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Minireview

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Purification of virus-like particles (VLPs) expressed in the silkworm *Bombyx mori*

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Abstract Virus-like particles (VLPs) are a promising and developing option for vaccination and gene therapy. They are also interesting as shuttles for drug targeting. Currently, several different gene expression systems are available, among which the silkworm expression system is known for its mass production capacity. However, cost-effective purification with high purity of the target protein is a particular bottleneck for this system. The present review evaluates the advances in the purification of VLPs, especially from silkworm larval hemolymph. Starting from applicable pre-treatments for VLPs over to chromatography methods and quality control of the purified VLPs. Whereupon the main focus is on the different chromatography approaches for the purification, but the structure of the VLPs and their intended use for humans make also the quality control important. Within this, the stability of the VLPs which has to be considered for the purification is as well discussed.

Keywords: chromatography · hemolymph · purification · silkworm · virus-like particle

Introduction

Virus-like particles (VLPs) are an interesting and expanding topic in biotechnology. They can be used as vaccines, for example, Engerix (hepatitis B virus) and Cervarix (human papillomavirus) from GlaxoSmithKline or Recombivax HB (hepatitis B virus) and Gardasil (human papillomavirus) from Merck and Co. Moreover, VLPs were shown to be capable of functioning as a drug delivery system (Deo et al. 2015). Although the origin of VLPs is their corresponding viruses, VLPs lack the genetic material. However, VLPs retain the ability to self-assemble from one or more subunit proteins and have

characteristics nearly similar to their viruses without the DNA or RNA. Thus, VLPs are merely empty vehicles and not infectious. This makes them intriguing for *in vivo* use because they maintain the ability to enter target cells. There are different types of structurally different VLPs (Lua et al. 2014): enveloped and non-enveloped VLPs (Fig. 1).

<< Figure 1>>

VLPs can have one or even multiple capsid proteins and some, such as the VLP from HIV (human immunodeficiency virus), have an envelope. They acquire their lipid bilayer from the cell membrane of their host cells. In this membrane, one or several novel proteins can be included during and after assembly (Palaniyandi et al. 2012). These proteins can be recognized as antigens by the immune system and trigger an immunological response (Deo et al. 2013). These with additional proteins modified VLPs are also highly usable as vaccine candidates.

Vaccination is a cheap and effective way to prevent diseases, but it takes approximately 6 months to cultivate the attenuated or inactivated wild-type viruses, excluding the batch controlled by the official authorities. This long production time impedes the flexibility of influenza vaccines. If there is an antigen switch from the predicted ones, the produced vaccine is less effective or even useless. VLPs have been shown to elicit a strong immune response and have the advantage of a shorter production time. The latter depends on the production system, but it is approximately 1–4 weeks. The time differs for each process, but it is approximately 1–2 weeks for insect cell- and silkworm-based systems if the recombinant bacmid has to be reproduced (Kato et al.

2010). Moreover, the whole production time is approximately 5 days in bacteria and 7 days in yeast.

Moreover, if there are any particular recognition proteins or antibodies in the membrane or capsid, it is possible to use them as drug delivery systems (Kato et al. 2015) because the VLPs are empty and can be filled with different compounds such as drugs. The anti-cancer drug doxorubicin was bind to the rotavirus VLPs, and lactobionic acid was used to particularly target the hepatoma cell line HepG2 (Zeltins 2013). Other studies showed that it was also possible to pack pharmaceutical drugs inside VLPs and target specific types of cells (Deo et al. 2015). Moreover, it was shown that nucleic acids, such as siRNA, could be incorporated into and transported via VLPs (Galaway and Stockley 2013). VLPs were functionalized with the single-chain variable fragment of humanized CC49 antibody, which was displayed on the surface of VLPs from *Rous sarcoma* virus and used to deliver an anti-cancer drug specifically to the colon carcinoma cell strain LS174T (Kato et al. 2015). Alternatively, the recombinant single chain fragment variable, which binds the tumor-associated glycoprotein 72 and recombinant human Inter leucine-2 in the lipid bilayer of *Rous sarcoma* VLP (Deo et al. 2016), can be anchored. This VLP specifically targets colon cancer cells, thus attracting macrophages to them.

