Recombinant virus-like particle-based vaccines are composed of viral structural proteins and mimic authentic native viruses but are devoid of viral genetic materials. They are the active components in highly safe and effective vaccines for the prevention of infectious diseases. Several expression systems have been used for virus-like particle production, ranging from Escherichia coli to mammalian cell lines. The prokaryotic expression system, especially Escherichia coli, is the preferred expression host for producing vaccines for global use. Hecolin, the first licensed virus-like particle vaccine derived from Escherichia coli, has been demonstrated to possess good safety and high efficacy. In this review, we focus on Escherichia coli-derived virus-like particle based vaccines and vaccine candidates that are used for prevention (immunization against microbial pathogens) or disease treatment (directed against cancer or non-infectious diseases). The native-like spatial or higher-order structure is essential for the function of virus-like particles. Thus, the tool box for analyzing the key physicochemical, biochemical and functional attributes of purified virus-like particles will also be discussed. In summary, the Escherichia coli expression system has great potentials for producing a range of proteins with self-assembling properties to be used as vaccine antigens given the proper epitopes were preserved when compared to those in the native pathogens or disease-related target molecules.

**INTRODUCTION**

Vaccination is the most efficient way to control and prevent infectious diseases. Currently, the majority of licensed vaccines produced by traditional technologies are either live-attenuated or inactivated, although both may present safety issues (such as reversion to virulence and residual virulence).\(^1\) In the 1970s, scientists discovered that a single key protein from a virus could be a vaccine antigen.\(^2\) Almost a decade later, the first genetically engineered vaccine using recombinant gene expression technology was produced in the prokaryotic microbe.\(^3\) With the advent of modern molecular biology, recombinant subunit vaccines have flourished in human vaccinology. Virus-like particles (VLPs) are composed of the virion-building proteins of a virus and spontaneously self-assemble into particles without incorporating the infective viral genome.\(^5\) Thus, VLPs are extremely promising vaccine candidates due to their native-like and non-infective properties. VLPs can induce both innate and adaptive immune responses and have shown to be highly immunogenic in animals and humans.\(^5\)\(^,\)\(^6\) The approved VLP-based vaccines have been produced in yeast,\(^7\)\(^,\)\(^8\) insect, bacteria Escherichia coli (E. coli),\(^9\)\(^,\)\(^10\) plant, and mammalian cells,\(^11\)\(^,\)\(^12\) ranging from prokaryotic to eukaryotic expression systems.

Bacteria, especially E. coli, have been widely used for producing recombinant proteins. The first recombinant pharmaceutical approved for the treatment of diabetes, recombinant human insulin (Humulin-US/Humuline-EU), was obtained from E. coli.\(^13\) Numerous recombinant protein-based products derived from E. coli have been approved for therapeutic use, including cytokines, hormones, growth factors, serine proteases, and fusion proteins.\(^14\) The food and drug administration and European medicines agency have approved 151 protein-based recombinant drugs, 45 (29.8%) of which are produced using products derived from E. coli.\(^15\) From 2010 to July 2014, almost 33% of the approved recombinant biopharmaceutical in the United States and EU were obtained from Chinese hamster ovary cells, while 29% and 16.5% were obtained from E. coli and yeast, respectively.\(^6\) Thus, E. coli is still a widely used host for the production of protein-based biopharmaceuticals. Of the 174 different types of VLPs successfully produced, approximately 28% were produced in bacterial systems, 20% in yeast systems and 28% in insect systems.\(^17\) As of 2015, over 50 VLP-based vaccines or vaccine candidates, derived from different expression hosts, have been licensed or are under clinical development (Fig. 1, Supplementary Table 1S). Hecolin was the first VLP-based vaccine against hepatitis E virus (HEV) obtained from E. coli and was licensed by the FDA of China, or CFDA, in 2011.\(^18\)\(^,\)\(^19\) Currently, scientists in academia and industry are actively seeking ways to produce more cost-effective VLP-based vaccines, particularly low-cost vaccines for distribution in developing countries.

