Development of picornavirus-like particle vaccines

Vaccination has been the most effective and economical way of combating the spread of infectious disease. Virus-like particles (VLPs) against human and animal diseases caused by picornaviruses are actively being researched and developed as potential vaccines. Promising immunogenicity and protective efficacy data has been reported for VLPs produced using various expression systems. The translation of these VLPs into vaccine products requires further bioprocess development, underpinned by advanced analytical VLP characterization techniques. We overview approaches taken to effectively produce and purify these multiprotein VLPs to achieve the desired immune responses. The opportunities for developing VLP bioprocessing technologies for cost-effective and efficient vaccine manufacturing are highlighted.

Picornaviruses are positive-stranded RNA viruses that belong to the family Picornaviridae. Among the several genera in this family, the commonly known ones are Enterovirus and Aphthovirus due to the human and animal diseases caused by these viruses. Poliovirus and viruses causing hand-foot-and-mouth disease (HFMD) fall under Enterovirus genus, while foot-and-mouth disease virus (FMDV) is classified under Aphthovirus. In recent years, developing new vaccine technologies to combat polio, HFMD and FMD have gained attraction.

Poliovirus, the causative agent of poliomyelitis, is the most recognized virus of human enteroviruses because of the historically devastating disease and well publicized Global Polio Eradication Initiative by WHO [1,2]. Inactivated poliovirus vaccine and live attenuated oral poliovirus vaccine have dramatically reduced the spread of polio. As illustrated in Figure 1, poliomyelitis is currently reported only in Pakistan, Afghanistan and Nigeria. However, vaccine-derived polio outbreaks due to use of oral poliovirus vaccine is a concern [3]. The use of live poliovirus for the production of vaccine is not without risk, and its use should be eliminated to achieve global eradication of polio.

In the last decade, regular epidemics of HFMD, typically caused by Enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16), in the Asia-Pacific region has garnered public health attention [4–6]. HFMD affects young children and can cause severe neurological diseases, and is potentially fatal in children. In recent years, large outbreaks have occurred in Mainland China, with cumulative number of reported cases higher than 4 million for 2012 and 2013 (WHO). HFMD cases were also reported in Australia, Hong Kong, Japan, Malaysia, Republic of Korea, Singapore, Taiwan, Thailand and Vietnam [1,7]. The countries affected by HFMD are represented in Figure 1. To date, no therapeutics or preventive vaccines are available for HFMD, and general hygiene is the only form of control.

FMDV is a pathogen that has a significant economical impact on livestock [8,9]. The virus is highly contagious, causes vesicular disease in cloven-hoofed animals, and is a major threat to livestock worldwide. Severe outbreaks in South Korea and Japan (year 2000), and the 2001 epidemic in UK, France and the Netherlands had led to significant economic loss [9]. Vaccinations with inactivated whole virus and control by animal
The widespread use of poliovirus vaccine has declined the incidence of poliomyelitis worldwide and is currently reported only in Pakistan, Afghanistan and Nigeria. On the contrary, HFMD has been a major public health issue mostly across the Asia-Pacific region. The cumulative numbers of reported HFMD cases (2012 and 2013) in the most affected countries are presented. HFMD: Hand-foot-and-mouth disease.

<table>
<thead>
<tr>
<th>Countries</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td>2,198,442</td>
<td>1,855,457</td>
</tr>
<tr>
<td>Japan</td>
<td>71,144</td>
<td>300,314</td>
</tr>
<tr>
<td>Viet Nam</td>
<td>Not available</td>
<td>71,627</td>
</tr>
<tr>
<td>Singapore</td>
<td>37,125</td>
<td>31,780</td>
</tr>
<tr>
<td>Macao</td>
<td>2005</td>
<td>2166</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>514</td>
<td>1630</td>
</tr>
</tbody>
</table>

Culling during an outbreak have not been satisfactory, thus tremendous amount of time, effort and money has been invested in developing alternative vaccine strategies.

Vaccination is by far the most effective and economical way of controlling the spread of any infectious diseases in human and animal. Although vaccines consisting of inactivated or live attenuated viruses have proven to be effective, their safety has always been questioned. Although rarely, live attenuated pathogens can revert to original pathogenic form and cause disease in those immunized and their contacts. Safer vaccine strategies such as recombinant subunit vaccine, epitope peptide vaccine and DNA vaccine have been developed [10,11]. However, their immunogenicity is generally low in comparison to inactivated or live attenuated vaccines. On the other hand, the demonstrated success by licensed virus-like particle (VLP) vaccines, as safe and effective vaccines, has propelled the development of VLP vaccines against picornavirus.

VLPs are highly ordered macromolecular structures of viral proteins. They are structurally similar and immunologically stimulative as their native virus but are without the viral infectivity [10,12–14] as they lack viral genome. VLPs have been proven to induce both innate and adaptive immunity, generating immune responses comparable to inactivated and attenuated virus vaccines [15–19]. VLP-based vaccines for polio, HFMD and FMD have been actively researched and were reviewed recently [8]. As a multiprotein VLP, picornavirus VLP is structurally more complex to produce in comparison to all currently licensed single-
protein VLP vaccines. In this review, we discuss the developments and progress with taking picornavirus-like particle from research to vaccine manufacturing. Here, we highlight the challenges and opportunities associated with the processing of picornavirus VLPs for translation into vaccine products.