VLPs have a broad application spectrum and offer a unique approach for gene therapy. Using recombinant VLPs for the HIV CD4-specific receptor and transporting a thymidine kinase, showed that VLPs could specifically kill HIV infected cells under ganciclovir treatment. Moreover, using HIV VLPs, it was possible to target resting CD4 T cells and manipulate the gene through gene silencing (Geng et al. 2014). This clearly demonstrates that VLPs are modifiable.

Interestingly, some types of VLPs, such as hepatitis B VLPs, are stable to alkaline treatment, which can be used in their preparation (Strods et al. 2015). However, even with the progress in technology, it is still difficult to cost-effectively prepare VLPs with high purity without contamination by nucleic acids and viruses.

This review focuses on the purification of VLPs particularly from the hemolymph of the silkworm *Bombyx mori*. This expression system is promising, but the purification process is still troublesome and requires methods with high recovery ratio without destroying VLPs.

Production of recombinant VLPs

Recombinant VLPs can be produced in different host expression systems, which should be carefully selected for the purpose (Zeltins 2013). It is possible to use a bacterial system such as *E. coli*, but bacterial systems cannot undertake post-translational modifications and have problems with protein solubility. Nevertheless, it is a well-known accepted type of system and is widely used. Yeast strains, such as *Pichia*, are an emerging platform to produce VLPs. Unlike *E. coli*, yeasts do not form inclusion bodies and can undertake introduction of post-translational modifications. However, they lack the high production capacity of *E. coli* and are incapable of supporting the assembly of multimers in some cases, but this does not indicate that their production capacity is generally low. Their production capacity for recombinant proteins is normally better than that insect or mammalian cell-based systems, if the recombinant proteins don't necessitate this kind of systems. Although *E. coli* and yeast are still the preferred platforms for some protein expression groups, mammalian recombinant proteins often require mammalian cells for optimum yields and activity.

Another option is insect cell-based systems. The Sf9 cell line, which originated from the ovary of *Spodoptera frugiperda* or the High Five line, is widely used. These systems are also capable of undertaking post-translational modification, have a high growth rate and can be used for large-scale production. However, they require the construction of a vector from the recombinant baculovirus containing the gene of interest (Krammer et al. 2010).

Moreover, plants may be used to produce VLPs, as they present a cost-efficient and scalable alternative. Therefore, the leaves of *Nicotiana benthamiana* were infected to produce the two types of VLPs of the non-enveloped polyomavirus protein VP1. The wild-type yielded 58 µg VLPs/ fresh weight tissues g and the polybasic motif deleted type 81 µg VLPs/ fresh weight tissues g (Catrice and Sainsbury 2015).

Mammalian cell lines have also been used. Although the mammalian cell lines are complex in construction and application, they are fully capable of post-translational modification and of assembling multimers. It was demonstrated that cell-free systems could be used for the production of VLPs, even in complicated cases where the production of the proteins is toxic for the host cell (Zeltins 2013).

Production of recombinant VLPs in silkworm

Asian countries possess the infrastructure for producing VLPs from silkworms, which are comparatively cheap to use and require an simple, artificial diet (Kato et al. 2010). This system has a high production capacity for a low cost as high productions from their protein of interest were reported (Mitsudome et al. 2014).

The fifth instar silkworm is injected with *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid on the second day, and the protein from the hemolymph is collected 4

to 6 days after injection. The entire process for protein expressing including bacmid preparation takes approximately 1–2 weeks (Kato et al. 2010). Another advantage of this system, also for insect cells, is that the used baculovirus, which can be hard to separate from the product during purification, is not infectious for mammalian cells and could even be an adjuvant for vaccinations. Thus, the desired gene, after PCR amplification, is introduced into a recombinant bacmid vector amplified in *E. coli* as a large plasmid. This is known as the BmNPV bacmid expression system and expedites the isolation and purification of the recombinant bacmid compared with the conventional baculovirus expression vector system (required several rounds of amplification in *Bombyx mori* cells), which usually takes 3–6 months. An additional production system related to the silkworm is the production of the silkworm *pupae*. VLP production has also been reported for this system (Nerome et al. 2017); however, it is still not sufficient, as in terms of production and purification the advantages/disadvantages of this silkworm system have yet to be explored.