VLP-based vaccines are pre- eminent candidates for vaccination because of their high immunogenicity and good safety performance.\(^5\)\(^,\)\(^20\) VLP-based vaccines derived from E. coli are more cost-effective than those derived from insect cells or yeasts during industrial production. However, only one VLP-based vaccine, Hecolin, fully derived from E. coli has been approved for use in humans.\(^20\) Currently, a number of recombinant specific E. coli strains have been developed with the intention of achieving high-yield and high-quality protein production. Proper folding that allows the formation of specific structures is essential for the
HEV vaccine

The HEV, the causative agent of hepatitis E, is the sole member of the genus *Hepeivirus* within the family *Hepeviridae* and transmits primarily in a fecal-oral manner. HEV infection is a serious threat to public health, especially in developing countries. Mammalian HEV is classified into four major genotypes, but only one serotype. This opens up the opportunity for the development of a univalent, broad-spectrum HEV vaccine. HEV is a 34-nm, non-enveloped, positive-sense single-stranded RNA icosahedral virus with an approximately 7.2-kb genome containing three open reading frames (ORFs). These ORFs encode a number of different proteins for various biological functions, among which ORF2 (660 amino acid) encodes the sole capsid protein, pORF2.30

Hecolin, the first prophylactic hepatitis E vaccine, was licensed in 2011 and launched in 2012 in China.19 It is also the world’s first *E. coli*-derived VLP-based vaccine synthesized on a commercial scale.10 The neutralizing and immunodominant epitopes from HEV genotype 1 were present on the surface of p239 VLPS.3 The high efficacy of the HEV vaccine was demonstrated by a randomized, double-blind, placebo-controlled phase III clinical trial; a follow-up study subsequently demonstrated long-term efficacy of up to 4.5 years after the initial vaccination.12 Product consistency was demonstrated through comprehensive characterization of antigens in Hecolin. The comparable p239 VLPS characteristics of the antigen produced at different scales indicated that the antigen manufacturing process was robust and scalable.33 The vaccine contains 30-μg truncated capsid protein formulated with aluminum adjuvants.10 Zhang et al. have demonstrated the...
| Vaccine name       | Company/Institution                                      | VLP platform | Vaccine antigen                                                                 | Clinical Trial/Approved | Reference or clinical trial identifier (NCT) * |
|-------------------|---------------------------------------------------------|--------------|--------------------------------------------------------------------------------|----------------------------|-----------------------------------------------|
| **Prophylactic vaccines** |                                                          |              |                                                                                  |                            |                                               |
| HEV Hecolin       | Xiamen Innovax Biotech Co., Ltd (Xiamen, China)         | HEV          | HEV capsid polypeptide                                                           | Licensed                  | 18, 19                                        |
| HPV HPV16/18      | Xiamen University, Xiamen Innovax Biotech Co., Ltd      | HPV          | HPV16/18 L1 major capsid protein                                                 | Phase III                 | NCT01735006                                  |
| HPV6/11           | Beijing Wantai Biological Pharmacy Enterprise Co., Ltd (Beijing, China) | HPV          | HPV6/11 L1 major capsid protein                                                  | Phase II                  | NCT02710851                                  |
| ACAM-FLU-Aa       | Sanofi Pasteur                                          | HBcAg        | Influenza A M2e                                                                   | Phase I                   | NCT00819013                                  |
| gH1-Qbetaa        | A*STAR and Cytos Biotechnology                          | Bacteriophage Qj | globular head domain (gH1) of haemagglutinin (HA)                             | Phase I                   | 61                                            |
| MalariVax (ICC-1132)a | Apovia                                               | HBcAg        | *Plasmodium falciparum* circumsporozoite protein                                | Phase I                   | NCT00587249                                  |
| **Therapeutic vaccines** |                                                          |              |                                                                                  |                            |                                               |
| HBV AX203 (HeberNasvac)b | The Center for genetic Engineering and Biotechnology, Cuba | HBV          | HBsAg/HBcAg                                                                     | Licensed                  | 65, 66                                        |
| Allergic rhinitis and asthma | Cytos Biotechnology                                    | Bacteriophage Qj | G10 (CpG DNA)                                                                  | Phase II                  | NCT00890734                                  |
| Malignant melanoma | Cytos Biotechnology                                    | Bacteriophage Qj | Melan-4, G10 DNA (CpG)                                                          | Phase II                  | NCT00651703                                  |
| Alzheimer’s disease | Cytos Biotechnology                                    | Bacteriophage Qj | Aβ1-6 epitope                                                                    | Phase II                  | NCT01097096                                  |
| Hypertension      | Cytos Biotechnology                                    | Bacteriophage Qj | Angiotensin II                                                                   | Phase II                  | NCT0050786                                   |
| Nicotine addiction | Cytos Biotechnology                                    | Bacteriophage Qj | Nicotine hapten                                                                  | Phase II                  | NCT01280968                                  |
| Type II diabetes mellitus | Cytos Biotechnology                                   | Bacteriophage Qj | IL-1β                                                                            | Phase I                   | NCT00924105                                  |

