Video Article

Expression and Purification of Virus-like Particles for Vaccination

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Abstract

Virus-like particles (VLPs) and subviral particles (SVPs) are an alternative approach to viral vaccine design that offers the advantages of increased biosafety and stability over use of live pathogens. Non-infectious and self-assembling, VLPs are used to present structural proteins as immunogens, bypassing the need for live pathogens or recombinant viral vectors for antigen delivery. In this article, we demonstrate the different stages of VLP design and development for future applications in preclinical animal testing. The procedure includes the following stages: selection of antigen, expression of antigen in cell line of choice, purification of VLPs/SVPs, and quantification for antigen dosing. We demonstrate use of both mammalian and insect cell lines for expression of our antigens and demonstrate how methodologies differ in yield. The methodology presented may apply to a variety of pathogens and can be achieved by substituting the antigens with immunogenic structural proteins of the user's microorganism of interest. VLPs and SVPs assist with antigen characterization and selection of the best vaccine candidates.

Video Link

The video component of this article can be found at https://www.jove.com/video/54041/

Introduction

Virus-like particles (VLPs) are an approved technology for human vaccination. In fact, some of the more contemporarily licensed vaccines, including the human papillomavirus (HPV) and hepatitis B (HepB) vaccines employ this approach. VLPs are formed from structural proteins capable of self-assembly. The assembled particles mimic viral morphologies, but cannot infect or replicate because they lack viral genomes. VLPs can be expressed and purified from a number of prokaryotic and eukaryotic systems. A review of the literature revealed that different expression systems are employed at the following rates: bacteria - 28%, yeast - 20%, plant - 9%, insect - 28%, and mammalian - 15%[1]. Of note, HPV vaccines based on L1 capsid protein are produced in yeast (Gardasil) or in an insect cell system (Cervarix)[2]. HepB vaccines, Recombivax and Engerix-B, are also produced in yeast, and are composed of HepB surface antigen[3,4].

We use mammalian and insect cell expression systems to produce VLPs requiring co-expression of multiple structural proteins for assembly. Our work focuses on designing, producing, and purifying VLP-based vaccines against human pathogens: influenza virus, respiratory syncytial virus (RSV), dengue virus (DENV), and chikungunya virus (CHIKV). Our methods are flexible enough to allow for co-expression of the multiple structural proteins from multiple expression plasmids, or a single expression plasmid (**Figure 1**). Previously, we produced and purified H5N1 VLPs assembled from the co-expression of plasmids encoding influenza hemagglutinin (HA), neuraminidase (NA), and matrix (M1) in human embryonic kidney 293T cells[5,6]. The genes were codon-optimized for expression in mammalian cells and cloned into pTR600, a eukaryotic expression vector containing the cytomegalovirus immediate-early promoter plus intron A for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal for termination of transcription[7]. A similar approach using the three-plasmid co-expression of HA, NA (**Figure 1A**) and an alternative viral matrix protein, HIV Gag p55, was used for generation of human seasonal influenza subtype H3N2 VLPs in this study and has been shown to generate VLPs of similar size as influenza particles[8]. Although influenza vaccines predominantly elicit anti-HA antibodies, the addition of influenza neuraminidase mediates sialidase activity to enable VLP budding from transfected cells[9] and also present additional immunogenic targets. To produce RSV VLPs, we also selected the unrelated core of HIV Gag to design prototypical vaccines that present exclusively RSV surface glycoproteins to further demonstrate flexibility of VLP formation using HIV Gag, as previously described and characterized by electron microscopy[6,10]. Others have previously shown that VLPs presenting RSV glycoproteins can be assembled using various viral components from Newcastle Disease virus (NDV)[11], and influenza matrix[12]. Full-length surface glycoprotein sequences were utilized in this study to retain native conformations that may be necessary for functional receptor binding and antibody recognition assay through enzyme-linked immunosorbent assay (ELISA).

