Received: 15 June 2018 | Revised: 8 November 2018 | Accepted: 29 November 2018

DOI: 10.1002/bit.26890

REVIEW

WILEY BIOTECHNOLOGY BIOENGINEERING

Synthetic biology for bioengineering virus-like particle vaccines

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Funding information
Australian Research Council, Grant/Award Number: DP160102915; European Commission, Grant/Award Number: 602640; Portuguese Fundação Fundação para a Ciência e a Tecnologia, Grant/Award Numbers: IF/01704/2014, EXPL/BBB-BIO/1541/2013, SFRH/BD/90564/2012

Abstract

Vaccination is the most effective method of disease prevention and control. Many viruses and bacteria that once caused catastrophic pandemics (e.g., smallpox, poliomyelitis, measles, and diphtheria) are either eradicated or effectively controlled through routine vaccination programs. Nonetheless, vaccine manufacturing remains incredibly challenging. Viruses exhibiting high antigenic diversity and high mutation rates cannot be fairly contested using traditional vaccine production methods and complexities surrounding the manufacturing processes, which impose significant limitations. Virus-like particles (VLPs) are recombinantly produced viral structures that exhibit immunoprotective traits of native viruses but are noninfectious. Several VLPs that compositionally match a given natural virus have been developed and licensed as vaccines. Expansively, a plethora of studies now confirms that VLPs can be designed to safely present heterologous antigens from a variety of pathogens unrelated to the chosen carrier VLPs. Owing to this design versatility, VLPs offer technological opportunities to modernize vaccine supply and disease response through rational bioengineering. These opportunities are greatly enhanced with the application of synthetic biology, the redesign and construction of novel biological entities. This review outlines how synthetic biology is currently applied to engineer VLP functions and manufacturing process. Current and developing technologies for the identification of novel target-specific antigens and their usefulness for rational engineering of VLP functions (e.g., presentation of structurally diverse antigens, enhanced antigen immunogenicity, and improved vaccine stability) are described. When applied to manufacturing processes, synthetic biology approaches can also overcome specific challenges in VLP vaccine production. Finally, we address several challenges and benefits associated with the translation of VLP vaccine development into the industry.

KEYWORDS
capsomere, computational, omics technologies, synthetic biology, vaccine, virus-like particle

1 | INTRODUCTION

Virus-like particles (VLPs) are self-assembling complexes of capsid proteins that mimic the overall structure of their parental virus. Void of viral genetic material, these noninfectious particles possess biologically desirable traits that are attributed to the particulate viral structure (Grgacic & Anderson, 2006; Pattenden, Middelberg, Niebert, & Lipin, 2005). Of particular interest is their efficient

recognition, cellular uptake, and processing by host immune systems. VLP technology is a rapidly expanding field, which aims to embrace such features to achieve specific biological outcomes. VLPs are amenable to a broad range of modifications including encapsulation, chemical conjugation, and genetic manipulation (Roldão, Mellado, Castilho, Carrondo, & Alves, 2010). This versatility of VLPs, combined with the natural ability to package and deliver nucleic acids has prompted their use as biodegradable delivery agents for gene therapy (Takamura et al., 2004; Tegerstedt, Franzen, et al., 2005). The successful packaging of peptides, proteins, and synthetic drugs into VLPs has revealed a prospective role in drug delivery (Kaczmarczyk, Sitaraman, Young, Hughes, & Chatterjee, 2011; Zdanowicz & Chroboczek, 2016). In the diagnostic field, VLPs containing gadolinium have shown potential as molecular imaging contrasting agents (Schwarz & Douglas, 2015) while the application of VLPs as research surrogates to study the clearance of live viruses has also been demonstrated (Johnson, Brorson, Frey, Dhar, & Cetlin, 2017; Loisy et al., 2005). At the forefront of VLP technology impact, however, is vaccinology.

Licensed prophylactic VLP vaccines, such as Gardasil®, Cervarix®, Hecolin®, and Porcilis PCV®, demonstrate that VLP vaccines are safe and effective. VLP technology can overcome numerous drawbacks associated with traditional methods of vaccine production; specifically, the infectious nature associated with live and inactivated vaccines and the lengthy production time. Synthetic biology, the redesign and construction of novel artificial biological organisms, pathways or processes, is revolutionizing vaccine production. When applied to VLPs, synthetic biology allows for more precise and predictable control over the composition and assembly of the viral capsid. This, in turn, widens the range of unrelated antigenic modules that can be incorporated. Consequently, vaccines can be engineered for prophylactic or therapeutic means (Jennings & Bachmann, 2009) to match specific viral strains (Schwartzman et al., 2015) or to generate multivalent (Pushko et al., 2011) or broadly cross-protective (Ben-Yedidia, 2011) vaccines. Moreover, new bioprocessing modalities including in vitro assembly (Chuan, Fan, Lua, & Middelberg, 2010) and cell-free expression (Bundy, Franciszkowicz, & Swartz, 2008), open the way to reimagined vaccine production processes.

2 | DESIGN TOOLS FOR MODERN VACCINES

With the ability to integrate biological data and computational analysis, synthetic biology has significantly contributed to move vaccine development beyond the constraints of Pasteur, assisting in the design of novel biological systems with enhanced efficacy and safety as well as reducing vaccine production times (Ruder, Lu, & Collins, 2011). With today's focus gearing towards using VLPs, not only as vaccines of unmodified viral assemblies against parental viruses but also as scaffolds for display of heterologous antigens (Lua et al., 2014; Roldão et al., 2010), synthetic biology has become a centerpiece in VLP vaccine engineering. Seconded by a multitude of tools, such as omics technologies, structural biology, system immunology, and bioinformatics and computational biology, one can now screen for pathogen-specific antigens with high immunogenic potential and apply that information to rationally design modern VLP vaccines (Figure 1).

2.1 | Omics technologies

Influenza is one of the best examples on how omics, more precisely genomics, is revolutionizing vaccine design. Dedicated databases detailing complete and accurate influenza genomic information have been created and can be readily accessed worldwide (McHardy & Adams, 2009). In the last decade, this vast wealth of data has been translated into knowledge, which, in conjunction with synthetic biology, has aided the design of VLP vaccine candidates (Prabakaran et al., 2010; Pushko et al., 2011; Pushko et al., 2017). These novel biological entities are undoubtedly safer with the potential to be more broadly reactive than their previous counterparts (i.e., traditional, commercially available live attenuated influenza vaccines).

Several genomic applications aid in the identification of novel antigens (Liao et al., 2017), namely, reverse vaccinology and comparative and functional genomics. Reverse vaccinology combines genomics with proteomics and bioinformatics to identify virtually all potentially protective antigens from coding regions within the genome (Bambini & Rappuoli, 2009; Rappuoli, 2007). Comparative genomics is primarily used to design broadly protective vaccines as it allows comparison of conserved and variable open reading frames within the same species. Functional genomics allows the identification of protein function based on reverse genetic evaluation (mutations and knockouts) or gene expression analysis (transcriptomics; Bagnoli et al., 2011; Bambini & Rappuoli, 2009; Rappuoli, 2007; Sette & Rappuoli, 2010). Although genomics per se has contributed significantly to the design of many vaccine candidates, such as influenza (Prabakaran et al., 2010; Pushko et al., 2011; Pushko et al., 2017), malaria (Draper et al., 2015; Y. Wu, Narum, Fleury, Jennings, & Yadava, 2015), and human immunodeficiency virus (HIV; Calazans, Boggiano, & Lindsay, 2017), its full potential for vaccine development can only be realized when integrated with proteomics and immunomics (Bagnoli et al., 2011). Moreover, the coordinated deployment of these omics technologies along with miniaturized bioprocess screening in, for example, microbioreactor and microfluidic cell culture systems can aid developability assessment leading to accelerated manufacture (Bhambure, Kumar, & Rathore, 2011; Gong & Lei, 2014; Hemmerich, Noack, Wiechert, & Oldiges, 2018).