*References or NCT numbers (registered at https://clinicaltrials.gov) are provided. Chimeric VLP-based vaccines: VLPs as vaccine platforms display heterologous epitopes or antigens on their surface by the way of genetic fusion or chemical conjugation. Hepatitis B virus surface antigen (HBsAg) and hepatitis B core antigen (HBcAg), were expressed in yeast (Pichia pastoris) and E. coli, respectively.
cost, are thus excluded low-income regions, where cervical vaccines, all produced in eukaryotic systems with high production for structural and functional characterization of VLPs. Biochemical: matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) subsequently into VLPs. Currently, three prophylactic HPV vaccines are based on VLPs, Gardasil-4 (a quadravalent HPV16/18/6/11 vaccine produced in yeast), Gardasil-9 (a 9-valent HPV16/18/31/33/45/52/6/11 HPV vaccine produced in yeast), and Cervarix (a bivalent HPV 16/18 vaccine expressed via insect cells). Papillomavirus virions like epitopes are present on the surface of (a bivalent HPV 16/18 vaccine expressed via insect cells). The mini-VLP in the figure is the structure mode of HPV59, which was adapted from Structure, Li et al. HPV vaccines HPVs, non-enveloped double-stranded DNA viruses, are the causative agents of cervical cancer. Papillomavirus virions consist of two structural proteins, L1 and L2; the major structural protein is L1, which is able to self-assemble into pentamers and subsequently into VLPs. Currently, three prophylactic HPV vaccines are based on VLPs, Gardasil-4 (a quadravalent HPV16/18/6/11 vaccine produced in yeast), Gardasil-9 (a 9-valent HPV16/18/31/33/45/52/6/11 HPV vaccine produced in yeast), and Cervarix (a bivalent HPV 16/18 vaccine expressed via insect cells). Clinical trials have shown that all three vaccines consistently induced production of protective and neutralizing antibodies to prevent infection. These vaccines are generally well tolerated. However, their high production and delivery costs are significant barriers to worldwide implementation. The globally licensed HPV vaccines, all produced in eukaryotic systems with high production cost, are thus excluded low-income regions, where cervical cancer results in higher mortality. Thus, there is a pressing need for more cost-effective vaccines.

Xiamen Innovax Biotech has used E. coli to produce a low-cost HPV vaccine based on L1 VLPs. L1 is the HPV major structural protein (the other minor capsid protein is L2). A bivalent HPV vaccine (Types 16, 18), based on these VLPs, has been developed and has been shown to be safe and highly immunogenic in preclinical studies. The data indicated that HPV16/18 VLPs were obtained from a prokaryotic expression system with desired immunogenicity. The results of a phase I safety trial showed that the E. coli expressed recombinant HPV 16/18 bivalent vaccine candidate is well tolerated in healthy women, with just few minor adverse events attributable to the vaccination were observed. The immunogenicity of vaccine was demonstrated in healthy young women in a phase II clinical trial. A large-scale phase III efficacy trial was initiated in November 2012 in China (NCT01735006). Additionally, another bivalent HPV vaccine candidate (Types 6, 11) obtained from E. coli is currently undergoing a phase II clinical trial (NCT02710851) (Table 1). The success of HPV L1 VLPs and HEV p239 obtained from E. coli indicates that this microbe-based vaccine technology may facilitate the development of cost-effective vaccines and bring benefits to people in developing countries.

Other prophylactic vaccines VLPs have been used as a vaccine delivery platform to increase the immunogenicity of antigens. Several chimeric VLP vaccine candidates are listed in Table 1. In addition to the HEV vaccine and HPV vaccines mentioned above, a malaria vaccine and two
influenza VLP-based vaccines expressed by the E. coli system were reported.