**Structure of picornavirus**

Picornaviruses are nonenveloped capsids of icosahedral symmetry, 25–35 nm in diameter, containing a single-stranded positive sense RNA (7.2 to 8.5 kb) [20,21]. The viral genome consists of the three polyprotein regions, namely P1, P2 and P3 (Figure 2A), translated as a single polyprotein. The P2 and P3 regions encode seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) that are essential for viral replication and virulence. P1 region encodes a precursor protein P1, typically about 90 kDa, which is proteolytically processed into structural proteins VP0, VP3 and VP1 (protomer) by the virally encoded protease. As the three **structural proteins** are never separated, the protomer is considered as a P1 monomer. The protomers assemble into a pentameric intermediates, and 12 pentamers further assemble into an icosahedral procapsid structure (Figure 2B). This is followed by a final encapsidation step, where the viral RNA is encapsidated into the procapsid, while VP0 is further cleaved into VP4 and VP2. A fully assembled virus contains 60 copies of VP1 and VP3, 58–59 copies of VP2 and VP4 and 1–2 copies of VP0. Among the four structural viral proteins, VP1, VP2 and VP3 are exposed on the surface of the virus and are responsible for host antibody response [22].

The understanding gained from native picornavirus structure allows the engineering of VLPs, using either the **cell-assembled** or cell free **in vitro** assembly approach, to produce them efficiently in heterologous hosts. Recombinant VLP production requires only the structural proteins and the presence of protease for processing of P1. For example, P1–2A and 3C protease of FMDV are co-expressed in mammalian cell [25,26], and this was further cemented when bacterial-produced CVA16 subunit proteins (VP0, VP3 and VP1) were unable to induce neutralization antibodies [27]. Therefore, maintaining conformational VLPs to elicit protective immune responses is critical. The expression host impacts significantly both upstream and downstream processes. The scalability, safety profile and economical industrial production of a system are important factors for vaccine manufacturing. Successful attempts have been made using yeast [28,29], insect cell [26,30–31] and mammalian cell [27] for the construction of picornavirus VLPs within cells. *Escherichia coli* has successfully produced FMDV VLPs via **in vitro** assembly of bacterial-derived viral structural proteins [32]. However, there is no reported enterovirus VLPs derived from this bacterial system.

Although picornavirus VLPs have been successfully expressed in several systems, there are considerable pitfalls in quantifying VLP yield, hence it is difficult to assess the merits and demerits of a particular expression system for VLP production. Comparisons can be drawn at the expression stage based on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis, where viral structural proteins are semiquantified. It is important to note that an increase in the expression of viral structural proteins may not necessarily indicate an increase in VLP formation [16]. Production of multiprotein VLPs, such as picornavirus VLPs, throws up a level of complexity as the expressed viral structural proteins need to assemble in precision and at a defined ratio into VLPs. SDS-PAGE and western blot analysis do not provide sufficient information to differentiate between unassembled, partially assembled and fully assembled viral structures. ELISA is often used for quantification of the assembly efficiencies of VLPs [33], however, ELISA is not a suitable assay for unpurified samples. Therefore, careful interpretation of analytical data is critical during optimization studies for the determination of expression hosts and parameters.
Figure 2. Picornavirus structure and genome representation. (A) Diagrammatic representation of key features from all genera of picornavirus. (B) Structural viral proteins VP0, VP3 and VP1 assemble to form a protomer. A pentamer is made of five protomers and twelve copies of pentamer come together to form the viral capsid. VP0 is cleaved into VP4 and VP2 in the final capsid assembly. VP1, VP2 and VP3 are exposed on the surface of the capsid, whereas VP4 is on the internal side of the capsid. (A) Reproduced with permission from [23] © Nature Publishing Group (2005). (B) Reproduced with permission from [24] © Swiss Institute of Bioinformatics (2010).

Microbially-produced picornavirus-like particles

E. coli system is fully exploited for fast, cheap and scalable production of recombinant proteins [34]. Hepatitis E virus VLPs (Hecolin®, Xiamen Innovax Biotech Co. Ltd) is manufactured using E. coli. Rapid progress has been made with the E. coli system for production of modular VLPs and VLP-subunit vaccines against influenza and Group A Streptococcus [35–38].

Preliminary studies on bacterial expression of FMDV VLP lead to the development of SUMO-fused viral structural protein system that resulted in soluble expression of viral structural proteins and significant inhibition of protein aggregation [39]. Guo et al. demonstrated further improvement in expression (3–4× higher yield) and FMDV VLP assembly, by co-expressing the structural proteins VP0, VP3 and VP1 in E. coli BL21 (DE3), and successfully in vitro assembled the purified heterotrimeric viral structures into VLPs [32]. Guinea pigs, swine and cattle immunized with the cell-free in vitro assembled FMDV VLPs were totally protected against challenged with FMDV. An E. coli production platform, combined with cell-free in vitro assembly technology, offers low-cost efficient VLP production ideal for FMDV veterinary vaccine manufacturing.
Table 1. Development of picornavirus-like particles in baculovirus-insect cell expression system.