Immunomics specifically addresses the interface between the host immune system and the pathogen proteome. It studies the subset of pathogen-derived proteins or epitopes that are recognized by the host immune system. This information can be used to validate antigens identified from in silico and/or in vitro approaches by evaluating whether they are targets of clinically relevant immune

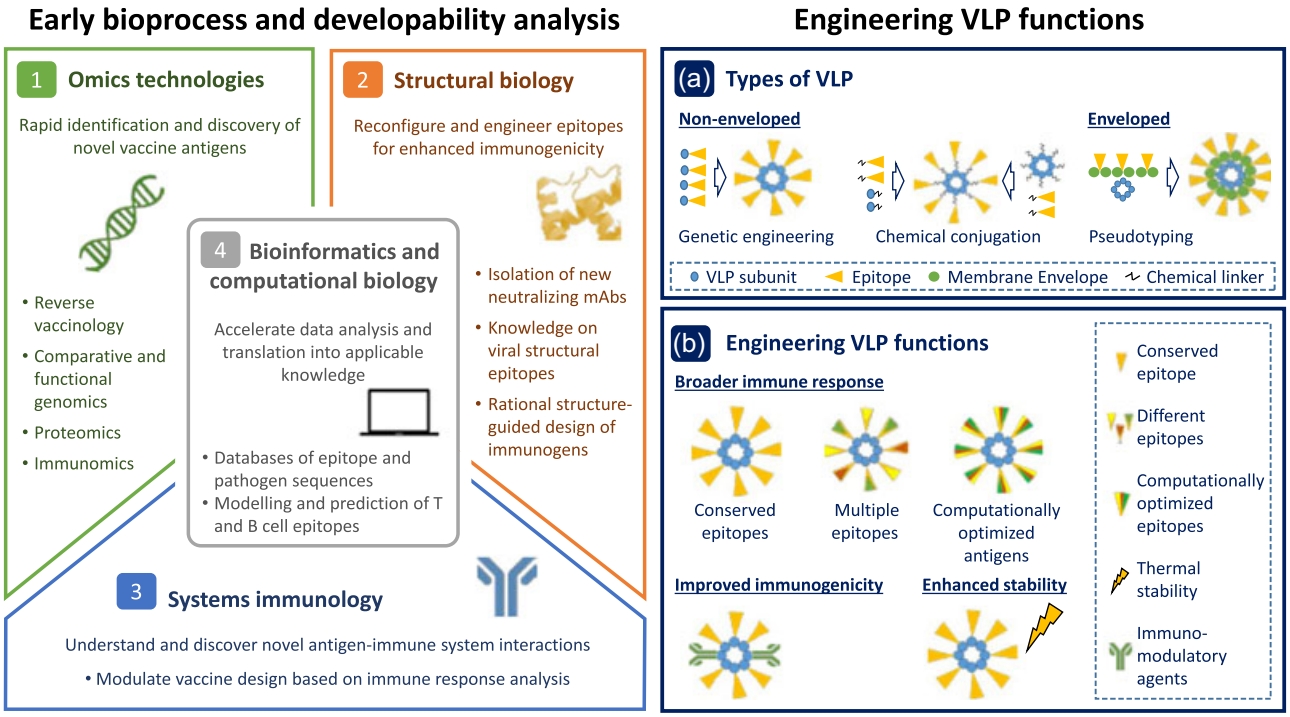

FIGURE 1 Design tools for VLP vaccine engineering. Multitude of tools and recent advances in synthetic biology enable screening for pathogen-specific antigens with high immunogenic potential and engineering of VLP function. (1) Omics technologies enable rapid identification and discovery of novel/potential vaccine antigens. (2) Structural biology and (3) system immunology assist rational reconfiguration and engineering of epitopes/VLPs for enhanced immunogenicity. (4) Bioinformatics and computational biology accelerate data analysis and translation into applicable knowledge. (a) Engineering VLP function on different types of VLP. While nonenveloped VLPs are commonly engineered using genetic engineering or chemical conjugation, enveloped VLPs rely on pseudotyping for function engineering. (b) VLPs can be engineered to offer broader immunogenicity, improved immunogenicity, or enhanced stability. Broadly immunogenic VLPs can be obtained by displaying multiple antigenically distinct epitopes (Pushko et al., 2011; Schwartzman et al., 2015), highly conserved epitopes (Krammer, 2015; Wiersma et al., 2015), or computationally optimized epitopes (Carter et al., 2016) within a single VLP. Improving VLP immunogenicity can be achieved by incorporating immunomodulatory agents, such as dendritic cells targeting antibodies into particles structure (Rosenthal et al., 2014). VLP stability can be enhanced by modulating particles formulation (Collins et al., 2017; Lua et al., 2014). VLP: virus-like particle [Color figure can be viewed at wileyonlinelibrary.com]

responses (i.e., they stimulate the production of specific cytokines or activate specific cell types; Kuleš et al., 2016). An example of immunomics application to VLP vaccine design is GlaxoSmithKline's RTS,S vaccine, the most advanced malaria vaccine candidate. RTS,S is a chimeric VLP in which a large portion of the C-terminal of *Plasmodium falciparum*-derived circumsporozoite protein (CSP) is displayed on a protein scaffold (hepatitis B surface antigen; Y. Wu et al., 2015). In combination with systems biology (genomics and proteomics), immunomics was used to highlight regions within CSP that were immunogenic and protective in humans and, thus, indispensable to incorporate into a chimeric VLP. From the many candidates identified, only one proved to be successful in Phase IIa/b efficacy trials (Draper et al., 2015; Kazmin et al., 2017).

2.2 | Structural biology

Structural information can be used to reconfigure and engineer conserved epitopes to expose areas that exhibit high immunogenicity or to insert multiple immunodominant epitopes within the same VLP platform (Liljeroos, Malito, Ferlenghi, & Bottomley, 2015). These strategies can broaden the immune response or enhance the existing response to weak immunogenic antigens. Identifying conformational epitopes and studying their interactions with the immune system can provide significant information for rational antigen design (Anggraeni et al., 2013; Mulder et al., 2012).

Encouraged by recent results from HIV-1 VLPs (C. Zhao, Ao, & Yao, 2016), structural biology is emerging as a powerful tool to assist in the rational design of a modern HIV VLP vaccine. Novel protective epitopes can now be identified in conformational epitope mapping studies via structural biology (Liljeroos et al., 2015; Malito, Carfi, & Bottomley, 2015). In addition, broad and potent HIV antibodies discovered in the pool of antigen-specific memory B cells using structural biology, highlight novel sites of vulnerability on HIV envelope glycoprotein epitope (J. Huang et al., 2014). When incorporated into HIV-1 VLPs, these antigens can be strategically modified to insert the extended 2F5, 4E10 epitope and membrane proximal external region (MPER) of HIV-1 gp41 (Zhai, Zhong, Zariffard, Spear, & Qiao, 2013) providing a better display of the conserved CD4 binding site and capturing broadly neutralizing antibodies (Ingale et al., 2014).

