant, which serves as a physical barrier, preventing intermolecular collisions and thus minimizing aggregation of HPV VLPs. The disassembly and reassembly process was key to improving both the stability (3mo+ at 37°C) and the in vitro potency of the vaccine. In addition, the in vivo immunogenicity of the vaccine was improved by 10-fold as shown by mouse potency studies.

The limitations of live viruses or bacteria as vectors include their limited DNA carrying capacity, toxicity, immunogenicity, the possibility of random integration of the vector DNA into the host genome, and their high cost (Khatri et al., 2008; Mills, 2009). Due to this, non-viral vectors have been further focused as an alternative in delivery systems and include cationic polymers such as polyethylenimine (PEI), polylysine (PLL), cationic peptides, and cationic liposomes (Martin and Rice, 2007). Currently, the main goal is to optimize the transfection efficiency of the above options. The routes of administration and formulation of DNA vaccines affect the therapeutic response by altering the immune pathway; the highest efficacy was achieved after in vivo electroporation and gene gun delivery. One team applied electroporation to delivery of a DNA-based HIV vaccine and encouraging mice data are presented (Yan et al., 2011).

Another main goal of formulation and delivery design for vaccines intended for both the developed and the developing world is to improve convenience and patient accessibility through unrefrigerated transportation and storage, needle-free administration, and dose minimization (Aunins et al., 2011).

Due to the concern over the health effects, many vaccine manufacturers have moved away from the use of thimerosal as a preservative and, in general, no preservative is used for single dose vials or syringes. Some manufacturers are using 2-phenoxyethanol to prevent the growth of gram-negative bacteria in single-dose vaccines such as DTaP, Hepatitis A, and inactivated polio vaccine (Bae et al., 2009). The development of a multi-dose formulation for Prevnar 13™ using 2-Phenoxyethanol (2-PE) at a concentration of 5 mg per dose was stable and met recommended criteria for antimicrobial effectiveness over a 30-month period (Khandke et al., 2011).

Despite significant efforts during development to optimize excipients for product stabilization, many vaccines do not exhibit long-term stability in aqueous solutions, especially if they cannot be stored uninterrupted in cold temperatures (difficult in developing countries). For this reason, many vaccines are lyophilized to a powder formulation. Lyophilization, however, can alter a variety of product characteristics, including pH and solute concentration, which may adversely affect the vaccine antigens (Chen et al., 2010; Jennings, 2002). In addition, development time to establish the optimal lyophilization cycle can be lengthy and costly.

The traditional additives (lyoprotectants) for lyophilization of biological agents include sugars and sugar alcohols, which encase the proteins in glassy state structures providing a more rigid, thermodynamically stable state (Amorij et al., 2007; Mouradian et al., 1984). In some cases, polymers or amino acids have been combined with the carbohydrate formulations for improved stability in a continuous crystalline network, although this reportedly led to loss of

product in some situations (Zeng et al., 2009). Aridis pharmaceuticals has improved on this by developing a stabilization technology that uses small molecular weight plasticizers in combination with traditional sugar stabilizers to fill in any remaining molecular gaps, further reducing molecular vibrations that lead to degradation. They report that this technology increased the temperature required for storage and distribution from below freezing to 25°C with a shelf life of several years (Cicerone et al., 2003; Cicerone and Soles, 2004). Stabilitech has developed another stabilization technology involving the use of chemical excipients that mimic proteins found in various types of seeds during the desiccation stage (e.g., passion flower, chrysanthemum). From a panel of proprietary excipients, Stabilitech performs multivariate analyses to determine the optimal combination to provide the best stabilization. The use of these novel excipients during lyophilization resulted in minimal product loss for an adenovirus vaccine stored at 37°C for 3 months (Drew, 2011a,b).

Vaccine delivery by needle and syringe has several disadvantages including: potential needle-stick injuries and bloodborne pathogen transmission, needle phobia, and inefficient immunological response due to lower density of APCs relative to skin. There are several alternatives to intramuscular injection currently being explored. One is the Nanopatch™, a dry-coated microprojection array skin patch that delivers vaccines intracutaneously in approximately 5 min (Corbett et al., 2010). The needle density was optimized to target immune cells in the epidermis and dermis. Both Fluvax 2008® (unadjuvanted trivalent influenza vaccine) and Gardasil® (HPV vaccine with alum adjuvant) have been administered in mice using this technology, resulting in higher neutralizing antibody titers than intramuscular injection. Another team has made important incremental steps in the evolution of microneedle technology (Kim et al., 2009; Lee et al., 2008). Their first approach was based on stainless steel microneedles dipped into the vaccine, which has since evolved into a design that encapsulates molecules within microneedles. The microneedles subsequently dissolve within the skin for bolus or sustained delivery and do not leave behind biohazardous or sharp medical waste. Challenges include device manufacturing and designing a package that is compact yet robust enough for shipment.

