Published in final edited form as:

Curr Protoc Microbiol. ; 30: Unit–18.2.. doi:10.1002/9780471729259.mc1802s30.

Newcastle Disease Virus-Like Particles: Preparation, Purification, Quantification, and Incorporation of Foreign Glycoproteins

Lori W. McGinnes¹ and Trudy G. Morrison^{1,2,*}

¹Department of Microbiology and Physiological Systems, University of Massachusetts Medical School 55 Lake Avenue North, Worcester, MA 01655

²Program in Immunology and Virology, University of Massachusetts Medical School 55 Lake Avenue North, Worcester, MA 01655

Abstract

Virus-like particles (VLPs) are large particles, the size of viruses, composed of repeating structures that mimic those of infectious virus. Since their structures are similar to that of viruses, they have been used to study the mechanisms of virus assembly. They are also in development for delivery of molecules to cells and they are used in studies of the immunogenicity of particle-associated antigens. However, they have been most widely used for development of vaccines and vaccine candidates. VLPs can form upon the expression of the structural proteins of many different viruses. This chapter describes the generation and purification of VLPs formed with the structural proteins, M, NP, F and HN proteins, of Newcastle disease virus (NDV). Newcastle disease virus-like particles (ND VLPs) have also been developed as a platform for assembly into VLPs of glycoproteins from other viruses. This chapter describes the methods for this use of ND VLPs.

Keywords

Virus-like particles; Newcastle disease virus; paramyxovirus; chimera glycoproteins

Introduction

Virus-like particles, VLPs, are formed upon the expression of some or all of the structural proteins of a virus (Jennings and Bachmann 2008). Expression of structural proteins of both enveloped and nonenveloped viruses can form VLPs. VLP structures, as well as mechanisms of assembly and release from cells, mimic that of cognate infectious virus. However, VLPs do not contain a viral genome.

VLPs have many potential uses (Ludwig and Wagner 2007). They are powerful tools for the study of mechanisms and requirements for the assembly of specific viruses (for example, (Schmitt et al. 2002; Takimoto et al. 1998; Takimoto et al. 2001)). They are potential means

*Telephone: 508 856-6592; Fax: 508 856-5920; trudy.morrison@umassmed.edu.

for delivery of molecules to cells (Kaczmarczyk et al.). They are useful in defining immune responses to specific antigens presented in a particulate form (for example, (Schmidt et al. 2012)). However, their widest use, thus far, has been as vaccines and vaccine candidates (reviewed in (Jennings and Bachmann 2008; Kang et al. 2009)). Indeed, two VLPs are now licensed for use as human vaccines, the hepatitis B virus and the papilloma virus vaccine (Harper et al. 2006). Furthermore, there are many preclinical studies and clinical trials of a wide variety of VLPs, which are potential vaccines for human use.

Expression of the structural proteins of many different viruses can result in formation of VLPs. Here we focus on VLP generation and purification of particles formed with the structural proteins of Newcastle disease virus, an avian paramyxovirus. The assembly and release of Newcastle disease virus-like particles (ND VLPs) is quite efficient (Pantua et al. 2006). As a result, quantitative amounts of these particles are relatively easy to generate even from transiently transfected cells (McGinnes et al. 2010). They can be purified using protocols adapted from virus purification protocols and the purified VLPs have minimal cell protein contamination. Basic Protocol 1 describes the generation of ND VLPs using transient transfection of an avian cell line. Methods for large-scale preparation of quantitative amounts of VLPs are described as well as methods for smaller scale preparations (Alternate Protocol 1) useful for more analytical analyses. Protocols for preparing radioactively labeled particles are included (see Support Protocol 1). Basic Protocol 2 and Alternate Protocol 2 describe purification protocols for both large and small-scale particle preparations. Basic Protocol 3 and Alternate Protocols 3 and 4 describe several different methods for quantification of the content of VLP preparations. Methods include protein staining, autoradiography, and western blots.

Not all virus systems will produce VLPs, while others produce VLPs at very low efficiency. Because of their efficiency of production, ND VLPs have been adapted for use as a platform for the assembly into particles of antigens from a wide variety of viruses including genetically complex viruses. For example, ND VLPs can assemble with the entire ectodomains of glycoproteins from respiratory syncytial virus (McGinnes et al. 2011; Murawski et al. 2010; Schmidt et al. 2012), influenza viruses, cytomegalovirus, and West Nile virus (Morrison, unpublished observations). Basic Protocol 4 describes the general methods for assembly of proteins from other viruses into ND VLPs.

Basic Protocol 1: Large Scale Generation of ND VLPs

Newcastle disease VLPs are assembled and released from cells expressing the viral M, NP, HN and F proteins (Pantua et al. 2006). This protocol describes a method for the large-scale generation of VLPs from cells expressing only these four proteins. This protocol is used when quantitative amounts of VLPs are required. Protein expression is accomplished using transient transfection of tissue culture cells with four plasmids containing the cDNA sequences encoding each of these viral proteins. VLPs, assembled on the plasma membranes of these cells, are released into the cell supernatant.

Materials

Incubator for tissue culture cells (maintained at 37.5°C with 5% CO₂ and humidified)

Biosafety hood (with airflow vented through a HEPA filter)

Tissue culture flasks—T150

Pipettes (sterile)

Micropipettes and tips (1-20 μ l, 1-200 μ l, 1-1000 μ l)

Tissue culture cells (ELL-0 are preferable but COS7, CHO, or 293T cell lines are acceptable. All are available at atcc.org)

Cell culture media such as DMEM with appropriate additions for the cells to be used OptiMEM (Life Technologies)

Fetal Bovine Serum (heat inactivated at 56°C for 30 minutes, see Appendix 2A)

Lipofectamine (Life Technologies) or similar transfection reagent

Plasmids containing cDNAs encoding NDV NP, M, F, and HN proteins (prepared using Qiagen Endo-free kit or the equivalent) (Use of the pCAGGS vector as the expression vector is highly recommended. This plasmid may be obtained from the Belgian Coordinated Collection of Microorganisms, BCCM/LMBP) (bccm.lmbp@dmbr.UGent.be)

Protocol Steps (Based on one T150 flask)

1. The day before transfection, seed T150 flasks so they will be 60-65% confluent the next morning.
2. Set up transfection mix. Per T150 flask, in a 15 ml tube mix 1.6 mls OptiMEM and 8 μ g each DNA (NDV NP, M, F and HN). In a separate tube mix 3.2 mls OptiMEM and 80 μ l lipofectamine. Incubate both tubes at room temperature for 15 minutes. Add the lipofectamine to the DNA and mix together. Incubate at room temperature for 45 minutes.

Note: Scale up for number of T150 flasks to be used. A good starting point is 24 T150 flasks for a VLP preparation.

3. Wash cells 2 times with 15 mls OptiMEM.
4. Add 5.6 mls OptiMEM to DNA/Lipofectamine transfection mix and add the mixture to cells growing in a T150 flask.
5. Incubate at 37° C for 5 hours.
6. Remove DNA/Lipofectamine in the cell supernatant and add 25 mls fresh cell culture media containing fetal calf serum. Incubate at 37° C.

Monitor the pH of the media from the beginning of transfection to the last VLP harvest, replacing media if it becomes too acid.

