

# Bioengineering and Bioprocessing of Virus-Like Particle Vaccines in *Escherichia coli*



Rufika S. Abidin and Frank Sainsbury

## Contents

1	Introduction .....	272
2	VLPs and Immunogenicity .....	273
3	Bioengineering Strategies for Surface Presentation .....	274
3.1	Genetic Fusion .....	275
3.2	Chemical Conjugation .....	277
3.3	Biochemical Conjugation .....	278
3.4	Capsid Protein Stoichiometry .....	279
3.5	Encapsulation .....	280
4	Bioengineering Strategies for Bioprocess Optimization .....	281
4.1	Upstream Bioprocess Optimization .....	281
4.2	Downstream Bioprocess Optimization by High-Throughput Screening .....	282
4.3	Platform Development to Reduce Production Cost .....	283
5	Conclusions and Perspective .....	284
	References .....	284

**Abstract** Virus-like particles (VLPs) are macromolecular assemblies of recombinant viral structural proteins. Self-assembling with high precision, the repetitive nature of VLPs, their size, and particulate form result in the capacity for effective stimulation of humoral and cellular immune responses. VLP vaccines have achieved commercial success for protection against cognate viruses. However, there is a rich field of research dedicated to harnessing the immune-stimulatory properties of VLPs for the presentation of heterologous antigens for protection and treatment of chronic and infectious diseases. Peptides, proteins, carbohydrates, and small molecules have been converted into effective immunogens via presentation on various VLP platforms. In each case, unique bioengineering challenges must be overcome to allow

---

R. S. Abidin  
Hasanuddin University Medical Research Center, Hasanuddin University, Makassar, Indonesia

F. Sainsbury (✉)  
Centre for Cell Factories and Biopolymers, Griffith Institute for Drug Discovery, Griffith University, Nathan, QLD, Australia  
e-mail: [f.sainsbury@griffith.edu.au](mailto:f.sainsbury@griffith.edu.au)

the recovery, assembly, and structural fidelity of the VLP platform, as well as the immunogenicity of the antigen. This review highlights some of the biomolecular engineering approaches that are being employed to effectively present diverse biological molecules and chemical moieties on VLP platforms and bioprocessing strategies for their efficient recovery from the prokaryotic expression host *Escherichia coli*.

## 1 Introduction

Virus-like particles are generally derived from the structural proteins of viruses, which self-assemble into spherical or rod-shaped structures, mimicking the original virus. A VLP does not contain other viral components such as pathogenic proteins or genetic material and is thus nonpathogenic and noninfectious (Teunissen et al. 2013; Lua et al. 2014; Rodriguez-Limas et al. 2013). VLPs can be constructed from the capsid proteins of enveloped or non-enveloped viruses. In the case of enveloped viruses, a lipid bilayer coats the capsid supporting the presentation of glycoproteins embedded in the membrane (Kushnir et al. 2012). The existence of naturally occurring VLPs was first reported in 1965 (Blumberg et al. 1965), and their ability to induce cellular and humoral immunity was subsequently identified in 1968 (Bayer et al. 1968). The development of genetic engineering further allowed the expression and purification of synthetic VLPs (Hagensee et al. 1993; Kirnbauer et al. 1992; Li et al. 1997), which enabled characterization and understanding of their assembly (Li et al. 1997; Salunke et al. 1986). Over the course of more than 4 decades, at least 110 VLPs from 75 different viral families have been constructed and evaluated (Zeltins 2013; Yan et al. 2015). VLPs are deemed an ideal biological vehicle due to their biocompatibility, solubility, uptake efficiency, and their capability for targeted delivery and drug loading (Yan et al. 2015). Amenable to both genetic engineering and chemical modification, recombinant VLPs have been developed for various uses such as antigen carriers (Babin et al. 2013; Kawano et al. 2014; Wibowo et al. 2012) or as carriers of other cargos such as drugs (Glasgow and Tullman-Ercek 2014), proteins (Abbing et al. 2004), and both oligonucleotides and plasmid DNA (Braun et al. 1999).

To date, there are three VLP-based vaccines that have been officially approved for clinical use (Kushnir et al. 2012). The first to be approved was the recombinant hepatitis B (HB) VLP expressed in yeast (Recombivax HB, Merck), approved by the Food and Drug Administration (FDA) for use as a vaccine in 1986. The second was recombinant HPV VLP vaccine produced either in yeast (Gardasil, Merck), approved by the FDA in 2006, or in insect cells (Cervarix, GlaxoSmithKline), approved by the FDA in 2009. The third was a recombinant hepatitis E (HE) VLP vaccine produced in *Escherichia coli* (Hecolin, Xiamen Innovax Biotech), approved by Chinese FDA in 2011 (Rodriguez-Limas et al. 2013). Of note, is the fact that these VLP vaccines are all active against the cognate virus. The inherent immunogenicity of VLPs, related to their size and structure, suggests that they also make

promising platforms for the presentation of heterologous antigens either by genetic fusion, chemical conjugation, or encapsidation. Such bioengineering efforts to expand the use of VLP platforms produced in *E. coli* as vaccines for both infectious and chronic diseases are the focus of this review.

## 2 VLPs and Immunogenicity

VLP-based vaccines are engineered to carry heterologous antigens with the goal of stimulating one or both branches of adaptive immunity: humoral or cellular immunity. VLP vaccine candidates carrying non-proteinaceous antigens primarily aim to stimulate an antibody response. Humoral immunity relies on antibody production, which is initiated by B-cell receptor (BCR) recognition of foreign antigen (Murphy et al. 2008). BCR binds specifically and sterically to a surface-exposed region of the antigen known as the antigenic determinant or B-cell epitope. In proteins, the antigenic determinant may be a conformational epitope, in that it relies on the tertiary or quaternary structure of the antigen. Or, an epitope could simply be a string of amino acids, which is thus known as a continuous or linear epitope. Heterologous peptide antigens displayed on a VLP carrier usually come in the form of identified conformational or linear epitopes fused or conjugated to the VLP (Lee et al. 2016). The display of conformational epitopes poses a significant protein engineering challenge (Lua et al. 2014) and will be discussed in later sections.