The mucosal immune system is an attractive target site for vaccines since many pathogens infect the host at M-cells in the mucosal surface. Mucosal delivery, which does not require a needle, is already being used for several vaccines (Chadwick et al., 2009; Dennehy, 2007). For example, the live attenuated influenza vaccine FluMist® (MedImmune) is given as a nasal spray (Belshe et al., 1998). The mucosal route of delivery may contribute to the heterovariant cross-protection seen with both of these vaccines by inducing broader immunity, including mucosal immunoglobulin A. Mucosal delivery is also being studied for several other potential vaccines directed against diseases such as HIV infection and tuberculosis. Vaccination at mucosal surfaces can induce local protection at the infection sites, as well as inducing systemic immunity; however, direct antigen delivery through an oral or nasal route generally leads to weak induction of immunity. Efficacy can be improved by co-administering or fusing the antigen with a strong mucosal adjuvant, such as cholera toxin (CT) of *Vibrio cholera* or heat labile toxin subunit B (LTB) of *E. coli*. One team recombinantly produced LTB in soybean plants that stimulated the antibody response against a co-administered antigen by 500-fold (Moravec et al., 2007). This technology could serve as a cost-effective efficient platform for generating oral vaccines in the future.

Particle-mediated delivery systems can enhance mucosal vaccination by protecting immunogenic material during delivery, providing targeted delivery systems, and allowing incorporation of adjuvant material (Chadwick et al., 2009). Encapsulation into microspheres or a multi-phase systems such as water-oil-water multiple emulsions, or biologically-active polymers can shield the antigens from the gastrointestinal tract. The intestinal residence time can be extended using specific muco- or bio-adhesins that bind to the intestinal mucus or to the epithelial cells in the intestine.

The FluGEM™ vaccine from Mucosis (Groningen, The Netherlands) is an intranasal influenza vaccine, based on their Mimopath™ technology, which recently entered PhI trials. This technology improves vaccine potency via the formulation of *Lactococcus lactis* bacteria into non-living bacterial-like particles that can showcase pathogen or tumor antigens on their surface. Mucosis is also developing two additional vaccine products: PneuGEM™, a pneumococcal vaccine, and SynGEM™, a vaccine to prevent RSV viral infection.

Globalization and Regulatory Influences

An estimated 2.5 million people die globally each year from vaccine-preventable diseases, many of these attributable to available vaccines not being fully utilized. This underuse mainly occurs in developing countries due to low income, lack of medical infrastructure, and lack of cold chain facilities. Innovations that could improve this situation include: high-yield production technologies to reduce vaccine costs, improved adjuvants and combination vaccines that reduce the number of shots required, packaging that reduces the required space for a vaccine in the cold chain, and heat-stable formulations that allow for excursions from cold chain systems. These innovations would also benefit developed nations by reducing the healthcare cost burden and improving patient compliance.

The 2010 National Vaccine Plan (NVP) stresses the need for rapid, flexible, and cost-effective manufacturing methods (U.S. Department of Health & Human Services, 2010). Since the last NVP was issued in 1994, new vaccine supply concerns have arisen, including pandemic influenza and bioterrorism threats, which require surge capacity. Creating regional capacity will allow for fast and relatively

inexpensive vaccine production in times of need and eliminates many supply-chain issues. New approaches to vaccine manufacturing are needed, along with improved delivery methods, stability profiles, and quality testing procedures. That same year, Health and Human Services also allocated $2 billion to overhaul the pandemic response process. This overhaul will involve investment in regulatory science and review, a reassessment of policy, and a more effective use of public-private partnerships. The need for a regulatory overhaul is not only recognized by the United States, there is a critical worldwide need for improved access to vaccines (Milstien et al., 2006). This globalization of vaccines will also involve the slow and arduous processes of regulatory harmonization, widespread agreement on intellectual property rights, and the involvement of developing-country manufacturers in vaccine supply.

Many regulations are more stringent for vaccines than biotech products, largely because vaccine processes are perceived as not well characterized. This is due in part to the fact that vaccines involve many technology platforms, making it difficult to standardize production processes. This may lead to a unique facility for each vaccine family, resulting in a long lead-time for construction of a commercial-scale facility. Phase III clinical trials require doses made in large-scale manufacturing facilities to be representative of the final process. If process changes are made, clinical bridging studies are required to demonstrate equivalence. The impact of this is that sound process decisions must be made early in development. This leads to a higher level of risk, since an early large capital investment in facilities is required. This risk can be mitigated by the use of disposable or single-use technology.