Alternate Protocol 1: Small Scale VLP Generation

This alternative protocol describes methods for generation of VLPs on a small scale. Small-scale generation of VLPs is used when quantitative amounts of VLPs are not required. For

example, this protocol is appropriate to determine the effects on VLP assembly of specific mutations in the expressed proteins or to determine time courses of VLP generation.

Materials

All materials from Basic Protocol 1 except T150 flasks

Plus:

6-well Tissue Culture Plates (or individual 35 mm plates)

Protocol Steps (based on one 35 mm plate)

1. The day before transfection, seed 6-well plates so they will be 60-65% the next morning.
2. Transfect one 35 mm plate (60-65% confluent) with DNAs of interest. Mix 0.5 μ g of each DNA (NP, M, F, HN) with 0.1 ml of OptiMEM. In a separate tube mix 6 μ l of Lipofectamine with 0.2 ml of OptiMEM. Incubate at room temperature for 15 minutes. Mix DNA and lipofectamine mixtures together and incubate at room temperature for 45 minutes. Wash cells twice with 2 ml OptiMEM and remove. Add 0.7 ml OptiMEM to the transfection mix and add to the cell monolayer. Incubate at 37°C for 5 hours. Remove DNA/lipofectamine and add 2 ml of complete cell culture media. Incubate at 37°C. VLPs are harvested at 72 hours.

Check cells at 40-48 hours. If the media is getting too acidic, remove 1 ml and keep at 4°C, add 1 ml fresh media and continue incubation to 72 hours. Combine all supernatants for VLP purification.

Support Protocol 1: Radioactive labeling of VLPs

In some instances it is useful to be able to visualize all components of a VLP made on a small-scale. This visualization may be accomplished by autoradiography of radioactively labeled VLP proteins separated on a polyacrylamide gel. This approach may be necessary if no antibody exists for the VLP protein in question or if the amount of a protein incorporated into the VLP relative to the core NP and M proteins is to be assessed. This protocol describes how protein components of the VLP may be radioactively labeled during their generation.

Materials

All materials required for Alternate Protocol 1

DMEM without methionine and cysteine (Life Technologies)

Dialysed fetal calf serum (Life Technologies)

³⁵S cysteine/methionine (Perkin/Elmer Express ³⁵S protein labeling mix or comparable)

- 1 Follow steps 1 and 2 in Alternate Protocol 1.

- 3 At 40 hours post transfection, wash the plate twice with DMEM without methionine and cysteine and containing dialyzed fetal calf serum (complete media without methionine and cysteine).
- 4 Add 0.5 ml of complete media minus methionine and cysteine.
- 5 Add ^{35}S methionine/cysteine (approximately 10-20 μCi).
- 6 Incubate cells for 8 hours at 37°C.
- 7 Remove media and replace with complete media containing methionine and cysteine.
- 8 Incubate for an additional 24 hours at 37°C.

Follow all safety protocols required for handling radioactive material.

Basic Protocol 2: Large Scale Purification of VLPs

This protocol describes the purification of a large-scale preparation of ND VLPs. The protocol is designed to purify the VLPs for use as immunogens and, therefore, is designed to minimize contamination of host cell molecules. The protocol involves multiple differential centrifugations. Cell supernatants containing released VLPs are cleared of cells and large-sized cell debris by low speed centrifugation. The VLPs in the cleared supernatants are then concentrated by pelleting in an ultracentrifuge. The resuspended VLPs are then purified on sequential sucrose gradients illustrated in Figure 1. The first gradient, sedimentation through 20% sucrose to a 65% sucrose pad, removes soluble material as well as non-lipid associated aggregates (illustrated in Figure 1, step 1). The second, flotation into a sucrose gradient centrifuged to equilibrium, removes additional non-lipid associated particulate material and minimizes non-VLP associated lipid material (illustrated in Figure 1, step 2). The third optional gradient, a more rigorous equilibrium gradient centrifugation, separates authentic VLPs from lighter density lipid vesicles as well as other non-VLP lipid associated material (illustrated in Figure 1, step 3).

Materials

Supernatants containing large scale amounts of VLPs (Basic Protocol 1)

Beckman GS-6R Centrifuge (or comparable)

Adaptors and 250 ml sterile/disposable conical bottles (or comparable)

Beckman Ultracentrifuge (or comparable)

Beckman Type 19 rotor and Type 19 bottles and Deldrin Cap Assemblies (or comparable)

Pipettes (5, 10, and 25 ml sterile)

Beckman SW28.1, SW41, SW50.1 rotors, buckets and tubes (30 ml, 17 ml, 11 ml, 5 ml tubes) or equivalent

Glass dounce homogenizer (10 ml) with a tight fitting pestle, pre-chilled on ice. 10%, 20%, 25%, 35%, 45%, 50%, 55%, 65%, 80% sucrose solutions in TNE Buffer (weight/volume)

2ml tubes stable at -80°C

-80°C freezer

Bio-safety hood vented through a HEPA filter

Protocol Steps (based on yields from 24-30 T150 flasks)

Clarify Cell Supernatants

1. Remove cell supernatants from transfected T150 flasks at 48, 72, 96 and 120 hours post transfection, replacing with fresh 25 ml of media at each collection.

Maximal release of VLPs begins at 48 hours and proceeds until at least 96 hours.

2. Place the harvested fluid directly into chilled 250 ml sterile centrifuge bottles.

Supernatants can be stored several days in the refrigerator.

3. Centrifuge the pooled supernatants in a Beckman GS-6R centrifuge, pre-cooled (4°C), using adapters, at 5,000 rpm for 15 minutes.

This spin will remove large sized cell debris and detached cells.

Concentrate VLPs (Pellet VLPs from harvested fluid)

4. Decant the supernatant into sterile, pre-cooled Beckman Type 19 bottles.

5. Add media to completely fill all bottles.

These bottles have a tendency to collapse if not completely filled.

6. Add the cap assembly to each bottle.

Do not autoclave Deldrin cap assemblies. Sterilize with ethanol and UV light.

7. Pellet VLPs for 8 hours (minimum) or overnight in Type 19 rotor in a Beckman ultracentrifuge at 16,000 rpm, 4°C .

8. Pour off supernatant. The supernatant is discarded.

The VLPs are in the pellet.

Purify and Concentrate VLPs

9. Resuspend all pellets in combined total of 22 ml of TNE buffer.

The pellet can be sticky. A dounce homogenizer is useful to resuspend all the particulate material. Homogenize with 10 strokes of the homogenizer.

10. Layer the supernatant (11 ml per gradient) over a discontinuous sucrose gradient formed with 2 ml of 65% sucrose in TNE buffer and 4 ml of 20% sucrose in TNE buffer. Make the gradients in a small (17 ml) (SW28.1) clear ultracentrifuge tube.

Prepare two gradients for material from 24-30 T150 flasks.

Be sure the gradients all have the same weight prior to centrifugation.

- 12** Centrifuge the gradients for 6 hours at 24,000 rpm, 4°C, in a Beckman SW28.1 rotor, small buckets.
- 13** The white “fluffy” layer between the 65-20% sucrose layers is the partially purified VLPs. Remove the top 13-14 ml of the gradient and discard. Collect the VLP layer into a sterile tube by pipetting the next 2-3 ml (containing the “fluffy” material) from gradient. Discard the tube with the remaining 1 ml or so of 65% sucrose.

- 14** Add 80% sucrose to bring VLPs, collected from 20%/65% interface (step 13) to approximately 60% with respect to sucrose.

For use as immunogens, VLPs are further purified by flotation centrifugation to equilibrium in a continuous sucrose gradient.