Our examples for use of single plasmid expression systems to generate particles are DENV and CHIKV. In the case of DENV, we can produce subviral particles (SVPs) with no capsid in 293T cells from a single plasmid containing a prM/E structural gene expression cassette[13]. The term SVP is used to denote the lack of a core or capsid protein in the assembly of viral structural proteins. CHIK VLPs can also be produced using a single plasmid containing a CHIKV structural gene cassette, encoding capsid and envelope proteins, or in insect cells by infecting with a recombinant baculovirus encoding the same structural gene cassette optimized for insect cell expression (**Figure 1B-C**).

The end result of the expression approaches discussed above is the release of VLPs into cell culture medium that can then be purified via ultracentrifugation through a 20% glycerol cushion. In this report, we present methods to express and purify these VLPs from mammalian and insect cell systems.

Protocol

1. Mammalian Expression System for Generation of Influenza H3N2 VLP

1. Subclone viral structural glycoproteins hemagglutinin (HA), neuraminidase (NA), and human immunodeficiency virus (HIV) Gag p55 into eukaryotic expression vectors, such as previously described pTR600.[7]
2. Amplify DNA in chemically competent Escherichia coli (*e.g.*, DH5α) and isolate transfection-grade plasmid using a plasmid purification kit as per manufacturer's instructions. Amount of DNA is dependent on yield and user's needs.
3. Maintain mammalian cell line, 293T, in growth media containing 10% fetal-bovine serum (FBS) at 37 °C with 5% CO_2 in a humidified incubator.
4. Grow cells such that a T-150 tissue culture flask contains 45-75 x 10^6 cells per flask such that flask obtains complete cell adherence and 85-95% cell confluency by the following day. Inspect cells under the microscope for desired confluency.
   Note: High cell density is typically recommended to yield optimal protein expression with minimal cytotoxicity when using commercial transfection reagents however over-confluency may result in accumulation of cellular waste by-products and reduce cell viability.
5. On the day of transfection, prepare liposome and DNA mixture as per the manufacturer's recommendations and transfect DNA cells with a DNA composition of 1:1:2 of HA:NA:Gag with a total DNA quantity of 40 µg per T-150 flask. Dilute DNA and liposome solutions in serum-free transfection media without antibiotics such that each flask contains a total volume of 20-30 ml.
   Note: Ratio of gene constructs may require user optimization. Although not demonstrated here, a similar transfection procedure has been performed with respiratory syncytial virus (RSV) F and HIV Gag at a 1:1 ratio. For DENV and CHIKV single expression plasmids, a total of 20 µg of DNA per T-150 flask are used for optimal expression. DNA:transfection reagent ratios are user optimized. Use recommended transfection medium based on transfection method, *i.e.*, polycationic lipid transfections recommend reduction or absence of fetal bovine serum thus media may be supplemented with growth hormones and trace elements to support growth in the absence of serum.
6. Return flasks to 37 °C with 5% CO_2 incubator. For a volume of 200 ml, use 9 T-150 flasks. The cells are maintained in transfection culture medium until the day of VLP harvest.
7. Harvest culture supernatant from cells after 72-96 hr post-transfection depending on antigen, or when cell viability has decreased to 70-80%, as estimated by microscopic inspection. Transfer supernatant into 50 ml conical tubes and spin the cells down at 500 × g for 5 min at 4 °C to pellet cellular debris.
   Note: Replacement of 3 day supernatant with fresh pre-warmed, serum-free transfection media to adherent cells will yield a second lot of VLPs with similar or slightly reduced yield and should be optimized by user.
8. Pool supernatant and filter through a 0.22 µm pore membrane before sedimentation via ultracentrifugation.
9. Test the hemagglutination activity of the HA-expressing VLPs by standard hemagglutination assay[14] using 0.8% turkey or mammalian red blood cells or proceed with antigen-specific ELISA. See **Figure 2** for representative results.

