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Review

Use of baculovirus expression system for generation of virus-like particles: Successes and challenges

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ABSTRACT

The baculovirus expression system (BES) has been one of the versatile platforms for the production of recombinant proteins requiring multiple post-translational modifications, such as folding, oligomerization, phosphorylation, glycosylation, acylation, disulfide bond formation and proteolytic cleavage. Advances in recombinant DNA technology have facilitated application of the BES, and made it possible to express multiple proteins simultaneously in a single infection and to produce multimeric proteins sharing functional similarity with their natural analogs. Therefore, the BES has been used for the production of recombinant proteins and the construction of virus-like particles (VLPs), as well as for the development of subunit vaccines, including VLP-based vaccines. The VLP, which consists of one or more structural proteins but no viral genome, resembles the authentic virion but cannot replicate in cells. The high-quality recombinant protein expression and post-translational modifications obtained with the BES, along with its capacity to produce multiple proteins, imply that it is ideally suited to VLP production. In this article, we critically review the pros and cons of using the BES as a platform to produce both enveloped and non-enveloped VLPs.

Contents

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Introduction

Since 1983, when the baculovirus expression system (BES)[1] was first used to express human beta interferon in insect cells [1], the BES has become a versatile and robust eukaryotic expression system for foreign protein expression. Two prototype members of the genus *Alphabaculovirus* [2] are broadly utilized in the BES as vectors to produce heterologous proteins in insect cells or silkworm larvae: namely *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and to a lesser extent *Bombyx mori* nucleopolyhedrovirus (BmNPV). Either can express foreign genes under the control of highly expressed very late promoters, including polyhedrin (polh) and 10-kDa fibrous polypeptide (p10) promoters. The most commonly used lepidopteran insect cell lines are derived from *Spodoptera frugiperda* (*Sf*9 and *Sf*21) and *Trichoplusia ni* (*Tn*5, commercially known as High Five™), which grow optimally at 27 °C and do not require CO_2, making scale up of protein production feasible for most laboratories. Baculovirus expression of heterologous genes permits multiple post-translational modifications, like folding, oligomerization, phosphorylation, glycosylation, acylation, disulfide bond formation, proteolytic cleavage and so on, which are similar or identical to those occurring in mammalian cells. These advantages over prokaryotic expression systems make it possible to express multiple proteins simultaneously in a single infection and to obtain multimeric proteins sharing functional similarity with their natural analogs. Thus, the BES has been broadly used for the production of heterologous proteins and even the generation of virus-like particles (VLP) in laboratories [3,4], as well as for the development of subunit vaccines, including VLP-based vaccines, in the vaccine industry [5,6].

VLPs are composed of viral capsid proteins that self-assemble into particles closely resembling the natural virions from which they derive. VLPs are replication as well as infection incompetent, due to the absence of any infectious genetic material [7]. A close resemblance to native viruses in molecular scaffolds enables VLPs to elicit both humoral and cellular immune responses even without adjuvant. VLPs have been constructed through co-expression and then self-assembly of their components in *Escherichia coli* (*E. coli*), yeasts, mammalian cells and insect cells. As a powerful eukaryotic expression system post-translationally modifying and processing the foreign proteins expressed *in vitro* or *in vivo*, the BES plays a critical role in self-assembly and release of VLPs. However, many studies have demonstrated the inability of lepidopteran cells to synthesize mammalian-type N-glycans [8], which was a limitation of the conventional BES [9]. In addition, the stoichiometry of VLP components, the self-assembly efficiency of structural proteins, and the budding process of enveloped VLPs are determined by the BES. In this article, we critically reviewed the pros and cons of using the BES to construct both enveloped and non-enveloped VLPs.

BES as a platform for protein expression

Several commercially available BES kits (*e.g.*, BaculoGold™, BD Biosences; *Flash*BAC, Oxford Expression Technologies; BacPAK™, Clontech) use the conventional method of homologous recombination *in vitro*. However, the final virus stock unavoidably contains a mixture of parental and recombinant viruses, so a plaque-assay is required to purify the recombinant baculovirus [10]. An alternative approach has been developed [11] to circumvent this problem by generating a recombinant baculovirus using site-specific transposition with Tn7 to insert foreign genes into bacmid (baculovirus plasmid) propagated in *E. coli*. Based on the principle of site-specific transposition, a rapid and efficient BES (Bac-to-Bac®, Life Technologies) that can be used as an alternative way to generate recombinant baculoviruses was developed and is now widely used as another commercial BES. Additionally, a modified baculovirus vector harboring a mammalian promoter, known as BacMam vector, has been turned into a transient expression vector for gene delivery and high-level screening in mammalian cells [12]. The BacMam system combines the advantages of viral transient expression, ease of generation and a broad cell tropism, enabling rapid, efficient and flexible gene over-expression experiments to be performed in various mammalian cells [13].

To date, the BES has been used to manufacture several biologicals, including the interferon [14], antigen [15] and vaccine [16]. One was GlaxoSmithKline's Cervarix™ (GSK, Rixensart, Belgium), a VLP-based bivalent human papillomavirus vaccine against cervical cancer, which was approved for human use in the USA in 2009 [17]. In the veterinary field, Porcilis® Pesti (Intervet) and Bayovac® CSF E2 (Bayer) are the first two licensed subunit vaccines produced by the BES. Either consists of an envelope glycoprotein of the classical swine fever virus as the antigen. In addition, FluBlok®, a seasonal influenza subunit vaccine for adults, was approved by the FDA in January, 2013. It is tailored annually to provide protection against the latest strains of influenza by containing the corresponding hemagglutinin (HA) antigens produced by the BES [18,19].

Generation of VLPs using BES

Many viral structural proteins have an intrinsic ability to spontaneously self-assemble into VLPs (Table 1) when expressed in insect cells by co-expression or co-infection with recombinant baculoviruses. Like parental viruses, many VLPs generated by the BES are enveloped, meaning that the capsids are coated with a lipid membrane known as the envelope, which is derived from the plasma membrane of insect cells. The other VLPs, known as the non-enveloped VLPs, contain no lipid membrane and are formed by only one or more major structural proteins. Owing to the difference in structures, there are differences in the assembly mechanisms for enveloped and non-enveloped VLPs.

Enveloped VLPs

In general, self-assembly of structural proteins into enveloped VLPs (Fig. 1A) includes two steps, namely capsid (or matrix) formation and then membrane enclosure for further budding (Fig. 2). Due to intrinsic properties of the lipid membrane and surface glycoprotein, the generation of enveloped VLPs in insect cells is more complicated than that of non-enveloped VLPs. However, efficient budding of enveloped VLPs from insect cells has been reported from time to time [30,31,36,38,39,45,46]. For example, using a quadruple baculovirus recombinant, Latham and Galarza (2001) initially showed that co-expression of four structural proteins of influenza virus, the HA, neuraminidase (NA), matrix protein M1 and M2 ion channel protein, was sufficient for the self-assembly and release of VLPs from surface of insect cells. Furthermore, the VLPs closely resembled the authentic virions in size, morphology, and in the fine structure of the surface spikes [3].