**Malaria vaccine.** Malaria, caused by the *Plasmodium* parasite, is a serious public health problem in the tropics.51 There is no highly effective vaccine for malaria.52 A chimeric VLP-based vaccine candidate, Malarivax (ICC-1132), consists of a hepatitis B virus core VLPs produced in *E. coli*, displaying malaria epitopes (the *Plasmodium falciparum* circumsporozoite) on their surface. The results of a phase I trial showed clinical efficiency against malaria parasites.53 No subsequent clinical data were published.

**Influenza vaccines.** Due to viral drifts and shifts, a particular influenza vaccine cannot provide long-term immunity.54 A microbial platform may rapidly provide a vaccine to combat seasonal influenza epidemics.55 The anti-influenza A M2e-HBc vaccine candidate, ACAM-FLU-A, was produced by *E. coli*. Recombinant hepatitis B core antigen (HBcAg), as a carrier VLP, is one of the main structural antigens of HBV.56 The M2 external domain is a relatively conserved epitope in both human and avian influenza A viruses that is present on the surface of HBcAg VLPs. The immunogenicity has been confirmed in a phase I clinical trial (NCT00819013).57 In addition, globular head domain (gH1)-Qbeta, a fully bacterially produced influenza vaccine, was obtained by chemically conjugating the gH1 of hemagglutinin (HA) from the pandemic A/California/07/2009(H1N1) influenza strain to the Qbeta VLPs. A phase I trial has demonstrated that gH1-Qbeta was able to induce high titer of anti-viral antibodies with a favorable safety profile.58

Therapeutic vaccines in clinical trials for human diseases

A combination vaccine used for chronic hepatitis B treatment, ABX203 (trade name HeberNasvac), is composed of hepatitis B virus surface (HBsAg) and core antigens (HBcAg), which are expressed in *Pichia pastoris* and *E. coli*, respectively.59 ABX203 has been shown to be effective and well tolerated in clinical trials.60 The Cuban regulatory authorities granted the Center for genetic Engineering and Biotechnology their first marketing authorization application for ABX203 in 2015.61 Additionally, a number of chimeric VLP vaccine candidates, chemically conjugated antigens to the RNA bacteriophage QB VLPs derived from *E. coli*, have been developed by Cytos Biotechnology AG (Switzerland) (Table 1). These chimeric VLP vaccine candidates are designed to target non-infectious diseases such as nicotine addiction, hypertension, cancer, diabetes, allergies, and Alzheimer’s disease. Results showed that the use of nicotine-Qβ VLPs, such as NicO02 (formerly CYT002-Nic002), have promoted long-term abstinence from smoking.62, 63 Similarly, a Qβ VLP conjugated with a modified Ang II peptide, CYT006-AngQβ, were developed as an anti-hypertensive vaccine.64 Additionally, CYT004-MelQB10 (NCT00651703), CYT103-IL1bQβ (NCT00924105), and CYT003- QB10 (NCT00890734), which are directed against malignant melanoma, Type II diabetes, allergic rhinitis, and asthma, respectively, are currently in various stages of clinical trials. CAD-106, in which Qβ VLP is covalently coupled to the Αβ1-6 peptide, is an immunotherapeutic vaccine for Alzheimer’s disease currently undergoing Phase II trials.65, 66

In addition, many *E. coli*-derived VLP-based vaccine candidates, against West Nile virus, foot-and-mouth disease virus, and hepatitis C virus also have been developed in preclinical studies. The potency of these *E. coli*-derived VLP antigens has been demonstrated in different animal models. The efficacy and safety of a vaccine need to be demonstrated for licensing in human use.67, 68 Post licensure, the quality of vaccines during manufacturing and storage should be assessed to ensure their safety and efficacy throughout the life cycle management of vaccine commercialization. Structural and functional assessment of VLPs is the most critical antigen characterization assays for recombinant protein based vaccines.