Structural biology is equally informative for VLP engineering as the capsid proteins forming each VLP need to be correctly folded to

TABLE 1 Nonenveloped virus-like particle platforms for the display of unrelated antigens

Platforms	Targets	Antigens	References
Bacteriophage AP205	HIV-1	HIV-1 gp41 epitopes	Pastori et al. (2012)
	Influenza	M2e	Tissot et al. (2008)
	Malaria	Circumsporozoite	Janitzek et al. (2016)
	Malaria	Pfs25 and VAR2CSA proteins	Thrane et al. (2016)
	Tuberculosis	Ag58A	Thrane et al. (2016)
	West Nile virus	Domain III of E glycoprotein	Spohn et al. (2010)
Bacteriophage Qβ	Allergenic	Allergen Der p 1	Kundig et al. (2006)
	Alzheimer's disease	Aβ1–6 (amyloid peptide)	Wiessner et al. (2011)
	HIV-1	CCR5 coreceptor	Hunter, Smyth, Durfee, and Chackerian (2009)
	Hypertension	Angiotensin II	Tissot et al. (2008)
	Influenza	Hemagglutinin (globular head)	Jegerlehner et al. (2013)
	Influenza	M2e	Bessa et al. (2008)
	Nicotine dependence	Nicotine	Maurer et al. (2005)
	Type 2 diabetes	Interleukin-1β	Spohn et al. (2010)
Bovine Papillomavirus	Alzheimer's disease	Amyloid β peptide	Li et al. (2004)
	HIV-1	CCR5 peptide	Chackerian, Lowy, and Schille (1999)
	HIV-1	V3 loop of HIV-1 gp120	X. S. Liu et al. (2002)
	HIV-1	HIV-1 gp41 neutralizing epitopes	Zhai et al. (2013)
	Human papillomavirus (HPV)	HPV 16 L2 neutralizing epitopes	Slupetzky et al. (2007)
Cowpea mosaic virus	Canine parvovirus	VP2 capsid protein	Langeveld et al. (2001)
	HIV-1	Glycoprotein 41 peptide	McLain, Porta, Lomonossoff, Durrani, and Dimmock (1995)
	Pseudomonas aeruginosa	CPMV-PAE5 peptide	Brennan et al. (1999)
	Staphylococcus aureus	Truncated D2-domain	Rennermalm et al. (2001)
Cucumber mosaic virus	Alzheimer's disease	Amyloid β peptides	Vitti et al. (2010)
	Hepatitis C virus	HCV-derived R9 and R10 mimotopes	Nuzzaci et al., (2007)
	Newcastle disease virus	Neutralizing epitopes	Y. Zhao and Hammond (2005)
Flock House virus	Anthrax	Von Willebrand A domain of ANTXR2 cellular receptor/protective antigen	Manayani et al. (2007)
	Hepatitis B and hepatitis C virus	Epitopes of hepatitis C virus and hepatitis B surface antigen	Chen et al. (2006)
	HIV-1	V3 loop of HIV-1 gp120 protein	Scodeller (1995)
	Influenza	A-helix epitope of HA2	Schneemann et al. (2012)
Hepatitis B core	Anthrax	Domain 4 epitope of the protective antigen (PA) of anthrax toxin	Bandurska et al. (2008)
	Anthrax	2β2–2β3 loop of PA	Yin et al. (2014)
	Dengue virus type 2	Envelope domain III	Arora, Tyagi, Swaminathan, and Khanna (2012); Arora, Tyagi, Swaminathan, and Khanna (2013)
	Enterovirus 71	SP55 and SP70 epitopes of enterovirus 71	Ye et al. (2014)
	Hepatitis C virus (HCV)	B- and T-cell epitopes of HCV	Mihailova et al. (2006)
	Influenza	M2e	De Filette et al. (2008); Fu et al. (2009)
	Lyme disease	OspA and variants of OspC	Nassal et al. (2008), (2005)
	Lyme disease	tHRF, Salp15, and Iric-1	Kolb, Wallich, and Nassal (2015)
	Malaria	CSP-specific B and T cell epitopes	Sällberg, Hughes, Jones, Phillips, and Milich (2002)
	Tuberculosis	CFP-10	Dhanasooraj, Kumar, and Mundayoor (2013)
Human papillomavirus	Human respiratory syncytial virus	Neutralizing epitopes	Murata, Lightfoote, Rose, and Walsh (2009)
Murine polyomavirus	Cancer (Breast)	Her2	Tegerstedt, Lindencrona, et al. (2005)
	Cancer (Prostate)	Prostate-specific antigen	Eriksson et al. (2011)
	Group A *Streptoccocus*	J8 peptide	Middelberg et al. (2011)
	Influenza	M2e	Wibowo, Chuan, Lua, and Middelberg (2013)
	Influenza	Helix 190 antigen	Anggraeni et al. (2013)
	Influenza	Hemagglutinin (globular head)	Waneesorn, Wibowo, Bingham, Middelberg, and Lua (2016)
	Rotavirus	VP8 antigen	Tekewe et al. (2015)
Tobacco mosaic virus	Foot and mouth disease	Foot and mouth disease peptides	L. G. Wu et al. (2003)
	Murine hepatitis	Murine hepatitis coronavirus	Koo et al. (1999)

(Continues)

TABLE 1 (Continued)

Platforms	Targets	Antigens	References
		neutralizing epitope	
	Poliovirus	Poliovirus type 3 epitope	Haynes et al. (1986)
	Pseudomonas aeruginosa	Peptide of outer membrane protein F	Staczek, Bendahmane, Gilleland, Beachy, and Gilleland (2000)
	Rabbit papillomavirus	L2 epitopes	Palmer et al. (2006)

ensure functionality, that is, induce a protective humoral immune response. The morphological characteristics of VLPs can be assessed through imaging technologies, such as cryoelectron microscopy, atomic force microscopy, and dynamic light scattering. These imaging methods provide essential data to solve the three-dimensional structure of a VLP, therefore, help to identify optimal VLP insertion sites for display of any given antigen. Many VLP platforms have been developed so far using this structure-based design approach. The most commonly reported are those derived from viral structures of hepatitis core antigen (HBcAg), murine polyomavirus (MuPyV), human papillomavirus (HPV), and bacteriophages Qβ and AP250 (Table 1).

2.3 | Systems immunology

Systems immunology is an emerging area of research that uses a broad and integrated, multilevel approach to study the immune system to identify immune correlates of protection or immunogenicity signatures (Davis, Tato, & Furman, 2017). Combined with recent technological advances in human immunology, systems immunology can provide guidance for rational vaccine design. Systems immunology allows the assessment of most cell types of the immune system (including specialized B and T white blood cells), their state, function, signaling molecules, and encoding genes (Bird, 2017). This accumulation of data captures a snapshot of the human immune system, providing valuable information for the creation of human immune response models that may later be translated into improved vaccine design (Davis et al., 2017). A systems immunology approach was previously undertaken to investigate the immune response to the live attenuated yellow fever vaccine YF-17D (Hou et al., 2017; Muyanja et al., 2014). Following immunization, comprehensive characterization of the immune system was performed providing insights into the vaccine's mechanism of action. A similar method was later applied to the malaria vaccine RTS,S, where a systems-level approach led to the identification of molecular and cellular signatures associated with protection and immunogenicity unique to this VLP-based vaccine (Kazmin et al., 2017). Systems immunology may also play an important role in designing vaccines capable of stimulating parts of the immune system not addressed by current vaccines (Davis et al., 2017). VLPs are ideal testing candidates for systems immunology approach due to their ability to stimulate B-cell-mediated immune responses, as well as CD4 proliferative responses and cytotoxic T lymphocyte responses (Gause et al., 2017).