Non-enveloped VLPs

Compared with the enveloped VLPs, the generation of non-enveloped VLPs with single capsid (Fig. 1B) should be less

[1] Baculovirus expression system (BES); virus-like particles (VLP); *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV); *Bombyx mori* nucleopolyhedrovirus (BmNPV); polyhedrin (polh); hemagglutinin (HA); neuraminidase (NA); endoplasmic reticulum (ER); European Molecular Biology Laboratory (EMBL); multiplicities of infection (MOI); Flock House nodavirus (FHV); time of infection (TOI); peste des petits ruminants virus (PPRV)

Table 1
Generation of VLPs using baculovirus expression system.

Virus	Classification of VLP	Cells	Recombinant proteins	Expression strategies	Refs.
Feline leukaemia virus	Enveloped VLP	*Sf*9 cells	Gp85 and gag	Co-infection	[20]
Norwalk virus	Non-enveloped VLP	*Sf*9 cells	Capsid protein	Single infection	[21]
Porcine parvovirus	Non-enveloped VLP	*Sf*9 cells	VP2	Single infection	[22]
Bluetongue virus	Non-enveloped VLP	*Sf*21 cells	VP2, VP6, VP7 and NS1	Co-expression	[23]
Poliovirus	Non-enveloped VLP	*Sf*21 cells	VP0, VP3 and VP1	Co-infection	[24]
Human papillomavirus	Non-enveloped VLP	*Sf*9 cells	L1 and L2	Co-expression	[25]
herpes simplex virus	Non-enveloped capsid	*Sf*21 cells	VP23, VP5, VP22a, VP21&VP24, VP26 and VP19C	Co-infection	[26]
Rotavirus	Non-enveloped VLP	*Sf*9 cells	VP2, VP6, VP7 and VP4	Co-infection	[27]
Porcine parvovirus (LCMV)*	Non-enveloped VLP	*Sf*9 cells	VP2 containing LCMV epitope	Single infection	[28]
African horse sickness virus	Non-enveloped VLP	*Sf*9 cells	VP3 and VP4	Co-infection	[29]
Human immunodeficiency virus	Enveloped VLP	*Sf*9 cells	Gag and gp120	Co-expression	[30]
Human severe acute respiratory syndrome coronavirus	Enveloped VLP	*Sf*21 cells	Spike, membrane and envelope proteins	Co-infection	[31]
Human astrovirus	Non-enveloped VLP	*Sf*9 cells	Complete ORF2	Single infection	[32]
Enterovirus-71	Non-enveloped VLP	*Sf*9 cells	P1 and 3CD	Co-expression or co-infection	[33]
Feline calicivirus	Non-enveloped VLP	*Sf*9 cells	Capsid	Single infection	[34]
Simian virus 40	Non-enveloped VLP	*Sf*9 cells	VP1, VP2 and VP3	Single infection or co-infection	[35]
Rift Valley fever virus	Enveloped VLP	*Sf*9 cells	Gn, Gc and N	Co-expression	[36]
Porcine circovirus	Non-enveloped VLP	*Tn* cells	VP2	Single infection	[37]
Avian influenza virus	Enveloped VLP	*Sf*9 cells	HA, NA and M1	Co-expression	[38]
Ebola virus	Enveloped VLP	*Sf*9 cells	VP40 and GP	Co-infection	[39]
Encephalomyocarditis virus	Non-enveloped VLP	*Sf*9 cells	P1–2A-3C	Single infection	[40]
Rous sarcoma virus	Enveloped VLP	*Tn* cells and SL	Gag	Single infection	[41]
Japanese encephalitis virus	Enveloped VLP	*Sf*9 cells	prM and gE	Co-infection	[42]
Coxsackievirus A16	Non-enveloped VLP	*Sf*9 cells	P1 and 3CD	Co-expression	[43]
Foot-and-mouth disease virus	Non-enveloped VLP	*Sf*9 cells	P1–2A-3C	Single infection	[44]
Chikungunya virus	Enveloped VLP	*Sf*21 cells	Structural polyprotein	Single infection	[45]

* Chimeric VLP; *Sf*: *Spodoptera frugiperda*; *Tn*: *Trichoplusia ni*; LCMV: lymphocytic choriomeningitis virus; ORF: open reading frame; SL: silkworm larvae; VLP: virus-like particle.

challenging, as most of these VLPs are composed of numerous copies of the same protein subunit and have icosahedral symmetry. Owing to the uncomplicated structures of parental viruses, expression of one capsid protein alone in insect cells is enough to cause the formation of VLPs (Fig. 2) that are morphologically and antigenically similar to native viruses [21,34,47,48]. Papillomavirus VLPs, produced by over-expression of the major capsid L1 protein in insect cells, represent the most studied example as a prophylactic vaccine among non-enveloped VLPs [6,7].

The other non-enveloped VLP, multilayered VLP (Fig. 1C), relies on the simultaneous expression of diverse structural proteins within one insect cell, so several technical problems seem to affect their generation, in comparison with those formed by only one capsid protein. Fortunately, baculovirus multiple gene transfer vectors pAcAB3 and pAcAB4 have been commercialized to facilitate the insertion of three or four foreign genes respectively into one AcMNPV genome by a single co-transfection experiment [23]. Complex self-assembly of multilayered VLPs in insect cells has been efficiently achieved for members of the family *Reoviridae*, such as bluetongue [49–51], African horse sickness [29] and rotavirus [27,52] VLPs.

Major properties of BES: promoting generation of VLPs

Over the past 20 years, the BES has become the most widely used system for the production of VLPs. It has the capacity to produce recombinant proteins at a high level and further to perform certain post-translational modifications, thus to some extent retaining the biological activity of original proteins. Consequently, it is natural to consider this system used for the production of VLPs. However, some essential properties of the BES, such as unspecific proteolysis, on the contrary, impede the production of VLPs. In this section, we discussed the pros and cons of such a system used to generate a variety of VLPs.

Polh and p10 promoters

Expression of the polh and p10 genes is regulated by the corresponding strong promoters, namely the polh and the p10 promoters. In comparison with other baculoviral promoters, both very late promoters provide abundant transcription of very late genes. The polyhedrin transcript represented approximately one-quarter of the viral polyadenylic acid-containing RNAs at 27 h post infection [53], thereby ensuring the production of the corresponding proteins in large amounts in the very late phase of infection. Furthermore, both the polh [54] and the p10 [55] genes are dispensable for the life cycle of baculoviruses. Thus, replacement of either of the gene coding sequences with that of another foreign gene in a recombinant baculovirus usually enables extensive expression of the heterologous protein, even more than 50% of the total protein in insect cells [56]. In general, the p10 promoter is more active at an earlier time post infection and reaches a lower maximum level than the polh promoter [57]. Under non-optimal conditions, the p10 promoter, however, would yield a higher level of expression than the polh promoter-driven expression [58,59].