- 15** In a SW41 clear ultracentrifuge tube, layer VLPs on top of a pad (0.5 ml) of 80% sucrose in a SW41 centrifuge tube. Overlay with 3.5 ml of 50% and then 2 ml of 10% sucrose solutions in TNE buffer.
- 16** Centrifuge the gradients overnight (18 hours) at 38,000 rpm at 4°C in a Beckman ultracentrifuge using SW41 rotor.
- 17** The VLPs will band at the 50%-10% interface and can be seen as a white “fluffy” layer. Collect top 1 ml of the gradient and discard. Collect next 1-2 ml of VLP material and place in a 15 ml tube.

After this equilibrium centrifugation, sometimes two bands form. The bottom band (seen at the 60%-50% interface), while it does contain VLPs, is considerably contaminated with cell debris. The majority of the VLPs are in the top band. The purified VLPs can be entirely removed in 1-2 ml.

- 18** (Optional) For further purification, concentrated VLPs may be fractionated by more rigorous equilibrium centrifugation. Concentrated VLPs are diluted in TNE to 10% sucrose (final volume 5 ml). Continuous sucrose gradients, 20-65% in TNE, are formed with a gradient maker. Alternatively, to make this gradient, slowly add successively 1.5 ml each of 65%, 55%, 45%, 35%, 25% sucrose solutions in TNE buffer to a SW41 washed, clear ultracentrifuge tube (11 ml). Allow the sucrose solutions in the tube to diffuse by incubating the tubes at 4°C overnight. Load 2.5 ml diluted VLPs on top of the gradient and centrifuge at 28,000 rpm for 18 hours. Collect VLP band as in step 17.
- 19** Bring collected VLPs up to 10 ml with TNE buffer (in order to dilute the sucrose to below 25%). Place 5 ml into each of two SW50.1 tubes. Centrifuge the VLPs 6 hours at 35,000 rpm to pellet.

This centrifugation serves to concentrate the VLPs.

- 20 Remove the supernatant from tubes and resuspend the VLPs in 10-20 μ l of 10% sucrose in TNE buffer per T150 flask.

VLP Storage

- 21 Aliquot the VLPs into vials and store at -80°C
VLPs stored in this way are stable. VLPs are also stable through multiple freeze-thaw cycles

Alternate Protocol 2: Small Scale VLP Purification

This protocol describes methods for purification of small-scale preparations of VLPs. Applications that can use small scale production of VLPs often do not require the rigorous VLP purification since detection of protein content is usually accomplished by Western blots or immunoprecipitation with specific antibodies. Thus this protocol is a shortened and modified version of Basic Protocol 2.

Materials

Supernatants containing small-scale amounts of VLPs (Alternate Protocol 1)
Beckman GS-6R Centrifuge (or comparable)
Beckman Ultracentrifuge (or comparable)
SW50.1 rotor and buckets or comparable
SW50.1 tubes
Pipettes (5, 10, and 25 ml sterile)
20% and 65% sucrose solutions dissolved in TNE Buffer (weight/volume)

Protocol Steps (based on yields from one 35 mm plate)

1. Collect supernatants from cells transfected as in Alternate Protocol 1 (combine all collected supernatants). Pellet cell debris by subjecting the supernatant to centrifugation at 5000 rpm for 15 minutes.
2. Layer supernatant on top of 2.5 ml of 20% sucrose in a SW50.1 centrifuge tube. Centrifuge gradient at 35,000 rpm for 6 hours.
The VLPs will be in the pellet. Alternatively, for increased purity, VLPs can be banded at a 60%-20% sucrose interface as in Basic Protocol 2 step 10-13 adjusting volumes for an SW50.1 centrifuge tube.
3. Remove the supernatant and resuspend the pelleted VLPs in 100 μ l TNE buffer or gel sample buffer.
4. Store the VLPs at -20°C .
VLPs may be frozen in aliquots to avoid repeated freeze thawing. However, the VLPs are quite stable after multiple freeze-thaw cycles.

Basic Protocol 3: Quantification of Protein Content of VLPs

For large-scale preparations, particularly, it may be important to quantify the protein content of the VLP preparations. Total protein content can be readily determined using standard protein assays such as the Bradford assay (kits are available for these assays, for example BioRad Bradford Quickstart Assay Kit). However, the concentration of a particular protein may also be important. For example, when VLPs are used as an immunogen, the amount of a particular protein injected into an experimental animal will be important in assessing immune responses to that protein. This protocol describes one method for quantifying the concentration of a particular VLP associated protein in a purified preparation.

Materials

Large scale purified VLPs (Basic Protocol 2)

Polyacrylamide gels (Life Technologies or comparable)

Polyacrylamide Gel sample buffer

Polyacrylamide Gel Running buffer

Silver stain kit (Thermo Scientific)

Coomassie stain kit (Life Technologies or BioRad or comparable)

Protein standard (such as BSA Bio-Rad Quickstart)

Gel scanner (Syngene G Box or comparable)

Rotator (platform shaker)

1. Prepare a stock solution of a protein standard, as, for example, bovine serum albumin (1.0 mg/10 ml of water).
2. Separate proteins present in 1, 5, and 10 μ l of the large scale VLP preparation on a polyacrylamide gel. On the same gel run, 2, 5, 10, 20 μ l of the protein standard stock solution to generate a standard curve.

Because 1-10 μ l of an undiluted stock of VLPs can overload the polyacrylamide gel, depending upon the gel thickness and sample well size, it is often necessary to prepare a 10-fold dilution of the VLP stock and use 1, 5, and 10 μ l of the diluted stock for electrophoresis.

3. Stain the protein bands using a protein-specific stain such as silver stain or Coomassie Brilliant Blue, following manufacturers' instructions. Agitate gels during staining protocols using a rotator. See Figure 2 for an illustration of a silver stain of a polyacrylamide gel containing proteins in ND VLPs.

Different proteins stain with different efficiencies depending upon the protein stain. Thus a rigorous assessment of the concentration of a particular protein will require separation of VLP proteins on several different polyacrylamide gels in order to accomplish different protein staining protocols.

4. Quantify the protein bands in question using appropriate instrumentation that will detect and measure the stain used.

Gel scanners are available for this application. In the absence of such instrumentation, an approximation of protein content can be made by scanning the stained gel, encased in plastic, on an office printer/scanner (For example, Epson or Hewlett Packard) and then quantifying the intensity of the bands using Photoshop.

5. Using the standard curve generated with a protein standard, estimate the concentration of VLP associated protein for each volume of VLP in order to calculate the concentration of the protein in the undiluted VLP stock.

Alternate Protocol 3: Use of Western Blots for Characterization of VLP Protein Content

If a purified protein version of a particular VLP protein is available or if a source of this protein with a known concentration is available, the concentration of a particular VLP associated protein can be determined using Western Blots. This protocol also requires an antibody specific for the protein in question.

In the absence of a source of purified protein, this protocol can also be used to assess the relative incorporation of different versions of the same protein into VLPs.