2. CHIK VLP Expression Using Baculovirus/Insect Cell System

1. Generate recombinant baculovirus expressing chikungunya virus capsid and envelope proteins (C-E3-E2-6K-E1) from S-27 strain using a commercial baculovirus expression system.
2. Culture *Spodoptera frugiperda* Sf9 cells in serum-free Sf9 growth medium with 100 U/ml penicillin and 100 µg/ml streptomycin at 28 °C. Use 3-5 x 10^5 c/ml to initiate spinner flask cultures for suspension cell growth. Passage routinely when they reach cell densities of 2-4 x 10^6 c/ml (every 3-4 days).
3. Culture Sf9 cells in suspension in spinner flasks with stirring at 130 rpm on a multipoint stirrer plate system. For proper aeration, maintain culture volumes at no more than half the volume of the spinner flask.
4. For expression of CHIK VLPs, infect Sf9 cells at a density of 2 x 10^6 cells/ml with recombinant baculovirus at a multiplicity of infection of 1 and return to 28 °C incubator. Typically, infect one or two spinner flasks, containing 250 ml Sf9 cells.
   Note: While we do not use this method, Sf9 cells may be cultured in shaker flasks at 28 °C using a shaker platform.
5. Harvest cultures after 72-96 hr post-infection, or when cell viability has decreased to 70-80% as determined by Trypan Blue Exclusion according to manufacturer's protocol.
   Note: The cells continue to proliferate while infected and there is a ~80% viability in Sf9 cultures infected with recombinant CHIK VLP baculovirus at 3 days post-infection (dpi). Baculovirus-infected cells are larger in appearance. Morphology may also change from round to oblong. In late-stages of baculovirus infections, the cells began to lyse.
6. Transfer cultures directly from suspension culture into 50 ml conical tubes and spin the cells down at 500 x g for 5 min at 4 °C.
7. Collect supernatants and filter through a 0.22 µm pore membrane before sedimentation via ultracentrifugation.

3. Sedimentation/Purification of VLP/SVPs

1. Sterilize 25 mm x 89 mm open-top ultracentrifuge tubes with 70% ethanol in biosafety hood. Ensure the ethanol has dried off before use.
2. Load up to 32 ml of supernatant into a clean tube. A minimal volume of 25 ml is recommended to prevent collapse and damage of inadequately filled ultracentrifuge tubes.
3. Carefully underlay supernatants with sterile 3 ml 20% glycerol in PBS (v/v). Make sure tubes are balanced.
4. Spin at 135,000 x g for 4 hr at 4 °C.
5. Aspirate supernatant, ensuring the pellet does not dislodge from tube.
6. Resuspend sedimented VLPs at bottom of the tubes with sterile PBS by vigorously pipetting up and down. The amount of PBS needed to resuspend VLPs depends on VLP total protein yield and downstream applications. Typically, resuspend each VLP pellet in at least 100 µl.

7. Store samples 4 °C for short-term storage and -80 °C storage for long-term storage.

4. Determining Protein Yields and Specific Antigen Yields

1. Determine the total protein content using a commercial protein quantification method, such as BCA assay as per manufacturer's protocol.
2. To assess specific antigen content, perform direct enzyme-linked immunosorbent assay (ELISA) by coating serial dilutions of standard antigen and 2-5 µg of total protein sample on to an ELISA 96-well flat bottom plate.
    1. Follow with antigen-specific antibodies to probe the presence of the antigens on the plate and use conventional ELISA development substrates to produce detectable absorbance for microplate reader. See **Figure 2** for representative results of HA and ELISA assay for a sample HA-expressing VLP; many combinations of HA and NA are possibly but yields may differ upon HA-NA compatibility[15].
    Note: Antigen-specific antibodies have variable affinities and it is recommended that endpoint-dilution titration of antibodies be determined before performing VLP quantification. Commercial antibodies will have suggested concentration or dilution ranges for use in ELISA, as well as suggested protocols.