The strong polh promoter probably overwhelms the processing capacity of the endoplasmic reticulum (ER) in insect cells, and in contrast, the weaker p10 promoter allows production of a biologically active glycoprotein [60]. Therefore, it would be possible to infer an improvement in complex glycosylation of foreign proteins required for the generation of VLPs, if the p10 promoter was used instead of the polh promoter. Genetic engineering of baculovirus

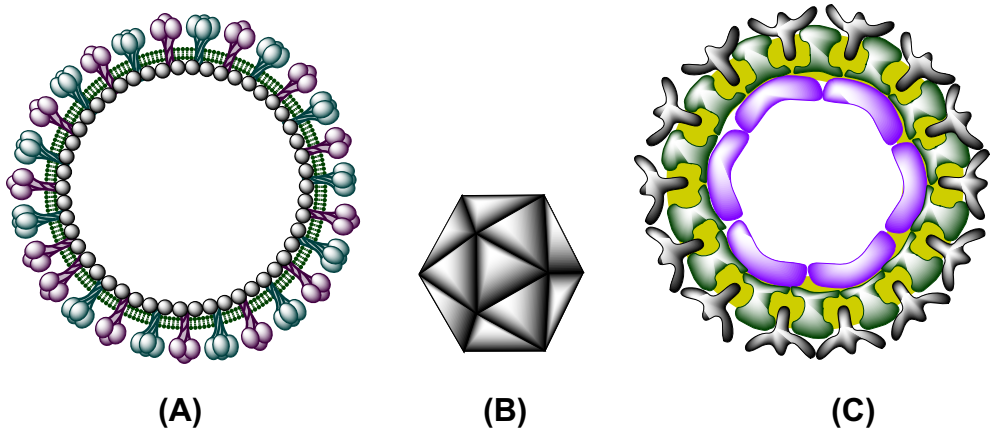

Fig. 1. Schematic representation of enveloped VLP (A), structurally simple VLP (B) and multilayered VLP (C).

facilitates both the insertion of foreign genes into these downstream promoter regions and the subsequent high level expression as well as self-assembly of heterologous proteins. A baculovirus with a certain gene present behind both promoters produced relatively more recombinant protein in host cells than those viruses driven with the polh or p10 promoters alone [59]. Nevertheless, the two promoters are activated at very late time post infection when the host machinery for post-translational modifications is

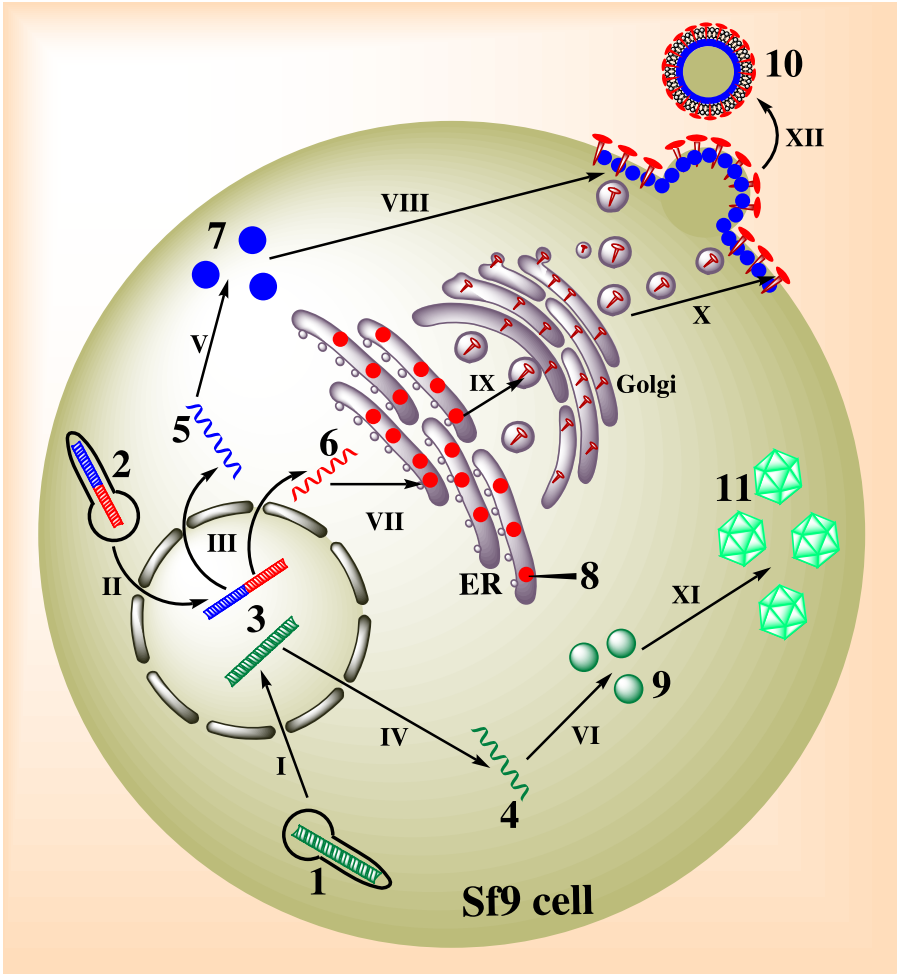

Fig. 2. Main stages of enveloped and non-enveloped VLPs formations in insect cell. 1 and 2, recombinant baculoviruses; 3, baculoviral genome; 4, 5 and 6, mRNAs; 7, capsid or matrix protein; 8, membrane protein; 9, capsid protein; 10, enveloped VLP; 11, non-enveloped VLP; ER, endoplasmic reticulum. I and II, baculoviral genomes gain access to the nucleus. III and IV, full-length mRNAs are exported from the nucleus. V, VI and VII, the mRNAs are translated into structural proteins. VIII, matrix protein subunits are transported inside the plasma membrane. IX and X, the membrane proteins are transported through the Golgi apparatus onto the plasma membrane. XI, capsid protein subunits are assembled into non-enveloped VLPs. XII, an enveloped VLP buds from the surface of cell.