Cellular immunity, on the other hand, relies on the activation of T cells by T-cell receptor (TCR) recognition of the antigenic determinant displayed on the surface of antigen-presenting cells (APCs). In this case, the antigenic determinant is a combination of a major histocompatibility complex (MHC) molecule and a string of amino acids derived from the antigen, the T-cell epitope (Murphy et al. 2008). Cytotoxic T-cell (Tc) epitopes are processed via cytosolic proteolysis as a part of the MHC Class I pathway, while helper T-cell (Th) epitopes are processed via lysosomal proteolysis as a part of the MHC Class II pathway. Tc epitopes are usually 8–10 amino acids long, while Th epitopes are usually longer (10–15 amino acids), and both are often hydrophobic (Mitic et al. 2014). For the delivery of heterologous Tc or Th epitopes by VLP vaccines, the peptides can either be displayed on the VLP surface or encapsidated within the interior of a VLP. However, the hydrophobicity of T-cell epitopes imposes stringent constraints on the location and context of epitope insertion to permit efficient expression and recovery of recombinant subunits and on the assembly and stability of VLPs.

The versatility of VLPs as heterologous antigen vaccine carriers is enhanced by the inherent properties of the particles that determine their immunostimulatory capacity. Principally, the repetitive nature of VLP structures activates innate as well as adaptive immunity by recognition of repetitive patterns as a characteristic of foreign entities (Bachmann and Jennings 2010). The repetitive structure of VLPs is due to the assembly of identical VLP subunits composed of one or more proteins. Therefore, VLP subunits can be manipulated to display foreign antigens in a

repetitive array that mirrors the structure on which it is presented. The multivalent display of heterologous antigens on the surface of a VLP augments humoral immunity by BCR cross-linking, which has been shown to strengthen B-cell activation and, in the case of self-antigen presentation, break B-cell tolerance (Bachmann and Jennings 2010; Schiller and Chackerian 2014). Furthermore, optimal BCR-mediated B-cell activation depends on antigen spacing. A distance of 5–10 nm between antigens is optimal for B-cell activation (Jegerlehner et al. 2002; Bachmann and Zinkernagel 1997), and, while this is not achievable with non-repetitive soluble antigens, the geometry of VLPs generally directs such spacing. In addition, there is some evidence to say that the repetitive pattern also permits complement activation, which can also enhance B-cell responses (Barrington et al. 2001).

The size of VLPs (20–200 nm) also contributes to their inherent immunogenicity. On one hand, their size allows them to migrate directly to secondary lymphoid organs where they may present intact antigens with a native configuration to B cells in the germinal center, which is a prerequisite for the stimulation of antibody production (Swartz 2001; Reddy et al. 2006; Oussoren et al. 1997). On the other hand, the size of VLPs also enables efficient uptake by professional antigen-presenting cells (APCs), especially dendritic cells. APC stimulation activates the MHC Class II pathway to further support the humoral response and antibody production by B cells. However, APC stimulation also activates the MHC Class I pathway, either directly or via cross-presentation, to support the cytotoxic activity of T cells (Bachmann and Jennings 2010). Recombinant icosahedral capsids may also have an empty cavity, which opens the possibility of loading immunogenic cargos such as polypeptides and nucleic acids. Uptake of VLPs can result in the intracellular release of their cargo, which is essential for cross-presentation and the stimulation of cellular immunity.

### 3 Bioengineering Strategies for Surface Presentation

Bioengineering approaches to presenting pathogenic antigens on heterologous VLP platforms must consider several factors such as immunogenicity, structural preservation, upstream and downstream bioprocess development, scalability, cost of production, and stability/shelf life. To improve humoral immunogenicity, antigens should be presented on the surface of the VLPs and their conformation preserved in the case of discontinuous epitopes. To improve cellular immunogenicity, vaccine design and VLP selection must ensure that the VLPs are taken up by APCs and are correctly processed by the MHC Class I or Class II pathways. Furthermore, VLP-based vaccine development must ensure that foreign antigen coupling does not interfere with assembly competency of the VLP and does not cause aggregation. In this review, we will focus on bioengineering design to improve immunogenicity and structural preservation while touching on the other factors briefly.

### 3.1 Genetic Fusion

VLPs subjected to bioengineering via genetic means generally consist of capsid protein monomers or assembly subunits, called capsomeres, composed of homomultimers. For stimulation of antibody production, antigenic sequences are fused to surface-exposed regions of the capsid monomer. To achieve this, the fusion of peptide or subunit antigens can be located at surface-exposed loops, the N-terminal domain, or the C-terminal domain of the capsid monomer, depending on the structure of the VLP platform used.

#### 3.1.1 Genetic Fusion to Surface-Exposed Loops

Antigen insertion on heterologous VLPs is frequently done by replacing an immunodominant region of a surface-exposed loop with the antigenic sequence (Chackerian et al. 1999; Cheong et al. 2009), or simply by inserting the antigen without native amino acid removal (Kawano et al. 2014; Tumban et al. 2011; Zamora et al. 2006; Anggraeni et al. 2013; Rivera-Hernandez et al. 2013; Abidin et al. 2015; Tekewe et al. 2015; Pattinson et al. 2019). This approach often poses a bioengineering challenge as the removal of native regions of the capsid monomer or the addition of foreign antigens has been shown to interfere with the assembly VLPs. For example, while the removal of different immunodominant regions in the bovine papilloma virus (BPV) L1 protein has been reported not to interfere with assembly (Chackerian et al. 1999), replacing these regions with the heterologous CCR5 extracellular loop resulted in the loss of assembly competency in three out of four constructs depending on which native immunodominant region was replaced. Conversely, removal of hepatitis B virus surface antigen (Hbs-Ag) Tc epitopes resulted in a loss of assembly in three out of four constructs, while replacement with an influenza M1 Tc epitope could restore assembly for one of these constructs. Moreover, the one Hbs-Ag construct that retained the ability to assemble after native Tc epitope removal subsequently lost its ability to assemble following foreign epitope insertion (Cheong et al. 2009). Nevertheless, insertion of nine amino acid N-terminus of A $\beta$  protein into an immunodominant loop of BPV L1 (Zamora et al. 2006) and insertion of conserved regions of HPV L2 protein into the AB loop of PP7 bacteriophage (Tumban et al. 2011) did not seem to interfere with assembly. These examples underline that while removal of capsid regions and replacing them with foreign sequences are likely to affect capsid protein assembly, such an impact is not predictable, and insertion without removal of native sequences is often less likely to interfere with assembly.