The correct identification of epitopes that stimulate an immune response is crucial for the design of novel immunogens. These epitopes are regions within the antigen that are recognized by B- and T-cell receptors (Patronov & Doytchinova, 2013). Potential B- and T-cell epitopes can be mapped using bioinformatics and computational biology tools, however, without recent improvements in immunological characterization methods, the identification of epitopes that optimally stimulate the human immune response becomes challenging (Rappuoli, Bottomley, D'Oro, Finco, & De Gregorio, 2016). The ability to isolate memory B cells through high-throughput FACS followed by in vitro culture enables secretion of sufficient amounts of recombinant monoclonal antibodies or antigen-binding fragments to allow for antigen screening in binding and functional assays (J. Huang et al., 2013). The ultimate goal being the incorporation of specific antigenic regions on a VLP scaffold. Thus, improving vaccine specificity for generation of effective vaccines. An example is the above-mentioned novel vulnerability sites on HIV envelope glycoprotein that is used for HIV VLP vaccine design (J. Huang et al., 2014; Zhai et al., 2013).

2.4 | Bioinformatics and computational biology

Bioinformatics and computational biology tools have the potential to accelerate data analysis and translate results into applicable knowledge, fostering the discovery of new lead antigens by reducing the number of empirical experiments (He & Xiang, 2013). Vaccine design is an inherently complex and laborious process but software, algorithms, and databases outlined below have the potential to streamline vaccine development via identification of candidate antigens that may otherwise have been overlooked.

Epitope mapping is essential for designing vaccines capable of mounting a robust T- and B-cell response. The discovery of such epitopes relies upon immunological prediction software, such as netMHC, SYFPEITHI, EpiJen, or Epivax, as described in other studies (Soria-Guerra, Nieto-Gomez, Govea-Alonso, & Rosales-Mendoza, 2015). For T-cell epitope prediction, as are many software available that can reach up to 95% of positive predictive values (Soria-Guerra et al., 2015). In addition, there are interface platforms, such as MHCBench (Salomon & Flower, 2006) allowing direct evaluation of various Major Histocompatibility Complex (MHC)-binding peptide prediction algorithms. Interface platforms prove valuable to users unsure of which prediction model best suits their needs. For B-cell epitope prediction, available software is supported by algorithms that can reach up to 25% for discontinuous B-cell antigens (e.g., COBEpro [Sweredoski & Baldi, 2009], BCPRed and FBCPred [El-Manzalawy, Dobbs, & Honavar, 2008]) or up to 95% for continuous B-cell antigens (e.g., EPMeta [Liang et al., 2010]) of positive predictive values.

Bioinformatics and computational biology can also assist in the discovery of conserved epitopes through sequence variability analysis. This is particularly relevant when dealing with pathogens capable of evading the immune system due to their high mutation rates. The Protein Variability Server (PVS) is a valuable tool for the identification of such conserved epitopes, which can facilitate the development of broadly protective vaccines (Garcia-Boronat, Diez-Rivero, Reinherz, & Reche, 2008). In this study, PVS was used to identify a conserved fragment in the ectodomain of HIV-1-gp41. Several databases retain classified and well-curated data from experimentally verified vaccines and/or vaccine components and, thus, are useful tools for vaccine design. The Immune Epitope Database and Analysis Resource (Peters et al., 2005; https://www.iedb.org) collects all published experimental data characterizing immune epitopes and the context in which the molecular structure is recognized as an epitope by the immune receptors. IEDB has over 88,382 epitopes and multiple tools to identify B- and T-cell epitopes (Vita et al., 2015). The Syfpeithi database (Schuler, Nastke, & Stevanovikc, 2007; http://www.syfpeithi.de) has information on MHC classes I and II anchor motifs and binding specificity. The Conformational Epitope Database offers information about protein conformation. The AntigenDB (http://www.imtech.res.in/raghava/antigendb/) stores sequences, structures, origins, and epitopes of pathogen antigens (Ansari, Flower, & Raghava, 2010). The Computationally Optimized Broadly Reactive Antigen (COBRA) methodology was recently developed to overcome the challenges associated with antigenic diversity in influenza subtypes (Carter et al., 2016). This in silico approach, which uses consensus building to generate a number of antigen candidates termed COBRA antigens, was used to identify HA antigens that were broadly protective against H1N1 strains. Display of these antigens on VLPs demonstrated immunogenicity and efficacy in a murine model validating this technology.

3 | ENGINEERING VLP FUNCTIONS

With the advent of novel technologies for screening and discovery of immunogenic antigen targets and the advances in synthetic biology one can now engineer VLP function and design vaccines with increased or broader immunogenicity and/or improved stability (cold-chain free; Figure 1; Collins, Snaith, Cottingham, Gilbert, & Hill, 2017; Lua et al., 2014). VLPs can be engineered to enhance and tune the immune response to vaccination. Using synthetic biology tools, one can harbor immunogens inside or outside the VLP that upon vaccination are capable of triggering an early innate immune response that enhances vaccine effectiveness by increasing vaccine's uptake (Rosenthal, Chen, Baker, Putnam, & DeLisa, 2014). These immunomodulatory agents (e.g., pattern recognition receptor ligands, dendritic cells targeting antibodies, and endoplasmic reticulum targeting peptides) boost the immune response mainly through expansion of specific CD8+ T cells and the production of cytokines (without any long-lasting effects).

VLPs are divided into two main groups, enveloped and nonenveloped. Enveloped VLPs are self-assembling capsids, which acquire a lipid layer when budding from their host cells. This layer is absent in nonenveloped VLPs (Mateu, 2011). Insertion of heterologous antigens (otherwise known as modularization) into nonenveloped VLPs is mainly achieved through genetic fusion or chemical conjugation (Peacey, Wilson, Baird, & Ward, 2007). The size of the insert has implications for VLP assembly and correct presentation of the antigen. Small peptide epitopes are easily inserted into VLP structures without affecting VLP assembly, which can often occur when modularizing whole or large protein domains. Genetic fusion is the most popular method despite being time-consuming and error-prone (Mateu, 2011). Chemical conjugation supports the insertion of large antigens in preformed VLPs and these modifications are performed on naturally occurring conjugation sites using chemical crosslinkers or enzymes (Patel & Swartz, 2011; Tang, Xuan, Ye, Huang, & Qian, 2016). The downside of chemical conjugation is the incurred cost as it requires the production of both the VLP and the epitope(s) as well as conducting the chemical conjugation (Chackerian, 2007; Smith, Hawes, & Bundy, 2013). Recently developed technologies have started to address the cost and technological challenges. An example is the "Plug and Display" system, a technology based on two proteins, "the tag" and "the catcher," which react irreversibly when in close proximity of each other. When "the catcher" is fused to a VLP and "the Tag" is fused with a vaccine target, these form a two-component VLP vaccine ready to use (Brune et al., 2016). Examples of nonenveloped VLPs used to display foreign antigens are HBcAg (Chu et al., 2016), HPV L1 protein (Slupetzky et al., 2001), and Qβ (O'Rourke, Peabody, & Chackerian, 2015).