<table>
<thead>
<tr>
<th>Virus-like particle vaccines against FMDV and Poliovirus</th>
<th>Cell line</th>
<th>Promoter (gene)</th>
<th>Harvest site</th>
<th>Yield</th>
<th>VLP purification</th>
<th>Analysis</th>
<th>Animal test</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMDV Sf21</td>
<td>P&lt;sub&gt;ph&lt;/sub&gt; (L-P1-2A-3C)</td>
<td>Cell</td>
<td>NR</td>
<td>Sucrose gradient</td>
<td>Immunoprecipitation</td>
<td>NR</td>
<td>[47]</td>
<td></td>
</tr>
<tr>
<td>FMDV Hi5</td>
<td>P&lt;sub&gt;ph&lt;/sub&gt; (P1-2A)</td>
<td>Cell and supernatant</td>
<td>NR</td>
<td>Sucrose gradient</td>
<td>ELISA, TEM, WB and IFA</td>
<td>Guinea pigs, elicited neutralizing antibodies</td>
<td>[48]</td>
<td></td>
</tr>
<tr>
<td>FMDV Sf9 &amp; Hi5</td>
<td>P&lt;sub&gt;ph&lt;/sub&gt; (VP1-2A-VP3)</td>
<td>Cell and supernatant</td>
<td>0.1 mg/l</td>
<td>NR</td>
<td>ELISA, TEM and WB</td>
<td>NR</td>
<td>[49]</td>
<td></td>
</tr>
<tr>
<td>FMDV Sf9</td>
<td>P&lt;sub&gt;ph&lt;/sub&gt; (P1-2A-3C)</td>
<td>Cell</td>
<td>30 mg/l</td>
<td>Ammonium sulphate precipitation and gel filtration</td>
<td>ELISA, TEM and WB</td>
<td>Cattle, potency value (PD&lt;sub&gt;50&lt;/sub&gt;) 5.01, elicited neutralizing antibodies</td>
<td>[50]</td>
<td></td>
</tr>
<tr>
<td>FMDV Sf9</td>
<td>P&lt;sub&gt;ph&lt;/sub&gt; (P1-2A-3C)</td>
<td>Cell</td>
<td>2.1 g/l 2.6 g/l</td>
<td>Sucrose gradient</td>
<td>ELISA, TEM and WB</td>
<td>Guinea pigs, humoral and cell mediated immune responses, protection viral against challenge</td>
<td>[51]</td>
<td></td>
</tr>
<tr>
<td>FMDV Sf9, T. ni and T. niao38</td>
<td>P&lt;sub&gt;ph&lt;/sub&gt; (P1-2A-3C)</td>
<td>Cell</td>
<td>NR</td>
<td>Sucrose gradient</td>
<td>TEM and WB</td>
<td>NR</td>
<td>[52]</td>
<td></td>
</tr>
<tr>
<td>FMDV Sf9</td>
<td>P&lt;sub&gt;ph&lt;/sub&gt; (P1-2A-3C)</td>
<td>Cell</td>
<td>NR</td>
<td>Sucrose gradient</td>
<td>Crystallography</td>
<td>NR</td>
<td>[53]</td>
<td></td>
</tr>
<tr>
<td>PV Sf9</td>
<td>P&lt;sub&gt;ph&lt;/sub&gt; (P1-P2-P3)</td>
<td>Cell</td>
<td>10–90 μg/10&lt;sup&gt;9&lt;/sup&gt; cells</td>
<td>CsCl gradient</td>
<td>TEM and WB</td>
<td>Mice, elicited potent antibody responses</td>
<td>[54]</td>
<td></td>
</tr>
<tr>
<td>PV Sf9</td>
<td>P&lt;sub&gt;ph&lt;/sub&gt; (VP0)</td>
<td>Cell</td>
<td>NR</td>
<td>CsCl gradient</td>
<td>TEM and WB</td>
<td>NR</td>
<td>[55]</td>
<td></td>
</tr>
</tbody>
</table>

VLP vaccines against enterovirus & coxsackievirus

| EV71 Sf9                                               | P<sub>ph</sub> (P1) | Cell | NR | NR | TEM and WB | NR | [55] |
| EV71 Sf9                                               | P<sub>ph</sub> (P1) | Cell | 10 mg/10<sup>4</sup> cells | Sucrose and CsCl gradient | TEM and immunolabeling | NR | [56] |
| EV71 Sf9                                               | P<sub>ph</sub> (P1) | Cell | 10 mg/l | CsCl gradient | SDS-PAGE and WB | Mice, humoral and cellular immune responses, protection against viral challenge | [57] |
| EV71 Sf9                                               | P<sub>ph</sub> (P1) | Supernatant | 64 mg/l | CsCl gradient | TEM and WB | Mice, elicited neutralizing antibodies | [45] |

VLP vaccines against enterovirus & coxsackievirus (cont.)