Purification

The purification of proteins from the silkworm is still a major problem for a wider application. A few studies have described the general purification of proteins from the silkworm, and a few report the attempt to establish an up-scalable process for their purification. Protein tags are often used for purification (Morokuma et al. 2017) or non-scalable processes such as sucrose gradient centrifugation (Deo et al. 2011). The purification of human papilloma virus 6b L1 VLPs using heparin affinity chromatography and sucrose gradient centrifugation as comparison showed stable VLPs, but still with a

high amount of host proteins (Palaniyandi et al. 2012) (Supplementary Fig. S1). Although scalable processes are used, the improvements in yield and purity have not yet been investigated. One study aimed to investigate the antigenicity and the receptor binding capacity of the recombinant hemagglutinin, not the purification optimization and therefore the yield of 500 µg/30 silkworms using Fetuin-agarose and size exclusion chromatography (SEC) was satisfying (Dong et al. 2013). Instead of purification optimization, another study solely focused on the expression, characterization and hemagglutinin inhibition of rat α2,6-sialyltransferase in silkworm hemolymph. Using ammonium sulfate as pre-treatment and FLAG-Tag affinity chromatography for purification, a yield of 2.2 mg /11 ml silkworm hemolymph (recovery around 64 %) was obtained. In some cases benzonase was used, which is expensive and increases the cost of purification, but the possible benefit has to be calculated for each case independently (Steppert et al. 2016). Normally the purification from VLPs, or even viruses, does not differ from the general purification procedure for recombinant proteins. However, VLPs are large biomolecules that behave differently in standard chromatography material, and their stability in salts or a pH environment can be more critical compared to smaller proteins (Nestola et al. 2015).

Pre-treatment

Before chromatographic purification, a clarification step is recommended in the current downstream processing for biopharmaceutical products to remove large aggregates and cell debris to avoid clogging or fouling of the column in later stages. It also can make the whole purification process more efficient, if applicable. Centrifugation and filtration are the commonly used pretreatment methods, and the latter is easier to scale up and

facilitates a defined cut-off for the particle size. It can be also recommended to investigate the use of other precipitation methods for VLPs, such as the use of polyethylene glycol (PEG) or ammonium sulfate, which can reduce the chromatography steps and are cost effective if the virus or VLP can withstand the treatment. Besides ammonium sulfate and PEG, polyethylene imine (PEI) can be used for the precipitation. PEI is a basic, positively charged polymer and is therefore useful for the precipitation of the negatively charged DNA. Acid proteins also precipitate in lower salt concentrations (0.1 M NaCl); therefore, higher salt concentrations are used to prevent this protein precipitation. The human influenza virus was purified from mammalian cells using the statistical approach of a Design of Experiments for the investigation of the ideal PEI condition. A 0.045 % (w/v) of 2 kDa PEI with a high salt concentration could reduce the DNA to 15 % and recover 86 % of the virions. However, PEI can interact with assays and has *in vivo* toxicity; therefore, a low molecular PEI is favored, and it should be separated in later purification steps (Kröber et al. 2010).

Purification using chromatography

After pre-treatment steps are chosen, a chromatography step is recommended, but the chromatography purification of proteins and VLPs in different expression systems has been often shown, but rarely for silkworm larval hemolymph (Table 1 and Supplementary Table S1) (Steppert et al. 2016).

<<Table 1>>

On the other hand, VLPs have the same properties as their origin viruses; therefore, it is possible for the purification to comply with the purification protocols of the virus. Furthermore, an increase in the purification yield is the main goal during the purification of viruses (Du et al. 2017; Leuchs et al. 2017). Therefore, this kind of knowledge can be transmitted to VLP purification. However, only a few studies have explored this aspect. Mundle et al. (2016) showed the potential of newer chromatography material, such as the core bead technology from GE Healthcare LifeScience, for the intermediate purification of enveloped viruses. This system uses the principle of SEC and has a cut off of 700 kDa. Proteins larger than 700 kDa cannot enter the pores of the shell to interact with the core and remain in the flow through. The respiratory syncytial virus, produced in a Vero cell line, was purified in this manner from the supernatant with a yield of approximately 50–60 %.