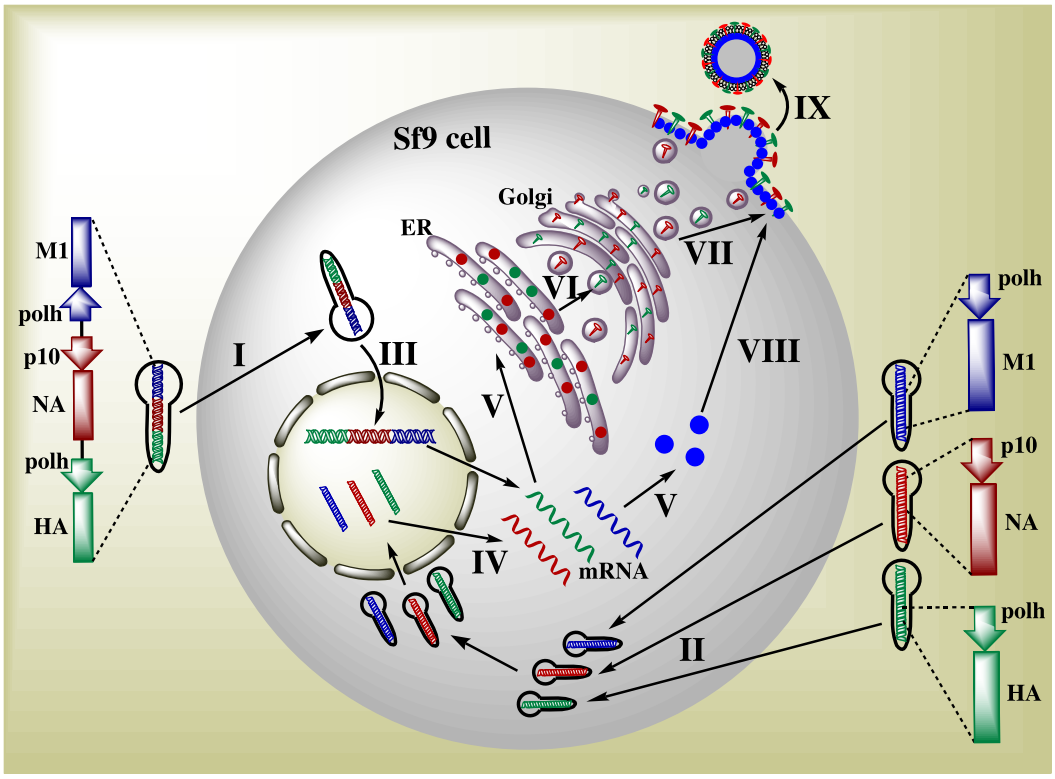

Fig. 3. Main stages of co-expression (I) and co-infection (II) to generate influenza VLP in insect cell. III, baculoviral genome gains access to the nucleus. IV, full-length mRNAs are exported from the nucleus. V, the mRNAs are translated into structural proteins of influenza virus. VI and VII, the HA and NA are transported through the Golgi apparatus onto the plasma membrane. VIII, the M1 is transported inside the plasma membrane. IX, a mature VLP buds from the surface of cell. ER, endoplasmic reticulum; HA, hemagglutinin; M1, matrix 1 protein; NA, neuraminidase; polh, polyhedron promoter; p10, p10 promoter.

no longer working properly due to gradual liquefaction of insect cells [61,62], so the insect cells may not be able to achieve self-assembly of VLPs exactly. This may be a problem if the formation of VLPs depends on proper post-translational modifications.

Monocistronic and polycistronic structures

VLPs constructed by the BES often consist of more than one protein, which can be expressed either with multiple baculoviruses each carrying a single foreign gene (monocistronic BES), or with a single baculovirus carrying multiple foreign genes (polycistronic BES) [63]. Based on both systems, two viral expression strategies to generate complex VLPs are feasible: infection with multiple monocistronic baculoviruses (co-infection) and infection with a single polycistronic baculovirus (co-expression) (Fig. 3). In contrast, structurally simple VLPs, such as circovirus [37] and parvovirus [22] VLPs, require only a single infection with monocistronic baculoviruses.

To date, a variety of BES kits based on mono-, bi-, tri- and quadcistronic expression vectors have been commercially available around the world, including the Invitrogen™ Bac-to-Bac® system, the BD BaculoGold™ system and the Oxford Expression Technologies flashBAC™ system, facilitating the generation of numerous VLPs. As described previously, the quadruple (pAcAB4) expression vector utilizing the polh and the p10 promoters has been constructed and commercialized, where each pair of different promoters was juxtaposed in identical orientation and the same promoters were segregated in opposite orientation. Using the quadruple vector, bluetongue virus double shelled VLPs consisting of four proteins were originally synthesized [23]. However, competition presumably occurs among promoters when different heterologous proteins are simultaneously co-expressed by one recombinant baculovirus in the same cells [64], thus inhibiting, to some extent, the protein expression and VLP production. Additionally, a novel and versatile BES, MultiBac, has been developed to allow simultaneous expression of multiple proteins in a single cell, which could be used to produce protein complexes and to recapitulate metabolic pathways [65–69]. The MultiBac system has been set up as an open-access platform technology at the European Molecular Biology Laboratory (EMBL) in Grenoble, France [66].

Genetic modification of baculoviruses

Baculovirus cathepsin (*cath*), the papain-like cysteine protease, if deleted, has no significant effect on viral growth or polyhedron production in insect cells, indicating that the *cath* is not essential for viral replication *in vitro* [70]. The other important enzyme, chitinase (*chiA*), in conjunction with the *cath*, promotes liquefaction of the host in the latter stages of infection and then results in release of viruses to infect more cells [61]. Like the *cath*, the *chiA* is dispensable for virus propagation in cell culture. Genetic modification based on *chiA* and/or *cath* deletions hampered the liquefaction of hosts to some extent, and thereby improved the expression of complex proteins, which has been confirmed by many research groups [71–74]. For example, Hitchman et al. (2010) constructed a baculovirus mutant devoid of both the *chiA* and the *cath* greatly enhancing levels of protein production for secreted, nuclear and cytoplasmic proteins [71,72]. However, it is not clear yet whether baculovirus mutants are used to generate envelope VLPs at a higher level than those produced by the conventional BES.

Protein folding post translation

Structurally complicated VLPs often are composed of different subunits depending on each other for correct folding. In yeasts and mammalian cells, molecular mechanisms of protein folding have been identified in the ER [75], whereas in insects this issue has remained underexplored. Although heterologous protein folding in insect cells is more similar to mammalian cells than bacteria and yeasts, it is not always identical [76] and, for construction of VLPs, this may be critical. Enveloped VLPs generally contain membrane proteins, whereas expression of membrane-active proteins in infected insect cells is possibly hindered by the misfolding of polypeptides, resulting in the accumulation and/or precipitation of non-functional protein in or at the ER [77].