CVA16: Coxsackievirus A16; DLS: Dynamic light scattering; EV71: Enterovirus 71; Hi5: High Five insect cell line; IFA: Immunofluorescence assay; NR: Not reported; P10: Baculovirus P10 promoter; PCMV: Cytomegalovirus promoter; Pph: Baculovirus polyhedrin promoter; SEM: Scanning electron microscopy; Sf9: Spodoptera frugiperda; TEM: Transmission electron microscopy; WB: Western blot.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell line</th>
<th>Promoter (gene)</th>
<th>Harvest site</th>
<th>Yield</th>
<th>VLP purification</th>
<th>Analysis</th>
<th>Animal test</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV71</td>
<td>Sf9 and Hi5</td>
<td>P_{Pph} (P1-3CD)</td>
<td>Supernatant</td>
<td>0.2–0.3 mg/l</td>
<td>Hydroxyapatite chromatography</td>
<td>ELISA and TEM</td>
<td>Macaque monkeys, humoral and cellular immune responses, induced neutralizing antibodies</td>
<td>[57]</td>
</tr>
<tr>
<td>EV71</td>
<td>Sf9</td>
<td>P_{P10} (P1)</td>
<td>Cell</td>
<td>NR</td>
<td>Sucrose gradient</td>
<td>ELISA, TEM and WB</td>
<td>Mice, elicited neutralizing antibodies</td>
<td>[58]</td>
</tr>
<tr>
<td>EV71</td>
<td>Hi5</td>
<td>P_{Pph} (P1)</td>
<td>Supernatant</td>
<td>5–10 mg/l</td>
<td>Hydroxyapatite chromatography</td>
<td>TEM, WB and DLS</td>
<td>NR</td>
<td>[3]</td>
</tr>
<tr>
<td>CVA16</td>
<td>Sf9</td>
<td>P_{Pph} (P1)</td>
<td>Cell</td>
<td>3 mg VP0/l</td>
<td>Sucrose cushion and sucrose gradient</td>
<td>ELISA, TEM, WB and IFA</td>
<td>Mice, elicited neutralizing antibodies, protection against viral challenge</td>
<td>[26]</td>
</tr>
<tr>
<td>CVA16</td>
<td>Sf9</td>
<td>P_{PCMV} (P1)</td>
<td>Supernatant</td>
<td>NR</td>
<td>Sucrose cushion and CsCl gradient</td>
<td>Analytical ultracentrifugation and Cryo-EM</td>
<td>Mice, elicited neutralizing antibodies</td>
<td>[5]</td>
</tr>
<tr>
<td>CVB3</td>
<td>Sf9</td>
<td>P_{P10} (VP3 and VP4), P_{P10} (VP1 and VP2)</td>
<td>Cell</td>
<td>400 μg/10^9 cells</td>
<td>Sucrose gradient</td>
<td>TEM, WB and IFA</td>
<td>Mice, elicited neutralizing antibodies, protection against viral challenge</td>
<td>[59]</td>
</tr>
<tr>
<td>CVB3</td>
<td>Sf9</td>
<td>P_{Pph} (P1)</td>
<td>Supernatant</td>
<td>0.5 mg/l</td>
<td>Polyethylene glycol precipitation and ion exchange chromatography</td>
<td>TEM, SEM, WB and DLS</td>
<td>Mice, humoral and cellular immune responses, elicited neutralizing antibodies</td>
<td>[60]</td>
</tr>
</tbody>
</table>

CVA16: Coxsackievirus A16; DLS: Dynamic light scattering; EV71: Enterovirus 71; Hi5: High Five insect cell line; IFA: Immunofluorescence assay; NR: Not reported; P10: Baculovirus P10 promoter; PCMV: Cytomegalovirus promoter; Pph: Baculovirus polyhedrin promoter; SEM: Scanning electron microscopy; Sf9: Spodoptera frugiperda; TEM: Transmission electron microscopy; WB: Western blot.
Gram-per-liter levels of viral capsid protein are achievable in this system [40]. This recent development on the use of E. coli for FMDV VLP production is very promising and has the potential to be extended to other viruses in this family, such as poliovirus, EV71 and CVA16.

Another microbial system noteworthy for VLP production is yeast expression system. Yeast is a high producer and offers cost-effective scalability, which has been proven for licensed human papillomavirus (HPV) quadrivalent VLP vaccine (Gradasil®, Merck & Co, NJ, USA) and hepatitis B surface antigen VLP vaccine. Intriguingly, success with using Saccharomyces cerevisiae to produce picornavirus VLPs is limited. Yeast-derived VLPs of EV71 [28], CVA16 [41] and poliovirus [29] are morphologically, compositionally and antigenically similar to the respective native virus. The VLPs are immunogenic and elicited protective immune responses. However, disappointingly low VLP yields ranging from 0.1 to 1 mg/l were obtained [28,29]. Further developments to increase productivity are essential before industrial yeast production of enterovirus VLPs is feasible.