Otherwise Ion exchange chromatography (IEX) is one of the most universally used principles and is often used during the first step of capturing. (Sviben et al. 2017) investigated the purification benefit for the enveloped measles virus (MeV) and mumps virus (MuV) using IEX and hydrophobic chromatography. The best result using IEX for MeV was achieved with a large channel monolith QA column (BIA Separations, Slovenia), pH 7.3 and 50 mM phosphate buffer. For MuV, the recovery was very low and was therefore not further investigated. Both viruses have a recovery of approximately 60 % in an OH column (BIA Separations, Slovenia), with 1.0 M ammonium sulfate in 50 mM HEPES, pH 7.3 as the loading buffer. The amount of ammonium sulfate influences the recovery of the infective viruses. The viruses bind less to the column at a lower concentration, but a higher concentration resulted in less infective viruses. Moreover, it

is interesting to use the flow through mode instead of the bind and elute mode of the traditional chromatography approach and material (Lee et al. 2016; Mundle et al. 2016). A Q anion exchange chromatography (AIEX) material modified with the inert polymer polyoligoethyleneglycol methacrylate was used for the steric exclusion of hepatitis B core VLPs, which resulted in a negative chromatography mode, and with an additional heat treatment, a purity of approximately 87.5 % was achieved (Lee et al. 2015). This approach was also successfully used in other studies. Detailed information about the utilization of the negative chromatography mode has been described in a review article by (Lee et al. 2014). Moreover, steric exclusion chromatography is interesting because PEG 6000 is unable to interact with macromolecules, the target, and induces at a higher concentration a thermodynamically unstable state that is released when the macromolecules bind to the surface of the column. This favors larger molecules so that the smaller molecules can be washed away. After washing, the bound macromolecules can be removed by decreasing the PEG concentration (Marichal-Gallardo et al. 2017).

Another common principle is SEC which is, with some exceptions, usually used as a polishing step in a chromatography purification protocol. Moreover, hydroxyapatite chromatography (HAC) has unique characteristics beneficial for protein purification and is also applicable for VLPs. Both methods were used for the purification of non-enveloped enterovirus 71 VLPs from insect cells in a protocol established using tangential flow filtration. The HAC alone had a VLP recovery of approximately 100 % and 17 % removal of the total protein. During the SEC step, 5 % of the total protein could be removed, but the VLP recovery was 55 %, which was the lowest in this protocol. The total VLP recovery was 36 %, and the purity was 83 %, which is still not practical for

wider use in the industry, particularly because it was a clean cell supernatant compared with silkworm hemolymph, but this result is an improvement nevertheless (Lin et al. 2015). Two SEC columns were also used for the purification of the BmNPV from the silkworm larval hemolymph which resulted in a high recovery or in a high removal of host proteins, but not both achievements simultaneous with only one of the columns. Moreover, the HAC should be also applicable for enveloped VLP, since dengue virus type 2 ThNH7/93 was purified with a recovery around 60 % from the supernatant of C6/36 cells and still could retain his infectivity which indicates that the membrane was intact (Kurosawa et al. 2012).

Viruses and their VLPs have sizes from approximately 30 nm to 300 nm and, therefore, have limited interaction with the classical chromatography material. The packed porous beds have a much smaller pore size so that the particles cannot diffuse into them. This makes the utilization of the traditional materials not useless, specifically not with smaller particles, but their use remains restricted. An approach to tackle this issue includes the use of monolith columns, which consist of packed beds of a single block of modified polymethacrylate with a larger pore size. This results in better resolution and recovery, higher capacity, and faster flow rates. These materials have already shown promising results in recent years (Steppert et al. 2017; Sviben et al. 2017).

Stability of VLPs

Another black box is the stability of the VLP. It is assumed that non-enveloped VLPs are more stable than enveloped VLPs, which are almost the same as for viruses. For the enveloped virus, the optimum pH is approximately 7–8 and the temperature range is from 0° to 30°C because the viruses are more likely to aggregate at a lower pH and a higher

temperature (Hu et al. 2011). The ionic strength/salt concentration was also shown to be a major factor; therefore, the stability ranges of the non-enveloped VLPs are larger. Enveloped VLPs are more fragile regarding their envelope, which should be particularly considered for precipitation. PEG and ammonium sulfate were implicated in the precipitation and purification of viruses without an envelope. However, ammonium sulfate is known to destroy the lipid bilayer, but apparently, low concentrations ranging from 5–10 % (w/v) or up to 2 M ammonium sulfate are tolerated without destroying the envelope (Steppert et al. 2017).