**STRUCTURAL AND FUNCTIONAL ASSESSMENT OF VLPs**

*E. coli*-derived HEV p239 VLPs and HPV VLPs consist only of the viral capsid protein without incorporating genetic materials but retain a conformation similar to that of the native virus.59, 60 Generation of functional antibodies is dependent on the correct antigen conformation and native-like epitopes being present on the surface of VLPs.70 VLPs containing virion-like epitopes can be acquired via antigen-presenting cells and then induce a protective humoral immune response.60, 61 Thus, recombinant VLPs must be correctly folded to ensure their function by inducing a protective humoral immune response. The spatial or higher-order structure of the vaccine antigen is the basis of the various biological functions of protein-based vaccines. Quantitative analysis of the functional epitopes on VLPs using monoclonal antibody-based assays can be an advantageous way to ensure vaccine safety and efficacy.61 Multifaceted analytical approaches, such as biochemical, biophysical, and immunochimical methods (Fig. 2), have been well established and are widely used for the evaluation of three different licensed recombinant VLP-based vaccines: hepatitis B vaccine, hepatitis E vaccine, and HPV vaccine.62 The identification of the primary structure indicated that the target protein composed of VLPs was successfully expressed. Biochemical characterization includes the primary amino acid sequence, molecular weight, isoelectric point, and purity of the VLPs.70, 71, 72 The secondary and tertiary structures of the VLPs can be measured by circular dichroism and ultraviolet spectroscopy.63 Mass spectrometry is an indispensable analytical technique used to determine the mass of proteins and their amino acid composition.64, 65 This tool is useful for process monitoring and demonstrating final product consistency at single amino acid level. SDS-PAGE, the most common method that used to determine the purity, integrity and molecular weight of the purified antigen.65, 66 The morphological characteristics and the state of the VLP are amenable to imaging by transmission electron microscopy (TEM).67, 68, 69 Functional epitopes have been applied for assessment of the antigenicity of HBV, HPV, and HEV VLPs using a panel of specific monoclonal antibodies.92, 93 Monoclonal antibodies have been developed as specific probes to identify and characterize virion-like epitopes.34, 94, 95 Binding activity to a certain neutralizing probe can serve as an excellent surrogate marker for in vivo immunogenicity or vaccine efficacy. Currently, various immunoassays have been applied for assessment of the antigenicity of HBV, HPV, and HEV VLPs using a panel of specific and functional monoclonal antibodies (Fig. 2). These methods include one-site binding and label-free SPR technology. The solution competition ELISA (IC50) and sandwich ELISA are critical in vitro relative potency assays (IVRP assays) generally is a sandwich-type
immunoassay that uses neutralizing monoclonal antibodies to measure the concentration of functional epitopes in the vaccine sample. The IVRP assay has been shown to have a good correlation with mouse potency in Gardasil-4. Thus, mouse potency can be replaced by IVRP for release and stability testing, as well as monitoring of the production process.

**DISCUSSION**

VLPs have been widely used in vaccinology. The next generation of VLP-based vaccine candidates must be creative in form and function to satisfy diverse needs. Several viral structures produced in *E. coli*, such as HBCAg, AP250, murine polyomavirus and HPV, have been used for vaccine platforms. Vaccinologists can now add heterologous epitopes or antigens to these VLPs from different origins achieved by genetically fusing or chemical conjugation. Middelberg et al. have developed in vitro cell-free assembly of modular VLPs based on murine polyomavirus capsid proteins expressed in *E. coli* as vaccine carriers to enhance immune responses, especially to weakly or non-immunogenic antigens. These modular VLPs have potential for use as a vaccine platform to increase the efficacy and stability and to allow for more versatile display of antigens. Re-engineering or grafting epitopes in chimeric VLPs may widen the coverage spectrum compared to monovalent vaccines. Several chimeric VLP vaccine candidates have been developed by chemically conjugating foreign antigens to RNA bacteriophage Q8 VLPs obtained from *E. coli*. These chimeric VLP vaccines are currently in clinical development. The yield of Q8 VLP production in *E. coli* is higher than that in yeast. RNA bacteriophage VLPs naturally encapsidated ssRNA in *E. coli*, such that it could influence the immune bias when used in mouse immunizations, with a shift from IgG1 to IgG2a compared to VLPs without RNA, indicating that the Th1-based immune response has occurred. The Th1-type immune response is essential for the control of intracellular pathogens and could be an ideal platform for future prophylactic (malaria, HIV, Herpes viruses) and therapeutic vaccine applications (cancer and chronic hepatitis). VLPs derived from a given viral protein or as displaying bionanoparticles of foreign epitopes could enhance the immunogenicity of the B-cell epitopes on the particle surface or modulate the Th1- and/or Th2-immune response due to the the nature of the B-cell or T-cell epitopes built in via recombinant technology as well as the protein-based particles.