Bioengineering strategies to maintain assembly competency and to maintain antigen conformational structure have been reported in the literature. For example, Anggraeni et al. (Anggraeni et al. 2013) compared two bioengineering approaches when inserting the H190 loop of the influenza hemagglutinin (HA) onto the HI loop of the murine polyomavirus (MPyV) VP1 major capsid protein. Both computational

modeling and biochemical analysis confirmed that the presentation of a dual tandem repeat of H190 on VP1 surface-exposed loop produced more stably assembled VLPs in comparison to antigen presentation with flanking linker sequences. The dual tandem repeat approach also showed better antibody reactivity, which indicated that this approach might have better preserved the H190 antigen conformation. The introduction of flexible peptide linker sequences is a common strategy to reduced interference of the inserted sequence with capsid protein structure. Flanking GGG linker sequences may have helped the insertion of a hydrophobic influenza M1 Tc epitope on the surface-exposed DE and HI loops of the SV40 capsid protein, as the recombinant VLP assembled well when expressed in insect cells (Kawano et al. 2014). However, insertion of the same epitope on HI loop on the closely related MPyV VP1, expressed in a bacterial host, resulted in aggregation of the capsid subunit (Abidin et al. 2015). The different outcomes of the modification of these closely related VLP platforms may be due to the difference in the expression host. While the modified SV40 VP1 was expressed in insect cells and assembly occurred in vivo, the modified MPyV VP1 was expressed in a bacterial host and purified as capsomeres for further assembly in vitro. This underlines the important role of expression host and downstream processes in recombinant VLP bioengineering. In this case, to overcome aggregation of purified capsomeres in vitro, the inserted hydrophobic Tc epitope was flanked with double charged amino acid (DD) (Abidin et al. 2015).

### 3.1.2 Genetic Fusion to the N-Terminus or C-Terminus

Antigen fusion to free termini of a capsid monomer is possible and, in the case of longer sequences or structured domain insertions, preferable for antibody stimulation if either terminal domain is surface-exposed. Reported VLP platforms with surface-exposed N-terminal domain include the alfalfa mosaic virus (AIMV) (Yusibov et al. 1997), potato X virus (PVX) (Marusic et al. 2001), and the MS2 bacteriophage (Tumban et al. 2012). Fusion of the V3 loop of an HIV-1 protein or a rabies virus derived glycoprotein to the N-terminus of AIMV, and insertion of a linear B-cell epitope of the HIV gp41 protein onto the N-terminus of PVX, both expressed in plants, did not seem to interfere with assembly and stimulated considerable antibody production against the respective antigens. On the other hand, of four different HPV L2 epitopes separately fused to the N-terminus of the bacteriophage MS2 coat protein, three resulted in more regular recombinant VLPs, while one did not form VLP at all (Tumban et al. 2012). The higher proportion of constructs that form stable VLPs may have been aided by the application of a particular bioengineering strategy where the coding sequences of two capsid monomers were connected as a dual tandem repeat. This was based on structural studies which demonstrated that the C-terminus of one monomer is in close proximity with the N-terminus of an adjacent monomer in an assembled MS2 VLP. To avoid disruption in assembly by steric hindrance, the heterologous antigen was inserted only at the N-terminus of the upstream capsid monomer.

The C-terminal domains of VLP components have also been modified to stimulate either the humoral or cellular immunity. For humoral immunity, the C-terminal domain needs to be externally presented or at least available for the presentation of fused antigenic sequences on the surface. Li et al. (2021) inserted model linear T- and B-cell epitopes on the surface-exposed C-terminus of the P22 capsid protein. P22 VLPs assemble *in vivo* during expression in *E. coli* into stable VLPs, and the vaccine candidates produced stimulated high antibody titer and allowed T-cell antigen cross-presentation for a strong T-cell response.

Comparison of genetic fusion at different regions of the same capsid protein has provided more insights on the bioengineering of antigens on heterologous VLP platforms. Research reported in the literature once more highlight that the formation of stable recombinant VLP vaccines depends very much on the compatibility of the inserted antigens with the site of insertion and their effect on the overall structure of the recombinant VLP. Babin et al. (2013) compared influenza NP Tc epitope fusion to the N-terminal or the C-terminal domains of the papaya mosaic virus (PapMV) capsid protein. Fusion to the N-terminus yielded higher expression levels of the recombinant VLPs produced than fusion to the C-terminus. Interestingly, since fusion into the N-terminus domain results in a loss of the localization signal needed for effective expression of the original plant expression host, expression host was changed *E. coli* host where *in vivo* assembly was retained and expression yield was improved.

### 3.2 Chemical Conjugation

In addition to biological approach via genetic fusion, presentation of antigens on the surface of heterologous VLP platforms can be achieved by chemical conjugation. The advantage of chemical conjugation is the ability to not only conjugate protein antigens but also antigens of other biochemical nature such as alkaloids and polysaccharides. There are many ways to conjugate a heterologous antigen to the surface of VLPs depending on the nature of the antigen (Pokorski and Steinmetz 2011). It is most frequently carried out via common bioconjugation chemistries exploiting addressable, or surface-exposed, cysteine or lysine residues. N-hydroxysuccinimide (NHS) and maleimide reactive groups can form covalent linkages with side chains of lysine or cysteine residues, respectively, under aqueous and generally benign conditions that preserve antigenicity.

The versatility of the chemical conjugation approach has been shown in examples using the Q $\beta$  bacteriophage VLP. The alkaloid nicotine was conjugated via a succinimate linker, leading to an anti-smoking vaccine candidate that reached clinical trials (Maurer et al. 2005). The success of this approach was a result of the high density of nicotine presented on the Q $\beta$  VLP surface (565 per VLP) and subsequently high antibody response. A variation of this approach is to first introduce or modify chemical functionality, permitted further flexibility and control. Yin et al. (2013) conjugated the tumor-associated carbohydrate antigen (TACA) to

introduce alkyne groups on the VLP surface via the copper (I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC), or “click” chemistry. Using this approach they were able to conjugate TACA to Q $\beta$  VLPs at different densities and demonstrate that rather than the amount of the antigen, it is the density and organized display of the antigen that play an important role in eliciting antibody production and IgG isotype switching.

Chemical conjugation can also be a useful strategy for peptide and protein display. Pastori et al. (2012) used a hetero-bifunctional linker to conjugate several alpha-helix regions of the HIV gp41 protein with the A205 bacteriophage VLP. The linker contains an NHS-ester on one end designed to bind with an introduced lysine on the surface of the A205 bacteriophage VLP, while on the other end contains a maleimide designed to bind with a terminal cysteine of different variants of the alpha-helical antigen. The conjugated VLP vaccine candidate was formed via successive mixing of the vaccine candidate components. However, in this case the number of antigens and their density on the surface of the VLP was dependent on the length and the amino acid composition of the conjugated antigen, resulting in limited control over the number and density of surface antigens. In another example, a malaria antigen, circumsporozoite protein (CSP), was conjugated as an entire protein domain to Q $\beta$  VLPs (Khan et al. 2015), resulting in a much higher antibody response than that against the nonconjugated soluble protein. As noted earlier, genetic fusion of long or structure antigenic sequences is unpredictable and frequently disrupts VLP assembly. Since chemical conjugation is performed post-assembly, it provides a means to overcome this limitation for larger antigens. Furthermore, it enables the presentation of antigens with posttranslational requirements such as glycosylation not possible in prokaryotic cells whereby antigens can be produced in suitable eukaryotic cells for subsequent conjugation to a VLP platform.