This problem has been addressed by co-expressing chaperones or foldases that enabled correct folding and post-translational processing, and prevented the accumulation of non-functional proteins [77–83]. Two main classes of chaperones investigated for use in the BES fall into two groups, namely those located in the ER and those in the cytosol. Calnexin, calreticulin and binding immunoglobulin protein are chaperones located in the ER where their expression has desirable effects on heterologous protein assembly; in the cytosol, Hsp70 has proven to be effective in increasing production efficiency by reducing the formation of aggregates, which would have otherwise been degraded [63]. In addition to these chaperones, a stably transformed insect cell line has been developed by engineering the folding pathway of insect cells, showing improved folding of a recombinant membrane protein. NinaA (neither inactivation nor afterpotential A) has been found to function in the ER and to directly interact with rhodopsin 1 ensuring correct folding of this membrane-bound protein in the rhabdomere of the fly [84]. Lenhard and Reiländer (1997) transformed *Sf*9 cells with a gene encoding NinaA of *Drosophila melanogaster*, and demonstrated that the new insect cell line produced more functional, plasma membrane-localized human dopamine transporter than the old one [77]. The co-expression of chaperones and foldases may complement other approaches, such as the development of alternative insect cell lines to optimize the BES for generating high yields of VLPs.

Glycosylation of recombinant proteins

Baculovirus-mediated expression in insect cells has become well-established for the production of recombinant glycoproteins. Since several glycoproteins could readily be produced in many insect cell lines compared with mammalian cells [85], these insect cell lines were widely employed for glycoprotein expression [86–88]. Nevertheless, the conventional BES may not be the best tool for producing glycoproteins to generate complex VLPs especially for pharmaceutical purposes, as the insect cells-produced glycoproteins have clearly different N-glycans from those produced by mammalian cells [8,89,90]. Completion of N-glycans in insect and mammalian cells appears to follow a similar initial pathway but diverge at subsequent processing steps (Fig. 4) [89,91]. Due to insufficient expression of multiple processing enzymes responsible for generating complex-type structures and metabolic enzymes involved in generating appropriate sugar nucleotides, N-glycans from insect cells are not usually processed to terminally sialylated complex-type structures but are generally instead modified to paucimannose structure [92]. Few studies have reported the use of O-glycosylation sites in insect cells [93,94]. For instance, human interferon-α 2 expressed in Sf9 insect cells was O-glycosylated at

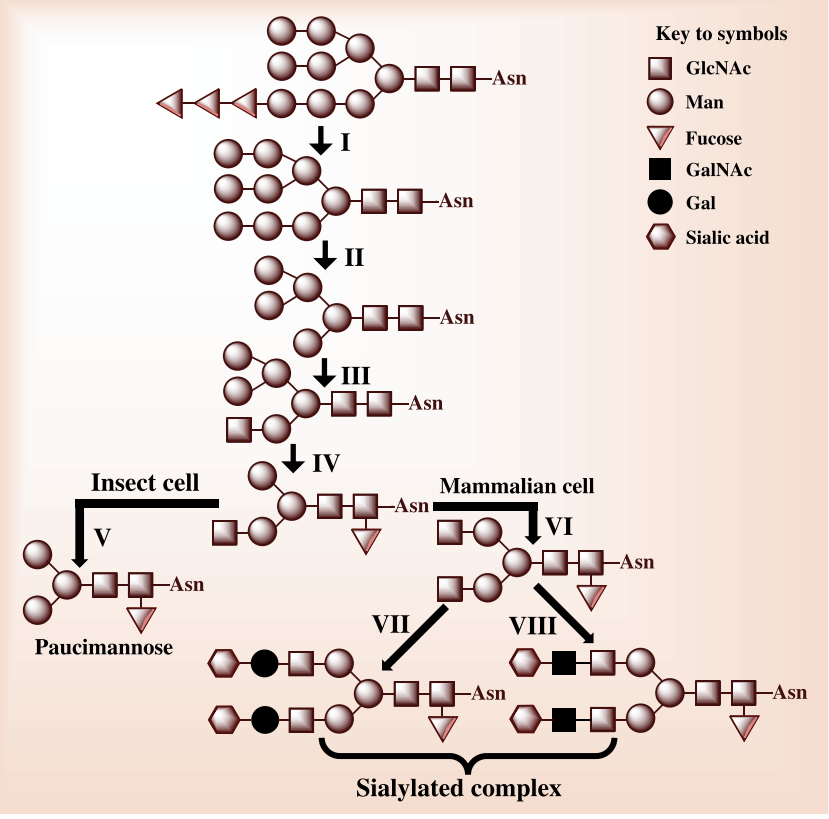

Fig. 4. Protein N-glycosylation pathways in insect and mammalian cells. The processing pathways in both cells share a common intermediate but diverge at subsequent processing steps. I: α1,2-glucosidase I and α1,3-glucosidase II; II: α-mannosidase I (in rough endoplasmic reticulum and Golgi apparatus); III: N-acetylglucosaminyltransferase I; IV: α-mannosidase II and fucosyltransferase; V: N-acetylglucosaminidase; VI: N-acetylglucosaminyltransferase II; VII: N-acetylgalactosyltransferase and sialyltransferase; VIII: galactosyltransferase and sialyltransferase. Adapted from [89] with permission from Elsevier (License No.: 3135990712390).

the same position as the natural one [95]. However, like the N-glycosylation, the O-glycosylation potential depends on culture medium and insect cell types. One of the most predominant and consistent changes in the O-glycosylation potential of insect cells would occur, if the culture medium is altered. Such a resulting change may be attributed to the alteration of the corresponding glycosyltransferase activities with variation of culture medium constituents [96].

In previous studies [97–100], methods involving genetic modification of either insect cells or baculoviruses have been used to address these problems. For the modification of cells, a new cell line could be developed by glycoengineering insect cell lines with mammalian genes encoding protein N-glycosylation functions under the transcriptional control of constitutive promoters [9]. Okada et al. (2010) prepared and characterized *Sf*21 cells by transfection with a rat cDNA for β1,4-N-acetylglucosa-minyltransferase III (GnT-III), showing that the GnT-III transfection has had the potential to be an effective approach in humanizing the N-glycosylation of lepidopteran insect cells [97]. For the modification of baculoviruses, a more sophisticated version of the MultiBac system was created by integrating sequences encoding Caenorhabditis elegans N-acetylglucosaminyltransferase II and bovine β1,4-galactosyltransferase I into the backbone of a baculovirus genome, resulting in a recombinant baculovirus designated as SweetBac. It has proven to be effective in the production of mammalianized glycoproteins in insect cells [98]. More recently, a new cell line, SfSWT-4, was generated by transforming *Sf*9 cells with six mammalian genes and has proven to produce sialylated glycoproteins when cultured with the sialic acid precursor, N-acetylmannosamine. Further, a daughter cell line, SfSWT-6, was isolated by super-transforming the SfSWT-4 with a human cytosine-5′-monophospho-sialic acid transporter gene. The resulting SfSWT-6 cells had higher levels of cell surface sialylation and also supported higher levels of recombinant glycoprotein sialylation, particularly when cultured with low concentrations of N-acetylmannosamine [101].