Materials

Large-scale purified VLPs (Basic Protocol 2) or small-scale purified VLPs (Alternate Protocol 2)

Polyacrylamide gels (Novex, Life Technologies, or comparable)

Polyacrylamide gel sample buffer

Polyacrylamide gel running buffer

Western Blot transfer system (iBlot gel transfer system, from Invitrogen, or comparable)

Western Blot chemiluminescence detection reagents (ECL from GE Health Care, or comparable)

PBS-0.5% Tween 20

Nonfat powdered milk

Specific protein standard

Protein specific antibodies

X Ray Film (Thermo Scientific CL-XPosure Film or comparable or instrument designed to detect Western blots as, for example, Syngene G box)

Rotator

1. Follow steps 1 and 2 of Basic Protocol 3 except use the specific purified protein in question as standard.
2. Transfer separated proteins from gel, using iBlot system or comparable, onto a PVDF membrane (included in the iBlot Western Blot system) according to manufacturer's directions.

The iBlot Western Detection System is a dry transfer system that works quite well. Other types of Western Blot systems including semi-dry transfer or wet transfer can be used. See Basic Protocol 4 in Unit 14A.2 or CPMB Unit 10.8.

3. Detect the protein in question on the membrane, using antibody specific for that protein, in the following steps:

The PVDF membrane should be continuously agitated during each step by placing it in a plastic box or a sealed plastic bag on a rotator.

- a. Incubate PVDF membrane (blot) containing proteins in PBS containing 0.5% Tween and 10% powdered milk for 1-4 hours at room temperature or 4° C overnight.
 - b. Incubate blot in PBS-0.5% Tween containing diluted primary antibody (1/1000-1/5000 dilution) for 1 hour at room temperature or overnight at 4°C.
 - c. Wash blot extensively with PBS-0.5% Tween.
 - d. Incubate blot for 1-2 hours at room temperature with diluted secondary antibody (anti-mouse or anti-rabbit antibody depending upon the primary antibody used) coupled to HRP at the dilution recommended by the supplier (often about 1/40,000 dilution).
 - e. Wash blot extensively in PBS-0.5% Tween.
 - f. Incubate blot with chemiluminescence detection reagents as recommended by manufacturer.
4. Detect and quantify the signals generated from the Western Blot using X-ray film or using instrumentation designed to detect Western blot signals.

Gel scanners are available for this application. In the absence of such instrumentation, an approximation of band intensity can be made by scanning the X-Ray film used to detect signal on an office printer/scanner (For example, Epson or Hewlett Packard) and then quantifying the intensity of the bands using Photoshop. In detection of signal, insure that the signal obtained is in the linear range of the film or the instrumentation.
 5. Follow step 5 in Basic Protocol 3 for quantification of the protein in question if a protein standard is available.

Alternate Protocol 4: Use of autoradiography for assessment of protein incorporation into VLPs

In the absence of an antibody specific for a VLP associated protein, assessment of the incorporation of a protein into VLPs, relative to other versions of that protein or relative to the NP and/or M protein core components of the VLPs, can be accomplished by detecting radioactively labeled proteins in purified VLP proteins. Support Protocol 1 describes the generation of radioactively labeled VLPs. This protocol describes the detection and quantification of the radioactively labeled VLP proteins.

Materials

VLPs prepared in Support Protocol 1 and purified by Alternate Protocol 2

Polyacrylamide gels and buffers as described in Alternate Protocol 3

Additionally:

Equipment for drying polyacrylamide gels (Bio-Rad or comparable)

X ray film for detection of radioactively labeled proteins (Kodak* BioMax* XAR*

Film from Carestream Health, 5 × 7 in. catalogue no.: 05-728-36, or comparable)

1. Separate proteins in 1, 5, and 10 µl of a radioactively labeled VLP preparation on a polyacrylamide gel.

The proteins in radioactively labeled VLPs can be directly separated on polyacrylamide gels or, alternatively, the VLPs can be solubilized in, for example, 1% Triton X-100 and the proteins then immunoprecipitated with protein specific antibody prior to separation on polyacrylamide gels, as described in Pantua, et al 2006.

2. Dry the gel according the manufacturer's instructions.
3. Detect radioactively labeled proteins by autoradiography by placing, in a light tight room, the dried gel onto X ray film and then placing the film/gel in a light-tight envelope. The film should be firmly held against the completely dry gel by placing the light tight envelop between two rigid boards and the boards should be held together with clamps. Signals are enhanced by storing the film/gel at -80°C. Exposure times can vary depending upon the levels of radioactivity in the VLPs. Typically exposure times are from 12 to 72 hours. The film is developed in an X-ray film processor. (Alternatively, radioactively labeled proteins can be detected using appropriate instrumentation).
4. Quantification of signals detected upon the gel exposure to X ray film can be accomplished as described in step 4 of Alternate Protocol 3.

Relative incorporation of different proteins into VLPs can be directly compared by this method. Calculations must take into consideration the methionine and cysteine contents of proteins being compared.

Basic Protocol 4: Incorporation of foreign glycoproteins into ND VLPs

Full-length, foreign glycoproteins expressed with the NDV proteins are minimally incorporated into ND VLPs (unpublished observations). Specific, directed incorporation of a foreign glycoprotein ectodomain into ND VLPs can be achieved by constructing a chimera protein gene composed of sequences encoding the foreign protein sequences fused to those encoding the TM and CT domains of the appropriate NDV glycoprotein (McGinnes et al. 2011; Morrison 2010; Murawski et al. 2010). Both type 1 and type 2 glycoproteins can be assembled into these particles. The NDV F protein is a type 1 glycoprotein, thus foreign type 1 glycoprotein ectodomains can be fused to the transmembrane (TM) and cytoplasmic tail (CT) domains of the F protein. The NDV HN protein is a type 2 glycoprotein and type 2 glycoprotein ectodomains can be fused to the CT and TM domains of the HN protein (see Figure 3).

Protocol 4 describes the logic for the construction of the chimera glycoproteins. Any candidate chimera proteins resulting from this construction must be characterized for cell surface expression since incorporation of the protein into the ND VLP platform requires that the chimera be expressed efficiently on cell surfaces. This protocol describes a method for analysis of the efficiency of that surface expression. Once candidate chimera proteins are identified, incorporation of chimera glycoproteins into VLPs is accomplished as described in Alternate Protocols 1 and 2. Efficiency of incorporation is assessed as described in Alternate Protocols 3 or 4. Once chimera proteins with optimal efficiency of incorporation into VLPs are defined, quantitative amounts of VLPs containing chimera proteins are generated, purified, and quantified as described in Basic Protocols 1, 2, and 3 respectively.

Materials

All materials from Alternate Protocols 1 and 2

Plus:

Restriction enzymes

Ligase

Agarose gels

Competent E coli cells (DH5 α) (New England Biolabs)

Ampicillin containing bacterial selection LB (Luria Broth-Difco) agar plates

6-well Tissue Culture Plates

PBS-CM (PBS + 0.1 mM CaCl₂ + 1 mM MgCl₂)

Sulfo-NHS-SS-Biotin (Pierce)

Tween 20

Cell lysis buffer

Sodium dodecyl sulfate (10% stock solution)

Neutravidin Agarose Resin (Pierce)

Polyacrylamide Gels

Polyacrylamide Gel Sample Buffer

Protein Molecular Weight Markers

Reagents for Western Blots as described in Alternate Protocol 3

Microfuge

Rotator

Plasmid DNA Purification Kit (Qiagen Endo Free or comparable)

Construction of chimera proteins

1. For incorporation of a type 1 glycoprotein into ND VLPs, design chimera protein junctions using NDV F protein TM domain sequences and the sequences at the carboxyl terminus of the ectodomain of the foreign glycoprotein.