### 3.3 Biochemical Conjugation

Recently, a new conjugation strategy using the SpyTag-SpyCatcher system to display antigens on heterologous VLP has been reported. The SpyTag and SpyCatcher elements are optimized split components derived from the second immunoglobulin-like collagen adhesion domain (CnB2) from the fibronectin-binding protein FbaB of *Streptococcus pyogenes*. The folding of the CnB2 domain is known to be stabilized by an isopeptide bond between the C-terminal aspartate and a lysine in the protein, catalyzed by adjacent glutamine. Zakeri et al. (2012) split the C-terminal  $\beta$  strand of CnB2 which contains the aspartate (SpyTag) from the rest of the protein domain (SpyCatcher). Further modification and optimization (Zakeri et al. 2012; Li et al. 2014) resulted in accelerated binding just by mixing. The resulting amide bond is formed under physiological conditions, yet is resistant to extreme pH, temperature, and diverse buffer conditions and allowed the use of these split components as tag partners.



Thrane et al. (2016) tested several combinations of the tag partners on the surface of the A205 bacteriophage. They either inserted SpyCatcher to the N-terminus (SpyCatcher-VLP), SpyTag on the N-terminus (SpyTag-VLP), or both the N and C-terminus (2xSpyTag-VLP) of the A205 capsid protein. They paired these modified VLPs with their respective tag partners fused separately at either the N- or C-terminus of a variety of antigens. The antigens tested included a total of 12 antigens derived from malaria, tuberculosis, and cancer, the size of which ranged from 15 to 118 kDa. Their research showed that the coupling efficiency of a variety VLP-antigen combination using the SpyTag-SpyCatcher tag partners resulted in a 22–88% coupling efficiency, where the number of conjugated antigens decreased with increasing antigen size. Moreover, they concluded that SpyTag-VLP is preferable for small antigen display, while SpyCatcher-VLP is preferable for large antigen display. The variety of SpyCatcher-SpyTag antigen-tagged VLPs elicited high IgG titer and, in the case of self-antigens, were able to break B-cell tolerance. Similar results with malaria antigens have also been reported by Brune et al. (2016), and Janitzek et al. (2016) tested this strategy further by fusion of whole CSP with SpyCatcher and conjugating this large whole protein antigen into a SpyTag-VLP. Interestingly, while the SpyTag fused A205 VLP was produced in a bacterial host, the CSP-SpyCatcher antigen was produced in insect cells for the desired posttranslational modification important for antibody recognition. The development of the SpyCatcher-SpyTag tag partner strategy opens up new possibilities in displaying conformational antigens, especially large antigens, onto the surface of heterologous VLP platforms.

### 3.4 Capsid Protein Stoichiometry

Aside from modification at the capsid monomer protein level, bioengineering strategies to develop effective vaccines also encompass modification at the VLP assembly level. This includes, for example, presentation of multiple antigens or a combination of differential capsid monomers or subunits to form mosaic VLPs. Mosaic VLPs are VLPs composed of differently modified monomers or subunit proteins. Mosaic VLPs are developed for different purposes, such as to restore assembly lost due to steric hindrance or other factors, to broaden immune response, and to reduce surface hydrophobicity-related aggregation.

Tyler et al. (2014) used the co-expression strategy to assemble mosaic VLPs containing two different antigens for broader cross-protection against heterologous HPV strains. They did this by co-expressing two dual tandem repeats of either the PP7 or MS2 capsid monomer, where each of the dual tandem repeats contains different HPV L2-derived antigens. Their experiments showed that the co-expression of dual tandem repeats, each displaying different antigens, resulted in recombinant mosaic VLPs which elicited broader cross-protection against a wider spectrum of heterologous HPV strains in comparison to their single dual tandem repeat designs.

Co-expression of modified and unmodified capsid monomers or subunit have also been applied to prevent surface crowding and steric hindrance or to reduce surface hydrophobicity to prevent aggregation. Though not in an explicit vaccine context, steric hindrance that interfered with assembly was experienced by Brown et al. (2009) and Pokorski et al. (2011) when they inserted the Z protein or the epidermal growth factor (EGF), respectively, onto Q $\beta$  particles assembled in vivo. Co-expression of the modified and unmodified Q $\beta$  monomers restored the assembly competency of recombinant mosaic VLPs. The former was able to bind IgG with high capacity (Brown et al. 2009), and the latter was shown to specifically react with EGF receptor on mammalian cells (Pokorski et al. 2011).

Tekewe et al. (2017) co-expressed unmodified MPyV VP1 with modular MPyV VP1 displaying the rotavirus VP8\* antigen on the surface in a bacterial host. Co-expression in the bacterial host resulted in mosaic pentameric capsomeres that could then be assembled into recombinant mosaic VLPs in vitro. Without the co-expression strategy, the pentameric subunit experienced aggregation due to the hydrophobic nature of the antigen, and mosaic subunit formation with unmodified VP1 reduced aggregation and restored the capacity for assembly. The resulting recombinant mosaic VLP induced a high level of VP8\* antibodies in immunized mice. The ability of expression host cells to accommodate in vivo assembly is preferable in several ways such as shorter bioprocess steps and the inclusion of nucleic acid which may stimulate the innate immunity via TLR activation (Bessa et al. 2009). However, platforms that employ in vitro assembly provide the ability to control the composition of the mosaic VLPs as in the above example and, theoretically, the ability to modulate encapsulation of antigens inside VLPs.

### 3.5 Encapsulation

Bioengineering strategy to encapsulate antigens inside the cavity of heterologous VLP platform could be an option for antigens which do not need to be externally displayed to stimulate the adaptive immunity. This is especially preferable for antigens to stimulate the cellular immunity due to several reasons. First, T-cell epitopes are mostly hydrophobic, and their display on the surface of the VLPs is prone to aggregation. Second, T-cell epitope recognition does not depend on antigen conformation but rather on the ability to be taken up by APCs, especially dendritic cells, and be presented by either the MHC Class I and Class II pathways. Third, to ensure that the antigen is able to reach the intracellular compartments, encapsulation would be preferable to avoid extracellular antibody neutralization. Fourth, the ability to encapsulate larger protein antigen consisted of T-cell epitopes specific to a variety of HLAs does not only prevent hydrophobicity-related aggregation but also protect broadly against heterologous strains and a broader population coverage.