Representative Results

VLP yields were variable by viral antigen construct design. In this protocol, we have demonstrated use of insect and mammalian cells for production of SVP or VLPs in supernatant and purification by ultracentrifugation. Four subtypes of DENV prM/E structural gene expression cassettes were used to construct the versions of DENV SVPs (demarcated as 1-4) in **Table 1** and demonstrate a range between 1.1-2.6 mg of total protein in 0.6 ml volumes. For VLPs that require a three gene constructs, we found the optimized DNA transfection ratio of 1:1:2 for HA:NA:Gag respectively to synthesize influenza VLPs; in contrast, a single plasmid expressing multiple chikungunya viral proteins was adequate for the generation of recombinant baculovirus and mammalian CHIK VLP. Additionally, a dual-plasmid transfection procedure is also possible with mammalian cells, as such with RSV F and Gag transfected at a 1:1 ratio, and yields approximately 0.01 mg/ml of total cell culture supernatant. As shown in the **Table 1**, CHIK SVP yield from Sf9 cells can range between 0.008-0.016 mg total protein/ml of supernatant volume, while production through mammalian 293T yields a 10-fold reduction of protein. Between the two versions of H3N2 VLPs using different wild-type, full-length HA plasmids, the H3N2 VLP Sample 2 had a reduced yield of both total protein and specific HA content. Other sample VLP HA quantities are shown in **Figures 2** and **3** and illustrate how HA and ELISA are used to estimate surface content on the VLPs. As with a conventional direct ELISA method, a standard curve is generated using known concentrations of a recombinant HA to compare ELISA reactivity to sample H3 VLPs loaded onto an ELISA plate. ELISA or similar immunoassay are recommended for quantification of VLPs that have readily available monoclonal antibodies with known crossreactivity to a well-conserved epitopes, but may not be available for all antigens. RSV F is well-characterized, therefore an ELISA using monoclonal anti-RSV F antibody is also applicable for quantification of surface F on pelleted RSV F VLPs. In addition to antigen functionality and antigen recognition by specific antibodies, VLP preparations can be structurally assessed by electron microscopy. **Figure 4** is a representative image showing that HA (Gag-core) influenza VLPs are successfully expressed, assembled, and purified as VLPs.

Description	Culture Vol (ml)	Harvest[a]	Total Volume	Total Protein Yield (mg)	Yield (mg)/Vol (ml)	Specific antigen content
DENV1 SVP	293T186	4 d	0.6 ml	1.115	0.006	n.d.
DENV2 SVP	293T/186	4 d	0.6 ml	2.5	0.0134	n.d.
DENV3 SVP	293T/186	3 d	0.6 ml	1.536	0.0083	n.d.
DENV4 SVP	293T/186	4 d	0.6 ml	2.613	0.014	n.d.
CHIK VLP	Sf9/186	3 dpi	0.6 ml	1.503	0.0081	n.d.
CHIK VLP	Sf9/500	4 dpi	2.0 ml	8.276	0.0166	n.d.
CHIK VLP	293T/186	3 d	0.6 ml	0.296	0.0016	n.d.
H3N2 VLP 1	293T/216	3 d	0.6 ml	1.25	0.0058	1.9 µg HA/µl
H3N2 VLP 2	293T/216	3 d	0.6 ml	0.956	0.0044	0.96 µg HA/µl
H3N2 VLP 3	293T/216	3 d	0.6 ml	1.6	0.0074	1.2 µg HA/µl
H3N2 VLP 4	293T/216	3 d	0.6 ml	1.54	0.0071	0.98 µg HA/µl
RSV F VLP	293T/216	3 d	0.6 ml	2.3	0.0106	0.15 µg RSV F/µl

[a] d = days post-transfection, dpi = days post-infection

Table 1: Subviral and virus-like particle yields by construct. Representative data of VLP volumes, total protein yield (determined by conventional BCA assay) or specific antigen content (determined by ELISA) is shown among a variety of SVP/VLP constructs. Yields are variable due to difference in SVP/VLP assembly and cell type, and maximum yields may require user optimization. The various versions of either DENV1-4 differ based on serotype, while the CHIK VLPs use the same sequence but preparations differ in either volume or cell culture type. The different versions of H3N2 VLPs (1-4) express four unique HA sequences that share a HA-specific ELISA reactive epitope and were co-expressed with the same NA and Gag core. The RSV F VLP, which is generated from a dual-plasmid transfection of RSV F and Gag, was synthesized and RSV F content was approximated using RSV F specific ELISA assay.