See Figure 3 for the suggested fusion junctions. Three alternatives that have been successful with specific foreign glycoproteins are illustrated. Alternatively, changing the specific NDV sequences (such as the TSTSA sequence at the junction of the NDV F ectodomain and TM domain) at the junction sites have also been successful. The carboxyl terminus of the ectodomain of the foreign protein at the fusion junction may also be varied.

2. For incorporation of a type 2 glycoprotein into ND VLPs, design of chimera protein junctions using NDV HN protein TM domain sequences and the amino terminal sequences of the ectodomain of the foreign glycoprotein.

See Figure 3 for the suggested fusion junction. This junction has been successful for a number of different type 2 glycoproteins.

3. Generate chimera protein genes. This can most easily be accomplished by ordering the codon-optimized gene encoding the chimera protein from one of numerous companies offering such services (for example, GeneWiz, Gene Art, Celtek Genes). Request the DNA be inserted into the expression plasmid of choice, for example, the pCAGGs vector.

4. Alternatively, the chimera gene can be made using standard PCR protocols as documented in McGinnes, et al, 2011 and Murawski, et al, 2010.

For type 2 glycoproteins, the HN protein CT and TM domain sequences should be generated by PCR using primers that will encode restriction enzyme sites Xho I at the 5' end and Not I at the 3' end. The ectodomain of the type 2 glycoprotein of interest should be generated by PCR with primers containing restriction sites Not I at the 5' end and Msc I (or any restriction site generating blunt ends upon digestion) at the 3' end.

Care should be taken to insure that ligation of the Not I sites at the ends of the HN TM domain and the glycoprotein ectodomain will result in a protein sequence in frame without addition of extra amino acids. Use of computer programs to identify

restriction sites to insert or mutate without changing translation are useful (for example, EMBOSS 6.3.1:silent).

For type 1 glycoproteins, the F protein TM and CT domain sequences should be generated by PCR using primers with restriction enzyme sites Sna B1 at the 5' end and Msc I at the 3' end (as in Figure 3, alternate 1). The sequences of the ectodomain of a glycoprotein of interest should be generated by PCR using primers containing Xho I at the 5' end and Sna B1 at the 3' end.

Care should be taken to insure that ligation of the Sna B1 sites at the ends of the F protein TM domain and the glycoprotein ectodomain will result in a protein sequence in frame without addition of new codons (and, therefore, additional amino acids at the chimera junction).

5. Generate plasmid DNA encoding candidate chimera type 2 protein by ligating pCAGGs vector cut with Xho I and Msc I with the HN CT and TM domain sequences (flanked by Xho I and Not I sites and the sequences encoding the ectodomain of the type two protein of interest (flanked by Not I and Msc I sites). Transform competent *E. coli* with ligated molecules and select ampicillin resistant colonies.

Generate plasmid DNA encoding candidate chimera type 1 protein by ligating pCAGGs vector cut with restriction enzymes Xho I and Msc I with DNA sequences encoding the ectodomain of the type 1 glycoprotein of interest, flanked by Xho I and Sna B1 sites, and DNA sequences encoding the TM and CT domains of the NDV F protein, flanked by Sna B1 and Msc I sites.

6. Prepare plasmid DNA stocks from *E. coli* transformants and verify candidate chimera genes by sequencing the DNA inserted into pCAGGs vector.
7. Generate large-scale preparations of plasmid DNA encoding candidate chimera genes.

Assessment of surface expression of candidate chimera proteins

Transfection of cells

- 8 The day before transfection, seed 25 mm plates so cells will be 60-65% confluent the next morning.
- 9 Transfect cells growing in 35 mm plates with plasmids encoding chimera candidate proteins using Alternate Protocol 1.

Include transfection of cells with the wild type version of the foreign protein as a positive control for surface expression.
- 10 Incubate transfected cells at 37°C for 40 to 48 hours.

Biotinylation of cell surfaces

- 11 Wash cell monolayer 3 times with PBS-CM (cold).

- 12** Add 1 ml of PBS-CM and incubate at room temperature for 10 minutes, then remove PBS-CM.
- 13** Add 1 ml PBS-CM + 0.5 mg/ml Sulfo-NHS-SS-Biotin. Incubate at room temperature for 30 minutes.

Sulfo-NHS-SS-Biotin will covalently link the biotin to primary amines such as the side chain of lysines in proteins. By incubating the reagent (which does not cross cell membranes) with intact cells, only cell surface proteins are modified by biotin and the biotin thus serves as a marker for cell surface expression.
- 14** Remove buffer with Sulfo-NHS-SS-Biotin, add 2 mls complete cell culture media and incubate 5 minutes at room temperature, remove and wash cells 3 times with PBS-CM.
- 15** Lyse cells with 400 μ l of cell lysis buffer (without reducing agent).

The presence of reducing agent will cleave the biotin from proteins.

Immunoprecipitate cell surface proteins containing covalently linked biotin

- 16** Add 100 μ l of cell extract to a fresh microfuge tube and add 50 μ l of washed Neutravidin agarose resin.

To wash neutravidin-agarose resin, mix bottle well, remove what is needed (e.g. 50 μ l x number of samples). Pellet resin in a microfuge at 10,000 rpm for 30 seconds. Wash 2 times with PBS containing 0.5% Tween20. Resuspend to original volume in PBS + 0.5% Tween20. Streptavidin-resin can also be used in place of neutravidin-agarose.
- 17** Mix extract-resin mixture at 1 hr at room temp (or overnight at 4°C).

Mixing can be accomplished automatically by using a rotator. Biotinylated surface proteins are bound to the neutravidin coupled to resin.
- 18** Pellet resin-biotinylated protein complexes at 10,000 rpm for 30 seconds in a microfuge, remove supernatant, and wash resin-protein complexes 3 times with 1 ml of PBS containing 0.5% Tween20 + 0.2% SDS. After the final wash, pellet the resin-protein complexes and remove all traces of supernatant.
- 19** Resuspend the pellet in 30 μ l Gel Sample Buffer (without reducing agent if the protein is to be detected using the biotin label), boil 5 minutes, and pellet resin.

The biotinylated cell surface proteins are in the supernatant. Do not use reducing agent in the gel sample buffer if the protein is to be detected using the biotin label since the biotin will be removed. If the protein is to be detected by Western blots using protein specific antibody or by autoradiography of radioactively labeled proteins, then reducing agent in the sample buffer will not affect protein detection.
- 20** Load 15-30 μ l of the supernatant onto a polyacrylamide gel. Load aliquots of total cell extract in parallel lanes. After electrophoretic separation of proteins, transfer separated proteins to a PVDF membrane. Perform a standard western

blot to detect proteins of interest using appropriate antibody as described in Alternate Protocol 3. Alternatively, all cell surface biotinylated proteins can be detected by Western blots using HRP coupled neutravidin for detection or by autoradiography of radioactively labeled proteins.

Quantitative analyses of amounts of surface protein (biotinylated molecules precipitated with neutravidin-agarose) and total cell protein (in total cell extracts) allow determination of the efficiency of surface expression of the chimera protein. Inclusion of control monolayers transfected with the cDNAs encoding the wild type foreign protein will serve as a positive control for efficient surface expression. Chimera proteins efficiently expressed on cell surfaces (comparable to wild type protein) are candidates for inclusion into ND VLPs.