The MPyV platform has been shown to provide programmed encapsulation of heterologous proteins and even to co-encapsulation of multiple proteins during in vitro assembly (Dashti et al. 2018). Incorporation is driven by specific interactions

between the pentameric capsomeres and the C-terminus of the minor capsid protein, VP2 (Abbing et al. 2004; Boura et al. 2005; Pleckaityte et al. 2015), and examples where production and in vivo assembly have been carried out in eukaryotic cells show the potential of this platform as carriers of encapsulated antigens. Eriksson et al. (2011) fused the prostate-specific antigen (PSA) to the C-terminus of VP2 and demonstrated that while no PSA-specific antibodies were produced, dendritic cells loaded with PSA-MPyV VLPs strengthen protection against PSA-expressing tumors. Cellular immunity such as Tc and Th response was also high in loaded dendritic cell treatment, indicating efficient delivery of the antigen to the cytosol of these cells. Similar results were shown when HER2, a breast cancer antigen, was fused to MPyV VP2 instead of PSA (Tegerstedt et al. 2007).

Co-encapsulation of two different proteins, such as the M and M2 proteins of the respiratory syncytial virus (RSV) inside the P22 VLP, has also been reported (Schwarz et al. 2016). The P22 VLP is able to encapsulate heterologous proteins fused to the C-terminus of the scaffold protein that binds to the interior of the capsid during assembly. Schwarz et al. concatenated the M and M2 gene for encapsulation in this manner. Despite the quaternary structure of the fusion, VLPs of well-rounded and correct sizes bearing both the CP and M/M2-SP proteins were assembled in vivo when co-expressed in *E. coli* (Schwarz et al. 2016). Administration of the M/M2-P22 VLP alone resulted in reduced lung viral titer in RSV challenged mice and elevated Tc response. However, it was not known whether loading M/M2 P22 VLP into dendritic cells prior to immunization in mice would improve protection and Tc response.

## 4 Bioengineering Strategies for Bioprocess Optimization

As has been described throughout this review, the bioengineering of VLPs to incorporate heterologous antigens often poses challenges to be addressed for successful manufacturing. These challenges include improvement in overall bioprocess development; overcoming biosynthetic challenges such as soluble protein yield, aggregation, upstream, and downstream method selection and validation; and reducing overall production cost. A few example approaches to address these challenges are described in this section that demonstrate the effectiveness of designing process optimization experiments with increased throughput.

### 4.1 *Upstream Bioprocess Optimization*

The optimal result in protein expression at the upstream bioprocess depends on many interacting factors such as temperature, oxygen availability, initial culture concentration, culture acidity, additives, inducer concentration, induction time, and harvest time. Assessing these factors one by one or in combination in laboratory scale would

be resource, labor, and time-consuming. Therefore, a high-throughput method to assess different culture condition combinations at once at milliliter scale would be a versatile and informative option to optimize upstream bioprocess.

In producing vaccine candidates based in MPyV VP1, Lad Effio et al. (2016a) examined a triplicate of 16 culture conditions varied by pH, the presence of additives, 2 different inducer concentrations, and 2 different induction times in a miniaturized 48 well plates which were then incubated at 3 different temperatures and 2 different shaking speeds. The high-throughput multiparametric culture conditions screen aimed to discover the best culture condition for the expression of untagged MPyV VP1 protein in *E. coli*, adapted from GST-tagged MPyV VP1 upstream platform previously reported (Chuan et al. 2008). The results showed that the best culture condition for the untagged protein was different from the previously reported optimum condition for GST-tagged MPyV VP1. The selected condition was scalable in 2.5 L culture flasks, and the amount of purified VP1 obtained from this laboratory scale expression was similar to quantitated soluble VP1 derived from the miniaturized culture. This work showed that a high-throughput culture condition screen can be a robust approach for bacterially produced VLP subunits and would be useful in upstream bioprocess optimization of other microbially expressed industrial proteins.

#### **4.2 Downstream Bioprocess Optimization by High-Throughput Screening**

Along with upstream bioprocess optimization, high-throughput approaches to optimize downstream bioprocess in bioengineering heterologous antigens of VLP platforms have also been reported. Abidin et al. (2015) reported the use of a multiparametric high-throughput buffer screen to determine the most suitable condition to prevent aggregation of MPyV capsomeres displaying hydrophobic epitopes. The buffer screen tested 40 buffer conditions in a miniaturized 96-well format examining 6 different additives across 5 different pH. The results, based on spectrophotometry and light scattering analysis, showed that the addition of L-arginine (L-Arg) was the most promising additive to recover soluble capsomere during tag removal before in vitro assembly. Tekewe et al. (2015) also reported light scattering-based rapid screening method to select appropriate additives to stabilize MPyV capsomeres displaying rotaviral epitopes, though at lower throughput.

The development of VLP-based vaccines necessitates a robust VLP purification method to isolate VLPs assembled in vivo or in vitro. An ideal VLP purification method would have a good resolution to separate between aggregates, VLPs, and unassembled VLP subunits as well as capacity for rapid throughput for condition screening. The current methods to assess the quality of developed VLP purification methods involve TEM and light scattering analysis. Ladd Effio et al. (2016b) recently reported a novel high-throughput interlaced size exclusion-ultrahigh-

performance chromatography (SE-UHPLC) which allows rapid assessment of VLP purification conditions. The interlaced SE-UHPLC was designed to shorten analysis time by overlapping the hold-up phase of one sample application with the lag phase of the next sample application, reducing run times to approximately 3 minutes per sample. The analysis tool was validated with multiple commercially available VLP vaccines as well as *E. coli*-produced hepatitis B and MPyV VLPs. The particle size distribution and aggregate percentage analysis of the iSE-UHPLC were compared with dynamic light scattering (DLS) and TEM analysis, and it was shown that the iSE-HPLC was more sensitive in detecting aggregates and more accurate in calculating particle size. The iSE-HPLC analysis tool was then used to assess four VLP purification methods and conduct stability studies.

### 4.3 Platform Development to Reduce Production Cost

One of the important aspects of vaccine development, besides as a preventive and therapeutic measure, is the reduction in production cost and time. Production cost and production time are necessary aspects to ensure that the vaccines are affordable, widely distributable, and timely available.