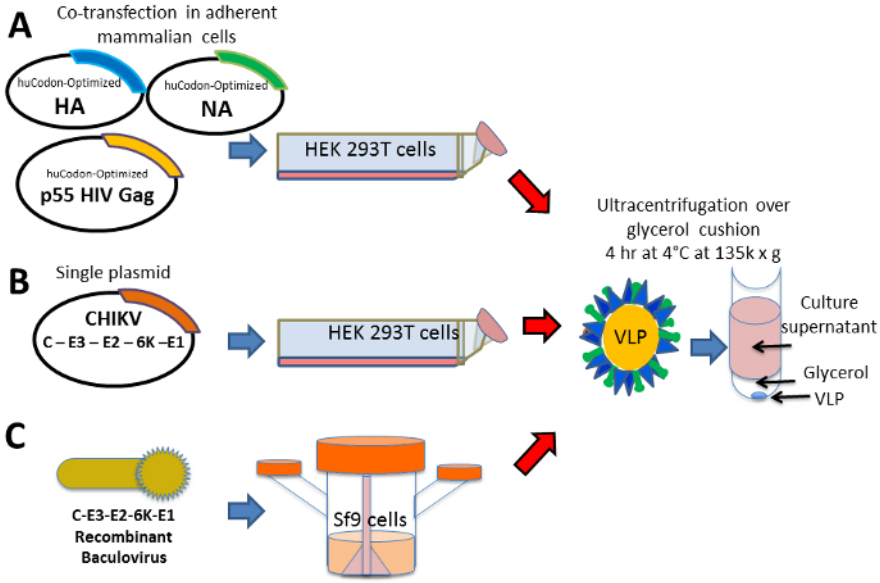
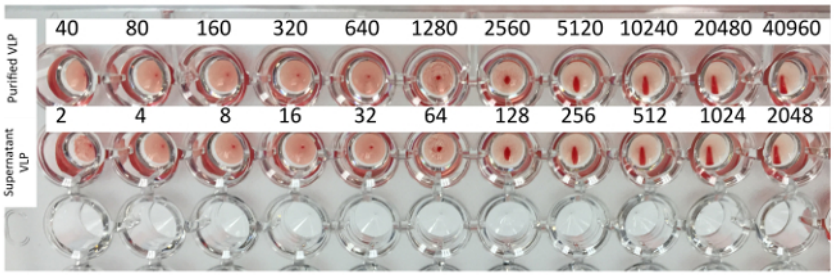
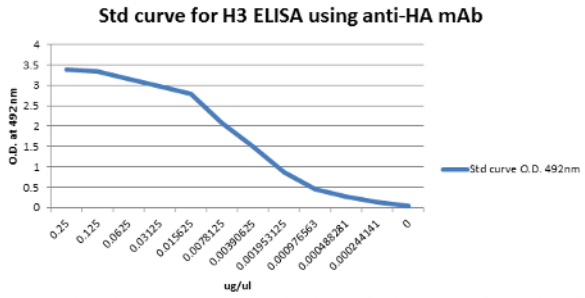


Figure 1: Approach - VLP design, synthesis, and purification. Schematic representation of plasmid gene designs for expression of viral structural proteins that will form VLPs: (**A**) co-expression of multiple structural proteins by co-transfection of three plasmids in 293T cells, (**B**) expression of structural proteins from a single gene cassette encoded in a single plasmid in 293T cells, and (**C**) recombinant baculovirus encoding CHIK structural proteins is used to infect Sf9 cells cultured in spinner flasks to promote high density cell growth in suspension. The VLPs are harvested from the cell culture media, clarified of cell debris, and separated from non-particles via sedimentation by ultracentrifugation on a 20% glycerol cushion. Please click here to view a larger version of this figure.



	HA titer (0.8% turkey RBCS, ½ h)	BCA concentration	HA ELISA titer
Purified VLP	2560 (1280)	1.9ug/ul	0.5 ug HA/ul
Unpurified, supernatant VLP	64	N/D	N/D

Figure 2: Example Hemagglutination assay of H3 VLPs. A conventional hemagglutination assay was performed on a representative sample of H3N2 VLP and its corresponding clarified supernatant; H3N2 HA assay can be performed using 0.8% turkey red blood cells or guinea pig red blood cells (not shown). Total protein content is estimated using the standard BCA assay on the purified VLP and was not performed or recommended for clarified supernatant. ELISA was performed on the purified VLP and HA content was estimated using a recombinant HA standard of known concentration. Please click here to view a larger version of this figure.