A number of viruses consist of envelope glycoproteins, which are the primary target of protective immunity. Glycosylation generally affects protein folding, localization, solubility and antigenicity [102]. For many viruses, mutation of special glycosylation sites may be highly detrimental to the antigenicity and immunogenicity of glycoproteins [103–107]. Therefore, the inability to produce glycoproteins with structurally authentic mammalian N- or O-glycans, to a certain extent, limits the application of the BES for production of VLP-based vaccines. Undoubtedly, the variation of antigenic properties of VLPs is an awfully serious issue for VLP-based vaccines. Interestingly, for influenza VLPs, "removal" of structurally nonessential glycans on VLPs surface glycoproteins may be a very effective and general approach for VLP-based vaccine design. Truncation of the N-glycan structures on HA can increase sialic acid binding affinities [104], and furthermore these structures are similar to those of insect cell-type N-glycans, which thereby can facilitate the uptake of influenza VLPs by antigen-presenting cells [108]. To date, enveloped influenza VLPs have been developed by biopharmaceutical companies and were demonstrated to induce protective immunity during preclinical and clinical studies [109–115].

Disulfide bond formation post translation

Disulfide bonds can be employed in a variety of viruses, such as hepatitis B virus [116], hepatitis C virus [117] and papillomavirus [118], to covalently cross-link the monomers of their capsids, contributing to virion stability. Accordingly, the impact of disulfide bonds on VLP stability varies with the location and the number of disulfide bonds, which can increase the thermal stability from just a few degree Celsius to over 50 °C [119]. The improved thermal stability would be advantageous for most applications, especially based on VLP-based vaccines, which can be used in absence of a reliable cold chain in many tropical or subtropical regions.

Foreign proteins expressed in insect cells may form disulfide bonds each other, which are necessary for formation of VLPs. For example, VP60, a unique capsid component of the rabbit hemorrhagic disease virus (RHDV), can be expressed in insect cells and assemble without the need of any other viral component to form VLPs, further structurally and immunologically indistinguishable from the RHDVs. More importantly, a 120 kDa protein, the possible dimer of VP60 formed via disulfide bridging, is revealed in VLP and RHDV samples when they are boiled and dissociated under non-reducing conditions [120]. Likewise, Sapp et al. (1995) studied the disulphide bonding between L1 proteins and the association of L2 proteins with capsomers using VLPs obtained in insect cells by co-expression of the L1 and L2 proteins of human papillomavirus type 33, indicating that approximately 50% of the L1 protein molecules in VLPs formed disulphide bonded trimers [121].

Disulfide linkages between cysteine residues stabilize VLPs. Simian virus 40 VP1 protein when substituted at cysteine residues and then expressed in insect cells could assemble into VLPs containing intermolecular disulfide linkages, one of which was critical for maintenance of these VLPs at low calcium ion concentrations and contributed to their stabilization against dissociation to pentamers by preventing the release of calcium ion [122]. A disulfide-bonded dimer of the core protein of hepatitis C virus is also important for VLP production [123], which is achieved by expression of the core protein alone in insect cells [124]. All of these studies indicated that the BES was a preferred platform for the production of VLPs whose physical and chemical stabilities, at least in part, relied on the disulfide bonds.

Proteolytic processing post translation

Several signal peptides deriving from vertebrate proteins can normally be cleaved in insect cells [125,126]. Proper cleavage of signal peptides in baculovirus-infected cells is inferred from the size of the product or its presence in the plasma membrane or extracellular culture fluid. Many studies have included amino acid sequence analysis of the N-terminus of the baculovirus-produced protein to confirm that the signal peptide cleavage site is identical to that observed in the original source [127]. However, other heterologous signal peptides may be inefficiently recognized by the protein translocation machinery in lepidopteran insect cells. The postulate has been verified by a previous study [128], revealing that secretion of plant propapain expressed in insect cells was improved by replacement of its native signal peptide with that from honeybee mellitin. Conversely, the other study has demonstrated that insect-derived signal peptides and/or prosequences could not always enhance the expression and/or secretion of foreign secretory pathway proteins in the BES, suggesting that the inability of insect cells to recognize the processing signals efficiently was probably not the major factor preventing high level production of foreign proteins [129].

Theoretically, the inefficient recognition of heterologous signal peptides could limit the amount of newly recombinant proteins that entered the secretory pathway [129], thereby impeding the generation of VLPs when composed of such proteins. After cleavage of the signal peptide, further proteolytic processing occurs predominantly at basic amino acid residues in insect cells [130] and possibly causes the degradation of foreign proteins that are already expressed, thereby hindering the formation of VLPs once again. This assumption was also confirmed by Cruz et al. (1999) who indicated that proteolytic degradation of Pr55gag particles (HIV-1 core-like particle) obviously occurred in the supernatant and inside insect cells when such cells were infected in the late exponential