### Insect cell-based picornavirus-like particles

Baculovirus-insect cell expression system is the system of choice for the production of complex multiprotein VLPs. This technology offers many advantages for industrial production of high value vaccines [42] and is being used to produce several licensed vaccines. Cervarix® (GlaxoSmithKline, Australia) HPV VLP vaccine and the world’s first recombinant influenza vaccine, FluBlok® (Protein Sciences Corporation, CT, USA), are produced using insect cells. Many VLP-based vaccine candidates that are currently in clinical trials are insect cell-derived [43]. Baculovirus-insect cell system is also commonly employed for the production of veterinary vaccine products [44], such as E2 glycoprotein for classical swine fever virus (Bayovac CSF, Bayer Leverkeusen Inc.).

Table 1 presents the different picornavirus VLPs that are expressed using the baculovirus-insect cell expression system. As shown, two commonly used cell lines are Spodoptera frugiperda SF9 and Trichoplusia ni High Five™. The effect of cell line on VLP assembly and yield has not been investigated closely, although yield differences for EV71 VLPs have been reported [45]. Higher extracellular VLP yields were obtained in SF9 cells than High Five when 3CD protease was placed under CMV promoter. Protein Sciences Corporation has reported higher recombinant protein yields with their superior clonal expresSF+ cells [46], however, VLP production in this cell line has not been reported. Testing of this FDA-qualified industrial cell line for multiprotein VLP expression is warranted.

<table>
<thead>
<tr>
<th>Key term</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-infection</td>
<td>Simultaneously infecting a single insect cell with two or more recombinant baculoviruses to enable co-expression of multiple proteins in one cell...</td>
</tr>
</tbody>
</table>

An inherent difficulty with the co-expression of FMDV VLPs in insect cells is the toxicity of 3C protease to host cells [52]. Processing of P1 or P1–2A polyprotein requires 3C protease of FMDV. Variability of FMDV VLP yields was observed and the cause uncovered as the overexpression of 3C protease. In the case of enterovirus VLP production, the overexpression of 3CD protease leads to truncation of structural protein VP1, and consequently lowers VLP yields [56]. Several strategies were tested to balance the processing of P1 polyprotein and the expression of the protease. Downregulation of 3C protease by incorporating frame shift elements has shown to improve FMDV VLP yields [53]. Other strategies including weaker promoters (e.g., CMV and p10) to drive expression of 3CD protease [45] and incorporation of internal ribosome entry site [61] were also investigated.

For enterovirus VLPs, two strategies have been commonly employed for the co-expression of P1 and 3CD, namely co-infection with more than one recombinant baculovirus or a single recombinant baculovirus carrying all genes of interest. Chung et al. tested the efficiency of both expression strategies and observed that both methodologies resulted in correct processing of P1 polyprotein into structural proteins VP0, VP1 and VP3 [56]. However, the infection with a single recombinant baculovirus (P1–3CD) yielded more VLPs. Similar observations were reported for CVA16 VLPs [26]. With the co-infection methodology, a subset of cell population may be infected with only one of the two baculoviruses, and consequently no VLP formation was obtained. A single infection with a recombinant baculovirus carrying both P1 and 3CD overcomes the heterogeneity of infection, thus resulting in proper processing of P1 polyprotein in all infected cells and leading to a higher VLP yield.

Protease 3CD cleaves the P1 polyprotein into structural proteins VP0, VP3 and VP1 and these proteins self-assemble into VLPs in the cytoplasm of infected insect cells. VLPs have been purified from both the cells and culture supernatant (see Table 1). One group purified CVB3 VLPs from the cells [59], whereas another group purified the same VLPs from the culture supernatant [60]. Unlike the enveloped VLPs, enterovirus VLPs do not require the budding from host cells to mature as VLPs. Thus, the mechanism of release for these nonenveloped VLPs into the culture supernatant is unknown. However, the choice of promoter used to drive the expression of 3CD protease was reported...
Figure 3. Process flow diagram on downstream processing of baculovirus-insect cell derived VLPs. VLPs can be purified from the infected insect cells or from culture supernatant. Ultracentrifugation with sucrose and/or cesium chloride gradient is commonly used to purify VLPs. Large-scale production of VLPs will require the use and development of chromatographic techniques to purify VLPs from contaminants.

VLP: Virus-like particle.

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to impact the localization of mature EV71 VLPs. To obtain secreted version of EV71 and CVA16 VLPs [5], 3CD protease was placed under CMV promoter instead of p10 promoter, based on the findings of EV71 VLPs [45].

The research group in Taiwan has purified EV71 VLPs from both fractions [25,31,45,55–57], however, comparison on the quality and stability of VLPs obtained from within the cells and the supernatant has not been reported. The localization of assembled VLPs, thus the harvest site, has a significant impact on downstream processing strategies (Figure 3). Baculovirus virions commonly co-purified with VLPs from the supernatant [11,43,62–63], and considerable effort is invested to remove them as these virions have an adjuvanting effect on vaccine candidates and also affect the immunogenicity of VLPs. To overcome this problem, a vp80 deletion in baculovirus was engineered to eliminate the production of baculovirus virions [64]. This newly engineered baculovirus could greatly simplify downstream processing of insect-cell based products by eliminating the production of baculovirus virions in the culture supernatant.