The benefits of disposable or single-use bioreactors (SUBs) include lower facility capital and operation costs, shorter changeover times, increased productivity, lower risk of bacterial or mold contamination, and less scrap. They increase flexibility in the manufacturing suite, enabling multiple processes using the same equipment without cross-contamination risks or scale issues. GE manufactures Wave Bioreactors^{TM}, available in working volumes up to 500 L, which are typically used in process development and in seed train expansion during manufacturing. Several companies have developed stirred-tank SUBs in volumes up to 2,000 L including Xcellerex, Thermo Scientific (Marlborough, MA), and Sartorius Stedim (Goettingen, Germany). These stirred-tank SUBs have overcome the mass transfer limitations of wave-style disposable bioreactors and offer improved agitation, sparging, and feeding options that rival stainless steel bioreactors. This has enabled their use for growth of animal cells where mixing demands for oxygen transfer are modest, making them suitable for many vaccine applications. The stirred-tank SUBs can also be used for bacterial fermentation in some cases, although the higher energy input required for bacterial fermentation often necessitates cooling, making scale-up less straightforward than for animal cells. Despite their suitability for animal cell processes, the current volume limitations may hinder the wide-spread acceptance of SUBs in cases where large volumes are required, limiting their use to seed train expansion (Nienow, 2006). Many companies are incorporating single-use technologies into their production processes. For example, Bavarian Nordic (Kvistgaard, Denmark) manufactures a new investigational modified *vaccinia* Ankara smallpox vaccine in GE Wave bioreactor bags (Board on Life Sciences, 2011).

Several companies offer single-use mixers and downstream purification systems as well. Sartorius Stedim and Natrix (Burlington, Canada) both sell single-use, disposable anion-exchange membrane adsorption cartridges, which can be used for DNA and host cell protein removal or viral clearance. Likewise, Pall Corporation (Port Washington, NY) offers a similar disposable membrane product specifically designed for DNA removal. Other companies including GE Healthcare (Little Chalfont, UK), Millipore (Billerica, MA), BioFlash Partners (Worcester, MA), and Tarpon Biosystems (Marlborough, MA) have developed pre-packed and pre-sanitized disposable-format chromatography columns. Most of these columns were designed for polishing applications.

The last decade has seen a regulatory push towards enhanced process understanding and process control. New regulatory initiatives such as Process Analytical Technology (PAT), Quality by Design (QbD), and Real-time Release (RTR) will impact the future of vaccine process development, trending towards platform technologies. PAT is a regulatory framework of guidelines that encourages innovation, as it will be necessary to respond to the challenges of new technologies and ways of doing business (e.g., personalized medicine and pandemic response). Gains in quality, safety, and efficiency are expected to include: cycle time reduction with the use of on-, in-, or at-line measurements and controls, prevention of rejects, implementation of RTR, improved safety with increased use of automation, improved efficiency from continuous processing. These gains are expected to be realized by regulatory inspectors, manufacturers, and patients.

QbD involves creation of a design space that is a multidimensional combination of material attributes and process parameters that have been demonstrated to provide assurance of quality. Process changes within an approved design space do not require additional regulatory submissions and approval, allowing flexible processes. Creating a design space involves identifying process parameters, which can influence product quality and then performing a multivariate analysis to locate the ranges in which process parameters can be varied without affecting the product quality.

RTR involves basing the release of a product on process understanding rather than end-product testing or batch analysis. This requires identifying the critical process parameters (CPPs) and understanding their influence on the critical quality attributes (CQAs). During processing, a risk-based control strategy must be in place to ensure that

the process is maintained within the boundaries of the design space.

Streefland et al. have published several papers on applying the concepts of PAT and QbD to the *Bordetella pertussis* vaccine for whooping cough (Streefland et al., 2007, 2009; van de Waterbeemd et al., 2009). This bacterial whole-cell vaccine relies on conventional vaccine technology that is subject to inherent variability, yet the critical process parameters were still easily defined within a design space once the CQAs were understood. In this example, the main CQA was the outer-membrane protein composition and the CPPs were the dissolved oxygen level and the concentration of key nutrients lactate and glutamate. Near Infrared spectroscopy (NIR) was simultaneously used to monitor other process parameters, such as optical density and particle size, as a PAT application to allow RTR. Integrating and recording this data will build a more accurate design space over time.

Conclusion

The evolution of vaccines (e.g., live attenuated, recombinant) and vaccine production methods (e.g., in ovo, cell culture) are intimately tied to each other. As vaccine technology has advanced, the methods to produce and analyze vaccines have advanced in parallel. Drivers for technology innovation include the goal of making vaccines safer and more immunogenic in a cost effective way that allows for worldwide distribution. Advances in biology also result in the identification of new targets for vaccine research and these opportunities are often developed given the universal need and desire to improve human health and to gain protection from avoidable disease. These advances, along with improvements in disposable equipment design, are paving the way towards vaccines that are more accessible to developing countries and in pandemic outbreaks, as well as being more convenient and safe to use. As initiatives such as PAT and QbD gain widespread acceptance for highly purified vaccines, we can anticipate more streamlined regulatory approvals ultimately improving the global disease burden.

References

Aggarwal K, Jing F, Maranga L, Liu J. 2011. Bioprocess optimization for cell culture based influenza vaccine production. Vaccine 29(17):3320–3328.