The commercial VLP-based vaccines have been constructed through eukaryotic or prokaryotic systems. A brief comparison among different expression systems with respect to their applications in producing recombinant VLPs has been summarized in Table 2. As a manufacturing platform, *E. coli* faced several obstacles, which may limit its application in protein-based biopharmaceuticals. Their limitation factors included: (1) lack of ability to produce the correct disulfide bonds, (2) fail to produce recombinant proteins with mammalian-like post-translational modifications; (3) the problems of protein solubility, and (4) the presence of endotoxins (lipopolysaccharide, LPS). Post-translational modifications play an important role in protein folding, processing, and stability, as well as final biological activity and even the immunogenicity of the protein. Disulfide bond formation, glycosylation, phosphorylation and proteolysis play a crucial role in biological activity of some recombinant proteins. *E. coli* cannot synthesize useful HBsAg particles, probably because of unfavorable environmental conditions, such as pH and redox potential, or lipid compositions within the bacterium. Studies have also shown that HBCAg phosphorylation is essential for viral replication and capsid formation. Therefore, the expressed proteins may be insoluble, unstable or inactive without post-translational modifications. *E. coli* stands out as the expression system for the production of small recombinant proteins without post-translational modifications. However, Spiess et al. developed an approach for the efficient generation of non-immunogenic, stable bspecific antibodies with a natural IgG architecture by co-culture of bacteria (*E. coli*) expressing two distinct half-antibodies. This technology provides a rapid generation of biospecific antibodies with natural architecture from any two existing antibodies for academic research and industrial development.

Currently, numerous mutant *E. coli* strains have been developed to improve the different protein expression (representative examples are shown in Table 3). Origami™ (DE3) is mutated in glutathione reductase and thioredoxin reductase to promote target protein disulfide bond formation. Several strategies were applied to solve the insolubility of proteins by adjusting culture
supports for recombinant VLP-based vaccines to ensure their analytical methods for antigen characterization provide important future vaccines via structure-based modular design. Different interests to a well-behaved VLP display vector are platforms for mimicking viral capsids or chimeric VLPs by grafting epitopes of facilitated the production of low-cost vaccines for global use. VLPs suggested that using microbial synthesis has the potentials to derived VLPs (Hecolin) as recombinant vaccine antigens suggested that using microbial synthesis has the potentials to derived VLPs (Hecolin) as recombinant vaccine antigens native-like epitopes during manufacturing, storage and transport.

In summary, several promising E. coli-derived VLP-based vaccines or vaccine candidates, directed against both infectious and non-infectious diseases, have been currently commercialized or are being developed in the clinical testing stage. The success of E. coli-derived VLPs (Hecolin) as recombinant vaccine antigens suggested that using microbial synthesis has the potentials to facilitate the production of low-cost vaccines for global use. VLPs mimicking viral capsids or chimeric VLPs by grafting epitopes of interests to a well-behaved VLP display vector are platforms for future vaccines via structure-based modular design. Different analytical methods for antigen characterization provide important supports for recombinant VLP-based vaccines to ensure their efficacy and safety, and most importantly the preservation of native-like epitopes during manufacturing, storage and transportation of the vaccines. Further improvements on the E. coli platform could be achieved by genetically modify the expression host for achieving certain specific goals, such as protein expression with post-translational modifications. Better understanding of protein production and self-assembly would facilitate scale up and better process control at commercial production scale. As a result, rapid and inexpensive VLP-based vaccine production could be realized for global accessibility.

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AUTHORS CONTRIBUTIONS

Q.Z. conceptualized the review and X.H., X.W., J.Z., N.X., and Q.Z. wrote the manuscript. All authors improved the manuscript before it was submitted by the corresponding author.

COMPETING INTERESTS

The authors declare no competing interest.

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