Enveloped VLPs can present heterologous membrane proteins (i.e., glycoproteins) in their native configuration on top of a self-assembling capsid protein(s) without the need to engineer both epitope and capsid structures in a process called pseudotyping (Chua et al., 2013; Kirchmeier et al., 2014). With pseudotyping, one can alter the VLP stability or even its tropism (Cronin, Zhang, & Reiser, 2005; K. Palomares et al., 2013). In addition, transmembrane domains within foreign viral envelope proteins can be replaced with transmembrane domains of specific viruses (e.g., vesicular stomatitis virus) to improve pseudotyping efficiency and immunogenicity (Kirchmeier et al., 2014). Enveloped VLPs, such as retro- and lenti-VLPs, have shown promise as vaccines candidates against diseases, such as Influenza, Malaria, or Dengue virus (Chua et al., 2013; Pitoiset, Vazquez, & Bellier, 2015).

The engineering of VLP has long been a complex process and often unsuccessful, as the insertion of small peptides can disrupt the VLP structure. The field has evolved and now VLP chimeras are used in both fundamental and applied research (Mateu, 2011; Murata et al., 2009). A successful example of the insertion of large peptides is the VLP derived from flock house virus that was engineered to carry a receptor domain and could be used as an anthrax antitoxin as well as a vaccine (Manayani et al., 2007).

Circulating viruses that exhibit high antigenic variability and high mutation rates, such as influenza, pose substantial challenges for current vaccination strategies (Wibowo et al., 2014). Novel strategies are, therefore, under development to create broadly cross-protective vaccines. Highly conserved antigens have been identified between divergent influenza viruses. These broadly reactive antigens, which are located within the membrane proximal stalk domain of the hemagglutinin protein have been inserted into VLPs and demonstrate success as vaccines candidates (Ben-Yedidia, 2011; Krammer, 2015; Wiersma, Rimmelzwaan, & de Vries, 2015). Broad protection is also possible through the simultaneous display of multiple, antigenically distinct (Prabakaran et al., 2010; Pushko et al., 2011; Schwartzman et al., 2015), or chimeric (Carter et al., 2016) HA antigens from different influenza subtypes within the same VLP.

4 | VLP-BASED PLATFORM TECHNOLOGY

The engineering of viral structural proteins to display heterologous antigens presents an opportunity to manufacture vaccines against unrelated viruses, as well as pathogens from other sources, that is, bacteria and parasitic protozoans. This versatile nature, coupled with the suite of VLPs amenable to modularization, makes VLPs ideal candidates for the development of vaccine platforms. Noninfectious, generic VLP platforms offer the potential for streamlined bioprocesses, parallel infrastructure, predictable biosafety, and regulatory practices (Charlton Hume & Lua, 2017), which, in turn, would significantly reduce vaccine production times. Given the lack of preparedness during recent global outbreaks of influenza A (H1N1) (Fineberg, 2014), Zika (Boeuf, Drummer, Richards, Scoullar, & Beeson, 2016), and Ebola virus (Coltart, Lindsey, Ghinai, Johnson, & Heymann, 2017), as well as Middle East Respiratory Syndrome (Okba, Raj, & Haagmans, 2017), VLP platform technologies are pertinent to address the rapid spread of emergent viruses.

4.1 | Platform capabilities

Several viral structural proteins and bacteriophages are currently under development as vaccine platforms to target a variety of pathogens (Tables 1,2). Synthetic biology approaches have widened the scope of potential antigens for modularization and show that platforms can be modified to overcome specific challenges associated with VLP vaccine manufacturing (Figure 2). Nonenveloped VLPs are structurally less complex than their enveloped counterparts and can be produced in prokaryotic and lower order eukaryotic systems making them easily scalable, cost-effective, and rapid to manufacture. The presence of a lipid bilayer in enveloped VLPs necessitates the use of eukaryotic hosts for expression, which increases the overall production time and cost. Nonetheless, unlike nonenveloped VLPs, enveloped VLPs permit presentation of antigenic modules that require membrane-association.

High immunogenicity is a desirable vaccine attribute that potentially translates to protectivity against target infections. To enhance immunogenicity, modularized antigens must be strategically inserted to maximize their presentation to the immune system. Modularization of peptides to the N- or C-termini of viral proteins has been previously reported (De Filette et al., 2008; Haynes et al., 1986). However, low surface expression or the inability of peptides to adopt native conformation can result in weak immunogenicity at these insertion sites (Schödel et al., 1992). Platforms based upon HBcAg, papillomavirus (bovine and human), Flock House virus (FHV), and others, are thus engineered to take advantage of surface-exposed loops that demonstrate enhanced immunogenicity (Murata et al., 2009; Slupetzky et al., 2007; Ye et al., 2014). Peptides inserted into exposed loops protrude from the surface of VLPs making them more accessible to the immune system. Short peptides displaying relatively simple structures pose little problem for modularization within exposed loops, yet those with more complex structures require further platform engineering (Anggraeni et al., 2013). Incorporation of epitope scaffolds into exposed loops of VLPs maintain the structural properties of complex peptides as shown with the presentation of a 20aa A-helix of the influenza HA2 chain on the FHV platform (Schneemann et al., 2012). This strategy, however, requires detailed structural knowledge of the chosen peptide and the identification of a suitable scaffold fragment. Antigens inserted into the immunodominant loops of the HBcAg platform require closely juxtaposed N- and C-termini to maintain VLP integrity (Walker et al., 2011). This imposes considerable limitations upon antigen choice. To support the modularization of antigens that possess an extended structure, a SplitCore system has thus been devised (Walker et al., 2011). Dividing the HBcAg core protein within the immunodominant c/e1 loop yields two fragments, which are able to form VLPs when coexpressed. Fusion of antigen to the c/e1 termini of either fragment before coexpression enables modularization of antigens that may otherwise have been structurally incompatible.

Display of whole protein domains on the surface of VLPs allows presentation of multiple antigenic epitopes and increases the likelihood that epitopes will adopt their native conformation. Their large size, however, can cause steric hindrance resulting in compromised VLP assembly (Lua, Fan, Chang, Connors, & Middelberg, 2015). As such, several strategies have been developed to specifically modularize large antigens. Long, flexible glycine-rich linkers were engineered to flank the antigen in the c/e1 loop of the HBcAg platform, allowing spatial separation and enabling proteins of up to 238aa to be inserted (Kratz, Bottcher, & Nassal, 1999). Though effective, optimal linker length for different antigens needs to be empirically determined. Steric hindrance can also be addressed in some cases by reducing the antigen content on the surface of the VLP. For example, Peyret et al. (2015) engineered a single polypeptide composed of an unmodified HBcAg fused to a chimeric HBcAg. A dual expression construct was also devised to coexpress unmodified murine polyomavirus (MuPyV) VP1, with antigen-modularized VP1 displaying an 18 kDa rotavirus antigen (Lua et al., 2015; Tekewe, Fan, Tan, Middelberg, & Lua, 2017). Although such methods can lead to successful VLP assembly, it is possible that increasing the antigen mass, whereas reducing the antigen number may be reflected in lower immunogenicity, as is seen