µg/µl	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	BLANK

	1	2	3	4	5	6	7	8	9	10	11	12	
A	3.361	3.334	3.154	2.969	2.782	2.081	1.506	0.862	0.454	0.262	0.153	0.039	492
B	3.393	3.357	3.164	2.979	2.796	2.087	1.519	0.868	0.461	0.264	0.1	0.04	492
C	2.261	2.528	1.978	2.028	1.85	1.923	2.488	1.936	2.418	0.041	0.042	0.041	492
D	2.242	2.526	2.029	1.979	1.753	1.909	2.636	1.831	2.657	0.034	0.036	0.037	492

Wells A&B	Std curve O.D. 492nm	3.377	3.3455	3.159	2.974	2.789	2.084	1.5125	0.865	0.4575	0.263	0.1265	0.0395
	Concentration (µg/µl)	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	BLANK
	H3 VLPs examples:	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9			
Wells C&D	average O.D. 492	2.2515	2.527	2.0035	2.0035	1.8015	1.916	2.562	1.8835	2.5375			
	concentration (µg/µl)	0.0117	0.015	0.006	0.006	0.007	0.0072	0.015	0.0072	0.015			
	x Dilution factor (x50)	**0.585**	**0.75**	**0.3**	**0.3**	**0.35**	**0.36**	**0.75**	**0.36**	**0.75**	FINAL CONCENTRATION (µg/µl)		

Figure 3: Example H3 ELISA for quantification of H3 content in H3 VLPs. An ELISA plate was coated with serial dilutions of a recombinant HA standard of known concentration and diluted H3N2 VLP samples generated from different HA sequences (Samples 1-9). Correlating the concentration (µg/µl) of the standard to the optical density values obtained at 492 n.m. (O.D.), concentrations are obtained by deducing the x-value that intersects the linear portion of the standard curve. Dilutions of recombinant HA standard and unknown samples were loaded in duplicate in wells A& B or C & D, respectively. The final concentration of the VLP sample is determined by multiplying the concentration by the dilution factor of the VLP sample. Please click here to view a larger version of this figure.

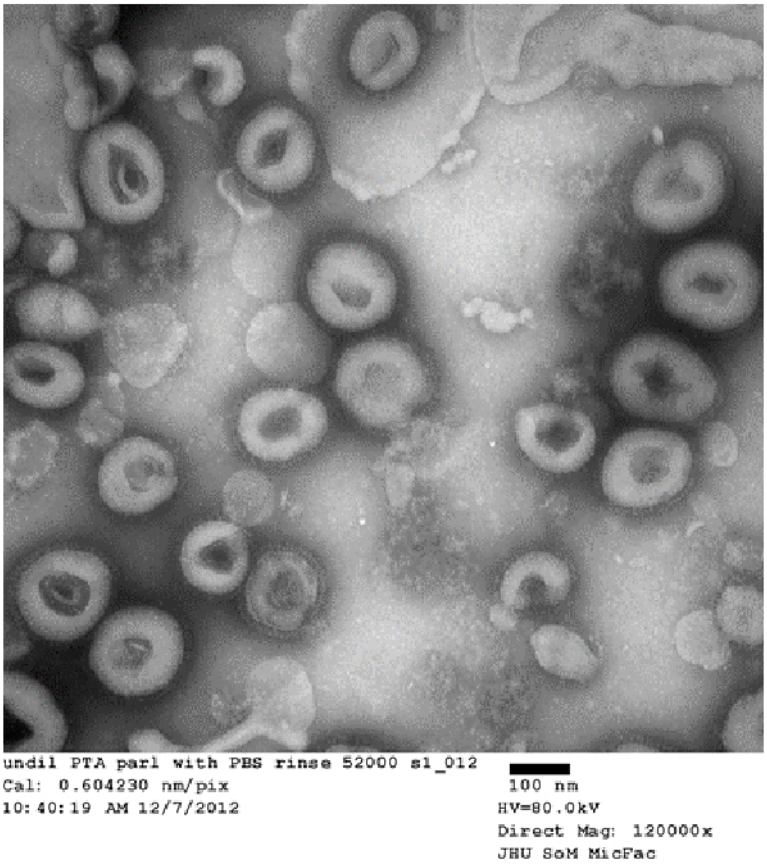


Figure 4: Electron microscopy of HA-VLPs using Gag-core. Representative electron microscopy image of purified influenza HA VLPs. VLPs assemble into spherical particles coated with HA. Please click here to view a larger version of this figure.