A bacterial platform and process was developed for the production of the MPyV VLP platform as a commercially viable system using *E. coli* as the expression host (Chuan et al. 2008; Liew et al. 2010). Briefly, at laboratory scale, capsomeres are produced in bacteria (Chuan et al. 2008; Middelberg et al. 2011) and harvested as capsomeres by affinity chromatography and further purified by gel filtration after tag removal (Lipin et al. 2009). The capsomeres can be maintained in capsomere form or assembled into VLP in vitro using optimized buffer conditions (Chuan et al. 2010). The laboratory scale production has subsequently been translated into a large-scale process, involving fed-batch *E. coli* expression with a volumetric protein yield of 4.38 g/l which is 15-fold laboratory scale production (Liew et al. 2010). Capsomere purification can also be achieved by ion exchange chromatography (IEX) (Chuan et al. 2014), and assembly into VLP can be performed by either diafiltration or dilution methods (Liew et al. 2012a; Liew et al. 2012b). Optimized assembly buffer for the assembly via diafiltration increased yield by 42–56% in comparison to laboratory scale assembly by dialysis (Liew et al. 2012a). Analysis of this extensive process development estimated the cost of optimized large-scale production of capsomeres and VLPs at 500 L and 1500 L volume, respectively, to be at less than 1 cent (USD) per dose (50 µg per dose). Moreover, it was predicted that in a 10 k-L production scale, 320 million capsomere or VLP vaccine doses could be produced within 2.3 or 4.7 days, respectively (Chuan et al. 2014). In addition, an ammonium sulfate-based precipitation method to harvest MPyV capsomeres to skip laborious and costly chromatographic process has also been developed (Wibowo et al. 2015). The precipitation method was successfully used to purify mosaic capsomeres, and in vitro assembled VLPs elicited high titer antibody in immunized mice (Tekewe et al. 2017). Although this approach is not suitable for human vaccine production

due to unacceptable levels of host cell contaminants, it may stimulate use in veterinary settings and demonstrate the remarkable capacity of prokaryotes for cost-effective vaccine production.

## 5 Conclusions and Perspective

VLPs have a now long track record as successful subunit vaccines. Although those VLPs that are currently available commercially as vaccines are designed to raise immunity against the virus from which they are derived, there is a growing interest in the development of VLPs as carriers of heterologous antigens. Their inherent physical properties, leading to high immunogenicity, make them ideal platforms to develop as vaccines against not just infectious diseases but also nonpathogen-related cancers and other chronic diseases and conditions. They are amenable to precise modification by genetic or chemical means and are characterized by structural fidelity. As we have described herein, the use of prokaryotes, specifically *E. coli*, to produce VLPs provides an accessible and low-cost source of VLPs or VLP subunits for further development. One of the drawbacks of marketed VLPs vaccines has been relatively high cost, limiting their immediate use to developed countries. Bioengineering and bioprocessing improvements, including the use of prokaryotic production hosts such as *E. coli*, aim to reduce the cost and improve the efficacy of VLP vaccines. While challenges remain with regard to the manufacture of VLP vaccines incorporating heterologous antigens, a myriad of bioengineering approaches combined with recent developments in bioprocessing hold great promise.

## References

- Abbing A, Blaschke UK, Grein S, Kretschmar M, Stark CM, Thies MJ, Walter J, Weigand M, Woith DC, Hess J, Reiser CO (2004) Efficient intracellular delivery of a protein and a low molecular weight substance via recombinant polyomavirus-like particles. *J Biol Chem* 279(26): 27410–27421
- Abidin RS, Lua LH, Middelberg AP, Sainsbury F (2015) Insert engineering and solubility screening improves recovery of virus-like particle subunits displaying hydrophobic epitopes. *Protein Sci* 24(11):1820–1828
- Anggraeni MR, Connors NK, Wu Y, Chuan YP, Lua LH, Middelberg AP (2013) Sensitivity of immune response quality to influenza helix 190 antigen structure displayed on a modular virus-like particle. *Vaccine* 31(40):4428–4435
- Babin C, Majeau N, Leclerc D (2013) Engineering of papaya mosaic virus (PapMV) nanoparticles with a CTL epitope derived from influenza NP. *J Nanobiotechnol* 11:10
- Bachmann MF, Jennings GT (2010) Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* 10(11):787–796
- Bachmann MF, Zinkernagel RM (1997) Neutralizing antiviral B cell responses. *Annu Rev Immunol* 15:235–270