TABLE 2 Enveloped virus-like particle platforms for pseudotyping

Platforms	Targets	Antigens	References
BIV Gag	Influenza	Hemagglutinin (HA) (subtypes) and neuraminidase (NA)	Pushko et al. (2017); Tretyakova et al. (2016)
HBsAg	Dengue virus	Envelope domain III	Harahap-Carrillo, Ceballos-Olvera, and Valle (2015)
	Malaria	Circumsporozoite protein	Stoute et al. (1997)
HIV1-Gag	Dengue virus	Envelope domain III	Chua et al. (2013)
	West Nile virus	Glycoprotein E	
	Influenza	HA and NA	Carter et al. (2016)
Influenza M1	Influenza	HA subtypes	Schwartzman et al. (2015)
	Influenza	M2	Song et al. (2011)
	Influenza	NA	Ben-Yedidia (2011); Wiersma et al. (2015)
	Respiratory syncytial virus	RSV A2 fusion	Kim et al. (2015)
Murine leukemia virus Gag	Cancer	Melanoma antigens	Kurg et al. (2016)
	Human cytomegalovirus	Glycoprotein B	Kirchmeier et al. (2014)
	Rift Valley fever virus	Glycoproteins G_N, G_C, and nucleoprotein N	Mandell et al. (2010)
	Influenza	HA and NA	Haynes et al. (1986)

with the synthetically derived RTS,S malaria vaccine (Pitoiset et al., 2015). Nevertheless, this trade-off is inevitable as the mass of the antigen increases relative to the carrier, necessitating an understanding of the optimization domain.

Display of large antigens has been reported using novel platforms based upon the HPV 16 L1 protein and the *Acinetobacter* phage, AP205 (Thrane et al., 2015; Thrane et al., 2016; Brune et al., 2016). The AviTag and SpyTag/SpyCatcher platforms allow the conjugation of antigens post VLP assembly through biotin-streptavidin reactions (AviTag) or spontaneous formation of an irreversible isopeptide bond (SpyTag/SpyCatcher). With linkers already predefined, platforms remain constant permitting high throughput screening of vaccine candidates. In addition, independent production of the antigen in a different expression system is possible and of particular interest, if posttranslational modifications are critical for immunogenicity, provided these do not sterically inhibit attachment.

Encapsulation of host-cell proteins and nucleic acid during cell-based VLP assembly can alter immunogenicity and contribute significantly to the batch-to-batch variation of vaccine preparations. Specific limitations regarding host contaminants are clearly stipulated in licensing regulations. Host contaminants can be removed by VLP disassembly and ex vivo reassembly, as necessitated for licensing of HPV L1 VLPs (McCarthy, White, Palmer-Hill, Koenig, & Suzich, 1998). Unfavorably, this complicates downstream processing and adds to manufacturing costs. Synthetic modification of the HPV L1 and MuPyV VP1 proteins has led to the development of capsomere platforms (Middelberg et al., 2011; Schadlich et al., 2009). Removal of the carboxyl termini from each protein yields capsomeres incapable of forming VLPs in vivo. MuPyV capsomeres display increased stability, although less immunogenic than VLPs when administered with adjuvant they are just as effective (Middelberg et al., 2011). Furthermore, a quantitative process study reported that when produced in *E. coli*, the MuPyV capsomere platform is capable of producing 320 million vaccine doses in 2.3 days at low cost highlighting its suitability as a rapid response and low-cost vaccine platform (Chuan, Wibowo, Lua, & Middelberg, 2014).

The safe and robust influenza M1 platform further illustrates the aforementioned, broadly immunoprotective capabilities of VLP platforms. Multiple subtypes of HA antigens have been displayed on M1-VLPs either individually (Schwartzman et al., 2015) or simultaneously (Pushko et al., 2011; Sequeira et al., 2017; Tretyakova, Pearce, Florese, Tumpey, & Pushko, 2013). M1-VLP based vaccines afford protection against virus challenge in multiple species (Liu, Massare, et al., 2015; Pyo et al., 2012) and have been the subject of recent influenza phases I and II clinical trials (NCT01897701 and NCT02078674). The vast majority of studies producing M1-VLPs utilize either mammalian cell lines (transient or stable expression) or insect cell lines (mainly transient expression using the baculovirus expression system, IC-BEVS). Synthetic biology has recently been applied to the IC-BEVS platform to overcome process-related drawbacks. When produced in IC-BEVS, VLPs and baculovirus are not easily separated owing to current limitations in purification and analytical techniques. A recently developed biorthogonal labeling strategy enables distinction between baculovirus and HA containing M1-VLPs. Thus, allowing greater control over vaccine contaminants (Carvalho et al., 2016). This system involves fluorescent labeling of an azide-tagged noncanonical amino acid, which is incorporated into the HA protein within the enveloped VLP. Combinatorial analysis using size exclusion chromatography (SEC), confocal microscopy, and flow cytometry demonstrated the possibility of obtaining VLPs independently of baculovirus. At present, this synthetic modification holds promise as an analytical tool at the laboratory level. However, given the use of fluorescent reagents and their cost, further development is required to address practicality at the industrial scale.