Amorij JP, Meulenaar J, Hinrichs WLJ, Stegmann T, Huckriede A, Coenen F, Frijlink HW. 2007. Rational design of an influenza subunit vaccine powder with sugar glass technology: Preventing conformational changes of haemagglutinin during freezing and freeze-drying. Vaccine 25(35):6447–6457.

Amorij JP, Huckriede A, Wilschut J, Frijlink HW, Hinrichs WL. 2008. Development of stable influenza vaccine powder formulations, challenges and possibililties. Pharm Res 25:1256–1273.

Armstrong ME, Giesa PA, Davide JP, Redner F, Waterbury JA, Rhoad AE, Keys RD, Provost PJ, Lewis JA. 1993. Development of the formalin-inactivated hepatitis A vaccine, VAQTA, from the live attenuated virus strain CR326F. J Hepatol 18(2):S20–S26.

Aunins JG, Lee AL, Volkin DB. 2000. The biomedical engineering handbook, vol. II. In: Bronzino JD, editor. Vaccine production. Boca Raton, FL: CRC Press. p 1–16.

Aunins JG, Laska ME, Phillips BR, Otero JM. 2011. Chemical engineering perspectives on vaccine production. Chem Eng Prog 107(11):34–37.

Bae KD, Choi J, Jang Y, Ahn S, Hur B. 2009. Innovative vaccine production technologies: The evolution of value of vaccine production technologies. Arch Pharmacal Res 32(4):465–480.

Bakker WAM, Thomassen YE, van't Oever, Westdijk AG, van Oijen J, Sundermann MGCT, van't LC, Veld P, Sleeman E, van Nimwegen FW, Hamidi A, Kersten GFA, van den Heuvel N, Hendriks JT, van der Pol LA. 2011. Inactivated polio vaccine development for technology transfer using attenuated Sabin poliovirus strains to shift from Salk-IPV to Sabin-IPV. Vaccine 29(2):188–7196.

Barrett PN, Mundt W, Kistner O, Howard MK. 2009. Vero cell platform in vaccine production: Moving towards cell culture-based viral vaccines. Expert Rev Vaccines 8(5):607–618.

Belshe RB, Mendelman PM, Treanor J, King J, Gruber WC, Piedra P, Bernstein DI, Hayden FG, Kotloff K, Zangwill K, Iacuzio D, Wolff M. 1998. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine in children. N Engl J Med 338:1405–1412.

Board on Life Sciences. 2011. Protecting the frontline in biodefense research: The special immunizations program. Washington, DC: National Research Council.

Bolhassani A, Safaiyan S, Rafati S. 2011. Review: Improvement of different vaccine delivery systems for cancer therapy. Mol Cancer 10(1):1–3.

Bright RA. 2011. Review of new vaccine platforms and influenza vaccine pipeline. Influenza Division, Biomedical Advanced Research and Development Authority, United States Department of Health and Human Services (Presentation from http://www.who.int/influenza_vaccines_plan/resources/bright.pdf).

Buckland BC. 2005. The process development challenge for a new vaccine. Nat Med 11(4):S16–S19.

Carnes AE, Williams JA. 2007. Plasmid DNA manufacturing technology. Recent Pat Biotechnol 1(2):1–16.

Chadwick S, Kriegel C, Amiji M. 2009. Delivery strategies to enhance mucosal vaccination. Expert Opin Biol Ther 9:427–440.

Cheetham PSJ, Dunnill P, Lilly MD. 1982. The characterization and interconversion of three forms of cholesterol oxidase from *Nocardia rhodochrous*. Biochem J 201:515–521.

Chen XS, Garcea RL, Goldberg I, Casini G, Harrison SC. 2000. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. Molecular Cell 5:557–567.

Chen D, Kapre S, Goel A, Suresh K, Beri S, Hickling J, Jensen J, Lal M, Preaud JM, Laforce M, Kristensen D. 2010. Thermostable formulations of a hepatitis B vaccine and a meningitis A polysaccharide conjugate vaccine produced by a spray drying method. Vaccine 28:5093–5099.

Cicerone MT, Soles CL. 2004. Fast dynamics and stabilization of proteins: Binary glasses of trehalose and glycerol. Biophysical J 86(6):3836–3845.

Cicerone MT, Tellington A, Trost L, Sokolov A. 2003. Substantially improved stability of biological agents in dried form: The role of glassy dynamics in preservation of biopharmaceuticals. BioProc Int 6(1): 36–47.

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

Cooper D, Yu X, Sidhu M, Nahm MH, Fernsten P, Jansen KU. 2011. The 13-valent pneumococcal conjugate vaccine (PCV13) elicits cross-functional opsonophagocytic killing responses in humans to *Streptococcus pneumoniae* serotypes 6C and 7A. Vaccine 29(41):7207–7211.