- Barrington R, Zhang M, Fischer M, Carroll MC (2001) The role of complement in inflammation and adaptive immunity. *Immunol Rev* 180:5–15
- Bayer ME, Blumberg BS, Werner B (1968) Particles associated with Australia antigen in the sera of patients with leukaemia, Down's Syndrome and hepatitis. *Nature* 218(5146):1057–1059
- Bessa J, Jegerlehner A, Hinton HJ, Pumpens P, Saudan P, Schneider P, Bachmann MF (2009) Alveolar macrophages and lung dendritic cells sense RNA and drive mucosal IgA responses. *J Immunol* 183(6):3788–3799
- Blumberg BS, Alter HJ, Visnich S (1965) A "new" antigen in leukemia sera. *JAMA* 191:541–546
- Boura E, Liebl D, Spisek R, Fric J, Marek M, Stokrova J, Holan V, Forstova J (2005) Polyomavirus EGFP-pseudocapsids: analysis of model particles for introduction of proteins and peptides into mammalian cells. *FEBS Lett* 579(29):6549–6558
- Braun H, Boller K, Lower J, Bertling WM, Zimmer A (1999) Oligonucleotide and plasmid DNA packaging into polyoma VP1 virus-like particles expressed in *Escherichia coli*. *Biotechnol Appl Biochem* 29:31–43
- Brown SD, Fiedler JD, Finn MG (2009) Assembly of hybrid bacteriophage Qbeta virus-like particles. *Biochemistry* 48(47):11155–11157
- Brune KD, Leneghan DB, Brian IJ, Ishizuka AS, Bachmann MF, Draper SJ, Biswas S, Howarth M (2016) Plug-and-display: decoration of virus-like particles via isopeptide bonds for modular immunization. *Sci Rep* 6:19234
- Chackerian B, Lowy DR, Schiller JT (1999) Induction of autoantibodies to mouse CCR5 with recombinant papillomavirus particles. *Proc Natl Acad Sci U S A* 96(5):2373–2378
- Cheong WS, Reiseiger J, Turner SJ, Boyd R, Netter HJ (2009) Chimeric virus-like particles for the delivery of an inserted conserved influenza A-specific CTL epitope. *Antivir Res* 81(2):113–122
- Chuan YP, Fan YY, Lua LH, Middelberg AP (2010) Virus assembly occurs following a pH- or Ca<sup>2+</sup>-triggered switch in the thermodynamic attraction between structural protein capsomeres. *J R Soc Interface* 7(44):409–421
- Chuan YP, Lua LH, Middelberg AP (2008) High-level expression of soluble viral structural protein in *Escherichia coli*. *J Biotechnol* 134(1–2):64–71
- Chuan YP, Wibowo N, Lua LHL, Middelberg APJ (2014) The economics of virus-like particle and capsomere vaccines. *Biochem Eng J* 90:255
- Dashti NH, Abidin RS, Sainsbury F (2018) Programmable in vitro coencapsidation of guest proteins for intracellular delivery by virus-like particles. *ACS Nano* 12(5):4615–4623
- Eriksson M, Andreasson K, Weidmann J, Lundberg K, Tegerstedt K, Dalianis T, Ramqvist T (2011) Murine polyomavirus virus-like particles carrying full-length human PSA protect BALB/c mice from outgrowth of a PSA expressing tumor. *PLoS One* 6(8):e23828
- Glasgow J, Tullman-Ereck D (2014) Production and applications of engineered viral capsids. *Appl Microbiol Biotechnol* 98(13):5847–5858
- Hagensee ME, Yaegashi N, Galloway DA (1993) Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *J Virol* 67(1):315–322
- Janitzek CM, Matondo S, Thrane S, Nielsen MA, Kavishe R, Mwakalinga SB, Theander TG, Salanti A, Sander AF (2016) Bacterial superglue generates a full-length circumsporozoite protein virus-like particle vaccine capable of inducing high and durable antibody responses. *Malar J* 15(1):545
- Jegerlehner A, Storni T, Lipowsky G, Schmid M, Pumpens P, Bachmann MF (2002) Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation. *Eur J Immunol* 32(11):3305–3314
- Kawano M, Morikawa K, Suda T, Ohno N, Matsushita S, Akatsuka T, Handa H, Matsui M (2014) Chimeric SV40 virus-like particles induce specific cytotoxicity and protective immunity against influenza A virus without the need of adjuvants. *Virology* 448:159–167
- Khan F, Porter M, Schwenk R, DeBot M, Saudan P, Dutta S (2015) Head-to-head comparison of soluble vs. Q $\beta$  VLP circumsporozoite protein vaccines reveals selective enhancement of NANP repeat responses. *PLoS One* 10(11):e0142035

- Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT (1992) Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc Natl Acad Sci U S A* 89(24):12180–12184
- Kushnir N, Streatfield SJ, Yusibov V (2012) Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. *Vaccine* 31(1):58–83
- Ladd Effio C, Baumann P, Weigel C, Vormittag P, Middelberg A, Hubbuch J (2016a) High-throughput process development of an alternative platform for the production of virus-like particles in *Escherichia coli*. *J Biotechnol* 219:7–19
- Ladd Effio C, Oelmeier SA, Hubbuch J (2016b) High-throughput characterization of virus-like particles by interlaced size-exclusion chromatography. *Vaccine* 34(10):1259–1267
- Lee KL, Twyman RM, Fiering S, Steinmetz NF (2016) Virus-based nanoparticles as platform technologies for modern vaccines. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 8(4):554–578
- Li L, Fierer JO, Rapoport TA, Howarth M (2014) Structural analysis and optimization of the covalent association between SpyCatcher and a peptide tag. *J Mol Biol* 426(2):309–317
- Li M, Cripe TP, Estes PA, Lyon MK, Rose RC, Garcea RL (1997) Expression of the human papillomavirus type 11 L1 capsid protein in *Escherichia coli*: characterization of protein domains involved in DNA binding and capsid assembly. *J Virol* 71(4):2988–2995
- Li W, Jing Z, Wang S, Li Q, Xing Y, Shi H, Li S, Hong Z (2021) P22 virus-like particles as an effective antigen delivery nanopatform for cancer immunotherapy. *Biomaterials* 271:120726
- Liew MW, Rajendran A, Middelberg AP (2010) Microbial production of virus-like particle vaccine protein at gram-per-litre levels. *J Biotechnol* 150(2):224–231
- Liew MWO, Chuan YP, Middelberg APJ (2012a) Reactive diafiltration for assembly and formulation of virus-like particles. *Biochem Eng J* 68:120–128
- Liew MWO, Chuan YP, Middelberg APJ (2012b) High-yield and scalable cell-free assembly of virus-like particles by dilution. *Biochem Eng J* 67:88–96
- Lipin DI, Raj A, Lua LH, Middelberg AP (2009) Affinity purification of viral protein having heterogeneous quaternary structure: modeling the impact of soluble aggregates on chromatographic performance. *J Chromatogr A* 1216(30):5696–5708
- Lua LH, Connors NK, Sainsbury F, Chuan YP, Wibowo N, Middelberg AP (2014) Bioengineering virus-like particles as vaccines. *Biotechnol Bioeng* 111(3):425–440
- Marusic C, Rizza P, Lattanzi L, Mancini C, Spada M, Belardelli F, Benvenuto E, Capone I (2001) Chimeric plant virus particles as immunogens for inducing murine and human immune responses against human immunodeficiency virus type 1. *J Virol* 75(18):8434–8439
- Maurer P, Jennings GT, Willers J, Rohner F, Lindman Y, Roubicek K, Renner WA, Muller P, Bachmann MF (2005) A therapeutic vaccine for nicotine dependence: preclinical efficacy, and phase I safety and immunogenicity. *Eur J Immunol* 35(7):2031–2040
- Middelberg AP, Rivera-Hernandez T, Wibowo N, Lua LH, Fan Y, Magor 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
- Mitic NS, Pavlovic MD, Jandrlic DR (2014) Epitope distribution in ordered and disordered protein regions - part a. T-cell epitope frequency, affinity and hydrophathy. *J Immunol Methods* 406:83–103
- Murphy K, Travers P, Walport M, Janeway C (2008) *Janeway's Immunobiology*, 7th edn. Garland Science, New York
- Oussoren C, Zuidema J, Crommelin DJ, Storm G (1997) Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. Influence of liposomal size, lipid composition and lipid dose. *Biochim Biophys Acta* 1328(2):261–272
- Pastori C, Tudor D, Diomedea L, Drillet AS, Jegerlehner A, Rohn TA, Bomsel M, Lopalco L (2012) Virus like particle based strategy to elicit HIV-protective antibodies to the alpha-helic regions of gp41. *Virology* 431(1–2):1–11