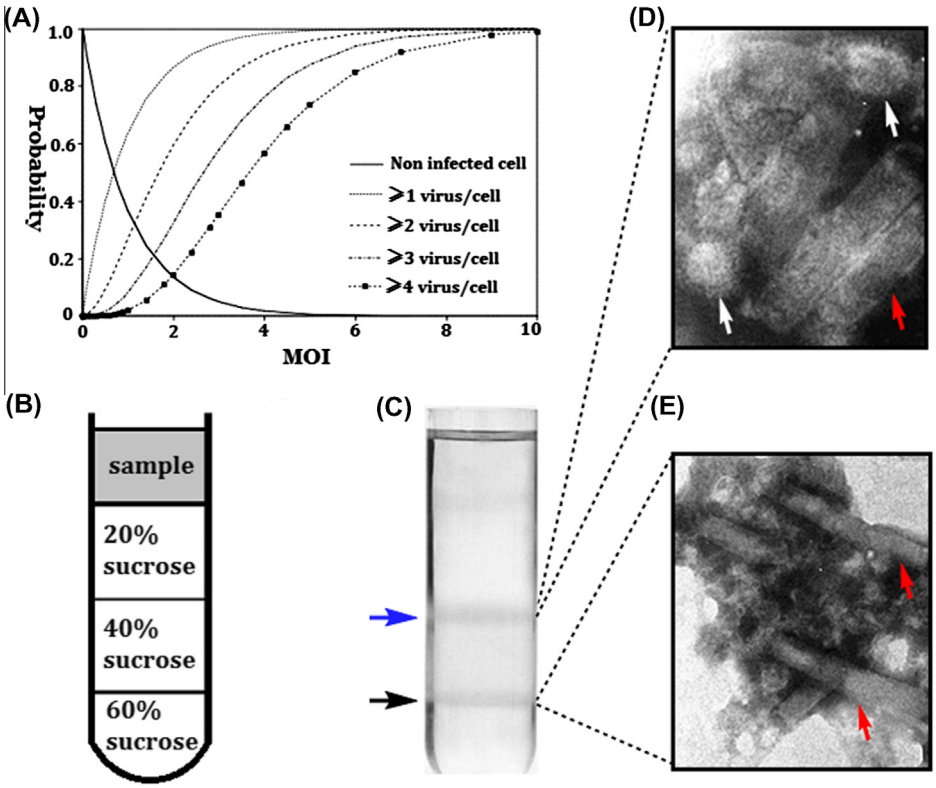

Fig. 5. Production optimization and ultracentrifugation purification of VLPs according to the Poisson distribution and sucrose density gradient centrifugation, respectively. Probability of infection of a population according to the Poisson distribution (A). MOI, multiplicities of infection. Adapted from [52] with permission from Elsevier (License No.: 3136000207794). A discontinuous sucrose density gradient was prepared by layering successive decreasing sucrose densities solutions upon one another (B). The sample was obtained from culture supernatant of *Sf*9 cells, infected with recombinant baculoviruses expressing major structural proteins of a peste des petits ruminants virus (PPRV). Two white discs of protein formed between the 40% and 60% (black arrowed), as well as between the 20% and 40% (blue arrowed) sucrose layers after ultracentrifugation (C). Electron micrographs of ultracentrifugation-purified products collected from upper (D) and lower (E) protein layers. The PPRV VLPs and baculoviruses were indicated by the white and red arrows, respectively.

growth phase or with very low multiplicities of infection (MOI) [131].

Fortunately, for generation of influenza VLPs, proteolytic cleavage of the influenza HA expressed in insect cells are retarded and less efficient, and a substantial fraction of the HA can persist in uncleaved form [132]. Furthermore, the proper proteolytic processing does not impair its binding capacity but does membrane fusion [108,133]. Additionally, there were several other studies [134,135] independently to show that proteolytic processing contributed to generating VLPs in insect cells. Expression of immature precursor and subsequent proteolytic processing seem to be an effective strategy in formation of VLPs [136]. When the Flock House nodavirus (FHV) coat protein (CP) precursor (CP-alpha) alone was expressed in insect cells, virus-like precursor particle could be formed. It normally matured by proteolytic cleavage of protein alpha into polypeptide chains beta and gamma. The mature VLP was morphologically indistinguishable from authentic FHV. Alteration of proteolytic cleavage by mutations was responsible for defective VLPs, some of which possessed unusual structural features [135].

Host factors

In addition to recombinant baculoviruses, insect cells are the other important player in defining the efficiency of VLP production. To date, hundreds of cell lines from lepidopteran insects have been established. Despite use of the same BES, differences between distinct insect cell lines may cause the differences in the efficiency of VLP production. For example, VLPs of influenza subtypes H1 [110], H3 [137], H5 [138] and H9 [139] have been produced by co-expression of structural proteins in insect cells, all of which were the Sf9 cells. Nevertheless, comparison of VLP production in terms of yield and quality between *Sf*9 and *Tn*5 lines revealed dramatic differences in baculovirus background as well as in yield and density of VLPs: the *Tn*5 cells produced homogenous VLPs carrying more HA than their *Sf*9-derived counterparts and resulted in a much lower virus background of the final VLP preparation [140]. Significantly, the commercially available VLP-based vaccine, Cervarix™, was also produced in the *Tn*5 cells [141]. Additionally, one research group demonstrated that the silkworm could produce higher levels of recombinant proteins than *Sf*9 cells, although the level of expression of soluble protein was higher in *Sf*9 cells than in the silkworm for all proteins except the membrane proteins [142]. These differences most likely reflect the great level of complexity of the biological systems in silkworms, compared with that in *Sf*9 cells. Despite the high-yield expression of recombinant proteins and especially membrane proteins in silkworms, it remains unclear whether the generation of VLPs *in vivo* is better than *in vitro*.

It is widely believed that synonymous codon usage of highly expressed genes is strongly biased and tends to match the more abundant tRNAs. Previous research [143,144] on codon usage in a variety of baculoviruses suggested that co-adaptation between baculovirus codon usage and tRNA availability in insect cells affected protein expression and VLP formation. Unfortunately, there are few reports of comparative VLP production in different insect cell lines, so we still do not know which factors are responsible for potential differences in the self-assembly mechanism of proteins in various insect cells.

Bioprocess considerations for production of VLPs

Although the BES has been broadly adopted for the basic construction of VLPs in different laboratories, upstream and downstream processing issue should conform to industry or, at least, basic research standards. These parameters should include MOI, TOI (time of infection), agitation and aeration rates, and dissolved oxygen tension among others. In addition, different strategies for production and purification may affect the quality of VLPs to a great extent. In this section, we briefly discussed many bioprocess considerations for production of VLPs.

Co-expression and co-infection

At first glance, choosing co-expression, co-infection or both (Table 1) for production of VLPs may appear arbitrary, whereas it probably has a serious impact on heterologous protein expression in insect cells. Co-expression using polycistronic baculoviruses has been explored as a strategy to overcome the limitations inherent in the co-infection, namely the uneven distribution of individual virus infections in insect cell populations [63]. As shown in the Fig. 3, co-expression ensures that every protein necessary for the formation of VLPs is produced in a single infected cell. This principle is significant for the formation of structurally complex VLPs in the perspective of biological stoichiometry. However, polycistronic baculoviruses are often unstable and therefore it is difficult to achieve equivalent expression of different genes in one insect cell. In contrast, one advantage of the co-infection over the co-expression is that monocistronic baculoviruses can be rapidly produced if new genotypes or serotypes appear, and only one or two baculoviruses need to be replaced, reducing additional validation requirements for the formation of VLPs [145].

Two different strategies were compared for the production of triple layered rotavirus VLPs composed of three structural proteins (VP2, VP6 and VP7): co-infection with three monocistronic baculoviruses and co-expression with one tricistronic baculovirus. The results showed that the co-expression was more efficient for production of such VLPs than the co-infection, which could be attributed to three major factors: (1) higher DNA replication rates presented by the tricistronic vector than the monocistronic vectors, (2) invariant mRNA stability for all three mRNAs, and (3) an excess of VP7 over VP6, both of which were produced by the co-expression, being more approximately equivalent to the VP7/VP6 stoichiometric ratio in the native virons [146]. Nonetheless, because the number of insect cells co-infected by all three baculoviruses could not be (or was not) measured, it was difficult to determine whether co-infection produced VLPs at a lower level. Nonetheless, another comparison [147] of the two strategies for the production of rotavirus VLPs indicated that virus DNA replication and transcription rates were appropriately 50% slower in the co-expression than in the co-infection experiments, which was contrary to the results of [146].