Corbett HJ, Germain JP, Fernando JP, Chen X, Frazer IH, Kendall MAF. 2010. Skin vaccination against cervical cancer associated human papillomavirus with a novel micro-projection array in a mouse model. PLoS ONE 5(10):e13460.

Cox MMJ, Hollister JR. 2009. FluBlok, a next generation influenza vaccine manufactured in insect cells. Biologicals 37:182–189.

Cox MMJ. 2010. Preparing for the next pandemic: Rapid ''novel'' influenza vaccine product. The Vaccine Renaissance Conference IV. (Presentation: obtained personally).
Curling J, Gottschalk U. 2007. Process chromatography: Five decades of innovation. Biopharm Int 20(10):70–94.
Dekleva ML. 1999. Vaccine Technology. New York: Wiley & Sons. p 2611–2617.
Dennehy PH. 2007. Rotavirus vaccines: An update. Vaccine 25(16):3137–3141.
Dimond PF. 2010. What comes after dendreon's provenge? Genetic Engineering and Biotechnology News. Web 03 Mar 2011.
Ding Y, Chuan YP, He L, Middelberg APJ. 2010. Modeling the competition between aggregation and self-assembly during virus-like particle processing. Biotechnol Bioeng 107(3):550–560.
Division of Cell and Gene Therapies, Office of Cellular, Tissue, and Gene Therapies. 2010. CMC Review BLA 125197. web. 07 July 2011. Obtained from: http://www.fda.gov/downloads/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/UCM214287.pdf
Douek D, Nabel G. 2011. Vaccines. Immunol Rev 239:5–7.
Drew J. 2011a. A Broadly Applicable Stabilisation Technology for Vaccines and Biopharmaceuticals. web: 23 Nov 2011. obtained from: http://www.stabilitech.com/sites/default/files/Stabilitech_NonConf_Aug2011.pdf
Drew J. 2011b. Method for preserving polypeptides using sugar and polyethyleneimine. U.S. Patent 20110236412.
Eisenstein M. 2011. Vaccines: A moving target. Nature 474:S16–S17.
Extance A. 2011. Cell-based flu vaccines ready for US prime time. Nat Rev Drug Discov 10:246.
Frasch C. 2009. Preparation of bacterial polysaccharide–protein conjugates: Analytical and manufacturing challenges. Vaccine 27(46):6468–6470.
Freitas S, Canario S, Santos JA, Prazeres DMF. 2009. Alternatives for the intermediate recovery of plasmid DNA: Performance, economic viability and environmental impact. Biotechnol J 4:265–278.
Geels M, Ye K. 2010. Developments in high-yield system expressed vaccines and immunotherapy. Recent Pat Biotechnol 4(3):189–197.
Genzel Y, Reichl U. 2009. Continuous cell lines as a production system for influenza vaccines. Expert Rev Vaccines 8(12):1681–1692.
George M, Farooq M, Dang T, Cortes B, Liu J, Maranga L. 2010. Production of cell culture (MDCK) derived live attenuated influenza vaccine (LAIV) in a fully disposable platform process. Biotechnol Bioeng 106(6):906–917.
Gregersen JP, Schmitt HJ, Trusheim H, Bröker M. 2011. Safety of MDCK cell culture-based influenza vaccines. Future Microbiol 6(2):143–152.
Hagen AJ, Oliver CN, Sitrin RD. 1996. Optimization of Poly(ethylene glycol) precipitation of hepatitis A virus used to prepare VAQTA, a highly purified inactivated vaccine. Biotechnol Prog 12(3):406–412.
Hagen AJ, Oliver CN, Sitrin RD. 1997. Optimization and scale-up of solvent extraction in purification of hepatitis A virus (VAQTA®). Biotechnol Bioeng 56(1):83–88.
Hardy E, Martinez E, Diago D, Diaz R, Gonzalez D, Herrera L. 2000. Large-scale production of recombinant hepatitis B surface antigen from *Pichia pastoris*. J Biotechnol 77(2–3):157–167.
Hoare M, Levy MS, Bracewell DG, Doig SD, Kong S, Titchener-Hooker N, Ward JM, Dunnill P. 2005. Bioprocess engineering issues that would be faced in producing a DNA vaccine at up to 100 m^3 fermentation scale for an influenza pandemic. Biotechnol Prog 21(6):1577–1592.
Iaccino E, Schiavone M, Fiume G, Quinto I, Scala G. 2008. The aftermath of the Merck's HIV vaccine trial. Retrovirology 5:56.
Jennings TA. 2002. Lyophilization. In: Introduction and basic principles. Boca Raton, Florida: CRC Press. p 1–646.
Jones K. 2008. Biomaterials as vaccine adjuvants. Biotechnol Prog 24:807–814.
Kabanova A, Margarita I, Berti F, Romanoa MR, Grandia G, Bensia G, Chiarota E, Proietti D, Swennena E, Cappellettia E, Fontania P, Casinia D, Adamoa R, Pintoa V, Skibinskia D, Capoa S, Buffia G, Gallottaa M, Christ WJ, Campbell AS, Penac J, Seebergerd PH, Rappuolia R, Costantinoa P. 2010. Evaluation of a group a Streptococcus synthetic oligosaccharide as vaccine candidate. Vaccine 29(1):104–114.