Assessment of the efficiency of incorporation of a chimera protein into VLPs

21 Generate of VLPs containing chimera proteins using Alternate Protocol 1.

22 Purify VLPs containing chimera proteins using Alternate Protocol 2

Assess the efficiency of incorporation of a chimera protein into ND VLPs using Alternate Protocols 3 or 4.

Reagents and Solutions

Phosphate buffered saline (PBS)

145 mM NaCl

7.6 mM K₂HPO₄

2.4 mM KH₂PO₄

pH 7.2

PBS-CM

PBS containing 1mM CaCl₂ and 1mM MgCl₂

TNE Buffer

0.150M NaCl

0.025 M Tris-HCl, pH 7.4

0.005 M EDTA

Fetal bovine serum (FBS) or bovine serum depending upon requirements of cells (complement inactivated by incubation at 56°C for 30 minutes)

Dialyzed fetal calf serum or bovine serum (Life Technologies)

Tissue Culture Media such as Dulbecco's Modified Eagles Medium (DMEM)

DMEM with additions

1× DMEM

fetal calf serum, 10% (numerous sources including Life Technologies) or dialyzed fetal calf serum (10%)

penicillin and streptomycin (pen/strep) (can be obtained as a pre-made stock solution from Life Technologies)

10,000U/ml of penicillin and 10,000 µg/ml streptomycin

vitamins (obtained as a 100x stock solution from Life Technologies)

glutamine (obtained as a 100x stock solution from Life Technologies)

Other additions to the media will depend upon the requirements of the cells used. For example, some cells require added amino acids (nonessential amino acids).

10%, 20%, 25%, 35%, 45%, 50%, 55%, 65%, and 80% sucrose in TNE Buffer (weight/volume)

Cell lysis buffer

RSB buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl 1.5 mM Mg Cl₂,) 8.5 ml

Triton X-100--1 ml of a 10% stock solution

Na deoxycholate--0.5 ml of a 10% stock solution

N' ethylmalaimide--25 mg

Gel sample buffer (2×)

Glycerol 2ml

0.5 M Tris-HCl, pH 6.8

Bromphenol Blue 0.4 ml of a 10% solution

10% sodium dodecyl sulfate

optional β mercaptoethanol (0.1 M)

Gel running buffer (choose buffer appropriate to gels being used) Buffer for Tris-Glycine Gels

Tris g

28.35 g Glycine

250 ml water

Commentary**Background Information**

VLPs are particles with sizes similar to authentic virus and, like virus particles, contain repeating protein complexes in ordered arrays on their surfaces and in their cores similar to

those of infectious viruses (reviewed in Jennings and Bachmann 2008; Noad and Roy 2003). VLPs derived from nonenveloped virus proteins are empty capsids structurally similar to those of infectious virus. VLPs derived from enveloped virus proteins may contain surface glycoproteins that are properly folded and inserted into membranes in repeating arrangements typical of the enveloped virus. Internal core or capsid proteins in enveloped VLPs are also likely folded and assembled typical of a virus. The protocols described here were developed for the production and purification of VLPs formed with structural proteins of Newcastle disease virus, an enveloped virus.

Newcastle disease virus (NDV) is a paramyxovirus. Paramyxoviruses are enveloped, negative-stranded RNA viruses (Collins and Crowe 2007; Karron and Collins 2007; Lamb and Parks 2007). All paramyxovirus virions contain three membrane proteins. Two of these proteins are glycoproteins, an attachment protein, termed HN protein for Newcastle disease virus, and a fusion (F) protein. The third membrane protein is a nonglycosylated matrix protein (M protein), which lines the inner surface of the membrane. The virus also contains three core proteins, the nucleocapsid protein (NP), which binds to the RNA genome, as well as a phosphoprotein (P) and the viral polymerase, the L protein. It has been reported that paramyxovirus VLPs can be produced upon expression of the M protein or M protein and various combinations the glycoproteins and NP (Ciccanelli and Basler 2006; Coronel et al. 1999; Li et al. 2009; Patch et al. 2007; Schmitt et al. 2002; Sugahara et al. 2004; Takimoto et al. 2001). Indeed, cells expressing the NDV HN, F, NP, and M proteins release particles that both structurally and functionally resemble virus particles (McGinnes et al. 2010; Pantua et al. 2006). What distinguishes ND VLPs from other paramyxovirus VLPs and, indeed, from many other types of VLPs, is their efficiency of release (Pantua et al. 2006). As a result, quantitative amounts of these particles are relatively easy to prepare even from transiently transfected cells (McGinnes et al. 2011; McGinnes et al. 2010). They can be purified using protocols modified from those used for virus purification and the purified VLPs showed minimal cell protein contamination. Furthermore, the ratios of viral proteins were similar to those in virus particles.

ND VLPs contain biologically active glycoproteins, indicating that they have folded into an authentic conformation during VLP assembly. Similar to virion associated HN protein, the VLP associated HN protein mediates cell binding and possesses neuraminidase activity (McGinnes et al. 2010). ND VLPs have hemagglutinating titers comparable to equivalent amounts of virus (McGinnes et al. 2010). Typical of virion associated F protein, the VLP associated F protein can direct the fusion of the VLP membrane with red blood cell membranes (McGinnes et al. 2010).

In a murine model, ND VLPs are effective immunogens. Levels of soluble antibodies, characterized by ELISA and by neutralizing antibody titers, resulting from ND VLP immunization were as high or higher than those resulting from immunization with inactivated virus (McGinnes et al. 2010). Furthermore, ND VLPs stimulated T cell responses at levels slightly higher than those stimulated by the vaccine virus (McGinnes et al. 2010).

The protocols described here can be readily adapted for VLPs produced by proteins from other enveloped viruses. Indeed, we have used these protocols for the generation of quantitative amounts of VLPs formed by expression of West Nile Virus structural proteins (unpublished observations).

The protocols described here are not the only ones suitable for generation and purification of VLPs. The generation of VLPs requires significant levels of expression of the viral structural proteins in cells that will efficiently assemble and release these particles. The protocol described here utilizes transient transfection of avian cells. While avian cells most efficiently release these particles, other cell types, such as COS7 cells or 293T cells, can be used with only a slight decrease in yields (McGinnes et al. 2011; Murawski et al. 2010). Another widely used method for the production of VLPs is the use of baculovirus-insect cell expression systems (for example, (Kang et al. 2009; Quan et al. 2011; Ye et al. 2006). Baculoviruses can be engineered to encode VLP proteins. Infection of insect cells with these viruses often results in very efficient release of VLPs. This protocol is an excellent method for cost effective, large-scale production of VLPs. Indeed, this is a preferred method if the particular proteins are efficiently released as VLPs from insect cells. Use of baculovirus-insect cell systems for VLP production, however, must take into consideration that VLP preparations can be contaminated with infectious baculoviruses. In addition, the glycosylation of glycoproteins in insect cells is somewhat different than that in mammalian or avian cells. Furthermore, there are VLPs not efficiently released from insect cells infected with recombinant baculoviruses, including ND VLPs. Thus alternative methods are required for production of these VLPs, one of which is described here.

The protocol for purification of VLPs described here utilizes differential centrifugation. However, other protocols utilizing column chromatography and size exclusion chromatography are possible.