As for rotavirus VLPs, it is difficult to propose an ideal strategy for the production of influenza VLPs. In our laboratory, avian influenza VLPs have been constructed by co-expression of HA, NA and M1 in *Sf*9 cells, and we have demonstrated that the resulting VLPs could induce efficient immune responses in chicken (data not shown). In contrast, other studies reported successful production of influenza VLPs via the co-infection of a *Sf*9 cell culture with a combination of bi- and mono-cistronic [113] or mono- and mono-cistronic baculoviruses [148]. Unfortunately due to the absence of recombinant baculoviruses expressing HA, NA and M1, respectively, it is not possible to directly compare the two different production strategies for producing avian influenza VLPs.

Optimizing production of VLPs by mathematical models

To date, different mathematical models of baculovirus infection have been established to optimize protein expression or VLP construction in insect cells [149–153]. The MOI should be selected carefully in order to achieve optimal production of VLPs. The use of MOI to manipulate recombinant protein concentration and to optimize VLP formation relies on the probability of infection described by a Poisson distribution, which is a discrete probability distribution that expresses the probability of a given number of events occurring in a fixed interval of time. The Poisson distribution can be used to describe the probability (P) of one insect cell initially infected by w recombinant baculoviruses, and it has the following form [52]:

$$P(w, MOI) = \left[\left(\frac{MOI^w}{w!}\right)e^{-MOI}\right]$$

The Poisson distribution is graphically represented in the Fig. 5A, where the probability of infection of a population is shown, indicating that the MOI determines the population fraction initially infected by w baculoviruses. The mathematical method describing the probability of virus infection was initially reported by Licari and Bailey (1992) who proposed an insect cell-baculovirus model that simulated cell population dynamics, extracellular virion densities, and heterologous product titers in reasonable agreement with experimental data for a wide range of MOI and TOI [149]. In the meantime, a predictive kinetics-based model was developed to describe the infection of insect cells with baculoviruses in a continuously operated reactor configuration [154], and subsequently, another model was established to predict the cell population dynamics, production of recombinant protein and infective extracellular virus progeny [155].

For production of VLPs, the MOI could be optimized by a probabilistic model developed by Tsao et al. as early as 1996. It has proven to correlate well with experimental results, thereby providing a better understanding of co-infections using the BES to produce parvovirus VLPs [156]. Even though the probabilistic model could be used for a steady-state co-infection analysis for prediction of VLP composition, there were no models of the VLP assembly process until a kinetic and statistical-thermodynamic model was established for baculovirus infection and VLP assembly in suspended insect cells. This mathematical model could be used to characterize baculovirus infection, protein synthesis and more importantly VLP assembly in insect cells. The complete model suggested that the formation of infectious bursal disease virus VLPs was thermodynamically favorable and predicted well the baculovirus infection in individual cells or in cell population as a whole [152]. This model can potentially be used to further describe and optimize VLP formation for other virus pathogens. Despite the establishments of mathematical models for baculovirus infection for a long time, the total number of reports on application of models to optimize the production of VLPs is relatively scarce over the past decade.

Purification of VLPs

Baculoviruses have shown adjuvant activity and if not removed or inactivated, would induce undesirable synergistic effects on the target VLP-based immunologic response [157]. Therefore, before VLPs can be used for scientific and especially medical purposes, they must be biophysically or biochemically separated from baculoviruses, which are co-produced as by-product in large amounts in insect cells and culture supernatant [158]. Undoubtedly, if VLPs share structural similarity with recombinant baculoviruses, the

removal of baculoviruses to a great extent complicates the purification process.

Sucrose [31,159,41,160] or cesium chloride [24,33,161] gradient ultracentrifugation is generally considered to be chemically and physically appropriate for purification of VLPs, but this general approach is labor-intensive, time-consuming and scale-restricted [162], and can be associated with unexpected batch-to-batch variation. Although several reports have shown that gradient ultracentrifugation could be employed to purify rotavirus VLPs, it provided only low yield and failed to remove impurities from the final products [163]. Such a phenomenon of incomplete removal of impurities was also found in a recent experiment, whereby we tried to purify peste des petits ruminants virus (PPRV) VLPs from culture supernatant of *Sf*9 cells, which were infected with recombinant baculoviruses expressing PPRV major structural proteins. However, the purified products by sucrose density gradient centrifugation have proven to be mainly mixed with recombinant baculoviruses (Fig. 5D).

In addition to the method of gradient ultracentrifugation, many purification processes as stepwise unit operations were developed, based either on centrifugation, precipitation and ultrafiltration/diafiltration [164], or on depth filtration, ultrafiltration and size exclusion chromatography [165]. More recently, an anion exchange-based purification method for norovirus VLPs was reported. This novel method consisted of polyethylene glycol precipitation followed by a single anion exchange chromatography step, which could be completed within one day to purify norovirus VLPs produced using insect cells. More significantly, high product purity could be obtained by the method and the final products still contained fully assembled, mono-dispersed VLPs [166].

Biophysical methods concerning electrostatic technology need to be exploited to allow separation of both particles, whereas this may be a challenge if VLPs is also enveloped. Alternatively, purified VLPs can be chemically inactivated to eliminate baculovirus infectivity [157], but this strategy may alter the antigenicity of VLPs. In addition to biophysical and biochemical separations, the use of *vp80* gene-deleted baculovirus vectors has been presented as a novel strategy that would greatly simplify the downstream processing of biopharmaceuticals produced in insect cells, as it could prevent the release of progeny baculoviruses into the culture supernatant [167]. While there have not yet been any reports of VLP production using such gene-deleted baculoviruses, this new strategy would hold promise for the industrial production of VLPs in the near future.

Conclusions

In 1983, the first report on foreign protein expression in insect cells stimulated great interest in use of the BES for recombinant protein production. A large variety of VLPs have been produced using the BES, mainly owing to the high expression levels of foreign proteins and proper post-translational modifications in insect cells. The two VLP-based vaccines manufactured using the BES have been commercialized and proven to confer strong immune responses *in vivo*. Nonetheless, the differences in essential mechanisms between insect and mammalian cells, such as glycosylation and folding of heterologous proteins post translation, may hinder the formation of VLPs, especially structurally complicated VLPs. In addition, several manufacturing issues need to be addressed to achieve high-quality VLP upstream and downstream processing. The production of structurally complicated VLPs will benefit from improvements in downstream processes that could improve recovery yields without compromising VLP quality [168]. In conclusion, a long-term goal to accelerate the manufacturing capability of the BES for VLPs should be to revolutionize the conventional BES in order to improve protein quality (such as optimizing folding, enhancing glycosylation and preventing degradation) and to stabilize protein quantity over longer periods of time.

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