Quality Control

Most likely cell-assembled VLPs still contain host DNA or host proteins even after purification, which not only can disturb the structure of the VLP but also is disadvantageous for pharmaceutical use, which requires the least possible contamination. In this case, sucrose gradient centrifugation could separate the empty VLPs from the loaded VLPs. Only for the non-enveloped VLPs, a disassembly/reassembly step is possible because, so far, it is not possible to reconstruct the plasma membrane after destruction. After reassembly, less contaminating DNA (Xue et al. 2017) and better stability and biophysical properties of the VLPs are obtained, and the VLPs could elicit a stronger immune response (Zhao et al. 2012).

After purification, the structure and purity of the VLPs must be checked. This should be done with SDS-PAGE and staining and, if specific antibodies are available, via Western blot or ELISA. Since these methods measure all VLPs, including the destroyed ones, a visual confirmation from electron microscopy such as transmission electron microscopy (TEM) is necessary, particularly for the enveloped ones because of the

stability of the envelope. In addition to the already mentioned ELISA and TEM, other methods for characterizing the VLPs include dynamic light scattering for size, circular dichroism, fluorescence spectroscopy and, if necessary, the hemolysis assay (Catrice and Sainsbury 2015). Further analytical methods depend on the purpose of the purified VLPs. A high purity is important for use in the human body, and this can be analyzed with HPLC, in addition to other methods. Furthermore, it was shown that the utilization of capillary zone electrophoresis is useful for the analysis of VLPs because of its excellent resolution and separation quality (Hahne et al. 2014). Of course, mass spectrometry can be used to detect the desired modification of the VLP surface. The trace of the target protein during the different purification steps for the calculation of the final yield or overall loss is still a challenge. The separate acquisition of the concentration of the target protein during each purification step is usually not possible.

Ideally, a purification process should apply to all viruses or VLPs, but this is challenging given the unique properties of the particle. It more likely is possible to choose specific model particles, establish purification protocols and use these protocols with slight variations for the purification of similar particles.

Conclusion

There are several expression systems for producing VLPs and the production of VLPs in the silkworm *Bombyx mori* showed higher yield, except for special proteins which require mammalian cells for the production. Though, the purification of the silkworm hemolymph is still a major challenge, there is neither a general purification procedure nor a satisfying protocol yet for obtaining particular VLPs with a high yield from the hemolymph. Moreover, there aren’t so many studies to improve the purification of

VLPs from all expression systems. As we showed, mainly sucrose gradient ultracentrifugation is still used in the purification of VLPs, but this method has often a high loss and insufficient purity. Chromatography methods as addition or standalone show a promising outcome in purity and yield, but the recovery is with 16 – 60 % still too low. For the future we expect an increasing exploration of chromatography methods in combination with several kind of pre-treatments to remove so much host proteins as possible before the chromatography steps.

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Supplementary material

Supplementary Table S1 Some purifications from proteins from the silkworm or purifications from viruses and virus-like particles (VLPs) from other expression systems

Supplementary Fig. S1 The heparin affinity purification

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Table 1 Purifications of viruses or virus-like particles (VLPs) from the haemolymph of the silkworm.

Virus	Envelope	DNA/ RNA	Expression system	Compartment	Purification method	Yield (Purification)	Reference
Foot-and-mouth disease virus	none	(+)ssRNA	Eri silkworm (*Samia cynthiaricini*)	Haemolymph	Fetuin-agarose affinity chromatography and SEC	2.12 mg/ml	(Kumar et al. 2016)
Rous sarcoma virus-gag VLP	yes	(+)ssRNA	*Bombyx mori*	Haemolymph	Sucrose gradient centrifugation	384 mg/10 silkworms	(Deo et al. 2011)
Rotavirus VP2, VP6 and VP7 proteins (VLP) + 3 fluorescence proteins	none	dsRNA	*Bombyx mori*	Haemolymph	CsCl gradient centrifugation	12.7 µg/silkworm	(Yao et al. 2012)
Rotavirus VP2 and VP6 proteins (VLP)	none	dsRNA	*Spodoptera frugiperda*	Haemolymph	Sucrose gradient centrifugation	0.4 µg/silkworm	(Molinari et al. 2008)

Legend for figure

Fig. 1 Schematic view of enveloped and non-enveloped virus-like particles (VLPs). The enveloped VLP acquires its lipid bilayer from the host cell. One frequent form of the capsid from a non-enveloped VLP is the icosahedron, as shown. It is possible to modify the VLPs using coexpression with a different kind of (surface) protein. Some proteins are automatically incorporated into the membrane or capsid; others need to be fused with them.