Discussion

We have used the techniques described above to successfully express and purify SVPs and VLPs composed of multiple structural proteins for various pathogens. In general, we use mammalian expression systems to generate our VLPs. However, in our hands, mammalian-cell derived CHIK VLP yields were low. CHIK VLP yield was more robust when using A recombinant baculovirus-insect cell system. In general, baculovirus-insect cell systems yield higher amounts of recombinant proteins, which may result from the higher cell densities that can be cultured in spinner flasks versus tissue culture flasks, and also due to high level of expression of foreign genes while under the control of the baculovirus polyhedrin promoter.[16] However, a disadvantage of using baculovirus is the very presence of baculovirus in VLP preparations, which has adjuvant activity, and skew immunological results in vaccine studies[17] We also found variability in the yield of influenza VLPs depending on the HA and NA combinations used; H3N2 VLP2 had relatively reduced yield compared to H3N2 VLP1, potentially due to less efficient VLP assembly or NA-compatibility[15] and remains to be further investigated. Optimization of DNA constructs and transfection ratios remain critical steps for ensuring optimal VLP yields. We opted to use a mammalian vector pTR600 consistently among our mammalian-based VLPs because expression is generally efficient and subcloning is convenient with multiple restriction enzymes. Ratios of plasmids may be adjusted for user's requirements to improve VLP yields. However, as discussed, current limitations of the mammalian VLP production remain with cost of transfection and the cell growth capacity in current tissue culture flasks. Although not performed in this protocol, we have observed additional successful production of VLPs in transfected cells when media is replenished with fresh growth media (*e.g.*, OptiMEM) after the first 72 hr harvest and subsequent collection of VLPs after an additional 72 hr, although yields may be slightly reduced as compared to the first collection of VLPs.

We demonstrate a procedure for expressing viral structural proteins in either insect or mammalian cell cultures to release VLPs in the supernatant for semi-purification by ultracentrifugation. The SVP/VLPs are generally stable for use in antigen immunoassays and vaccination studies, and pose fewer safety threats as compared to live viral particles, particularly for select agents or high-pathogen avian influenza (HPAI) strains[8,18], which require Biosafety Level 3 (BSL-3) laboratory conditions[19]. VLP production is relatively simple and often yields particles that may be recognized by antibodies elicited against native viral structural proteins, not exclusively denatured linear epitopes. VLPs serve as a useful platform for eliciting specific immune responses to the antigen and may be a promising candidate in vaccine development.

This protocol demonstrates two approaches for transfecting viral genes in the mammalian system. In this illustration, two influenza genes, HA and NA, are co-transfected with the HIV Gag to generate a VLP that has a Gag internal core, with HA and NA assembled on the surface. The substitution of Gag instead of influenza Matrix 1 (M1) demonstrates flexibility of Gag-based VLPs with unrelated viral glycoproteins, such as RSV F and potentially others. Alternatively, the synthesis of CHIKV utilizes a structural gene cassette, which expresses the necessary components for

SVP self-assembly, with CHIKV E proteins presented on the surface of a center composed of C protein. These two approaches demonstrate the flexibility of VLP synthesis, which requires minimal structural viral proteins to assemble from transfected cells.

Finally, while we presented general protocols and representative results for expression and purification of H3N2, RSV, DENV, and CHIKV, these procedures are easily amenable for generation of VLPs using other viral proteins. These procedures have been successfully used by our group in the generation of other influenza virus subtypes including H1, H5, and H7 viruses. Moreover, the single plasmid, prM/E design used for generation of DENV SVPs can be adapted for generation of SVPs for other members of the Flavivirus family that include West Nile and Japanese encephalitis viruses. Similarly, methods of CHIK VLP production can be used for other members of the Togaviridae family, including the Venezuelan, East, and West equine encephalitis viruses.

Disclosures

Authors have nothing to disclose.

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