- Pattinson DJ, Apte SH, Wibowo N, Chuan YP, Rivera-Hernandez T, Groves PL, Lua LH, Middelberg APJ, Doolan DL (2019) Chimeric murine polyomavirus virus-like particles induce plasmodium antigen-specific CD8+ T cell and antibody responses. *Front Cell Infect Microbiol* 9:215
- Pleckaityte M, Bremer CM, Gedvilaite A, Kucinskaite-Kodze I, Glebe D, Zvirbliene A (2015) Construction of polyomavirus-derived pseudotype virus-like particles displaying a functionally active neutralizing antibody against hepatitis B virus surface antigen. *BMC Biotechnol* 15:85
- Pokorski JK, Hovlid ML, Finn MG (2011) Cell targeting with hybrid Qbeta virus-like particles displaying epidermal growth factor. *Chembiochem* 12(16):2441–2447
- Pokorski JK, Steinmetz NF (2011) The art of engineering viral nanoparticles. *Mol Pharm* 8(1): 29–43
- Reddy ST, Rehor A, Schmoekel HG, Hubbell JA, Swartz MA (2006) In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. *J Control Release* 112(1): 26–34
- Rivera-Hernandez T, Hartas J, Wu Y, Chuan YP, Lua LH, Good M, Batzloff MR, Middelberg AP (2013) Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS). *Vaccine* 31(15):1950–1955
- Rodriguez-Limas WA, Sekar K, Tyo KE (2013) Virus-like particles: the future of microbial factories and cell-free systems as platforms for vaccine development. *Curr Opin Biotechnol* 24(6):1089–1093
- Salunke DM, Caspar DL, Garcea RL (1986) Self-assembly of purified polyomavirus capsid protein VP1. *Cell* 46(6):895–904
- Schiller J, Chackerian B (2014) Why HIV virions have low numbers of envelope spikes: implications for vaccine development. *PLoS Pathog* 10(8):e1004254
- Schwarz B, Morabito KM, Ruckwardt TJ, Patterson DP, Avera J, Miettinen HM, Graham BS, Douglas T (2016) Virus like particles encapsidating respiratory syncytial virus M and M2 proteins induce robust T cell responses. *ACS Biomaterials Sci Eng* 2(12):2324–2332
- Swartz MA (2001) The physiology of the lymphatic system. *Adv Drug Deliv Rev* 50(1–2):3–20
- Tegerstedt K, Franzen A, Ramqvist T, Dalianis T (2007) Dendritic cells loaded with polyomavirus VP1/VP2Her2 virus-like particles efficiently prevent outgrowth of a Her2/neu expressing tumor. *Cancer Immunol Immunother* 56(9):1335–1344
- Tekewe A, Connors NK, Sainsbury F, Wibowo N, Lua LH, Middelberg APJ (2015) A rapid and simple screening method to identify conditions for enhanced stability of modular vaccine candidates. *Biochem Eng J* 100:50–58
- Tekewe A, Fan Y, Tan E, Middelberg AP, Lua LH (2017) Integrated molecular and bioprocess engineering for bacterially produced immunogenic modular virus-like particle vaccine displaying 18 kDa rotavirus antigen. *Biotechnol Bioeng* 114(2):397–406
- Teunissen EA, de Raad M, Mastrobattista E (2013) Production and biomedical applications of virus-like particles derived from polyomaviruses. *J Control Release* 172(1):305–321
- Thrane S, Janitzek CM, Matondo S, Resende M, Gustavsson T, de Jongh WA, Clemmensen S, Roeffen W, van de Vegte-Bolmer M, van Gemert GJ, Sauerwein R, Schiller JT, Nielsen MA, Theander TG, Salanti A, Sander AF (2016) Bacterial superglue enables easy development of efficient virus-like particle based vaccines. *J Nanobiotechnol* 14:30
- Tumban E, Peabody J, Peabody DS, Chackerian B (2011) A pan-HPV vaccine based on bacteriophage PP7 VLPs displaying broadly cross-neutralizing epitopes from the HPV minor capsid protein, L2. *PLoS One* 6(8):e23310
- Tumban E, Peabody J, Tyler M, Peabody DS, Chackerian B (2012) VLPs displaying a single L2 epitope induce broadly cross-neutralizing antibodies against human papillomavirus. *PLoS One* 7(11):e49751
- Tyler M, Tumban E, Peabody DS, Chackerian B (2014) The use of hybrid virus-like particles to enhance the immunogenicity of a broadly protective HPV vaccine. *Biotechnol Bioeng* 111(12): 2398–2406

- Wibowo N, Chuan YP, Lua LH, Middelberg AP (2012) Modular engineering of a microbially-produced viral capsomere vaccine. *Chem Eng Sci* 103:12–20
- Wibowo N, Wu Y, Fan Y, Meers J, Lua LH, Middelberg AP (2015) Non-chromatographic preparation of a bacterially produced single-shot modular virus-like particle capsomere vaccine for avian influenza. *Vaccine* 33(44):5960–5965
- Yan D, Wei YQ, Guo HC, Sun SQ (2015) The application of virus-like particles as vaccines and biological vehicles. *Appl Microbiol Biotechnol* 99(24):10415–10432
- Yin Z, Comellas-Aragones M, Chowdhury S, Bentley P, Kaczanowska K, Benmohamed L, Gildersleeve JC, Finn MG, Huang X (2013) Boosting immunity to small tumor-associated carbohydrates with bacteriophage qbeta capsids. *ACS Chem Biol* 8(6):1253–1262
- Yusibov V, Modelska A, Steplewski K, Agadjanyan M, Weiner D, Hooper DC, Koprowski H (1997) Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1. *Proc Natl Acad Sci U S A* 94(11):5784–5788
- Zakeri B, Fierer JO, Celik E, Chittock EC, Schwarz-Linek U, Moy VT, Howarth M (2012) Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proc Natl Acad Sci U S A* 109(12):E690–E697
- Zamora E, Handisurya A, Shafti-Keramat S, Borchelt D, Rudow G, Conant K, Cox C, Troncoso JC, Kirnbauer R (2006) Papillomavirus-like particles are an effective platform for amyloid-beta immunization in rabbits and transgenic mice. *J Immunol* 177(4):2662–2670
- Zeltins A (2013) Construction and characterization of virus-like particles: a review. *Mol Biotechnol* 53(1):92–107