There are limited reports in the literature on the process development of VLP production of picornavirus. Chung et al. has reported an extracellular EV71 VLP yield of 64.3 mg/l by optimizing parameters such as host cells (Sf9 cells), cell density (4 × 10⁶ cells/ml), culture mode and dissolved oxygen (DO = 30%) [45]. The yield from the bioreactor was 43-fold greater than the yield obtained in spinner flasks (1.5 mg/l) [45]. Lin et al. produced EV71 VLPs in a stirred tank bioreactor (Biostat® B, Sartorius) by infecting High-Five™ cells with a single baculovirus (P1–3CD) at a multiplicity of infection of 10 when the cell density reached 3 × 10⁶ cell/ml [31]. Optimum time of harvest in this case was determined to be 30% drop in cell viability, resulting in 5–10 mg/l yield.
The cell-assembled in vivo VLP assembly is well exploited for the production of a range of VLPs, but is still poorly understood. The accumulation of structural proteins in the appropriate stoichiometric ratio for precise assembly of VLPs is critical [16]. A major drawback for cell-aid assembly is the incorporation of host cell contaminants (DNA and proteins) within assembled VLPs, which leads to processing complexity [65]. Insect cell-derived HPV VLPs overcomes this issue by expressing a truncated L1 structural protein to prevent VLP assembly in the cell [66]. These highly purified pentameric L1 viral structures are assembled in vitro into VLPs under appropriate buffer conditions. Extending this approach to picornavirus VLPs, there is an opportunity to engineer and express in vivo assembly incompetent structural proteins that can be purified away from the host cell contaminants before controlled in vitro assembly into VLPs. Studies such as rational based structure approach for VLP vaccine development may provide important insights for developing in vitro VLP assembly [5,53].

**Downstream processing of insect cell-based picornavirus VLPs**

Downstream processing is the most critical step in biopharmaceutical manufacturing because the economics of the entire production process is determined at this stage [67]. Purification of VLPs has a level of complexity in comparison to the purification of protein products, and processing complexities do lead to higher production cost. VLPs are hollow particles and the structure integrity of VLPs is critical for their immunostimulatory function. During the VLP purification process, attaining high purity and maintaining the structural architecture of VLPs at all stages of purification are equally important. Developing efficient VLP downstream processing strategies will require an understanding of the physical, chemical and biological characteristics of VLPs, which can be exploited during these processes [68].

The complexity of a purification process largely depends on the host contaminants from which the desired product is separated away. Apart from the baculovirus particles, other major contaminants observed in insect cell-derived VLPs are host proteins such as tubulin, actin, Hsp70 and several housekeeping proteins [69]. As discussed in earlier section, the choice of vector, promoter and cell line can significantly assist in the removal of such host contaminants. For example, using an engineered baculovirus (vp80 deletion) can eliminate the production of baculovirus virions in the culture supernatant, thereby significantly reducing the effort invested for their removal [64]. Another problem associated with purifying cell-assembled picornavirus VLPs is the encapsulation of host cell contaminants in the hollow centres of VLPs [65]. Commercial HPV VLP vaccine, Gradasil (Merck & Co.), overcomes this roadblock by in vitro VLP disassembly and reassembly, to remove the host contaminants. Development of similar bioprocess strategy for picornaviruses requires a bioprocess-relevant mathematical model for thorough understanding of VLP assembly mechanism [65]. Such a mathematical model may allow the prediction of VLP disassembly and reassembly outcome. Although this disassembly and reassembly processing can enhance the product quality [70], it can also increases VLP production costs significantly.

The purification strategy for cell-assembled VLPs is often driven by the pathway taken by the structural proteins to form VLPs (Figure 3). Although VLPs of picornaviruses are structurally identical, their sites of harvest do vary as illustrated in Table 1. The mechanism of picornavirus VLPs release into the culture supernatant remains unclear but was preferred by some for the ease of purification [45]. Currently, a basic laboratory scale purification of picornavirus VLPs from culture supernatant involves a clarification process to remove cell debris and large aggregates by centrifugation, followed by tangential flow filtration for concentrating the clarified material. This concentration is very useful in reducing the culture volume for further processes and hence significantly reduce the overall time and material cost involved in downstream processing. Moreover, crossflow membranes allow gentle handling of VLPs, avoiding structural disruption of VLPs, thus tangential flow filtration is the preferred method for VLP concentration [71].

A cell lysis step is required to release intracellular VLPs from cells [4,27,56,72]. Cells are lysed either mechanically (homogenization/sonication), chemically or enzymatically [73], resulting in the release of VLPs along with host intracellular proteins. Modern homogenizers are often continuous and are ideal for large-scale cell lysis. This is followed by DNA diges-

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**Key terms**

**Tangential flow filtration:** A filtration technique in which the feed solution is tangentially passed across the surface of the filter membrane. Particles that are smaller than the membrane pore size will pass through the membrane (permeate), while the remaining materials are retained in the feed solution (retentate). This is also known as crossflow filtration.

**Colloidal stability:** colloidal particles may remain dispersed in a solution over a period of time if the repulsive inter-particle forces are stable. This is referred to as colloidal stability. Unstable suspension occurs when particles start to aggregate, as the repulsive forces are attractive.
amino) and charged calcium and phosphate ions on actions between charged protein groups (carboxyl or HAP chromatography is based on nonspecific inter-
tion of buffer for cell lysis is critical, as the buffer (salt and pH) affects the structural and colloidal stability of VLPs.