Figure 1 was made with ChemOffice 2016, Microsoft Word 2016 and Windows Photo.

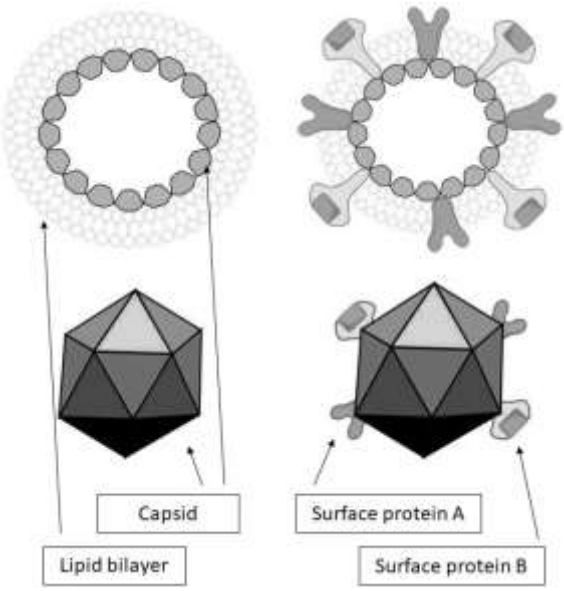

Fig. 1

Figure 1 was made with ChemOffice 2016, Microsoft Word 2016 and Windows Photo.

Supplementary file

Minireview

Biotechnology Letters

Section: Bioprocessing and Bioengineering

Purification of virus-like particles expressed in the silkworm *Bombyx mori*

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Supplementary Table S1. Example purifications from proteins from the silkworm or purifications from viruses and VLPs from other expression systems

Protein	Expression system	Compartment silkworm	Purification method	Yield	Reference
Interleukin 21	Silkworm larvae/pupae	Haemolymph / Pupae	CIEX	50 µg/ml	(Muneta et al. 2004)
RGNNV capsid protein (VLP)	*S. cerevisiae* Y2805	----------	Heparin affinity chromatography	1.1 mg from 125 ml culture	(Kim et al. 2016)
Mumps and Measles Virus	Vero cell	----------	AIEX and hydrophobic monolith chromatography	Method depending	(Sviben et al. 2017)
PBMHP-12 and BmJHBP (Haemolymph proteins)	Silkworm	Haemolymph	SEC and AIEX	0.6 mg /ml	(Pietrzyk et al. 2011)
Endo-β-N-Acetylglucosaminidase H	Silkworm / Bme21 cells	Haemolymph	His-TAG (Nickel) Affinity chromatography	3 µg/ml /----	(Mitsudome et al. 2014)
N-tagged and C-tagged rhb4GalT1	Silkworm	Haemolymph	Nickel and Strep-TAG affinity chromatography	124 µg/ 10 ml and 239 µg /10 ml	(Morokuma et al. 2017)
Hemagglutinin (H5N1)	Silkworm	Haemolymph	Fetuin-agarose Affinity chromatography and SEC	500 µg / 10 Larvae	(Dong et al. 2013)
N-terminal and C-terminal tagged forms of rhIL-2	Silkworm, Silkworm cell line	Haemolymph, Cell supernatant	His-TAG and Strep-TAG Affinity chromatography	30 and 163 µg /ml haemolymph	(Hino et al. 2016)
HIV-1 gag VLP	CHO	----------	AIEX monolith	~20 – 49 % method depending	(Steppert et al. 2016)
Hepatitis B Surface Antigen VLP	*Pichia pastoris*	----------	AIEX, isopycnic density ultracentrifugation, SEC	50 mg HBsAg/ 1 l = Recovery 3 %	(Gurramkonda et al. 2013)