Kazaks A, Balmaks R, Voronkova T, Ose V, Pumpens P. 2008. Melanoma vaccine candidates from chimeric hepatitis B core virus-like particles carrying a tumor-associated MAGE-3 epitope. Biotechnol J 3(11):1429–1436.
Kee GS, Pujar NS, Titchener-Hooker NJ. 2008. Study of detergent-mediated liberation of hepatitis B virus-like particles from *S. cerevisiae* homogenate: Identifying a framework for the design of future-generation lipoprotein vaccine processes. Biotechnol Prog 24(3):623–631.
Khandke L, Yang C, Krylova K, Jansen K, Rashidbaigi A. 2011. Preservative of choice for Prev(e)nar 13™ in a multi-dose formulation. Vaccine 29(41):7144–7153.
Khatri K, Goyal AK, Vyas SP. 2008. Potential of nanocarriers in genetic immunization. Recent Pat Drug Deliv Formul 2:68–82.
Kim YC, Quan FS, Yoo DG, Compans RW, Kang SM, Prausnitz MR. 2009. Improved influenza vaccination in the skin using vaccine coated microneedles. Vaccine 27(49):6932–6938.
Knuf M, Kowalzik F, Kieninger D. 2011. Comparative effects of carrier proteins on vaccine-induced immune response. Vaccine 29(31):4881–4890.
Kong S, Rock CF, Booth A, Willoughby N, O'Kennedy RD, Relton J, Ward JM, Hoare M, Levy SL. 2008. Large-scale plasmid DNA processing: Evidence that cell harvesting and storage methods affect yield of supercoiled plasmid DNA. Biotechnol Appl Biochem 51(1):43–51.
Lander RJ, Winters MA, Meacle FJ, Buckland BC, Lee AL. 2002. Fractional precipitation of plasmid DNA from lysate by CTAB. Biotechnol Bioeng 79(7):776–784.
Lee JW, Park JH, Prausnitz MR. 2008. Dissolving microneedles for transdermal drug delivery. Biomaterials 29(13):2113–2124.
Lee CH, Kuo WC, Beri S, Kapre S, Joshi JS, Bouveret N, LaForce FM, Frasch CE. 2009. Preparation and characterization of a highly immunogenic meningococcal group A conjugate vaccine for use in Africa. Vaccine 27(5):726–732.
Lees A, Sen G, LopezAcosta A. 2006. Versatile and efficient synthesis of protein-polysaccharide conjugate vaccines using aminooxy reagents and oxime chemistry. Vaccine 24(6):716–729.
Li S, Locke E, Bruder J, Clarke D, Doolan DL, Havenga MJE, Hill AVS, Liljestrom P, Monath TP, Naim HY, Ockenhouse C, Tang DCC, Van Kampen KR, Viret JF, Zavala F, Dubovsky F. 2007. Viral vectors for malaria vaccine development. Vaccine 25(14):2567–2574.
Lightfoot EN, Moscariello JS. 2004. Bioseparations Biotechnol Bioeng 87(3):259–273.
Liniger M, Zuniga A, Naim HY. 2007. Use of viral vectors for the development of vaccines. Exp Rev Vaccines 6:255–266.
Lis JT, Schleif R. 1975. Size fractionation of double-stranded DNA by precipitation with polyethylene glycol. Nucleic Acids Res 2(3):383–389.
Liu J, Shi X, Schwartz R, Kemble G. 2009. Use of MDCK cells for production of live attenuated influenza vaccine. Vaccine 27(46):6460–6463.
Maranga L, Aunins JG, Zhou W. 2005. Characterization of changes in PER.C6 trade mark cellular metabolism during growth and propagation of a replication-deficient adenovirus vector. Biotechnol Bioeng 90(5):645–655.
Martin ME, Rice KG. 2007. Peptide-guided gene delivery. AAPS J 9:18–29.
Martino M. 2011. Cancer vaccine market to hit $7B by 2015. FierceVaccines. web 18 July 2011.
Metz B, van den Dobbelsteen G, van Els C, van der Gun J, Levels L, van der Pol L, Rots N, Kersten G. 2009. Quality-control issues and approaches in vaccine development. Expert Rev Vaccines 8(2):227–238.
Middleberg APJ, Rivera-Hernandez T, Wibowo N, Lua LHL, Fan Y, Major G, Chang C, Chuan YP, Good MF, Batzloff MR. 2011. A microbial platform for rapid and low-cost virus-like particle and capsomere vaccines. Vaccine 29(41):7154–7162.
Mills KHG. 2009. Designer adjuvants for enhancing the efficacy of infectious disease and cancer vaccines based on suppression of regulatory T cell induction. Immunol Lett 122:108–111.
Milstien JB, Kaddar M, Kieny MP. 2006. The impact of globalization on vaccine development and availability. Health Aff 25(4):1061–1069.
Montagnon BJ, Fanget B, Vinvent-Falquet JC. 1984. Industrial scale production of inactivated polio virus prepared by culture of Vero cells on microcarrier. Rev Inf Dis 6(S2):S341–S344.