Ultracentrifugation with sucrose and/or cesium chloride gradient has been extensively used to purify VLPs for research studies. Researchers working on FMDV VLP vaccine development have reported only ultracentrifugation purification process (Table 1). However, moving toward large-scale VLP production, it is practically noneconomical to use ultracentrifugation as the method is labor-intensive, time-consuming and also exhibits unexpected batch-to-batch variation [68]. This in turn can influence the final quality of purified picornavirus VLPs. Although large-scale influenza VLPs have been purified using continuous ultracentrifugation [74], the scalability of this technique is not cost efficient. To take picornavirus VLPs from research to vaccine manufacturing, it will require researchers to investigate beyond ultracentrifugation and develop economical downstream processing strategy that strikes a balance between cost (economical scale-up), purity (VLP quality) and immunogenicity (potency).

Chromatographic separation techniques are the preferred purification methods for biopharmaceutical products. In the literature, there are limited reports on chromatography purification of picornavirus VLPs (Table 1). Developing a capture step as primary purification step in VLP purification will allow a bioprocess engineer to obtain reasonable purity and concentration of the desired product. Hydroxyapitate chromatography (HAP) purification of EV71 VLPs purification has been investigated [31]. The mechanism of HAP chromatography is based on nonspecific interactions between charged protein groups (carboxyl or amino) and charged calcium and phosphate ions on the resin. Proteins are selectively adsorbed onto the column and eluted by varying salt concentration [75]. Elution buffers in HAP purification are usually mild and hence less likely to affect the structural and colloidal stability of VLPs. Exploring HAP as a primary purification step in other picornavirus VLPs downstream processing will require optimization, however this can potentially replace the ultracentrifugation purification [67].

Ammonium sulphate and polyethylene glycol precipitation serve as another attractive purification strategy. Precipitation techniques in combination with chromatographic techniques have been attempted to successfully purify picornavirus VLPs. For example, CVB3 VLPs have been purified from the clarified cell culture supernatant by polyethylene glycol precipitation followed by ion exchange chromatography (IEX), resulting in pure and stable CVB3 VLPs [60]. The antibodies raised by these CVB3 VLPs showcased a strong neutralizing capacity against the homologous CVB3 strain. In another report, FMDV VLPs with a potency value (a measure of the vaccine candidate’s ability to initiate immune response) of 5.01, were purified by ammonium sulphate precipitation followed by gel filtration [50]. These recent successful developments in chromatographic and nonchromatographic methods for downstream processing of picornavirus VLPs are very promising and could be extended to other viruses of this family.

Recent advances in the development of new chromatography media for VLP purification such as convective interaction media (CIM monoliths, BIA Separations) and CaptoCore™ (GE, Healthcare, Australia) offer more economical scale-up options [5,68]. Monolith columns have been successfully used for purifying large macromolecular complexes. A rigid and stable structure, well defined pore size distribution (1.2 μm or more), large surface area for binding and improved matrix accessibility to macromolecules such as VLPs are important characteristic features of monolith columns. High flow rates, low-pressure drop, enhanced mass transport by convection and significant decrease in diffusion time are some of the important advantages offered by monolith columns over traditional chromatographic matrices [76]. Koho et al. have demonstrated the use of CIM IEX media functionalized with either quaternary amine or sulfate (SO3) for successful purification CVB3 VLPs [60]. However, like HAP, this technology has not been applied to other viruses of picornavirus family. The other attractive chromatographic matrix alternative is the Capto™Core media. The CaptoCore allows VLPs to be purified in flow-through mode because this technology excludes VLPs from interacting with an interior adsorptive matrix that binds small and soluble contaminating proteins [15]. The flow-through mode purification will allow the VLPs to remain in the same buffer environment which could be advantageous. While currently there is limited data on picornavirus VLP purification using such modern technologies, employing these emerging innovative matrices could offer a more economical, scalable and less labor intense purification strategy for industrial production of picornavirus VLPs in the near future.
Characterization of picornavirus-like particles

VLPs have the propensity to aggregate particularly in nonoptimal conditions. Consequently, the characterization of VLP vaccine candidates is important during vaccine development to ensure VLP quality and consistency. Following compositional analysis of VLPs using SDS-PAGE and western blot, transmission electron microscopy (TEM) has been the first choice by researchers for visual confirmation, morphological characterization and size determination of picornavirus VLPs [26,30–31,52]. Researchers have started to look beyond TEM for the characterization of EV71 and CVB3 VLPs, using dynamic light scattering (DLS) to investigate stability and aggregation [31,60]. However, DLS is highly sensitive to large scattering particles and is unable to resolve polydispersity. The availability of superior and robust analytical techniques, such as electrospray differential mobility analysis (ES-DMA) and asymmetric flow field-flow fractionation with multiangle light scattering detection (AFFFF–MALS or AF4–MALS, Figure 4), that offers high resolution biophysical characterization of nonenveloped VLPs [15,18,77] should be applied to picornavirus VLPs to detect batch-to-batch variations, degradation or aggregation during storage. These latest analytical tools can also assist in evaluating the performance of various excipients in enhancing the stability of picornavirus VLPs during formulation studies, and in monitoring the thermal stability of VLPs [19]. Furthermore, these techniques could be employed to support rational engineering of VLPs to enhance their thermal stability, as reported for FMDV empty capsids [53], thus removing cold-chain requirement for vaccine transport and storage. A detailed review on VLP physical characterization has been reported elsewhere [15]. Use of such powerful analytical techniques along with improvements in choice of animal models, quantification antigenic content and evaluation of protective efficacy is essential for the development of picornavirus VLP-based vaccines.