Hepatitis B VLP	*E. coli*	----------	Negative Chromatography with Poly(oligo(ethylene glycol) methacrylate) (POEGMA)	~ 66 %	(Lee et al. 2015)
Fused capsid protein from enterovirus 71	*E. coli*	----------	2x AIEX	16.3 %	(Xue et al. 2017)
Hepatitis core particle	*Pichia pastoris*	----------	AIEX	3 mg/ 1 g wet cells	(Freivalds et al. 2011)
Rabbit hemorrhagic disease virus capsid protein VP60 (VLP)	Silkworm pupae	Pupae	Sucrose gradient centrifugation	10^7 HA units/pupa	(Zheng et al. 2016)
Japanese encephalitis virus E protein (VLP)	Silkworm pupae	Pupae	Sucrose gradient centrifugation	316 µg/pupae and 528 µg/pupae	(Matsuda et al. 2017)
3 H7 and 2 H5 influenza haemagglutinin VLPs	Silkworm pupae	Pupae	Sucrose gradient centrifugation	Estimated 408,58 µg/pupae	(Nerome et al. 2017)
E nterovirus 71 VLP	High Five™ cells	----------	TFF, HAC, TFF, SEC, TFF	77,456 µg VLPs/L (recovery 36 %, purity 83 %)	(Lin et al. 2015)
Own c-type lysozym	Eri silworm *(Samia cynthiaricini*)	Haemolymph	Heat treatment, CIEX, RP-HPLC, SEC	----------	(Fujimoto et al. 2001)
H5 Influenza haemagglutinin VLPs	Silkworm pupae	Pupae	Sucrose gradient centrifugation	2,000 µg/pupae	(Nerome et al. 2015)

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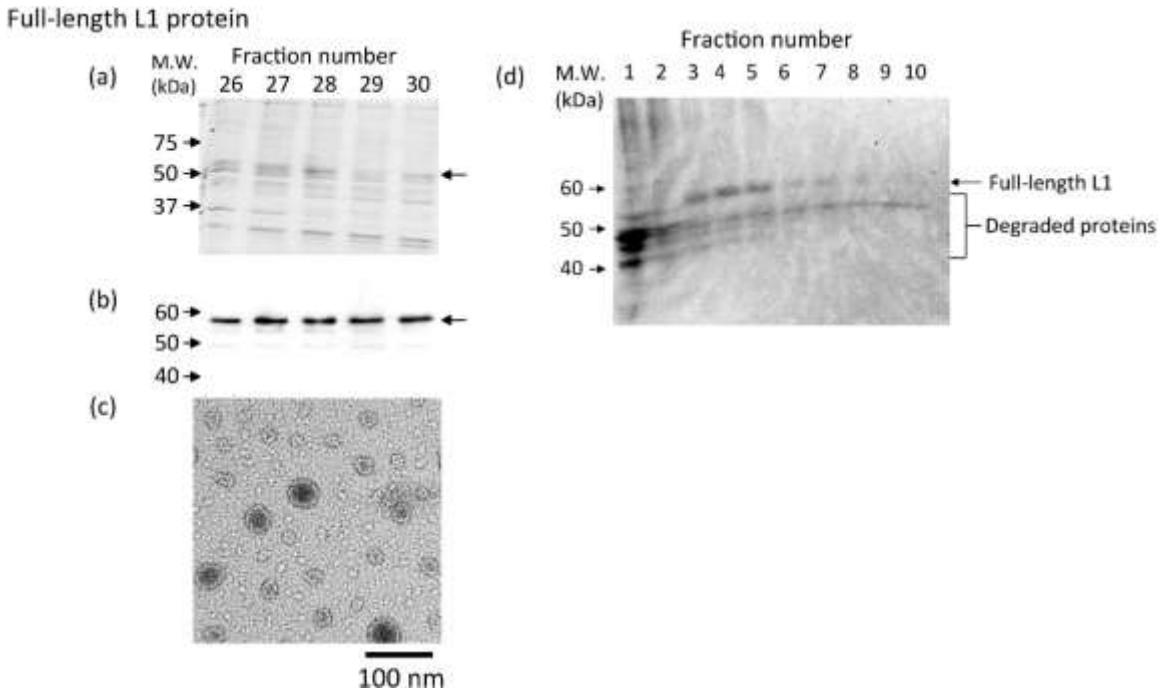

Supplementary Fig. S1 The heparin affinity purification of human papillomavirus L1 protein still shows many host proteins in SDS-PAGE stained with CBB (A) and Western blot (B), which also, together with electron microscopy (C), show stable L1 VLPs. Compared to this result, it is visible from the Western blot of the sucrose gradient that the degradation is stronger (D). The pictures are all modified from Palaniyandi M. et al. (2012) under license with BioMed Central Ltd. (https://springerplus.springeropen.com/articles/10.1186/2193-1801-1-29).

Supplementary Fig. 1 was made with Microsoft Power point 2016 and freeware pdf24.

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