Not all virus systems can be adapted to produce virus-like particles. Other systems do not yield VLPs at levels sufficient for their use for various applications, notably as vaccine candidates (for example, (McGinnes et al. 2011). To overcome this problem established, well-characterized VLPs have been adapted for assembly of foreign glycoproteins and peptides into particles. Several different approaches have been reported. One approach, described here, uses directed incorporation of a foreign protein into ND VLPs by creating chimera proteins with NDV domains. Alternatively, the passive incorporation of wild type, intact proteins into VLPs has been reported. For example, influenza HA glycoprotein has been incorporated into a VLP formed with lentivirus gag (Haynes et al. 2009). The RSV G and F proteins have been passively incorporated into influenza VLPs (Quan et al. 2011). The efficiency of this passive incorporation is not well characterized. Passive incorporation of wild type foreign glycoproteins into ND VLPs is extremely inefficient requiring the use of chimera proteins described here.

In another approach, Immunodominant molecules can be chemically cross-linked to the surfaces of VLPs (reviewed in (Jennings and Bachmann 2008)). Alternatively, key epitopes of a target virus have been genetically fused to a structural protein of a well-characterized VLP. If a sequence of a protein has been identified as a domain that stimulates neutralizing

antibody responses, incorporation of this domain into a VLP platform could stimulate these antibodies. Incorporation of T cell epitopes could enhance the ability of the VLPs to stimulate cell-mediated immune responses to specific pathogens. There are many variations on this approach (for example (Deml et al. 2005; Fu et al. 2009; Grgacic and Anderson 2006; Halsey et al. 2008; Jennings and Bachmann 2008; Li et al. 2009; Sadeyen et al. 2003; Saini and Vratil 2003)) but most of these chimera VLPs have been reported for lentivirus gag, the HBcAg, or the HBsAg VLPs.

Critical Parameters/Troubleshooting

Problem	Possible Cause	Solutions
1. Inefficient VLP release	cell type used	Use ELL-0 cells
	condition of cells	Cells need to be in log phase of growth at time of transfection
	cells passaged multiple times may have altered phenotypes	Use a new clone of cells
	inefficient transfection	Use different transfection protocol
		Clean up plasmid preparations poor cell condition
	VLPs are rebinding to cell monolayers	Inhibit VLP binding to cells cell monolayers using, for example, inclusion in cell supernatants neuraminidase or heparin
	Cell pH	Monitor pH of cell supernatant after transfection, replacing when media becomes too acid
	Poor incorporation of a glycoprotein into VLP	Poor surface expression Adjust chimera construction by changing junction sequences
	Detection of protein	Use another antibody for detection Use another stain for protein detection
	Host cell contamination	Condition of cells Monitor pH of media Eliminate last VLP collections Add an additional sucrose gradient to purification protocol

Anticipated Results

For preparation of ND VLPs, transfections of 60-75 T150 plates will yield approximately 1.5 ml of 2-4 mg/ml of total protein of purified VLPs.

Time Considerations

Large Scale Transfections—Seeding of cells for transfection and transfection will take 2 days. Harvesting of cell supernatants containing VLPs proceeds for 96-110 hours.

Small Scale Transfections—Times are the same as for large-scale transfections although harvesting of cell supernatants can be terminated after 72 hours.

Large Scale VLP purification—The time required for the multiple centrifugations is between 40 and 50 hours.

Small Scale VLP purification—This more modest purification requires approximately 7 hours.

Quantification of Protein Content of VLPs—This protocol requires polyacrylamide gel electrophoretic separation of proteins as well as protein detection in gels using silver stained gels or Coomassie Brilliant Blue stained gels. Both can be accomplished in 8 hours.

Quantification of Protein Content by Western Blots—The protocols involved will require approximately 2 days.

Construction of chimera proteins—Depending upon the complexity of the construction, this protocol can be accomplished in 1-2 weeks.

Assessment of Surface Expression—This protocol requires 48 hours for transfection and incubation of cells. Biotinylation, immunoprecipitation, and Western blots will require a minimum of 2 days.

Acknowledgments

The work described here was supported by grants AI30572 (TM), AI093791 (TM) and U19AI057319 (A. Rothman) (awarded to the University of Massachusetts Center for Translational Research in Human Immunology and Biodefense) from the National Institutes of Health; The Massachusetts Technology Transfer Center; The Worcester Foundation; and Novavax, Inc.

Literature Cited

- Ciccanelli MJ, Basler CF. Mutation of the YMXL in the Nipah virus matrix protein abrogates budding and alters subcellular localization. *Virology*. 2006; 80:12070–12078.
- Collins, PL.; Crowe, JE. Respiratory syncytial virus and metapneumovirus. 5 edn.. LippincottWilliams and Wilkins; Philadelphia: 2007.
- Coronel EC, Murti KG, Takimoto T, Portner A. Human parainfluenza virus type I matrix and nucleocapsid genes transiently expressed in mammalian cells induce release of virus-like particles containing nucleocapsid structures. *J. Virol.* 1999; 73:7035–7038. [PubMed: 10400805]
- Deml L, Speth C, Dierich MP, Wolf H, Wagner R. Recombinant HIV-1 Pr55gag virus-like particles: potent stimulators of innate and acquired immune responses. *Molecular Immunology*. 2005; 42:259–277. [PubMed: 15488613]
- Fu T-M, Grimm KM, Citron MP, Freed DC, Fan J, Keller PM, Shiver JW, Liang X, Joyce JG. Comparative immunogenicity evaluations of influenza A virus M2 peptide as recombinant virus like particle or conjugate vaccines in mice and monkeys. *Vaccine*. 2009; 27:1440–1447. [PubMed: 19146898]
- Grgacic EVL, Anderson DA. Virus-like particles: Passport to immune recognition. *Methods*. 2006; 40:60–65. [PubMed: 16997714]
- Halsey RJ, Tanzer FL, Meyers A, Pillay S, Lynch A, Shephard E, Williamson A-L, Rybicki EP. Chimeric HIV-1 subtype C Gag molecules with large in-frame C-terminal polypeptide fusions form virus-like particles. *Virus Research*. 2008; 133:259–268. [PubMed: 18329748]