Moravec T, Schmidt MA, Herman EM, Woodford-Thomas T. 2007. Production of *Escherichia coli* heat labile toxin (LT) B subunit in soybean seed and analysis of its immunogenicity as an oral vaccine. Vaccine 25(9):1647–1657.

Mouradian R, Womerseley C, Crowe LM, Crowe JH. 1984. Preservation of functional integrity during long term storage of a biological membrane. Biochem Biophys Acta 778(3):615–617.

Murphy JC, Winters MA, Watson MP, Konz JO, Sagar S. 2005. Monitoring of RNA clearance in a novel plasmid DNA purification process. Biotechnol Prog 21:1213–1219.

Nienow AW. 2006. Reactor engineering in large scale animal cell culture. Cytotechnology 50:9–33.

Onions D, Cote C, Love B, Toms B, Koduri S, Armstrong A, Chang A, Kolman J. 2011. Ensuring the safety of vaccine cell substrates by massively parallel sequencing of the transcriptome. Vaccine 29(41): 7117–7121.

Parkin DM. 2006. The global health burden of infection-associated cancers in the year 2002. Int J Cancer 118:3030–3044.

Pejawar-Gaddy S, Finn O. 2008. Cancer vaccines: Accomplishments and challenges. Crit Rev Oncol Hematol 67:93–102.

Plummer E, Manchester M. 2010. Viral nanoparticles and virus-like particles: Platforms for contemporary vaccine design. Rev Nanomed Nanobiotechnol 3(2):174–196.

Polakova I, Pokorná D, Dušková M, Šmahel M. 2009. DNA vaccine against human papillomavirus type modifications of the E6 oncogene. Vaccine 28(6):1506–1513.

Price A. 2011. New technologies to meet the challenge of pandemic influenza. BioProc Int 9(8):40–45.

Rappuoli R. 1990. New generation vaccines. In: Woodrow GC, Levine MM, editors. New and improved vaccines against diphtheria and tetanus. New York: Marcel Dekker, Inc. p 251–264.

Rathore AS, Yu M, Yeboah S, Sharma A. 2008. Case study and application of process analytical technology (PAT) towards bioprocessing: Use of on-line high-performance liquid chromatography (HPLC) for making real-time pooling decisions for process chromatography. Biotechnol Bioeng 100(2):306–316.

Rathore AS, Parr L, Dermawan S, Lawson K, Yuefeng L. 2010. Large scale demonstration of a process analytical technology application in bioprocessing: Use of on-line high performance liquid chromatography for making real time pooling decisions for process chromatography. Biotechnol Prog 26(2):448–457.

Rinaudo CD, Telford JL, Rappuoli R, Seib KL. 2009. Vaccinology in the genome era. J Clin Invest 119(9):2515–2525.

Roy P, Noad R. 2008. Virus-like particles as a vaccine delivery system. Human vaccines 4:5–12.

Schuster SJ, Neelapu SS, Gause BL, Muggia FM, Gockerman JP, Sotomayor EM, Winter JN, Flowers CN, Stergiou AM, Kwak LW. 2009. Idiotype vaccine therapy (BiovaxID) in follicular lymphoma in first complete remission: Phase III clinical trial results. J Clin Oncol 27(abstr2):18s.

Shank-Retzlaff ML, Zhao Q, Anderson C, Hamm M, High K, Nguyen M, Wang F, Wang N, Wang B, Wang Y, Washabaugh M, Sitrin R, Shi L. 2006. Evaluation of the thermal stability of GARDASIL®. Hum Vaccin 2(4):147–154.

Shaw AR. 2006. Alternative methods of making influenza vaccines. In: Engineering and vaccine production for an influenza pandemic. NAE: The Bridge. 36(3).

Shi L, Sings HL, Bryan JT, Wang B, Wang Y, Mach H, Kosinski M, Washabaugh MW, Sitrin R, Barr E. 2007. GARDASIL®: Prophylactic human papillomavirus vaccine development—From bench top to bedside. Clin Pharmacol Ther 81(2):259–264.

Singh R, Kostarelos K. 2009. Designer adenoviruses for nanomedicine and nanodiagnostics. Trends Biotechnol 27:220–229.

Singhvi R. 2010. Manufacturing Enabled Global Influenza Vaccine Solution. All Things Considered, Arlington, VA (Presentation: obtained from R. Singhvi).

Stadler J, Lemmens R, Nyhammar T. 2004. Plasmid DNA purification. J Gene Med 6:S54–S66.