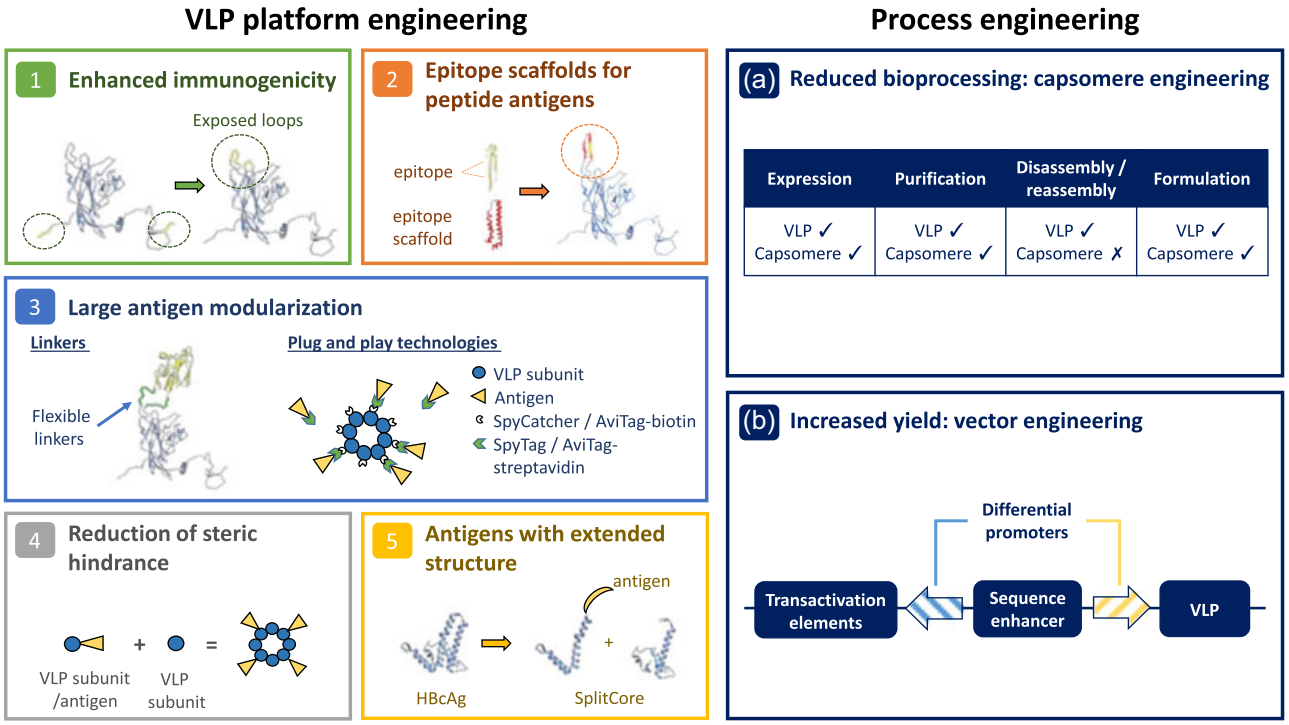

FIGURE 2 Application of synthetic biology to VLP vaccine platforms. (1) Enhanced immunogenicity of peptides is achieved through their insertion into exposed loops of viral capsid proteins (Murata et al., 2009; Slupetzky et al., 2007; Ye et al., 2014). (2) The structural properties of complex peptides are maintained through the incorporation of epitope scaffolds into exposed loops (Schneemann et al., 2012). (3) Large antigens are modularized onto VLP vaccine platforms using long flexible linkers to maintain structural separation between the viral capsid protein and the antigen (Kratz et al., 1999); or onto preformed VLPs using plug and play technologies, such as SpyCatcher/SpyTag (Brune et al., 2016) and AviTag (Thrane et al., 2015). (4) Dual expression of modified and unmodified viral capsid proteins reduces steric hindrance and permits VLP assembly (Tekewe et al., 2017). (5) The SplitCore system permits modularization of antigens with an extended structure through the coexpression of modified and unmodified HBcAg core fragments (Walker, Skamel, and Nassal, 2011). (a) Synthetic production of capsomeres minimizes host cell contaminants reducing required bioprocessing steps (Chuan et al., 2010). (b) Synthetic engineering of baculovirus vectors can increase VLP expression yield (Gómez-Sebastián et al., 2014; Y. K. Liu et al., 2015; Y. V. Liu et al., 2015). VLP: virus-like particle [Color figure can be viewed at wileyonlinelibrary.com]

5 | CONSIDERATIONS FOR VLP PRODUCTION

The choice of expression host and culture parameters can greatly influence expression yield (Kushnir, Streatfield, & Yusibov, 2012; Lua et al., 2014; Roldão et al., 2010; Vicente, Roldão, Peixoto, Carrondo, & Alves, 2011). *E. coli* is far superior to other expression systems in terms of cost, yield, and speed making it a popular choice for the production of single capsid protein nonenveloped VLPs (X. Huang, Wang, Zhang, Xia, & Zhao, 2017). The inability of *E. coli* to perform posttranslational modifications means that complexity is minimized. In cases where posttranslational complex modification is required, IC-BEVS is used to manufacture licensed VLP vaccines, such as Cervarix® and Porcilis PCV®, yet vaccine costs are high, owing partly to low production yield. Synthetic engineering of baculovirus vectors using combinations of transcriptional or translational elements can increase yield in this system (Gómez-Sebastián, López-Vidal, & Escribano, 2014; Liu, Zhang, et al., 2015). Similarly, a synthetically designed expression cassette, containing rearranged genetic regulatory elements, transcription factors IE1 and IE0 and a transcription enhancer sequence linked to differential promoters, increased both expression and cell viability of porcine circovirus VLPs and rabbit hemorrhagic disease VLPs in IC-BEVS (López-Vidal et al., 2015).

Maintaining VLP integrity is another key consideration. In multi-protein capsids, specific protein ratios are required to prevent aggregation of unassembled VLPs (L. A. Palomares & Ramirez, 2009; Roldão et al., 2012). Protein stoichiometry can be controlled in IC-BEVS and mammalian expression systems through manipulating multiplicity of infection, transfection ratios, or thermodynamics (Arevalo, Wong, & Ross, 2016; L. A. Palomares & Ramirez, 2009; Roldão et al., 2012) though it involves increased process cost. Different strength promoters can also be synthetically integrated into multicistronic vectors resulting in differential expression of individual proteins (Jere, O'Neill, Potgieter, & van Dijk, 2014). However, given that optimal ratios vary depending on the specific VLP, individual optimization is required. To surpass the drawbacks of traditional coinfection strategies and/or larger, unstable viral vectors, a modular expression system in which the number of genes to be expressed is rationally distributed between a recombinant viral construct

and a stable cell line can be adopted. Such a strategy has proven already to be successful for the production of multivalent influenza VLPs using an insect High Five cell-based platform (Sequeira et al., 2017).

Vaccine production scale, cost, and purity vary depending on VLP purification processes. Centrifugation, depth, and tangential flow filtration, used for initial clarification and concentration steps, are easily scaled. At the industrial level, chromatographic methods are used to remove host cell and DNA impurities as density gradients and ultracentrifugation are not easily scalable. Anion exchange and SEC are time-consuming and costly, highlighting the need for more efficient and cost-effective methods. Nonchromatographic strategies based upon aqueous two-phase systems (ATPS), a process presently used for enzyme production at the industrial level, are being developed for VLP purification. High yields of rotavirus VLPs have been purified from insect cell supernatant using ATPS, although purity was relatively poor (Benavides et al., 2006). More recently, single and multistep ATPS were used to purify human B19 parvovirus-like particles from insect cell lysates (Effio et al., 2015). Purity levels were greater than 90%, yet this appeared to be at the expense of yield. Monolith technology presents a rapid and scalable method that offers distinct advantages over the classical packed-bed chromatography (Vicente et al., 2011). HBsAg VLPs from yeast homogenate was effectively purified using a hydroxyl derivatized monolith (Burden, Jin, Podgornik, & Bracewell, 2012) and when compared to density gradient centrifugation, an anion-exchange monolith yielded 220-fold more HIV-1 gag VLPs from Chinese hamster ovary (CHO) cell supernatant (Steppert et al., 2016). Sulfated cellulose membrane absorbers offer significant improvements over conventional ion exchangers membrane absorbers (Carvalho, Fortuna, et al., 2017). As they are easily scaled and reduce the number of required processing steps they may qualify as a generic purification platform for VLP-based vaccines. These technologies hold promise for large-scale VLP purification, warranting further investigation.

VLP characterization is a critical step in analyzing VLP stability and integrity. Additional information can be obtained regarding host- and product-derived impurities, both of which have significant impacts on vaccine efficacy and biosafety. Robust analytical tools are used in tandem to provide comprehensive characterization data. Traditional methods, such as western blot analysis, SEC, and ultracentrifugation are time-consuming. Others, such as transmission electron microscopy demand high investment costs, extensive preparation work, and specific expertize. Methods allowing swift analysis are currently under development. Tekewe et al. (2015) developed a high throughput method based upon dynamic light scattering technology that permits rapid analysis of capsomere stability based on their hydrodynamic radius. A size exclusion ultrahigh performance liquid chromatography method was also developed in which VLP samples were analyzed in 3.1 min using an interlaced injection technique, allowing accurate quantitation of VLP aggregates (Effio, Oelmeier, & Hubbuch, 2016). More recently, a universal label-free analytical tool for influenza VLPs quantification based on biolayer interferometry technology applied on an octet platform was developed (Carvalho, Moleirinho, et al., 2017). Overall, these analytical methods support the rapid characterization of large sample sets aiding more efficient vaccine development.