Conclusion & future perspective

There is a growing interest in developing VLP-based vaccines for polio, HFMD and FMD. Currently, production of picornavirus VLPs is mainly in laboratory research scale and significant milestones must be accomplished for the translation of these VLP products. Bioprocess engineering and development will no doubt lead to a cost-effective and scalable manufacturing process for VLPs of picornavirus. The recent success in attaining protective efficacy using bacteria-derived FMDV VLPs production has paved the way to deploying the well-exploited and scalable E. coli
manufacturing system for producing other VLPs of picornavirus. In addition, we can expect continuous developments on the VLP production workhorse, the baculovirus-insect cell expression system, which will greatly benefit enterovirus VLP vaccine development. Eliminating packaged contaminants from cell-assembled VLPs continues to pose challenges to downstream processes and subsequent vaccine approval. However, opportunities are present for in vitro VLP assembly processing and innovative bioengineering of subunits of VLP to overcome these challenges. Use of latest chromatographic matrices such as monoliths, mixed-mode media and membranes for maximum VLP recovery is anticipated as many of these VLP candidates move toward the translational pathway. Vaccine characterization with established high-resolution analytical techniques to ensure VLP product quality, consistency and structural integrity is important in underpinning picornavirus VLP product development. Harnessing the knowledge gained in recent developments for a diverse range of VLPs, and with the latest VLP technologies, we can expect picornavirus VLPs progressing toward clinical testing.

Financial & competing interests disclosure
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

Executive summary

Introduction
• Success of licensed virus-like particle (VLP) vaccines as safe and effective vaccines has propelled the development of VLP vaccines against picornavirus.
• Multiprotein picornavirus VLP is structurally more complex to produce in comparison to all currently licensed single-protein VLP vaccines.

Structure of picornavirus
• Picornaviruses are small, nonenveloped, icosahedral particles consisting of three polyprotein regions, namely P1, P2 and P3, that are translated as a single poly protein.
• The P1 region encodes the viral structural proteins (VP1-VP4) that assemble to form a protomer. Five protomers assemble together to form a pentamer and 12 copies of pentamer come together to form the viral capsid.

Cell-free & cell-assembled picornavirus-like particles
• Picornavirus VLPs have been successfully expressed using different systems, namely E. coli, yeast, insect cells and mammalian cells.
• Recent developments on the use of E. coli and in vitro VLP assembly for foot-and-mouth-disease virus (FMDV) VLP production is promising and has the potential to be extended to other picornaviruses.
• The promoter used to drive the expression of 3CD protease has an impact on the localization of mature enterovirus 71 (EV71) and coxsackievirus A16 (CAV16) VLPs produced using baculovirus-insect cell expression system.

Downstream processing of insect cell-based picornavirus VLPs
• Ultracentrifugation with sucrose and/or cesium chloride gradient has been extensively used to obtain picornavirus VLPs.
• Chromatography techniques such as Hydroxyapatite chromatography and IEX are attractive alternatives and modern technologies such as CIM monoliths and Capto™Core could be further explored. Removal of host cell contaminants from within the VLPs presents a challenge to process development.

Characterization of picornavirus-like particles
• Immunological responses are directly related to the physical properties of VLPs, and hence biophysical characterization for picornavirus VLPs is essential. Advanced high resolution analytical tools such as ES-DMA and AFFFF-MALS, coupled with TEM, can provide accurate quantitative and qualitative data on purified VLPs.

References
Papers of special note have been highlighted as:
• of interest; •• of considerable interest
• This article provides a detailed description of virology, molecular epidemiology of enterovirus 71 and documents the outbreaks in the Asia-Pacific region over the last decade.
This review article provides a description of biophysical characterization techniques that can be applied to accurately characterize VLPs.


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This research article demonstrates the application of SUMO fusion protein approach to produce foot-and-mouth disease virus VLPs in bacterial expression system. Microbial production of virus-like particle vaccine protein at gram-per-litre levels. Saccharomyces cerevisiae elicit protective immunity against Coxsackievirus A16 in mice. Recombinant protein vaccines produced in insect cells. Vaccine-like particles by a SUMO fusion protein approach in Escherichia coli. This article demonstrates the application of SUMO fusion protein approach to produce foot-and-mouth disease virus VLPs in industrial expression system. Recombinant protein vaccines produced in insect cells. Recombinant protein vaccines produced in mammalian cells. Recombinant protein vaccines produced in bacteria. This research article demonstrates optimization of expression conditions to produce EV71 VLPs in a bioreactor.