Streefland M, van de Waterbeemd B, Happe H, van der Pol LA, Beuvery EC, Tramper J, Martens DE. 2007. PAT for vaccines: The first stage of PAT implementation for development of a well-defined whole-cell vaccine against whooping cough disease. Vaccine 25(16):2994–3000.

Streefland M, Van Herpen PFG, Van de Waterbeemd B, Van der Pol LA, Beuvery EC, Tramper J, Martens DE, Toft M. 2009. A practical approach for exploration and modeling of the design space of a bacteria vaccine cultivation process. Biotechnol Bioeng 104(3):492–504.

Stuve O, Eagar TN, Frohman EM, Cravens PD. 2007. DNA plasmid vaccination for multiple sclerosis. Arch Neurol 64(10):1385–1386.

Subramanian S, Kim JJ, Harding F, Altaras GM, Aunins JG, Zhou W. 2007. Scaleable production of adenoviral vectors by transfection of adherent PER. C6 cells. Biotechnol Prog 23(5):1210–1217.

Sureau P. 1987. Rabies vaccine production in animal cell cultures. Adv Biochem Eng Biotechnol 34:112–125.

Thiem VD, Lin FYC, Canh DG, Son NH, Anh DD, Mao ND, Chu C, Hunt SW, Robbins JB, Schneerson R, Szu SC. 2011. The Vi conjugate typhoid vaccine is safe, elicits protective levels of IgG anti-Vi, and is compatible with routine infant vaccines. Clin Vaccine Immunol 18(5):730–735.

Tritto E, Mosca F, Gregorio ED. 2009. Mechanism of action of licensed vaccine adjuvants. Vaccine 27(25–26):3331–3334.

Tsai CW, Duggan PF, Shimp RL, Miller LH, Narum DL. 2006. Overproduction of Pichia pastoris or Plasmodium falciparum protein disulfide isomerase affects expression, folding and O-linked glycosylation of a malaria vaccine candidate expressed in *P. pastoris*. J Biotechnol 121:458–470.

U.S. Department of Health & Human Services. 2010. Protecting the Nation's Health through Immunization: 2010 National Vaccine Plan. web 23 July 2011.

van de Waterbeemd B, Streefland M, Pennings J, van der Pol L, Beuvery C, Tramper J, Martens D. 2009. Gene-expression based quality scores indicate optimal harvest point in *Bordetella pertussis* cultivation for bacterial vaccine production. Biotechnol Bioeng 103(5):900–908.

Wang Z, Le G Shi Y, Wegrzyn G. 2002. Studies on recovered plasmid DNA from *Escherichia coli* by heat treatment. Process Biochem 38:199–206.

Wang K, Holtz KM, Anderson K, Chubet R, Mahmoud W, Cox MMJ. 2006. Expression and purification of an influenza hemagglutinin—One step closer to a recombinant protein-based influenza vaccine. Vaccine 24(12):2176–2185.

Wenger MD, DePhillips P, Price CE, Bracewell DG. 2007. An automated microscale chromatographic purification of virus-like particles as a strategy for process development. Biotechnol Appl Biochem 47(2):131–139.

Williams JA, Luke J, Langtry S, Anderson S, Hodgson CP, Carnes AE. 2009. Generic plasmid DNA production platform incorporating low metabolic burden seed-stock and fed-batch fermentation processes. Biotechnol Bioeng 103(6):1129–1143.

Winters MA, Richter JD, Sagar SL, Lee AL, Lander RJ. 2003. Plasmid DNA purification by selective calcium silicate adsorption of closely related impurities. Biotechnol Prog 19:440–447.

Xie L, Metallo C, Warren J, Pilbrough W, Peltier J, Zhong T, Pikus L, Yancy A, Leung J, Aunins JG, Zhou W. 2003. Large-scale propagation of a replication-defective adenovirus vector in stirred-tank bioreactor PER.C6™ cell culture under sparging conditions. Biotechnol Bioeng 83(1):45–52.

Yan J, Corbitt N, Pankhong P, Shin T, Khan A, Sardesai NY, Weiner DB. 2011. Immunogenicity of a novel engineered HIV-1 clade C synthetic consensus-based envelope DNA vaccine. Vaccine 29(41):7173–7181.

Young D. 2011. BARDA bestows $215 million flu vaccine contract on Novavax, VaxInnate. Scrip Clinical Research. web: 09 July 2011. http://www.scripintelligence.com/home/BARDA-bestows-215-million-flu-vaccine-contract-on-Novavax-VaxInnate-312018

Zeng Y, Fan H, Chiueh G, Pham B, Martin R, Lechuga-Ballesteros D, Truong VL, Joshi Sangeeta, Middaugh CR. 2009. Towards development of stable formulations of a live attenuated bacterial vaccine: A preformulation study facilitated by a biophysical approach. Hum Vaccin 5(5):322–331.