- Harper DM, Franco EL, Wheeler CM, Moscicki A-B, Romanowski B, Roteli-Martins CM, Jenkins D, Schuid A, Clemens SAC, Dubin G. Sustained efficacy up to 4-5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised trial. *Lancet*. 2006; 367:1247–1255. [PubMed: 16631880]
- Haynes JR, Dokken L, Wiley JA, Cawthon AG, Bigger J, Harmsen AG, Richardson C. Influenza-pseudotyped Gag virus-like particle vaccines provide broad protection against highly pathogenic avian influenza challenge. *Vaccine*. 2009; 27:530–541. [PubMed: 19026705]
- Jennings GT, Bachmann MF. The coming of age of virus-like particle vaccines. *Biol. Chem.* 2008; 389:521–536. [PubMed: 18953718]
- Kaczmarczyk SJ, Sitaraman K, Young HA, Hughes SH, Chatterjee DK. Protein delivery using engineered virus-like particles. *Proceedings of the National Academy of Sciences*. 2012; 108:16998–17003.
- Kang S-M, Song J-M, Quan F-S, Compans RW. Influenza vaccines based on virus-like particles. *Virus Res.* 2009; 143:140–146. [PubMed: 19374929]
- Karron, RA.; Collins, PL. Parainfluenza viruses. In: Knipe, DM.; Howley, PM., editors. *Fields Virology*. Vol. 1. LippincottWilliams & Wilkins; Philadelphia: 2007. p. 1497-1526.
- Lamb, RA.; Parks, GD. Paramyxoviridae: The Viruses and Their Replication. In: Knipe, DM.; Howley, PM.; Griffin, DE.; Lamb, RA.; Martin, MA.; Roizman, B.; Strauss, SE., editors. *Fields Virology*. Fifth Edition edn. Vol. 1. LippincottWilliams & Wilkins; Philadelphia: 2007. p. 1450-1496.
- Li M, Schmitt PT, Li Z, McCrory TS, He B, Schmitt AP. Mumps Virus Matrix, Fusion, and Nucleocapsid Proteins Cooperate for Efficient Production of Virus-Like Particles. *J. Virol.* 2009; 83:7261–7272. [PubMed: 19439476]
- Ludwig C, Wagner R. Virus-like particles--universal molecular toolboxes. *Cur. Opinion Biotech.* 2007; 18:537–545.
- McGinnes LW, Gravel KA, Finberg RW, Kurt-Jones EA, Massare MJ, Smith G, Schmidt MR, Morrison TG. Assembly and immunological properties of Newcastle disease virus-like particles containing the respiratory syncytial virus F and G proteins. *J. Virol.* 2011; 85:366–377. [PubMed: 20980510]
- McGinnes LW, Pantua H, Laliberte JP, Gravel KA, Jain S, Morrison TG. Assembly and biological and immunological properties of Newcastle disease virus-like particles. *J. Virol.* 2010; 84:4513–4523. [PubMed: 20181713]
- Morrison TG. Newcastle disease virus-like particles as a platform for the development of vaccines for human and agricultural pathogens. *Future Virol.* 2010; 5:545–554. [PubMed: 21339837]
- Murawski MR, McGinnes LW, Finberg RW, Kurt-Jones EA, Massare M, Smith G, Heaton PM, Fraire A, Morrison TG. Newcastle disease virus-like particles containing respiratory syncytial virus G protein induced protection in BALB/c mice with no evidence of immunopathology. *J. Virol.* 2010; 84:1110–1123. [PubMed: 19889768]
- Noad R, Roy P. Virus-like particles as immunogens. *Trends in Microbiol.* 2003; 11:438–444.
- Pantua HD, McGinnes LW, Peeples ME, Morrison TG. Requirements for the assembly and release of Newcastle disease virus-like particles. *J Virol.* 2006; 80:11062–11073. [PubMed: 16971425]
- Patch JR, Crameri G, Wang LF, Eaton BT, Broder CC. Quantitative analysis of Nipah virus proteins released as virus-like particles reveals a central roll for the matrix protein. *Virol. J.* 2007; 4:1–12. [PubMed: 17204159]
- Quan F-S, Kim Y, Lee S, Yi H, Kang S-M, Bozja J, Moore ML, Compans RW. Virus-like Particle Vaccine Induces Protection Against Respiratory Syncytial Virus Infection in Mice. *J. of Infect. Diseases.* 2011; 204:987–995. [PubMed: 21881112]
- Sadeyen J-Ré, Tourne S, Shkreli M, Sizaret P-Y, Coursaget P. Insertion of a foreign sequence on capsid surface loops of human papillomavirus type 16 virus-like particles reduces their capacity to induce neutralizing antibodies and delineates a conformational neutralizing epitope. *Virology.* 2003; 309:32–40. [PubMed: 12726724]
- Saini M, Vrati S. A Japanese Encephalitis Virus Peptide Present on Johnson Grass Mosaic Virus-Like Particles Induces Virus-Neutralizing Antibodies and Protects Mice against Lethal Challenge. *J. Virol.* 2003; 77:3487–3494. [PubMed: 12610124]

- Schmidt MR, McGinnes LW, Kenward SA, Willems KN, Woodland RT, Morrison TG. Long term and memory immune responses in mice against Newcastle disease virus-like particles containing respiratory syncytial virus glycoprotein ectodomains. *J. Virol.* 2012; 86:11654–11662. [PubMed: 22896618]
- Schmitt AP, Leser GP, Waning DL, Lamb RA. Requirements for budding of paramyxovirus simian virus 5 virus-like particles. *J. Virol.* 2002; 76:3952–3964. [PubMed: 11907235]
- Sugahara F, Uchiyama T, Watanabe H, Shimazu Y, Kuwayama M, Fujii Y, Kiyotani K, Adachi A, Kohno N, Yoshida T, Sakaguchi T. Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein. *Virology.* 2004; 325:1–10. [PubMed: 15231380]
- Takimoto T, Bousse T, Coronel EC, Scroggs RA, Portner A. Cytoplasmic domain of Sendai virus HN protein contains a specific sequence required for its incorporation into virions. *Journal of Virology.* 1998; 72:9747–9754. [PubMed: 9811709]
- Takimoto T, Murti KG, Bousse T, Scroggs RA, Portner A. Role of matrix and fusion proteins in budding of Sendai virus. *J. Virol.* 2001; 75:11384–11391. [PubMed: 11689619]
- Ye L, Lin J, Sun Y, Bennouna S, Lo M, Wu Q, Bu Z, Pulendran B, Compans RW, Yang C. Ebola virus-like particles produced in insect cells exhibit dendritic cell stimulating activity and induce neutralizing antibodies. *Virology.* 2006; 351:260–270. [PubMed: 16678231]

Large Scale Purification on Sucrose Gradients

Small Scale Purification on a Sucrose Gradient

Figure 1. Diagram of sucrose gradients used to purify VLPs

Large-scale purification of VLPs requires two to three sequential sucrose gradients, which are diagramed. In step one, VLPs, pelleted from the cleared cell supernatants, are resuspended in TNE buffer and placed on top of layers of 65% and 20% sucrose, and then subjected to centrifugation for 6 hours at 24,000 rpm (step 1). The white fluffy layer between the 65% and 20% layers is harvested. In step 2, the VLPs from step 1 are subjected to flotation into a gradient. The VLPs, made approximately 60% with respect to sucrose, are placed on top of an 80% sucrose pad and then over layered with 50% sucrose and then 10%

sucrose. The gradients are centrifuged at 38,000 rpm for at least 18 hours. The VLPs form a white layer in between the 50% and 10% layers (step 2). For additional purification, the VLPs can be subjected to sedimentation in a continuous sucrose gradient (Step 3). Small-scale purification of VLPs can be accomplished by centrifugation of VLPs through a 20% sucrose layer into a pellet as diagramed at the bottom of the figure. Alternatively, step 1 of the large-scale purification can be done.

Figure 2. Silver stain of a preparation of ND VLPs

Proteins in purified VLPs were separated by polyacrylamide gel electrophoresis in a 10% gel in the absence of reducing agent. Molecular weight protein markers were electrophoresed in an adjacent lane. The resulting gel was stained using a Silver staining kit, and the stained gel was scanned on an Epson printer/scanner. NDV proteins: M, matrix protein; NP, nucleocapsid protein; Fnr, fusion protein not reduced (disulfide linked F₁ and F₂); HN, hemagglutinin-neuraminidase protein, which forms a disulfide linked dimer under nonreducing conditions.

Figure 3. Protein junctions in chimera proteins

The top panel shows linear diagrams of type 1 glycoproteins with the ectodomain of NDV F protein replaced with that of protein X. The panel shows several alternative NDV F protein sequences at the chimera junction.

The bottom panel shows linear diagrams of type 2 glycoproteins with the ectodomain of the NDV HN protein replaced with that of protein X. The panel shows the HN protein sequence at the junction of the chimera protein.