6 | VLP VACCINE TRANSLATION

From R&D to licensing, new vaccines can take up to two decades to come to fruition and are estimated to cost from USD 500 million to over 1 billion (S. A. Plotkin, Mahmoud, & Farrar, 2015). VLP vaccines have been under development for >30 years (Lua et al., 2014). Today there are six commercially licensed VLP vaccines and over 110 VLP vaccine trials (completed and current) documented at www.clinicaltrials.gov. Over 50% of these trials are designed to assess VLP vaccine candidates against various cancers (where Merck Sharp & Dohme Corp. and GlaxoSmithKline plc. are the leading sponsors) and over 20% against influenza (where Novavax, Inc. and Medicago Inc. dominate the trials). VBI Vaccines Inc. and Takeda Pharmaceutical Co. Ltd. are currently conducting trials for vaccines against cytomegalovirus and norovirus, respectively. India-based manufacturers, CPL Biologicals Pvt. Ltd. have developed Cadiflu-S, the world's first VLP influenza vaccine to successfully complete phase 3 trials (http://cadilapharma.com). These multistage clinical trials assess safety and efficacy and determine the suitability of vaccine candidates for licensing approval. Successful translation of vaccines, however, depends upon viable vaccine manufacturing processes to ensure reliable vaccine supply and to minimize production costs.

VLP vaccine platforms provide opportunities to overcome several limitations of traditional vaccine manufacturing. Substantial variation between antigen characteristics of traditional vaccines and their infectious nature adds significantly to production cost as they demand specialized production facilities and tailored manufacturing processes (S. Plotkin, Robinson, Cunningham, Iqbal, & Larsen, 2017). The standardized production of noninfectious, generic vaccine platforms (used to display various antigens) would reduce process variation (i.e., platform remains constant) and provide grounds to establish multi-product facilities. Streamlining of upstream and downstream processes is likely to increase efficiency, reduce laboratory waste, and permit cost savings through bulk buying of equipment and reagents (Konstantinov & Cooney, 2015). Predictable bioprocesses may allow the standardization of facility infrastructure and the use of single-use technologies, already in use for some VLP vaccines (Roldão et al., 2010). Disposable technologies offer several advantages over traditional stainless steel equipment including lower initial investment and operating costs, elimination of cross-contamination, and flexibility in terms of scale (Lopes, 2015; Shukla & Gottschalk, 2013). Combining disposable technologies with VLP platforms offer advantages on multiple levels and would facilitate VLP translation into the industry.

6.1 | Regulation and economics

The increased cost and regulatory uncertainty associated with producing new vaccines have long been challenges for vaccine manufacturers

(Ulmer, Valley, & Rappuoli, 2006). The intricacies of vaccine manufacturing demand a substantial amount of expertize and contribute significantly to cost (S. Plotkin et al., 2017). Expenses associated with vaccine R&D, testing and manufacturing as well as a poor profit margin compared with current drug markets, have led to a dramatic fall in funding from profit-driven pharmaceutical companies (Offit, 2005). However, this decline is thought to be premature. As demonstrated throughout this review, VLP technologies are advancing rapidly with a strong focus on developing platform capabilities and improving VLP production yields to enable rapid production of safer and cheaper vaccines, particularly when combined with single-use bioprocessing systems. This progress is set to continue with financial investment provided from "not-for-profit," sources, such as national governments, international organizations, and philanthropic bodies. In particular, Gavi, the vaccine Alliance (Balaji, 2004) and the Bill & Melinda Gates Foundation (www.gatesfoundation.org) are motivated to deliver affordable vaccines to third world countries that are the world's biggest vaccine market. To ensure vaccine purity, safety, efficacy, and stability, regulatory authorities govern every stage of vaccine manufacturing from raw materials and production processes to clinical trials and beyond. This includes the assessment and certification of safe and viable manufacturing processes (S. Plotkin et al., 2017). Meeting regulatory requirements can be a complicated process, particularly when manufacturing vaccines for overseas markets or utilizing newly developed bioprocesses. By streamlining upstream and downstream processes and utilizing a generic VLP base, VLP platform technology is expected to reduce the regulatory load of individual vaccines given that regulations for the base and its purification will become well characterized. VLP platforms may even fast track vaccine delivery in response to pandemic circumstances.

7 | FUTURE PERSPECTIVE/CONCLUDING REMARKS

Vaccinology is experiencing an impressive technological revolution, enabling to move vaccines development beyond the rules of Pasteur (empirical approach), using data-rich disciplines, such as systems biology, immunology, or computational biology to assist rational vaccines design (modern approach). Indeed, the last decade has witnessed a trend toward the use of alternative vaccine designs to attenuated pathogens, having VLPs emerged as a powerful and versatile platform for their production. Today, VLPs are being used not only as vaccines of unmodified viral assemblies against parental viruses but also as scaffolds for displaying heterologous antigens. In addition, tremendous investments have been made to develop new technologies capable of (a) deciphering pathogen biology and vaccine mechanistic responses, and (b) storing and curating extracted data into biological references databases. Machine learning algorithms can then use this information for epitope prediction and structure-based modular design. Complemented with synthetic biology, this information will provide the basis for (a) engineering VLP functions and (a) developing generic VLP platforms offering not only the potential for streamlined bioprocesses, parallel infrastructure, and predictable biosafety but also the ability to manufacture vaccines against unrelated viruses or pathogens from other sources (i.e., bacteria and parasitic protozoans). Although unable to deliver any marketable product to date, these technologies have massive potential to provide, in near future, solutions against untargeted infectious agents (e.g., antibiotic-resistant bacteria, HIV, malaria, or tuberculosis), and most importantly to reduce vaccine development times and manufacturing cost associated with current vaccine platforms.

ACKNOWLEDGMENTS

The authors acknowledge the support from Australian Research Council (ARC Discovery DP160102915), European Commission (Project EDUFLUVAC, Grant No. 602640), and Portuguese "Fundação para a Ciência e a Tecnologia" through the following programs: FCT Investigator Starting Grant (IF/01704/2014), Exploratory Research and Development Project EXPL/BBB-BIO/1541/2013, and PhD fellowship SFRH/BD/90564/2012. iNOVA4Health Research Unit (LISBOA-01-0145-FEDER-007344), which is co-funded by Fundação para a Ciência e Tecnologia / Ministério da Ciência e do Ensino Superior, through national funds, and by FEDER under the PT2020 Partnership Agreement, is also acknowledged.

CONFLICTS OF INTEREST

The University of Queensland (UQ) filed patents on the use of MuPyV as a vaccine platform. L. H. L. L. and A. P. J. M. contributed to those patents and, through their employment with UQ, hold an indirect interest in this intellectual property.

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How to cite this article: Charlton Hume HK, Vidigal J, Carrondo MJT, Middelberg APJ, Roldão A, Lua LHL. Synthetic biology for bioengineering virus-like particle vaccines. *Biotechnology and Bioengineering*. 2019;116:919–935. https://doi.org/10.1002/bit.26890